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Phthalate is Associated With Insulin Resistance in Adipose Tissue of Male Rat: Role of Antioxidant Vitamins

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ABSTRACT

Diethyl hexyl phthalate (DEHP) is a plasticizer, commonly used in a variety of products, including lubricants, perfumes, hairsprays and cosmetics, construction materials, wood finishers, adhesives, floorings and paints. DEHP is an endocrine disruptor and it has a continuum of influence on various organ systems in human beings and experimental animals. However, specific effects of DEHP on insulin signaling in adipose tissue are not known. Adult male albino rats of Wistar strain were divided into four groups. Control, DEHP treated (dissolved in olive oil at a dose of 10, and 100 mg/kg body weight, respectively, once daily through gastric intubations for 30 days) and DEHP + vitamin E (50 mg/kg body weight) and C (100 mg/kg body weight) dissolved in olive oil and distilled water, respectively, once daily through gastric intubations for 30 days. After the completion of treatment, adipose tissue was dissected out to assess various parameters. DEHP treatment escalated H_2O_2 and hydroxyl radical levels as well as lipid peroxidation in the adipose tissue. DEHP impaired the expression of insulin signaling molecules and their phosphorelay pathways leading to diminish plasma membrane GLUT4 level and thus decreased glucose uptake and oxidation. Blood glucose level was elevated as a result of these changes. Supplementation of vitamins (C & E) prevented the DEHP-induced changes. It is concluded that DEHP-induced ROS and lipid peroxidation disrupts the insulin signal transduction in adipose tissue and favors glucose intolerance. Antioxidant vitamins have a protective role against the adverse effect of DEHP. J. Cell. Biochem. 114: 558–569, 2013.

KEY WORDS: DIETHYL HEXYL PHTHALATE (DEHP); ANTIOXIDANT VITAMINS; INSULIN SIGNAL TRANSDUCTION; ADIPOSE TISSUE

Phthalates are used in a variety of products, including lubricants and cosmetics. DEHP is an endocrine disruptor, (a benzene dicarboxylic acid ester) which is typically added to building materials and medical devices made from polyvinyl chloride (PVC) to increase flexibility. DEHP is used as a plasticizer in medical devices such as storage containers, bags and tubing; it can leach from these devices into infusate (e.g., pharmaceuticals, blood, blood products, parenteral nutrition solutions) [Shea, 2003]. DEHP may represent between 20%, and 40% of the finished weight of plastics [Jaeger and Rubin, 1973]. The potential for human exposure by oral, dermal inhalation, and intravenous means is high. The general population is exposed to DEHP through food, water, and air by inhalation, and ingestion [Meek and Chan, 1994].

Adipose tissue is a complex, essential and highly active metabolic and endocrine organ. It is essential for normal glucose homeostasis and a role in inflammatory processes has been proposed [Trayhurn and Beattie, 2001]. Several evidences support the hypothesis that the adipose tissue is the primary site where the early metabolic disturbances leading to the development of insulin resistance occurs: (1) The high correlation between disturbances in normal metabolism of adipose tissue (obesity, lipodystrophy) and the manifestation of insulin resistance indicates the importance of

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adipose tissue in its etiology [Kahn and Flier, 2000]. (2) Mice with fat-specific disruption of GLUT4 gene show impaired glucose tolerance [Abel et al., 2001], indicating the vital role of the adipose tissue for whole-body glucose disposal. (3) Insulin resistance, and impaired insulin action occur early in adipose tissue; long before glucose intolerance develops [Smith, 2002].

Vitamin C (ascorbic acid) is an essential micronutrient required for normal metabolic functioning of the body and it is a well-known antioxidant. Vitamin C may help to prevent the oxidative damage to organs such as the eyes, brain, and kidneys that frequently occur in type-II diabetics. Supplementation of antioxidant vitamins significantly accelerated the regeneration of injured seminiferous epithelium in DEHP treated animals, suggesting the therapeutic effect of vitamins on DEHP-induced aspermatogenesis [Ablake et al., 2004]. Vitamin E is the generic term for a group of compounds known as tocopherols, and tocotrinols. The major function of vitamin E is its antioxidative property. It is the main chain-breaking antioxidant present in biological membranes. It was reported that administration of vitamin E prevented DEHP-induced deleterious effects like degenerative changes in the brain, and thyroid and decrease in the level of serum insulin [Dhanya et al., 2004]. In vitro studies have shown that vitamin E increased activity of superoxide dismutase (SOD), catalase activities, malondialdehde (MDA) and conjugated dienes. DEHP treated rats show decreased concentration of glutathione, and vitamin E in the liver tissue [Conway et al., 1989].

The DEHP-fed rats had an altered glucose tolerance associated with abnormal glucose intermediate metabolites in liver, and skeletal muscle. Reduction in muscle glucose & lactate transport, hexokinase & hepatic glucokinase activities, and glycogen synthesis was also recorded in DEHP-fed rats [Martinelli et al., 2006]. Administration of DEHP to adult male rats interfered with carbohydrate metabolism by reducing the blood-glucose utilization, hepatic glycogenesis, and glycogenolysis [Mushtaq et al., 1980]. Stahlhut et al. [2007] reported that the elevated concentration of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult males. DEHP exposure has also been shown to reduce the circulating hormone levels such as insulin and testosterone and increase in estrogen level in males [Gayathri et al., 2004]. The importance of optimal level of insulin and sex steroids in the regulation of glucose homeostasis is well recognized [Livingstone and Collison, 2002]. The DEHP-induced insulin deficiency and decreases in testosterone (T)/estrogen (E) ratio are suggestive of the diabetogenic effects of DEHP.

Although, many polyvinylchloride (PVC) based endocrine disruptors including DEHP may exert effects on endocrine development and function, few studies have addressed their toxic effects on insulin resistance and their possible correlation with metabolic disorders. We have shown previously that DEHP exposure to Chang liver cells leads to dose-dependent decrease in insulin receptor and glucose oxidation. However, effect of DEHP on insulin signaling molecules has received only little attention and it is attested by the limited reports available on this aspect. The aim of the present study was to evaluate the effects of DEHP on insulin-signaling molecules, ROS generation, and lipid peroxidiation in adipose tissue as well as the functional aspect of glucose uptake and oxidation. Additionally, we have evaluated the protective role of antioxidant vitamins (C & E) against DEHP-induced toxicity.

MATERIALS AND METHODS

MATERIALS

All chemicals and reagents used in the present study were of molecular and analytical grade; and they were purchased from Sigma Chemical Company; Amersham Biosciences (UK); and Sisco Research Laboratories (Mumbai, India). Total RNA isolation reagent (TRIR) and one-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit were purchased from ABgene (UK) and Qiagen (Germany), respectively. The insulin receptor, insulin receptor substrate (IRS-1), GLUT4, RPL-19, β-actin primers and the β-actin monoclonal antibody were purchased from Sigma Chemical Company, Polyclonal insulin receptor β-subunit, IRS-1, p-IRS-$1^{Ser636/639}$, p-IRS-1^{Tyr632}, Akt and p-Akt^{Ser473}, β-arrestin-2, p-$GLUT4^{ser488}$, GLUT4 and SREBP-1c antibodies were purchased from Santa Cruz Biotechnology, Inc. AS160 monoclonal antibody was purchased from Cell Signaling Technology, Inc. ^{14}C-glucose and ^{14}C-2-deoxyglucose were purchased from the Board of Radiation and Isotope Technology (Mumbai, India). Glucose estimation kit was supplied by Linear Chemicals (Barcelona, Spain).

