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Regulation of glucose metabolism by Alox8

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REGULATION OF GLUCOSE METABOLISM BY *Alox8*

By

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AN ABSTRACT OF THE THESIS OF

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Type II diabetes is one of the leading cause of morbidity in the U.S. and other parts of the world. Insulin resistance which precedes Type II diabetes is a complex state of the body where the body fails to respond to insulin. Its complexity lies in its multifactorial origin that is to say various environmental and polygenic components come into play. Here we try to dissect one of these components – '***Alox8***' in transgenic mice and try to see if it affects blood glucose homeostasis. Comparison of glucose tolerance and insulin sensitivity among sixteen mice comprising of six wild type, five heterozygous and five knockout mice with respect to ***Alox8*** gene showed that wild type mice had relatively more glucose tolerance than knockout mice and this corresponded with relatively more insulin sensitiveness of wild type mice with respect to the knock out. However, these findings were not significant statistically at p=0.05. In search of any relevant biological significance, periodic acid schiff staining of the liver sections from these mice in three independent repeated experiments revealed that the knockout phenotype led to accumulation of glycogen deposits as compared to the wild type mice an indication of insulin resistance.

Taken together, our data suggests that these findings when extrapolated to human which carries ***ALOX15B*** instead of mice orthologue ***Alox8***, could lead to a benefit of administration of

lower doses of insulin in the wild type phenotype as compared to its polymorphic alleles carrying individuals.

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ABBREVIATIONS

AA/ AAc -arachidonic acid

AGEs- Advanced glycation end products

ANOVA- One- way analysis of variance

AUCs- Area under curves

cAMP -cyclic adenosine monophosphate

COXs -Cyclooxygenases

cPLA2α- Phospholipase A_2

CRE - cAMP- response element

CYP- Cytochrome P450

cysLTs - cysteinyl-LTs

DAG - Diacylglycerol

DHETs -vicinal-dihydroxyeicosatrienoic acids

EETs - Epoxyeicosatrienoic acids

8-(*S*)-HPETE -8-hydroperoxy-5,9,11,14- eicosatetraenoic acid

FFA - Free fatty acid

5(*S*)-HETE- 5(*S*)-hydroxy-6,8,11,14- eicosatetraenoic acid

FLAP - 5-LO activating protein

GAD_{65}- Glutamic acid decarboxylase

GSIS - Glucose stimulated insulin secretion

Gsk3-Glycogen synthase kinase 3

HE – Hematoxylin and eosin

HODE - Hydroxyoctadecadienoic acid

HPETE - Hydroperoxyeicosatetraenoic acid

IDDM- Insulin dependent diabetes mellitus

IFG - Impaired fasting glucose

IGF - Type I insulin-like growth factor

Igf1r- Type I insulin-like growth factor receptor

IGT – Impaired glucose tolerance

iNOS - Inducible nitric oxide synthase

Insr - Insulin receptor

IPITT - Intraperitoneal Insulin tolerance test

Irr - Insr-related receptor

IRS - Insulin receptor substrate

KO- Knockout

LACUC - Committee on the Use and Care of Laboratory Animals

LDL- Low density lipoprotein

LOXs- Lipoxygenases

LTs - Leukotrienes

LX - Lipoxin

MAPK- Mitogen activated protein kinases

PAS - Periodic acid-schiff

PASD- PAS- diastase

PG- Prostaglandin

PGD2- Prostaglandin D_2

PGDS- PGD-synthase

PGE_2- Prostaglandin E_2

PGES- PGE-synthase

$PGF_{2\alpha}$- Prostaglandin $F_{2\alpha}$

PGFS- PGF-synthase

PGI_2-Prostacyclin

PGIS- Prostacyclin-synthase

PI3K - Phosphatidylinositol-3-kinase

PKC - Protein kinase C

PMNs- Polymorphonuclear leukocytes

PPARs - Peroxisome proliferator activated receptors

PUFAs - Polyunsaturated fatty acids

ROS - Reactive oxygen species

sEH- soluble epoxide hydrolase

13-HPODE - 13- hydroperoxyoctadecadienoic acid

13-(*S*)-HODE- 13-hydroxyoctadecadienoic acid

20-HETE - 20- hydroxyeicosatetraenoic acids

Tx- Thromboxane

TxA2- Thromboxane A_2

TxAS- Thromboxane A-synthase

TZDs -Thiazolidinediones

WT- Wild type

CHAPTER 1

INTRODUCTION

1.1) Metabolism of Arachidonic acid

"Eicosanoids" (Greek: eikosi – twenty) are metabolites of 20 C polyunsaturated fatty acids (PUFAs) generated primarily due to the action of three distinct enzymes : Lipoxygenases (LOXs), Cyclooxygenases (COXs) and Cytochrome P450 (CYP) which we collectively propose to be called as " Eicoxygenases" on arachidonic acid (AA) (1). The biosynthesis of eicosanoids in mammalian cells generally begin due to the activation of phospholipase A_2 (cPLA2α) which releases the substrate AA from sn-2 position of glycerophospholipids (2, 3) of the cell membrane (1, 4, 5) in response to various stimuli (6) that usually occur under inflammatory conditions (5) such as growth factors , cytokines (5,7) oxidative stress, complement C5b-9, hypoxia, mechanical stretch, endothelin, angiotensin II, vasopressin (7) and wound (8) (figure 1).

LOX enzymes occur in various forms of life including bacteria (9, 10), fungi, plants and animals (11) but not in archaea (12). LOX (EC. 1.13.11.12) consists of single polypeptide chain of MW ~ 75-80 kDa (12) folded into two domains: a noncatalytic N-terminal β- barrel domain and a catalytic C-terminal domain (10, 12). The non-heme iron is held in the catalytic domain of the enzyme by interaction with conserved histidines (H361, H366, H541, H545) and a conserved carboxylic group of isoleucine moiety at the C- terminal end (11,12). Oxidation of the iron to ferric state is required for catalysis (12), figure 2.

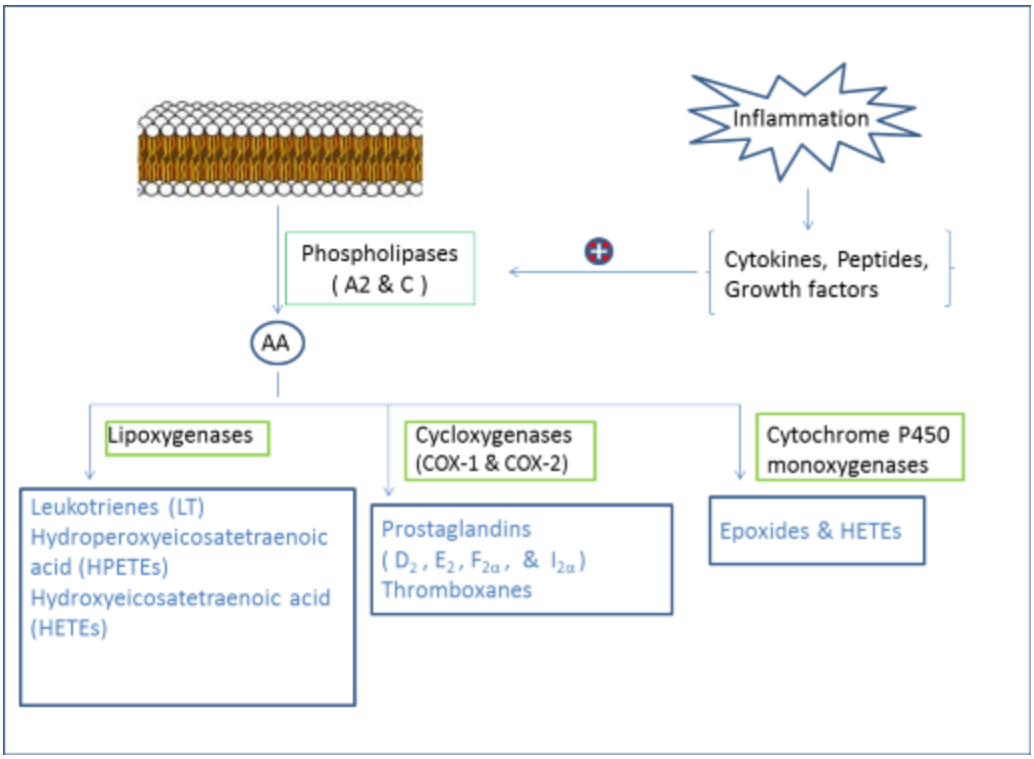

Fig.1 Fate of AA due to the action of " eicoxygenases" .

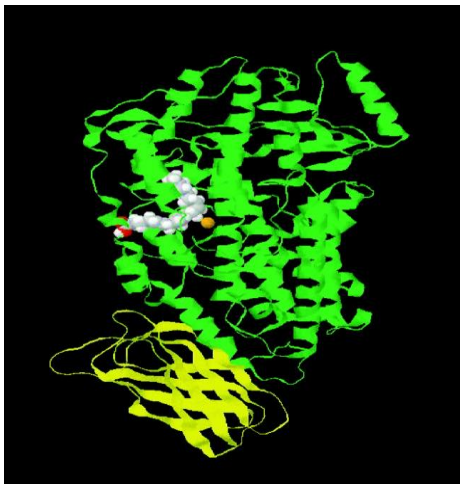

Fig. 2 A 3D structure of 15-LOX showing N-terminal β- barrel domain and a C-terminal catalytic domain and a non heme iron shown as a purple ball (Adapted from Kuhn *et al*., 2002).

Seven genes encode LOXs in mouse whereas six homologues and an expressed pseudogene (***ALOX12P2***) exist in humans (5, 12, 14) figure 3.

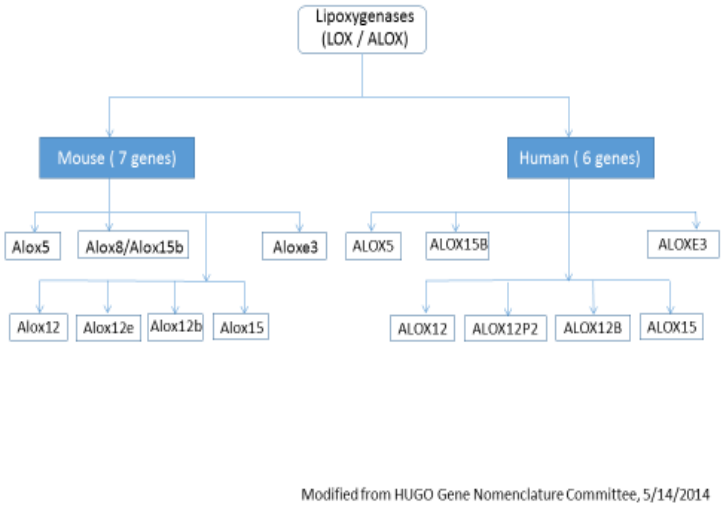

Fig. 3 Comparison of lipoxygenase gene family between two species viz. Mouse and Human.

These non-heme iron containing enzymes carry out the insertion of hydroperoxy group (13) into *cis* double bonds (12) or 1Z, 4Z- pentadiene moieties of polyunsaturated fatty acids (10) like AA (7, 15) and linoleic acid (14) with varying stereoconfiguration (*S* or *R*) (16). According to the position of insertion in AA, these are classified as 5-LO, 8-LO, 12 LO and 15-LO (15, 16, 17). The initial reaction of LOXs with AA, results in corresponding hydroperoxyeicosatetraenoic acid (5-, 8-, 12- or 15- HPETE) whereas with linoleic acid (LA) 9- or 13-hydroperoxyoctadecadienoic acid are produced which undergoes further reduction by glutathione peroxidase to yield hydroxyeicosatetraenoic acid (5-, 8-, 12- or 15- HETE) (1) and hydroxyoctadecadienoic acid (9-or 13-HODE) respectively (9, 19) .

