Chemicals and supplies

All chemicals and reagents used in the study were of molecular and analytical grade; and they were purchased from Sigma Chemical Company, St. Louis, MO, USA; Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom; and Sisco Research Laboratories, Mumbai, India. $^{14}$C-glucose, $^{14}$C-2-deoxyglucose and Iodine-125($^{125}$I) were purchased from the Board of Radiation and Isotope Technology, Mumbai, India. Primers, porcine insulin, β-actin monoclonal antibody and DEHP were purchased from Sigma Chemicals Co., St. Louis, Missouri, USA. Total RNA isolation reagent [Trizol] from Invitrogen, M-MuLV Reverse Transcriptase from New England Biolabs, USA and MESA GREEN qPCR MasterMix for SYBR® Assay from Eurogentec, Europe. Polyclonal InsRβ (Insulin Receptor beta subunit), InsRβ$^{Tyr1162/1163}$, IRS-1 (Insulin Receptor Substrate), IRS-1$^{Tyr632}$, PTEN (Phosphatase and tensin homolog), β-arrestin-2, c-Src (cellular sarcoma), Akt1/2/3, Akt$^{Ser^{473}}$, Akt$^{Thr^{308}}$, Akt$^{Tyr^{315/316/312}}$, AS160 (Akt substrate160)$^{Thr^{642}}$, GLUT4 (Glucose Transporter4), GLUT4$^{Ser^{488}}$, Rab8A (Ras-related proteins in brain), Rab13 and α-actinin4 antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). AS160 monoclonal, mTOR (Mammalian target of rapamycin), PDK1 (Phosphoinositide-dependent kinase-1), AS160, HDAC2 (Histone deacetylase) and IRS-1$^{Ser^{636/639}}$ antibodies were purchased from Cell signaling technology (USA). Prestained protein molecular weight markers (low range and high range) were procured from Fermentas International Inc, Canada and Bio-Rad Laboratories Inc. India, respectively. Goat anti-Rabbit IgG HRP, Rabbit anti-Goat IgG
HRP and Goat anti-Mouse IgG HRP conjugated secondary antibodies were purchased from GeNei, Bangalore, India.

**Experimental Design**

As described under chapter I (Page 53 to 56)

**Insulin receptor assay**

Insulin receptor was quantified as per previously published method (Torlinska *et al.*, 2000).

**Radioiodination of Insulin**

The iodination procedure involves substitution of radioactive iodine into tyrosine moieties in peptides. Highly purified iodination grade porcine insulin (Sigma chemical company, USA) was iodinated using $^{125}$I by Chloramine-T method (Greenwood *et al.*, 1963) and purified by gel filtration on Sephadex G-25 column and subsequently Sephadex G-75 in 50mM phosphate buffer (pH7.5) containing 0.1% (w/w) BSA.

**Principle**

The principle of radioiodination involves exposure of the antigen for a defined short period to the oxidizing agent Chloramine-T and $[^{125}$I]. Covalent labeling of proteins directly with $[^{125}$I] involves oxidative generation of cationic iodine (I$^+$) and its spontaneous electrophilic addition to tyrosine residue and lesser extent to tryptophan and histidine residues. Depending upon the reaction conditions, one or two $[^{125}$I] atoms are incorporated into one or more tyrosine residues. The reaction is terminated before
purifying the product by adding sodium metabisulphite, which converts the residual $[^{125}\text{I}]$ iodine into $[^{125}\text{I}]$ iodide.

**Reagents**

1. **$[^{125}\text{I}]$ carrier free sodium iodide** with radioactivity of about 0.5mCi/reaction.

2. **Sodium phosphate buffer 0.05M (pH7.4):** 780mg sodium dihydrogen phosphate and 889mg disodium hydrogen phosphate were dissolved in 90ml distilled water. pH was adjusted to 7.4 with 0.1 N NaOH and final volume made up to 100ml with distilled water.

3. **Chloramine-T (freshly prepared):** 10mg of Chloramine-T was dissolved in 5ml of phosphate buffer.

4. **Hormone:** 1mg highly purified iodination grade porcine insulin (Sigma, USA) was dissolved in 1ml of 0.05 M phosphate buffer to get a concentration of 1µg/µl.

5. **1% Potassium iodide with 8% sucrose:** 100mg of potassium iodide and 800 mg of sucrose were dissolved in 10ml phosphate buffer.

6. **Barbitone buffer (0.07 M, pH8.6):** 7.216g sodium barbitone was dissolved in 490ml distilled water. The pH was adjusted to 8.6 and final volume was made up to 500ml with distilled water.

7. **Bovine serum albumin 1%:** 500mg was dissolved in 50ml baritone buffer.

8. **Preparation of Sephadex G-25 column:** Prepacked Sephadex G-25 PD 10 column (Pharmacia, LKB Technology, Sweden) was coated with 1% BSA and then eluted with 35ml of phosphate buffer to equilibrate the column.
9. **Preparation of Sephadex G-75 column:** 3g of Sephadex G-75 (Pharmacia, LKB Technology, Sweden) was mixed with 150ml of phosphate buffer. The mixture was allowed to stand at 4°C overnight. The column (20 x 1cm) was packed with Sephadex G-75 by keeping the glass wool at the bottom. The presence of air bubbles was removed by passing 25ml of phosphate buffer through the column. Finally, the column was rinsed with 1% BSA and was equilibrated with the same.

