Role of Double Stranded Rna Dependent Protein Kinase R in Diabetes: Mechanism and Prevention by Novel Pkr Inhibitors

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Role of Double Stranded Rna Dependent Protein Kinase R in Diabetes: Mechanism and Prevention by Novel Pkr Inhibitors** submitted by **Mary Priyanka Udumula** ID No.**2014PHXF0011H** forward of Ph.D of the Institute embodies original work done by her under my supervision.

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ABSTRACT

Due to increased consumption of fatty products and sugar rich carbohydrates metabolic disorders are prevalent at younger age. International Diabetes federation reported that India was standing in 2nd place among top ten countries with 90.3 million people suffering from diabetes, this may move to first place by 2045. So there is a need for search of new therapeutic agents. Glucose and Fructose are supplied as sweetners in many carbonated beverages and prepared foods. As diabetes is linked with stress, inflammation and nutrient factors. We further explored for new targets which were linking with all these factors. Researchers reported the role of PKR-/- in diabetes, they also reported the role of small molecule PKR inhibitors C16 and aminopurine in diabetes. But the exact mechanism underlying behind this is yet to be understood. So we investigated the role of PKR by supplying glucose (invitro) and fructose (invivo) as exogenous nutrient factors in type 2 diabetes. The aim of the project was to 1) To correlate the pathological process of diabetes with changes in PKR expression in different cell lines and the related signal pathways.2)To investigate the role of PKR in the development of high carbohydrate induced diabetes. 3) To investigate the potential of small molecule PKR inhibitors and examine their effectiveness in the prevention of development of diabetes. 4) To investigate the effect of small molecule PKR inhibitors role in diabetic complications(diabetic cardiomyopathy). We used skeletal muscle cell line for diabetic model, we measured PKR expression on incubation of h9c2 cells with HG(25mM) for 3,6,12 and 24h. In-vivo we gave High Fructose water to wistar rats for 6 weeks and a dose of streptozotocin to induced insulin resistance. Biochemical parameters such as body weight, plasma glucose and aspartate transaminase levels were measured. We also measured lipid parameters such as HDL, LDL, TG'S and Total Cholestrol. PKR expression was

maximum at 24 hours, PKR expression was measured using western blot, Q-PCR and immunocytochemistry. PKR downstream markers were also measured such as JNK and NFKB expression using Q-PCR, western blot and immunohistochemistry. Regular insulin signaling gets impaired in diabetes, so we next measured markers involved in insulin signaling pathway such as IRS-1, PI3-K and AKT. Glucose uptake was also measured by flowcytometry in and absence of insulin using 2NBDG. In in-vivo GLUT4 expression was measured as Glut 4 is an important transporter present in skeletal muscle which helps in glucose uptake. PdX an important marker present in pancreas which helps in beta cell maturation and insulin secretion, was also measured using Q-PCR. As ROS and apoptosis are major factors involved in many metabolic diseases, ROS and apoptosis levels was also measured using Flowcytometry and immunohistochemistry. As ROS production leads to autophagy, we next measured autophagy (vacuole formation) using rhodamine staining. We further investigated HG and HF treatment induced morphological changes in insulin signaling organs such as skeletal muscle, liver, pancreas and adipose tissue using H and E staining. Collagen deposition and fibrosis was also measuredusing Sirius red staining and massons trichome. Tunnel staining was also performed in pancreas to see if any apoptosis occurred. Next to explore the role of PKR in diabetic cardiomyopathy, Cardiomyocytes(H9C2) cell line was used for further screening of novel PKR inhibitors I3O and IHZ in diabetes and diabetic cardiomyopathy along with HG and HF in comparision with specific PKR inhibitor C16. IHZ in presence of HG was more significant in inhibiting PKR expression when compared to I3O incubated with HG. IHZ was more significant in inhibiting JNK, Caspase-3expression, ROS and apoptosis compared to C16.

Taken together, these findings suggest PKR pathway is likely to play a pivotal role in the progression of insulin resistance and beta-cell dysfunction and, thus, could be a potential therapeutic target for diabetes.

So Targeting PKR by safe and selective inhibitors can be an important strategy in combating metabolic disorders.

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To everyone those took part in this journey many, many thanks.

September, 2018 *MARY PRIYANKA UDUMULA*

DEDICATIONS

to

MY PARENTS AND ALMIGHTY

Who groomed me and made me who I am today and are

my real life mentors, the biggest role models and a true blessing from God

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LIST OF ABBREVIATIONS

WHO-	WORLD HEALTH ORGANISATION
IDF-	INTERNATIONAL DIABETES FEDERATION
IDDM-	INSULIN DEPENDENT DIABETES MELLITUS
NIDDM-	NON INSULIN DEPENDENT DIABETES MELLITUS
GDM-	GESTATIONAL DIABETES MELLITUS
T1DM-	TYPE 1 DIABETES MELLITUS
T2DM-	TYPE 2 DIABETES MELLITUS
STZ-	STREPTOZOTOCIN
ZFD-	ZUCKER FATTY DIABETIC RATS
GLUT-	GLUCOSE TRANSPORTERS
SGLTS-	SODIUM GLUCOSE TRANSPORTERS
IR-	INSULIN RECEPTOR
IRS-	INSULIN RECEPTOR SUBSTRATE
PTB-	PHOSPHOTYROSINE BINDING DOMAIN
PI3-K	PHOSPHOTIDYL IONOSITOL PHOSPHATE
L6-	SKELETAL MUSCLE MYOTUBES
PDX-1	PANCREATIC AND DUODENAL HOMEOBOX-1
HDL-	HIGH DENSITY LIPOPORTEIN

LDL-	LOW DENSITY LIPOPROTEIN
TG-	TRIGLYCERIDES
PKC-	PROTEIN KINASE C
DAG-	DI ACYL GLYCEROL
PKR-	PROTEIN KINASE R
DsRNA-	DOUBLE STRANDED PROTEIN KINASE R
DAI-	DOUBLE -STRANDED RNA ACTIVATED INHIBITOR
DsI-	DOUBLE -STRANDED RNA -DEPENDENT KINASE
P68-	DOUBLE -STRANDED RNA ACTIVATED P68 PROTEIN KINASE
Eif-2α	EUKARYOTIC INITIATION FACTOR
TLR-	TOLL LIKE RECEPTORS
LPS-	LIPO POLYSACCHARIDE
TNF-	TUMOR NECROSIS FACTOR
PACT-	PKR ASSOCIATED ACTIVATOR
PGDF-	PLATELET DERIVED GROWTH FACTORS
MAPK-	MITOGEN ACTIVATED PROTEIN KINASES
JNK-	C-JUN- N-TERMINAL KINASE (JNK)
IKK-	INHIBITORY KAPPA B KINASE (IKK).
HG-	HIGH GLUCOSE
I30-	INDIRUBIN 3 MONOOXIME

IHZ-	INDIRUBIN 3 HYDRAZONE
HFD-	HIGH FRUCTOSE DIET
H9C2-	CARDIOMYOCYTES
DCFDA-	DICHLOROFLUORESCIN DIACETATE
IGF-1	INSULIN GROWTH FACTOR
MDA-	MALONDIALDEHYDE
ROS-	REACTIVE OXYGEN SPECIES
STAT	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION
2-NBDG	2-(N-(7 Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose)
NO	NITRIC OXIDE
TCA	TRI CARBOXYLIC ACID CYCLE
RBD	RNA BINDING DOMAIN
RAX	PKR ASSOCIATED PROTEIN X
CAMK	CALMODULIN DEPENDENT PROTEIN KINASE
TUNEL	TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE dUTP NICK END LABELING
DAPI	4,6-DIAMINO-2-PHENYLINDOLE
PFA	PARA FORMALDEHYDE

FACS	FLOUROSCENCE ACTIVATED CELL SORTING
CDK	CYCLIN DEPENDENT KINASE
SGOT	SERUM GLUTAMIC OXALOACETIC TRANSAMINASE
Q-PCR	QUANTITATIVE POLYMERASE CHAIN REACTION
CHF	CONGESTIVE HEART FAILURE
TBARS	THIOBARBITURIC ACID REACTIVE SUBSTANCES
SDS	SODIUM DOCECYL SULPHATE

Chapter 1

INTRODUCTION

AND

LITERATURE REVIEW

World health organization internationally defines Diabetes mellitus as a heterogeneous group of disorders with multiple attribution/aetiology characterized by chronic hyperglycaemia (high blood glucose levels) which occurs due to disturbances in protein, fat and carbohydrate metabolism (WHO, 2006). Diabetes is caused by a complex interaction of environmental and genetic factors (Harrison Principle, 19th edition). Diabetes causes long term damage to the body leading to damage, dysfunction and failure of many organs. Diabetic symptoms include increased thirst (polydipsia), frequent need to urinate (polyuria), increase in hunger (polyphagia), weight loss and blurred vision. Diabetes when left untreated can lead to development of specific complications such as retinopathy with potential blindness, neuropathy leading to nerve damage , nephropathy leading to renal failure and increased risk of cardiovascular and cerebrovascular damage (Health line News letters). Insulin is believed to be central to diabetes, it is centere for regulating carbohydrate and fat metabolism. It is a peptide hormone secreted by β cells in response to blood glucose levels in pancreas, amino acids and nervous stimulation. Insulin secretion is essential for maintaining normal body functions. Insulin plays a vital role on different tissues of body with variety of effects. In the skeletal muscle it increases glucose uptake, K+ uptake, glycogen synthesis and protein synthesis. Insulin present in adipose tissue is responsible for increased glucose uptake, triglyceride deposition, glycerol phosphate synthesis, fatty acid synthesis, activation of lipoprotein lipase and inhibiting hormone-sensitive lipase (Rother,2007).

According to International Diabetes Federation (IDF) atlas 2017, 8.8% of 20-79 years age group that is 425 million people worldwide were suffering from diabetes. This number might raise to 629 million by 2045. (Thomas ER,2006; Gandica RG, 2015; Murphy R,2008) .

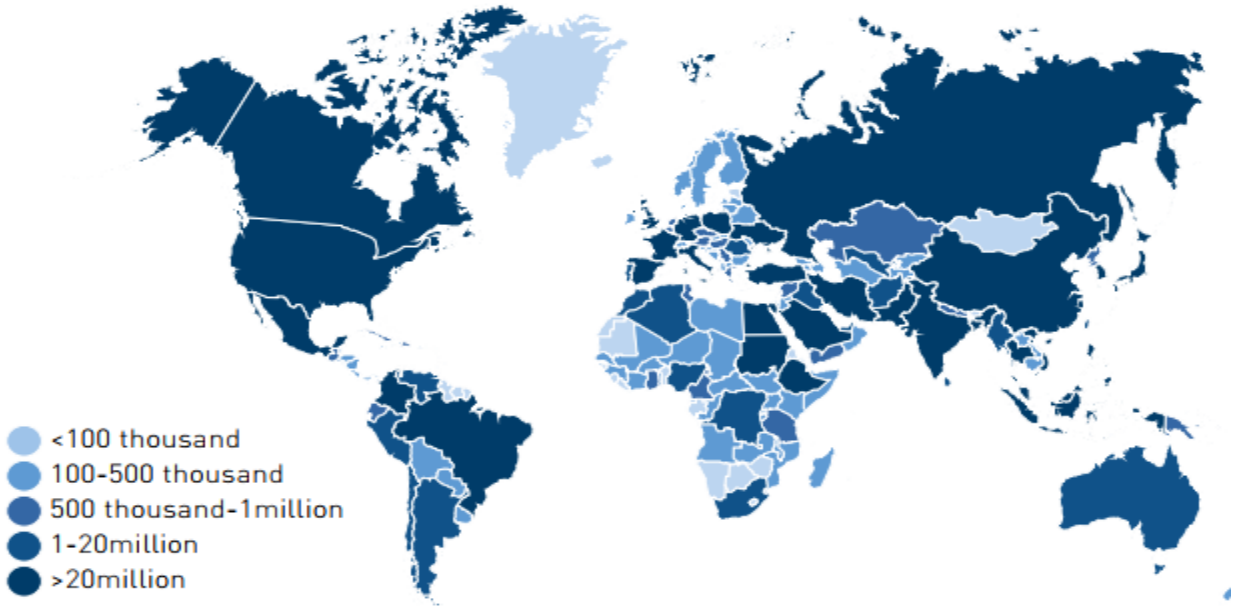


Fig 1.1 Estimated total number of adults (20-79 years) living with diabetes, 2017

According to IDF, India is reported second for number of people suffering with diabetes (90.2 million). This number was estimated to increase from 103.4 to 165.4 million and may stand first across the globe (IDF, 2017).