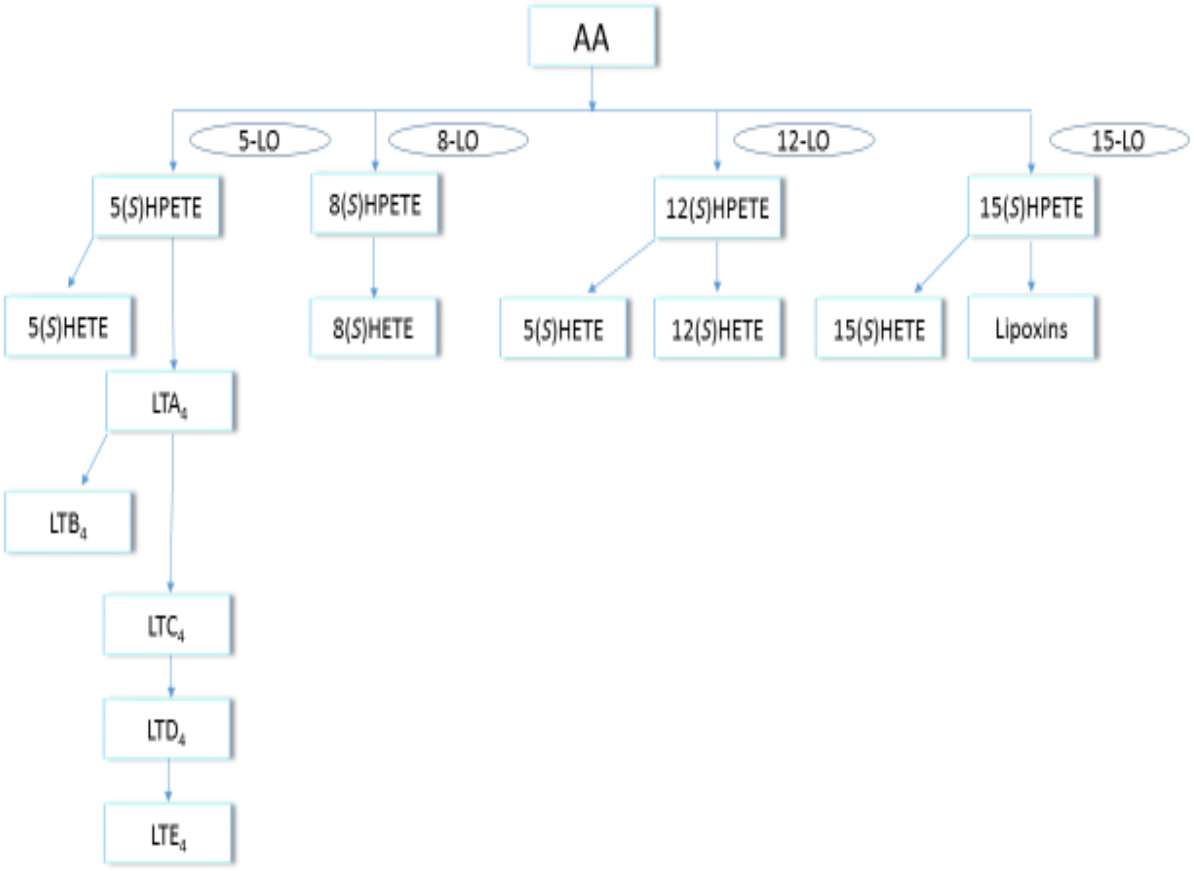

Fig. 4 Metabolism of AA via Lipoxygenase pathway.

LOX generated products act in an autocrine or paracrine fashion via interaction with G protein coupled cell surface receptors and nuclear receptor – peroxisome proliferator activated receptors (PPARs) (18). Activation of LOXs have been noted in numerous health conditions like atherosclerosis (14), diabetes (19, 20) and vascular remodelling (14). Based upon carcinogenicity, LOXs are classified as procarcinogenic which includes 5-, 8-, and 12- LO or anticarcinogenic (15-LO-2) (16). Hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs) are the two major metabolites derived from LOX action upon AA (3, 15) . HETEs can activate protein kinase C (PKC) and mitogen activated protein kinases (MAPK) and thereby activating

key transcription factors that promotes expression of genes involved in growth and inflammation (7).

The 5-LO which with the help of 5-LO activating protein (FLAP) interacts with AA (4,12,15) and metabolizes the latter into 5- (*S*)-HPETE (5-hydroperoxyeicosatetraenoic acid) which under further metabolism yields 5(*S*)-HETE (5(*S*)-hydroxy-6,8,11,14- eicosatetraenoic acid) (7) and LTA_4, a precursor of downstream LTs (4). LTA_4 under hydrolysis yields LTB_4. LTA_4 further can conjugate with glutathione to form cysteinyl-LTs (cysLTs), LTC_4, LTD_4 and LTE_4 (1,15, 21). As the name suggests, leukotrienes are the major products in white blood cells like eosinophils, monocytes/ macrophages, neutrophils and mast cells and they exert proinflammatory effects by inducing production of proinflammatory cytokines and also recruiting inflammatory cells in nearby tissues (1,15).

12-LO oxygenates polyunsaturated nonesterified fatty acid to produce 12-HPETE and 12-HETE (22). Three different types of 12- LO exists viz. leukocyte, platelet and epidermal (12, 15) which differ in sequence, catalytic activities and function (12). 12-LO is often designated by its stereoconfiguration as in 12*R*-LOX or 12*S*-LOX (12). 12(*S*) - HETE is a major LOX product in the pancreatic islet where it induces β-cell death (15). 12-LO products promote atherogenesis by enhancing the interaction of monocytes with vascular endothelium and participates in oxidative modification of lipoproteins and membrane lipids (24).

15-LO-1 (reticulocyte/ leukocyte 15-LO-1) metabolises AA into 15(*S*) HETE and lipoxin (LX) and occurs in polymorphonuclear leukocytes (PMNs), reticulocyte, eosinophils and airway epithelial cells (18). With LA, the product is 13-HPODE (13- hydroperoxyoctadecadienoic acid) and 13-(*S*)-HODE (hydroxyoctadecadienoic acid) (19, 20). One remarkable feature of 15-LO-1 is that it can convert LTA_4 into LXs (1). LXs are believed to be involved in wound healing and

resolution of inflammation unlike other eicosanoids which provoke the inflammatory process (1). Mammalian 15-LO-1 is known to play a significant role in the maturation of red cell by causing peroxidation of membrane lipids and thereby inducing structural changes. Besides it is also known to initiate atherosclerosis by oxidation of low density lipoprotein (LDL) (12). 15-LO-1 preferentially, does the conversion of linoleic acid to 13(*S*)-HODE and is known to have procarcinogenic role (16).

15-LO-2 (epidermis type) unlike 15-LO-1 converts AA exclusively to 15(S)-HpETE and 15(S)-HETE and occurs in prostate, skin and lung (23), cornea but absent in pheripheral blood leukocytes (11). 15-LO-2 shows only a partial sequence homology to 15-LO-1 and is remarkably distinct from the latter when it comes to chemical and enzymatic properties. Its role is speculated in relation to skin functionality and prostate cancer (11).

Human and rabbit reticulocyte 15-LO-1 as well as the murine/ rabbit leukocyte- type 12-LO have high homology and share a common enzymatic activity and hence are classified under 12/15 LO especially in rat and mouse (11, 17, 18). There exists no separate gene for leukocyte-type 12-LOX in humans. However separate genes encoding for both reticulocyte type 15-LO-1 and leukocyte type 12-LO exist in case of rabbit. Gene sequencing studies show that these isoforms share >99 % identity implying they might be the result of gene duplication (11). 12/15 LO unlike other LOXs can insert molecular oxygen at 12th and/or 15th position in AA (20:4) thereby producing corresponding hydroperoxide. Whereas with LA (18:2) the insertion occurs at 9th and/or 13th positions (24). In humans and rabbit 12/15 LO activity results in 15-HPETE as the dominant product hence the enzyme is preferably called as 15- LOX whereas, in case of rat, mouse, pig and cow, 12-LO activity is the dominant one (12, 14). The substrates for 12/15 LO may be either free unsaturated fatty acids or fatty acids in phospholipids and cholesteryl esters

(18, 26). Peroxidation of polyunsaturated fatty acids present in the biomembrane causes alteration in the membrane structure contributing to cellular remodeling (14). 12/15 LO occur in dendritic cells, differentiated macrophages, inflamed endothelial and smooth muscle cells and in some tumors (26). 12/15 LO in mammals regulate MAPK, PKC, small GTPases like Ras and Rho A and NF-kB (26). 12/15-LO can mediate oxidation of LDL promoting atherosclerosis (7,8).

In mouse, AA when metabolized by 8(*S*)- lipoxygenase (8-LO) leads to the formation of 8-hydroperoxy-5,9,11,14- eicosatetraenoic acid (8-(*S*)-HPETE) which is further reduced to 8(*S*)-HETE. Based on sequence identity, structure and chromosomal location, human epidermis type 15(*S*)-LO-2 which is located in chromosome 17p13.1 is considered as the orthologue of mouse 8-LO located in chromosome 11, central region although they insert dioxygen at different positions . However, the gene encoding 8-LO is designated as ***Alox 15b*** commemorating 15-LO-2 encoding ***ALOX 15B*** of humans. 8(*S*)-HETE in rats and mice has been detected in corneal epithelium and skin like its human orthologue 15-LO-2 . 8(*S*)-HETE plays a role in terminal differentiation of keratinocytes (25).

Prostanoids are arachidonic acid derived metabolites due to the acting upon by the enzyme –cyclooxygenase (COX/PTGs) which by itself exists in two isoforms viz. COX-1 and COX-2 (8,25,28,29,30) . COX-1 is encoded by ***cox-1*** gene whereas ***cox-***3 splice variant and ***cox-2*** encode COX-2 (30). Besides being regulated post-transcriptionally, STAT-1, STAT-3, NF-kB, NF-IL6 induce COX-2 via *cis*-acting elements whereas other stimuli upregulate ***cox-2*** via cAMP- response element (CRE), peroxisome-proliferator-activated receptor-responsive element and CCAAT enhancer (26). The two isoforms have comparable activity albeit expressed differentially with ***cox-1*** being constitutive in most tissues and ***cox-2*** being inducible

(1,7,8,15,25,28,29,31,32). However, recent findings show that ***cox-2*** is constitutive in kidneys (2,22,25), brain, tracheal epithelium, some endothelial cells (25) and pancreatic islets (7,15,31,33,34). COX-1 performs housekeeping functions as in cytoprotection of the gastric mucosa, regulation of platelet aggregation (8) and renal water balance (25). However, COX-2 is involved in pathophysiological processes like angiogenesis, inflammation and tumorigenesis and is induced by mitogens, inflammatory mediators (8) hyperglycemia (27, 29) lipopolysaccharides and chemicals (34). AA gets converted first to prostaglandin (PG)G_2 via bis-oxygenase activity of the enzyme which is then subsequently converted to endoperoxides (PGH_2) due to peroxidase COX- activity (8) which upon further acting by specific synthases yields respective prostanoids/ prostaglandins like : prostacyclin (PGI_2), thromboxane A_2 , prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (28, 32). Five G-protein coupled seven transmembrane receptors are engaged in interacting with prostanoids. These are: DP, EP, FP, IP, and TP which interact with prostaglandins D_2, E_2, $F_{2\alpha}$, I_2 (prostacyclin) and thromboxane A_2 respectively (4). Some prostanoids however may engage with nuclear receptors such as PPAR δ and PPAR γ. There are four subtypes of EP receptors viz. EP_1, EP_2, EP_3, and EP_4. The G-protein coupled signaling cascade initiated by each prostanoids is distinct. For instance, EP_3 receptor interaction to an inhibitory G protein reduces cyclic adenosine monophosphate (cAMP) synthesis. Whereas IP, DP, EP_2 and EP_4 receptors coupled to stimulatory G protein results in signaling cascades due to increasing cAMP rather than inducing calcium mobilization as with TP, FP, and EP_1 receptors. Depending upon the type of prostanoids and receptor interactions, the role played by prostanoids is diverse (8). The biosynthetic pathways for prostanoids is shown in figure 5 below (28).

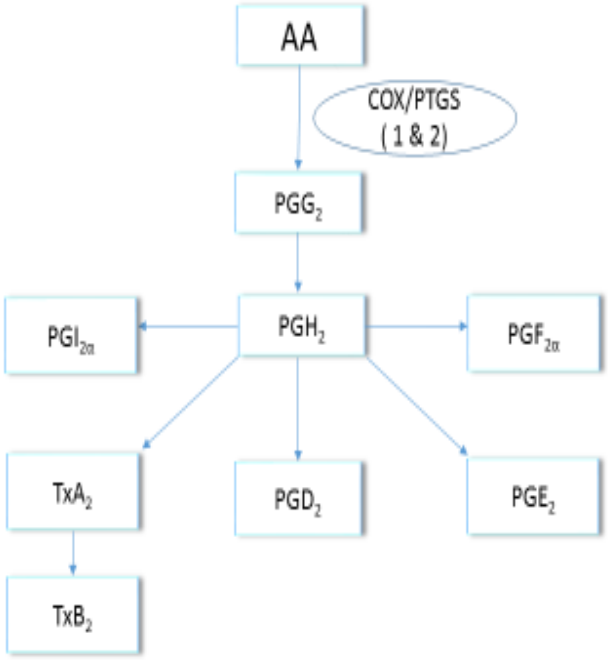

Fig.5 Biosynthetic pathways for prostanoid from arachidonic acid.

