Chapter 1

Atherogenic and anti-atherogenic genes in white adipose tissue of db/db mice, an animal model of obese diabetic and insulin resistance

1.1 Introduction

Research from recent decades has drawn a completely new picture of adipose tissue as an endocrine and secretory organ that is subject to complex regulation and involved in multiple pathologies [Miner, 2004]. In addition, visceral fat is thought to have a greater impact on lipid & glucose metabolism and cardiovascular health than subcutaneous fat [Atzmon et al., 2002].

Adipose tissue is both a dynamic endocrine organ, as well a highly active metabolic tissue. Fat produces and secretes inflammatory factors, which are well known to play important roles in insulin resistance, type 2 diabetes and atherosclerotic process. Collectively, these factors are called adipocytokines or adipokines. These include TNF alpha, leptin, plasminogen activator inhibitor-1 (PAI-1), IL-6, resistin, and angiotensinogen [Ahima & Flier, 2000]. Serum adipokine levels are elevated in humans and animals with excess adiposity [Yudkin et al., 1999; Samad et al., 1996 & 1997; Zhang et al., 1996], and visceral fat appears to produce several of these adipokines more actively than subcutaneous adipose tissue [Dusserre et al., 2000; Fried et al., 1998; Eriksson et al., 2000; Giacchetti et al., 2002]. The secreted FFAs, IL-6 and TNF alpha cause insulin resistance in adipocytes through activation of several serine-threonine kinase pathways, such as IKK beta and JNK1, which interfere with insulin signaling via serine phosphorylation and subsequent inactivation of IRS-1 [Kyriakis & Avruch, 2001; Aguirre et al., 2000]. In addition, IKKβ and JNK1 activate transcription factors that increase expression of cytokines and inflammatory genes [Yuan et al., 2001; Kim et al., 2001; Hiroshumi et al., 2002]. This vicious cycle amplifies inflammation and accentuates insulin action. Another key signaling molecule, Toll like receptors (TLRs) were demonstrated to be key molecules in the induction of inflammation in response to microbial products [Medzhitov, 2000] and dietary lipids [Lee et al., 2001]. A recent report suggested that the activation of TLR4 via saturated fatty acids promotes insulin resistance [Shi et al., 2006]. Furthermore Poulain- Godefroy et al reported that TLR4 receptor regulates the maintenance of preadipocyte status and inflammatory

environment encountered in inflamed white adipose tissue [Poulain- Godefroy & Froguel, 2007]. Reduction in fat mass correlates with decrease in the serum levels of many of proinflammatory adipokines [Dandona et al., 1998; Ziccardi et al., 2002; Primrose et al., 1992; Folsom et al., 1993; Itoh et al., 2002], implying that approaches designed to promote fat loss should be useful in attenuating the proinflammatory milieu associated with obesity.

Since major function of the adipose organ is to store excess energy as triglycerides, particularly under conditions of nutrient excess [Flier, 1995] in response to prolonged periods of calorie excess, the adipose organ may become overloaded and unable to recruit new fat cells, resulting in adipose tissue hypertrophy of existing fat cells and increased ectopic fat deposition in tissues such as skeletal muscle, liver, myocardium, and pancreas. There is clearly evident in Pima Indians with larger abdominal fat cells who are insulin resistant and more likely to develop diabetes than those with smaller fat cells [Paolisso et al., 1995; Weyer et al., 2000]. While it is believed that excess adipose tissue is associated with insulin resistance. It is also now recognized that a lack of adipose tissue is similarly associated with insulin resistance and increased risk for development of type 2 diabetes [Reitman et al., 1999; Shimomura et al., 1999; Kim et al., 2000]. Similarly, in humans, lipodystrophy is associated with insulin resistance and often type 2 diabetes [Garg, 2000]. Recently, Danforth [Danforth, 2000] proposed that impaired adipocyte proliferation and differentiation may cause the progressive filling of existing adipocytes, leading to overflow of excess calories as fat into other tissues and insulin resistance. Thus, it is hypothesized that insulin resistance and eventually full-blown type 2 diabetes can be triggered by a failure of new adipocytes to differentiate because of increase in proinflammatory condition.

To explore the role of adipokines and cytokines in development of metabolic syndrome, extensive transcriptional profiling studies were performed in white adipose tissue (WAT) taken from mice with obese diabetic (db/db) or lean C57BL/6 mice. In addition, tracked the transcriptional regulation of several genes

involved in adipocytes differentiation CAAT/enhancer-binding protein (C/EBP) alpha, PPAR gamma, adiponectin, adipsin and visfatin) and representative inflammation genes (TNF alpha, IL-6, COX-2 and TLR-4 in WAT with progression of obese (adipose mass) in db/db and their lean mice.

Selection of animal model: db/db mice have a mutation in the leptin-receptor gene, resulting in deletion of the cytoplasmic tail needed for intracellular signaling [Lee et al., 1996]. They became obese and develop hyperinsulinemia, hyperglycemia, and insulin resistance [Halaas et al., 1995; Lee et al., 1996]. At four weeks of age animal became obese while no significant change in glucose however twelve weeks old became obese and significant increase in glucose and insulin.

1.2. Materials and Methods

1.2.1 Animals

All animal experiments were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, using protocols approved by the Institutional Animal Ethics Committee. White adipose tissue and blood samples were obtained from 4 and 12-week-old female mice from the following strains: db/db (n=6) and C57BL/6 (n=6) mice. Mice were housed in individually ventilated cages and given pelleted food (Standard Rodent diet, NIN, Hyderabad, India) and water ad libitum, and were maintained at 25 ± 3°C and 50–70% humidity with a 12 h light–dark cycle.

1.2.2 Collection of adipose tissue and blood

Animals of each group were anaesthetized using ether as anaesthesia, the abdomen was opened and the periovarian, retroperitoneal, mesenteric and subcutaneous fat pads were removed and weighed [Remesar et al., 2002]. Samples of retroperitoneal WAT were flash frozen in liquid nitrogen for quantitative real-time PCR (qRT-PCR) analysis. Blood samples were collected and serum was separated and stored at –70°C for biochemical estimation.