ANIMALS AND TREATMENT WITH DIETHYL HEXYL PHTHALATE

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethical Committee (IAEC No. 03/030/07). Healthy adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180–200 g (100 days old) were used in the present study. Animals were housed in polypropylene cages under specific humidity (65 ± 5%) and temperature (21 ± 2°C) with constant 12 h light, and 12 h dark schedule. They were fed with standard rat pelleted diet (Lipton India, Mumbai, India), and clean drinking water was made available ad libitum. Rats were divided into four groups each consisting of six animals.

Group I: Control (vehicle treated).
Group II: DEHP treated (dissolved in olive oil at a dose of 10 mg/kg body weight, daily at 10 AM through gastric intubation for 30 days).
Group III: DEHP treated (100 mg/kg body weight, daily at 10 AM through gastric intubations for 30 days).
Group IV: DEHP and vitamin E (dissolved in olive oil at a dose of 50 mg/kg body weight), and vitamin C treated (100 mg/kg body weight dissolved in distilled water daily at 10 AM through gastric intubation for 30 days).

At the end of treatment, animals were anesthetized with sodium thiopental (5 mg/kg, i.p), and 20 ml of normal saline was perfuse through the left ventricle, to clear blood from the liver, and other organs. Visceral adipose tissue was dissected out and used for the assay of various parameters.

PLASMA GLUCOSE

Blood samples were also collected after overnight fasting in microfuge tubes containing EDTA by puncturing the orbital sinus

with the help of heparinized microhematocrit capillary tubes. Plasma was separated from blood by centrifugation for 10 min at 800*g* at 4°C within 30 min to prevent autoglycolysis by leukocytes. Plasma glucose was estimated by glucose oxidase–peroxidase method (CPC diagnostics, Spain). The coefficient of variations was 1.8%. Results are expressed as mg/dl.

SEMI-QUANTITATIVE RT-PCR

Total RNA was isolated from control and experimental samples, concentration and purity of RNA were determined spectrophotometrically at $A_{260/280}$ nm. The purity of RNA obtained was 1.8–1.9. The yield of RNA is expressed in microgram (μg). Total RNA (2 μg) extracted from adipose tissue of control and experimental animals were reverse-transcribed in a reaction volume of 20 μl using 1 μM Oligo-dT primer, 0.5 μM dNTPs, 10 U ribonuclease inhibitor and 4 U Omniscript reverse transcriptase (Qiagen, Hilden). The reaction was carried out in an eppendorf autorisierter (Hamburg, Germany) thermocycler (37°C for 60 min). The resulting cDNAs were stored at −20°C until used for RT-PCR. The rat-specific primer sequences used in this study for RT-PCR are listed in Table I. Briefly 10 μl of each RT-PCR products was analyzed by gel electrophoresis on a 2% agarose gel. The molecular size of the amplified products (GLUT4, IR, IRS-1, β-actin, and RPL-19) were determined by comparison with molecular weight marker (100 bp DNA ladder) run in parallel with RT-PCR products. Then the gels were subjected to densitometric scanning (Bio Rad) to find out the OD units of each band and then normalized against that of internal control (β-actin or RPL19).

IMMUNOBLOT ANALYSIS

Isolation of plasma membrane and cytosolic fractions. Plasma membrane and cytosolic fractions from adipose tissue of control and experimental animals were prepared as described previously [Dombrowski et al., 1996]. Briefly, tissues were homogenized in buffer A containing 10 mM $NaHCO_3$ (pH 7.0), 250 mM sucrose, 5 mM NaN_3, protease inhibitor cocktail and 100 μM PMSF using a polytron equipped homogenizer at a precise low setting on ice. The resulting homogenate was clarified at 1,300*g* for 10 min at 4°C. The resultant supernatant was centrifuged at 20,000*g* for 30 min at 4°C. The supernatant was used for cytosolic fraction and pellet was resuspended in buffer A, and applied on discontinuous sucrose gradients (25%, 32%, and 35%, w/w), and centrifuged at 150,000*g* for 16 h at 4°C. Plasma membrane at the 25–32% interface were recovered, diluted with sucrose-free buffer A and centrifuged at 190,000*g* for 1 h at 4°C. Pellets were resuspended in buffer A, and protein concentration was estimated using BSA as a standard. GLUT4 and insulin receptor protein levels were measured in both plasma membrane and cytosolic fractions and translocation was evaluated by the difference in protein levels in cytosol and membrane fractions.

NUCLEAR LYSATE PREPARATION FOR MATURE SREBP-1c DETECTION

Adipose tissue were homogenized for 15–30 s using a homogenizer in 1.5 ml homogenization buffer [20 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 0.25 M sucrose, 10 mM EDTA, 10 mM EGTA, proteases (1 M dithiothreitol and 0.1 M PMSF), and 1× protease inhibitor cocktail (5 mM leupeptin, 5 mM pepstatin, 5 mM chmostatin, and 5 mM aprotinin)]. The homogenates were kept on ice for 10 min and spun down for 5 min at 2,500*g* at 4°C. The pellet was resuspended in 1 ml homogenization buffer including protease inhibitor cocktail and nuclei were spun down at 1,000*g* for 5 min at 4°C. The pellet was resuspended with 300 μl buffer C [20 mM Hepes (pH 7.6), 2.5% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail] [Im et al., 2006].

PREPARATION OF TISSUE LYSATE

Adipose tissues were homogenized in buffer containing 20 mM Tris–HCl (pH 7.8), 300 mM NaCl, 2 mM ethylene diamine tetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 2% NP-40, 0.2% SDS, 0.2% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 25 mM sodium pyrophosphate, 40 mM β-glycerophosphate, 2 mM Na_3Vo_4 and protease inhibitor cocktail (Sigma–Aldrich, India) using a polytron equipped homogenizer at a precise low setting on ice. The homogenate was centrifuged at 1,300*g* for 10 min at 4°C. The supernatant was centrifuged at 12,000*g* for 15 min at 4°C. The resultant supernatant was sampled as a total protein for IRS-1, phosphorylated IRS-1, Akt, phosphorylated Akt, AS160 -arrestin-2 and then the protein concentration was estimated using BSA as a standard. Briefly, each sample (25 μg) was subjected to heat denaturation at 96°C for 5 min with Laemmli buffer. The proteins were resolved by SDS–PAGE on 10% polyacrylamide gels and then transferred to PVDF membrane

TABLE I. List of Primer Sequences Used in the Study

Genes	Sense Primer, Anti-sense primer	Amplicon size (bp)	Accession no.
Glucose transporter 4 (GLUT4)	5′-GGG CTG TGA GTG AGT GCT TTC–3′ 5′-CAG CGA GGC AAG GCT AGA–3′	150	NM_012751.1
Insulin receptor (IR)	5′-GCC ATC CCG AAA GCG AAG ATC-3′ 5′-TCT GGG TCC TGA TTG CAT-3′	224	NM_017071.2
Insulin receptor substrate (IRS-1)	5′-GCC AAT CTT CAT CCA GTT GCT -3′ 5′-CAT CGT GAA GAA GGC ATA GGG-3′	336	NM_012969.1
β-actin	5′-GCC ATG TAC GTA GCC ATC CA-3′ 5′-GAA CCG CTC ATT GCC GAT AG-3′	374	NM_031144.2
60S ribosomal protein L19 (RPL-19)	5′-CTG AAG GTC AAA GGG AAT GTG-3′ 5′-GGA CAG AGT CTT GAT GAT CTC-3′	194	XM_001061918.2