CYP monooxygenases, first described in 1980 consists of two enzymatic pathways-the epoxygenases and the hydroxylases (2,34). Epoxyeicosatrienoic acids (EETs) are metabolites of AA due to the action of epoxygenases and are endothelial in origin and plays diverse spectrum of roles from activating Ca^{2+} -sensitive K^{+} channels thereby inducing hyperpolarization (29, 30, 31) based vascular dilatation of coronary, renal and cerebral arteries to anti-inflammatory effects in such tissues (32) and proliferation of endothelial, epithelial and smooth muscle cells (33, 38). The four *cis*- regioisomers of EETs (based on location of double bond across which epoxide is added) that are produced from AA by the action of cytochrome P450 (P450 or CYP)

epoxygenases of 2C and 2J classes (34) in the presence of NADPH and oxygen (52) are : 5,6-EET, 8,9-EET, 11,12-EET, and 14, 15-EET (6,36,38,41) which gets metabolized via β-oxidation into shorter chain fatty acids and vicinal-dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) in mammalian system (38, 43). Each EET regioisomer exists in two enantiomeric forms based on how epoxide group is attached to the double bond viz. *R/S* and *S/R* thus giving rise to eight different EETs each with different biological effects (42). Whereas the epoxygenases results in EETs from AA metabolism, ω- hydroxylases belonging to the classes CYP4A and CYP4F (22) in extrahepatic tissues also lead to the formation of 7-, 10-, 12-, 13-, 15-, 16-, 17-, 18- HETEs and 19- and 20- hydroxyeicosatetraenoic acids (19- and 20-HETE) (2,36,42). Because of various cardiovascular protective effects of EETs, the therapy for treating such cardiovascular diseases is centered on targeting sEH and thereby stabilizing EETs (36). The summary of CYP based metabolism of AA is depicted in figure 6.

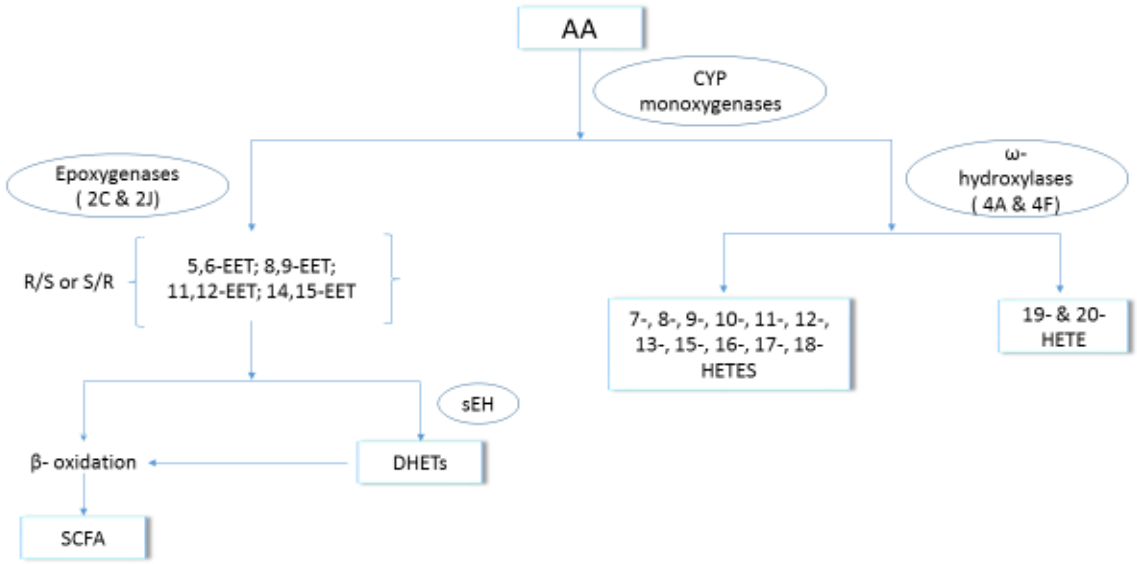

Fig. 6 Metabolism of AA via CYP pathway.

1.2) Diabetes

According to International Diabetes Federation, the global prevalence of diabetes was 382 million in the year 2013. Hyperglycemia due to insulin secretory defect or failure of insulin action or both results in diabetes mellitus and under chronic conditions leads to damage or dysfunction of eyes, kidneys, blood vessels, heart, nerves (35, 36). Various risk factors of diabetes are: sedentary life style, hypertension, dyslipidemia, family history of diabetes, history of gestational diabetes and polycystic ovary syndrome (37). Diabetes often leads to poor prognosis of cardiovascular diseases like atherosclerosis and hypertension (7, 38, 39, 40, 41) and renal diseases (42). > 700, 000 annual deaths in the U.S. are attributed to diabetes induced cardiovascular diseases and further it has been estimated that the subclinical states of glucose intolerance will increase (50) 40% death risk due to cardiovascular complications in adults as compared to 110% risk among overt diabetics independent of other risk factors for cardiovascular diseases (49). It has been estimated that the global burden of diabetes would be increased to 439 million adults by 2030 and this would be mainly due to increase in cases among developing countries rather than in developed countries. The reason being obvious decrease in physical activity but increase in obesity (43). Impaired glucose tolerance (IGT) is common (1.5 times Type II diabetes) in the U.S. accounting 11.2% adults of age 20 to 74 years (44). IGT but not impaired fasting glucose (IFG) has been identified to be an independent risk factor for cardiovascular diseases (45). However, IFG and IGT are used to predict risk of developing diabetes (46, 105) as they occur as an intermediate stages in the natural history of diabetes mellitus (47). Hyperglycemia induces mitochondria to overproduce reactive oxygen species (ROS) which leads to induction of tissue damages through following five pathways: 1) increased formation of intracellular AGEs (advanced glycation end products) 2) increased expression of

the receptor for AGEs and its activating ligands 3) activation of protein kinase (PK) C isoforms 4) increased flux of glucose and other sugars through the polyol pathway and 5) hexosamine pathway overactivity (31, 46).

1.2.1) Type I diabetes

Type I diabetes also known as insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes accounts for 5-10% of all diabetics results from immune attack to the pancreatic beta cells (45, 48). Increase in autoantibodies to insulin, glutamic acid decarboxylase (GAD_{65}) , tyrosine phosphatases IA-2 and IA-2β (45), carboxypeptidase-H, islet cell antigen (ICA)-69, GM gangliosides and SOX13 (59) are markers of beta cells destruction . Both genetic and environmental factors come into play in the onset of Type I diabetes (59, 105).

The onset of Type I diabetes is characterized by infiltration of inflammatory cells (29,49, 50) particularly CD_4^+ and CD_8^+ T cells, monocytes and macrophages (31, 58, 59) which release various cytokines like IL-1 β that induce expression of COX-2 that result in production (35) of proinflammatory mediators like prostaglandins and thromboxanes and iNOS (inducible nitric oxide synthase) that produce NO radical. It is due to these metabolites that leads to destruction (34,62) and apoptosis of pancreatic islets (51) . 70-80 % of β cell mass is lost in type I diabetes (52). Affected individuals depend upon exogenous source of insulin for life and are at high risk of developing serious cardiovascular and microvascular complications (60).

1.2.2) Type II diabetes

Around 285 million adults globally suffer from type II diabetes (105) also known as adult onset diabetes (45). This form of diabetes which is most prevalent account approximately 90-95% of total diabetics (45). Type II diabetes or non-insulin dependent diabetes mellitus is a result

of insulin resistance and β- cell dysfunction (35,53, 54, 105). The latter process encompasses phenomena like: loss of glucose sensing and thereby decrease in glucose stimulated insulin secretion (GSIS), increased basal insulin secretion, decrease in insulin content of the islet, altered gene transcription, changes in intracellular signaling intermediates (65) and loss of β- cell mass (35, 65). It has been observed that there is 25- 50 % loss of β- cell mass in Type II diabetes (15) but this is not due to autoimmune destruction (45). Initially, there is hyperinsulinemia to maintain normoglycemia but later on due to progressive insulin resistance and β- cell destruction leads to hyperglycemic state (15). Chronic exposure to elevated levels of glucose and free fatty acid (FFA) induce β- cell apoptosis particularly via ER-stress which is independent of IL-1β, NF-kB or NO and is unique from β- cell death during type I diabetes (64). However, some study has shown that elevated level of glucose also has been shown to induce IL-1β production by the beta cells and there by leading to their own apoptosis (29) this contrast with other findings where no induction of IL-1β due to high glucose was noticed (64).

Release of insulin under glucose stimulation by the pancreatic beta cells is a biphasic process. The first phase that lasts 10-15 minutes is extremely dependent upon elevated level of intracellular $[Ca^{+2}]_i$ level. Whereas the amplifying signals resulting from glucose metabolism initiates the second phase and is less oscillatory $[Ca^{+2}]_i$ dependent. Partial or complete defect in the first phase of insulin secretion marks the onset of type II diabetes whereas defect in second phase occurs secondary but is prominent too (66). Although type II diabetic patients, have normal level or elevated level of insulin at the baseline, they cannot respond to higher glucose level due to defect in insulin secretion (45).

Type II diabetes is a major risk factor for health complications like hypertension, cardiovascular diseases, dyslipidemia, infections, renal failure, blindness and amputations due to

microvascular complications (45, 105). Obesity is often associated with type II diabetes (15, 55). However, a distinct link of genetic trait to this form of diabetes has not been delineated (45).

1.3) Mode of Insulin action

Insulin acts by binding to the growth factor receptor tyrosine kinases subfamily of cell surface receptor which includes insulin receptor (***Insr***), type I insulin-like growth factor (IGF) receptor (***Igf1r***) and Insr-related receptor (***Irr***). Although being an structural analogue, the roles played by these receptors vary although intracellular signaling cascades coincide, with ***Insr*** being involved in fuel metabolism and ***Igf1r*** in growth. Insulin, Igf1 and Igf2 activate the former two receptors but not the third one making it an orphan receptor (56). The glucose homeostasis maintenance due to insulin is a result of its pivotal role intertwined among metabolism of three major nutrients: carbohydrate, protein and fats by acting upon liver, muscle and fat cells. Insulin stimulates liver cells to uptake glucose and store as glycogen but at the same time preventing gluconeogenesis and glycogenolysis. Whereas in case of muscle and fat cells, it stimulates uptake, storage and use of glucose.

Upon binding of insulin to its receptor, the tyrosine residue of the intracellular region of the receptor gets phosphorylated thus activating receptor tyrosine kinase activity. This activity leads to phosphorylation of protein tyrosine which inturn initiates a signaling cascade. Of interest is the phosphorylation of serine kinase at the serine residue. This activates the latter which phosphorylates protein phosphatase 1 at the serine residue and activates it. The activated, protein phosphatase1 has dual functions: first, it dephosphorylates the glycogen synthase~Pi to glycogen synthase which causes increase in glycogen synthesis and second, there occurs dephosphorylation of phosphorylase kinase~Pi to phosphorylase kinase which leads to inhibition of glycogenolysis. This occurs usually in the liver and muscle cells where as in case of fat cells,

lipogenesis is encouraged, this tissue specific discrepancy is due to the fact that there occurs disparity of enzymes involved in the lipogenesis among these tissues with much abundance of these enzymes occurring in the fat cells (57) (figure 7).

The binding of the insulin to its receptor also initiates other signalling pathways that are of interest. For instance, the activated tyrosine receptor activity leads to the phosphorylation of Src and Insulin receptor substrate (IRS) which inturn activate GRB2. GRB2 and SOS, two exchange factors then form a complex which inturn activate Ras ⟶ Mitogen activated protein (MAP) kinase pathway thereby stimulating growth and proliferation of cells. The IRS ⟶ phosphatidylinositol-3-kinase (PI3K) pathway leads to activation of downstream kinases like pdk1 which in turn phosphorylates and activates serine/ threonine kinases like isoforms of Akt. Akt inturn phosphorylates, glycogen synthase kinase 3 (Gsk3), cGMP-inhibitable phosphodiesterase b and Foxo transcription factors which leads to stimulation of glycogen synthesis, inhibition of lipolysis and gene expression. PI3K has been stated as necessary but not sufficient cause for glucose transporter translocation. A new PI3K independent pathway for insulin dependent uptake of glucose is based on activation of protooncogene c-Cbl. It is believed that this or related pathway aids in glucose transporter recycling by remodeling cortical actin filaments with possible involvement of atypical myosin isoforms (68).