**Procedure**

Iodination was performed in 1.5ml glass tube at 20-22°C. The following steps were performed in a sequence rapidly:

- 0.5 mCi (6μl) carrier free $[^{125}\text{I}]$ was taken in the reaction vial containing 25μl 0.05M phosphate buffer.
- 5μg insulin (5μl) was added to the vial and mixed gently.
- The iodination reaction was initiated by adding 15μl (30μg) Chloramine-T.
- The contents were mixed gently by mild tapping of the vial and incubated for 45sec at 25°C.
- At the end of incubation, the reaction was stopped by adding 50μl of Sodium metabisulphite solution and 100μl of 1% potassium iodide with 8% sucrose and mixed well.
- The reaction mixture was immediately transferred in to equilibrated Sephadex G-25 column to remove unreacted iodide.
➢ The column was then eluted with phosphate buffer. Ten drops of elutant was collected in about 30 polypropylene tubes precoated with 1% BSA and from that 10µl was taken for counting the radioactivity in a gamma counter for 60sec.

➢ Two peaks of radioactivity were detected, when the eluted tubes were counted and plotted in a graph paper. The first peak was the protein peak and the second peak was salt peak. The protein peak tube was taken for receptor assay as this tube contained the most immunoreactive and least damaged labeled hormone.

**Purification of radioiodinated insulin**

➢ The protein peak fraction (500µl) obtained was incubated with equal volume of normal serum at room temperature for 2h.

➢ After incubation, the reaction mixture was transferred to the pre-equilibrated sephadex G-75 column and eluted with 0.05M phosphate buffer. 10drops of elutants was collected in about 20 polypropylene tubes precoated with 1% BSA and from that 10µl was taken for counting the radioactivity in a gamma counter for 60sec.

➢ Two peaks of radioactivity were obtained, when the elutants were counted and plotted in a graph paper. The first peak represents the damaged radiolabeled insulin fraction and the second peak represents the intact radiolabeled insulin fraction. The intact radiolabeled insulin fraction was used for the assessment of insulin receptor concentration.
**Calculation of specific activity**

To determine the approximate specific activity of iodinated hormone, the following procedure was followed.

\[
\text{Specific activity of the hormone} = \frac{\text{Counts incorporated into protein}}{\text{Total counts}} \times \frac{\mu\text{Ci of } ^{125}\text{I}}{\mu\text{g of Hormone}}
\]

Total counts (TC) include the radioactivity contained in all the polypropylene tubes as well as in the pipette tips and reaction vial after transferring the reaction mixture into the Sephadex column (residual count). The specific activity of iodinated hormone was 60-80µCi/µg.

**Isolation of plasma membrane**

According to previously published method, the plasma membrane (Dombrowski *et al.*, 1996) was isolated as described under chapter I.

**Saturation analysis**

In order to determine the quantity of labeled hormone to be used for the assessment of insulin receptor, saturation analysis was done by keeping the membrane preparation (100µg protein) constant and increasing amount of labeled hormone \[^{125}\text{I}\]-insulin (10,000, 20,000, 40,000, 80,000, 1,60,000 CPM) was added in triplicate and incubated at 4°C for 24h. A saturation curve was drawn by plotting tracer concentration in X-axis and % binding in Y-axis.
**Measurement of insulin binding**

Plasma membrane insulin receptors in skeletal muscle was quantified by the method of Habberfield *et al.* (1986). Membrane preparation (100 µg protein) was incubated at 4°C for 24h with the saturating concentration (40,000 CPM) of $^{125}$I-labeled porcine insulin in a final volume of 0.5ml 0.04M Tris buffer (pH7.4) containing 0.1% BSA. Nonspecific binding (NSB) was determined in the presence of excess (1000 fold) unlabelled insulin. Bound and free fractions of insulin were separated by centrifugation at 20,000 × g for 8min and then the radioactivity of the pellets was determined using a gamma counter. The specific binding (SB) is calculated by subtracting the NSB from total binding. The concentration of insulin receptor was then calculated and expressed as fmol/mg protein.

**Real-time PCR**

As described under chapter I. Real-time PCR was used to measure mir143 & mRNA of InsR, IRS-1, Akt and GLUT4. Total RNA was extracted from gastrocnemius muscle using Trizol reagent. RNA quality was calculated by measuring the $A_{260/280}$ nm. The purity of RNA obtained was 1.8-1.9 and integrity of the RNA was validated by running samples on 1% formaldehyde agarose gel. The yield of RNA was expressed in µg. cDNA was synthesized from 2µg of total RNA using M-MuLV Reverse Transcriptase according to the manufacturer’s protocol. Real time-PCR was carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad, USA). Reaction was performed using MESA Green PCR master mix (It 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 pairs. Relative amount of each mRNA and miRNA was normalized to the β-actin and U6 RNA. The data were analyzed by comparative CT method and the fold change is calculated by $2^{-\Delta \Delta CT}$ method (Schmittgen and Livak, 2008) using CFX Manager Version 2.1 (Bio Rad, USA).

**Oligonucleotide primers**

The following specific oligonucleotide primers for rat were used for the generation of cDNAs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon size (bp)</th>
<th>Sense primer, Anti-sense primer</th>
<th>GenBank Accession No.</th>
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<td>InsR</td>
<td>225</td>
<td>5’-GCCATCCCGAAAGCGAAGATC-3’</td>
<td>NM_017071.2</td>
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<td></td>
<td></td>
<td>5’-TCTGGGGAGTCTGATTGCAT-3’</td>
<td></td>
</tr>
<tr>
<td>IRS-1</td>
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<td>5’-AAAGCACTGTGACACCGGAA-3’</td>
<td>NM_012969.1</td>
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<tr>
<td></td>
<td></td>
<td>5’-ACACGGTTTCAGAGCAGAGG-3’</td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td>146</td>
<td>5’-GGAAAGCCTTCAGTTTGATCCCAA-3’</td>
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<td>5’-AGTGGAAATCCAGTTCCGAGCTTG-3’</td>
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<td></td>
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<td>5’-CAGCTTGGGAGCCCGATGCC-3’</td>
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</tr>
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</table>
Western blot analysis

As described under chapter I (Page 78 to 87).

