Glucose homeostasis

Glucose is an essential metabolic fuel for the brain under physiological condition. It is the fundamental source of energy for all eukaryotic cells and multiple mechanisms have evolved for its synthesis, storage and metabolism (Bryant et al., 2002; Watson et al., 2004). In humans, although all cells use glucose for their energy needs, the main consumer under basal conditions is the brain (Bryant et al., 2002). Whole body glucose homeostasis depends upon the balance between hepatic glucose output and glucose utilization by insulin-dependent tissues (skeletal muscle and adipocytes) and insulin-independent tissues (brain and splanchnic organs) (DeFronzo et al., 1985; Wallberg-Henriksson, 1987). The majority of endogenous glucose production is derived from liver (approximately 85%) and the remaining amount is produced by the kidney (Gerich et al., 2001). Under basal and post-absorptive conditions, approximately half of the hepatic glucose production is derived from glycogenolysis and half from gluconeogenesis (Rothman et al., 1991). Of the glucose that is taken up by peripheral tissues, the majority (~80%) is disposed of in skeletal muscle (DeFronzo et al., 1985; Mandarino et al., 2001) with only a small amount (approximately 4-5%) is being metabolized in fat tissue (Virtanen et al., 2001). Increase in glucose level stimulates the secretion of insulin and suppresses the secretion of glucagon (Atkinson and Maclaren, 1994).

The three major tissues largely responsible for clearing glucose from the blood in healthy individuals are liver, muscle and adipose tissue and all become insulin resistant in type-2 diabetes (Cornier et al., 2008; Turcotte and Fisher, 2008; Staiger et al., 2009).
has been demonstrated that targeted inactivation of the insulin receptor gene specifically in the liver leads to diabetes-like symptoms in mice and suggest that insulin has a direct role in regulating liver metabolism (Michael et al., 2000; Rutter, 2000; Frost and Olson, 2011). The liver facilitates sustaining normal blood glucose concentration in the fasting and postprandial states (Wasserman, 2009). Decreased level of insulin effect on the liver leads to glycogenolysis and an increase in hepatic glucose production (Moore et al., 1998; Wahren and Ekberg, 2007). Abnormalities of triglyceride storage and lipolysis in insulin-sensitive tissues such as the liver are an early manifestation of conditions characterized by insulin resistance and are detectable earlier than fasting hyperglycemia (Lewis et al., 2002; Xu et al., 2006; Lee et al., 2009; Morton and Schwartz, 2011). In another study, development of insulin resistance in the liver involves selective inactivation of the capacity of insulin to block hepatic glucose production and the ability of the hormone to stimulate fatty acid synthesis is retained (Shimomura et al., 2000; Holland and Summers, 2008; Lin and Sun, 2010).

Gluconeogenesis is a life-sustaining process for maintaining blood glucose levels within the physiologic range and providing the solitary fuel source for the brain, testes, and erythrocytes during starvation (Sonksen and Sonksen, 2000; Kim et al., 2011). Gluconeogenesis occurs mainly in the liver in a metabolic pathway that is tightly regulated by insulin (Desvergne et al., 2006; Kim et al., 2011). When hepatic insulin signalling goes awry, gluconeogenesis becomes uncontrolled, resulting in excessive glucose production and causing fasting hyperglycemia in diabetes (Kim et al., 2011). While it has variety of functions, the liver plays a distinctive role in managing
carbohydrate metabolism by retaining glucose concentrations in an optimum range (Xu et al., 2011).

Skeletal muscle is quantitatively the most important tissue target of glucose postprandially and accounts for 70–80% of insulin-stimulated glucose uptake (Bouche et al., 2004; DeFronzo and Tripathy, 2009; Abdul-Ghani and DeFronzo, 2010; Glatz et al., 2010). Skeletal muscle insulin resistance is considered to be the initiating or primary defect that is evident decades before β-cell failure and overt hyperglycemia develops (Lillioja et al., 1988; Warram et al., 1990; Matthaei et al., 2000; Das and Elbein, 2006; Semenkovich, 2006). In insulin resistant states, such as type-2 diabetes and obesity, insulin-stimulated glucose disposal in skeletal muscle is markedly impaired (Smith, 2002; Kashyap et al., 2005; Defronzo, 2009; Abdul-Ghani and DeFronzo, 2010; Timmers et al., 2012). The decreased insulin-stimulated glucose uptake is due to impaired insulin signalling and multiple post-receptor intracellular defects including impaired glucose transport and phosphorylation, and reduced glucose oxidation and glycogen synthesis (Cusi et al., 2000; Bajaj and Defronzo, 2003; Bouzakri et al., 2005; Karlsson and Zierath, 2007).

**Mechanism of glucose transport**

In mammals, glucose is cleared from the blood stream by facilitative transporters, which comprise a family of highly related 12 transmembrane domain-containing proteins (Joost and Thorens, 2001; Joost et al., 2002; Augustin, 2010; Mueckler and Thorens, 2013). As facilitative carriers, the glucose transporter (GLUT) proteins transport glucose down its concentration gradient in an energy-independent manner (Shepherd and Kahn,
1999; Wood and Trayhurn, 2003). Most mammalian cell types are net consumers of glucose and maintain low intracellular glucose concentrations, thus favouring glucose entry (Bryant et al., 2002; Wood and Trayhurn, 2003; Watson et al., 2004; Mueckler and Thorens, 2013).

**Hormonal control of glucose homeostasis**

Glucose homeostasis is controlled primarily by the anabolic hormone insulin and also by insulin-like growth factors (Clemmons, 2004). Apart from insulin, many other hormonal factors regulate glucose homeostasis (Cryer, 1991, 1993). Several hormones (glucagon, catecholamines, glucocorticoid and growth hormone) oppose the action of insulin and they are known as insulin antagonistic or counterregulatory hormones (Baynes and Dominiczak, 2004).

**Insulin**

Insulin, the dominant glucose-lowering hormone, suppresses endogenous glucose production and stimulates glucose utilization by insulin-sensitive tissues, thereby lowering the plasma glucose concentration. Insulin is a polypeptide hormone, secreted from β-cells of pancreatic islets into the portal circulation and acts on the liver and peripheral tissues. It inhibits hepatic glycogenolysis and gluconeogenesis and in concert with other factors (including hyperglycemia and hypoglucagonemia), converts the liver into an organ of net glucose uptake and fuel storage (glycogen and triglycerides) (Cryer, 1991; Wahren and Ekberg, 2007). It also suppresses renal glucose production and stimulates glucose uptake, storage and utilization by tissues such as muscle and fat. In the post-absorptive state, insulin regulates the plasma glucose concentration primarily by
restraining hepatic glucose production (Rizza and Gerich, 1979). High levels, such as
doioe that occur after meals, are required to stimulate glucose utilization (Rizza and
Gerich, 1979). Conversely, decreased insulin secretion causes increased hepatic and renal
glucose production and decreased glucose utilization by insulin-sensitive tissue such as
muscle and thus tend to raise plasma glucose concentration. The rate of insulin secretion
is regulated by a number of factors, the most important one is glucose. A fall in the
plasma glucose concentration has an immediate inhibitory effect on insulin secretion,
thereby limiting a further fall in the plasma glucose level (Cryer, 1993).

**Sex steroids**

Extensive experimental evidences advocate that sex steroids and insulin interact in
their actions on tissues. At physiological levels, testosterone and estradiol are involved in
maintaining normal insulin sensitivity (Livingstone and Collison, 2002; Kapoor *et al.*, 2005; Song *et al.*, 2006). Steroids have a peripheral site of action (Polderman *et al.*, 1994) and since skeletal muscle is responsible for the majority of peripheral glucose
disposal, it appears that sex steroids have a direct action on skeletal muscle to modulate
insulin sensitivity. Administration of testosterone or its derivatives to women resulted in
impairled glucose tolerance and hyperinsulinaemia, indicative of insulin resistance
reported that testosterone deficiency-induced defective glucose oxidation in skeletal
muscles, liver and adipose tissue is mediated through impaired serum insulin level and its
receptor gene expression.
**Thyroid hormone**