1.4) Lipoxygenases and diabetes

12/15-LO has been shown to induce production of proinflammatroy cytokines and cause reduction in beta cell mass thereby causing type I diabetes in mice (60). 12-LO in mice makes pancreatic islets more sensitive to cytokines and enhance nitric oxide synthesis in peritoneal macrophage and thereby induce type I diabetes (58). 12-LO products have been implicated in the pathogenesis of both type I and type II diabetes. It has been demonstrated that 12-(*S*)- HETE

induce apoptosis in beta cells (15,17). However, no literature exists regarding the role of ***Alox8*** in glucose metabolism or diabetes. Further, the goal of the study was also supported by the fact that PPARα which is an endogenous ligand for 8(*S*)-HETE gets down regulated in zucker diabetic rats implying that 8-LO might play anti-diabetic role (8) . Hence to understand the role played if any by 8-LO in glucose homeostasis in mice, this study was conducted. The study revealed a biologically significant role of this enzyme in maintaining lower blood glucose level in mice.

CHAPTER 2

MATERIALS AND METHODS

2.1) Animals

Wild type (WT) mice C57BL6 and Alox8 $^{-/-}$ knockout (KO) mice were obtained from the Jackson Laboratories. These mice were used for breeding and the heterozygous off springs were interbred further to generate the KO mice required for the experiment. The mice were housed at room temperature under controlled light comprising 12: 12 (light : dark) cycle under pathogen free conditions with free access to normal feed (Purina Lab Diet 5001, Gateway Lab Supply) and water all the time. Mice were housed usually 1-3 per cage but not more than 5. All procedures including were carried out with total compliance to the guidelines of the National Institutes of Health and as per the protocols that were reviewed and approved by the committee on the Use and Care of Laboratory Animals (LACUC) of Southern Illinois University School of Medicine Springfield, Illinois.

2.2) Genotyping

Weaning and genotyping of the mice were carried out at the age of 21 days. Tail tissue was used to obtain DNA. Briefly, mice were anesthetized and tail tip less than 5 mm were sterilized and snipped off using a pair of sterilized scissors. The downstream extraction was done according to the protocols of available commercial kits (ArchivePure DNA Cell/Tissue and Tissue Kits, 5 PRIME). For genotyping following primers were used: Alox8wtF - 5'CCC AGA AAT ACA AAG GTT TAG ATT TT-3' ; Alox8wtR- 5'AGA CAA ATT GGT ACG GGG AAT GG-3' and Common-LoxP-F— 5' GAG ATG GCG CAA CGC AAT TAAT-3' ; CSD-Alox8-R— 5' AAG GCT CCT ACA GGT CTC TTT TGA CC-3' . The PCR product size for WT

was 700 bp whereas for KO it was 350 bp using the above sets of primers. The PCR cycling conditions used were as follows: Initial denaturation at 92 ºC for 2 minutes followed by 34 cycles of denaturation at 92 ºC for 30 seconds , annealing at 56 ºC for 30 seconds and extension at 72 ºC for 40 seconds followed by a final elongation at 72 ºC for 5 minutes. GoTaq® qPCR Master Mix (Promega) was used for the process.

2.3) Intraperitoneal Glucose tolerance test (IPGTT)

Age matched male mice (59) were fasted for 16 hours prior to the conduction of the test. The mice had however access to the water. On the test day, the weights of the mice were taken. Tip of the mouse tail was surfaced sterilised by wiping with 70% ethanol and then with a sterile razor blade, the tail was incisioned just enough to collect 5- 10 ul of blood from the tail vein . Baseline blood glucose was noted. Then the mice were injected peritoneally with glucose solution made in sterile 0.9 % normal saline. Whereas the control mice received only 0.9 % normal saline. The amount of glucose injected was at the rate of 2mg/ gram body weight or 1mg/ gram body weight depending upon the experimental purpose. However, no more than 200 µl of solution in toto was injected. After injection, the same tail wound was scrapped for further blood glucose testing every 5 min, 15 min, 30 min, 60 min and 120 min respectively. All the readings were noted. ReliOn® Prime glucose meter was used for the process.

2.4) Intraperitoneal Insulin tolerance test (IPITT)

Age matched male mice (71) were fasted for 5 hours prior to the conduction of the test. The mice had however access to the water. On the test day, the weights of the mice were taken. Tip of the mouse tail was surfaced sterilised by wiping with 70% ethanol and then with a sterile razor blade, the tail was incisioned just enough to collect 5- 10 µl of blood from the tail vein .

Baseline blood glucose was noted. Then the mice were injected peritoneally with porcine insulin (Sigma-Aldrich) solution made in sterile 0.9 % normal saline. Whereas the control mice received only 0.9 % normal saline. The amount of insulin injected was at the rate of 0.5 U/ Kg body weight . However, no more than 200 µl of solution in toto was injected. After injection, the same tail wound was scrapped for further blood glucose testing every 5 min, 15 min, 30 min, 60 min and 120 min respectively. All the readings were noted. ReliOn® Prime glucose meter was used for the process.

2.5) Histopathology

Mice from the three genotypes were euthanized and then sacrificed by cervical dislocation. Then immediately liver tissue was isolated and then fixed in 10 % formalin and then embedded in paraffin. The embedded blocks were sectioned at the thickness of 4 µm for staining via H & E (HE), periodic acid-schiff (PAS) and PAS- diastase (PASD) methods. And the readings were taken at the same exposure of light.

2.6) Statistical Analysis

Mean body weights and ages of the mice were compared among the genotypes by using unpaired Student's t-test. Area under curves (AUCs) were compared by using One- way analysis of variance (ANOVA). Values were expressed as mean ± SEM. P value of ≤ 0.05 was considered to be significant.

CHAPTER 3

RESULTS

3.1) Genotyping

All the mice were genotyped as per the protocol mentioned in the materials and methods section at the inception of the experiment. A representative genotyping profile of the three different genotypes is shown in figure 8.

3.2) Alox8 -/- mice has lower glucose tolerance

Sixteen mice with the following distributions: WT = 6; Het = 5 and KO = 5 were taken for the IPGTT. The age and weight distribution of the mice are shown in table 1. One-way ANOVA for the age distribution of the mice among the three different genotypes showed no significant difference, $p = 0.87$. However, the weight distribution showed a significant difference, One-way ANOVA $p= 0.017$ with the KO mice being slightly heavier than the WT. The blood glucose levels at various time points of the IPGTT for these mice is shown in table 2. The same thing is depicted in the form of line curve to see the dynamics of blood glucose levels with time in the three genotypes (figure 9).

Mean area under curve (AUC) values obtained from the glucose tolerance is shown in table 3. One-way ANOVA analysis of AUCs showed insignificance in blood glucose distribution among the three genotypes with p value of 0.124. However, there was a slightly higher glucose level trend among the KOs as compared to the WT mice (figure 10).

3.3) *Alox8* $^{+/+}$ mice are comparatively more responsive to insulin than the KO

Sixteen mice with the following distributions: WT = 6; Het = 5 and KO = 5 were taken for the IPITT. The age and weight distribution of the mice are shown in table 4. One-way ANOVA for the age distribution of the mice among the three different genotypes showed no significant difference, p = 0.88. However, the weight distribution showed a significant difference, One-way ANOVA p= 0.058 with the KO mice being slightly heavier than the WT.

The blood glucose levels at various time points of the IPITT for these mice is shown in table 5. The same thing is depicted in the form of line curve to see the dynamics of blood glucose levels with time in the three genotypes (figure 11).

Mean drop in blood glucose level five minutes after IPITT in three genotypes is shown in table 6. One-way ANOVA analysis of these mean drop in blood glucose level among three genotypes at five minutes time point after insulin administration showed insignificance with p value of 0.717. However, a rapid drop in blood glucose level among the WT mice as compared to the KOs was observed although statistically invalid (figure 12).

3.4) Histology

The pictures of the liver sections obtained from the three different genotypes of mice are shown as figures 13 and 14. The sections were stained by HE, PAS and PASD. HE staining of the liver sections showed no significant changes in the cell or nuclear morphology (figure 13). However, a trend in PAS stain intensity was observed among three genotypes in all three repeated batch of independent experiments (figure 14).

CHAPTER 4

DISCUSSION

Diabetes is a costly (partly because it is chronic) disease that claims huge economic loss to a nation (15, 60, 105) . In its subclinical stages, the disease is manifested in the form of impaired glucose tolerance or impaired fasting glucose (45, 47). All these together have been proved to be an independent risk factors for various cardiovascular (7, 48, 52, 70) and microvascular complications leading to severe health consequences like neuropathy , retinopathy (45), renal complications (52), amputations and so on (46) . Various genetic (eg. *PPARγ*, *ABCC8*, *KCNJ11*, *CALPN10* for type II diabetes) (61) and environmental factors have been linked to the predisposition of this malady (64). The important environmental factors that are linked strongly to diabetes are obesity and physical inactivity (54). Due to unhealthy diets , obesity, population growth and advancement in technologies, that causes reduction in physical activity in our daily lives, a increase in prevalence in diabetes in the upcoming years particularly in the developing nations has been predicted (54, 72).

Lipoxygenases particularly 12-LO (24, 70) and 12/15- LO (7, 60) have been correlated to cause type I diabetes. And this has led us to the belief that 15-LOX 2 might possibly play some role in glucose homeostasis. Here in this study, we tried to study the role of ***Alox8*** gene, which is a mouse orthologous for human 15-LO2, in glucose metabolism if there exists any.

Our hypothesis was also supported by the fact that our KO mice had significantly higher baseline blood glucose level than the WT ($p = 0.0072$) (Supplementary table 7). We began the study by looking for tolerance for glucose among the three genotypes. As shown in fig. 9, the trend in glucose metabolism over time were similar for both the WT and KO mice. Also, the

AUC for blood glucose level for KO was higher as compared to the WT although not statistically valid ($p = 0.124$) (figure 10).

We further tried to figure out if this poor tolerance of glucose in KO mice was due to resistance to insulin in this cohort of mice. For this we carried out insulin tolerance test, the trend in level of blood glucose after insulin administration is shown in figure 11. There are controls (0.9 % PBS) which shows increase in blood glucose level after injection in mice , this is due to the fact that trauma due to injections cause release of hormones like cortisol which leads to elevated blood glucose level . Since the half-life of insulin is ~ 10 minutes in mice, we tried to see if there is some effect of insulin within the first 15 minutes after its administration (71). Since the trend lines among the two genotypes showed some remarkable difference at 5 minutes and 15 minutes time points (fig. 11), we performed statistical analysis to see if the difference was significantly valid. However, difference in drop of blood glucose levels at both the time points of 5 minutes and 15 minutes revealed insignificance in the nature of data with corresponding one way ANOVA p values of 0.717 and 0.528 respectively. However, in conformity to the glucose tolerance test trend line, there was a slightly higher drop in blood glucose level among the WT mice as compared to that of the KO mice 5 minutes after insulin administration (figure 12). This data tells us that insulin may have biological significance in lowering blood glucose level among the WTs although not valid statistically.

Next we tried to question why was this effect of insulin acting so differently among the two, otherwise seemingly comparable, genotypes? The reason for this insulin resistance (IR) may be various. One clear reason for IR is obesity (26, 62, 63, 64). There occurs macrophage infiltration and inflammation (26,76) in the adipose tissue, this leads to inactivation of IRS by

cytokine-activated JNK, IKKβ and SOCS ultimately causing resistance to insulin action. 12/15-LO has also been shown to cause infiltration of macrophages to adipose tissue and thereby causing release of proinflammatory cytokines ultimately leading to whole body insulin resistance (26). Besides storage of excess calories in the form of fat , adipose tissue is a metabolically active site for synthesis and release of bioactive compounds like proinflammatory cytokines (TNF-α, IL- 1β, IL6, MCP1) , leptin, resistin, adiponectin, PAI-1, acute phase reactants, angiotensin II and so on . And it has been known that some of these compounds in high dose leads to the development of IR (65, 66, 67). FFA which increases with obesity is known to induce IR and drugs like thiazolidinediones (TZDs) are based on increasing insulin sensitivity by causing oxidation of FFA and thereby lowering their content. One mechanism of IR due to high FFA is believed to be due to intramyocellular and intrahepatic accumulation of triglycerides and metabolites of FFA reesterification pathway such as long chain Acyl-CoAs and diacylglycerol (DAG). And it has been shown that DAG leads to activation of protein kinase C (a serine threonine kinase) isoforms which cause decrease in tyrosine phosphorylation of the IRS thereby inhibiting the insulin signaling pathway. Also it has been known that FFA in adipocytes leads to oxidative stress by generating reactive oxygen species which causes deregulated generation of proinflammatory cytokines which participates in IR. Further, FFA in adipocytes, liver cells and pancreatic beta cells is known to cause ER stress which then activates JNK that leads to IR (78). Leptin, a fat derived hormone reduces intracellular fat accumulation. Hyperleptinemia, occurs early in obesity due to resistance of extra adipose tissues to leptin and thereby leading to lipid accumulation and lipotoxicity which promotes IR (68). Low –grade chronic inflammation is associated with IR and type II diabetes, and IL-6 which is produced during such inflammation has been known to interfere with the Insuin receptor downstream signaling leading to IR in liver

and adipose tissues (69). Study has shown that cells overexpressing 12/15-LO leads secretion of osteopontin and MCP-1 that cause IR (15). The existence of similar phenomenon cannot be denied with 8-LO. Lipocalin 2 that is produced from adipocytes, is known to be an independent risk factors for inflammation, IR and diabetes (79). It has now been realized that any obesity inducing genes are also the culprits for induction of IR (80). In this study although the mice from three different genotypes were of comparable weights at the time of weaning (data shown as supplementary, tables 8 and 9 and figure15) there was significant difference in the weights of the mice at the time of experiment (i.e. mice aged over 200 days) as mentioned previously. So, we think that the IR seen in this study might be due to increase in obesity in KO mice as compared to the WT mice. However, we were unable to perform body composition analysis at the moment.