2017			2045		
Rank	Country/territory	Number of people with diabetes	Rank	Country/ territory	Number of people with diabetes
1	China	114.4 million (104.1-146.3)	1	India	134.3 million (103.4-165.2)
2	India	72.9 million (55.5-90.2)	2	China	119.8 million (86.3-149.7)
3	United States	30.2 million (28.8-31.8)	3	United States	35.6million (33.9-37.9)
4	Brazil	12.5 million (11.4-13.5)	4	Mexico	21.8 million (11.0-26.2)
5	Mexico	12.0 million (6.0-14.3)	5	Brazil	20.3 million (18.6-22.1)
6	Indonesia	10.3 million (8.9-11.1)	6	Egypt	16.7million (9.0-19.1)
7	Russian Federation	8.5 million (6.7-11.0)	7	Indonesia	16.7million (14.6-18.2)
8	Egypt	8.2million (4.4-9.4)	8	Pakistan	16.1 million (11.5-23.2)
9	Germany	7.5 million (6.1-8.3)	9	Bangladesh	13.7 million (11.3-18.6)
10	Pakistan	7.5 million (5.3-10.9)	10	Turkey	11.2 million (10.1-13.3)

Table 1.A Top ten countries/territories for number of people with diabetes (20-79 years), 2017 and 2045

According to World Health Organisation (WHO) in 1980 and 1985 diabetes was classified in to two classes insulin-dependent diabetes mellitus (IDDM) or type 1 and non-insulin-dependent diabetes mellitus (NIDDM) or type 2, these classification encompasses both aetiological and clinical stages. Gestational diabetes mellitus (GDM) recently happening one is also one of the diabetes that occurs due to carbohydrate intolerance within the onset of pregnancy (Diabetes Care, 2017; Largay J, 2012; Evans JM, 2000; Bruno G; 2005)

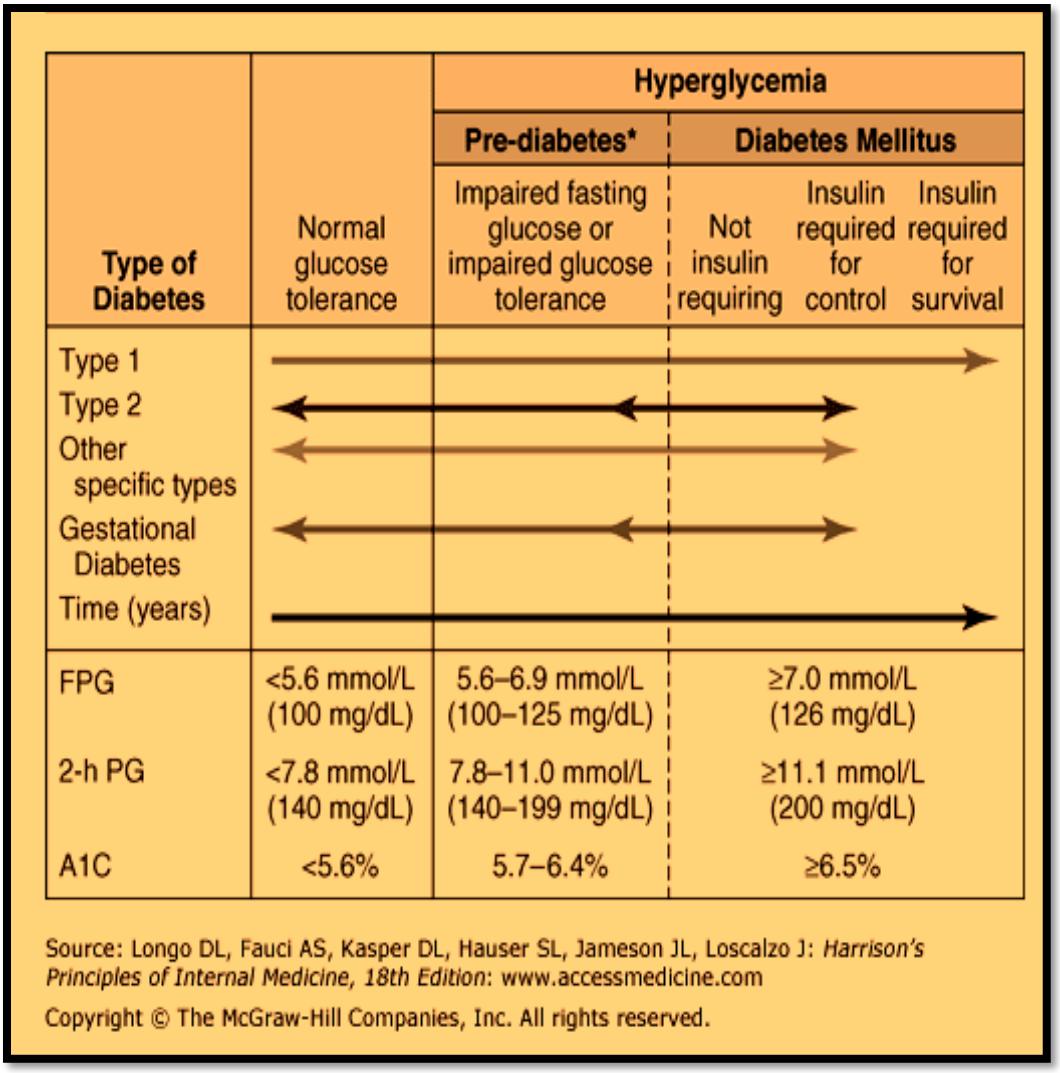


Table 1.B Types of diabetes with fasting glucose levels. This figure is reproduced from Principles of Internal Medicine

1.2 TYPE 1 DIABETES MELLITUS

Type 1 diabetes or insulin dependent diabetes also known as juvenile diabetes is caused when a body immune system attacks its own beta cells in pancreas which produces insulin. This reaction is an autoimmune reaction. Due to destruction of beta cells there will be less to no insulin production. The reason for this destructive process is not clear, however some studies

have reported combination of environmental factors such as viral infections, toxins and some dietary factors. Studies have also reported genetic susceptibility as a cause for type 1 diabetes (Maahs DM, 2010).

Autoimmunity a major reason for T1D has been identified by the presence of auto antibodies to islet and/or b-cell antigens. Other antibodies that are associated with T1DM are islet cell auto antibodies to glutamic acid decarboxylase , insulin auto antibodies and auto antibodies to trans membrane tyrosine phosphate. However in some cases of T1DM no antibodies for β cell were found (Gillespie, 2006). Recent studies in animals have shown the role of b-lymphocytes which act as self antigens against cytotoxic cells which destroy pancreatic beta cells. Streptozotocin (STZ)-induced diabetes is the most widely used model in laboratory setting. STZ an alkylating agent causes destruction of insulin producing β cells of the pancreas this is a general model used for inducing T1DM (Cefalu, 2006).

Pathophysiology of Type 1 Diabetes

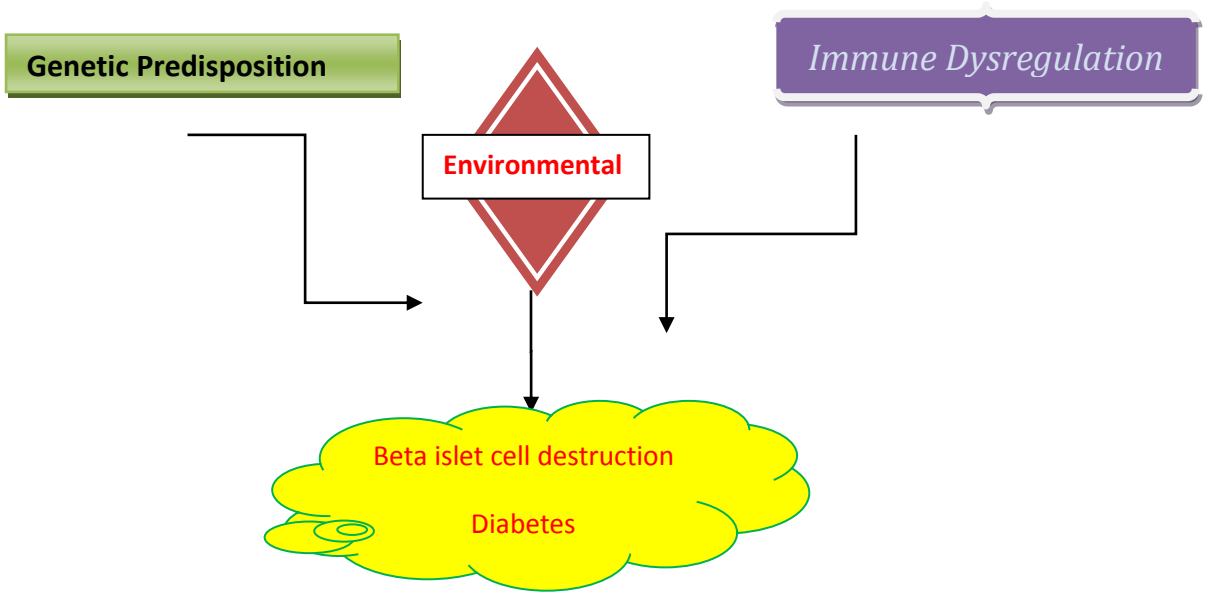


Fig 1.2 Path physiology of type 1 diabetes

Sympotms observed in type 1 diabetes condition

Abnormal thirst, Fatigue, Constant hunger, Frequent urination, Sudden weight loss, Bed wetting and Blurred vision.

1.3 TYPE 2 DIABETES MELLITUS

Type 2 diabetes/ non- insulin dependent diabetes mellitus (NIDDM). In this diabetes hyperglycaemia occurs due to in adequate production of insulin and inability of the body insulin sensitive tissues (liver, skeletal muscle and adipose) to respond to insulin. These conditions progressively lead to β–cell failure or dysfunction, which leads to a condition in which β–cells are no longer able to secret enough insulin due to defects in glucose-stimulated insulin secretion and/or reduction in β–cell mass (Rhodes CJ, 2005).

Pathophysiology of type 2 Diabetes

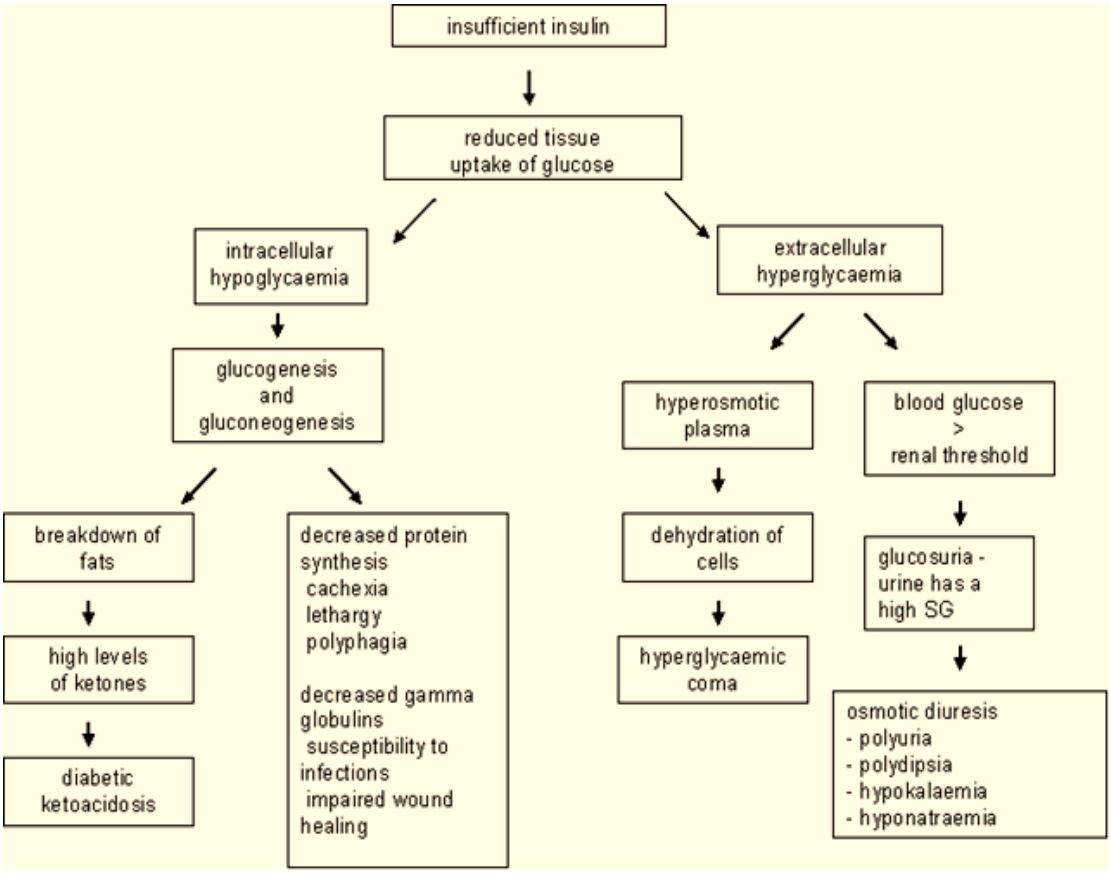


Fig 1.3 Path physiology of type 2 diabetes

When there is an inadequate production of insulin or when body peripheral tissues does not respond to insulin there will be less glucose uptake. This causes both intracellular hypoglycemia and extracellular hyperglycemia. When intracellular hypoglycemia occurs this leads to gluconeogenesis followed by break down of fats, high production of ketones leading to diabetic ketoacidosis. On the other hand when extracellular hyperglycemia occurs it causes hyper osmotic plasma levels followed by dehydration of cells and finally leading to hyperglycemia (Ley SH,2014).

Neubauer and Kulkarni (2006) described various animal models for type 2 diabetes. db/db mice a diabetes model, is created by inducing mutation in leptin receptor, on other side ob/ob model is an obese animal generated by developing mutation in leptin gene. Leptin is an obese hormone present in adipose tissue in response to fatty acids accumulated in cells. It depicts an important role in food intake, weight gain and in regulating energy. An another example for T2DM is Zucker diabetic fatty (ZDF) in which mutation is introduced in leptin receptor, where rat becomes obese very early (Peterson et al., 1990, Clark et al., 1983;). Decreased β cell mass is an non-obese model found in Goto Kakizaki (Goto and Kakizaki, 1981).

1.4 Dissimilarities in type 1 and type 2 diabetes

Type 1	Type 2	Gestational
Usually young	Usually older	First detected in pregnancy
Acute onset	Insidious onset	Routine testing
Insulin deficiency	Insulin resistance	Insulin resistance and placental hormones
Not overweight	Often overweight	Often overweight
Rare	Common	More common
Requires insulin for diagnosis	Diet and lifestyle changes can reverse it, oral medications	Diet and life style plus mediactions
Often random	Strong family history	Family history of T2DM

Table 1. C Differences between types of diabetes

1.5 Gestational Diabetes

Carbohydrate intolerance with onset or recognition of pregnancy is defined as gestational diabetes. Predominantly present as type 2 diabetes, it is difficult to identify the symptoms away from normal pregnancy symptoms. It is advised to pregnant women to go for glucose tolerance test during 24^{th}-28^{th} week of pregnancy period. Women during pregnancy period with hyperglycaemia are at higher risk for pregnancy outcomes such as respiratory syndrome, high blood pressure, insulin resistance, large baby called as foetal macrosomia and finally death.(International Book of Diabetes Mellitus,2015)