Thyroid hormone action has long been recognized as an important determinant of glucose homeostasis and it is known to affect most of the aspects of carbohydrate metabolism (Chidakel et al., 2005; Wang, 2013a; Moura Neto et al., 2014; Tereshchenko and Suslina, 2014). They appear to enhance the actions of epinephrine in stimulating glycogenolysis and gluconeogenesis and to potentiate the actions of insulin and glycogen synthesis and glucose utilization (Bilezikian and Loeb, 1983; Liu et al., 2003). It has been shown to increase glucose disposal *in vivo* (Casla et al., 1990) and to stimulate glucose transport in a rat liver cell line (Weinstein et al., 1990; Haber et al., 1995). In addition, thyroid hormone (T$_3$) has been shown to have a positive regulatory effect on GLUT4 mRNA and protein in rat cardiomyocytes and skeletal muscle of hyperthyroid rats (Weinstein et al., 1991; Gosteli-Peter et al., 1996). Consistent with these data, Richardson and Pessin (1993) have identified a T$_3$ response element in the promoter region of the GLUT4 gene between -517 and -237, which when placed in a luciferase reporter gene construct, displayed myotubes- specific activation by the T$_3$ receptor $\alpha_1$ in C2C12 mouse muscle cell line. Differentiation of the myotubes in the absence of T$_3$ resulted in a decreased expression of GLUT4 mRNA, suggesting T$_3$-dependent regulation of both GLUT4 mRNA levels as well as the luciferase reporter constructs in cultured myotubes (Richardson and Pessin, 1993).
Endocrine disrupting chemicals

Metabolic diseases have increased at an astounding rate in recent decades. Even though poor diet and physical inactivity are central drivers, these lifestyle changes alone fail to fully account for the magnitude and rapidity of the epidemic. Thus, attention has turned to identifying novel risk factors, including the contribution of environmental endocrine disrupting chemicals (EDCs). Epidemiologic and preclinical data support a role for various contaminants in the pathogenesis of diabetes (Kirkley and Sargis, 2014). However, well-established risk factors, such as diet, lifestyle, and genetics, cannot fully explain this phenomenon (Lin et al., 2011b). It has been suggested that this could, to some extent, be linked to the exposure to environmental pollutants (EDCs), which coincidentally increased during the same period (Feige et al., 2010; Lin et al., 2011b).

EDCs or endocrine disruptors (EDs) are ubiquitous compounds that can interfere (synthesis, secretion, transport, metabolism, binding, action and catabolism of natural hormones) with and alter the homeostasis of the endocrine system, resulting in long-term adverse effects on human and animal health (Diamanti-Kandarakis et al., 2009) through defaults in foetal development and postnatal life of their progeny (Hashimoto et al., 2006). EDs may thus interact with the endocrine system of animals and humans and exert this effect even if present in minute amounts (Brevini et al., 2005). The majority of currently identified EDCs are synthetic industrial chemicals which are widely used for daily purposes including pesticides, plasticizers and industrial by-products (WHO, 2002; Brevini et al., 2005).
**Phthalates**

Phthalates are a group of endocrine-disrupting chemicals commonly used to render plastics soft and pliable to rigid plastics such as PVC (Shea, 2003; Kobayashi et al., 2006). The primary use of phthalates is to serve as a plasticizer in the manufacture of vinyl products. Phthalates are high production volume chemicals and due to leaching these are ubiquitous environmental contaminants (ECB, 2008). They are found in a wide variety of common products including plastics, cosmetics, pharmaceuticals, baby care products, building materials, modelling clay, automobiles, cleaning materials and insecticides (Janjua et al., 2008).

**Di-2(ethyl hexyl) phthalate**

Di-2(ethyl hexyl) phthalate (DHEP) (Cas. No: 117-81-7) is an endocrine disruptor. It is a diester of phthalic acid and the branched-chain 2-ethylhexanol; colourless liquid plasticizer used in the industry to add flexibility, strength, optical clarity and resistance to broad-range of temperature variations to polyvinyl chloride (PVC) plastics (Kavlock et al., 2002b; Shea, 2003). DEHP may represent between 20 and 40% of the finished weight of the plastic (Jaeger and Rubin, 1973; Hauser et al., 2004). DEHP is one of the commonly used plasticizers which leaches from vinyl products due to their unbound nature (Pearson and Trissel, 1993). DEHP has been used as the most important plasticizer for more than 30 years (Helm, 2007). DEHP is among the most used plasticizers in the industry with an annual production of approximately 2 million tons which represent a quarter of all phthalates produced (Shelby, 2006) and the general population is being exposed to DEHP through air, water and food (Sharman et al., 1994) because of leaching
and off-gassing from products and emissions from industries (Feige et al., 2007; Feige et al., 2010; Casals-Casas and Desvergne, 2011). Phthalates bioaccumulate in invertebrates, fish and plants but do not biomagnify, because higher animals efficiently metabolize and excrete phthalates (Shea, 2003). Human exposure to DEHP begins while the child is still in the mother's womb as DEHP crosses the placenta because of its lipophilic nature resulting in foetal exposures (Enke et al., 2013).

**Physicochemical properties and toxic potential of DEHP**

Molecular weight of DEHP is 390.57 g/mol and the molecular formula is C_{24}H_{38}O_{4} (ECB, 2006). DEHP is a clear, oily liquid, highly lipophilic (fat soluble) and poorly soluble in water. Howard et al. (1985) determined a value of 160 µg/litre at 25°C in salt water. The boiling point is 385°C and the melting point is -50°C (Leyder and Boulanger, 1983).

![Structure of DEHP](image)

**Structure of DEHP**

Multiple studies have identified DEHP as an endocrine disruptor with antiandrogenic activity (Shelby, 2006). Endocrine disruptors are chemicals found in the environment that can interfere with the normal function of the endocrine system (Latini et
Since DEHP is not part of the covalent structure of the PVC, it can leach out from the polymer material and, in humans, can be absorbed through skin contact, oral exposure (Frederiksen et al., 2007), and inhalation (Heudorf et al., 2007; Huang et al., 2009; Huang et al., 2011). The typical human exposure is 3–30 μg/kg/day (Koch et al., 2003b; McKee et al., 2004) and is mainly from contact with consumer products. However, DEHP exposures of 20 mg/kg/day and higher have been described in workers at a PVC plant (Huang et al., 2011). DEHP and its metabolites have been found in semen (Phillips and Tanphaichitr, 2008), saliva (Silva et al., 2003), amniotic fluid (Silva et al., 2004; Huang et al., 2007), umbilical cord blood (Latini et al., 2003), human milk and baby formula (Frederiksen et al., 2007; Huang et al., 2009). This extensive biodistribution of DEHP and its metabolites present a particular threat to prenatal, neonatal, and infant development due to the high exposures and low body mass during early life (McKee et al., 2004). In this regard, enteral and parenteral infant feeding procedures using PVC containers and pipelines are sources of DEHP (Calafat et al., 2004). It has also been estimated that patients undergoing hemodialysis receive from 36–457 μg DEHP/kg/day (Doull et al., 1999), and that neonates receiving blood products may be exposed to 10–20 mg DEHP/kg/day (Loff et al., 2000). Higher levels of exposure in medical settings have been observed in neonatal intensive care unit patients (Green et al., 2005; Weuve et al., 2006) likely due to blood bags, tubing, and other medical equipment leaching large amounts of DEHP (Loff et al., 2000).
Metabolism of phthalate

Phthalates are rapidly metabolized in humans upon phase-I biotransformation to their respective monoesters, which depending on the phthalate, can be further metabolized to oxidative products of their lipophilic aliphatic side chain. Once absorbed, DEHP is metabolized (hydrolysed) into mono-2-ethylhexyl phthalate (MEHP) by intestinal esterases and lipases in the lining of the gut (Pollack et al., 1985), this metabolite might accumulate in liver via the organic anion transport system (Inoue, 1985). MEHP has antiandrogenic activity 10-times greater than DEHP (Frederiksen et al., 2007).

Monoesters and the oxidative metabolites of phthalates may be conjugated with glucuronide (phase-II biotransformation), and both free and conjugated metabolites can be excreted in the urine and feces (Albro et al., 1973; ATSDR, 2002). More precisely, the shorter-chain-length dialkyl phthalates (e.g. DEP and DBP) are predominantly metabolized by ester hydrolysis to the simple monoester phthalates, which are excreted in the urine, usually after glucuronidation (Albro et al., 1973; ATSDR, 2002). The longer-chain-length dialkyl phthalates, including DEHP, DnOP and DiNP, are similarly hydrolyzed to their respective monoester phthalates, but extensively further metabolized by oxidation of their alkyl side chain. Less than 10% of the original DEHP dose is excreted in urine as the monoester MEHP with the remainder being excreted as the secondary oxidized metabolites (Albro et al., 1973; Koch et al., 2003a; Koch et al., 2003b; Silva et al., 2003). The half-life values in blood are 23.8 h and 18.6 h for MEHP and DEHP, respectively (Oishi and Hiraga, 1982).
Genomic actions of phthalates

Phthalates with bioactive properties have been identified to activate the peroxisome proliferator activated receptor (PPAR) α and β nuclear receptors. PPARα heterodimerizes with retinoid X receptor (RXR) and activates peroxisome proliferator response elements (PPREs) in the promoter regions of targets genes. Activation of PPARα affects the lipid metabolism and PPARγ the adipocyte differentiation and development. MEHP has been found to be a potent activator of both PPARα and PPARγ (Hurst and Waxman, 2003). In adipose cells, MEHP a potent metabolite of DEHP, activates PPARγ and promotes fat formation through metabolic disruption and by affecting gene expression (Feige et al., 2007; Ellero-Simatos et al., 2011) via PPARs. In liver, another metabolically relevant organ, phthalates activate PPARα (Nr1c1) and behave as prototypical peroxisome proliferators (PP) (Lapinskas et al., 2005).