The other possibility for impaired insulin action in KO mice relative to WT might be due to defect in insulin receptor interaction. To elucidate the mechanism we performed glucose tolerance test again, but this time we halved the concentration of glucose administered i.e. 1mg per gram body weight of the mice. The age and weight distribution of the mice are shown as supplementary tables 10 and 11. There were no significant difference in weights and ages of the mice (one way ANOVA, $p > 0.05$). Comparison of AUC for glucose tolerance showed that WT mice were less glucose tolerant that the KO (figure 16) although this difference was not statistically significant (one –way ANOVA $p= 0.315$). This finding is just the opposite of what we found with administration of 2 mg/ gm BWt. of glucose (figure 10). One way of explaining this anomaly is by taking into account of insulin –receptor interaction constant K_d. We know that IR leads to hyperinsulinemia (47, 69, 70,75) here we assume the hyperinsulinemia is compensatory due to defect in insulin receptor interaction instead of other usual causes of IR.

Thus when 1mg per gram body weight of glucose is administered to the mice during IPGTT, the hyperinsulinemia in the KO mice gets rid of the excess glucose effectively than the WT mice which may have relatively less insulin flowing in the blood stream. But this is not the case when glucose is injected at the rate of 2 mg per gram body weight of the mice. In this case since the receptor insulin interaction is better in WT mice as compared to the KO, the effectiveness of insulin is more pronounced in the former although both the mice might have optimum or equivalent amount of insulin produced (figures 10, 12 and 16). However, we could not measure insulin levels during the tests. And there may also be a possibility that, signalling pathway downstream of insulin receptor interaction may have gone defective instead, which needs verification in future studies.

One important reason for the non-significant nature of the data presented in this study is due to the biased expression of ***Alox8*** in mice tissues. It is highly expressed in corneal epithelium however its mRNA has been detected in lung, colon, brain, footsole, forestomach or hair follicles (27). Also, antitumorigenic role of ***Alox8*** has been explored in relation to skin (9). Based on availability of data at present, we have no evidence that ***Alox8*** is highly expressed in liver, skeletal muscle or adipose tissue, the hot spots for the glucose storage and insulin action. Furthermore, its orthologue 15 LO-2 has been known to be highly expressed in platelets rather than any other sites (www.genecards.org). On these grounds, it is plausible to state that ***Alox8*** might play a biologically significant but statistically invalid role in lowering blood glucose level in mice.

Inaddition, PAS staining which is specific for glycogen showed a trend in the liver sections . Usually PAS staining can stain nonspecifically, hence to sort out specific binding with glycogen, PAS staining is followed by PASD after which only nonspecific stain will remain.

Hence, comparing the two staining slides we can figure out if glycogen was stored in the tissue. In our triplicate repeats of PAS (D) in three different genotypes of mice, the storage of glycogen was non-uniform (fig. 14). In case of row A, WT mice had highest glycogen storage intensity followed by KO and then Het. Whereas in case of rows B and C, there was an increase in intensity gradient while moving from WT to Het to KO with KO mice having the highest and WT mice with lowest PAS intensity. The reason for this discrepancy is obvious. WT liver responds to insulin better than the heterozygous or KO liver. This notion is also supported by the fact that liver from 80 % of diabetic patients often suffers from glycogen accumulation possibly due to long run of insulin insufficiency (71, 72). Further, in our three batches of independent experiments (A, B and C) only B and C gave the repetitive pattern of glycogen accumulation in liver, this is due to the fact that the role of ***Alox8*** in lowering blood glucose is biologically significant but not statistically.

CHAPTER 5

CONCLUSION

Type II diabetes which comprises approximately 95% of all the diabetic cases is a costly disease. The most amenable way of combating the disease is by taking life style modification therapy where people with the illness is suggested for consuming a balanced diet and taking a regular exercise (67). Besides there are currently available drugs that induces insulin sensitivity like Biguanides (Metformin) and TZDs (105) . Taken together our data suggests that KO mice is less responsive to insulin than the WT mice implying that the findings when extrapolated to human subjects may require less insulin administration in 15 LO-2 diabetics as compared to those carrying its polymorphic alleles. However, such claim requires further validation.

TABLES

Table 1. Distribution of ages and weights among the three genotypes.

Average					
Genotype	No.	Wt. (gms)	Age (days)	Std. Wt.	Std. Age
WT	6	30.67	288	2.16	25.56
HET	5	34.7	296.6	2.01	24.39
KO	5	33.46	287	1.91	43.41

Table 2. Blood glucose levels among the three genotypes during IPGTT.

	Baseline	5 min	15min	30min	60min	120min
WT	117.67	238.17	372.17	266.5	158	97.83
WT-C	107	160.33	156.67	164.67	121.33	96
Het	116.8	289.6	465.4	395	286.4	143.2
Het-C	142	201.6	179.8	199.2	161.8	153
KO	124.8	246.2	372.4	305.2	217.6	119.2
KO-C	129.8	160.8	166	181.8	156	108.8

Table 3. Mean AUC among the three genotypes during IPGTT.

Genotype	n	AVG	Std
WT	6	8653.75	4548.8
Het	5	20337	14737.4
KO	5	12072.5	3218.69

Table 4. Distribution of ages and weights among the three genotypes.

		Average			
Genotype	No.	Wt. (gms)	Age (days)	Std. Wt.	Std. Age
WT	6	33.48	301.17	2.81	24.79
HET	5	37.18	310.2	2.36	24.72
KO	5	35.84	301.8	1.49	44.22

Table 5. Blood glucose levels among the three genotypes during IPITT.

	Baseline(B)	5 min	15 min	30 min	60 min	120 min
WT	108.33	116.5	111.67	110.33	116.5	150.5
WT-C	135.33	192.67	200	203.67	168.83	138.83
Het	172.8	176.8	178.4	135.2	170	166.8
Het-C	182.6	223.2	249.8	254.6	206	171
KO	156.4	193.6	164	175.4	221.2	186
KO-C	156	230	275.8	246.8	176.8	167.6

Table 6. Mean drop in blood glucose level among the three genotypes five minutes after insulin administration via intraperitoneal route. C- control, T- test.

Δ 5min (C-T)	AVG	SE
WT	76.17	25.09
Het	46.4	43.49
KO	36.4	36.98

FIGURES

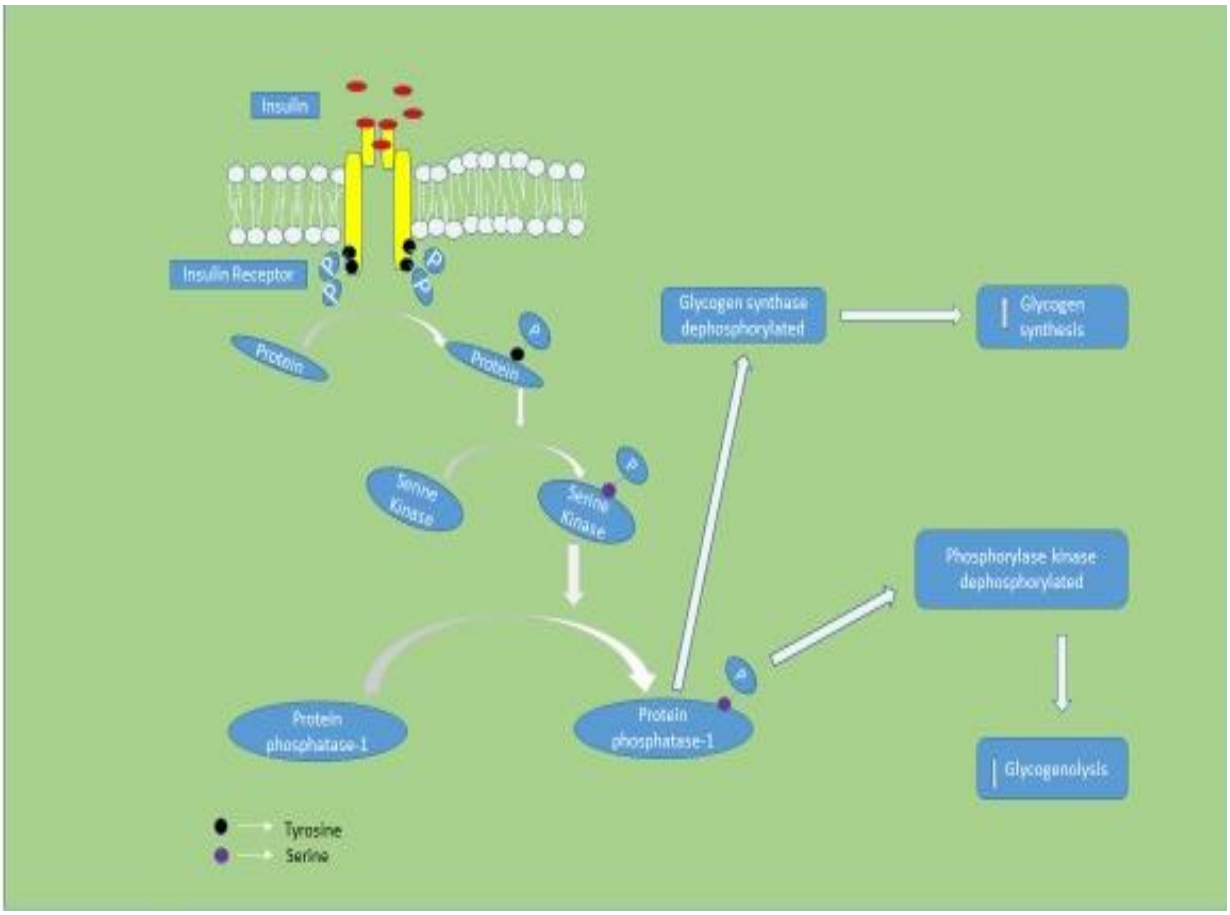

Fig. 7 Mode of insulin action in liver cell.