1.2.3 RNA analysis and quantitative real-time PCR

Samples of WAT were homogenized in TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) using a Mixer 301 (Retsch, Haan, Germany) and total RNA was extracted following the manufacturer's protocol. Then, 1 µg total RNA from each sample was taken for first-strand cDNA synthesis using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). An equal amount of cDNA from each sample was taken for qRT-PCR using ABIprism- 7300 real time PCR macine. FAM-labelled Taqman probes viz. C/EBP alpha, PPAR gamma, fatty acid binding protein (aP2), lipoprotein lipase (LPL), adiponectin, adipsin, COX-2, TLR-4, TNF-alpha and IL-6 (all from Applied Biosystems). Taqman Universal Mastermix (Applied Biosystems) was used for expression profiling of the aforementioned target genes.

1.2.4 Quantitation PCR method for visfatin was optimized in house

The optimal primer concentration of visfatin for qRT-PCR was determined using the following combinations of forward and reverse primers: 50/50, 50/300, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300 and 900/900 nmol/l. The concentration resulting in the lowest cycle threshold and best amplification efficiency was selected and used for qRT-PCR experiments (forward and reverse primers both 900 nmol/l). Amplification efficiency was determined by amplifying the different cDNA concentrations (10–100 ng) with the selected combination of forward and reverse primer. VIC-labelled mouse beta actin probe was co-amplified in each sample with every target gene(s) to normalise the results.

1.2.5 Histology

Tissue from animals was fixed in 10% formalin for 48h. Sectioning of the tissue was done with the help of cryo-microtome. The slides were stained with routine H& E staining method. Stained slides were mounted with DPX.

1.2.6 Serum measurements

Serum triglyceride (Pointe scientific, USA) levels were determined by the colorimetric method using biochemical kit. Serum glucose levels were determined by the glucose oxidase/peroxidase (GOD/POD) method using a commercially available kit (Ranbaxy Laboratories, Gurgaon, India). Insulin (Linco Research Inc., St Charles, MO, USA) and leptin (B Bridge, Mountain View, CA, USA) levels in the serum were determined by ELISA according to the manufacturers' protocols.

1.2.7 Data and statistical analysis

The data were expressed as the mean ± SEM. Comparisons were made with the one-way analysis of variance (ANOVA) followed by Tuckey test, with p values < 0.05 being considered statistically significant. Student t test was also used when two groups were compared in case of biochemical parameters.

1.3 Results

1.3.1 Comparison of body weights, adiposity and blood data between two age groups of db/db and C57BL/6J mice

The db/db mice of both age group had a significantly ($P<0.05$) higher adiposity as well as body weight when compared to their lean C57BL/6 mice (Table 1.1). As shown in table-1, four weeks old db/db mice had significant ($P< 0.05$) higher triglyceride and insulin levels but no significant difference on blood glucose concentrations as compared to C57BL/6, however twelve weeks old db/db mice had significantly ($P<0.05$) higher in triglyceride, glucose and insulin concentration when compared to the age matched C57BL/6 mice (Table 1.2). Twelve weeks old db/db mice had a significant ($P<0.05$) higher in visceral adiposity and body weight associated with triglyceride, glucose and insulin concentration compared to four weeks old db/db mice (Fig 1.1, Table 1.2). The leptin levels in db/db mice were significantly higher when compared with the lean C57BL/6 mice. However,

there was no significant change in leptin levels between the four and twelve week's old db/db mice.

Table 1.1 Serum biochemical parameters of four weeks old C57BL/6 and db/db mice.

Metabolic Parameters	C57BL/6 mice	db/db mice
Triglyceride (mg/dl)	75.6 ± 1.16	121.3 ± 6.72 *
Glucose (mg/dl)	96.2 ± 5.58	114.09 ± 7.41
Insulin (ng/ml)	0.21 ± 0.06	2.19 ± 0.21 *
Leptin (ng/ml)	3.2 ± 0.56	5.1 ± 0.63
Body weight (g)	11.8 ± 0.07	18.9 ± 1.15 *

Values are described as mean ± SEM. * $P < 0.05$ as compared to age matched C57BL/6 control. # Lean C57BL/6 mice had extremely small fat depots that were difficult in measure and quantify.

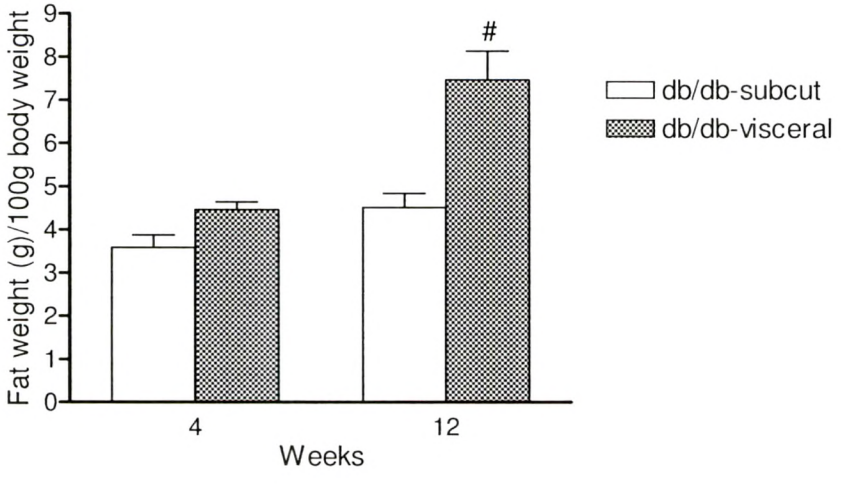

Fig 1.1: Analysis of the weight of different adipose tissue from 4 and 12 weeks old db/db mice. Each value represents mean ± S.E.M. (n = 6 mice/group). # $P < 0.05$ compared with 4 weeks old db/db mice.