Constitutive androstane receptor (CAR) and Pregnane X receptor (PXR) are also increasingly recognized as important regulators of genes involved in lipid and glucose homeostasis (Konno et al., 2008; Moreau et al., 2008). In particular, CAR activation reduces the expression of critical genes involved in fatty acid oxidation (Maglich et al., 2009), bile acid synthesis and gluconeogenesis. Gene expression profiling delineated the role of PPARα and revealed a PPARα-independent regulation of several prototypic CAR target genes. DEHP dose-dependently induced CYP2B6 in human primary hepatocyte cultures (Eveillard et al., 2009). This finding demonstrates that CAR also represents a transcriptional regulator sensitive to phthalates. CAR-mediated effects of DEHP provide a
new rationale for most endpoints of phthalates toxicity described previously, including endocrine disruption, hepatocarcinogenesis and the metabolic syndrome (Eveillard et al., 2009).

**Non-genomic actions of phthalates**

Phthalate induces free radical production *in vivo* by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that generates superoxide anion (Rusyn et al., 2001). Superoxide anion was rapidly transformed to hydrogen peroxide (H$_2$O$_2$) and then to hydroxyl radical. An increased production of H$_2$O$_2$ would lead to formation of highly reactive oxygen species (ROS). ROS production was totally dependent on DEHP-induced Ca$^{2+}$ influx, plausibly through the Ca$^{2+}$-mediated activation of NADPH complex (Brechard et al., 2008). It has been shown that DEHP increases the [Ca$^{2+}$]i of human granulocytes by promoting a Ca$^{2+}$ influx from the extracellular medium, an effect likely contributing to the proinflammatory activity of the plasticizer in granulocytes (Palleschi et al., 2009). ROS generation in living organism can react with macromolecules like lipids, proteins, nucleic acid and carbohydrate and damage the structure and function of the same (Perrone et al., 2008). DEHP promotes lipid peroxidation and incorporation of vitamin E along with DEHP into the culture medium containing hepatocytes counteracted these effects (Santhosh et al., 1998). Oxidative stress and ROS have been shown to directly affect DNA methylation pattern and histone organization, therefore tuning the expression of multiple genes (Cerda and Weitzman, 1997).
Effect of DEHP exposure on animal models

DEHP is not classifiable as a carcinogen in human by the International Agency for Research on Cancer (IARC, 2000), but it has been designated as a carcinogen in experimental animals (Kluwe, 1982; IARC, 2000). DEHP also caused liver tumors in PPARα-null mice, which lack the peroxisome proliferator-activated receptor α (which has been proposed to be involved in DEHP-induced tumorigenesis) (Ito et al., 2007). In addition, dietary exposure to DEHP caused benign testicular tumors (Leydig-cell tumors) (Voss et al., 2005) and benign pancreatic tumors (acinar-cell and islet-cell adenoma) in male rats (Rao et al., 1990; David et al., 2000). Initiation-promotion studies in two strains of mice provided evidence that DEHP acted as a promoter of liver tumors (IARC, 2000). Oral administration of DEHP to rats significantly increased the serum marker enzymes (glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and alkaline phosphatase), 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).

Available toxicological testing in animals and in vitro test, supported by limited human data, provide evidence linking DEHP and its metabolites to a wide range of adverse effects in the reproductive tracts, liver, lungs, kidney and foetus. A large number of animal studies, primarily in rodents have examined the developmental toxicity of DEHP through dietary or gavages which results in increased foetal mortality, malformed
limbs and somatic mutations (Tomita et al., 1982; Ritter et al., 1987; Tyl et al., 1988; Peters et al., 1997).

Mice fed with high doses of DEHP (12000ppm dry weight in food) showed renal toxicity after four to eight weeks of feeding and moderate lesions after 24 weeks including focal tubular degeneration atrophy (Ward et al., 1998). Intravenous administration of DEHP has been shown to cause respiratory distress, tracheal bleeding and inflammation, and subsequent death due to pulmonary edema in rats after large doses from 200-300 mg DEHP/kg body weight (Syracuse Research Corporation, 1982; Huber et al., 1996). Rock et al. (1987) found that increasing doses of MEHP significantly decrease the heart rate and blood pressure resulting in cardiac arrest.

Adverse effects of DEHP exposure may include reduced fertility, and sperm production in males and ovarian dysfunction in females (Reddy et al., 2006a; Reddy et al., 2006b; Pant et al., 2008; Roya et al., 2009). Human exposure to di-2-ethylhexyl phthalate (DEHP) occurs throughout life. Adverse effects in the reproductive system include changes in the testes, specifically the Sertoli cell, leading to reduced fertility and changes in sperm production in males(Poon et al., 1997; Foster et al., 2001; Park et al., 2002) and ovarian dysfunction and decreased hormone production in females (Davis et al., 1994; Lovekamp-Swan and Davis, 2003).

The Sertoli cells are the likely target for male reproductive toxicity of DEHP and MEHP (Agarwal et al., 1986; Douglas et al., 1986). Early studies in adult animals found that high levels of DEHP exposure (0.9-20g/kg body weight/day) resulted in a dose-
dependent atrophy of the seminiferous tubule and testis resulting in loss of spermatogenesis (Schaffer et al., 1945; Agarwal et al., 1986). Testicular toxicity is likely to be mediated by inhibiting follicle-stimulating hormone (FSH) signal transduction (Heindel and Chapin, 1989; Park et al., 2002).

Akingbemi et al. (2001) have shown that the effects of DEHP on Leydig cell steroidogenesis are influenced by the state of development at exposure. Chronic DEHP exposure has been found to increase serum concentration of both luteinizing hormone and sex hormones [testosterone and 17β-estradiol (E2)] in male and female rats, which suggest the possibility of multiple crosstalks between androgen, estrogen and steroid hormone receptors (Nilsson and Gustafsson, 2002; Akingbemi et al., 2004). Inhibition of steroidogenesis leads to low fetal testosterone levels, during sexual differentiation and inhibit fetal rat Leydig cell testosterone and insulin-like 3 (insl3) hormone levels resulting in malformations of androgen- and insl3-dependent tissues (Wilson et al., 2004) referred to as the ‘Phthalate syndrome’.

Parks et al. (2000) conducted a series of in vivo, ex vivo, and in vitro studies to examine mechanisms of DEHP-induced malformations in rat reproductive organs where treatment with DEHP at 750 mg/kg bw/day inhibited testosterone production in male pups during the period of sexual differentiation, and this inhibition was a likely cause of malformations observed in other studies. Malformations likely result from a mechanism that does not directly involve the androgen receptor. The in vivo anti-androgenic activities of DEHP could mediate through some of its secondary metabolites (MEHP) were
evaluated by Stroheker et al. (2005). DEHP and other phthalates do not bind the androgen receptor but suppress androgen-stimulated sexual differentiation and they are considered prototype antiandrogens (Gray and Beamand, 1984; Gray and Gangolli, 1986; Wolf et al., 1999; Gray et al., 2000). MEHP decreased the levels of mullerian inhibiting substance (MIS) and GATA-binding family4 (GATA-4) proteins in Sertoli cells and impaired Sertoli cell proliferation in the organ culture of E18 and P3 testes. In utero exposure to phthalate esters such as di-2-ethylhexyl (DEHP) or dibutyl phthalate (DBP) reduces nuclear receptors SF-1 mRNA, PPAR γ protein and expression of several genes involved in steroidogenesis in fetal rat testes (Borch et al., 2006).

Animal toxicological studies have also demonstrated endocrine-modulating effects of DEHP (Gray and Beamand, 1984; Gray and Gangolli, 1986; Gray et al., 2000). DEHP alters thyroid structure and activity (Hinton et al., 1986; Gray et al., 2000) in male Wistar rats and produces reproductive and developmental toxicities (Kavlock et al., 2002b). It interferes with steroid hormone action and affects the reproductive function (Sharpe, 2001). The potential public health risks associated with phthalates exposure not only include carcinogenesis (Ito and Nakajima, 2008) but also metabolic and endocrine disruption.