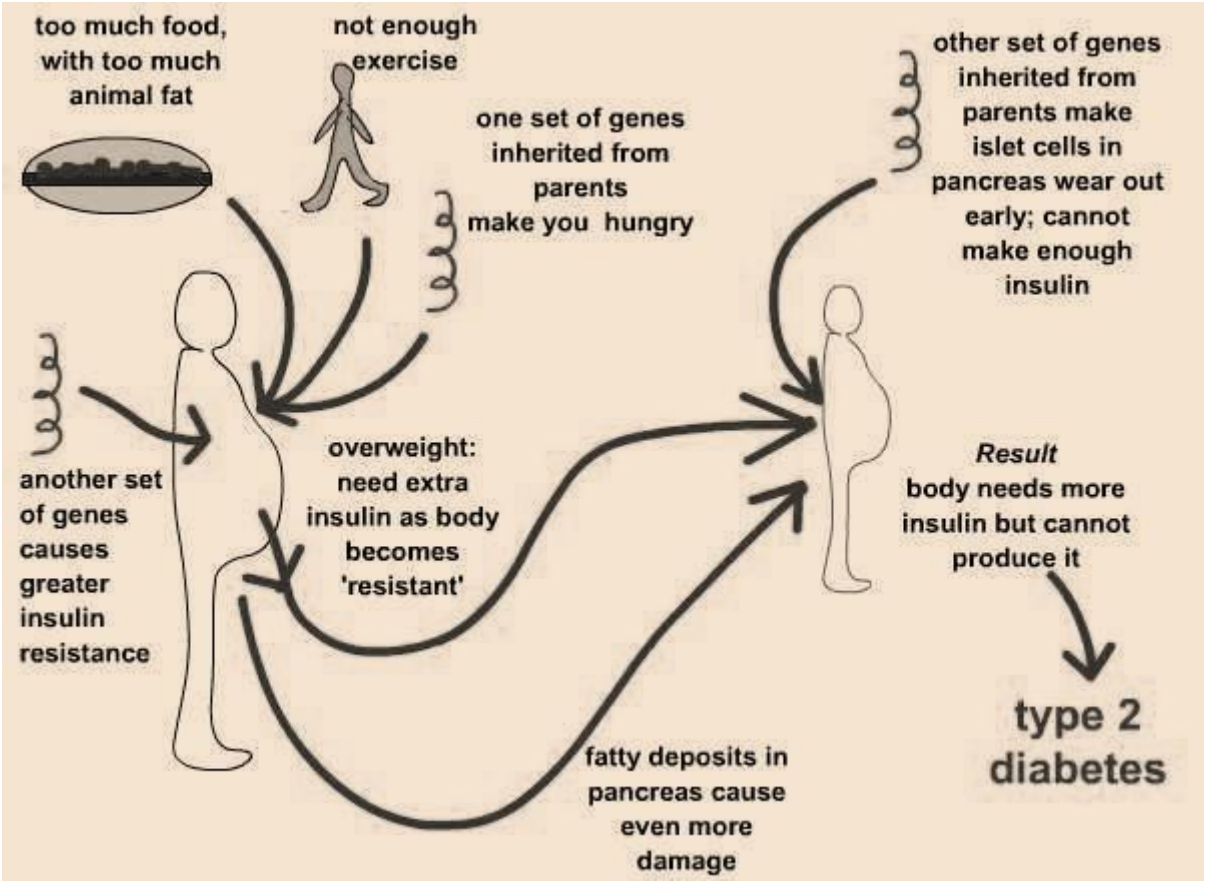


Fig 1.4 Pathophysiology of Gestational Diabetes. Various factors leading to GDM were explained

1.6 Glucose transport

Glucose is a , it is hydrophilic molecule which cannot enter the lipid layer of cell membrane so it needs some special transporters for facilitation across the membrane. There are 2 different type of glucose transporters, insulin dependent and insulin independent. Insulin dependent are GLUT transporters and insulin independent are sodium glucose transporters (SGLT'S) (Joost et al 2002, Scheepers et al 2004). SGLT'S transporters are mainly involved in absorption of glucose in intestine and kidney. GLUT's are facilitative glucose transporters. Recently 14 GLUT gebnes have been identified in which 11 genes transport sugar/ glucose across membrane (GLUT1-14). Each GLUT isotope has different function and affinity for various tissues (Scheepers *et al*, 2004). In skeletal muscle GLUT4 is the predominantly expressed isoform (Birnbaum, 1989; Fukumoto *et al*, 1989; James *et al*, 1989) whereas in pancreas GLUT2 is expressed more and GLUT4 is intercellularly localized and in response to insulin it translocates to the surface membrane. (Douen *et al*, 1990; Hirshman *et al*, 1990; Kristiansen *et al*, 1996; Ryder *et al*,2000).

1.7 Insulin Signaling

The Insulin receptor (IR) consists of four subunits with two transmembrane ß peptides connected to two extracellular insulin binding α peptides. ß subunits contains a tyrosine kinase domain on its top, these ß-subunits get activated when insulin binds to insulin receptors. this is called as autophosphoryltion. T h e Phosphorylated tyrosine residues which are present on the activated insulin receptor 's provide docking and binding sites for several downstream markers such as Grb2, Shc and the insulin receptor substrate (IRS) proteins.

When insulin receptor substrate (IRS) binds to the tyrosine residues (phosphorylated) with the help of phosphotyrosine binding domain (PTB), this further leads to mediation of

secondary signaling downstream markers. One of the important insulin signaling molecule is phosphatidylinositol 3 (PI3)-kinase (Cantrell, 2001). PI 3-kinase chain contains one catalytic subunit and one regulatory subunit , the 3'-OH position of the inositol ring present in inositol phospholipids of plasma membrane is phosphorylsed due to regulatory subunit. The substrate products that are generated as a result are phosphatidylinositol-3-monophosphate and phosphatidylinositol bisphosphate

Downstream Signaling of PI 3-kinase

AKT is also known as protein kinase B is one of the important downstream marker of PI3-K. AKT a serine threonine molecule, phosphorylases glycogen synthase kinase-3 which helps in translocation of GLUT4 molecule in adipocytes and skeletal muscle (Tanti *et al*, 1997). It also helps in glycogen synthesis and glucose transport in L6 myotubes (Ueki *et al*, 1998). Generation of phosphatidylinositol-3 monophosphate is responsible for translocation of GLUT4. Phosphorylation of serine 473 site is responsible for translocation of GLUT4 (Huang *et al*, 2005) which leads to uptake of glucose in adipose tissue and skeletal (Feng *et al*, 2004).

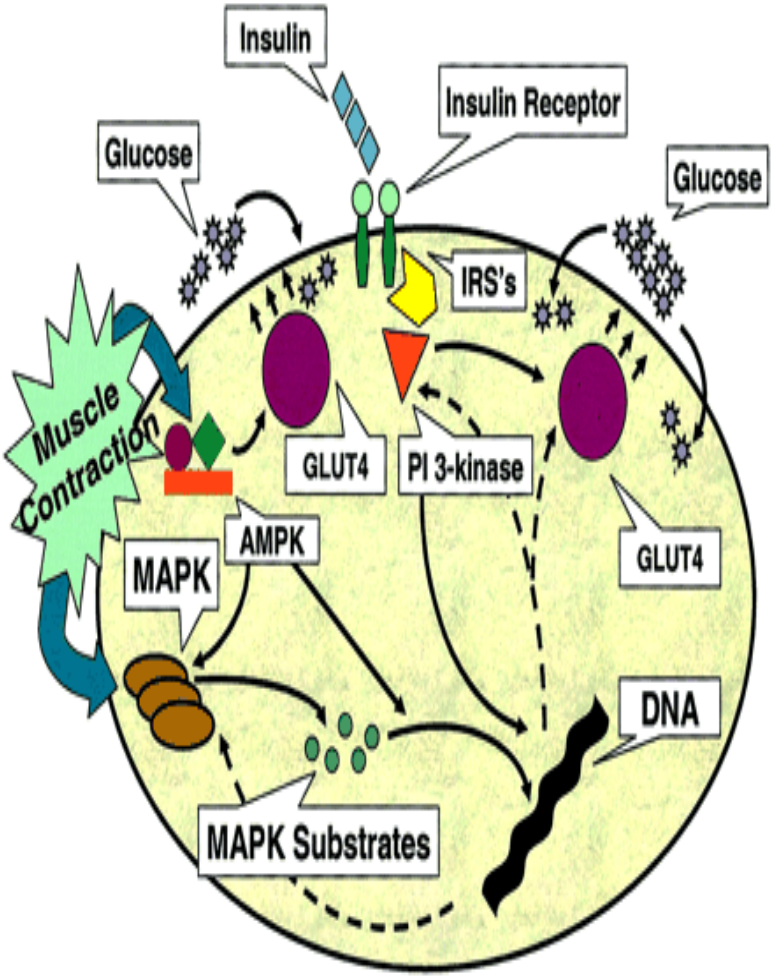


Fig 1.5 Insulin signaling in skeletal muscle General features of insulin signal transduction pathways. PI 3-kinase branch of insulin signaling regulates GLUT4 translocation and glucose uptake in skeletal muscle and NO production and vasodilation in vascular endothelium. MAP-kinase branch of insulin signaling generally regulates growth , mitogenesis and controls secretion of ET-1 in vascular endothelium.

Insulin signaling in Pancreas

Increased blood glucose enters pancreatic beta cells through GLUT2, once entered the glucose is oxidized to pyruvate and acetyl co A, this acetyl co A is used in TCA cycle. The resulting is increase in ATP levels. This ATP inhibits KATP channel resulting in depolarization of membrane leading to influx of Ca^{+2} which releases insulin to the blood (Thorens B, 2015).

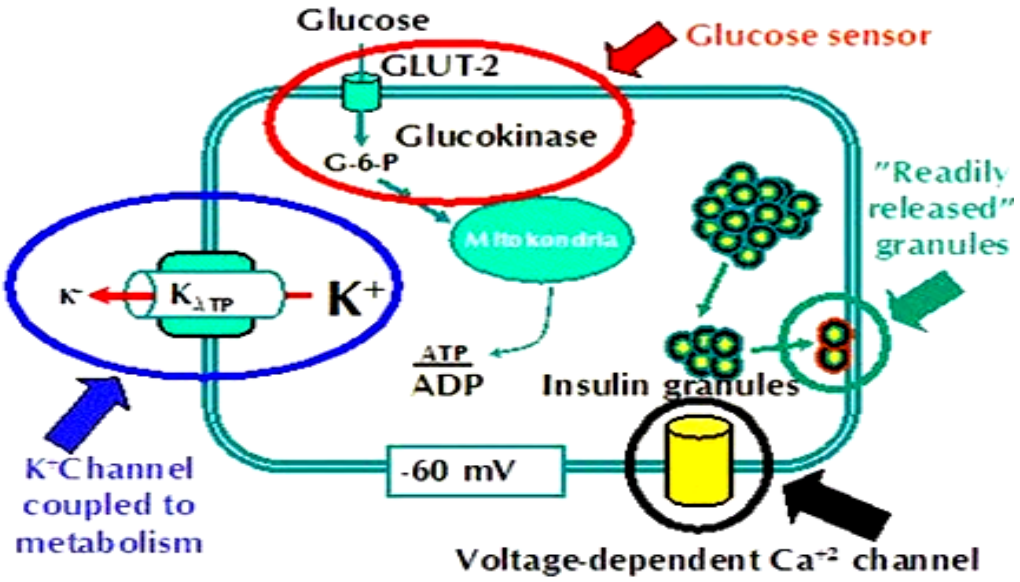


Fig 1.6 Insulin signaling in Pancreas

1.8 Insulin Gene Transcription

Insulin gene transcription includes many important factors such as pancreatic and duodenal homeobox-1 (PDX-1), also defined as an insulin promoter factor 1(IPF-1) (Leibowitz et al., 2001). It plays a crucial role not only in beta cell development but also in insulin secretion. Maf is a heterodimer associated with a glucose responsive element, which is present on the insulin promoter, A3.In normal condition PDX and MafA enter into nucleus of pancreatic beta cell and

bind to A and C sites of promoter region of insulin gene which supports in synthesis of insulinmRNA. Whereas in glucotoxicity or hyperglycemia conditions, PDX membrane will be absent so no binding to A and C regions andno insulin synthesis (Leibowitz et al., 2001, Ren et al., 2007..

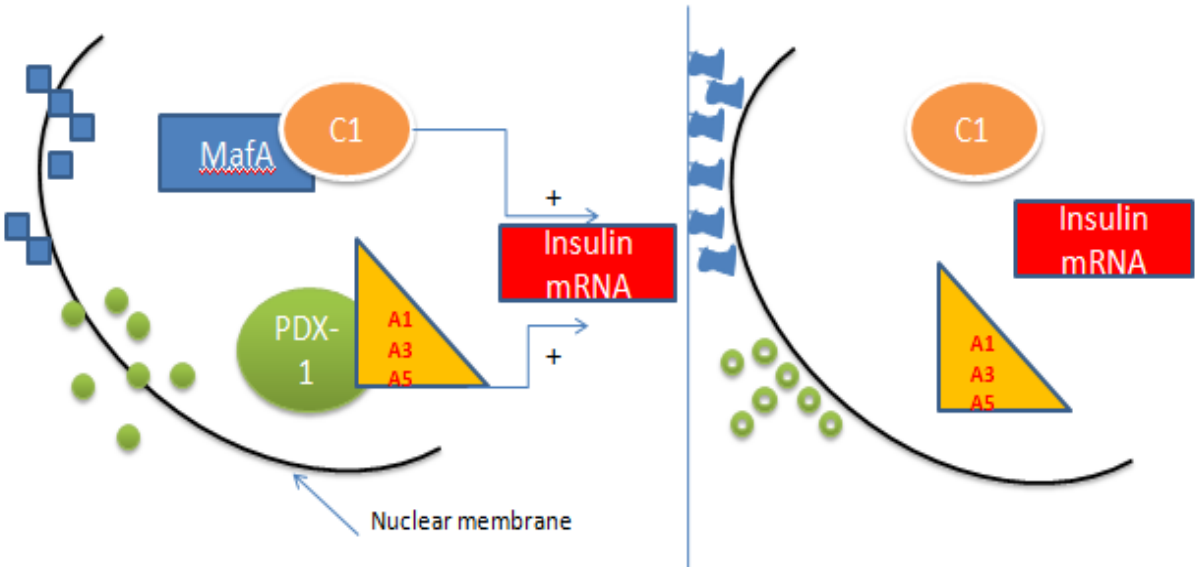


Fig 1.7 Insulin gene transcription

Insulin Resistance

Insulin resistance can be termed as a situation in which decreased response or no response to peripheral target tissues when there is normal levels of insulin in body. Insulin resistance plays an important role in the development of T2DM also associated for its responsibility in developing the metabolic syndrome, involving obesity and hypertension (Sechi & Bartoli, 1997). Defective glycogen synthesis in skeletal muscle has been reported as an important factor for development of insulin resistance (Bogardus et al., 1984; Kelly et al., 1996). Decreased phosphorylation of PI3-K, and IRS-1 is also an important factor for insulin resistance (Goodyear et al., 1995; Bjornholm et al., 1997; Smith et al., 1999). Some of the risk factors for insulin resistance are obesity, physically inactivity, gestational diabetes, high triglycerides, low HDL cholesterol, elevated

blood pressure and stroke.

1.9 Nutritional factors and its relation with NIDDM

Nutrition represents a lifestyle which can directly influence health, so healthy food intake and weight control should be mainly focused. The Western diets available now a days are rich in high content carbohydrates with aldo sugars and ketosugars are major cause for metabolic syndrome (Garg A,1994).

Carbohydrates and diabetes.