Table 1.2 Serum biochemical parameters of twelve weeks old C57BL/6 and db/db mice.

Metabolic Parameters	C57BL/6 mice	db/db mice
Triglyceride (mg/dl)	79.5 ± 1.1	132.8 ± 15.4 *
Glucose (mg/dl)	120.5 ± 6.2	430.8 ± 21.4 *#
Insulin (ng/ml)	0.21 ± 0.06	2.40 ± 0.19 *
Leptin (ng/ml)	89.2 ± 10.3	120.1 ± 5.6
Body weight (g)	25.4 ± 2.1	41.6 ± 2.2 *#

Values are described as mean ± SEM. * $P < 0.05$ as compared to age matched C57BL/6 control. # $P < 0.05$ when compared to 4 weeks old db/db.

1.3.2 Expression of genes involved in adipogenesis in the white adipose tissue (WAT) of C57BL/6 and db/db mice:

To examine the effect of adiposity on adipogenic markers, I determined the mRNA levels of classic markers of adipogenesis. Visceral adipose tissue of 4 weeks old db/db mice (early obesity) have lower mRNA expression of C/EBP α, PPAR gamma, aP2, LPL, adiponectin and visfatin, compared with the same age group of lean C57BL/6 mice. Among all the adipogenic markers, the lowest expression in WAT of db/db mice was found in C/EBP alpha which is a master regulator of adipogenesis. The C/EBP alpha mRNA levels in the diabetic mice were significantly ($P < 0.05$) lower (11.3 folds) as compared to the age matched (4 weeks) C57BL/6 mice. Suppression of C/EBP alpha mRNA expression was saturated even at 4 weeks old db/db mice when compared with 12 weeks old (Fig 1.2). PPAR gamma is another nuclear receptor involved in adipogenesis and possessing anti-inflammatory and insulin-sensitizing properties, was lowered (2.3 folds) significantly in 4 weeks old db/db mice when compared with age matched C57BL/6 mice (Fig 1.3).

Further, PPAR gamma mRNA levels in the 12 weeks old diabetic mice were significantly lower as compared to the age matched C57BL/6 mice ($P < 0.05$). In twelve weeks old db/db mice had a further reduction in the expression of PPAR gamma in WAT when compared with the 4 weeks old db/db mice.

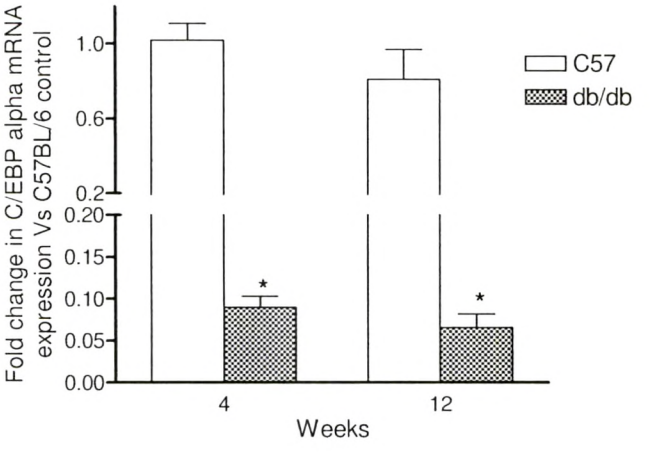

Fig 1.2: Analysis of the expression of C/EBP alpha mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with age matched C57BL/6 wild type mice.

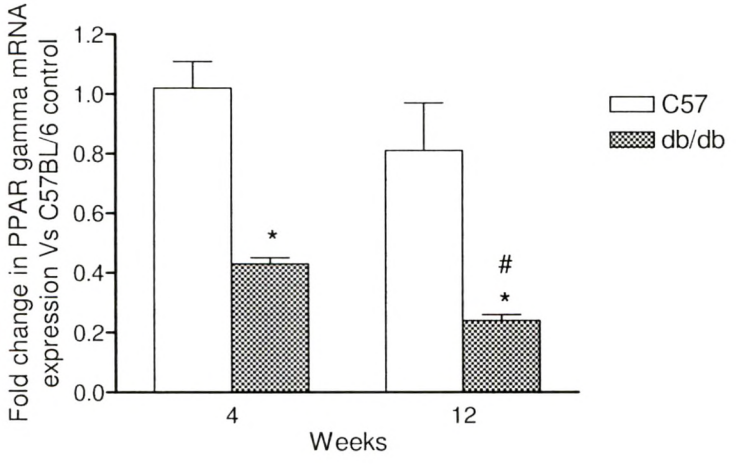

Fig 1.3: Analysis of the expression of PPAR gamma mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * P <

0.05 compared with age matched C57BL/6 wild type mice. # P < 0.05 compared with 4 weeks old db/db mice.

Further, expression of aP2, LPL and adiponectin which are target gene of PPAR γ were investigated. The expression of all adipocyte markers such as LPL, aP2 and adiponectin in WAT were lowered in db/db mice of both age group when compared to the same age group of their C57BL/6 mice. The aP2, LPL and adiponectin mRNA levels in the 4 weeks old db/db mice were significantly (8.5, 2.2 and 4.1 folds) lower respectively as compared to the age matched C57BL/6 mice (P< 0.05) (Fig 1.4, 1.5, 1.6). These expressions were further reduced in the 12 weeks old obese diabetic db/db mice when compared to their 4 weeks old db/db mice. However, in 12 weeks old C57BL/6 mice have higher adiponectin, adipsin and visfatin levels as compared to their 4 weeks old mice (Fig 1.6, 1.7, 1.8).