(Amersham Biosciences). The membrane was treated with blocking buffer containing 5% blocking reagent (Amersham Biosciences) in TBS-T for 1 h at room temperature followed by incubation with primary antibody to insulin receptor /IRS-1/p-IRS-1$^{Ser636/639}$/ p-IRS-1$^{Tyr\ 632}$/Akt/ phospho Akt^{Ser473}/AS160/β-arrestin-2/GLUT4/ p-GLUT4^{Ser488} at a dilution of 1:1,000 in TBS-T at room temperature for 1 h. The membrane was washed in three times with TBS-T and then incubated for 1 h in horseradish peroxidase (HRP)-conjugated mouse/rabbit secondary antibody, which was diluted 1:7,500 with TBS-T. The membrane was washed in three times with TBS-T and targeted protein was detected using Enhanced Chemiluminescence Reagents (ECL; Amersham Biosciences). The protein bands were captured using Chemidoc and quantified by Quantity one image analysis system (Bio Rad). Later, the membranes were incubated in stripping buffer (50 ml containing 62.5 mM Tris–HCl 62.5 mM (pH 6.8), 1 g SDS, and 0.34 ml β-mercaptoethanol) at 55°C for 40 min. Following this, re-probed the membrane using a β-actin antibody (1:2,000). As the invariant control, the present study used rat β-actin.

GLUCOSE UPTAKE

^{14}C-2-deoxyglucose uptakes were estimated by the method as described previously [Valverde et al., 1999]. Briefly, after control and experimental rats were anesthetized, adipose 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 (119 mM NaCl, 4.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 24 mM $NaHCO_3$, 12 mM HEPES, 0.1% BSA, and 2 mM sodium pyruvate) 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 (2 μU/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). Plates were supplied continuously with 95% O_2–5%CO_2 throughout the experiment and insulin was present during the wash and for measuring insulin-stimulated glucose uptake. Then, tissues were removed, rapidly rinsed in isotope-free KRB buffer, solubilized with 1 N NaOH. Radioactivity was counted using liquid scintillation counter. Results are expressed as CPM of ^{14}C-2-deoxyglucose uptake/10 mg tissues.

DETERMINATION OF ^{14}C-GLUCOSE OXIDATION

^{14}C-Glucose oxidation was estimated as per the standard method [Kraft and Johnson, 1972]. Briefly, 10 mg adipose tissue was weighed and placed in a 2 ml ampoule containing 170 μl DMEM (Dulbecco's modified Eagle's medium, pH 7.4), 10 IU penicillin in 10 μl DMEM, and 0.5 μCi ^{14}C-glucose. Then, the ampoules were aerated with a gas mixture (5% CO_2, 95% air) for 30 s and tightly covered with rubber cork containing CO_2 trap (a piece of filter paper was inserted into the rubber cork and 0.1 ml of diethanolamine 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 trap was replaced every 2 h. After removing the second trap, 0.01 ml of 1N H_2SO_4 was added to halt further metabolism and release of any residual CO_2 from the sample. The system was again closed for 1 h before the third and final trap is removed. All the CO_2 traps were placed in the scintillation vials containing 10 ml of scintillation fluid and counted in a Beta counter. Results are expressed as CPM of $^{14}CO_2$ released/mg tissue.

ESTIMATION OF GLYCOGEN

Glycogen was estimated by the method, which was previously described [Hasid and Abraham, 1957]. Five milligrams of adipose tissue were digested with 1 ml of 30% KOH for 20 min in a boiling water bath. The contents were cooled in an ice bath and 1.25 ml of 95% ethanol was added, thoroughly mixed and gently brought to boil in a hot water bath. This was cooled and centrifuged for 15 min at 3,000 rpm. The supernatant was decanted and the tubes were allowed to drain on a filter paper for few minutes. The precipitate was redissolved in 1 ml of distilled water, reprecipitated with 1 ml of 95% ethanol, centrifuged and drained as stated before. The precipitate was dissolved in 5 ml of distilled water and 10 ml of 0.2% anthrone reagent was added under ice-cold conditions. Five milliliters of distilled water and series of standards with a final volume of 5 ml were treated with anthrone reagent and subjected to the same procedure. The tubes were covered with glass marbles and heated for 10 min, in a boiling water bath. The contents were cooled immediately and the color was read at 680 nm. The amount of glycogen is expressed as mg/g wet tissue.

OXIDATIVE STRESS ASSESSMENT

Lipid peroxidation (LPO) was measured by the previous method [Devasagayam and Tarachand, 1987]. The melanodialdehyde (MDA) content of the sample is expressed as nmoles of MDA formed/min/mg protein. Hydrogen peroxide generation was assessed by the spectrophotometric method [Pick and Keisari, 1981]. The H_2O_2 content of the sample is expressed as μmol/min/mg protein. Hydroxyl radical ($OH^{\bullet -}$) production was quantified by the earlier method [Puntarulo and Cederbaum, 1988]. The hydroxyl radical content is expressed as μmol/min/mg protein.

STATISTICAL ANALYSIS

All the data are expressed as mean ± standard error of mean (SEM). Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student Newman–Keul's test for multiple post hoc comparison tests. The alpha level for the analysis was set at $P < 0.05$. All analyses were performed using computer-based software (SPSS 7.5 for windows Student version).

RESULTS

ANTIOXIDANT VITAMINS (C & E) PREVENTED INCREASES IN $OH^{\cdot}$, H_2O_2 AND MDA LEVELS IN ADIPOSE TISSUE OF DEHP TREATED RATS

Oxidative stress plays a major role in the development of insulin resistance. The hydroxyl radical, hydrogen peroxide and lipid per oxidation status in the adipose tissue are shown in Figure 1A–C. A significant increase in $OH^{\bullet}$, H_2O_2, and MDA was observed due to

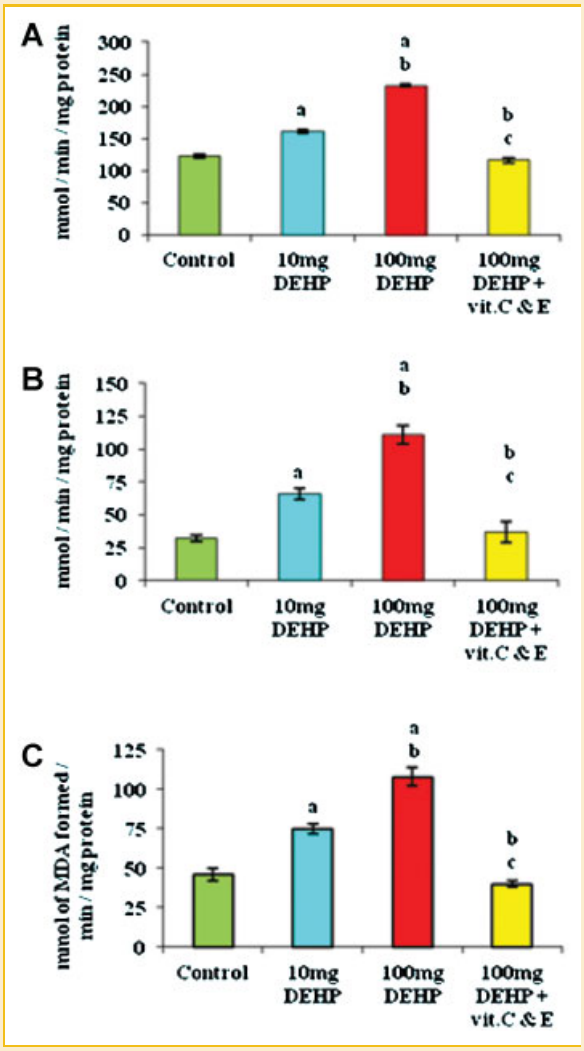

Fig. 1. Effect of di-2-ethylhexyl phthalate (DEHP) and vitamins (C & E) supplementation on H_2O_2 generation (A), Hydroxyl radical production (B), and Lipid peroxidation (C) in the adipose tissue of adult male rat. H_2O_2, hydroxyl radical and lipid peroxidation levels were assessed by spectrophotometric method. Each bar represents mean ± SEM of six animals. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

DEHP treatment (10 mg dose) and it was further increased in 100 mg DEHP treated group. In rats treated with antioxidant vitamins (C and E) along with 100 mg DEHP, their levels were maintained at control level.