Administration of DEHP to adult male rats interfered with carbohydrate metabolism by reducing the blood glucose utilization and hepatic glycogenesis and glycogenolysis in rat (Lake et al., 1975; Mushtaq et al., 1980; Mann et al., 1985). The DEHP-fed rats had an altered glucose tolerance associated with abnormal glucose
intermediate metabolites in liver and skeletal muscle. In these rats, hepatic glucose-6-phosphate, fructose-6-phosphate, pyruvate, lactate, glucose-1-phosphate and glycogen levels were decreased (Martinelli et al., 2006). At the same time, the glucose-6-phosphate level decreased while the pyruvate and lactate levels were increased in skeletal muscle (Martinelli et al., 2006). DEHP administration decreases the amount of glucose-1-phosphate (G1P) in rat testis, which is indicative of impaired glycogenolysis. As glycogen debranching enzyme (GDE) is required for the complete degradation of glycogen to glucose, it is proposed that DEHP and or its primary metabolite MEHP may modulate glycogenolysis in testis via inhibition of GDE (Kuramori et al., 2009).

At low doses, DEHP reduced the serum insulin and increased the blood glucose, triiodothyronine (T₃) and thyroxine (T₄) in rats. Moreover, DEHP-induced insulin deficiency and a decrease in testosterone/estrogen ratio are suggestive of the diabetogenic effects of DEHP (Gayathri et al., 2004). The importance of optimal level of insulin and sex steroids in the regulation of glucose homeostasis is well recognized (Barros et al., 2006; Song et al., 2006; Kelly and Jones, 2013; Gibb and Strachan, 2014). Testicular steroid hormone synthesis and development of reproductive system in males have been adversely affected by exposure to certain phthalates, including DEHP, di-butyl phthalate, benzyl-butyl phthalate, and di-isononyl phthalate (Parks et al., 2000).

Our previous observation of in vitro and in vivo experiments revealed that DEHP has negative impact on the number of insulin receptors and glucose oxidation in cultured Chang liver cells and L6 myotubes (Rengarajan et al., 2007; Rajesh and
Balasubramanian, 2013). In addition, DEHP exposure affects glucose uptake and oxidation in rat gastrocnemius muscle & adipose tissue and is mediated through enhanced lipid peroxidation, impaired insulin signalling and GLUT4 expression in plasma membrane (Srinivasan et al., 2011; Rajesh et al., 2013). A recent study from our laboratory indicated that lactational exposure of DEHP impairs insulin signal transduction and glucose oxidation in the cardiac muscle of F1 female albino rats (Mangala Priya et al., 2014).

**Human exposure to DEHP and health outcomes**

Di-2(ethyl hexyl) phthalate (DEHP) & its metabolites were the dominant compounds, collectively accounting for >95% of the total concentrations in the samples from seven Asian countries [China, India, Japan, Korea, Kuwait, Malaysia, and Vietnam] (Guo and Kannan, 2011). Apart from these Asian countries, other countries (US, Europe etc..) also measured phthalate in human circulation and correlated with various diseases or disorders.

Human studies have found that phthalates are associated with many of the same effects that have been observed in laboratory studies, including alterations in sex hormone levels, feminization of male genitalia and alterations in semen quality (Lee and Koo, 2007). *In utero* exposure to phthalates including DBP and BBP has been associated with feminization of male genitalia with a shortening of the ano-genital distance (Swan et al., 2005). Post-natal exposure to the phthalate metabolites of DiNP and DBP in breast milk has been associated with alterations in male hormone profiles in baby boys (Main et al.,
In adult men, phthalate exposures have been associated with poor sperm quality and DNA damage (Hauser et al., 2004). Occupational exposures to DBP and DEHP have been shown to be associated with alterations in testosterone levels (Pan et al., 2006). DEHP metabolites in semen of infertile men were negatively correlated with sperm concentration and motility but positively correlated with percentage (%) of abnormal sperm and these adverse effects might be ROS, LPO and mitochondrial dysfunction mediated (Pant et al., 2008).

Studies have shown that women with endometriosis had significantly higher DnBP, BBzP, DEHP, dimethyl phthalate (DMP), DEP and di-n-octal phthalate (DnOP) levels than controls and these exposure level was strongly correlated with the severity of endometriosis (Reddy et al., 2006a; Reddy et al., 2006b; Roya et al., 2008; Roya et al., 2009).

**Human exposure to DEHP and glucose homeostasis**

Phthalates have also been associated with diabetes in a study of Mexican women which showed the levels of three types of DEHP metabolites higher in adult women with diabetes than those without diabetes. The results suggest that phthalate exposures may play a role in diabetes development (Svensson et al., 2011).

In a study of Swedish elderly people, researchers found that 3 of 4 types of phthalate metabolites were associated with type-2 diabetes prevalence. The phthalate metabolites linked to diabetes included MMP, MiBP, and MEP, which are breakdown products of phthalates found in body care products. MiBP was related to poor insulin secretion, while
MMP and MEP were related to insulin resistance. The phthalate metabolite MEHP, which is a breakdown product of the plasticizer DEHP, was not associated with diabetes (Lind et al., 2012). In one human study, levels of several phthalate metabolites were associated with increased insulin resistance and abdominal obesity in US men (Stahlhut et al., 2007).

In another study of U.S. people aged 6-80, various phthalate metabolites were associated with higher body mass index (BMI) and waist circumference in men aged 20-59. Effects in women were not as consistent. In some ages, exposure was associated with lower BMI (Hatch et al., 2008). In Chinese school children, levels of certain phthalates were associated with increased BMI or waist circumference (Wang et al., 2013a; Zhang et al., 2014). In a large US population, certain phthalate metabolites were associated with an increased risk of overweight/obesity and BMI in black children, but not children of other ethnic groups (Trasande et al., 2013a). In New York city children, certain phthalate exposures measured at age 6-8 were associated with a higher body mass index and waist circumference one year later (Teitelbaum et al., 2012).

**Effect of DEHP exposure during development**

Endocrine-disrupting chemicals (EDCs) have been hypothesized to contribute to the high prevalence of diseases such as obesity, hypertension, and diabetes mellitus (Grun and Blumberg, 2009; Heindel and vom Saal, 2009; Latini et al., 2010). EDC exposure occurs during all phases of life. Early EDC exposure in the developing embryo may cause permanent metabolic alterations (Fowler et al., 2012), thus potentially contributing to development of metabolic disorders later in life.
Numerous rodent studies have shown clear anti-androgenic effects caused by developmental exposure to DEHP at medium and high levels, i.e. exposure above 100 mg/kg (Gray et al., 2000; Parks et al., 2000; Jarfelt et al., 2005; Borch et al., 2006), but only few studies on rat have investigated DEHP at lower doses (Arcadi et al., 1998; Akingbemi et al., 2001; Andrade et al., 2006). Wei et al. (2012) showed that early-life DEHP exposure retards the development of glomeruli, resulting in a nephron deficit, and subsequently hypertension onset later in life through the renin-angiotensin system in offspring. Recently, it has been shown that the maternal exposure to the phthalate affects blood pressure of adult male offspring (Hashimoto et al., 2006).

In utero exposure to DEHP induces both short- and long-term changes in adrenal aldosterone production and decreases mineralocorticoid receptor expression in the adult testis. Further, they suggested that the decreased mineralocorticoid receptor (MR), possibly epigenetically mediated, is a novel mechanism by which phthalates may affect diverse functions later in life (Withers et al., 1998; Kulkarni et al., 1999; Kubota et al., 2000; Ueki et al., 2006).

Besides being a potential risk for reproductive health, possibly in females as well as in males, DEHP and its metabolite MEHP exerts metabolic effects by activating peroxisome proliferator-activated receptors (PPARs) α and γ, key mediators of lipid metabolism and adipogenesis (Lapinskas et al., 2005; Feige et al., 2007; Feige et al., 2010). It is because of their high prevalence, obesity and the related disorder dyslipidemia have become global health risks and are sometimes termed an “obesity pandemic.” In
addition, dyslipidemia is associated with both obesity and subfertility (Carr and Brunzell, 2004; Pasquali and Gambineri, 2006; Schmidt et al., 2012).

In an experiment, when pregnant mice were exposed to environmentally relevant doses of DEHP, their F₁ offspring showed increased body weight and visceral fat deposits. Although F₁ mice were exposed to DEHP only *in utero* and during lactation, they observed metabolic changes in female mice (Kulkarni et al., 1999). Tuttle et al. (2001) have shown that *in utero* exposure to DEHP promotes local adipose and chronic low-grade systemic inflammation in adult male rat offspring. Further, those animals showed elevated C-reactive protein and TNF levels with increased CD163 in adipose tissue.