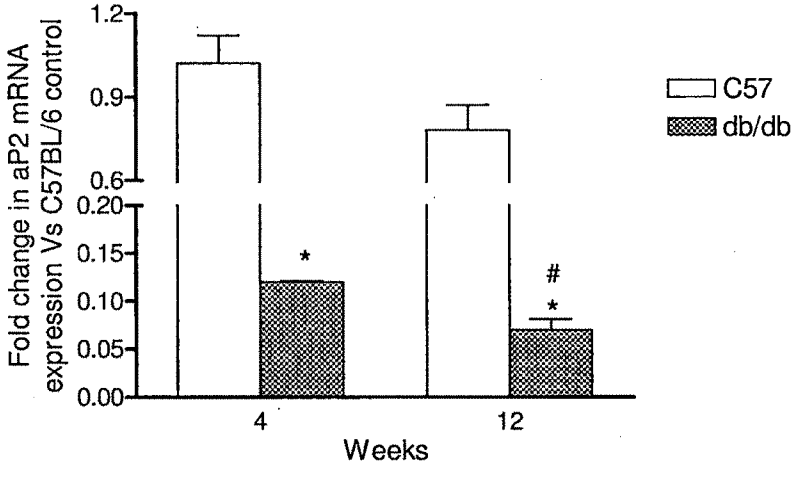

Fig 1.4: Analysis of the expression of aP2 mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (*n* = 6 mice/group). * P < 0.05 compared with age matched C57BL/6 wild type mice. # P < 0.05 compared with 4 weeks old db/db mice.

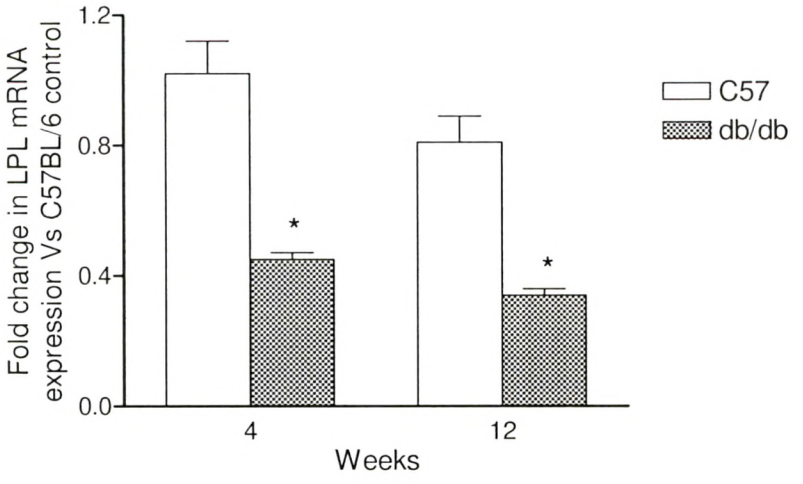

Fig 1.5: Analysis of the expression of LPL mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with age matched C57BL/6 wild type mice.

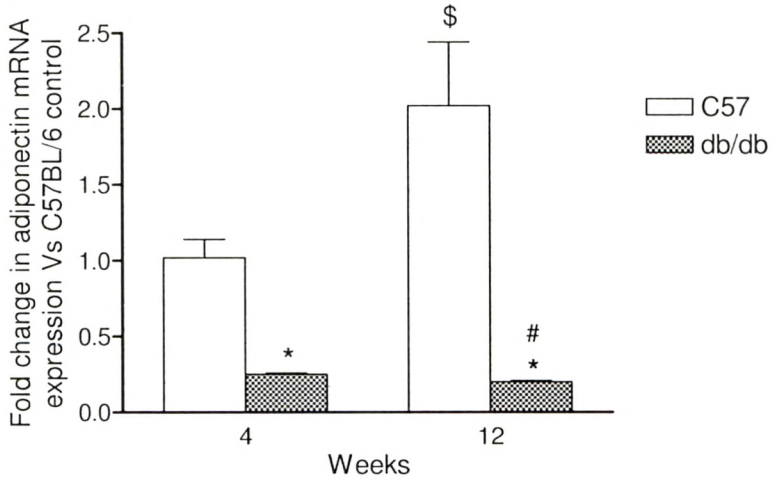

Fig 1.6: Analysis of the expression of adiponectin mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with age matched C57BL/6 wild type mice. # $P < 0.05$ compared with 4 weeks old db/db mice. $ $P < 0.05$ compared with 4 weeks old C57BL/6 mice.

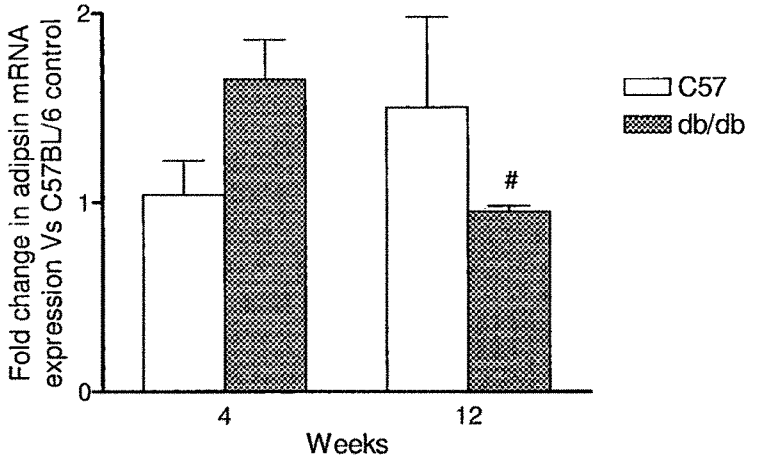

Fig 1.7: Analysis of the expression of adipsin mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). # $P < 0.05$ compared with 4 weeks old db/db mice.