FASTING BLOOD GLUCOSE AND GLYCOGEN CONTENT WERE AFFECTED BY DEHP

The dose-dependent effect of DEHP was reflected in fasting blood glucose. It was unaltered in 10 mg DEHP treated rats whereas, 100 mg DEHP treatment showed a significant increase in fasting blood-glucose level. Vitamin supplementation maintained the fasting blood-glucose level at par with control rats (Fig. 2A). DEHP treatment caused a dose-dependent significant decrease in glycogen level in adipose tissue (Fig. 2B). Conversely, vitamins supplementation increased the glycogen, compared to the control group ($P < 0.05$).

GLUCOSE UPTAKE AND OXIDATION IN ADIPOSE TISSUE WAS AFFECTED BY DEHP

Figure 2CD represents the dose-dependent effect of DEHP and vitamin (C & E) supplementation on ^{14}C-2-deoxyglucose uptake and ^{14}C-glucose oxidation in the adipose tissue of adult male rat; 10 mg DEHP treatment have shown a significant decrease in glucose uptake and oxidation in the adipose tissue and it was further decreased in 100 mg DEHP treated group. Vitamins (C & E) supplementation significantly increased the glucose uptake when compared with control. But in the case of glucose oxidation antioxidant vitamins was able to prevent diminution only partially.

ANTIOXIDANT VITAMINS PREVENTED THE DECREASE IN IR GENE EXPRESSION IN DEHP-TREATED RATS

Insulin receptor (IR) mRNA expression in the adipose tissue of adult male rat (Fig. 3A) registered a significant decrease at 10, and 100 mg doses of DEHP treatment whereas; vitamins (C & E) supplementation elevated IR mRNA expression more than that of control group. Plasma membrane IR protein in the adipose tissue was reduced significantly upon DEHP treatment (Fig. 3B). Antioxidant vitamins (C & E) supplementation group was able to prevent diminution only partially.

ANTIOXIDANT VITAMINS (C & E) PARTIALLY PREVENTED THE DEHP-INDUCED INHIBITION OF IRS-1 GENE EXPRESSION AND PHOSPHORYLATION

DEHP treatment significantly decreased the IRS-1 mRNA levels at 10, and 100 mg groups but the effects did not reach statistical significance within these groups whereas, vitamins have no protective role (Fig. 4A). DEHP treatment evoked a significant decrease in IRS-1 protein expression in adipose tissue (Fig. 4B) when compared to control. However, supplementation of vitamins (C & E) failed to maintain the IRS-1 protein level at par with control although, it was significantly higher than that of 100 mg treated group ($P < 0.05$). Figure 4C shows the dose-dependent effect of DEHP and vitamins (C & E) supplementation on pIRS-1$^{Ser\ 636/639}$ proteins level in the adipose tissue of adult male rat; 10 mg DEHP showed significant decrease in pIRS-1$^{Ser\ 636/639}$ protein level when compared with control but 100 mg DEHP treatment increased serine phosphorylation more than that of control rats. Supplementation of vitamins did not have any effect. DEHP treatment impaired the phosphorylation of IRS-1 at tyrosine 632 residues (Fig. 4D) at 10 and 100 mg doses indicating the dose-depended influence and simultaneous supplementation of antioxidant vitamins partially restored the level compared to control group.

EFFECT OF DEHP ON β-ARRESTIN2 PROTEIN

Figure 5A depicts the effect of DEHP and vitamins (C & E) supplementation on β-Arrestin2 protein. A significant decrease in the β-arrestin2 protein expression was seen only in 100 mg DEHP treated rats when compared to control. Supplementation of antioxidant vitamins (C & E) had no effect on the same.

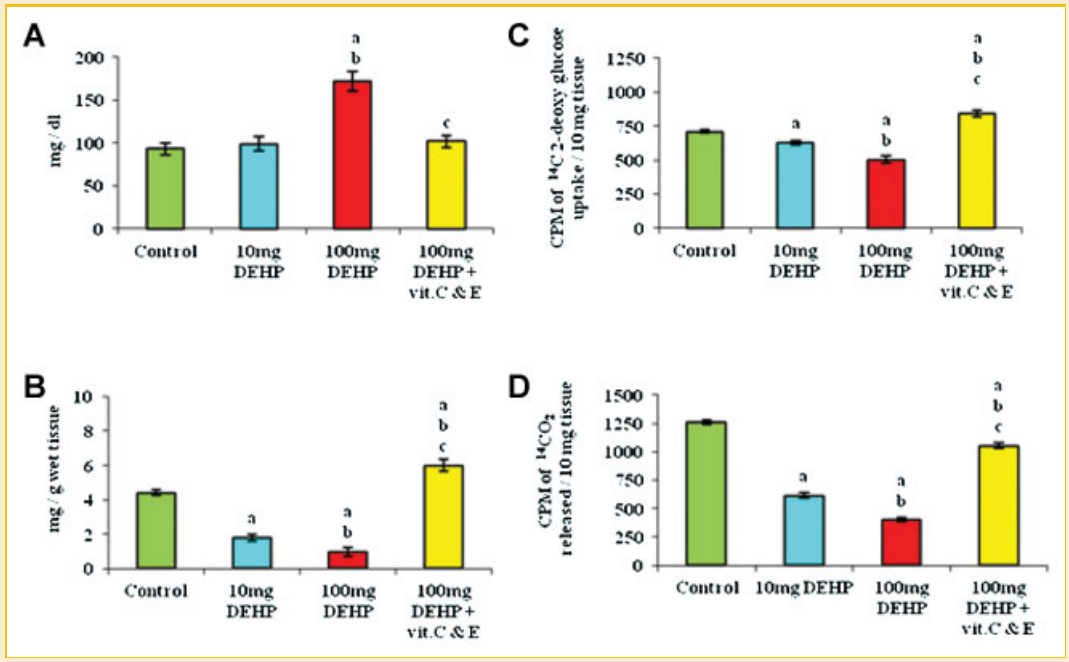

Fig. 2. Effect of di-2-ethylhexyl phthalate (DEHP) and vitamins (C & E) supplementation on fasting blood-glucose level (A), glycogen content (B), ^{14}C-2-deoxy glucose uptake (C), and ^{14}C-glucose oxidation (D) of adult male rat. Plasma glucose was estimated by glucose oxidase–peroxidase method and the coefficient of variation was checked. After overnight fasting, blood glucose was checked. Glycogen was estimated by anthrone method. Glucose uptake in adipose tissue was estimated by the ^{14}C-2-deoxy glucose uptake assay. Glucose oxidation was assessed using ^{14}C-glucose. Each bar represents mean ± SEM of six animals. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

DEHP HAS NO EFFECT ON AKT PROTEIN, BUT ALTERS ITS SERINE 473 PHOSPHORYLATION

The expression of Akt protein was unaltered in all the groups (Fig. 5B). DEHP treatment evoked a significant decrease in pAkt^{Ser473} in both 10, and 100 mg doses. Vitamins supplementation maintained the serine 473 phosphorylation of Akt at par with control group (Fig. 5C).

