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Diethyl hexyl phthalate-induced changes in insulin signaling molecules and the protective role of antioxidant vitamins in gastrocnemius muscle of adult male rat

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ABSTRACT

Diethyl hexyl phthalate (DEHP) is an endocrine disruptor, it influences various organ systems in human beings and experimental animals. DEHP reduced the serum testosterone and increased the blood glucose, estradiol, T_3 and T_4 in rats. However, the effect of DEHP on insulin signaling and glucose oxidation in skeletal muscle is not known. Adult male albino rats were divided into four groups: Group I: Control; Groups II and III: DEHP treated (dissolved in olive oil at a dose of 10 and 100 mg/kg body weight, respectively, once daily through gastric intubation for 30 days); and Group IV: DEHP (100 mg/kg body weight) plus vitamins 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 intubation for 30 days. On completion of treatment, animals were euthanized and perfused (whole body); gastrocnemius muscle was dissected out and subjected to assessment of various parameters. DEHP treatment increased the H_2O_2, hydroxyl radical levels and lipid peroxidation which disrupt the membrane integrity and insulin receptor. DEHP impaired the insulin signal transduction, glucose uptake and oxidation through decreased expression of plasma membrane GLUT4, which may partly be responsible for the elevation of fasting blood glucose level. The present study suggests that DEHP exposure affects glucose oxidation in skeletal muscle and is mediated through enhanced lipid peroxidation, impaired insulin signaling and GLUT4 expression in plasma membrane. Antioxidant vitamins (C and E) have a protective role against the adverse effect of DEHP.

Introduction

Insulin is an essential hormone for maintaining whole-body glucose homeostasis. In normal individuals, increased plasma glucose level stimulates the secretion of insulin from the β-cells of the pancreatic islets (Kahn, 1994), which in turn stimulates glucose transport into peripheral tissues. Insulin receptor is expressed in almost all mammalian tissues, highest concentrations are found in insulin target tissues like muscle, adipose tissue and liver (Cheatham and Kahn, 1995). Insulin binds to its receptor, leading to receptor autophosphorylation and activation of receptor tyrosine kinase, which in turn results in tyrosine phosphorylation of endogenous substrates including insulin receptor substrate (IRS). Subsequently, activation of PI3 kinase results in phosphorylation of phosphatidylinositol-4,5 bisphosphate to form phosphatidylinositol-3,4,5-triphosphate; this in turn, activates ser/thr kinase i.e. phosphoinositide dependent kinase-1 (PDK-1) (Shepherd et al., 1998). Activated PDK-1 or PIP3 phosphorylates Akt/PKB (ser/thr kinase). Akt plays a central role by phosphorylating one of its substrates, AS160 which is essential for effective translocation of glucose transporter protein (GLUT4) to the plasma membrane, for the transport of glucose into the cell (Larance et al., 2005). Skeletal muscles have a central role in the maintenance of glucose homeostasis and they are the predominant site for peripheral glucose utilization. Glucose transport in skeletal muscle is the rate-limiting step for glucose utilization under physiological condition (DeFronzo, 1997).

Diethyl hexyl phthalate (DEHP) is commonly used to confer flexibility to many polyvinyl chloride (PVC)-based plastics. Flexible materials containing DEHP and other phthalate compounds are used in food packages, toys, clothing, medical devices such as blood storage bags, intravenous fluid bags and many other products (Sunny et al., 2004). DEHP is an endocrine disruptor, which interferes with steroid hormone action and affects the reproductive function (Sharpe, 2001). Administration of DEHP to adult male rats interfered with carbohydrate metabolism by reducing the blood glucose utilization, hepatic

Abbreviations: DEHP, diethyl hexyl phthalate; IR, insulin receptor; IRS, insulin receptor substrate; AS160, Akt substrate 160; GLUT4, glucose transporter protein 4; LPO, lipid peroxidation; RIA, radioimmunoassay; RT-PCR, reverse transcriptase-polymerase chain reaction; OGTT, oral glucose tolerance test; CV, coefficient of variation; TRIR, total RNA isolation reagent; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; OH^*, hydroxyl radical; LPO, lipid peroxidation.

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glycogenesis and glycogenolysis (Mushtaq et al., 1980). The DEHP-fed rats 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 also recorded in DEHP-fed rats (Martinelli et al., 2006). A recent study from our laboratory showed that DEHP has adverse effects on insulin receptor and glucose oxidation in Chang liver cells in vitro suggested that DEHP exposure may possess a negative influence on glucose homeostasis (Rengarajan et al., 2007).

Women with endometriosis had higher plasma DEHP concentrations (median 0.57 mg/ml, IQR 0.06–1.23) than control women (0.18 mg/ml, IQR 0–0.44) ($p = 0.0047$) (Cobellis et al., 2003). High levels of serum DEHP and its metabolites level were also detected in thelarche (premature breast development) in young girl patients (Colon et al., 2000). Stahlhut et al. (2007) have reported that phthalates exposure may contribute to the population burden of obesity, insulin resistance and related clinical disorders in adult U.S. males. The exposure of animals to phthalate esters can result in a significant perturbation of normal metabolism in liver, heart, testes, adrenal gland, and brain and can affect blood lipid levels (Bell, 1982). The DEHP-induced insulin deficiency and a decrease in the testosterone (T)/estradiol (E) ratio are suggestive of the diabetogenic effects of DEHP. DEHP exposure alters the expression of the spermatogenesis- or steroidogenesis-related genes resulting in decreased sperm production in the testis (Lee et al., 2009). Oral administration of DEHP to rats significantly increased the serum marker enzymes, the level of total bilirubin and hepatic lipid peroxidation. The levels of serum protein, hepatic glutathione and ascorbic acid were also decreased (Jain et al., 2009).