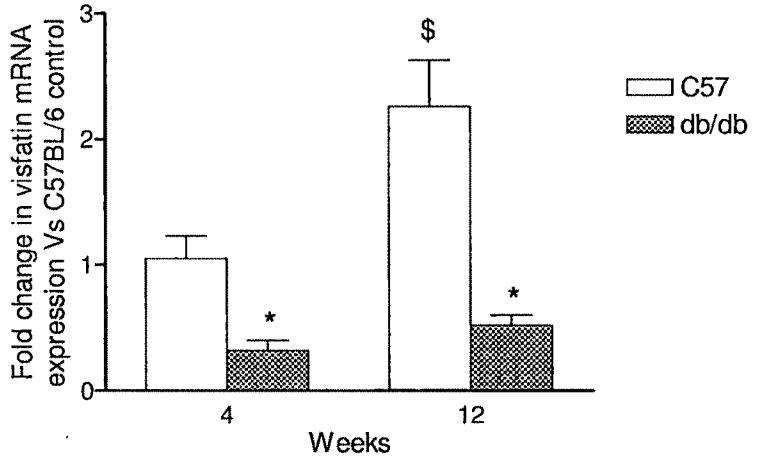

Fig 1.8: Analysis of the expression of visfatin mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with age matched C57BL/6 wild type mice. $ $P < 0.05$ compared with 4 weeks old C57BL/6 mice.

1.3.3 Expression of inflammatory molecules in the white adipose tissue of C57BL/6 and db/db mice

To determine whether increased adipose tissue mass exhibit increase in expression of inflammatory molecule which may affect the adipogenic potential. We determined the mRNA levels of classic markers of inflammation and found that visceral adipose tissue from 4 weeks old db/db mice have no change in the mRNA expression of IL-6, TNF-α, and COX-2 compared with those from same age group of lean mice (Fig. 1.9, 1.10, 1.11). Further we investigated the expression of TLR-4 a target gene which is involved in adipose inflammation. There was no significant difference between the adipose expression of TLR-4 of 4 weeks old db/db and C57BL/6 mice (Fig. 1.12).

However, higher expression of IL-6, TNF alpha, COX-2 and TLR-4 mRNA in WAT were found in 12 weeks old db/db mice, compared with age matched C57BL/6 mice (Fig. 1.9, 1.10, 1.11, 1.12). TNFalpha, IL-6 and COX-2 mRNA levels in the diabetic mice were also significantly higher (7.0, 4.5 and 2.1 folds) respectively as compared to 4 weeks old db/db mice ($P < 0.05$). Parallel to the increased levels of cytokine gene, adipose TLR-4 expression was significantly increased in 12 weeks old db/db mice when compared with 4 weeks old db/db mice.

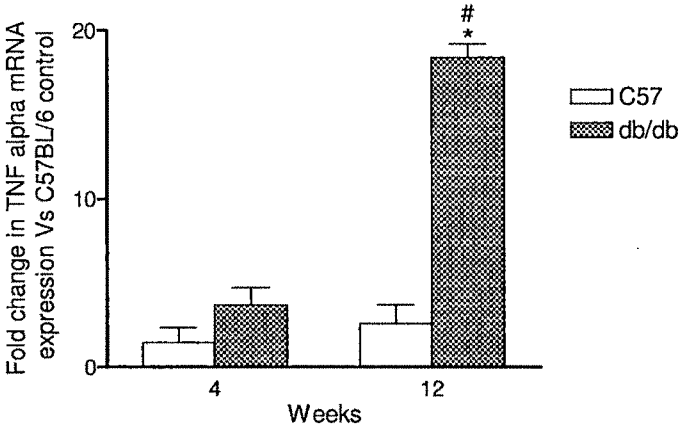

Fig 1.9: Analysis of the expression of TNF alpha mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P <$ 0.05 compared with age matched C57BL/6 wild type mice. # $P < 0.05$ compared with 4 weeks old db/db mice.

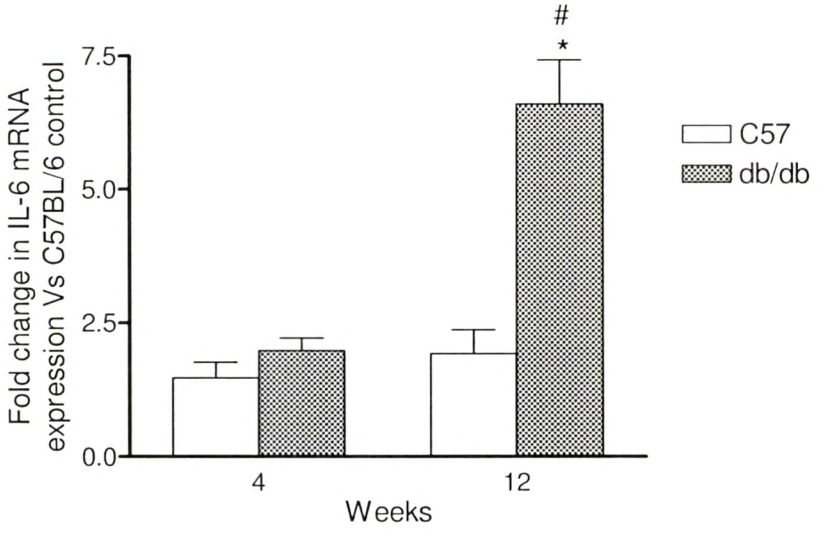

Fig 1.10 Analysis of the expression of IL-6 mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with age matched C57BL/6 wild type mice. # $P < 0.05$ compared with 4 weeks old db/db mice.