ANTIOXIDANT VITAMINS HAVE NO ROLE IN AS160 PROTEIN EXPRESSION OF DEHP TREATED RATS

DEHP treatment (10 mg dose) showed a significant decrease in AS160 protein level in the adipose tissue and it was further diminished in 100 mg DEHP treated group (Fig. 6). However, antioxidant vitamins supplementation was not able to prevent the diminution.

EFFECT OF DEHP ON TRANSCRIPTION FACTOR SREBP-1c

The mature transcription factor SREBP-1c level in nucleus is shown in Figure 7. A significant decrease in SREBP-1c was observed in the DEHP treated groups. In rats treated with antioxidant vitamins (C & E) along with 100 mg DEHP, the decrease in SREBP-1c was prevented partially.

EXPRESSION OF GLUCOSE TRANSPORTER4

Among the various isoforms of glucose transporter proteins GLUT4 is the one, which is insulin sensitive/insulin responsive transporter. The dose-dependent effect of DEHP and vitamin (C & E) supplementation on glucose transporter4 (GLUT4) mRNA expression in the adipose tissue of adult male rat is represented in Figure 8A. DEHP treatment (10 mg dose) significantly increased GLUT4 mRNA but 100 mg dose of DEHP did not have any effect on GLUT4 mRNA expression in adipose tissue. In rats treated with antioxidant vitamins (C & E) plus 100 mg DEHP, GLUT4 mRNA expression was maintained at control level. Figure 8B,C depicts the effect of DEHP and vitamins (C & E) supplementation on GLUT4 protein in the cytosolic fraction and plasma membrane. Both 10 and 100 mg DEHP treatment caused a significant decrease in cytosolic and plasma membrane GLUT4 content. However, rats treated with antioxidant vitamins (C & E) along with 100 mg DEHP maintained the same as coeval control in cytosol alone. GLUT4 protein level in plasma membrane was not fully maintained at control level in vitamins supplemented group. In addition, we investigated the phosphorylation of GLUT4 at serine 488. It was statistically increased in a dose-dependent manner (Fig. 8D). Vitamins supplementation partially prevented serine 488 phosphorylation of GLUT4 when compared to 100 mg DEHP. Nevertheless, vitamins have partial beneficial role on GLUT4 gene expression.

DISCUSSION

In the present study, DEHP treatment significantly increased the generation of hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and lipid peroxidation (LPO). It is well established that DEHP can induce ROS generation and lipid peroxidation [Rusyn et al., 2001; Palleschi

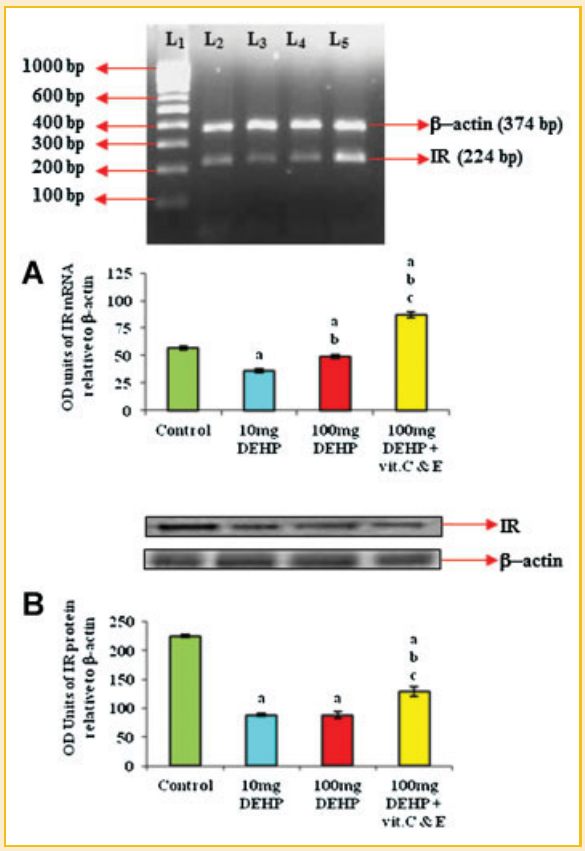

Fig. 3. Effect of antioxidant vitamins (C & E) against di-2-ethylhexyl phthalate (DEHP)-induced changes in the insulin receptor (IR) mRNA (A) and protein (B) levels in the adipose tissue of adult male rat. The mRNA expression was analyzed by isolating total RNA using total RNA isolation reagent (TRIR) and converting into complementary DNA (cDNA) using Reverse Transcriptase Polymerase Chain Reaction, identified on Agarose Gel Electrophoresis (AGE) and quantified by densitometric scanning (Bio Rad). Lane 1: 100 bp marker; Lane 2: Control; Lane 3: 10 mg DEHP; Lane 4: 100 mg DEHP; Lane 5: 100 mg DEHP + vitamins (C & E). IR protein was analyzed using Western Blot. Each bar represents mean ± SEM of three observations. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

et al., 2009]. Rats exposed to DEHP in diet showed a significant increase in the peroxisomal fatty acyl-CoA oxidation in liver [Osumi and Hashimoto, 1978]. The peroxisomal β-oxidation appears to be independent of the electron transport chain resulting in production of H_2O_2 by direct transfer of electrons to O_2. Any increased production of H_2O_2 would lead to formation of highly reactive oxygen species (ROS) and $OH^{\bullet}$ free radicals [Chance et al., 1979]. Phthalates also induce free radical production in vivo by activating NADPH oxidase complex, which generates superoxide anion [Rusyn et al., 2001]. ROS can also participate in diabetogenesis and development of late diabetic complications [Baynes, 1991]. Antioxidant vitamins (C & E) quench the free radicals produced by DEHP. In this regard, Dhanya et al. [2004] reported that simultaneous administration of vitamin E with DEHP significantly reduces the ROS-induced degenerative changes in the brain and thyroid gland. Vitamin E improves the free radical defense system potential and insulin sensitivity of rats fed with high fructose diet [Faure et al., 1997]. Ascorbic acid and alpha tocopherol may have a dual mode of action. They block the immediate rise in peroxides and the subsequent lipid peroxidation, and they may suppress the DEHP-induced activation of oxidative stress induced transcription factors such as NF-κB, and AP-1. It has been recently reported that NF-κB, and AP-1 implicated in the inducible expression of a variety of genes involved in response to oxidative stress and cellular defense mechanisms. Vitamins C & E inhibit the activation of NF-kB by multiple stimuli, including IL-1, and TNF. Vitamin C is in a unique position to scavenge aqueous peroxyl radicals before these destructive substances have a chance to damage lipids. It works along with vitamin E, a fat-soluble antioxidant and the enzyme glutathione peroxidase to stop free radical chain reactions. The decreased levels of hydrogen peroxide, hydroxyl free radicals and lipid peroxidation in vitamins (C & E) supplemented rats suggest the protective role of these vitamins against the toxic effects of DEHP.