Supplying glucose and fructose as an external nutritional factor, moves directly to liver, which is a main organ involved in metabolizing carbohydrates (Imamura F, 2015). When excess of these carbohydrates are carried into liver, this hepatic metabolism gets disturbed and this leads mainly to two consequences: One of them is glucose metabolism and its uptake pathways, the second major consequence is lipogenesis (denovo) and Triglyceride synthesis. Fructose in presence of fructokinase converts to fructose-1 phosphate and then through series of processess finally is metabolized to ATP and carbon dioxide. On the other hand excess fructose intake results in more production of hepatic triglycerides which reduces insulin sensitivity and finally leads to insulin resistance (Romaguera D, 2013)

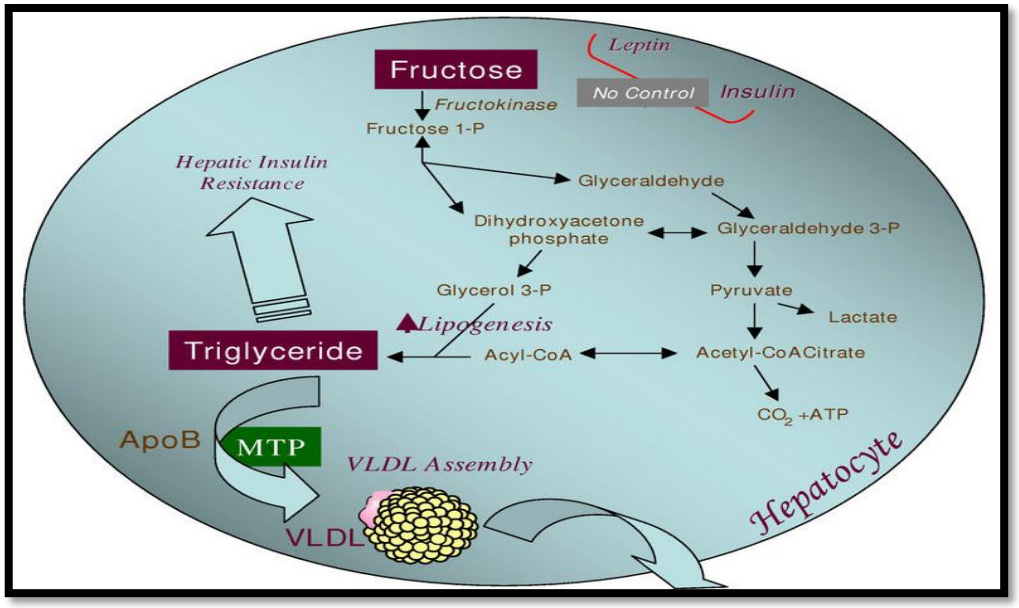


Fig 1.8 Carbohydrate intake and its relation to diabetes

The other mechanism reported was excess carbohydrates intake may increase the organ load followed by the production of superoxides and ROS, beta cell destruction, apoptosis , finally leading to insulin resistance (Imamura F, 2015)

Carbohydrate intake and its effect on liver

Excess carbohydrates and free fatty acids intake leads to increase in hepatic gluconeogenesis and lipogenesis. Gluconeogenesis is started with the formation of diacylglycerols (DAG'S) and later causes activation of protein kinase C (PKC) so the regular downstream insulin signalling pathway gets disturbed and glucose uptake does not occur. Denovo lipogenesis starts producing more amount of triglycerides and leads to insulin resistance (Aeberli I, 2013; Ameer F, 2014).

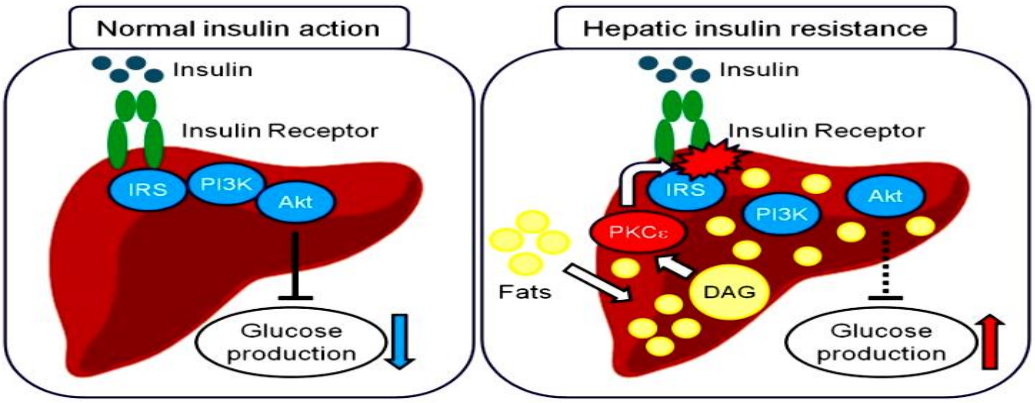


Fig 1.9 Carbohydrate intake and its effect on liver

Carbohydrate intake and its effect on skeletal muscle

Glucose when supplied in normal amount to body enters skeletal muscle with help of GLUT-4 transporters and then converts finally into pyruvate. This pyruvate enters mitochondria where oxidation occurs and KATP channels gets closed and Ca+2 channels open influx of calcium occurs and insulin is released through exocytosis. But when excess of carbohydrates are supplied cellular overload is formed and distrupts the regular mechanism (Aslesen R ,1998;Aselen R, 2001; Bouskila M, 2010) .

Carbohydrate intake and its effect on adipocytes.

When excess of carbohydrates are supplied extracellularly, fat cell hypertrophy and immune cell infiltration occurs. This is due to increase in accumulation of fatty acids in liver and increase in leptin levels and this ultimately leads to insulin resistance (Nolte and Karam, 2001).

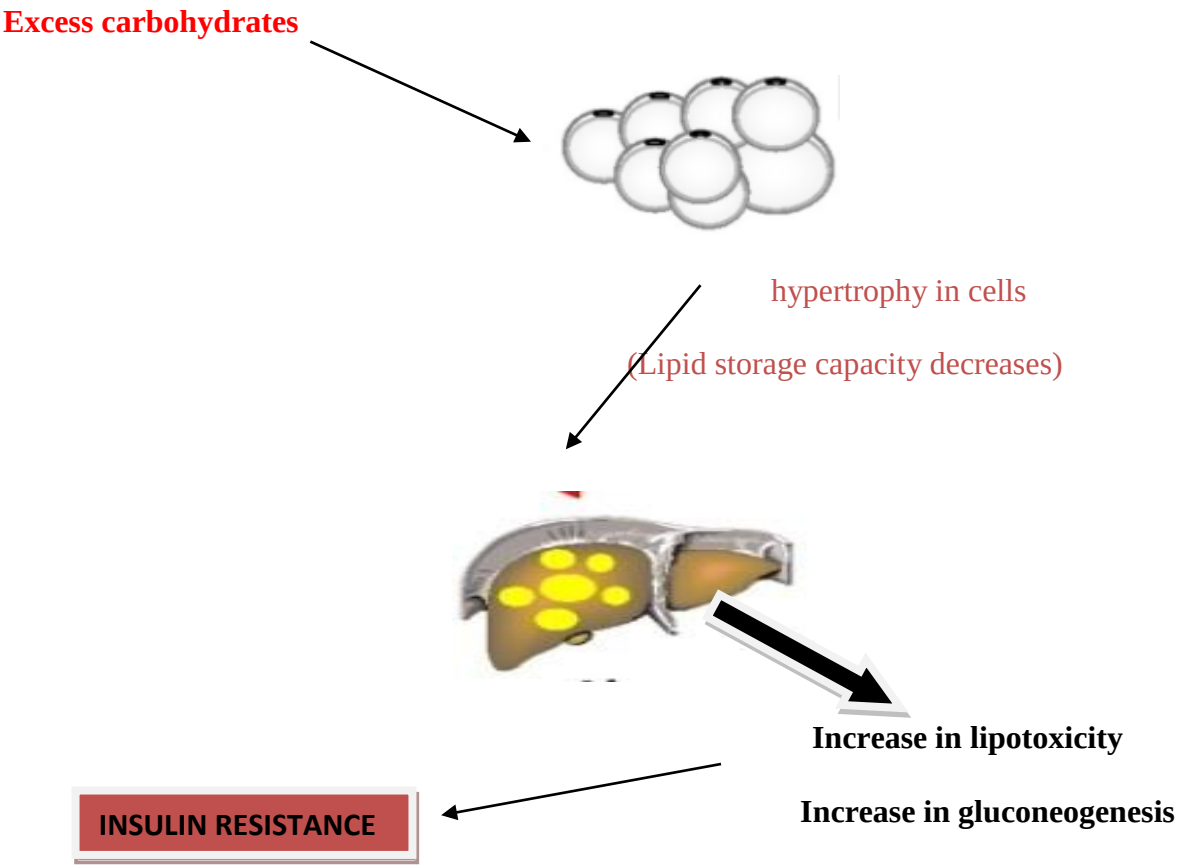


Fig 1.10 Carbohydrate intake and its effect on adipocytes

ANTI-DIABETIC DRUGS

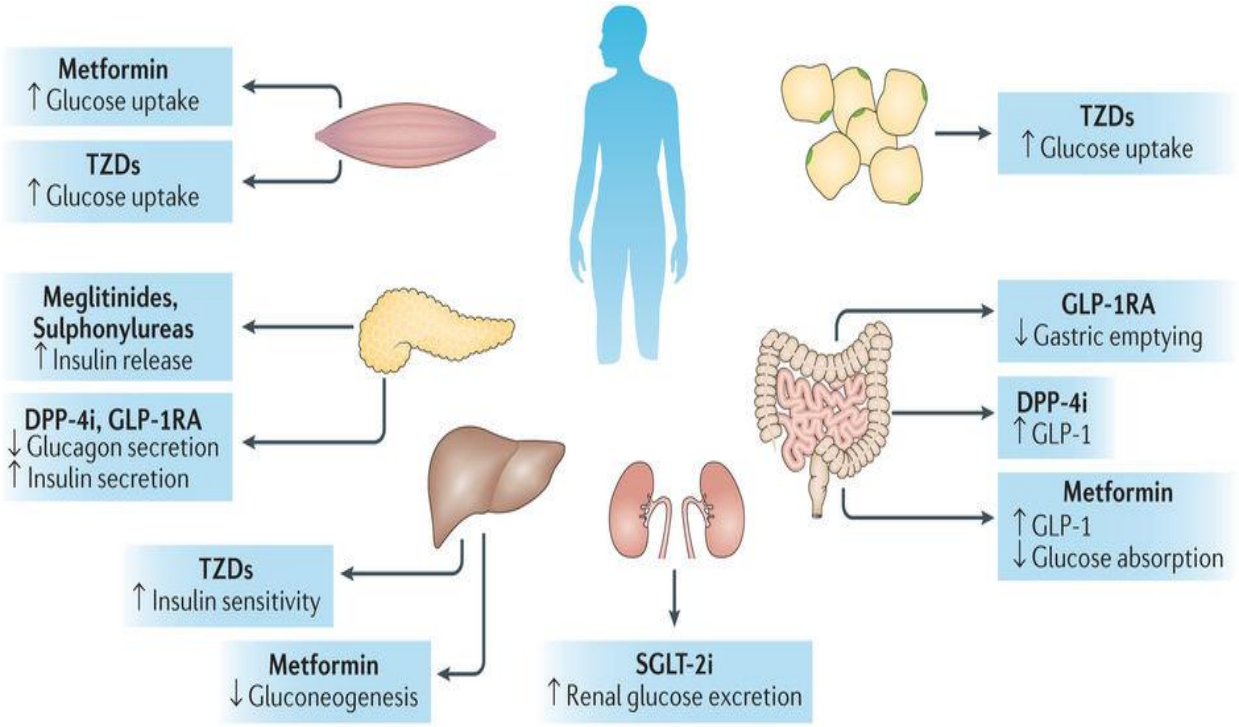


Fig 1.11 Types of anti-diabetic drugs and their mechanism of action. This figure is reproduced from an review article nature reviews, endocrinology

1.10 Literature review

Protein Kinase R (PKR)

PKRs function is well reported in inflammation and infections for its antiviral and antiproliferative effects. It is an important protein kinase responsible for cellular response but it also plays an important role in cell signaling during stress associated conditions. Metabolic syndrome provoke stress related responses in the body, one of them is PKR. It has been more than 20 years since protein kinase R was discovered (Clemens MJ,1993). Double stranded RNA dependent protein kinase R has now been standardized as PKR, however, earlier it was given name as double -stranded RNA activated inhibitor (DAI); double -stranded RNA -dependent kinase (dsl) and double -stranded RNA activated p68 protein kinase (p68) (Clemens MJ,1994).

Structure of PKR

PKR belongs to one of the small enzyme family having smallest alpha subunit of polypeptide chain of eukaryotic initiation factor. PKR homologs are not only identified in human they were identified in rat, mouse and rabbit. PKR in humans is encoded by a 551 aminoacid chain. PKR is a serine / threonine protein kinase. Its activity is regulated by its interaction with RNA. PKR has an C-terminal protein kinase domain and 20- kDa N-terminal RNA binding domain (RBD). The RNA binding domain contains two copies of the binding motif dsRBM I and dsRBM II, these are found in many DsRNA binding proteins. The aminoacid sequence for RBM1 and RBM2 motifs is 11-77 and 101-167 in 551 amino acid peptide respectively (Farrell, 1977; Levin DH,1980)

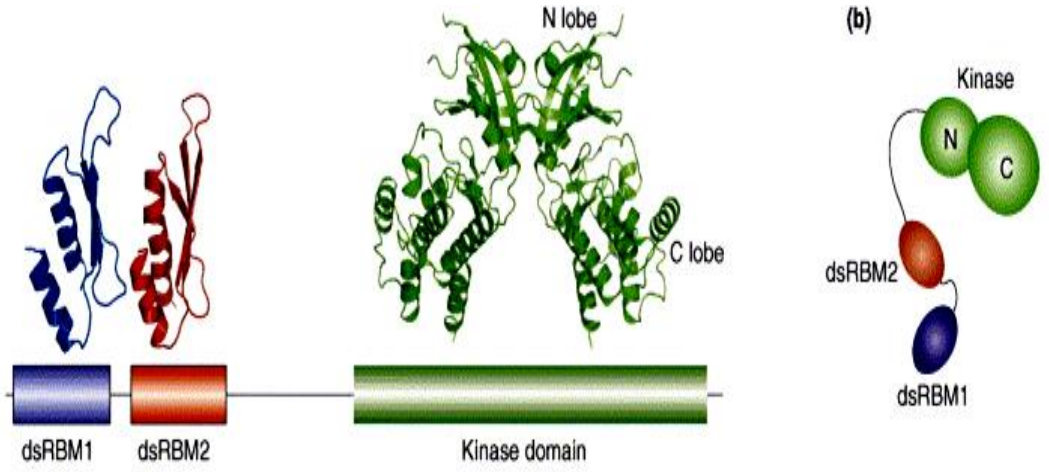


Fig 1.12 Structure of double stranded RNA dependent protein kinase R

PKR phosphorylates Eif-2α in response to stress signals, infections and nutrients. This along with other four mammalian kinases GCN2, PERK and HRI phosphorylate Eif-2α. (Barber GN, 1998; Harding HP,2000). Phosphorylation of serine residue at 51 position in eIF-2α prevents the further ongoing translation, and generally inhibits this step. PKR has the kinase domain (KD) at C terminal and dsRNA-binding domain (dsRBD) at N terminal that regulates its activity. As a result dsRNA acccumulates in pathogen or viral cells. This triggers phosphorylation of eif-2α and inhibits viral mRNA synthesis. This is the basic mechanism how PKR exerts its antiviral effects among all DNA and RNA viruses (Maclean, 1991; Malathi K , 2005);

In normal conditions, eIF2 forms a complex with methionyl t-RNA and GTP, where this complex further combines with 40S ribosomal subunit, This complete complex recognizes the start codon during translation. When large subunit 60S is combined with this trio complex, the GTP-eIF2 complex is hydrolyzed to a GDP complex. During any viral or pathogen infection eIF2 is phosphorylated to eIF2α form, here the exchange of GTP-eIF2 alpha complex to GDP is

inhibited. This results in further inhibition of translation as there as low GDP levels and thus prevents viral replication in cells (Behrmann IH,1994; Ben Asouli YY,2002).