Isolation of plasma membrane and cytosolic fractions

Plasma membrane (Dombrowski et al., 1996) and cytosolic fractions (Nevado et al., 2006) from gastrocnemius muscle of control and experimental animals were prepared as described previously. Protein concentration was estimated (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. InsRβ, pInsRβTyr1162/1163 levels were estimated in plasma membrane and GLUT4 level was estimated in both plasma membrane and cytosolic fractions. pGLUT4Ser488 and HDAC2 levels were estimated in cytosolic fraction. Results were normalized with β-actin (the phosphorylated form was normalized with the respective total protein).

Preparation of tissue lysate

Total tissue lysate from gastrocnemius muscle and islets of control and experimental animals were prepared as described previously (Bennett and Tonks, 1997), and protein concentration was estimated. Western blot was done to quantify IRS-1, pIRS-1Tyr632, pIRS-1Ser636/639, PTEN, β-Arrestin-2, c-Src, Akt1/2/3, pAktSer473, pAktThr308, pAktTyr315/316/312, mTOR, PDK1, AS160, pAS160Thr642, Rab8A, Rab13 and α-actinin4 from gastrocnemius muscle lysate. Rat β-actin was used as the invariant loading control.
Primary antibody

**InsR (Insulin Receptor)**

Insulin Receptor β (C-19) rabbit polyclonal antibody detects endogenous levels of total insulin receptor-β protein of rat, mouse and human. Polyclonal antibodies raised against a peptide mapping at the C-terminus of insulin receptor-β of human origin.

**Phospho-InsRβ (Tyr 1162/1163)**

Phospho-InsRβ (Tyr1162/1163) is a rabbit polyclonal antibody raised against a short amino acid sequence containing Tyr 1162 and Tyr 1163 phosphorylated InsRβ of human origin. phospho-InsRβ (Tyr1162/1163) is recommended for detection of Tyr1162/1163 dually phosphorylated InsRβ of mouse, rat and human origin.

**IRS-1 (Insulin Receptor Substrate-1)**

IRS-1(H-165) rabbit polyclonal antibody detects endogenous levels of total IRS-1 protein of rat, mouse and human. It does not cross-react with IRS-2, 3 or 4. Polyclonal antibodies are produced by immunizing rabbits with a synthetic peptide (KLH-coupled) derived from the carboxy-terminal sequence of human IRS-1.

**Phospho-IRS-1 (Tyr 632)**

Phospho-IRS-1 goat antibody detects endogenous levels of IRS-1 protein only when phosphorylated at Tyr632 of rat, mouse and human. Polyclonal antibodies are
produced by immunizing goat against short amino acid sequencing containing phosphorylated tyrosine 632 of IRS-1 of human origin.

**Phospho-IRS-1 (Ser 636/639)**

Phospho-IRS-1 (Ser636/639) Antibody detects endogenous levels of IRS-1 only when phosphorylated at Ser636/639 of rat, mouse and human. This antibody does not cross-react with other related phospho-proteins. Rabbit polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding serine 636/639 of human IRS-1.

**Akt**

Akt (H-136) rabbit antibody detects endogenous levels of total Akt protein of rat, mouse and human. Polyclonal antibodies are produced by immunizing rabbits with amino acids 345-480 of Akt1/2 of human origin.

**Phospho- Akt (Ser 473)**

Phospho-Akt (Ser473) rabbit polyclonal antibody detects endogenous levels of Akt protein only when phosphorylated at Ser 473 of rat, mouse and human. Polyclonal antibodies are produced by immunizing rabbits against short amino acid sequencing containing phosphorylated serine 473 of Akt-1 of human origin.
**Phospho-Akt (Thr 308)**

Phospho-Akt (Thr 308) rabbit polyclonal antibody detects endogenous levels of Akt protein only when phosphorylated at Thr 308 of rat, mouse and human. Polyclonal antibodies are produced by immunizing rabbits against short amino acid sequencing containing phosphorylated Thr 308 of Akt 2 of human origin.

**Phospho-Akt (Tyr 315/316/312)**

Phospho-Akt1/2/3 (Tyr 315/316/312) is a rabbit polyclonal antibody raised against a short amino acid sequence containing Tyr 315/316/312 phosphorylated Akt1/2/3 detects endogenous levels of Tyr 315 phosphorylated Akt1, correspondingly Tyr 316 phosphorylated Akt2 and correspondingly Tyr 312 phosphorylated Akt3 of mouse, rat and human origin.

**AS160**

AS160 (C69A7) rabbit monoclonal antibody detects endogenous levels of total AS160 protein of rat, mouse and human. Monoclonal antibody is produced by immunizing rabbits with a synthetic peptide (KHL-coupled) derived from the sequence around Ala195 of human AS160.

**Phospho-AS160 (Thr 642)**

Phospho-AS160 (Thr642) rabbit polyclonal antibody detects endogenous levels of AS160 protein only when phosphorylated at Thr642 of rat, mouse and human. Polyclonal
antibodies are produced by immunizing animals with a synthetic peptide corresponding to the sequence around Thr 640 of human AS160.

**α-actinin-4**

α-actinin-4 (B-12) is a mouse monoclonal antibody specific for an epitope mapping between amino acids 861-891 at the C-terminus of α-Actinin of human origin which detects endogenous level of α-actinin-4 protein of rat, mouse and human.

**Rab8A**

Rab 8A (P-16) is an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of Rab 8A of human origin which detects endogenous level of Rab8A protein of rat, mouse and human.

**Rab13**

Rab 13 (K-15) is an affinity purified goat polyclonal antibody raised against a peptide mapping within an internal region of Rab 13 of human origin which detects endogenous level of Rab13 protein of rat, mouse and human.