Our recent findings showed that the lactational exposure of DEHP impairs insulin signal transduction and glucose oxidation in the cardiac muscle of F₁ female albino rats (Mangala Priya et al., 2014). It has been reported that developmental DEHP exposure disrupted the pancreas and altered whole body glucose homeostasis (Lin et al., 2011b). Furthermore, it has been shown that exposure to phthalates reduces fetal insulin level in rat (Boberg et al., 2008), which may lead to insulin resistance in adulthood (Holemans et al., 2003) and be a risk for diabetes.

Several lines of evidence have indicated that conditions experienced during early development play a powerful role in influencing both short and long-term health of individuals and programming (Daitoku et al., 2011) certain chronic diseases, including cardiovascular disease, metabolic disease and chronic kidney disease in adulthood.
(Kulkarni et al., 1999; Andrade et al., 2006; Ueki et al., 2006; Grande et al., 2007; Wu et al., 2009; Wu et al., 2010b; Hayashi et al., 2012; Xie et al., 2012; Hao et al., 2013; Kay et al., 2013; Mankidy et al., 2013; Zhang et al., 2013a; Hayashi et al., 2014; Kay et al., 2014; Veeramachaneni and Klinefelter, 2014).

Women who deliver prematurely have, on average, up to three times the phthalate level in their urine compared to women who carry to term (Zhang et al., 2009; Zhang et al., 2014). Studies reveal the presence of various phthalate monoesters in breast milk, including MEHP, the primary metabolite of DEHP, and presence of some of these phthalate monoesters seems to be correlated with increased levels of sex hormone-binding globulin (SHBG), luteinizing hormone (LH) and the ratio of LH/free testosterone in boys at 3 months of age (Main et al., 2006; Meeker et al., 2009; Fromme et al., 2011). Furthermore, Swan et al. (2005) have reported an association between phthalate exposure and shortened anogenital index (anogenital distance/weight) in infant boys whose mothers had elevated urinary phthalate metabolites during pregnancy. These studies clearly show that humans are exposed to phthalates during sensitive periods of development and indicate that exposure to phthalates may cause developmental effects in infants.
Studies have shown that DEHP is an endocrine disruptor and environmental toxicant as it affects endocrine glands, liver and kidney. Further, the incidence of type-2 diabetes is in the rising trend due to a mixture of factors including revolutionized lifestyle. There are epidemiological and experimental data demonstrating that exposure to DEHP has a negative influence on glucose homeostasis. Based on the available reports, it is hypothesized that DEHP exposure during gestation may impair glucose homeostasis in F₁ offspring as a result of endocrine pancreas dysfunction as well as defective insulin signal transduction and GLUT4 expression in the gastrocnemius muscle. Hence, the current investigation was designed to evaluate the effects of gestational exposure to DEHP on β-cell function and insulin signal transduction in gastrocnemius muscle of F₁ rat offspring. The first chapter deals with the gestational exposure to DEHP on pancreatic insulin gene expression, glucose-induced insulin secretion and insulin sensitivity in F₁ offspring. Whereas, the focus of second chapter is on insulin signalling molecules and glucose oxidation in gastrocnemius muscle. The third chapter is concerned with the gestational exposure to DEHP on GLUT4 epigenome in gastrocnemius muscle of F₁ offspring.
Type-2 diabetes mellitus (T2DM) is much more common than type-1 diabetes mellitus (T1DM). T2DM is generally associated with increased insulin resistance and often obesity, and is associated with certain susceptibility genes that tend to be different from the genes associated with T1DM (Raj et al., 2009). While T2DM is historically a disease seen in adults, children, even younger than 10 years old, are now developing it (Pettitt et al., 2014).

More and more children, meanwhile, have characteristics features of both T1DM and T2DM, including autoantibodies to β-cells, as well as signs of increased insulin resistance or obesity. Some researchers call this overlapping diabetes phenotype typical of both T1DM and T2DM as "double" or "hybrid" diabetes (Pozzilli et al., 2007; Pozzilli et al., 2011). In fact, a large number of people with diabetes may have both T1DM and T2DM associated processes contributing to their diabetes (Tuomi, 2005). Interestingly, one research group took another look at the diagnosis of children with diabetes (mostly type 1), after an average of 7 years. Twenty percentage of the patients had a different diagnosis that what was originally given, after reevaluation. And, 10% of the children were determined to have both type-1 and type- 2, a "mixed diabetes phenotype" (Lipton et al., 2011). A German study of children diagnosed with type-2 diabetes found that 15% tested positive for islet autoantibodies (Awa et al., 2013).

In US children diagnosed with diabetes, 55% could be classified as autoimmune and insulin sensitive (traditional type-1), and 16% as non-autoimmune and insulin resistant (traditional type-2). Almost 20% had autoimmunity and insulin resistance; signs
of both T1DM and T2DM, perhaps including those who are overweight with T1DM. Then 10% were non-autoimmune but insulin sensitive, perhaps indicating a different type altogether (Dabelea et al., 2011).

Insulin resistance and impaired β-cell function are hallmarks of T2DM. Many more people have insulin resistance than have T2DM, suggesting that insulin resistance is necessary but not sufficient to induce the onset of diabetes mellitus (Ferrannini, 1998; Kitamura, 2013). Genome-wide association studies have identified many susceptibility genes linked to diabetes mellitus, most of which are expressed in pancreatic β-cells and are thought to have roles in their function and growth (Grant et al., 2006; Miyake et al., 2008; Yasuda et al., 2008; Chadt et al., 2012). Furthermore, studies of postmortem and surgical pancreas specimens describe a 63% loss of β-cell mass in obese patients with T2DM, and a 41% loss in lean patients with T2DM, compared with weight-matched healthy individuals (Butler et al., 2003). Dysfunction of β-cell is, therefore, thought to have a primary role in the pathogenesis of T2DM.

**Development of endocrine pancreas in the rodent**

Development of the pancreas starts from a pool of common progenitor cells (multipotent endodermal progenitors) which will commit into duct cell, endocrine or acinar cell lineage (Pan and Wright, 2011; Arda et al., 2013). Thereafter, within the endocrine compartment, the cells will have to further differentiate into α, β, δ, PP and ε cells producing glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively (Murtaugh and Melton, 2003; Wilson et al., 2003; Jensen, 2004; Burlison et
This is regulated by the expression of distinct genes, under the control of a hierarchy of various and specific networks of transcription factors (Augustin, 2010; Arda et al., 2013).

The development of pancreas in rodents shows similarities to that in humans. However, while foetal β-cells are functioning as true endocrine cells at the end of the first trimester in human (Carruthers et al., 2009), this occurs only during the last third of gestation in rats. In response to signals coming from the mesodermal tissues, pancreatic morphogenesis begins at E9.5 days with two evaginations of foregut endoderm to form a dorsal, and then a ventral pancreatic bud. The dorsal and ventral buds fuse at E16.5. A branched structure is already distinguishable at E14.5 where endocrine cells can be identified by E15.5 (Jorgensen et al., 2007). The endocrine tissue is derived from epithelial duct cells (neogenesis). After divisions, the cells will form small clusters budding out the pancreatic ducts (Fowden and Hill, 2001). The vascularisation begins to invade these immature endocrine cell clusters that co-express several pancreatic hormones and neuropeptides and will become “islets of Langerhans” (Joost and Thorens, 2001; Rojas et al., 2010).

**Transcriptional control in endocrine pancreas development**

The classic way of specifying a particular cell fate of the pancreatic cell within the field of initially equivalent cells is the lateral specification mediated by the Notch-Delta serrate pathway (Murtaugh et al., 2003; Fortini, 2009; Guruharsha et al., 2012). In the pancreas, blocking the activation of the Notch receptor results in high Neurogenin-3 gene
expression, and promotes the endocrine fate. In contrast, cells with active Notch signalling adopt the acinar fate and/or remain as undifferentiated progenitor cells (Edlund, 2001). Pdx1 plays a central role in the β-cell differentiation (Seeberger et al., 2014). It initiates endocrine lineage commitment from cells within the pancreatic ducts (Reichert and Rustgi, 2011). It becomes restricted to differentiating β, δ and PP cell, during development and becomes restricted to mature β-cell. The final fate of individual endocrine cells is determined by the expression of a series of transcription factors specific for each type of endocrine cell. Some of them are early markers, such as Pax4, Nk2.2 and Nk6.1, co-expressed with neurogenin-3, others are late markers, such as Pax6, Isl1, Hb9 and Pdx-1 for the β cells (Augustin, 2010).