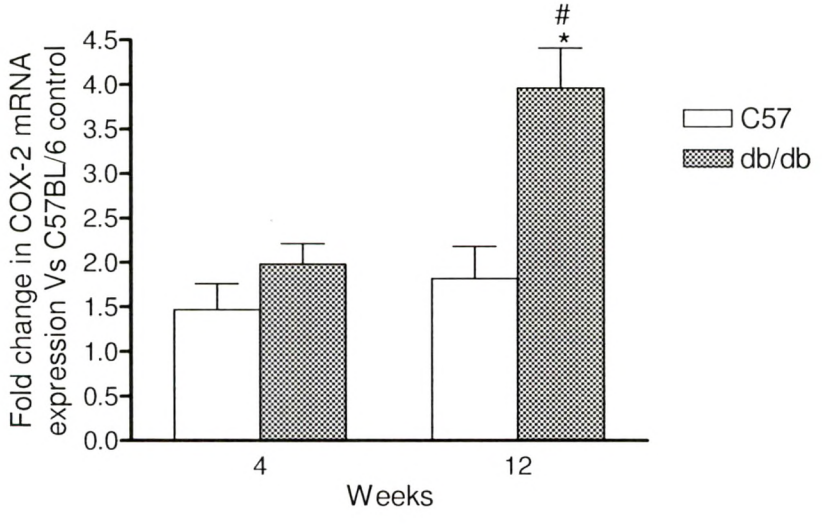

Fig 1.11 Analysis of the expression of COX-2 mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with age matched C57BL/6 wild type mice. # $P < 0.05$ compared with 4 weeks old db/db mice.

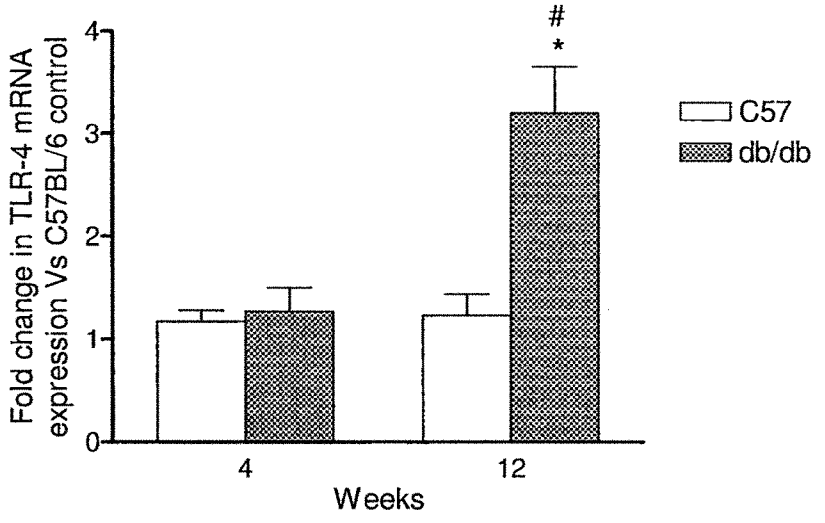

Fig 1.12: Analysis of the expression of TLR-4 mRNA in WAT of C57BL/6 and db/db mice at 4 and 12 weeks old. Fold change in all groups against the 4 weeks old C57BL/6 mice is represented in the bar diagram. Each value represents mean ± S.E.M. (*n* = 6 mice/group). * P < 0.05 compared with age matched C57BL/6 wild type mice. # P < 0.05 compared with 4 weeks old db/db mice.

1.3.4 Morphological changes of WAT

Hematoxylin and eosin (H&E) stains of mice showed a decreased number of adipocytes in visceral depots in the db/db mice of both age group when compared to the their same age group C57BL/6 mice, reaching statistical significance indicating that there was a significant increase in size of the adipocytes in db/db mice. Interestingly, few larger cells are appeared in 12 weeks old db/db mice but this was not significant when compared to 4 weeks old db/db mice. H & E stains did not show any macrophage present in WAT either in C57BL/6 or db/db mice of different age group. (Fig. 1.13)

1.3.5 Histopathological Examination

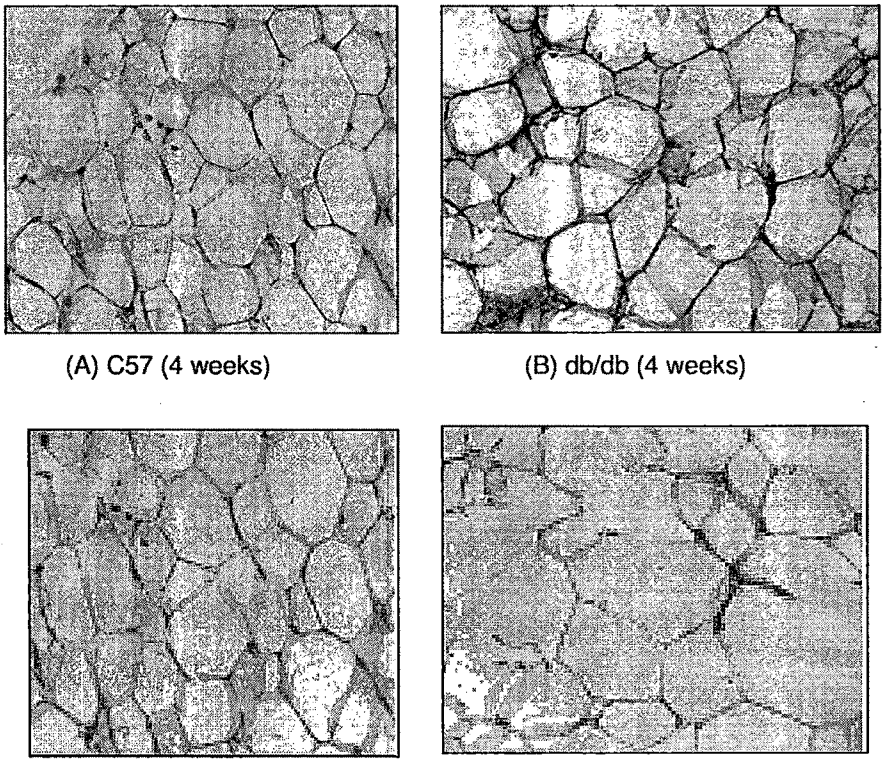

Figure 1.13. Hematoxylin and eosin stains of visceral adipose tissue of mice showing (magnification ×20). (Panel A) C57BL/6 (4 weeks) animal (Panel B) db/db (4 weeks) animal (Panel C) C57BL/6 (12 weeks) animal (Panel D) db/db (12 weeks) animal

1.4 Discussion:

Adipocytes serving to store lipids also function as an endocrine tissue by secreting adipokines, a number of inflammatory molecules believed to cause metabolic complications including insulin resistance [Rabe et al., 2008]. The incidence of T2D and cardiovascular risk increases with progression of obesity with aging. Here, it is hypothesized that secretion of these pro-inflammatory adipokines by adipose tissue may be up-regulated with aging and their up-regulation may affects the adipogenic potential. To evaluate the correlation

between aging associated adiposity, levels of pro-inflammatory cytokines and markers of adipogenesis, adipokine genes profiling in two different age groups were performed in genetically obese diabetic db/db mice, and compared them with the same age group of lean C57BL/6 mice.