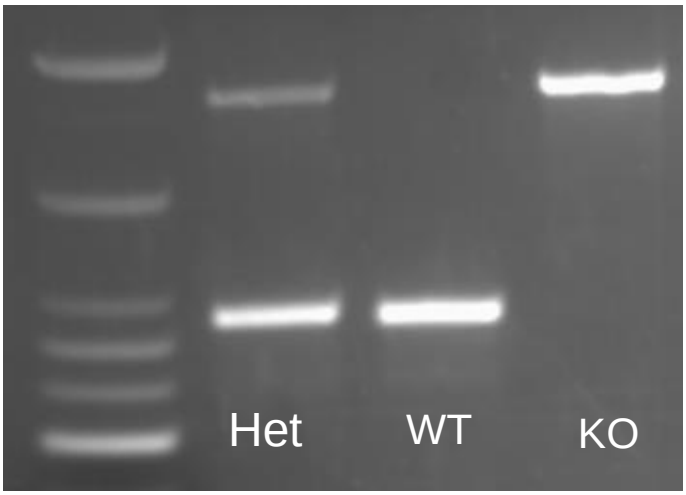

Fig. 8 Genotyping

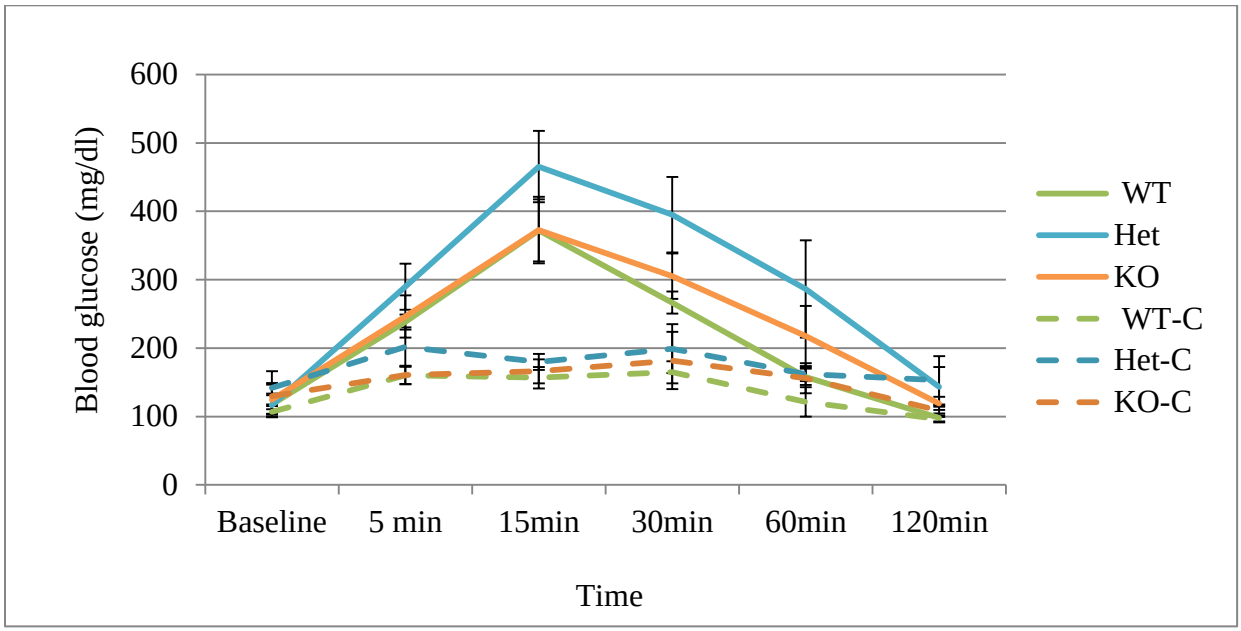

Fig. 9 Dynamics of blood glucose level with time among three genotypes during IPGTT. Error bars denote ± SEM and n = 6, 5, 5 for WT, Het & KO resp. . C stands for control of the respective genotypes.

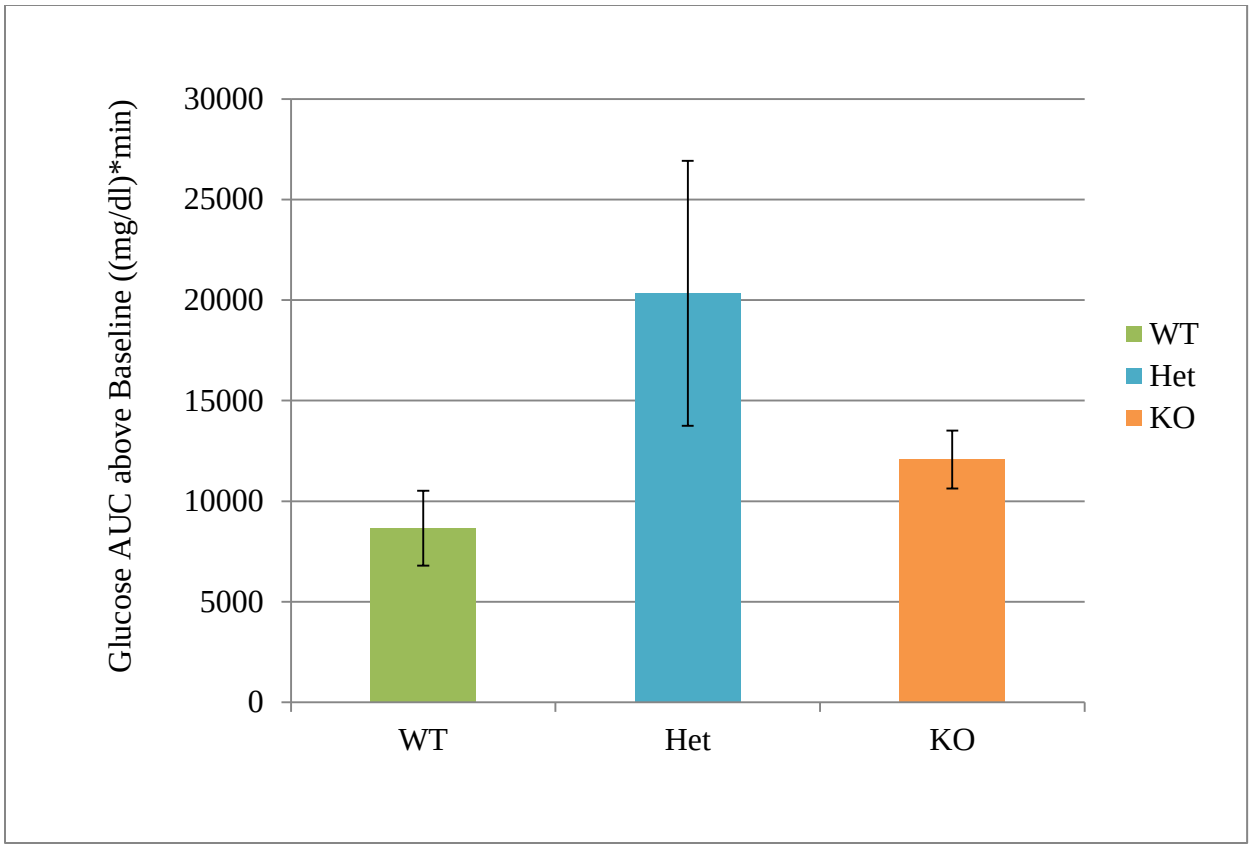

Fig. 10 AUC analysis of data obtained from IPGTT. Error bars denote ± SEM and n = 6, 5, 5 for WT, Het & KO resp. .

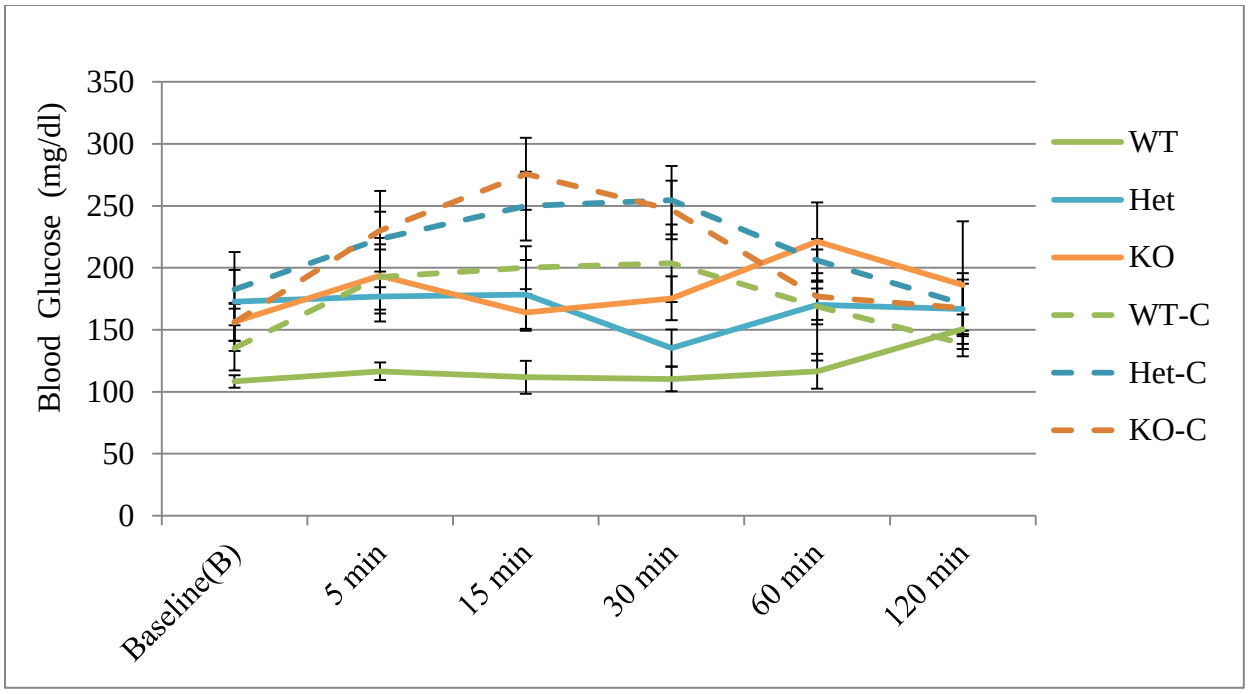

Fig. 11 Dynamics of blood glucose level with time among three genotypes during IPITT. Error bars denote ± SEM and n = 6, 5, 5 for WT, Het & KO resp. . C stands for control of the respective genotypes.

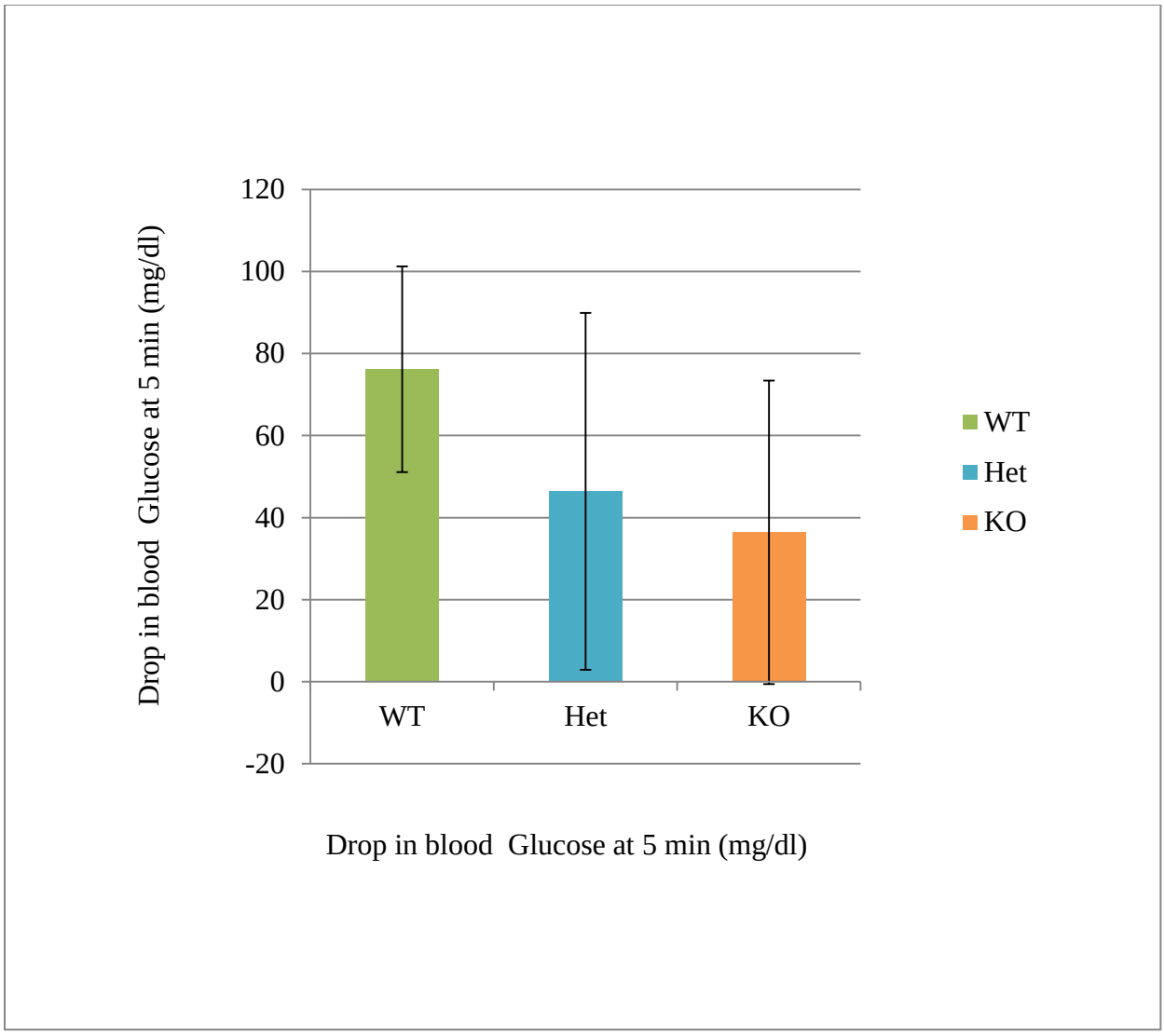

Fig. 12 Drop in blood glucose level five minutes interval after insulin administration among three genotypes during IPITT. Error bars denote ± SEM and n = 6, 5, 5 for WT, Het & KO resp. .