Peptide growth factors, such as PDGF, VEGF, FGF-7 that are expressed within the pancreatic stroma adjacent to the ductal epithelium contribute to endocrine cell formation and islet expansion (Lammert et al., 2001, 2003; Warzecha et al., 2004; Augustin, 2010; Nandy and Mukhopadhyay, 2011; Reusens et al., 2011). The ongoing proliferation and developmental differentiation of β cell, once formed is highly dependent on the expression of insulin like growth factors (IGFs) within the islets (Augustin, 2010; Nandy and Mukhopadhyay, 2011).

In rat and mouse, it is at the end of foetal period that the β-cell mass increases the most (Kaung, 1994). Following birth, this growth declines although the proliferation rate maintains. Therefore, a wave of apoptosis is suspected which should occur between 2 and 3 weeks postnatally (Scaglia et al., 1997). The loss of β cells during that time is
compensated by neogenesis. Although it was thought that in adult, new β cells derive mainly from stem cells, it seems now that they arise abundantly from pre-existing β cells that multiply (Dor et al., 2004). They may also be generated from trans-differentiation of acinar cell (Lardon and Bouwens, 2005). Thus, the β-cell mass at the end of development will determine the islet cell mass in adulthood. Anyway, the production of new β cells in adulthood is low. It is thus obvious that any deficiency occurring in utero or soon after birth will jeopardize the β-cell mass contributing to β-cell failure and favours glucose intolerance in adulthood (Prentki and Nolan, 2006; Augustin, 2010; Dumortier et al., 2011).

Transcription factors viz., MafA, Beta2 / NeuroD1, PDX1, Pax4, Pax6 and FoxO1 represent specific key players for early β-cell differentiation, mature β-cell function and identity. These factors cooperate to synergistically activate the transcription of insulin promoter in β-cell (Sosa-Pineda et al., 1997; Aramata et al., 2005; Cerf, 2006; Martinez et al., 2006; Daitoku et al., 2011; Hu He et al., 2011).

Musculoaponeurotic fibrosarcoma (Maf)

Pancreatic beta-cell-specific insulin gene expression is regulated by a variety of pancreatic transcription factors and the conserved A3, C1 and E1 elements in the insulin gene enhancer region are very important for activation of insulin gene (Kaneto et al., 2009b). Indeed, PDX-1 binding to the A3 element and NeuroD binding to the E1 element are crucial for insulin gene transcription. Transcription factor Maf also known as proto-oncogene c-Maf or v-Maf oncogene homolog is a transcription factor (Nishizawa et al.,
1989). MafA which is a basic-leucine zipper transcription factor of the AP-1 superfamily which binds towards C1 element region of insulin gene (Rocques et al., 2007) and functions as a potent transactivator (Kaneto et al., 2009b).

RIPE3b (in rat)/C1 (in human) is one of the most important cis-regulatory elements where Maf family members (MafA, MafB and c-Maf) regulates pancreatic-beta-cell-specific and glucose-regulated transcription of the insulin gene (Han et al., 2007). MafA and MafB were present within the nuclei of islet beta cells and not within pancreas acinar cells. Since MafA, MafB, and c-Maf are each capable of specifically binding to and activating insulin C1 element-mediated expression, it is suggested that all these factors play a role in islet beta-cell function (Matsuoka et al., 2003). The gene which encodes the RIPE3b/C1 activator has been cloned and identified as MafA (Kataoka et al., 2002). MafA was found to specifically localize in nuclei of insulin-positive cells and could activate the insulin gene promoter through interacting with Pdx-1 and Beta2 in a synergistic fashion. Additionally, up-regulation of MafA alone was sufficient to improve endogenous insulin mRNA levels (Matsuoka et al., 2003; Matsuoka et al., 2004).

Transcription factors are recruited to the insulin promoter region by MafA, and together with Pdx-1 and NeuroD1, this enables these transcription factors to exert strong synergistic effects and to markedly induce insulin gene expression (Kaneto et al., 2005). MafA, Pdx-1, and NeuroD1 also control glucose-regulated transcription of the insulin gene (Sharma and Stein, 1994; Marshak et al., 1996; Petersen et al., 1998a; Zhao et al., 2000). MafA was found to interact functionally with Pdx-1 and NeuroD1 to promote
synergistic activation of the insulin enhancer-driven reporter cell in non-β-cells and shown to play a direct and principal role in insulin gene activation in β-cell lines (Zhao et al., 2005a). MafA appears to act downstream to Nkx 6.1 and is only found in terminally differentiated β-cells (Matsuoka et al., 2004). MafA also regulates genes involved in beta-cell function such as Glucagon-like peptide 1 receptor, and prohormone convertase 1/3 (Aramata et al., 2007). MafA-deficient mice also display age-dependent pancreatic islet abnormalities. Further evidence revealed that insulin 1, insulin 2, Pdx1, Beta2 / NeuroD1, and GLUT2 transcripts are reduced in MafA-deficient mice. These results show that MafA is a key regulator of glucose-stimulated insulin secretion in vivo (Zhang et al., 2005) in adult β-cells. Neonatal β-cells do not secrete glucose-responsive insulin and are considered immature. Whereas, in vitro exposure of immature islets to triiodothyronine enhanced the expression of MafA, the secretion of glucose-responsive insulin, and the proportion of responsive cells, all of which are effects that were abolished in the presence of dominant-negative MafA. Thus, thyroid hormone can be considered a physiological regulator of functional maturation of β cells via its induction of MafA (Aguayo-Mazzucato et al., 2013). MafA is constitutively phosphorylated by GSK3β, and that phosphorylation is a prerequisite for rapid degradation of MafA under low-glucose condition. Thus, it is evident that glucose-sensing signalling pathway in islet beta cells that regulates insulin gene expression is mediated through the regulation of MafA protein stability (Han et al., 2007). MafA may qualify as a crucial master regulator of genes implicated in maintaining beta cell function, in particular the GSIS [Glucose stimulated insulin secretion] (Shao et al., 2009).
**Pancreatic and duodenal homeobox factor-1 (Pdx-1)**

Pdx-1 is considered to be the master transcription factor involved in early pancreatic development, β-cell differentiation and maintenance of the mature β-cell (Cerf et al., 2005). Pdx-1 has been reported to function in concert with other transcription factors in regulating the expression of insulin gene and several other islet-specific genes (German and Wang, 1994; Sharma and Stein, 1994; Naya et al., 1997; Kojima et al., 2003). Pdx-1 may be directly activated by the transcription factors NeuroD1 (Sharma et al., 1997), Hnf-1α and Hnf-3β (Ben-Shushan et al., 2001).

Pdx-1 directly regulates insulin gene transcription through formation of a complex with transcriptional co-activators on the proximal insulin promoter (Iype et al., 2005). The complex leads to enhancement of elongation by basal transcriptional machinery (Iype et al., 2005). The co-activator, p300, interacts with Pdx-1 and is believed to enhance insulin transcriptional activity through multiple mechanisms, including the recruitment and activation of components of the basal transcriptional machinery and histone/protein acetylation (Baynes, 1991; Qiu et al., 2002; Chakrabarti et al., 2003; Mosley et al., 2004; Qiu et al., 2004). Previous studies using the rat insulin promoter constructs showed that Pdx-1, E47 (Dumonteil et al., 1998) and NeuroD1 could interact synergistically to stimulate promoter activity (Glick et al., 2000). Pdx-1, MafA, and NeuroD1/E47 acting through the promoter proximal A1, C1, and E1 sites respectively, play an important role in maintaining basal promoter activity of insulin gene, with evidence of the transcription
factors having a synergistic effect on the human and rat insulin promoter (Stoffers et al., 2000).

In adult β-cells, Pdx-1 transactivates insulin (Ohlsson et al., 1993) and other genes involved in glucose sensing and metabolism (Kaneto et al., 2008) such as GLUT2 (Waebner et al., 1996), glucokinase (Watada et al., 1996), and Nkx 6.1 (Shih et al., 2002; Pedersen et al., 2005) genes. Additionally, specific removal of Pdx-1 in mice led to a severe diabetic phenotype due to β-cell dysfunction (Gannon et al., 2008). Importantly, heterozygous mutations in Pdx1 cause early and late onset forms of diabetes in humans (Khoo et al., 2012). These studies indicated that expression and/or activation of Pdx-1 in beta cells are reduced under diabetic conditions.

**Forkhead box O1 (FoxO1)**

Mammalian cells express four FoxO protein isoform: FoxO1, FoxO3a and FoxO4 (Daitoku et al., 2011) are transcription factors of the forkhead family which play critical role in cellular differentiation, proliferation, apoptosis and stress resistance, of which FoxO1 is the most abundant isoform in liver, adipose tissue and pancreatic β cells (Kitamura et al., 2002; Nakae et al., 2003). Further it is conventionally viewed as a regulator in glucose and lipid production in liver; food intake and cell differentiation in preadipocytes, myoblasts and vascular endothelium (Kitamura et al., 2002).