**PDK1**

PDK1 Antibody detects endogenous levels of total PDK1 protein of rat, mouse and human. Rabbit polyclonal antibodies is produced by immunizing animals with a synthetic peptide corresponding to residues surrounding the carboxy terminus of human PDK1.
**mTOR**

mTOR Antibody detects endogenous levels of total mTOR protein of rat, mouse and human. Rabbit polyclonal antibodies is produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Ser2481 of human mTOR.

**HDAC2**

HDAC2 Antibody detects endogenous levels of HDAC2 protein protein of rat, mouse and human. The antibody does not cross-react with other HDAC proteins. Rabbit polyclonal antibodies is produced by immunizing animals with a synthetic peptide corresponding to the carboxy terminus of the human HDAC2 protein.

**PTEN**

PTEN (138G6) rabbit mAb detects endogenous levels of total PTEN protein protein of rat, mouse and human. Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to the carboxy-terminal sequence of human PTEN.

**β- Arrestin-2**

**c-Src**

c-Src (N-16) rabbit polyclonal antibody detects endogenous levels c-Src protein of rat, mouse and human. Polyclonal antibodies are produced by immunizing rabbits against peptide mapping at the N-terminus of c-Src of the human origin.

**Glucose transporter 4 (GLUT4)**

GLUT4 (H-61) rabbit polyclonal antibody detects endogenous levels of total GLUT4 protein of rat, human and mouse. Polyclonal antibodies are produced by immunizing rabbits with amino acids 230-290 cytoplasmic Glut4 of human origin.

**β-actin**

β-actin antibody detects endogenous levels of total β-actin.

**Secondary antibody**

- Goat anti-Rabbit IgG-HRP conjugated secondary antibody was raised from Goat against Rabbit IgG.
- Goat anti-Mouse IgG-HRP conjugated secondary antibody raised from Goat against Mouse IgG.
- Rabbit anti-Goat IgG-HRP conjugated secondary antibody raised from Rabbit against Goat IgG.
Determination of Glucose uptake

$^{14}$C-2-deoxyglucose uptake in tissues was estimated by the method of Valverde et al. (1999) and Nevado et al. (2006).

**Principle**

The rate of glucose uptake was measured in terms of measuring the radiation emission from $^{14}$C-2-deoxyglucose in the tissue, which has the 2-hydroxyl group replaced by hydrogen, so that it cannot undergo further glycolysis or oxidation after entry in to the cell.

**Reagents**

1. **Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4)** (119 mM NaCl, 4.8 mM KCl, 1mM KH$_2$PO$_4$, 1.2mM MgSO$_4$, 1mM CaCl$_2$, 24mM NaHCO$_3$, 12 mM HEPES, 0.1% BSA, and 2mM sodium pyruvate): 695.4 mg NaCl, 35.78mg KCl, 13.6 mg KH$_2$PO$_4$, 29.6 mg MgSO$_4$, 14.7 mg CaCl$_2$, 201.6 mg NaHCO$_3$, 120.3 mg HEPES, 100mg BSA and 22mg sodium pyruvate were dissolved in 75 ml of distilled water, pH was adjusted and made upto 100 ml.

2. **$^{14}$C-2-Deoxyglucose (0.05 µCi)**: 20 µl of $^{14}$C-2-deoxyglucose (50 µCi) was mixed with 1980 µl of KRB buffer.

3. **Scintillation fluid**: 4 g of 2, 5 - Diphenyloxazole (PPO) and 400 mg of 1,4 - bis (5-phenyloxazole-2-yl) - benzene (POPOP) were dissolved in 1 liter of scintillation grade toluene, mixed well and kept in dark.

4. **1N NaOH**: 4g NaOH was dissolved in 100ml of distilled water.
**Procedure**

Skeletal muscle (fresh tissue) was dissected out and rapidly cut into pieces of 10 mg. The tissues were put into 12 well plate, containing 2 ml Krebs-Ringer bicarbonate (KRB) buffer supplemented with 8 mM glucose and incubated at 37°C for 60 min. Then, tissues were incubated for 20 min using KRB buffer supplemented with (for measurement of insulin-stimulated glucose uptake) or without (for measurement of basal glucose uptake) insulin (2µU/ml). Then, tissues were rinsed using KRB buffer and further incubated for 20 min at 37°C in 2 ml KRB buffer which contained 8 mM $^{14}$C-2-deoxyglucose (0.05 µCi). Then, tissues were removed, rapidly rinsed in isotope-free KRB buffer, solubilized with 1N NaOH. Radioactivity was counted using liquid scintillation counter. Results are expressed as counts per minute (CPM) of $^{14}$C-2-deoxyglucose uptake/ 10mg tissue.

**Determination of $^{14}$C-glucose oxidation**

$^{14}$C-glucose oxidation in tissues was estimated by the method of Johnson and Turner (1971) and Kraft and Johnson (1972).

**Principle**

The rate of glucose oxidation was measured in terms of release of $^{14}$CO$_2$ which is trapped by diethanolamine.

**Reagents**

1. Dulbecco’s Modified Eagle’s Medium + Nutrient Mixture F-12 HAM (DMEM)

   (pH 7.4) : 7.8g of DMEM/F-12 medium containing HEPES (5 mM), 600 mg of
sodium bicarbonate, 50 mg of benzathine penicillin, 50 mg of streptomycin and 25 mg of fungicide (Amphotericin B), pH was adjusted to 7.4 and the volume was finally made up to 500 ml with distilled water.

2. **14C-glucose (specific activity 310 mCi/mmol):** 20 µl of $^{14}$C-glucose was mixed with 180 µl of DMEM/F-12 (pH 7.4).

3. **Diethanolamine buffer (pH 9.8):** 10 ml of 60% diethanolamine was mixed with 4 ml of 6N HCl and 3 g of KHCO$_3$ was dissolved in this solution.

4. **1N H$_2$SO$_4$:** 1 ml of (36 N) concentrated sulphuric acid was made up to 36 ml with distilled water.