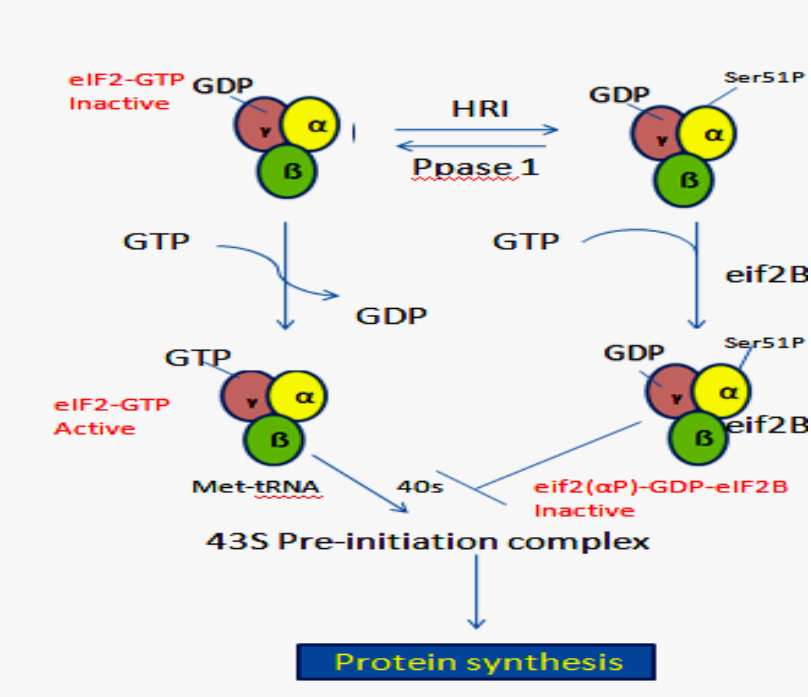


Fig 1.13 Translation regulation by PKR

PKR activation by homodimerisation and heterodimerisation

Dimerization is important for activation of PKR. Dimerisation can form protein-protein interactions with itself or other proteins. Studies reported that dimerisation can be formed due to dsRNA interaction between two PKR molecules. TAR-RNA binding is an example of PKR heterodimerisation (Romano PR, 1995; Carpick BW,1997)

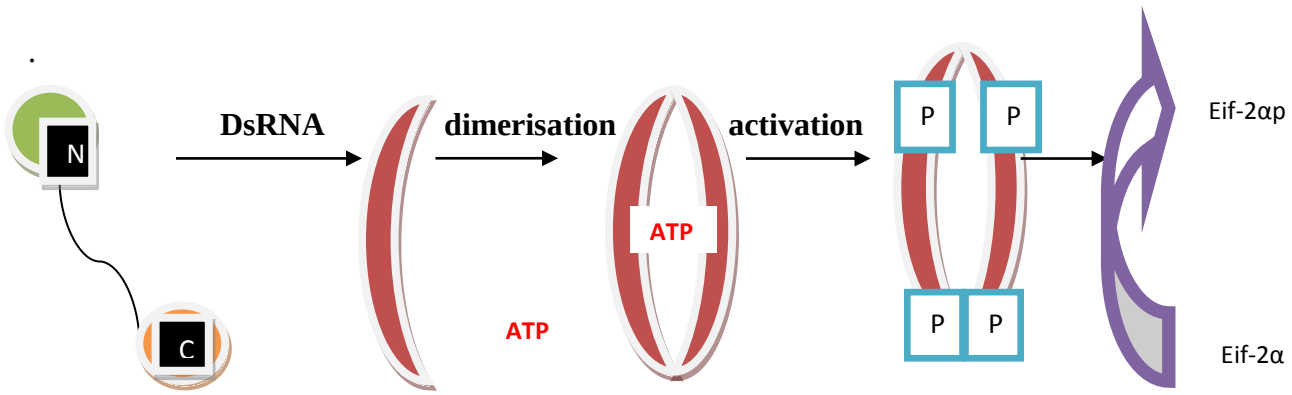


Fig 1.14 Dimerisation by PKR

PKR activation by toll like receptors

The toll receptor (TLR) family consists of a large number of members, plays an vital role in activating the innate immune response (Takedak,2003). Toll like receptors recognize different microbial products such as lipopolysaccharides (LPS), dsRNA, peptidoglycans and CpG motifs. The TLRs have four main domain adapters through which they function through. The main adapters are TIRAP, TRIF, MyD88, and TRAM. Followed by receptor-associated factor (TRAF) and tumor necrosis factor (TNF) and their domain adapters such as TRAF3 or TRAF6 (Gohda, 2004; Gunnery S, 1992), this further activates further signal transduction cascades that involves inflammatory pathway markers such as NF-κB, JNK, p38 and/ are activated. Due these inflammatory markers, cytokines and interleukins mainly interleukin-10 (IL-10) are secreted (Takedak,2003) further leading to antiviral activities.

Literature review emphasize that cells derived from PKR gene deficient mice showed improper or no response to various TLR domains. Studies also reported regarding reduced pro-inflammatory production such as cytokines in response to lipopolysaccharides. All these studies show that PKR acts as an interlinking connector in TLR signaling. PKR when combines with TIRAP in LPS induced wild type macrophages, one of the TLR domain adapter gets

phosphorylated, supporting an evidence that PKR is an effective component of the TLR4 signaling pathway (Horng, 2001).

PKR Induction/Activation in Response to Stress

A wide range of stresses induced by compounds upregulate PKR expression such as H_2O_2, arsenite and thapsigargin and the second messengers for example ceramide can promote PKR activation (Ruvolo,2001). PACT a plausible cellular activator activates PKR by phosphorylation. While PKR associated protein X (RAX) is counter part for PACT when an extra cellular stimulus is applied to maintain cellular stresses. Serine 18 site is responsible for phosphorylation through PACT/RAX activation of PKR. Reference

PKR activation by dsRNA

Different dsRNA molecules bind to PKR at N-terminal through dsRBD binding motif, this leads to PKR activation. dsRBD motifs are composed of 70 amino acids connected by 20 amino acid linker. The structure of the PKR dsRNA-binding domain was observed by NMR (nuclear magnetic resonance); 70amino acid chain consists of α-β-β-α folds of two pairs which are identical to each other whereas the 20-amino-acid connector is structured in a coil conformation (Nanduri SB,1998). These dsRBD motifs allow the dsRBD to circle around the dsRNA molecule which helps for RNA- protein interactions. dsRNA molecules shorter than 30 base pairs fail to activate PKR because they cannot bind. So short interfering RNA'S of length 19-29 basepairs do not bind with PKR, it was reported that optimal PKR activation occurs at a base level of 80 nucleotide length base pairs. THR 446 and THR451 sites are responsible for phosphorylation of PKR (Schmedt C,1995).

PKR upregulation by Cytokines and Growth factors

PKR once activated acts as a downstream marker for many growth factors, interferons, platelet derived growth factors (PDGF), tumor necrosis factor-1 and interleukins. PKR-TNF alpha signaling plays a crucial role in inflammatory pathway such as NFKB-JNK pathway. PKR gene knockout animals have showed less expression of NFKB (Mundschau LJ, 1995; Munoz C, 2005).

Regulation of Signal transduction by PKR

STAT (signal transducers and activators of transcription) proteins involve in a number of signaling cascades triggered by IFN and other cytokines (Darnell JE, 1997). Modulating STAT is a basic mechanism by which PKR controls IFN and dsRNA signaling pathways. PKR-STAT1 interaction is not kinase-substrate interaction , it also does not depend on catalytic site interaction. But the complex depends on dsRNA interaction. (Wong AHN, 1999). PKR−/− cells are false this is due to phosphorylation in STAT1 phosphorylation at Ser727. (Ramana CV,2000).

MAPK activation by PKR

Mitogen activated protein kinases is also an example of serine/threonine protein kinases which participate and regulate many cellular activities. Mammalian MAPK is divided in to p38, JNK and ERK. MAPK needs some specific kinases such as MAPK kinases (MAPKK) for its activation. p38 by MKK3 and MKK6, SAPK/JNK by MKK4 and MKK7and ERK by MEK1 and MEK2. These MAPKK needs to be further activated by special kinases such as MsAPKK kinases (MAPKKK), examples of MAPKK are MEKK1, MLK, TAK1, ASK1 and Raf (Goh JC, 2000).

JNK is expressed ubiquitously and is activated by stress, UV and gamma radiation. JNK is also activated by chemical inhibitors such as heat shock, anisomycin, hyperosmolarity, toxins, ischemia/reperfusion injury, T-cell receptor stimulation ,chemotherapeutic drugs, inflammatory cytokines and peroxide. p38 is activated in response to cell stress such as osmotic shock , UV irradiation, LPS , heat shock and cytokines such as IFN-γ, IL-1, or TNF-α (Goh JC,2000). PKR is a linker activator in many signaling cascades it involves many stress-activated protein kinases for activation of JNK and p38 (kyriakis JM,1996).

NFKB activation by PKR

NF-κB activation by PKR involves phosphorylation of serines 32 and 36 residues on Ikß. Phosphorylation leads to its degradation and finally translocation of NF-κB to the nucleus (Gill JJ, 1999). Phosphorylation on serine residues is responsible for IKK kinase activity. The IKK activity is responsible for PKR activation for upregulation of NF-κB, this is due to either by infection and vesicular stomatitis virus (VSV). (Gill JJ 1999; Gill JJ,2000)

PKR and High carbohydrates

High carbohydrates diet intake increases non insulin dependent type 2 diabetes and cardiovascular diseases in adults and children. These are found to be major global health issues with less treatment options. Under diabetic, metabolic and insulin resistant states, several inflammatory pathways related to stress gets activated leading to the activation of signaling molecules linked to inflammation like c-Jun- N-terminal kinase (JNK) and inhibitory kappa B kinase (IKK). It was reported by Nakamura that PKR can act as a link between major inflammatory signaling pathways involving in metabolic homeostasis, including JNK and IkB

kinase (IKK). PKR was mentioned as a core component of metabolic inflammasome, which means it connects inflammatory signaling with insulin action. Zamanian reported that PKR activates NFkB via TNF-α this work was done in null cell lines. Carvalho and his group used Pkr(-/-) and Pkr(+/+) mice to investigate the role of PKR in modulating insulin sensitivity, glucose metabolism and insulin signaling in liver, muscle and adipose tissue in response to a high-fat diet. They worked on insulin resistance (AKT, IRS-1). Pkr-/- mice were protected from high fat diet induced insulin resistance compared to pkr+/+ mice. All these studies show that pkr is linked with inflammation and insulin action. But the exact mechanism behind this was not known till date, The translation of these studies into the clinic is halted due to the lack of effective pharmacological agents. Thus the purpose of our study is to investigate whether PKR, plays a role in diet induced (high carbohydrate) type 2 diabetes and diabetic cardiomyopthay, the mechanisms involved and its prevention by using novel inhibitors of PKR.

1.11 PKR and its effect on Brain

1. In 2014 researchers reported that Eif2<α is involved in alzheimers disease (Masuo Ohno, 2014).

2. Studies were reported that using Protein Kinase R (PKR) Inhibitor C16 an imidazolo oxindale compound inhibits Neuroinflammation in a Neonatal Rat and Protects neonatal Hypoxia-Ischemia Brain damage (Jinglei Xiao, 2016)

3. In 2016 Hazel Laura submitted her doctoral thesis on the role of PKR activation and its relation with Alpha-Synuclein Expression and how it is influencing the formation of the Amyloid Precursor Protein (Hazel Laura Roberts, 2016)

1.12 PKR and its effect on Metabolic Syndrome

1. Young et al reported the importance of endoplasmic reticulum (ER) stress as an activator for angiotensin II-induced hypertension in central nervous system and PKR inhibits translation of of mRNA under endoplasmic stress condition for angiotensin induced hypertension (Young, 2009)

2. In 2009 researchers reported a study on analysis of IRS-PKR Signaling in hepatocytes (Ming Wu, 2009)

3. In 2010 Afnan submitted her doctoral thesis based on Characterization of the interaction between the adaptor protein Nck (tyrosine kinase adaptor protein 1 non catalytic region) and the protein kinase R in heart (Afnan Abu,2010)

4. Nakamura reported that Double-Stranded RNA-Dependent Protein Kinase acts as a link between pathogen induced infections and Stress and Metabolic Homeostasis (Nakamura, 2010)

5. Nakamura in 2013 reported the role of Small-molecule PKR inhibitors in improving glucose homeostasis in genetic obese diabetic mice (Nakamura, 2014)

6. In 2014 Velloso reported that removing a lipid sensor such as PKR improves diabetic(Type 2) condition. (Velloso, 2014)

7. In 2014 0sama reported the Potential role for small nuclear RNA's in activating PKR via metabolic stress (Osama,2014)

8. In 2015 Arti dhar has reported in a review regarding the role of PKR and its relation with the metabolic syndrome (Obesity, Diabetes and Cardio vascular diseases) (Arti, 2015).

9. In 2015 we reported the role of Small Novel Molecule Indirubin-3'-Oxime Inhibits Protein Kinase R through its Antiapoptotic and Antioxidant Effect in cultured Cardiac Myocytes (Udumula,2015).