Emerging evidence shows that mediators and effectors of insulin receptor signalling, including insulin receptor substrate 2, PI3K, 3-phosphoinositolide-dependent protein kinase 1, forkhead box protein O1 and Akt kinases, have important roles in β-cell
growth and function (Withers et al., 1998; Kulkarni et al., 1999; Kubota et al., 2000; Tuttle et al., 2001; Hashimoto et al., 2006; Ueki et al., 2006).

FoxO1 is phosphorylated by Akt / PKB kinases, leading to its translocation from the nucleus to the cytoplasm, which in turn inactivates FoxO1 transcriptional activity (Datta et al., 1999; Kops and Burgering, 1999; Nakae et al., 1999). However, FoxO1 is also phosphorylated by other kinases, including mitogen-activated protein kinases (also known as JNKs), inhibitor of nuclear factor κB kinase (NFκB), and cyclin-dependent kinase 2 (Hu et al., 2004; Huang et al., 2006; Martinez et al., 2008). Whether these kinases contribute to regulate beta cell function through FoxO1 is remained to be explored.

The negative regulation of FoxO1 transcriptional activity by insulin and IGF-1 was through phosphorylation by PI3-kinase/Akt pathway. This resulted in the export of FoxO1 from the nucleus to the cytoplasm (Brunet et al., 1999). Furthermore, down-regulation of pancreatic islet glucokinase activity through suppressing IGF-1R expression was reported and PI3K/Akt/FoxO1 pathway was involved in it (Yoshida et al., 2007).

Additionally, Pdx-1 is a master regulator of β-cell growth and function and was reported to be transcriptionally regulated by another forkhead transcription factor FoxA2 (HNF3β) (Lee et al., 2002). FoxO1 and FoxA2 share common DNA-binding sites in the Pdx-1 promoter. Thus, binding of FoxO1 inhibits Pdx1 transcription, which results in reduced β-cell proliferation whereas FoxA2 activates transcription when bound to the Pdx1 promoter (Kitamura et al., 2002). However, unexpectedly, β-cell-specific FoxO1 knockout mice do not exhibit increased β-cell mass, and their glucose tolerance is
unaltered (Kobayashi et al., 2012). Moreover, it was reported that FoxO1 localized to cytoplasm in Pdx-1-positive beta cells. On the contrary, in Pdx-1-negative beta cells, FoxO1 localized in the nucleus (Kitamura et al., 2002). This mutual exclusion of FoxO1 and Pdx-1 was consistent and thus FoxO1 is a negative regulator of Pdx-1.

**Paired box (Pax)**

Paired box (Pax) genes are a family of genes coding for tissue specific transcription factors containing a paired domain and usually a partial or complete homeodomain.

**Pax 4**

Pax 4 is one of the key transcription factors involved in the formation of β-cells during pancreatic development and islet cell differentiation. Mutations in the Pax 4 gene are associated with T2DM (Shimajiri et al., 2001; Kanatsuka et al., 2002), with heterozygous mutations in the Pax 6 gene linked to glucose intolerance in human carriers of these mutations (Yasuda et al., 2002). Four of the MODY transcription factors, Hnf-4α (MODY1), Hnf-1α (MODY3), Pdx-1 (MODY4), and NeuroD1 (MODY6) interact with the Pax 4 regulatory region, thus the simultaneous expression of these transcription factors are required for efficient Pax 4 transcription and may play a role in regulating tissue-specific regulation of Pax 4 (Kemp et al., 2003). In a study using a rat glucagon-producing cell line, Pax 4 was shown to act as a repressor of glucagon gene expression (Petersen et al., 2000). Furthermore, Pax 4 can inhibit the insulin promoter in the absence of Pax 6, suggesting an active repression mechanism of Pax 4 (Petersen et al., 2000). The
Pax 4 gene promoter contains several binding sites for Pax 4 itself, suggesting that Pax 4 inhibits its own expression i.e. a strong negative autoregulatory effect (Smith et al., 2000). Pax4 protects adult islets from stress-induced apoptosis by suppressing selective nuclear factor-κB target genes while increasing Bcl-2 levels.

**Pax 6**

Pax 6 appears to be necessary for the correct execution of β-cell differentiation (Wang et al., 2004) and is essential for the normal expression of final differentiation markers such as insulin and GLUT-2 (Ashery-Padan et al., 2004). Pax 6 regulates the C2 element of the insulin gene (Sander et al., 1997). GLUT2 protein was not detected in the Pax 6-deficient pancreas suggesting that Pax 6 plays a role in GLUT-2 regulation (Ashery-Padan et al., 2004). Pax 6 has been proposed to regulate Pdx-1 expression as Pax 6-binding sites have been detected in the Pdx-1 promoter (Samaras et al., 2002). Pax6 is crucial for β-cells through transcriptional control of key genes coding for proteins that are involved in insulin biosynthesis and secretion as well as glucose and incretin actions on β-cells. Further they evidenced that new Pax6 target genes coding for GK, Nkx6.1, cMaf, PC2, GLP-1R and GIPR which are all involved in β-cell function (Gosmain et al., 2012). Pax6 represents a key element for adult maintenance of glucose homeostasis and function of the endocrine pancreas and mature β-cell function (Hart et al., 2013).

**HNF-4α (Hepatocyte Nuclear Factor)**

HNF-4α binds to over 1,000 promoter element in pancreatic islets or 45% of transcribed genes, suggesting the HNF-4α is a global regulator of β-cell transcription (Odom et al., 2004) for
glucose and lipid homeostasis (Yamagata et al., 1996; Stoffel and Duncan, 1997). Further, HNF-4α activate GLUT2 gene expression in embryonic stem cells (Stoffel and Duncan, 1997) and β-cells (Wang et al., 2000). Additionally cell-specific HNF-4α deletion leads to impaired glucose tolerance (Gupta et al., 2005).

**MicroRNAs : post transcriptional gene regulation**

MicroRNAs (miRNAs) are a family of endogenous highly conserved, small non-protein-coding RNA species of 19-28 nucleotides in length that regulate post-transcriptional gene expression by targeting 3' untranslated region (3' UTRs) of messenger RNA (mRNA) and generally function as negative regulators of gene transcription by inhibiting translation and/or causing mRNA degradation (Cuellar and McManus, 2005; Li et al., 2014b). It has been demonstrated to play a key role in numerous physiological, developmental and metabolic processes, and their deregulation has been implicated in disease pathology-including that of obesity and diabetes (Hennessy and O'Driscoll, 2008; Lynn, 2009; Pandey et al., 2009; Zhao et al., 2009; van de Bunt et al., 2013). The abnormal activity of various miRNAs involved in pancreatic gene regulation, insulin secretion, beta-cell differentiation and regeneration, for example miR-375, contribute to T2DM pathophysiology (Kolfschoten et al., 2009; Zhao et al., 2010; Fernandez-Valverde et al., 2011; Guay et al., 2011; Joglekar et al., 2011; Mao et al., 2013; Li, 2014; Pullen and Rutter, 2014).
miR-375

miR-375 is one of the first miRNAs identified in the pancreas (Poy et al., 2004), and remains one of the best characterised in terms of function. It is expressed in the pancreas and pituitary gland, organs linked by their role in hormone secretion, and expression levels increase during pancreas organogenesis (Avnit-Sagi et al., 2009). Loss-of-function studies showed that miR-375 is essential for β-cell formation in zebrafish (Kloosterman et al., 2007). miR-375 knockout mice have decreased numbers of β-cells and increased numbers of α-cells. The increase in glucagon level, combined with the reduction in insulin level, results in hyperglycaemia. This shows the importance of miR-375 in the establishment of normal pancreatic cell mass through the targeting of a group of genes which control cellular growth and proliferation in the developing pancreas (Poy et al., 2009).

Overexpression of miR-375 suppresses glucose-induced insulin secretion, and conversely, inhibition of endogenous miR-375 enhanced the insulin secretion. Insulin secretion is modified by miR-375, independent of changes in glucose metabolism or intracellular Ca²⁺-signalling but correlated with a direct effect on insulin exocytosis. Myotrophin (Mtpn) was predicted to be and validated as a target of miR-375. Inhibition of Mtpn by small interfering (si)RNA mimicked the effects of miR-375 on glucose-stimulated insulin secretion and exocytosis (Poy et al., 2004). Further, it has been shown that miR-375 target the myotrophin (V1) (MTPN) gene, which encodes the myotrophin protein, that regulates hormone release and exocytosis (Li et al., 2010). miR-375 also
lowers the level of 3'-phosphoinositide-dependent protein kinase-1 (PDK-1) gene (El Ouaamari et al., 2008). Additional evidence for the involvement of miR-375 in pancreas development includes the fact that its expression is regulated by several transcription factors important in pancreatic development and function, including HNF6, INSM1, Ngn3, NeuroD1, and Pdx1 (Keller et al., 2007; Li, 2014).