5. **Scintillation fluid:** 4g of 2, 5 - Diphenyloxazole (PPO) and 400 mg of 1,4 - bis (5-phenyloxazole-2-yl) - benzene (POPOP) were dissolved in 1 litre of scintillation grade toluene, mixed well and kept in dark.

**Procedure**

Fresh gastrocnemius muscle (10 mg) were weighed and placed in a 2ml ampoule containing 170 µl of DMEM (pH 7.4), 10 IU penicillin in 10 µl of DMEM and 0.5 µCi of $^{14}$C-glucose. Then the ampoules were aerated with a gas mixture (5% CO$_2$, and 95% air) for 30 seconds and tightly closed with rubber cork containing CO$_2$ trap. A piece of filter paper was inserted into the rubber cork and 0.1 ml of diethanolamine buffer (pH 9.5) was applied to the filter paper before closing the ampoule. This closed system with CO$_2$ trap was placed in an incubator at 37°C. CO$_2$ traps were replaced every 2h. After removing second trap, 0.1ml of 1N H$_2$SO$_4$ was added to the ampoule to halt further metabolism and release of any residual CO$_2$ from the sample. The system was again closed for 1h before
the third and final trap was removed. All the CO\textsubscript{2} traps were placed in the scintillation vials containing 10ml of scintillation fluid and counted in a Beta counter. Results are expressed as CPM of $^{14}$CO\textsubscript{2} released / 10mg tissue.

**Estimation of glycogen**

Glycogen was estimated by the method of Hassid and Abraham (1957) & Roe and Dailey (1966).

**Principle**

The tissue sample containing glycogen is digested with 30% potassium hydroxide, and then precipitated with ethanol. The precipitate is treated with anthrone reagent and glucose in the hydrolysate is determined colorimetrically as reduced sugars.

**Reagents**

1. **Potassium hydroxide (KOH) 30%**: 30 g KOH was dissolved in 100 ml of double distilled water.

2. **95% Ethanol (v/v)**: 94.905 ml of 99.9% ethanol was made up to 100 ml with 5.095 ml double distilled water.

3. **Sulphuric acid (H\textsubscript{2}SO\textsubscript{4}) 95%**: 95 ml of concentrated sulphuric acid was made up to 100 ml with 5 ml of distilled water.

4. **Anthrone reagent 0.2%**: 0.2 g anthrone was dissolved in 100 ml of 95% sulphuric acid.
5. **Standard glucose (Stock standard):** 100 mg D-glucose was dissolved in 100ml distilled water.

6. **Working standard:** 0.025, 0.05, 0.1, 0.15, 0.2ml stock solutions were made up to 5ml to get concentrations of 25, 50, 100, 150, 200μg, respectively.

**Procedure**

Five mg tissue was digested with 1ml of 30% KOH for 20 minutes in a boiling water bath. The contents were cooled in an ice bath and 1.25ml of 95% ethanol was added, thoroughly mixed and gently brought to boil in a hot water bath. This was cooled and centrifuged for 15 minutes at 750xg. The supernatant was decanted and the tubes were allowed to drain on a filter paper for few minutes. The precipitate was redissolved in 1ml of distilled water, reprecipitated with 1ml of 95% ethanol, centrifuged and drained as stated before. The precipitate was dissolved in 5ml distilled water and 10ml of 0.2% anthrone reagent was added under ice-cold conditions. 5ml of distilled water and series of standards with a final volume of 5ml were treated with anthrone reagent and subjected to the same procedure. The tubes were covered with glass marbles and heated for 10 minutes, in a boiling water bath. The contents were cooled immediately and the colour was read at 680 nm. The amount of glycogen is expressed as mg/g wet tissue.
Statistical analysis

Statistical analyses were carried out using Prism 6.00 software (GraphPad Software for Windows, La Jolla, California, USA). All the data are expressed as mean ± standard error of mean (SEM). Male and female data were analysed separately. Differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by the Duncan’s multiple-range test for multiple post hoc comparison. In all cases $p<0.05$ was considered as statistically significant.
Effect of gestational DEHP exposure on insulin binding and insulin receptor protein expression in gastrocnemius muscle of F₁ offspring

Insulin binding was significantly declined dose-dependently in gastrocnemius muscle of F₁ offspring of both genders due to gestational DEHP exposure when compared to control (Figure 10A).

InsR mRNA (Figure 10B); plasma membrane InsRβ protein (Figure 10C) and its tyrosine 1162/1163 phosphorylated forms (Figure 10D) were reduced significantly compared to control in a dose-dependent manner upon gestational exposure to DEHP in both male and female offspring.

Effect of gestational DEHP exposure on of insulin receptor substrate-1 (IRS-1) expression and cytosolic HDAC2 protein in gastrocnemius muscle of F₁ offspring

IRS-1 mRNA in the gastrocnemius muscle of rat F₁ offspring at PND60 was unaltered upon gestational exposure to DEHP treatment (Figure 11A). In contrast to mRNA, IRS-1 protein in 10 and 100mg doses of DEHP exposure showed a significant decrease. Unlike males, all the doses of DEHP have caused significant reduction in IRS-1 protein in females (Figure 11B). Cytosolic HDAC2 protein level was increased dose-dependently irrespective of gender compared to control (Figure 11C).
Effect of gestational DEHP exposure on phosphorylated IRS-1 tyrosine 632 and Serine 636/639 proteins in gastrocnemius muscle of F1 offspring

pIRS-1\textsuperscript{Tyr632} level was significantly reduced in all doses but there is no difference among treatment groups compared to control in male offspring. However, in female offspring significant decrease in 10 and 100mg doses alone were noted (Figure 12A). Unlike tyrosine phosphorylation pIRS-1\textsuperscript{Ser636/639} was significantly increased in 100mg dose in both male and female offspring while, statistical significance was not achieved in 1 and 10mg doses (Figure 12B).