Insulin in response to an elevation in blood glucose plays a pivotal role in the control of glucose homeostasis. Though, the circulating level of insulin was not measured in the present study, the available report indicates that DEHP treatment decreases serum insulin level in rat [Gayathri et al., 2004]. In the present study, elevated fasting blood glucose was observed. The protective effect of vitamins (C & E) supplementation was seen in DEHP + vitamins treated rats. In this respect, enhanced glycogenolysis may also be responsible for the elevated blood glucose, as DEHP has been shown to reduce hepatic glycogen concentration. Additionally, DEHP caused a significant decrease in glycogen concentration, which corroborates the above findings [Martinelli et al., 2006]. Administration of DEHP to adult male rats interfered with carbohydrate metabolism by reducing the blood glucose utilization and hepatic glycogenesis and glycogenolysis [Mushtaq et al., 1980]. The protective effect of vitamins (C & E) against glycogen concentration was observed. To identify the molecular mechanism behind the elevated fasting blood-glucose and decline in the glycogen content, expression of insulin signaling molecules were assessed.

DEHP treatment significantly decreased insulin receptor mRNA expression suggesting that DEHP down regulate the transcription of the gene. DEHP has reduced the insulin receptor protein in adipose tissue suggesting its adverse effects. In support of this, previous studies from our laboratory showed that DEHP lowered insulin receptor concentration in cultured Chang liver cells [Rengarajan et al., 2007]. Reduction in the plasma membrane insulin receptor protein may be due to a defect at the level of translation or translocation of the protein as a result of DEHP treatment whereas simultaneous vitamins (C & E) supplementation has partially prevented the IR protein down regulation.

IRS-1 (Insulin receptor substrate-1) is a perfect docking scaffold protein of IR. In the current study, expression of IRS-1 mRNA was significantly increased in DEHP-treated rats when compared to control group. Vitamins supplementation has no effect on IRS-1 mRNA. DEHP-mediated diminution in insulin receptor protein is associated with decreased IRS-1 protein. IRS-1 has been reported to be degraded by ROS [Taniyama et al., 2005]. DEHP-induced ROS production might have degraded the IRS-1 in adipose tissue leading to reduce the IRS-1 protein content. These data imply that DEHP

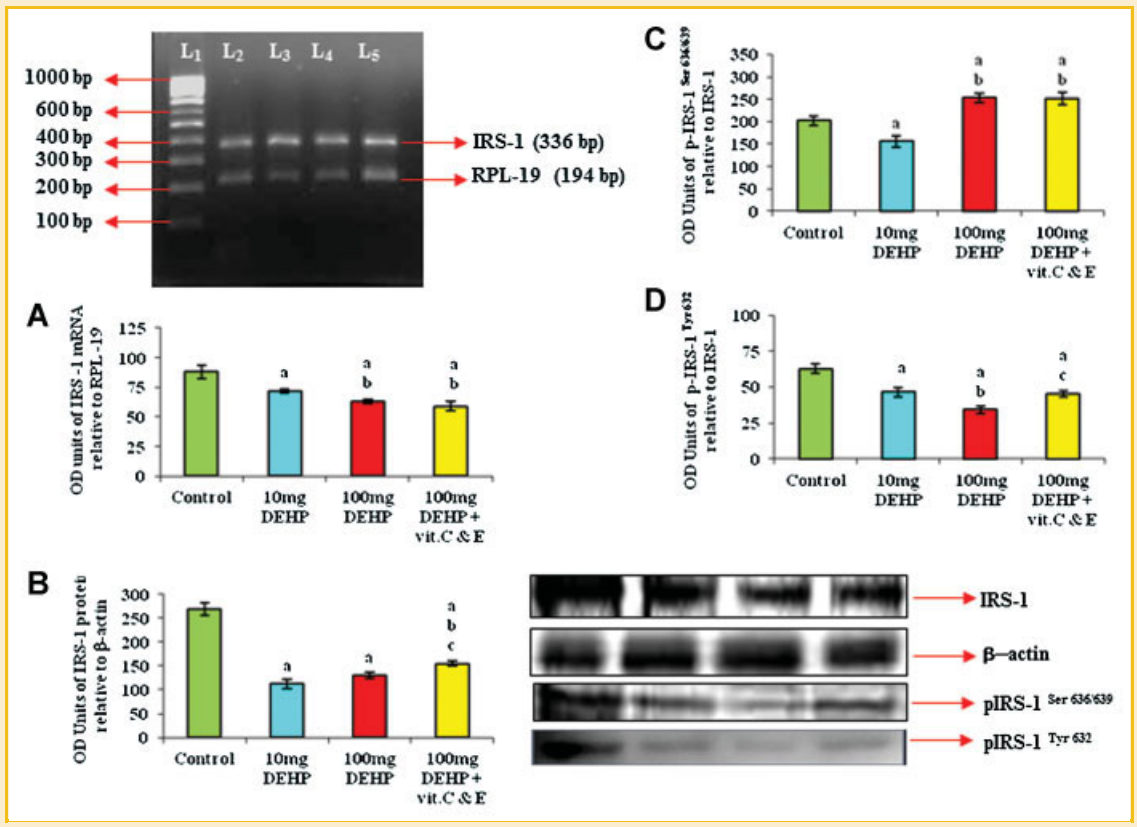

Fig. 4. Effect of antioxidant vitamins (C & E) against di-2-ethylhexyl phthalate (DEHP)-induced changes in insulin receptor substrate-1 (IRS-1) mRNA (A), and protein (B) phosphoIRS-1Ser(636/639) (C), and phosphoIRS-1Tyr632 (D) levels in the adipose tissue of adult male rat. Total protein concentration was determined prior to Western Blot analysis. Each bar represents mean ± SEM of three observations. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

may induce certain changes at post-translational level in IRS-1 that leads to degradation of the same. One of the first post-receptor events in insulin signaling is IRS-1 phosphorylation. Insulin receptor-mediated tyrosine phosphorylation of IRS-1 activates a metabolic signaling pathway by binding to the regulatory subunit (p85) of the PI3K. Ser636/639/307 is well-recognized phosphorylation sites in IRS-1, and the preponderance of evidence suggests that Ser636/639/307 phosphorylation can negatively influence insulin signaling including increased ubiquitin–proteasome degradation of IRS-1, reduced phosphorylation of IRS-1 on tyrosine and subsequent alteration of insulin-induced PI3-kinase activation [Bouzakri et al., 2003]. ROS and oxidative stress lead to activation of multiple serine/threonine kinase signaling cascades. These activated kinases can act on a number of potential targets in the insulin-signaling pathway, including the insulin receptor and the family of IRS proteins. An increase in serine phosphorylation of IRS-1, and IRS-2 decreases the extent of the activating tyrosine phosphorylation. Kinases that have been shown to be activated by oxidative stress

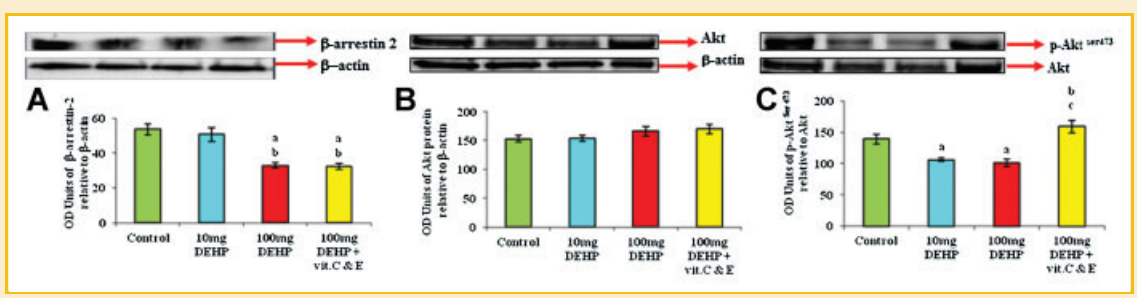

Fig. 5. Effect of antioxidant vitamins (C & E) against di-2-ethylhexyl phthalate (DEHP)-induced changes in β-Arrestin-2 (A), Akt protein (B), and phosphoAktSer473 (C) levels in the adipose tissue of adult male rat. Total protein concentration was determined prior to Western Blot analysis. Each bar represents mean ± SEM of three observations. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP, ccompared with 100 mg DEHP.