**Insulin gene expression**

Insulin is a critical hormone in the regulation of blood glucose levels. It is produced exclusively by pancreatic islet beta-cells. Beta-cell-enriched transcription factors, such as Pdx1, MafA, Pax4, Pax6, Beta2 / NeuroD and FoxO1 have roles in the activation of insulin gene promoter establishing beta-cell-specific insulin expression, and in the regulation of beta-cell differentiation (Aramata et al., 2007). In the adult, insulin is expressed almost exclusively in the β-cells of the pancreatic islets of Langerhans (Melloul et al., 2002), hence its name from Latin *insula* or “island.”

In the β-cell, sophisticated mechanisms have evolved to control insulin expression at the correct time and place during embryonic development (Halban et al., 2014). In the adult, related mechanisms and a variety of signalling pathways are involved in restricting insulin expression to β-cells and in coordinating insulin expression in response to diverse afferent signals (Melloul et al., 2002). Positive and negative crosstalk between the various signalling pathways, formation of homo- or heterodimers permitting individual transcription factors to act as activators, nonactivators or repressors, reversible phosphorylation of transcription factors, multiple isoforms of several transcription factors,
and synergistic interactions between certain combinations of transcription factors extend the gamut of signals influencing the regulation of insulin gene expression (Hay and Docherty, 2006; Halban et al., 2014).

Insulin transcriptional control is conferred by cis-acting regulatory sequences located within 300-400 bp from the transcription start site (German et al., 1995), which bind β-cell restricted and ubiquitous transcription factors (Melloul et al., 2002). Rat pancreatic beta-cell-specific expression was shown to be controlled by enhancer sequences lying between nucleotides -342 and -91 relative to the transcription start site (Whelan et al., 1989). The compact nature of the insulin promoter results in the close proximity of regulatory elements that can bind an extensive range of factors thereby permitting a multiplicity of outcomes through additive and synergistic interactions between the bound proteins (German et al., 1992; Glick et al., 2000; Qiu et al., 2002).

Insulin is a peptide hormone which consists of two polypeptide chains, the A- and B- Chains, linked together by disulfide bonds (Cecil and Weitzman, 1964). It is however first synthesized as a single polypeptide called preproinsulin in pancreatic β-cells. The preproinsulin becomes proinsulin in the rough endoplasmic reticulum (Ronald et al., 2005). The proinsulin undergoes maturation into active insulin through the action of cellular endopeptidases known as prohormone convertases (PC1 and PC2), as well as the exoprotease carboxypeptidase (Steiner and Oyer, 1967). The endopeptidases cleave at 2 positions, releasing a fragment called the C-peptide, and leaving 2 pairs of basic residues that are removed by the carboxypeptidase and the B- and A- chains, linked by 2 disulfide
bonds (Ronald et al., 2005). The resulting insulin is packaged inside mature granules (Suckale and Solimena, 2010).

**β-Cell Stimulus-Secretion Coupling**

Pancreatic β-cells are often referred to as “fuel sensors,” continually monitoring and responding to circulating nutrient levels, under the modulation of additional neurohormonal signals, in order to secrete insulin to best meet the needs of the organism (Newsholme et al., 2010). β-cell nutrient sensing involves notable metabolic activation, resulting in production of coupling signals that promote insulin biosynthesis and secretion (Nolan and Prentki, 2008; Newsholme et al., 2010; Newsholme and Krause, 2012). The primary stimulus of insulin secretion is glucose, and islet β-cells are particularly responsive to this important nutrient secretagogue, coupling metabolic and other stimuli with the insulin-secretory machinery (Waeber et al., 1997; Wollheim and Maechler, 2002; Pasternak et al., 2012; Meloni et al., 2013; Nie et al., 2013).

Elevation in blood glucose concentration results in rapid rise in intracellular glucose levels as glucose is transported across the β-cell plasma membrane. Glucose uptake and metabolism are two essential steps in the so-called “glucose-stimulated insulin secretion” (GSIS) pathway (Jensen et al., 2008; Huang and Joseph, 2014). GSIS represents the increase in insulin secretion over basal release in response to increased extracellular, and ultimately intracellular, glucose (Schuit et al., 2001; Fridlyand and Philipson, 2010; Fridlyand and Phillipson, 2011; Luni et al., 2012; Meloni et al., 2013). Glucose rapidly enters β-cells, through specific glucose transporters (GLUT1 in humans;
GLUT2 in rodents) (De Vos et al., 1995), after which it is swiftly phosphorylated by the enzyme glucokinase, which has a high Km for glucose (Wang and Iynedjian, 1997). These primary steps, particularly glucokinase, determine the rate of glucose utilization by the β-cell over a range of physiological glucose levels (3–20 mM) and the combination of transport and phosphorylation determines metabolic flux through glycolysis (Schuit et al., 2001; Fridlyand and Philipson, 2010; Porat et al., 2011).

β-cells in the islets of Langerhans release insulin in two phases. The first phase release is rapid triggered in response to increased blood glucose levels. The second phase is a sustained, slow release of newly formed vesicles triggered independently of glucose. (Schuit et al., 2001; Zhao and Keating, 2007). Glucose enters glycolysis and respiratory cycle, where multiple, high-energy ATP molecules are produced by oxidation, leading to a rise in the ATP: ADP ratio within the cell which in turn closes the ATP-sensitive potassium channel. This prevents potassium ions (K+) from leaving the cell by facilitated diffusion, leading to a buildup of potassium ions. As a result, the cytosolic portion of the cell becomes more positive with respect to the outside, leading to the depolarisation of the cell surface membrane. This depolarization opens voltage-dependent calcium channels (VDCC) (Seino et al., 2011) to increase the amplitude of free cytosolic Ca^{2+} levels ([Ca^{2+}]), which triggers fusion of the insulin granules with the cell membrane and the release of insulin, C-peptide and other molecules into the circulation by exocytosis (Boyd, 1992).
Early studies of EDC focused on identifying chemicals with the capacity to modulate sex steroid and thyroid hormone signalling; however, recent studies support that some chemicals may disturb signalling pathways critical for energy homeostasis (EPA, 1997). Phthalate exposure has been documented in pregnant women, breast milk and newborn (Main et al., 2006; Berman et al., 2009; Enke et al., 2013; Ferguson et al., 2014; Sathyanarayana et al., 2013; Tefre de Renzy-Martin et al., 2014; Cantonwine et al., 2014), but its impact on the development and function of β-cells are yet to be studied in detail. In this regard, Lin et al. (2011b) have shown that developmental exposure to DEHP gives rise to β-cell dysfunction and the whole body glucometabolic abnormalities in the rat.

β-cells of the pancreatic islets are highly specialized and high-throughput unit for the production of insulin, the key hormone for maintenance of glucose homeostasis (Magro and Solimena, 2013). Evidences indicate that genetic and environmental factors can lead to hyperglycemia, dyslipidemia, inflammation, and autoimmunity, resulting in β-cell dysfunction (Stahnke et al., 2014), thereby triggering the pathogenesis of T1DM / T2DM (Fu et al., 2013). DEHP exposure in critical periods of development can be a potential risk factor, at least in part, for developing diabetes. Considering the importance and limited research on human metabolic outcome as a result of phthalates exposure during β-cells development the present study was designed to evaluate the effect of gestational exposure of DEHP on the pancreatic β-cell function in rat F1 offspring. This chapter deals with the gestational exposure to DEHP on pancreatic insulin gene expression, glucose-induced insulin secretion and insulin sensitivity in F1 offspring.
Objectives

➢ To assess the effect of gestational DEHP exposure on glucose tolerance and insulin sensitivity in F₁ offspring.

➢ To identify the effect of gestational DEHP exposure on the expression of factors involved in the regulation of β-cell function.

Parameters studied

➢ Body weight, fat mass, lean body weight & pancreatic wet weight.

➢ Oral glucose tolerance & insulin tolerance.

➢ Serum insulin, pancreatic insulin concentration and insulin mRNA.

➢ MafA, Pax4, Pax6 & HNF-4α proteins.

➢ Pdx1, FoxO1(mRNA and protein) & pFoxO1Ser256 protein.

➢ GLUT2 (mRNA and protein) & Glucokinase (mRNA and enzyme activity).