Effect of gestational DEHP exposure on Akt expression and mir143 in gastrocnemius muscle of F1 offspring

Akt mRNA in the gastrocnemius muscle of F1 offspring at PND60 was reduced significantly in a dose-dependent manner upon in utero exposure to DEHP in both male and female offspring (Figure 13A) compared to control. Unlike mRNA level, Akt protein significantly decreased only in 100mg DEHP exposed group (Figure 13B). In utero DEHP exposure significantly escalated the mir143 level in a dose-dependent mode in both sexes at PND60 (Figure 13C).
Effect of gestational DEHP exposure on phosphorylated Akt serine 473, threonine 308 and tyrosine 315/316/312 proteins in gastrocnemius muscle of F₁ offspring

pAkt<sup>Ser473</sup> level was significantly reduced in all doses compared to control in male offspring. However, in female offspring there is no alteration in 1 and 10mg groups compared to control but a significant decrease at 100mg dose was recorded (Figure 14A). pAkt<sup>Thr308</sup> was significantly decreased in 10 and 100mg doses of DEHP treatment alone in both male and female offspring compared to control (Figure 14B). pAkt<sup>Tyr315/316/312</sup> was decreased in a dose-dependent manner similar to that showed the trend of Akt mRNA (Figure 14C).

Effect of gestational DEHP exposure on mTOR, PDPK1 and PTEN proteins in gastrocnemius muscle of F₁ offspring

mTOR protein level was significantly decreased dose-dependently in DEHP-exposed groups (Figure 15A). Surprisingly, no alteration was found in PDPK1 protein level (Figure 15B). PTEN protein level was significantly elevated in gastrocnemius muscle but, there is no dose-dependent difference among treatment groups compared to control (Figure 15C).
Effect of gestational DEHP exposure on β-arrestin2 and c-Src proteins in gastrocnemius muscle of F₁ offspring

Gestational exposure to DEHP significantly decreased the β-arrestin2 protein level in the gastrocnemius muscle of F₁ male and female rat offspring at PND60 in 10 and 100mg groups but, it did not get affected in 1mg treated group (Figure 16A). A significant decline in the level of c-Src protein (Figure 16B) was also observed in both gender.

Effect of gestational DEHP exposure on AS160 and phosphorylated AS160 threonine 642 protein levels in gastrocnemius muscle of F₁ offspring

Even though the effect of DEHP was not seen in AS160 protein level (Figure 17A), pAS160Thr642 level was significantly reduced in 100mg treated group but, there was no change in 1 and 10mg treated groups compared to control in female offspring (Figure 17B). Nevertheless, pAS160Thr642 level was dose-dependently decreased in gastrocnemius muscle of rat F₁ male offspring at PND60 (Figure 17B).

Effect of gestational DEHP exposure on Rab8A, Rab13 and α-actinin4 proteins in gastrocnemius muscle of F₁ offspring

Rab8A showed a marked decrease in 10 and 100mg DEHP exposed groups alone (Figure 18A). Whereas, Rab13 protein level was markedly decreased in a dose-dependent
manner compared to control (Figure 18B). Male offspring had significantly low α-actinin4 protein level in all groups compared with control but, in female offspring a significant decrease was recorded in 10 and 100mg DEHP exposed groups alone (Figure 18C).

**Effect of gestational DEHP exposure on expression of GLUT4 protein in gastrocnemius muscle of F1 offspring**

Among the glucose transporter proteins, GLUT4 is the one which is insulin responsive / insulin sensitive transporter. Both male and female rat F1 offspring showed a significant dose-dependent decline in GLUT4 mRNA compared to control group (Figure 19A). Cytosolic GLUT4 protein level (Figure 19B) also followed the same trend of mRNA. However, pGLUT4Ser488 was significantly increased in 10 and 100mg DEHP treated male and female offspring while no significant alteration was seen in 1mg group compared to control (Figure 19C). Plasma membrane GLUT4 protein level was significantly reduced in a dose-dependent manner compared to coeval control group (Figure 19D).
Effect of gestational DEHP exposure on glucose uptake, oxidation and glycogen concentration in gastrocnemius muscle of F₁ offspring

The eventual drive of insulin signalling is stimulation of glucose uptake from the circulation and subsequent oxidation at target tissues. To gain insight on the impact of developmental DEHP exposure on these processes, $^{14}$C-2-deoxy glucose uptake and $^{14}$C-glucose oxidation were studied. DEHP-exposed male and female F₁ offspring showed a significant dose-dependent decline in glucose uptake (Figure 20A) and oxidation (Figure 20B). This observation is in line with the decreased plasma membrane GLUT4. DEHP exposure caused a significant dose-dependent decline in gastrocnemius muscle glycogen concentration (Figure 20C), in both male and female rat offspring.
In response to elevated blood glucose, insulin has pleiotropic biological effect in virtually all tissues to control glucose homeostasis. In the present study, decreased serum insulin and elevated fasting blood glucose along with impaired glucose and insulin tolerance at PND60 of F₁ offspring exposed to DEHP during gestation were observed. In accordance with the present study other studies have shown 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 human. In addition, it has been shown that developmental DEHP exposure impairs glucose and insulin tolerance upto PND190 (Lin et al., 2011b).