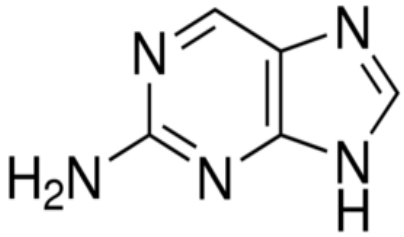
10. In 2016 researchers reported that Inhibiting PKR attenuates apoptosis and inflammation and protects neonatal cardiomyocytes against H2O2 (Yongyi Wang, 2016)

11. G.I Lancaster in 2016 reported that PKR is not a compulsion or mandatory for high-fat diet-induced obesity and its associated metabolic complications (G.I Lancaster, 2016).

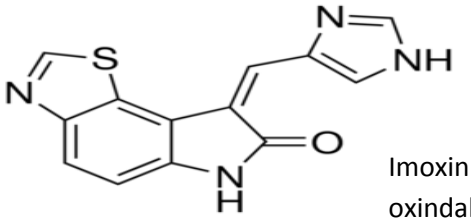
12. In 2017 we reported that High glucose impaired regular insulin signaling by upregulating PKR in L6 skeletal muscle cells (Udumula,2017)

1.13 PKR inhibitors

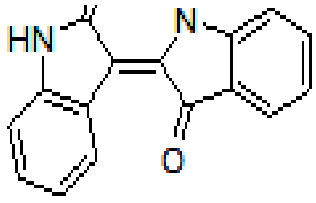
Till date PKR selective inhibitors reported are C16/ Imoxin and 2-Aminopurine. Recent studies reported 80 serine/threonine inhibitors through docking and statistical modelling. These 80 inhibitors are classified into (indol-3 yl)pyrimidin-2-amines, pyrazolo[3,4-d]pyrimidines pyrimido[5,4-b]indoles , 6-heterocyclylamino indazoles and triazolo[3,4-f]pyridazines, (3-methylene-5-oxo-pyrazolidin-4-ylidene)hydrazinosulfonamides. The other classes include very potent PKR inhibitors such as indirubin 3 oximes, CAMK inhibitor and JNK inhibitor analogs. All these compounds where screened for inhibitory concentration (IC50) levels using luciferative assay. 46 compounds with IC50 >100 µM, 20 compounds with 20<IC50 <100 µM , 6 compounds with <0.25 µM < IC50<20 µM and 7 compounds with IC50 <0.1 µM. So we selected two main compounds with <0.1 µM for further screening and evaluation (Bryk R, 2011).



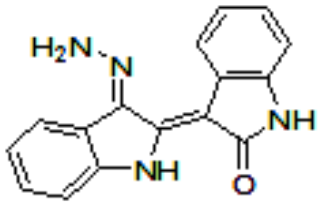
2-Aminopurine



Imoxin (Imidazolo oxindale)



Indirubin 3 oxime



Indirubin 3 hydrazone

Fig 1.15 Structures of PKR inhibitors

1.14 Gaps in Existing Research

High dietary carbohydrates intake is increasing the risk for type 2 diabetes and cardiovascular diseases in adults and children. Under metabolic conditions, several stress pathways involving inflammation gets activated which in turn lead to upregulation of inflammatory molecules like inhibitory kappa B kinase (IKK) and c-Jun- N-terminal kinase (JNK).

a) PKR acts as a link in connecting with many inflammatory signaling pathways involved in metabolic syndrome, including JNK and IkB kinase (IKK). In metabolic disorders, it is not known how these inflammatory signaling molecules are involved and disturbing the regular mechanism.

b) The translation of these studies into the clinic is halted due to the lack of effective pharmacological agents.Thus the purpose of our study is to investigate whether PKR, gets activated in high carbohydrate induced type 2 diabetes and diabetic vascular complications.

c) The mechanisms involved and its prevention by using novel inhibitors of PKR is unknown in normal conditions.

So our study shows an answer with an evidence for all these questions.

CHAPTER 2

HYPOTHESES AND OBJECTIVES

2.1 Rationale for hypotheses

The research on type 2 diabetes has been rising due to its dramatic importance in the last decade. Under metabolic (obese and diabetic) conditions, several inflammatory pathways related to stress get activated leading to upregulation of inflammatory signaling molecules like c-Jun- N terminal kinase (JNK) and inhibitory kappa B kinase (IKK). Inflammatory pathways play a vital role in developing diabetes by controlling the inflammatory responses present in metabolic tissues. These markers also inhibit insulin signaling and alter lipid and glucose homeostasis. Down regulation of these inflammatory networks protects body from obesity-induced insulin resistance and diabetes. PKR plays an vital role in nutrient induced obesity and pathogen or infections induced obesity. PKR thus acts as a key modulator in obesity linking chronic metabolic inflammation with insulin sensitivity and glucose homeostasis.

High glucose (HG) plays an crucial role in development of insulin resistance. Mechanisms underlying behind this is not clearly understood, it was reported that sustained hyperglycemia disturbs insulin-stimulated glucose utilization by peripheral tissues in muscle and fat, this was observed in animal models and humans. This is due to decreased ability of pancreatic b-cells to respond to hyperglycemia with acute insulin release. This is a hypothesized reason for the insulin resistance associated with uncontrolled type 1 diabetes and this leads to insulin resistance in type 2 diabetes (Yki, 1987). The other reason for Insulin resistance induced by high glucose might be due to insulin receptor substrate 1 protein depletion, this work was reported by renstrom in human adipocytes (Renstrom, 2007).

Double stranded PKR is a serine/threonine protein kinase which is activated by pathogens, several stress and nutrient factors. Among the very few substrates identified for the PKR kinase activity, the major one is the eukaryotic initiation factor 2a (eIF2a) responsible for

protein synthesis [13,14]. It has been depicted that PKR-mediated phosphorylation at serine 51 in eIF2a is a reason to inhibit viral protein synthesis in host cells, this is also a major reason in controlling endoplasmic reticulum (ER) homeostasis[15,16]. ER stress plays an vital role in the development of insulin resistance and diabetes by triggering JNK activity.

It was reported earlier that PKR was activated by several endoplasmic reticulum stresses and fatty acids . PKR when activated controls many major inflammatory signaling pathways such as JNK which is also required for inflammation (11-14). It has been reported earlier that PKR directly interacts with insulin receptor signaling mainly at IRS-1 near serine 301 site and inhibits insulin action (6). These studies have been reported in PKR knockout mice.

Till date there are only two specific PKR inhibitors available (Imoxin and 2-aminopurine). Both inhibitors are expensive and non-compliance with patients. These inhibitors were also reported for apoptosis. So there was an urgent need for synthesis of new inhibitors. Thus we synthesized new PKR inhibitors and screened for its effectiveness.

2.2 Hypotheses

(a) **Upregulation of protein kinase R, resulting from exogenous administration of high carbohydrate containing diet such as glucose or fructose activates inflammatory signaling pathways and causes Diabetes.**

(b) Novel PKR inhibitors such as Indirubin 3 hydrazone and indirubin 3 oxime along with selective PKR inhibitor, Imoxin (C16) can attenuate the upregulated PKR expression and its harmful effects on high carbohydrate induced apoptosis and reactive oxygen species generation.

2.3 Objectives and Experimental Approach

2.3.1. To correlate pathological process of diabetes with changes in PKR expression in in vitro in cultured cells and related signal pathways.

We performed experiments to investigate whether high glucose can upregulate PKR expression. Since previous studies have shown PKR upregulation can interfere with insulin signaling pathway. So the main aim was to investigate the role of PKR expression on exposing L6 muscle cells and H9C2 cardiacmyocytes with high glucose.

We collected HG and PKR inhibitors treated cell lysates at different time points and measured PKR expression using Q-PCR, western blot and immunohistochemistry. PKR expression was high at 24h. In-vivo studies were carried out using high fructose model, insulin signaling pathway markers such as GLUT4, IRS-1 were measured using Q-PCR, peripheral organs (liver, pancreas, adipose and skeletal muscle) were collected and studied for PKR expression.

2.3.2 To investigate the role of PKR in the development of high carbohydrate induced diabetes.

High glucose and high fructose are the main components of a high carbohydrate diet, which is believed to be an important cause of obesity, type 2 diabetes and the associated cardiovascular complications. The main aim of our study was to investigate if PKR is elevated under diabetic conditions, what would be the underlying molecular mechanism associated with it . Protein and mRNA samples were collected after exposing L6 muscle cells and H9C2 cardiomyocytes with high glucose. Samples were also collected from wistar rats treated with high fructose for 6 weeks. High fructose induced diabetes is a well known model. Expression

of inflammatory markers such as JNK and NF-kb were measured by RT-PCR and immunohistochemistry. mRNA expression of IRS-1, AKT,PI3-K, GLUT-4 and PDX was measured as these are the important markers for insulin signaling and synthesis.

Cell lysate and tissue homogenates were measured for reactive oxygen species levels using flowcytometry and spectrophotometer. For apoptosis, caspase-3expression, annexin-v assay and TUNEL assay was performed in cultured cells and tissue samples respectively.

2.3.3To investigate the potential of small molecule PKR inhibitors and examine their effectiveness in the prevention of development of diabetes

C16 an imidazolo oxindale compound is a standard selective PKR inhibitor. Previous studies have reported the effectiveness of C16 in PKR knockout mice (Nakamura, 2010; Wang, 2014). However till date there are no reports of C16 in diet induced models of diabetes. Due to its high cost, there is an urgent need for cost effective and selective PKR inhibitors. So currently number of studies are being done for further discovery of PKR inhibitors.

Till date, 80 serine threonine kinase inhibitors have been developed through statistical modeling. Based on IC50 values (<0.1uM), we selected 2 compounds for further biological evaluation. With different concentrations of I3O and IHZ, We performed immunohistochemistry to investigate an effective inhibitor in inhibitng PKR expression. The efficacy of these compounds was also compared with standard PKR inhibitor C16.

2.3.4 To investigate the effect of small molecule PKR inhibitors and their role in diabetes induced cardiovascular complications (diabetic cardiomyopathy)

To investigate the efficacy of novel PKR inhibitors in diabetic vascular complications we selected cardiomyocytes cell line (H9C2). Experiments such as western blot, RT-PCR and immunohistochemistry was performed on cardiomyocytes to see the changes of PKR expression in presence of high glucose alone and in combination with the PKR inhibitors. LDH an important marker for diabetic cardiomyopathy was used for evaluation. As high increase in ROS levels causes autophagy, Rhodamine staining was done to investigate whether HG can induce autophagy and PKR inhibitors can attenuate this or not. For apoptosis, Caspase-3 immunohistochemistry and RT-PCR was performed.

CHAPTER 3

GENERAL METHODOLOGY

3.1 Cell lines

Rattus novergicus skeletal muscle cell line L6 and H9C2 was procured from National Centre For Cell Sciences, Pune, India and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2. The cells were seeded in T25 flasks, with an equal amount of cells (10^6/ml) in each flask, and cultured to confluence. Cells were starved in FBS- free DMEM medium for 24 h prior to exposure to different treatments alone or in combination with high glucose (25mM) or PKR inhibitor imoxin (5 μM based on literature).

3.2 Immunofluorescence Staining

Cells were plated in six-well plates on cover slides. After treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked with 3% bovine serum albumin for 1 h and then incubated overnight at a dilution of 1:50 with the primary antibody (PKR, Caspase-3, JNK). The slides were then washed and incubated with a Texas red/ Alexa fluorconjugated secondary antibody for 1h. The slides were then mounted with the mounting media and counter stained with 6-diamidino-2-phenylindole (DAPI; Invitrogen) and analyzed under an Olympus FluoView FV500 laser confocal microscope (Olympus America) after adjustment for background staining.

3.3 Measurement of Reactive oxygen species.

The formation of peroxynitrite was determined by a DCFH assay. Cells were loaded with a membrane-permeable, non fluorescent probe 2,7′-dichlorofluorescin diacetate (CM-H2DCFDA, 5 μ mol/l) for 2 h at 37 ° C in FBS-free DMEM in the dark. After washing with PBS 3 times, cells were treated with or without different treatments for 24 h, and finally subjected to detection. Once inside the cells, CM-H2DCFDA becomes membrane-impermeable DCFH2 in the presence of

cytosolic esterases and is further oxidized by peroxynitrite to form oxidized DCF, which has detectable fluorescence. Oxidized DCF was quantified by monitoring the DCF fluorescence intensity.

3.4 **Western blotting**

Briefly, aliquots of cell and tissue lysates (50 μ g of protein each) were separated on 6-10% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and blocked with 5% nonfat milk in TBS-Tween buffer (20 mM Tris–HCl, pH 7.4, 135 mM NaCl, 0.1% Tween) for 1.5 h at room temperature. Next the membrane were e incubated with the appropriate primary antibodies, PKR (1:1000) and anti-β-actin(1:10000) (Santa Cruze Biotechnology, CA, USA) respectively, followed by incubation with horseradish peroxidase conjugated secondary antibody for 1 h. After extensive washing, immunoreactive protein was detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp.)

3.5 Real time quantitative PCR (RT-PCR)

Total RNA from cultured cells was isolated using RNA isolation kit. The primers for PKR, JNK, Caspase-3, Bax, Bcl-2 and NFkB were purchased from Sigma, (Sigma Aldrich, India). The real-time PCR was performed in an iCycler iQ apparatus in triplicates.

PKR	GCAGCAGTGGTTGGAAAAGA TGTTGCAAGGCCAAAGTCTC
NFKB	CCTCTACACATAGCGGCTGG GCACCTTGGGATGCGTTTTT

JNK	TGGATTTGGAGGAGCGAACT ACTGCTGTCTGTATCCGAGG
Caspase-3	TACCCTGAAATGGGCTTGTGT GTTAACACGAGTGAGGATGTG,
B-actin	GAGGCCCCTCTGAACCCTAA ACCAGAGGCATACAGGGACAA
AKT	TCACCTCTGAGACCGACACC ACTGGCTGAGTAGGAGAACTGG
IRS-1	GCCAATCTTCATCCAGTTGC CATCGTGAAGAAGGCATAGG
PI3-k	CATCATGGTGGGGCAAATCC ATCATCGACAGCAGGAGGAG

3.6 Glucose uptake assay

Cell homogenate was collected from L6 cells and incubated with and without insulin. Later these both samples were labeled with 2 deoxy glucose and analysed for glucose uptake using flowcytometry.

3.7 Measurement of apoptosis by annexin-v FITC assay

Cells were incubated for 24 hours with treatment groups and harvested under cool conditions. Annexin V conjugate(INVITROGEN) is diluted 1 in 100 in Annexin V binding buffer for each sample, each were kept at room temperature in the dark for around 20 minutes and to each sample 50-100µl of 50µg/ml propidium iodide (PI) solution and transferred onto ice. Apoptosis assay is performed using flow cytometry.