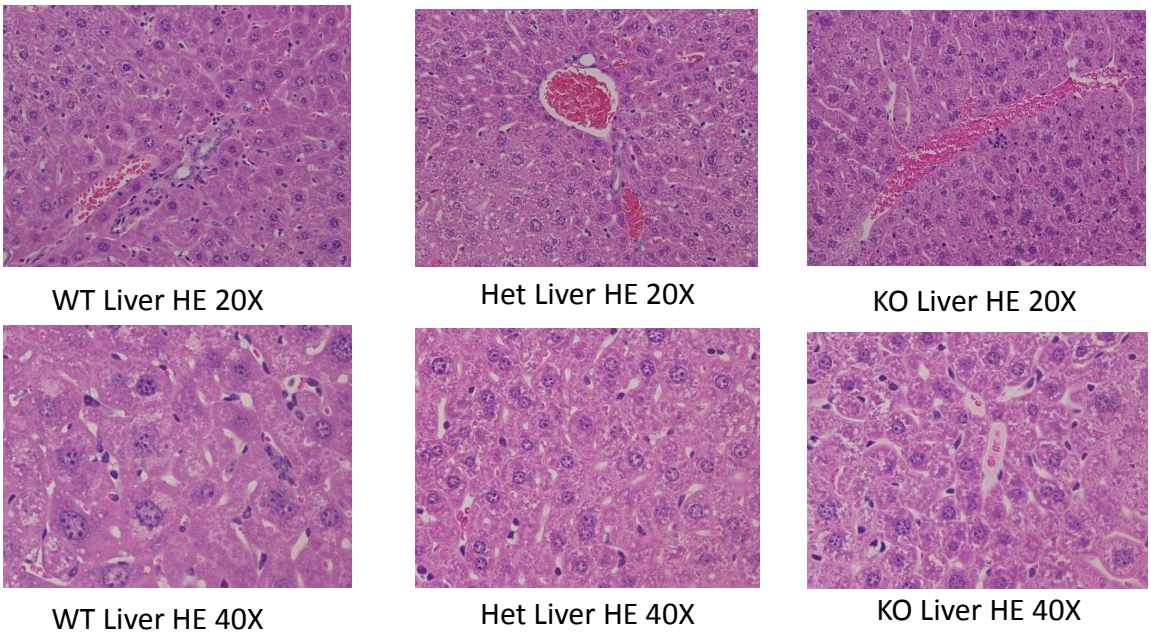

Fig. 13 Representative H & E staining of liver sections from three different genotypes.

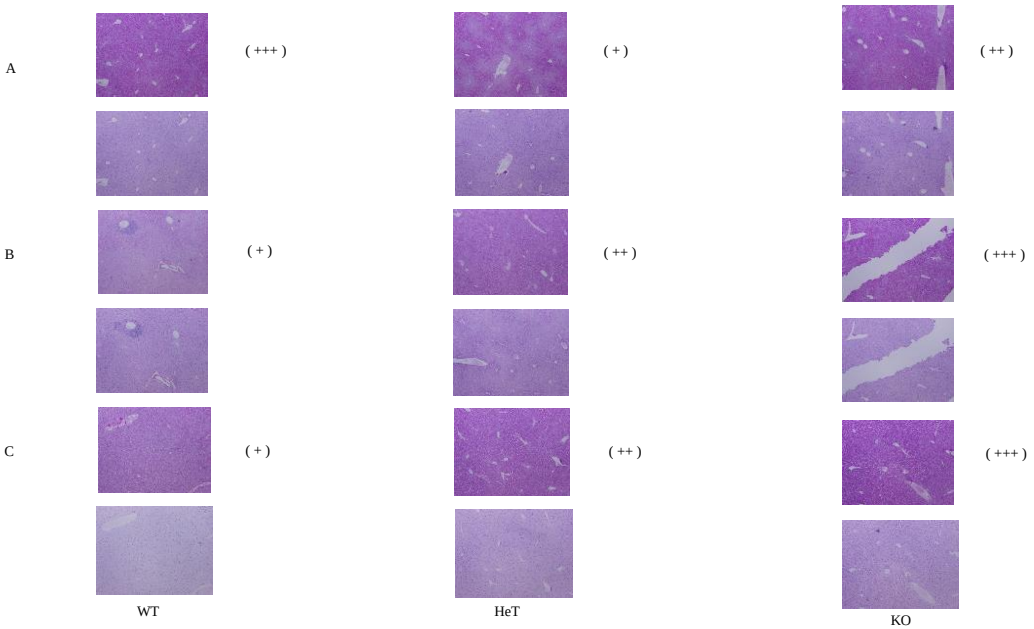

Fig. 14 PAS (upper row) and PAS-digestion (lower row) staining of liver sections from three different genotypes. (A, B & C denotes three different batches of experiments and + denotes degree of PAS intensity). Magnification 4X.

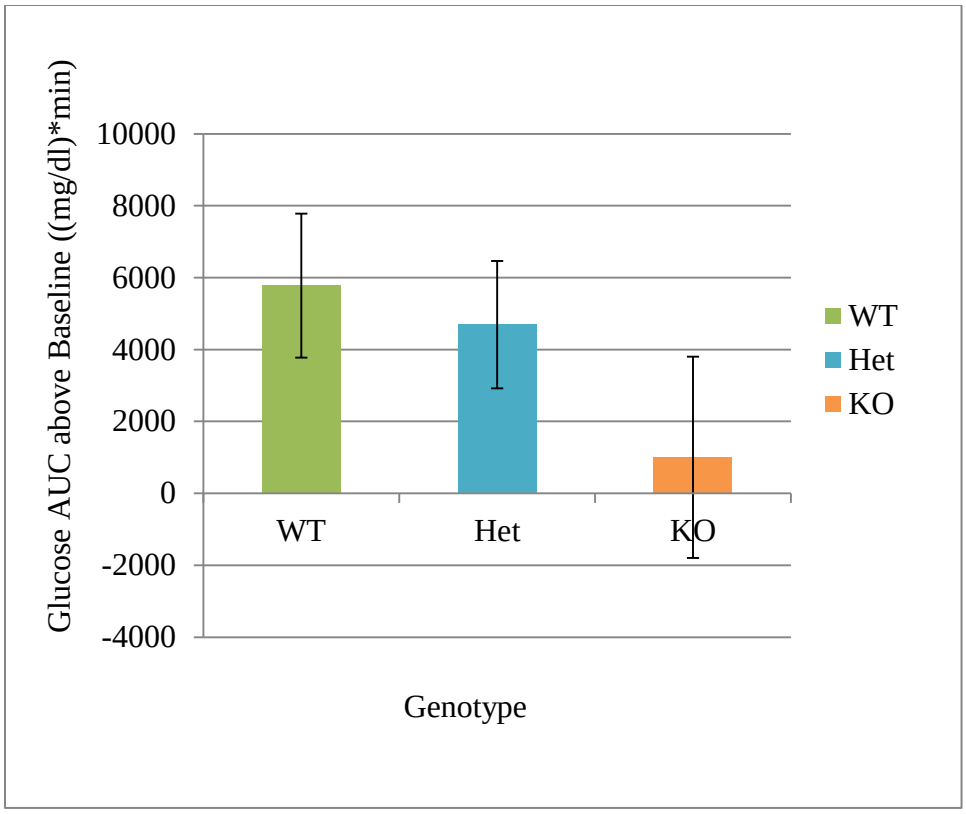

Fig. 16 AUC analysis of data obtained from IPGTT (1mg/gm BWt.) . Error bars denote ± SEM and n = 6, 5, 5 for WT, Het & KO resp. .

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APPENDICES

APPENDIX-A

SUPPLEMENTARY

GTT

	WT								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	266	Male	31	111	244	296	226	183	98
	266	Male	31	84	264	331	250	169	79
	266	Male	27	162	217	279	233	146	88
	321	Male	30	151	242	331	334	191	126
	314	Male	33.6	82	265	577	278	111	101
	295	Male	31.4	116	197	419	278	148	95
AVG	288	#DIV/0!	30.67	117.67	238.17	372.17	266.5	158	97.83
Std	25.56	#DIV/0!	2.16						
n	6		SEM	13.58	10.92	45.47	16.18	11.96	6.49

Control	WT								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	266	Male	31	89	193	139	174	95	96
	266	Male	31	112	106	136	134	112	101
	266	Male	27	103	175	156	136	94	82
	321	Male	30	120	166	159	240	228	105
	314	Male	33.6	81	144	120	147	99	101
	295	Male	31.4	137	178	230	157	100	91
AVG				107	160.33	156.67	164.67	121.33	96
Std									
n	6		SEM	8.39	12.71	15.78	16.22	21.49	3.43

	Het								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	322	Male	36.4	108	342	479	323	172	129
	266	Male	37.2	102	219	285	291	161	124
	314	Male	34	97	264	444	346	374	113
	276	Male	33.4	94	393	600	600	526	257
	305	Male	32.5	183	230	519	415	199	93
AVG	296.6	#DIV/0!	34.7	116.8	289.6	465.4	395	286.4	143.2
Std	24.39	#DIV/0!	2.01						
n	5								
			SEM	16.72	33.64	52.05	55.15	71.25	29.11

Control	Het								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	322	Male	36.4	120	154	206	138	137	107
	266	Male	37.2	96	133	169	142	142	94
	314	Male	34	136	191	139	249	168	138
	276	Male	33.4	122	240	197	151	171	290
	305	Male	32.5	236	290	188	316	191	136
AVG				142	201.6	179.8	199.2	161.8	153
Std									
n	5		SEM	24.36	28.61	11.90	35.68	9.96	35.27

	KO								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	322	Male	36	87	318	335	200	130	101
	322	Male	34.6	103	251	474	352	134	104
	221	Male	32	131	309	298	283	366	108
	266	Male	33.4	97	180	255	296	199	132
	304	Male	31.3	206	173	500	395	259	151
AVG	287	#DIV/0!	33.46	124.8	246.2	372.4	305.2	217.6	119.2
Std	43.41	#DIV/0!	1.91						
n	5								
			SEM	21.57	30.71	48.64	33.09	44.01	9.65

Control	KO								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	322	Male	36	81	118	108	103	133	96
	322	Male	34.6	127	175	178	152	220	123
	221	Male	32	100	195	160	344	178	87
	266	Male	33.4	150	173	166	144	160	120
	304	Male	31.3	191	143	218	166	89	118
AVG				129.8	160.8	166	181.8	156	108.8
Std									
n	5		SEM	19.28	13.54	17.67	41.88	21.92	7.25

	Glucose (mg/dL)						Area under the curve		
WT	0 min	5 min	15 min	30 min	60 min	120 min	Total	Baseline	Above Baseline
#1	111	244	296	226	183	98	22067.5	13320	8747.5
#2	84	264	331	250	169	79	21927.5	10080	11847.5
#3	162	217	279	233	146	88	19972.5	19440	532.5
#4	151	242	331	334	191	126	26220	18120	8100
#5	82	265	577	278	111	101	23685	9840	13845
#6	116	197	419	278	148	95	22770	13920	8850
								AVG	8653.75
								Std.	4548.80
								SEM	1857.04

	Glucose (mg/dL)						Area under the curve		
Het	0 min	5 min	15 min	30 min	60 min	120 min	Total	Baseline	Above Baseline
#1	108	342	479	323	172	129	27700	12960	14740
#2	102	219	285	291	161	124	22972.5	12240	10732.5
#3	97	264	444	346	374	113	35777.5	11640	24137.5
#4	94	393	600	600	526	257	55562.5	11280	44282.5
#5	183	230	519	415	199	93	29752.5	21960	7792.5
								AVG	20337
								Std.	14737.44
								SEM	6590.78

	Glucose (mg/dL)						Area under the curve		
KO	0 min	5 min	15 min	30 min	60 min	120 min	Total	Baseline	Above Baseline
#1	87	318	335	200	130	101	20170	10440	9730
#2	103	251	474	352	134	104	25135	12360	12775
#3	131	309	298	283	366	108	32447.5	15720	16727.5
#4	97	180	255	296	199	132	24355	11640	12715
#5	206	173	500	395	259	151	33135	24720	8415
								AVG	12072.5
								Std.	3218.69
								SEM	1439.44

ITT

	WT								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	280	Male	34.5	106	115	138	132	97	143
	280	Male	29.7	113	129	105	126	155	191
	280	Male	35.8	128	124	87	95	156	165
	334	Male	32.5	103	137	105	133	124	137
	326	Male	31.3	90	89	74	72	71	106
	307	Male	37.1	110	105	161	104	96	161
AVG	301.17		33.48	108.33	116.50	111.67	110.33	116.50	150.50
SEM	10.12		1.15	5.10	7.13	13.22	9.96	14.11	11.80