In the current investigation, the expression of important molecules involved in skeletal muscle insulin signalling showed an alteration at PND60 due to in utero DEHP exposure. Since initial interaction between insulin and its receptor on target cell surface is followed by a series of surface and intracellular steps that participated in the control of insulin action such as glucose clearance from circulation, insulin receptor is the master switch for insulin signal transduction (Accili et al., 1996; White, 1997; Dominici and Turyn, 2002) and therefore, reduction in the InsR expression and kinase activity account for insulin-resistant phenotype (Pessin and Saltiel, 2000). In utero DEHP-exposed group showed significantly reduced level of insulin binding, InsR mRNA, plasma membrane InsR protein and its phosphorylation at Tyr1162/1163 sites which may be the result of defective transcription / translation as well as post-translational modification.
IRS proteins have multiple tyrosine phosphorylation sites and once phosphorylated they act as regulatory docking proteins that direct the insulin signalling cascade towards either metabolic or mitogenic events by binding to numerous proteins containing Src-homology-2 (SH2) domains (Baumann et al., 2000; White, 2002; Cai et al., 2003). IRS-1 appears to have its major role in skeletal muscle whereas IRS-2 appears to regulate hepatic insulin action as well as pancreatic β-cell development and survival (Sesti et al., 2001). Defects in muscle IRS-1 expression and function have been reported in insulin-resistant states such as obesity and type-2 diabetes (Sesti et al., 2001). IRS-2 knockout study in mice (IRS-2−/−) showed insulin resistance and a defect in the insulin-stimulated signalling pathway in liver but not in skeletal muscle (Kubota et al., 2000; Kubota et al., 2003). Hence, IRS 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).

IRS-1 is a major docking substrate for the InsR and other tyrosine kinases in skeletal muscle. Therefore, in the present study the level of IRS-1 was examined. Gestationally DEHP-exposed F₁ offspring showed unaltered IRS-1 mRNA but a decrease in IRS-1 protein level suggesting that the DEHP site of action may be elsewhere at the translational and / or post-translational levels. Phosphorylation of IRS-1 leads to its activation which facilitates binding sites for regulatory subunit of PI3K and activates the Akt (Shepherd et al., 1998; Withers and White, 2000; Hirsch et al., 2007). In the current investigation, Tyrosine (632) and Serine (636/639) phosphorylated IRS-1 proteins were analysed. These two sites of phosphorylation are particular considering their opposing
effects on IRS-1 activation. While the phosphorylation of IRS-1 on tyrosine residues is required to mediate insulin signalling, phosphorylation of IRS-1 on certain serine residues (636 and 639) have been shown to terminate insulin effects (Esposito et al., 2001; Bouzakri et al., 2003). Acetylation of IRS-1 is permissive for tyrosine phosphorylation and facilitates insulin-stimulated signal transduction (Kaiser and James, 2004). Interestingly, *in utero* DEHP exposure elevated the HDAC2 level and diminished IRS-1$^{\text{Tyr632}}$ phosphorylation level compared to control irrespective of gender. However, phosphorylated IRS-1$^{\text{Ser636/639}}$ which impedes binding of downstream effectors and the negative regulator (PTEN) of intracellular levels of PIP3 were increased in DEHP-exposed groups which may contribute for decreased IRS-1 tyrosine phosphorylation. Hence, changes observed in protein may be an outcome of specific changes at the level of translational / post-translational modifications. Rather, the decrease in IRS-1 protein may also be the result of increased degradation of IRS-1. Evidence suggests that Ser336/639/307 are well-recognized phosphorylation sites in IRS-1, and it can negatively influence insulin signalling via increased ubiquitin-proteasome degradation of IRS-1, reduced tyrosine phosphorylation and subsequent alteration of insulin-induced PI3-kinase activation (Bouzakri et al., 2003).

β-Arrestin-2 [a molecular scaffold protein] mediates biological functions of G-protein coupled receptor by linking activated receptors with distinct sets of accessory and effector proteins, thereby determining the specificity, efficiency and capacity of signals (Luan et al., 2009). Interaction of Akt and c-Src with InsR depends on the expression level of β-Arrestin-2 (essential for proper insulin signalling and whole-body insulin
action) (Luan et al., 2009). β-Arrestin-2 regulates insulin action by scaffolding a complex containing insulin receptor, c-Src, and Akt/PKB (Sanders, 2001). This complex allows c-Src to phosphorylate Akt on tyrosine 315/326 residues, which are required for the subsequent phosphorylation of Akt at Threonine 308 and Serine 473 by PDPK1 and mTORC2, respectively (Alessi et al., 1997; Stephens et al., 1998; Balendran et al., 1999). Accordingly, β-Arrestin-2 knockout mice are insulin resistant, and over-expression of β-Arrestin-2 in mice causes increased insulin sensitivity (Luan et al., 2009). In the present study, β-arrestin-2 and c-Src proteins were found to be decreased in gastracnemius muscle suggesting that in utero DEHP exposure has significant negative influence over β-arrestin-2 signal complex in F1 offspring. The decrease in β-arrestin-2 and c-Src proteins in the skeletal muscle is suggestive of insulin resistant state in the F1 offspring exposed to DEHP during gestation.

Akt mRNA level was down regulated in DEHP exposed groups in both the gender. Surprisingly, in utero DEHP treatment significantly decreased the level of total Akt protein and activity-dependent Ser473 phosphorylation in a highest dose only but increased the miRNA143 level in a dose-dependent manner. Further, phosphorylation of Akt at threonine 308 & tyrosine 315/316/312 residues in DEHP-exposed offspring was significantly low compared to control. Akt is fully active only when it is phosphorylated on tyrosine 315/326 residue by c-Src which is prerequisite for threonine 308 and serine 473/474 phosphorylation (Hirsch et al., 2007; Luan et al., 2009). Eventhough there is no alteration in PDPK1 protein level, the reduction in Akt phosphorylation may be due to deficiency of β-arrestin-2 signal complex, mTOR & elevated mir143 level. At this
juncture, it is worth recalling the observation of miR-143-dependent downregulation of oxysterol binding protein-related protein (ORP) 8. Reduced ORP8 expression impairs the ability of insulin to induce Akt activation by increasing the serine-threonine protein phosphatase2 (PP2A) (Jordan et al., 2011). Even though ORP8 shows little or no expression in skeletal muscle, ORP6 (Lehto et al., 2004) and full-length ORP9L variants are highly expressed in skeletal muscle (Wyles and Ridgway, 2004) and this might have contributed for the reduction in phosphorylated Akt.