Higher visceral and subcutaneous white adipose tissue mass associated with increase in elevated levels of triglyceride and insulin but not glucose was observed in db/db mice at early age (4 weeks) compared to C57BL/6 mice. Increase in adipose tissue mass may arise from an increase in the number of adipose cells as a consequence of an enhanced adipogenesis or an increase in triglyceride storage in the preexisting adipocytes. It has been reported that C/EBP alpha and PPAR gamma [Tontonoz et al., 1994; Rosen et al., 2000; Cao et al., 1991; Shao et al., 1997] are two master genes that control adipocyte differentiation [Wu et al., 1999]. In our current study, lower mRNA expressions of C/EBP alpha and PPAR gamma were observed in db/db mice. Reduction in mRNA expression of PPAR gamma and C/EBP alpha in WAT supports an overall impairment of adipogenesis. In addition to that there was a decrease in matured adipocyte marker gene such as aP2, LPL and adiponectin mRNA expressions in WAT. These reduced expression matured adipocyte marker gene is likely to be resulted from the reduced expression of adipogenic genes PPAR gamma. PPAR gamma may be the main causative event of the coordinate impairment in the expression of genes encoding components of adipocyte metabolism or adipokines. Various adipokines such as adiponectin, adipsin [Sandouk et al., 1993; Wilkison et al., 1990], visfatin [Kralisch et al., 2005] were expressed at very low levels in pre-adipocytes and increased after differentiation *in vitro*. Lower expression levels of adiponectin and visfatin were found in WAT of db/db mice. Parallel reduction in expression of adipogenic transcription factors, mature adipocyte markers and adipokines indicate that the observed increase in adipose tissue mass in db/db mice is not due to an enhancement of pre-adipocyte differentiation into adipocytes. The increase in adipose tissue mass observed in db/db mice is accompanied by a cellular hypertrophy as evidenced by the enlargement of adipocytes in db/db mice when compared with C57BL/6.

Therefore, it conclude that the adipose tissue mass is more likely due to an increased triglyceride accumulation in the preexisting adipocytes rather than an increase in cell number or differentiation in db/db mice. To find out the molecular mechanism of reduced adipogenic potential in db/db mice, we investigated the expression of various inflammatory cytokine TNF alpha and IL-6 expression augmented due to increase in adipose mass and known to inhibits PPAR gamma and C/EBP alpha are key transcriptional factors for initiating adipocyte differentiation [Tontonoz et al., 1994; Rosen et al., 2000; Cao et al., 1991; Shao et al., 1997]. However no such over expression of TNF alpha, IL-6, COX-2 and TLR-4 in WAT of 4 weeks of db/db mice which act as anti-adipogenic [Cawthorn et al., 2007; Poulain- Godefroy et al., 2007; Chu et al., 2009 & 2010] were observed. It is likely that the increase in fat mass in early obesity in db/db mice is via the inhibition of adipogenic gene transcription, rather than the stimulation of anti-adipogenic gene expression. Increase in the leptin levels in db/db mice may be one of the possible explanations for the decrease in adipogenesis in early obesity.

However, in late stage of obese animals, significant upregulation of all the inflammation markers tested, TNF alpha, IL-6, COX-2 and TLR-4 in WAT was observed. An elevated level of glucose was observed in db/db mice when compared to lean C57BL/6 mice which were positively correlated with the expression of pro-inflammatory cytokines in WAT. Interestingly, there was significant higher in visceral adipose mass but not subcutaneous fat when compare to the 4 weeks old db/db mice which is positively correlated with enhanced the proinflammatory status. There is a handful literature suggesting that visceral adiposity confers more cardiovascular risk than peripheral adiposity. The comparison of pre-liposuction and post-liposuction values showed that removal of subcutaneous fat did not significantly alter insulin sensitivity, or change the plasma concentrations of CRP, IL-6, tumor necrosis factor-alpha, or adiponectin. It also did not produce significant changes in blood pressure, plasma glucose, insulin, or lipid concentrations those are the marker of cardiovascular disease. On the other hand, a number of studies have shown that

a reduction in visceral fat in animals and humans is associated with increased in insulin sensitivity, HDL cholesterol, and decreased in triglyceride and blood pressure [Thorne et al., 2002; Garbriely et al., 2002; Barzilai et al., 1999; Brochu et al., 2003]. In the present study, higher in visceral adiposity associated with 12 weeks old db/db mice emphasizing risk to cardiovascular disease.