3.8 Measurement of reactive nitrogen species

Levels of NO were detected by modified Greiss reagent. This assay depends on the enzymatic conversion of nitrate to nitrite in presence of nitrate reductase followed by the addition of 2,3-diaminonaphthalene, which converts nitrite to a fluorescent compound. Fluorescence intensity measurements of this compound accurately determine the nitrite (NO_2) concentration (excitation at 365 nm; emission at 450 nm).

3.9 Measurement of Lactate De hydrogenase

Cells were given treatment for 24 hours, later the supernatant was collected and incubated with working reagent for 5 minutes according to manufacturer's instructions and absorbance was taken using plate reader at 340nm**.**

3.10 Labelling of cells with Rhodamine for Autophagy

Cells were grown on coverslips and incubated with Rhodamine (100nm) for 30min in dark at 37°C and examined for autophagy under confocal microscope.

3.11 H and E staining for Cell integrity

Cells were stained with mayers hematoxylin and eosin for 5-10min and later washed with 2 changes of 95% alcohol later observed for changes in shape of the nucleus and cells under microscope.

3.12 Immunocytochemistry

Cells were cultured in 6 well plates after treatment cells were washed with PBS twice later fixed with 4% paraformaldehyde (PFA) in PBS at P^H 7.4 for 10 min, triton-x 0.1% for 2 min. Finally c FITC and DAPI solution of 1ug/ml conc is added into each well and excess stain is drained an observed for apoptosis with excitation 358nm and emission at 461nm under confocal microscopy.

3.13 Animals

5-6-week-old male wistar rats with a mean body weight (b.w.) of 180-220g were randomly divided into 4 groups of 6 animals in each group as follows: Normal control (NC), Diabetic control (DBC) with Fructose-20 (FR20), Fructose-20 and IHZ, IHZ alone. Animals were housed as 3 rats per polycarbonated cage in a temperature and humidity controlled room (22 ± 1°C, 45–60% humidity) with a set of 12 h light-dark cycle. The rats were fed a commercially available rat pellet diet ad libitum throughout the 6 weeks experimental period. Control groups were supplied with normal drinking water ad libitum. Diabetes control group were fed with FR20 were supplied with 20% fructose solution. The animals were maintained according to the rules and regulations of the Institutional ethical committee **(Ethical approval number: BITS-HYD/IAEC/2016/06)**

3.14 Histopathology

After sacrificing animals, pancreas and skeletal muscle were preserved in formalin solution for fixing, followed by processing the tissue with various concentrations of alcohol, xylene and paraffin. Paraffin blocks were prepared and histological sections were taken using Leica microtome. The sections were then deparaffinized with Xylene, rehydrated with alcohol and water. The rehydrated sections were stained using H&E, sirius red and tunnel staining, mounted with DPX mounting media and examined under the microscope at both high (40X) and low (20X) magnifications (Olympus BX51microscope, Tokyo, Japan).

CHAPTER 4 RESULTS

4.1 STATISTICAL ANALYSIS

4.2 In-vitro screening of novel PKR inhibitor I30 in HG induced PKR expression in Cardiomyocytes

4.1 Statistical Analysis

Data obtained from separate experiments are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance with post hoc Bonferroni's test. A p value of <0.05 was considered to be statistically significant.

Novel PKR inhibitor I3O synthesis

The synthesis of novel PKR inhibitor was given in scheme 1. In the first step of reaction we used anthranilic acid (1) with bromoacetic acid and potassium carbonate in presence of ethyl acetate to yield corresponding (carboxymethylamino) benzoic acid (2) which was then filtered and purified through column chromatography to obtain 1-acetyl-1*H*-indol-3-yl acetate (3). Later to cthis reaction indoline-2,3-dione (commercially purchased) (4) was added and dissolved in methanol in presence of potassium carbonate to yield indirubin 3 oxime (5).

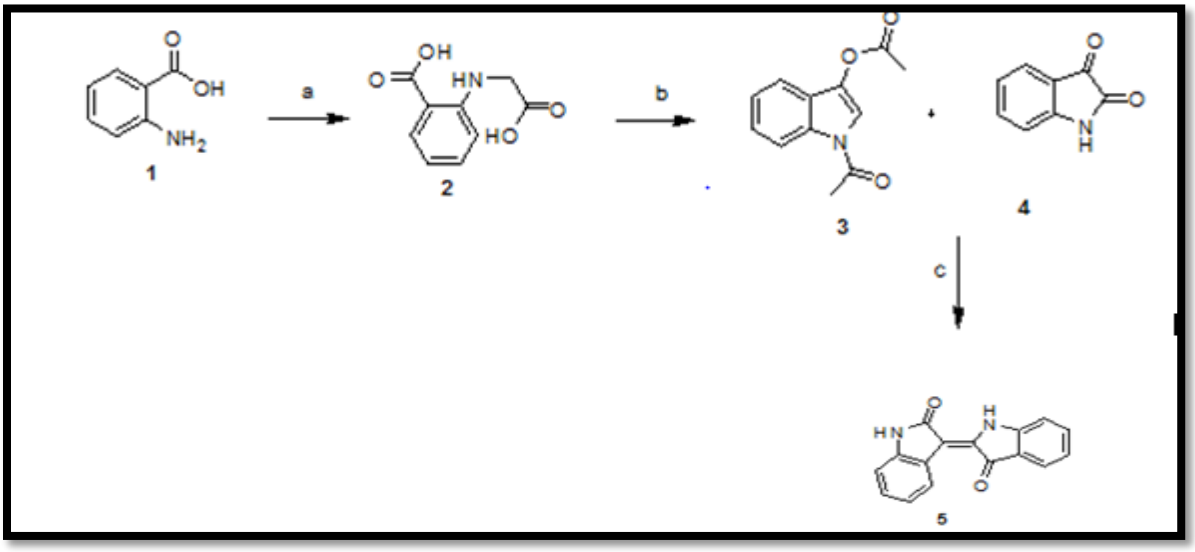


Table 4.A Schematic representation of Indirubin 3 Oxime synthesis

The research on type 2 diabetes and the associated cardiovascular complications, including cardiomyopathy has become increasingly important due to its dramatic rise in the last decade. Diabetic cardiomyopathy (DCM) is characterized by ventricular dilation, myocyte hypertrophy, prominent interstitial fibrosis and cardiomyocyte apoptosis (Wang *et al*,2006; Johnson RJ *et al*,2007). Under diabetic and insulin-resistant states, several stress and inflammatory pathways

get activated, which in turn lead to the activation of inflammatory signaling molecules like c-Jun-N-terminal kinase (JNK) and inhibitory kappa B kinase (Hotamisligil GS and Erbay E,2008). Recent efforts have been made to suppress diabetes and cardiomyopathy through pharmacological treatment; however, these treatments have had limited efficacy and serious side effects. Therefore, a major need is to identify safe therapeutic agents that reduce plasma glucose levels and inhibit DCM on a long-term basis. Recent studies have attached particular importance to double-stranded RNA (dsRNA)-dependent protein kinase R (PKR) pathway because of its activation in obesity-induced diabetic mice and in obese humans (Nakamura et al,2010; Hotamisligil GS, 2006). PKR is a serine/threonine protein kinase, activated by dsRNA, cytokines, stress signals, interferons. It plays an important role in the nutrient/pathogen sensing interface and acts as a key modulator of chronic metabolic inflammation, insulin sensitivity and glucose homeostasis (Hotamisligil GS, 2006). PKR is involved in several signal transduction pathways, like mitogen-activated protein kinase, nuclear factor of κB (NF-κB) and inhibitor of NF-κB. PKR is activated through autophosphorylation and once activated it phosphorylates certain substrates including the α-subunit of eukaryotic initiation factor 2 (Nakamura *et al*, 2010; Hotamisligil GS, 2006; Carvalho *et al*, 2012). PKR has also been reported to be activated by cellular stress, oxidative stress, metabolic stress, and inflammation (Stark GR *et al*,1998; Wang X *et al*, 2007; Lu B *et al*, 2012) and these stress signals have also been observed under diabetic and vascular complications (Saini AS, 2014). The PKR signaling pathway may therefore be an attractive target for the development of anti-diabetic and cardioprotective drugs. The aim of this study was to examine the effect of high glucose on rat cardiomyocytes and its relation with PKR expression.

4.2.1 Cell Culture

Rat cardiac myocyte cell line (H9C2) was obtained from the National Center for Cell Science, Pune, India and cultured in Dulbecco's Modified Eagle's Medium (DMEM). It contained 10% fetal bovine serum (FBS) at 37 ° C in a humidified atmosphere of 95% air and 5% CO2 . H9C2 cells were seeded either in 100 mm dishes for RNA/protein measurement or in 96-well plates for other assays, with an equal amount of cells (106/ml) in each well. For staining, cells were seeded on cover glass slides (2×10^6/ml). I3O (CAS Number 160807-49-8) was procured from Prof. Dharmarajan Sriram (BITS Pilani-Hyderabad Campus, India).

4.2.2 Measurement of HG induced PKR expression

Total RNA from cultured cells was isolated using RNA isolation kit (Qiagen, Germantown, Md., USA). The PKR expression was measured using western blotting and Q-PCR techniques. Novel PKR inhibitor I3O of different concentrations 3,10, 30µm was incubated for different time points 3,6,12 and 24 h along with HG of 25Mm. I3O of 10µm concentration for 24h time period has attenuated the HG induced PKR expression. The real-time PCR (RT-PCR) was performed in an iCycler iQ apparatus associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad Laboratories Ltd., India). Western blotting was detected by Enhanced Chemiluminescence Detection System (Amersham Biosciences Corp., USA) (Fig 4.1).

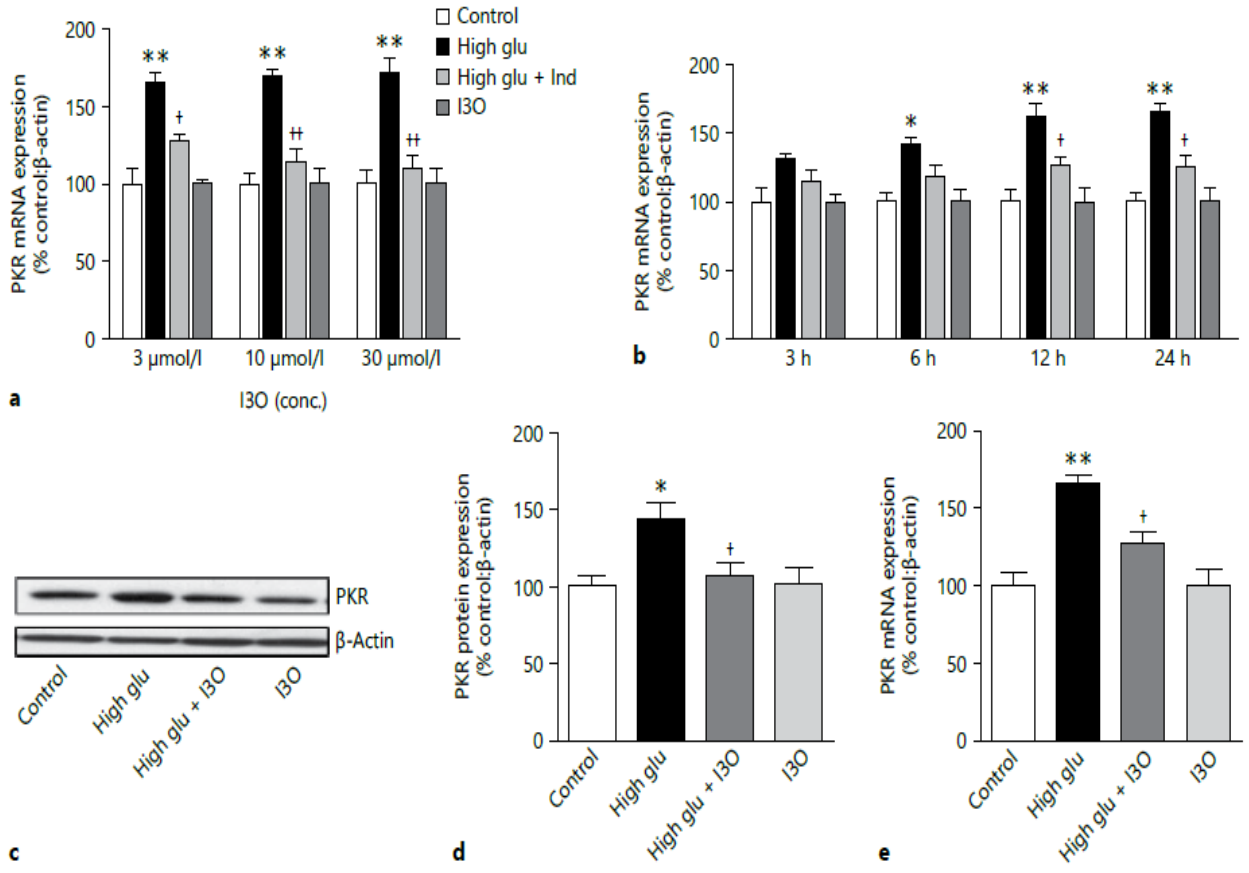


Fig 4.1. Exogenous high glucose increase PKR protein and mRNA levels in cultured cardiomyocytes, attenuation by I3O: cultured H9C2 cells were incubated with normal culture medium (control) or medium containing high glucose (25 mmol/l) for 3, 6, 12 and 24 h and 3, 10 and 30 μmol/l indirubin for 24 h for mRNA. Cultured H9C2 cells were incubated with normal culture medium (control) or medium containing glucose (25 mmol/l) (**a**) for 24 h for protein. Indirubin (10 μmol/l) was incubated alone or with glucose (25 mmol/l) (**a**) for 24 h. PKR mRNA was measured by RTPCR and PKR protein was measured by western blotting. n = 4 for each treatment. * $p < 0.05$, * * $p < 0.01$ vs. respective control. † $p < 0.05$ vs. respective glucose group.

4.2.3 Measurement of Reactive Oxygen Species

The formation of peroxynitrite was determined by a DCFH assay. Cells were loaded with a membrane-permeable, nonfluorescent probe 2 ′,7 ′ -dichlorofluorescin diacetate (CM-H2DCFDA, 5 µmol/l) for 2 h at 37 ° C in FBS-free DMEM in the dark. After washing with PBS 3 times, cells were treated with or without different treatments for 24 h, and finally subjected to detection. Once inside the cells, CM-H2DCFDA becomes membrane-impermeable DCFH2 in the presence of cytosolic esterases, and is further oxidized by peroxynitrite to form oxidized DCF, which has detectable fluorescence. HG (25mM) exposed cardiomyocytes has induced more ROS levels this was attenuated by I3O (10µm) along with HG (Fig 4.2). Oxidized DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm utilizing a Fluoroskan Ascent plate reader (Thermo Labsystem).