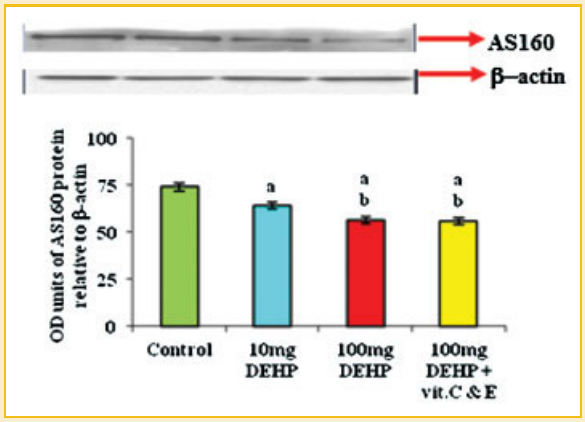

Fig. 6. Effect of antioxidant vitamins (C & E) against di-2-ethylhexyl phthalate (DEHP)-induced changes in AS-160 levels in the adipose tissue of adult male rat. Total protein concentration was determined prior to Western Blot analysis. Each bar represents mean ± SEM of three observations. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP.

include JNK, p38, MAPK, and IKKβ [Hirosumi et al., 2002]. In the present study, DEHP treatment increased the phosphorylation of IRS-1 at Ser636/639 and decreased it at Tyr632 implying the defective insulin signal transduction. Whereas, the partial protective effect of vitamins C and E supplementation was only seen in phosphorylation of IRS-1 Tyr632 site. In support of this, insulin resistance has been suggested to be a consequence of defects in the insulin-signaling cascade [Petersen and Shulman, 2006]. These defects are thought to be due to serine/threonine phosphorylation of IRS-1 by various kinases such as PKC and other serine/threonine kinases [Gual et al., 2005] that are activated by lipid metabolites such as DAGs and ceramides. Serine/threonine phosphorylation of IRS-1 results in reduced tyrosine phosphorylation and increased

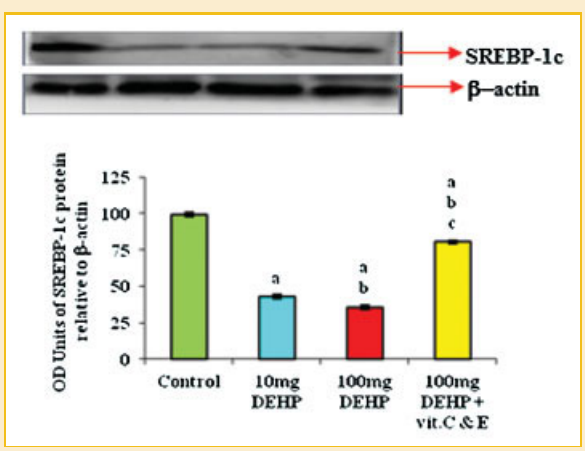

Fig. 7. Effect of antioxidant vitamins (C & E) against di-2-ethylhexyl phthalate (DEHP)-induced changes in mature SREBP-1c levels in the adipose tissue of adult male rat. Nuclear protein concentration was determined prior to Western Blot analysis. Each bar represents mean ± SEM of three observations. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP.

proteosomal degradation of the protein. This leads to lack of link between the upstream IR and downstream molecules resulting in impaired signaling.

Significant decrease in β-arrestin-2 protein level was observed after the 100 mg DEHP treatment rats but supplementation of antioxidant vitamins failed to maintain it at the control level. In this regard, it has been shown that β-arrestin-2 is severely down regulated in diabetic mouse models. Knockdown of β-arrestin-2 exacerbates insulin resistance, whereas administration of β-arrestin-2 restores insulin sensitivity in mice. Further, investigation reveals that insulin stimulates the formation of a new β-arrestin-2 scaffold recruiting Akt and Src to the insulin receptor. Loss or dysfunction of β-arrestin-2 results in deficiency of this signal complex and disturbance of the insulin signaling in vivo, thereby contributing to the development of insulin resistance and progression of the type-2 diabetes [Luan et al., 2009].

The tyrosine phosphorylated IRS provides SH_2-domain binding sites for the regulatory subunit of P13K, which activates the Akt. Akt is fully active only when it is phosphorylated on the $Thr^{308/309}$ and $Ser^{473/474}$ residues [Hirsch et al., 2007]. In the present study, DEHP did not influence Akt protein in all groups. However, Akt^{ser473} phosphorylation was diminished in 10 mg as well as 100 mg DEHP treated groups. Antioxidant vitamins supplementation had protective effect against DEHP-induced toxicity. Therefore, it is suggested that DEHP-mediated diminution in Insulin receptor and IRS-1 may be responsible for the impaired Akt^{ser473} phosphorylation.

AS160 contained six Akt consensus sequences that become phosphorylated in the insulin-treated adipocytes [Kane et al., 2002]. In the present study, AS160 protein level was significantly decreased in DEHP treatment but vitamins supplementation was not able to prevent the diminution. It has been well recognized that phosphorylated AS160 is essential to trigger insulin-stimulated translocation of GLUT4 and the associated increase in glucose uptake in adipose tissue. Further studies on AS160 phosphorylation would be interesting to understand the exact mechanism involved.

ADD1/SREBP-1c (adipocyte determination and differentiation dependent factor 1/sterol-regulatory-element-binding protein-1c) is a trans-acting factor that regulates transcription of genes involved in cholesterol and fatty acid synthesis [Horton and Shimomura, 1999] and is abundantly expressed in the liver and adipose tissue. In the liver, SREBP-1c has a well-documented role as an insulin-mediated transcriptional activator of genes involved in carbohydrate and lipid metabolism [Kim et al., 2004]. SREB-1c can activate GLUT4 gene expression by directly binding to the SRE (Sterol Response Element) in the GLUT4 promoter region [Im et al., 2006]. In the present study, the trans-acting transcription factor SREBP-1c is greatly reduced in dose-dependent manner.

GLUT4 exists in insulin-sensitive tissues mainly skeletal muscles and adipose tissue and is thus the major transporter protein responsible for insulin-mediated whole-body glucose uptake [Joost et al., 2002; Watson et al., 2004]. Translocation of GLUT4 is mediated through insulin-signaling pathway and any abnormality in this pathway results in insulin resistance and in turn type-2 diabetes [Watson et al., 2004]. In the present study, GLUT4mRNA expression was increased in low dose treated rats but in high dose, it was normal. Unlike GLUT4mRNA both cytosolic and membrane