These TNF-α and IL-6 are the mostly secreted from macrophages however, absence of macrophage in the H&E fat stain suggesting that the released cytokines are purely from adipose tissues. TNF alpha and IL-6 are the inflammatory cytokine that has been shown to be markedly increased in adipocytes of obese animals, and its neutralization by a soluble TNF alpha receptor leads to improved insulin sensitivity in these animals [Hotamisligil et al., 1993]. Few studies have found that TNF alpha can activate the Wnt signal during the early stages of adipogenesis and suppresses C/EBP alpha and PPAR gamma [Gustafson et al., 2006]. In the current study, suppression of C/EBP alpha mRNA expression in WAT was saturated even at 4 weeks old db/db mice. The enlargement of adipocyte was also observed at 4 weeks old db/db mice emphasizing that decrease in adipogenic potential even at 4 weeks db/db mice where there was no significant up regulation of pro inflammatory genes. An elevated level of inflammatory molecules in WAT of 12 weeks old db/db mice may further reduce the expression of PPAR gamma gene in adipose tissue. Adipogenic marker aP2, LPL, adiponectin and visfatin levels in WAT were also lowered in late stage (12 weeks) of db/db mice compared to the early (4 weeks) age of db/db mice. Furthermore, adipsin mRNA expression was decreased significantly as compared to the early age of db/db mice. Similarly Flier et al reported that, adipsin mRNA suppressed in two strains of genetically obese mice (db/db and ob/ob), compared to their wild type counterparts [Flier et al., 1987]. Therefore, elevated levels of TNF alpha and IL-6 and reduced levels of proinflammatory PPAR gamma in adipose tissue might be an important contributor of insulin resistance and higher incidence of T2D and cardiovascular risk as indicated by the elevated level of glucose, triglyceride and insulin levels in

db/db mice. Increase in TNF alpha, IL-6 are associated with the mRNA expression of TLR-4 and down regulation of anti-inflammatory PPAR gamma in WAT of db/db mice which further emphasizing the involvement of proinflammatory state with insulin resistance and type 2 diabetes and thus the metabolic syndrome.

The results of this study have clearly demonstrated that obese db/db mice have altered adipocytes released cytokine profile. There are numerous mechanisms by which obesity can adversely affect the vasculature and thereby increase cardiovascular complications. Systemic inflammation and the production of adipokines by adipose tissue may be considered as important mechanisms for the adverse effects of adiposity on the vessel wall. Released by adipose tissue these adipokines can target the liver through portal circulation, and, through changes in liver-derived lipoproteins, clotting factors and inflammatory factors impact the atherogenic environment of the vessel wall. In addition, adipokines have been shown to influence gene expression and cell function in endothelial cells, arterial smooth muscle cells, and monocytes/macrophages which are the major cell types of the artery wall and are key components for defending vessel wall homeostasis. IL-6 one of the adipose derived cytokine might play a key role in the development of coronary disease through a number of different mechanisms; metabolic, endothelial and coagulant. IL-6 increases the release of adhesion molecules by the endothelium and increases the hepatic release of fibrinogen, as well as having procoagulant effects on platelets. TNF-alpha is known to influence endothelial cell function [Bhagat and Vallance, 1997] and another study suggests that IL-6 may also induce endothelial expression of chemokines and adhesion molecules [Romano et al., 1997]. Thus the higher mRNA expression of these cytokines in adipose tissue may create atherorogenic condition. Further, the effects of these cytokines on triglyceride metabolism might further impair endothelial generation of nitric oxide, as consequence of non-esterified fatty acids [Robinson et al., 1995; Steinberg et al., 1997]. Both IL-6 and TNF alpha have been reported to inhibit LPL and stimulate lipolysis [Van Snick,

1990; Hardardo et al., 1994; Greenberg et al., 1992]. We also observed a lower expression of all adipocytes markers (aP2, LPL) and larger in adipocytes associated with db/db mice. The adipocytes markers such as aP2, CD36 and LPL are expressed in both adipocytes and macrophage. On the basis of in vitro studies it has been reported that expression of LPL, aP2, CD36 by the macrophage may promote foam cell formation and atherosclerosis and mice deficient of these genes are protected from atherosclerosis. So it is hypothesized that the abnormal regulation in adipogenic markers in macrophage in obese condition may promotes pro-atherogenic condition. Furthermore, PPAR gamma activators were reported to promote preadipocyte differentiation from aortic precursors [Reyes and Lazalde, 2007]. Perivascular/adventitial adipocytes have been reported to contribute to vascular tone and vascular pathology [Soltis and Cassis, 1991]. It is likely that perivascular/adventitial adipocytes may react like adipocytes present in other depots and such a dysregulation of the secretion adipogenic markers in aortic adipocytes may promotes maximum deposition of fat into adipocytes and accelerate the pro-atherogenic environment. This hypothesis, however, needs to be vigorously tested in different animal models of obesity, and other adipose tissue depots in contact with cardiovascular organs such as pericardial and pervascular.

Adiponectin an another adipokine In contrast to inflammatory cytokines such as contrast to IL-6 and TNF alpha, serum level adiponectin are significantly lower in men with coronary artery disease [Kumada et al., 2003]. Further, adiponectin levels were inversely correlated to progression of coronary artery calcium in both diabetic and nondiabetic subjects. Adiponectin levels after treatment with insulin sensitizers (PPAR gamma activators) have also been shown to be the best predictor of an improvement in carotid arterial wall stiffness in a group of subjects with type 2 diabetes [Araki et al., 2006]. Treatment of apoE-deficient mice with an adiponectin adenovirus has been shown to reduce plaque formation in the aortic sinus by 30% [Matsuzawa et al., 2004]. As might be expected based on the above observations, adiponectin may promote an antiatherogenic and antiinflammatory program of gene expression and function in

vessel wall cells [Chen et al., 2003; Motoshima et al., 2004]. A potential anti-inflammatory role of adiponectin is indicated by reduction of atherogenic lesions by PPAR gamma activators, which raise adiponectin levels.

In summary, I report, that an inflammatory state is developed within adipose tissue with aging as evidenced by higher expression of classic inflammatory mediators (TNF alpha, IL-6, COX-2 and TLR-4) and lower expression of the anti-inflammatory nuclear receptor PPAR gamma and adiponectin at 12 weeks old db/db mice. This state of inflammation ~~associated with~~ associated with higher visceral adiposity further increase the risk for cardiovascular disease. If adipocytes present in aorta react like adipocytes in other depots, a dysregulation of the secretion of these proinflammatory molecules can then be expected. Thus dysregulated local secretion of vasoactive and inflammatory molecules in obesity may play an important role for cardiovascular pathophysiology.