Control	WT								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	280	Male	34.5	112	151	196	124	133	107
	280	Male	29.7	216	212	255	266	224	167
	280	Male	35.8	157	316	174	162	194	116
	334	Male	32.5	115	154	249	323	155	161
	326	Male	31.3	93	158	155	199	172	128
	307	Male	37.1	119	165	171	148	135	154
AVG				135.33	192.67	200.00	203.67	168.83	138.83
			SEM	18.25	26.32	17.31	31.27	14.50	10.27

	Het								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	280	Male	38.8	120	236	213	186	172	157
	326	Male	38.6	135	116	135	152	124	122
	289	Male	33.2	127	164	164	119	92	173
	318	Male	36.8	331	202	267	114	342	241
	338	Male	38.5	151	166	113	105	120	141
AVG	310.2		37.18	172.8	176.8	178.4	135.2	170	166.8
SEM	11.06		1.06	39.89	20.15	27.76	14.98	44.88	20.39

Control	Het								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	280	Male	38.8	144	214	292	267	265	141
	326	Male	38.6	226	178	174	277	220	196
	289	Male	33.2	150	374	307	329	189	133
	318	Male	36.8	195	192	285	239	192	257
	338	Male	38.5	198	158	191	161	164	128
AVG				182.6	223.2	249.8	254.6	206	171
			SEM	15.54	38.79	27.83	27.56	17.21	24.71

	KO								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	235	Male	37	126	162	170	164	315	391
	280	Male	36.4	155	202	110	243	215	125
	338	Male	35.8	211	145	179	176	168	156
	338	Male	36.7	161	309	199	149	266	132
	318	Male	33.3	129	150	162	145	142	126
AVG	301.8		35.84	156.4	193.6	164	175.4	221.2	186
SEM	19.77		0.67	15.30	30.54	14.84	17.78	31.58	51.56

Control	KO								
	Age(days)	Sex	Weight(gms)	Baseline(B)	5 min	15 min	30 min	60 min	120 min
	235	Male	37	128	215	341	292	176	174
	280	Male	36.4	196	183	210	306	174	164
	338	Male	35.8	162	244	229	238	246	250
	338	Male	36.7	178	234	250	177	153	126
	318	Male	33.3	116	274	349	221	135	124
AVG				156	230	275.8	246.8	176.8	167.6
			SEM	15.01	15.14	28.98	23.62	18.86	22.89

WT					
Age(days)	Sex	Weight(gms)	5 min(C)	5 min(T)	C-T
280	Male	34.5	151	115	36
280	Male	29.7	212	129	83
280	Male	35.8	316	124	192
334	Male	32.5	154	137	17
326	Male	31.3	158	89	69
307	Male	37.1	165	105	60
				AVG	76.17
				SE	25.09

WT					
Age(days)	Sex	Weight(gms)	15 min(C)	15 min(T)	C-T
280	Male	34.5	196	138	58
280	Male	29.7	255	105	150
280	Male	35.8	174	87	87
334	Male	32.5	249	105	144
326	Male	31.3	155	74	81
307	Male	37.1	171	161	10
				AVG	88.33
				SE	21.61

Het					
Age(days)	Sex	Weight(gms)	5 min(C)	5 min(T)	C-T
280	Male	38.8	214	236	-22
326	Male	38.6	178	116	62
289	Male	33.2	374	164	210
318	Male	36.8	192	202	-10
338	Male	38.5	158	166	-8
				AVG	46.40
				SE	43.49

Het					
Age(days)	Sex	Weight(gms)	15 min(C)	15 min(T)	C-T
280	Male	38.8	292	213	79
326	Male	38.6	174	135	39
289	Male	33.2	307	164	143
318	Male	36.8	285	267	18
338	Male	38.5	191	113	78
				AVG	71.40
				SE	21.36

KO					
Age(days)	Sex	Weight(gms)	5 min(C)	5 min(T)	C-T
235	Male	37	215	162	53
280	Male	36.4	183	202	-19
338	Male	35.8	244	145	99
338	Male	36.7	234	309	-75
318	Male	33.3	274	150	124
				AVG	36.40
				SE	36.98

KO					
Age(days)	Sex	Weight(gms)	15 min(C)	15 min(T)	C-T
235	Male	37	341	170	171
280	Male	36.4	210	110	100
338	Male	35.8	229	179	50
338	Male	36.7	250	199	51
318	Male	33.3	349	162	187
				AVG	111.80
				SE	29.00

Table 7. Comparison of baseline blood glucose levels between two genotypes after 5 hours of fasting during IPITT.

WT	KO
106	126
113	155
128	211
103	161
90	129
110	

Table 8. Mean weaning (21 days) weight distribution between two sexes among the three genotypes of ***Alox8*** mice.

AVG	Genotype			SE			Std		
Wts.(gm)	WT	Het	KO	WT	Het	KO	WT	Het	KO
M	11.11	11.32	11.49	0.63	0.66	1.09	2.37	2.28	3.08
F	11.58	11.16	10.58	0.62	0.6	0.66	2.25	2	1.98

Table 9. Distribution of sexes among the three genotypes of ***Alox8*** mice at the time of weaning.

	Genotypes		
Sexes	WT	Het	KO
Male	14	12	8
Female	13	11	9

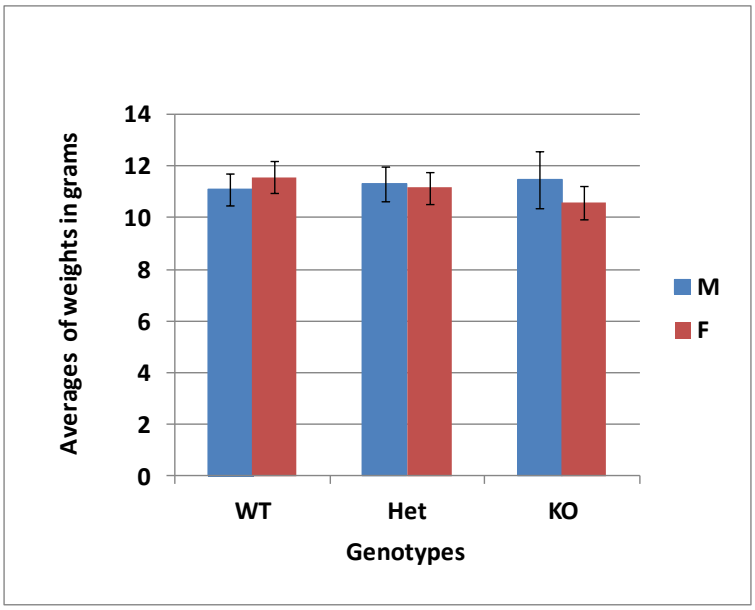
Table 10. Age and weight distribution among the three genotypes during IPGTT (1mg/ gm BWt.).

Genotype	Average Wt. (gms)	Age(days)	No.	Std Wt.	Std Age
WT	33.05	356.83	6	2.74	24.33
HET	35.86	363.4	5	4.09	22.97
KO	35.56	356.8	5	3.55	43.31

One way- ANOVA of ages in three groups , p = 0.926
One way- ANOVA of Wts in three groups , p = 0.355

Table 11. Dynamics of blood glucose level with time during IPGTT (1mg/ gm BWt.)

	Baseline	5 min	15min	30min	60min	120min
WT	138.17	252	285.67	200.33	182.67	127.5
Het	166.4	280.8	368.4	224.2	187.2	135.6
KO	211.2	294.6	289.2	284.2	211	136.8



One way ANOVA for weights of males p= 0.941
One way ANOVA for weights of females p= 0.553
students' t-test for weights in WT between sexes , p= 0.6124
students' t-test for weights in Het between sexes , p= 0.8663
students' t-test for weights in KO between sexes , p= 0.4747

Fig. 15 Comparison of weights distribution between sexes among three genotypes of ***Alox8*** mice at the time of weaning. Error bar denotes ±SEM. For n values see table 8 above.

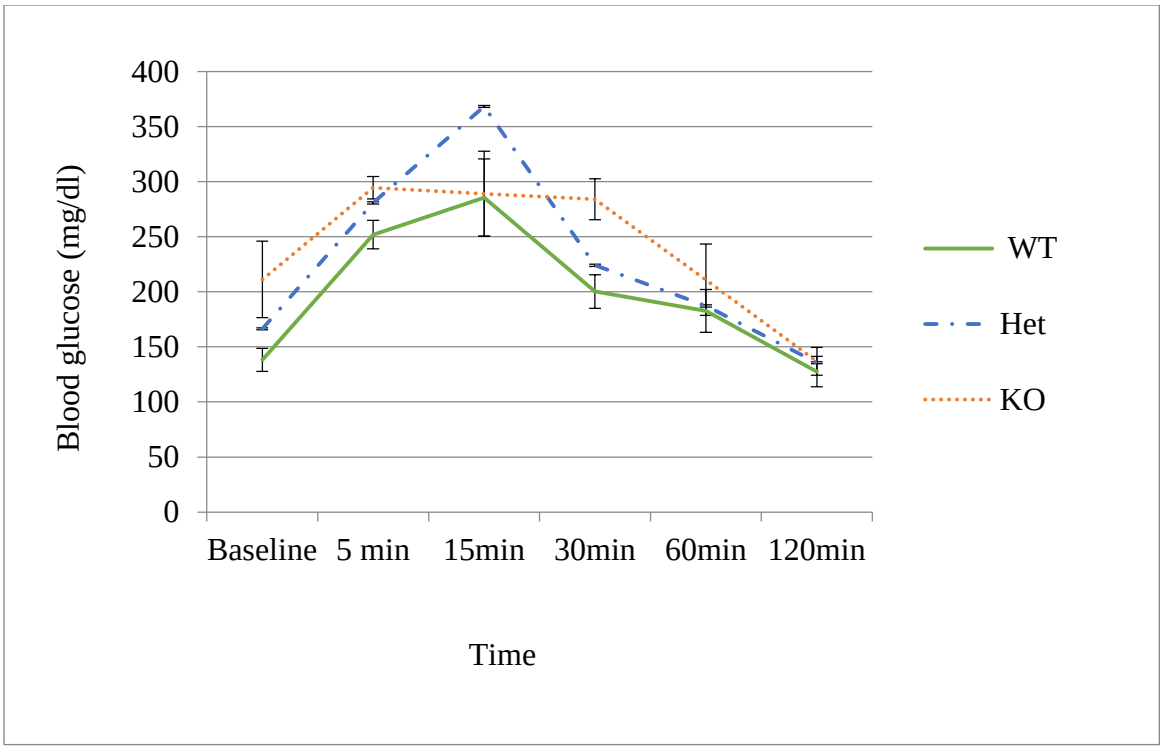


Fig. 17 Dynamics of blood glucose level during IPGTT (1mg/ gram BWt.).

IPGTT (1mg/gm BWt)

	Glucose (mg/dl)						Area under the curve		
WT	0 min	5 min	15 min	30 min	60 min	120 min	Total	Baseline	Above Baseline
#1	128	246	447	241	235	179	29120	15360	13760
#2	97	228	196	132	110	92	15082.5	11640	3442.5
#3	137	251	290	211	225	158	25462.5	16440	9022.5
#4	168	306	282	222	165	112	22020	20160	1860
#5	136	215	234	197	206	120	22180	16320	5860
#6	163	266	265	199	155	104	20287.5	19560	727.5
								AVG	5778.75
								Std.	4910.37
								SEM	2004.65

	Glucose (mg/dL)						Area under the curve		
Het	0 min	5 min	15 min	30 min	60 min	120 min	Total	Baseline	Above Baseline
#1	142	193	263	194	177	131	21350	17040	4310
#2	230	263	572	258	183	148	28177.5	27600	577.5
#3	182	311	306	202	183	123	23082.5	21840	1242.5
#4	158	359	470	272	224	116	28642.5	18960	9682.5
#5	120	278	231	195	169	160	22065	14400	7665
								AVG	4695.5
								Std.	3959.73
								SEM	1770.85

	Glucose (mg/dL)						Area under the curve		
KO	0 min	5 min	15 min	30 min	60 min	120 min	Total	Baseline	Above Baseline
#1	244	283	244	297	149	119	22740	29280	-6540
#2	132	290	202	306	158	119	22595	15840	6755
#3	174	333	321	244	237	170	28200	20880	7320
#4	176	274	256	239	186	111	22772.5	21120	1652.5
#5	330	293	423	335	325	165	35422.5	39600	-4177.5
								AVG	1002
								Std.	6267.90
								SEM	2803.09

APPENDIX-B

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