AS160, Akt substrate of 160 kDa, contains a Rab GTPase-activating protein (GAP) domain (Miinea et al., 2005). Unaltered total AS160 but diminished pAS160\textsuperscript{Thr642} level in gestational DEHP-exposed F\textsubscript{1} offspring were observed in the current study, indicating that phosphorylation of AS160 is dependent on 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 target Rab proteins for vesicle trafficking (Miinea et al., 2005). Since 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).

GLUT4 exists in insulin-sensitive tissues mainly skeletal muscles and is thus the major transporter protein responsible for insulin-mediated whole-body glucose uptake (Sano et al., 2003). GLUT4 mRNA expression has been reported to be reduced in type-2 diabetic subjects due to defective transcription of GLUT4 gene and alteration in the stability of its mRNA transcript (Zaid et al., 2008). In the present study, GLUT4 mRNA
and cytosolic GLUT4 protein were downregulated in gastrocnemius muscle of DEHP-exposed F₁ offspring. Significant reduction in both GLUT4 mRNA and cytosolic GLUT4 protein is suggestive of defective expression of GLUT4 gene.

Insulin-stimulated transport of glucose into muscle and fat cells is mediated by redistribution of GLUT4 (James et al., 1988; Birnbaum, 1989) from intracellular GLUT4 storages to the plasma membrane [PM] (Bryant et al., 2002). In this study, DEHP-exposed F₁ offspring showed decreased GLUT4 level in the PM of gastrocnemius muscle and this may be attributed to its defective expression as well as translocation to the plasma membrane.

Translocation of GLUT4 is mediated through insulin signalling pathway and any abnormality in this pathway leads to insulin resistance and in turn T2DM (Watson et al., 2004). 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). Increase in phosphorylation of GLUT4 was associated with decrease in the ability of insulin to stimulate glucose uptake in adipocytes (Begum et al., 1993). In the present study, phosphorylated GLUT4^{Ser488} was increased in DEHP-exposed F₁ offspring. This may be one of the reasons responsible for the defective GLUT4 translocation towards PM.

Rab proteins are small G-proteins and important regulators of insulin-stimulated GLUT4 translocation to the PM. It coordinates with myosin-Vb to mediate the final steps of insulin-stimulated GLUT4 storage vesicles (GSV) translocation to the PM.
(Ishikura and Klip, 2008). Previous studies have indicated that Akt phosphorylation of AS160, a GAP for Rabs, is required for GLUT4 translocation (Miinea et al., 2005). Based on their presence in GLUT4 vesicles and activity as AS160 GAP substrates, Rabs 8A and 13 are candidate Rabs. Among those Rabs, only the knockdown of Rab8A or Rab13 inhibited GLUT4 translocation (Ishikura and Klip, 2008; Sun et al., 2010). Rab8A and Rab13 are under the direct control of AS160 in muscle cells (Ishikura and Klip, 2008; Sun et al., 2010). Consistent with this, gestational DEHP exposed F1 offspring showed significant decline in Rab8A and Rab13 proteins and this might have contributed for impaired translocation of GSV. Further, reduction in α-actinin4 protein in the DEHP treated group was observed in the present study. It was shown that GLUT4 was colocalized with α-actinin4. α-Actinin4 knockdown study showed prevention of GLUT4-actin colocalization and GLUT4 was localized in a tight perinuclear location (Talior-Volodarsky et al., 2008), indicating the role of α-actinin4 in contributing GLUT4 traffic which is likely by tethering GLUT4 vesicles to the cortical actin cytoskeleton.

Insulin stimulates glucose uptake via translocation of GLUT4 (Etgen et al., 1996; Larance et al., 2008). GLUT4-dependent glucose uptake and oxidation is an essential functional process, which supplies energy to the cells to execute diverse functions (Huang and Czech, 2007). The rate of glucose oxidation in a cell depends on the rate of entry of glucose into the cell. In the present study, both processes declined in a dose-dependent manner. Reduced PM GLUT4 level leads to impaired glucose uptake and subsequent
oxidation. Skeletal muscles are the tissue that transforms chemical energy to mechanical work; glycogen is the principal storage form of glucose in animal cells and it is a main substrate during high intensity exercise (Hermansen et al., 1967; Romijn et al., 1993). Skeletal muscles the major tissue where insulin stimulates glucose uptake to remove glucose from the blood, and the glucose taken up is incorporated into glycogen (DeFronzo et al., 1981; Shulman et al., 1990). The logic link between glycogen content and insulin sensitivity is also supported experimentally (Jensen et al., 1997; Jensen et al., 2011). In the current study, reduced glycogen concentration was observed in DEHP-exposed F₁ offspring. It has been previously shown that DEHP exposure alters carbohydrate metabolizing enzymes (Martinelli et al., 2006). In diabetic condition, a major defect is that insulin-stimulated glucose uptake and glycogen synthesis is impaired in skeletal muscle (Shulman et al., 1990). The rate at which glucose is taken up from the blood by the skeletal muscle is the major determinant of insulin sensitivity (Hojlund and Beck-Nielsen, 2006; Jensen et al., 2011).

Most of the parameters studied showed similar trend in both sexes. However, in few parameters (IRS-1, IRS-1\textsuperscript{Tyr632}, Akt\textsuperscript{Ser473} and AS160\textsuperscript{Thr642}) though the protein levels and its phosphorylation were decreased, the dose-dependent reduction was not similar in both sexes.
INFERENCE

Gestational DEHP exposure predisposes F$_1$ offspring to glucometabolic dysfunction at adulthood by down regulating the expression of insulin signalling molecules in the gastrocnemius muscle.