Vitamin C (ascorbic acid) is an essential micronutrient required for normal metabolic functioning of the body as an antioxidant. Vitamin C may help to prevent the oxidative damage to organs such as eyes, brain and kidneys that frequently occur in type-II diabetes (Lee et al., 2007). The ability of vitamin C to donate electrons makes it a potent water-soluble antioxidant that readily scavenges free radicals such as molecular oxygen, superoxide, hydroxyl radical and hypochlorous acid. Vitamin C significantly decreases the adverse effect of oxidative damage to macromolecules like lipids, DNA and proteins, which are implicated in chronic diseases, such as neurodegenerative diseases (Ishihara et al., 2000). Supplementation of antioxidant vitamins accelerated the regeneration of injured seminiferous epithelium in DEHP-treated animals, suggesting that the vitamins have a therapeutic effect on DEHP-induced aspermatogenesis (Ablake et al., 2004).

Vitamin E is the major chain-breaking antioxidant present in biological membranes (Burton et al., 1983). Vitamin E interacts with the cell membrane, traps free radicals and inhibits reactive oxygen species-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of polyunsaturated fatty acid in membrane phospholipids. It acts as a donor antioxidant (reductant) reacting with peroxyl radicals to inhibit the propagation cycle of lipid peroxidation (LPO) in the cell membrane by scavenging peroxyl (RO*) and alkoxyl (ROO*) radicals (Ames et al., 1993). Dhanya et al. (2004) reported that administration of vitamin E prevents DEHP-induced deleterious effects, such as degenerative changes in the brain and thyroid.

Studies reveal that the polyvinyl chloride (PVC)-based endocrine disruptors, like DEHP affect the function of endocrine and other organs in human and experimental animal models. Further, it appears to possess a negative influence on glucose homeostasis. However, the effect of DEHP on insulin signaling molecules is not known. Therefore, the present study was designed to assess the DEHP-induced changes in serum hormones, insulin signaling molecules, GLUT4, glucose uptake and oxidation, in gastrocnemius muscle. Simultaneously, the protective role of antioxidant vitamins (C and E) in adult male albino rats was also assessed.

Materials and methods

Chemicals and supplies

All chemicals and reagents used in the present 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. Glucose estimation kit was supplied by Linear Chemicals, Barcelona, Spain. ^{14}C-glucose and ^{14}C-2-deoxyglucose were purchased from the Board of Radiation and Isotope Technology, Mumbai, India. Radioimmunoassay (RIA) kits for the assay of insulin and testosterone were obtained from Diasorin, Italy. Total RNA isolation reagent (TRIR) and one-step reverse transcriptase-polymerase chain reaction (RT-PCR) were purchased from A gene (UK) and Siegen (Germany). The insulin receptor (IR), IRS-1, GLUT4, RPL-19, β-actin primers and the β-actin monoclonal antibody were purchased from Sigma (USA). Polyclonal insulin receptor β-subunit, IRS-1, phospho IRS-1 (ser 636/639), phospho IRS-1 (tyrosine 632), Akt1/2/3 and phospho Akt (ser 473), β-arrestin-2, and GLUT4 antibodies were purchased from Santa Cruz Biotechnology (USA). Akt substrate 160 (AS160) monoclonal antibody was purchased from Cell Signaling Technology (USA).

Experimental design

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 to 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 °C ± 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 at a dose of 2 ml/kg body weight); 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 intubation for 30 days); and Group IV: DEHP (100 mg/kg body weight), 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). After the treatment period, animals were anesthetized with ether, blood was collected, sera separated and stored at −80 °C until the assay of hormones was performed. Skeletal muscle (gastrocnemius) was dissected out and subjected for the assay of various parameters.

Oral glucose tolerance test (OGTT) and plasma glucose

Rats of all groups were subjected to oral glucose tolerance test, two days prior to killing. All animals were fasted overnight and subjected to OGTT by giving an oral dose of glucose (2 g/kg body weight) after collecting blood by puncturing the orbital sinus, using heparinized microhematocrit capillary tube, for the estimation of fasting blood glucose. Blood samples were collected subsequently at 60 min, 120 min and 180 min and centrifuged for 10 min at 800 × g at 4 °C. This process was done within 30 min to prevent autoglycolysis by leukocytes. Plasma glucose level was estimated by glucose oxidase–peroxidase method (CPC diagnostics, Spain). Results are expressed as mg/dl.

Radioimmunoassay

Serum testosterone level was assayed using RIA kit obtained from Diasorin (Italy). The sensitivity of the assay was 0.02 ng/ml. The percentage of cross reactivity of testosterone antiserum to other steroids such as 5-dihydrotestosterone and androstenedione is 6.9%