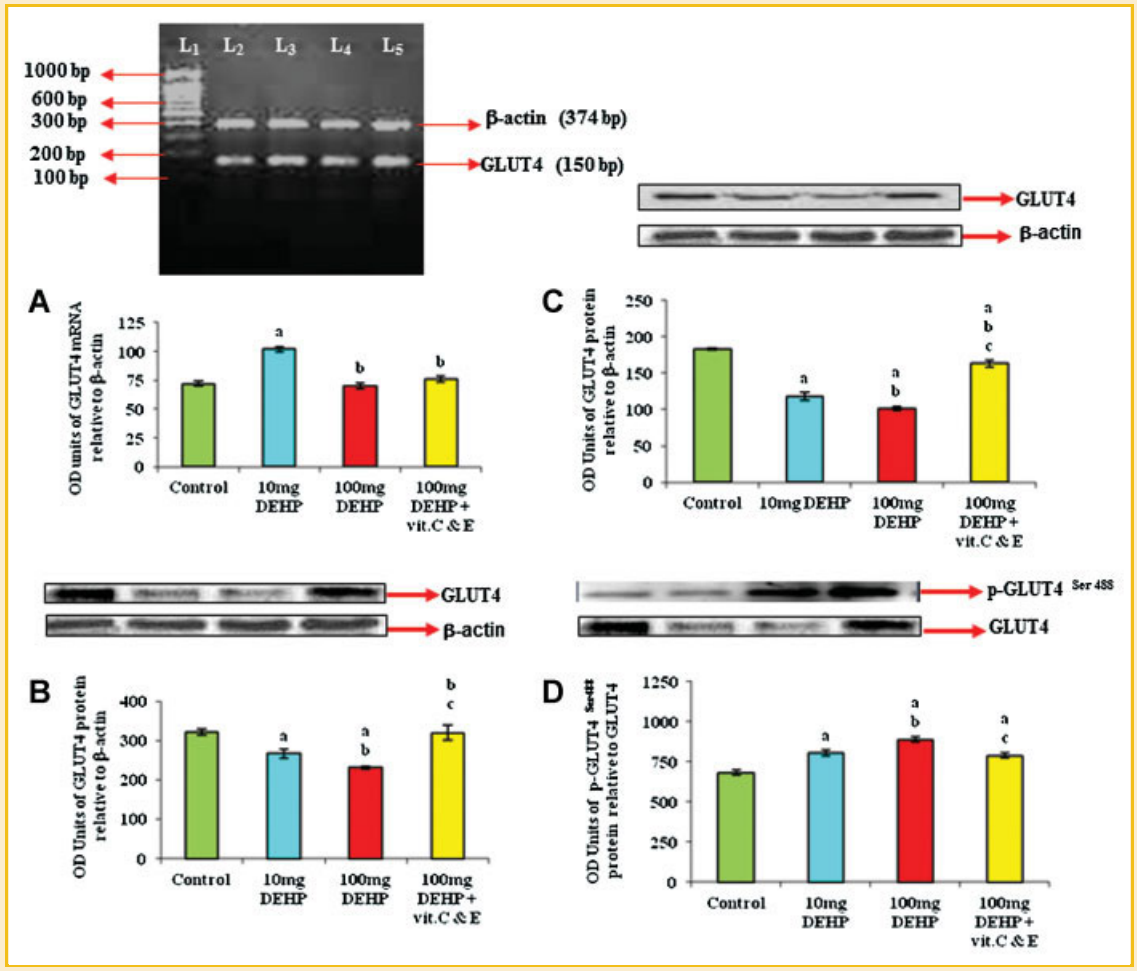

Fig. 8. Effect of antioxidant vitamins (C & E) against di-2-ethylhexyl phthalate (DEHP)-induced changes in the Glucose transporter-4 (GLUT4) mRNA (A), and protein levels in the cytoplasm (B), plasma membrane (C), and phosphoGLUT4Ser488 of adipose tissue of adult male rat. The mRNA expression was analyzed by isolating total RNA using total RNA isolation reagent (TRIR) and converting into complementary DNA (cDNA) using Reverse Transcriptase Polymerase Chain Reaction, identified on Agarose Gel Electrophoresis (AGE) and quantified it by densitometric scanning (Bio Rad). Lane 1: 100 bp marker; Lane 2: Control; Lane 3: 10 mg DEHP; Lane 4: 100 mg DEHP; Lane 5: 100 mg DEHP + vitamins (C & E). Total protein concentration was determined in cytoplasm and plasma membrane by spectrophotometric method prior to Western Blot analysis. Each bar represents mean ± SEM of three observations. Significance at $P < 0.05$, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

GLUT4 protein expression were decreased in DEHP treated rats. Insulin regulates GLUT4 expression [Jones and Dohm, 1997]. Though, the circulating level of insulin was not measured in the present study, the available report indicates that DEHP treatment decreases serum insulin level in rat [Gayathri et al., 2004]. In view of this finding, it is suggested that DEHP-induced sub-normal insulin could have contributed for the low levels of GLUT4 protein in cytosolic fraction of adipose tissue.

Further, decreased GLUT4 protein may be the consequence of decreased phosphorylation of Akt and its 160 kDa substrate (AS160) that is essential for the GLUT4 translocation from cytosol to the plasma membrane. Since the cytosolic GLUT4 is reduced in DEHP-treated rats, translocation to the membrane is also likely to be reduced. GLUT4 is a membrane-bound protein and is likely to get affected once the membrane integrity is lost [Joost and Thorens, 2001]. DEHP is shown to induce lipid peroxidation and this might have altered the membrane integrity resulting in reduced membrane-bound GLUT4. In the rats supplemented with vitamins C and E, the membrane GLUT4 level was maintained at normal level and this may be due to its protective action against lipid peroxidation by scavenging free radicals directly and thereby stabilizing membranes containing polyunsaturated fatty acids.

In the present study, phosphorylation of GLUT4 at serine 488 was statistically increased in DEHP treatment in a dose-dependent manner. Phosphorylation of GLUT4 decreases its intrinsic activity whereas under normal circumstances, insulin promotes dephosphorylation of GLUT4, which may be stimulating its intrinsic activity [Jones and Dohm, 1997]. Further, they reported that phosphorylation does not affect insulin-stimulated translocation of GLUT4 from the intracellular pool to the plasma membrane. Increase in phosphorylation of GLUT4 was associated with decrease in the ability of insulin to stimulate glucose uptake in adipocytes [Begum et al., 1993]. Eventually, vitamins supplemented group showed a statistically significant reduction in serine 488 phosphorylation of

GLUT4 when compared to DEHP exposed group. This signaling result in the dephosphorylation of GLUT4 to stimulate its intrinsic activity and translocation of GLUT4 containing vesicles to the plasma membrane and glucose uptake will take place to maintain glucose homeostasis.

Such alteration in the GLUT4 may typically originate from a blockade of sterol response element binding protein-1c (SREBP-1c) level in DEHP treated groups. Our comparison of transporter protein expression with gene expression data complies with this mechanism. SREBP-1c interacts with SRE in GLUT4, which control transcription of the GLUT4 gene in adipose tissue. In contrast, some discrepancies were observed in our study between mRNA and protein level of the key transporter GLUT4. This may be a consequence of its complex regulation with a significant contribution of post-transcriptional mechanisms.

Glucose uptake and oxidation is an important process, which provides energy to the cells to perform various functions. The rate of glucose oxidation in a cell depends on the rate of entry of glucose into the cell. In the present study, a dose-dependent reduction in the glucose uptake and oxidation was noted. Reduced membrane GLUT4 level leads to impaired glucose uptake and this may be responsible for the decreased glucose oxidation in the DEHP-treated group. Vitamins (C & E) supplementation maintained the glucose uptake and oxidation at control level suggesting their protective role against DEHP-induced changes.

In summary, the defective insulin signal transduction at the molecular level is responsible for the DEHP-induced glucose intolerance as a result of poor translocation of GLUT4, glucose uptake and subsequent oxidation in rat adipose tissue. Whereas, simultaneous supplementation of antioxidant vitamins C and E have beneficial roles against DEHP-induced effects like oxidative stress and thus fortifies the insulin-signaling transduction.

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