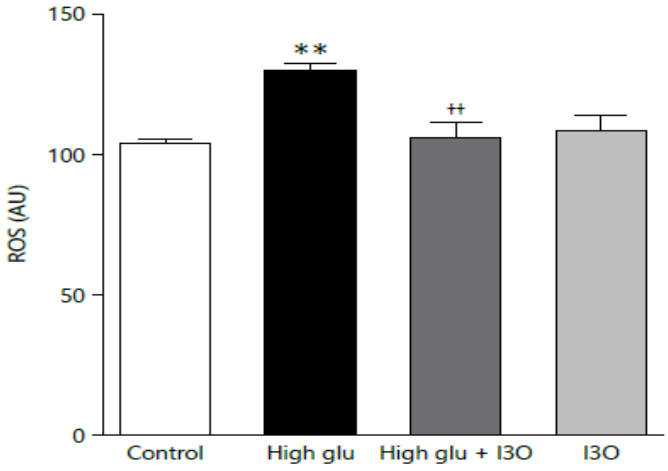


Fig 4. 2. ROS production in cultured H9C2 cells after treatment with high glucose, effect of I3O: incubation of cultured H9C2 with high glucose for 24 h increased ROS production, measured as oxidized DCF, which was attenuated by co-incubation with indirubin (10 µmol/l) n = 16 for each group. * * $p < 0.01$ vs. respective control. †† $p < 0.01$ vs. respective glucose.

4.2.4 Measurement of inflammatory and apoptotic markers

As upregulated PKR further activates inflammatory and apoptotic markers we next measured the expression of JNK, NFkB, Casp-3 and Bcl2 using Q-PCR studies. HG (25mM) has upregulated all these expressions where as I3O along with HG has significantly attenuated these upregulated expressions (Fig 4.3).

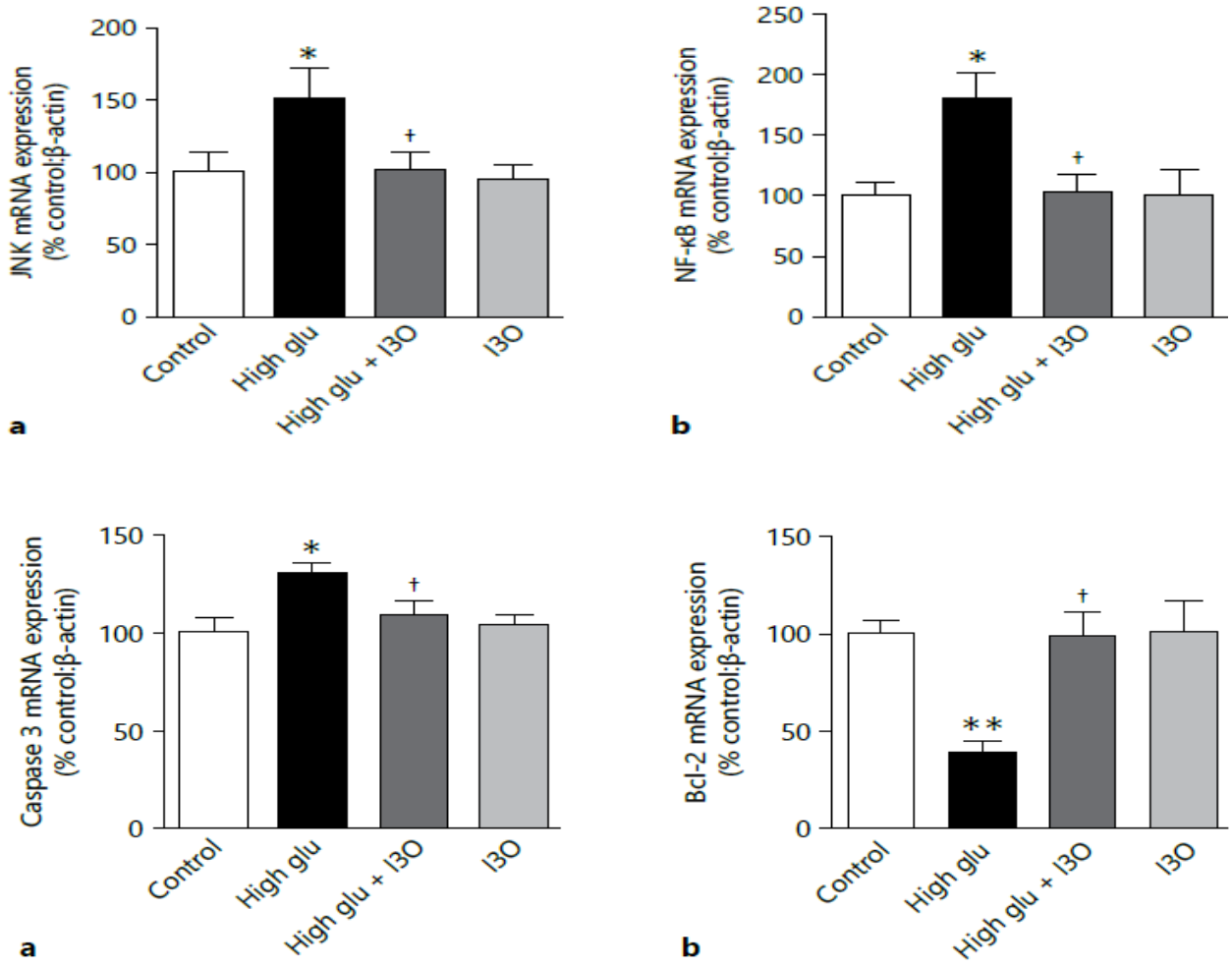


Fig 4. 3. JNK , NF-κB mRNA, Caspase-3 and Bcl-2 mRNA expression in cultured H9C2 cells after treatment with high glucose, effect of I3O : incubation of cultured H9C2 with high glucose for 24 h increased JNK, NF-κB, casp-3 mRNA expression, whereas Bcl-2 mRNA expression was decreased. which was attenuated by co-incubation of

incubation with indirubin (10 μmol/l). n = 3 for each group. * $p < 0.05$, * * $p < 0.01$ vs. respective control. † $p < 0.05$ vs. the respective glucose-treated group.

4.2.5 Summary

We studied the effect of HG on PKR protein/mRNA expression, reactive oxygen species (ROS) production and gene markers of inflammation, oxidative stress and apoptosis (JNK, NF-κB, Bcl-2, caspase-3) in high glucose-treated cardiomyocytes. Incubation of H9C2 cells with high glucose for 3, 6, 12, 24 h significantly increased mRNA expression of JNK, an inflammatory and apoptotic transcription factor and NF-κB, an oxidative stress marker gene were significantly increased in high glucose-treated H9C2 cells. High glucose treatment for 24 h also significantly increased caspase-3 mRNA, whereas Bcl-2 expression was significantly decreased. The effects of high glucose on PKR mRNA/protein expression, ROS production and proinflammatory and apoptotic gene markers were attenuated by I3O (10 μmol/l) co-incubated with high glucose.

4.3 In-vitro screening of novel PKR inhibitor indiribun 3 hydrazone (IHZ) on HG impaired insulin signaling pathway in L6 skeletal myotubes

The research on type 2 diabetes has become increasingly important due to its dramatic rise in the last decade. Under diabetic conditions, several stress and inflammatory pathways get activated which in turn lead to the activation of inflammatory signaling molecules like c-Jun-Nterminal kinase (JNK) and inhibitory kappa B kinase (IKK). These pathways play an important role in the development of diabetes by controlling the inflammatory responses in metabolic tissues, inhibition of insulin signaling, and alteration of glucose and lipid homeostasis (Hotamisligil GS, 2006; Hotamisligil GS and Erbay E, 2008; Hirosumi J *et al*, 2002; Kaneto H *et al;* 2004). Suppression of these broad inflammatory networks generally results in protection against obesity induced insulin resistance and diabetes (Hirosumi J *et al*, 2002; Kaneto H *et al;* 2004).

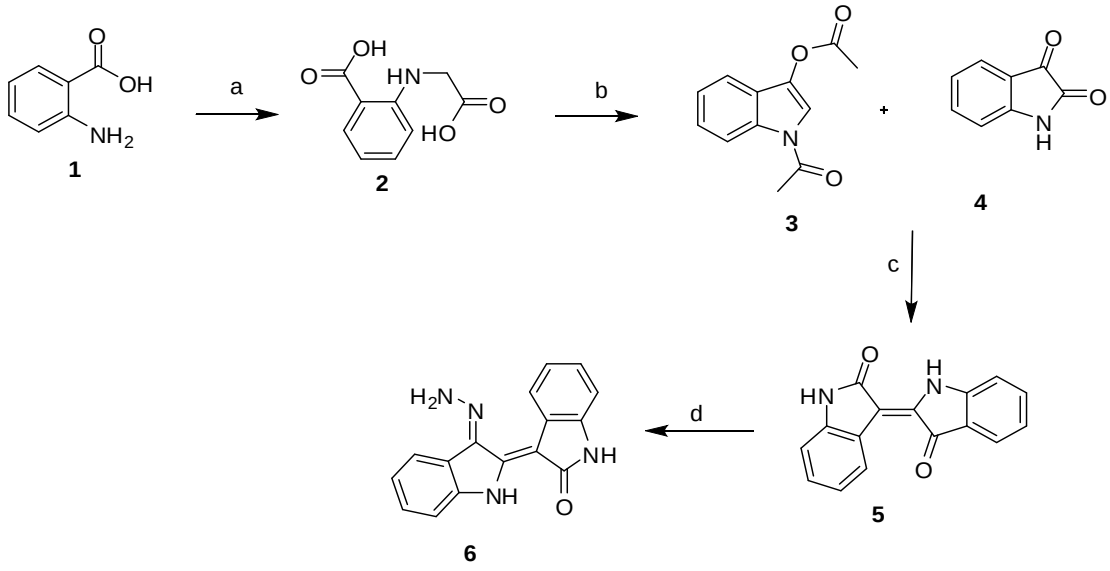
PKR is a serine/threonine protein kinase, activated by double-stranded RNA (dsRNA), cytokines, stress signals, interferon's and plays an important role in the nutrient/pathogen sensing interface and acts as a key modulator of chronic metabolic inflammation, insulin sensitivity and glucose homeostasis (Nakamura T et al, 2010; Carvalho MA et al, 2012; Nakamura T, 2014; Dhar A, 2016). It has been reported recently that PKR is involved in insulin resistance in peripheral tissues (Nakamura T et al, 2010; Carvalho MA et al, 2012; Nakamura T, 2014) as well as antiproliferative effect in pancreatic β cells (Song Y et al, 2015), representing a novel role of PKR in regulation of diabetes and metabolic disorders. These effects of PKR have been ascribed to its kinase catalytic activity and pharmacologically targeting PKR using small-molecule inhibitors of PKR kinase activity improved insulin sensitivity and glucose clearance in a mouse model of obesity as well as insulin resistance (Dhar A, 2016; Song Y *et al*, 2015; Lu *et al*, 2012). PKR is also reported to be activated by fatty acids and endoplasmic reticulum stress and controls major inflammatory signaling events such as JNK, and is also required for

inflammasome activity (Stark GR *et al*, 1998; Wang X *et al*, 2007). It has been reported earlier that PKR directly interacts with insulin receptor signaling constituents and inhibits insulin action. Increased PKR activity/expression is observed in liver and adipose tissue of mice with dietary and genetic obesity. Knockdown of PKR gene in mice have been shown to be protective against obesity induced insulin resistance and inflammatory changes (Nakamura T *et al*, 2010; Carvalho MA *et al*, 2012; Boden G *et al*, 2008; Gregor MF *et al*.,2009).

Recent efforts have been made to suppress diabetes through pharmacological treatment; however, these treatments have had limited efficacy and serious side effects. Therefore, a major need in the treatment of diabetes is to identify for a safe therapeutic agents that reduces plasma glucose levels on a long term basis. Despite all the studies done so far, the molecular and cellular mechanism underlying the role of PKR in insulin resistance and diabetes is not fully understood. Till today there are no reports investigating the role of PKR in L6 muscle cells. Since PKR is significantly activated during human obesity, importantly in adipose and liver tissues, thus, raising the possibility that PKR may exemplify a suitable target for drug development against diabetes (Song Y *et al*, 2015, Cai D *et al*, 2005). Thus, the aim of the present study was to examine the role of PKR and novel PKR inhibitors I30 and IHZ in L6 muscle cells and underlying molecular mechanism.

The synthesis of novel PKR inhibitor IHZ was given in scheme .

In the first step of reaction we used anthranilic acid (1) with bromoacetic acid and potassium carbonate in presence of ethyl acetate to yield corresponding (carboxymethylamino) benzoic acid (2) which was then filtered and purified through column chromatography to obtain 1-acetyl-1*H*-indol-3-yl acetate (3). Later to this reaction indoline-2,3-dione (commercially purchased) (4) was added and dissolved in methanol in presence of potassium carbonate to yield indirubin (5), resultant indirubin was dissolved in hydrazine and ethanol. finally, resulting compound was washed and filtered by column chromatography to obtain indirubin 3 hydrazone (IHZ) (6).



Scheme 1. Reagents and conditions: (a) 2-bromoacetic acid, K_2CO_3 , DMF, 4h,110^oC; (b) Acetic anhydride, 135-140^oC, 10h; (c) K_2CO_3, CH_3OH,rt (d) $NH_2NH_2.H_2O$, C_2H_5OH, 80^oC, reflux

Table 5.A Schematic representation of IHZ sy

4.3.1 Cell culture

Rattus novergicus skeletal muscle cell line (L6) was procured from National Centre For Cell Sciences, Pune, India and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37℃ in a humidified atmosphere of 95% air and 5% CO2. The cells were seeded in T25 flasks, with an equal amount of cells (106 /ml) in each flask, and cultured to confluence. Cells were starved in FBS- free DMEM medium for 24 h prior to exposure to different treatments alone or in combination with high glucose (25mM) or PKR inhibitor imoxin (5 μM based on literature), I30 (10μM) and IHZ (10μM).

4.3.2 Novel PKR inhibitors vs specific inhibitors in attenuating HG induced KR expression in L6 cells.

The PKR expression as detected by western blot was significantly increased in high glucose treated cultured L6 muscle cells in comparison to untreated control cells (Fig. 4.4). The effects of high glucose were attenuated significantly by selective PKR inhibitor imoxin and IHZ co-treated with HG.(Fig 4.4) compared to I30.

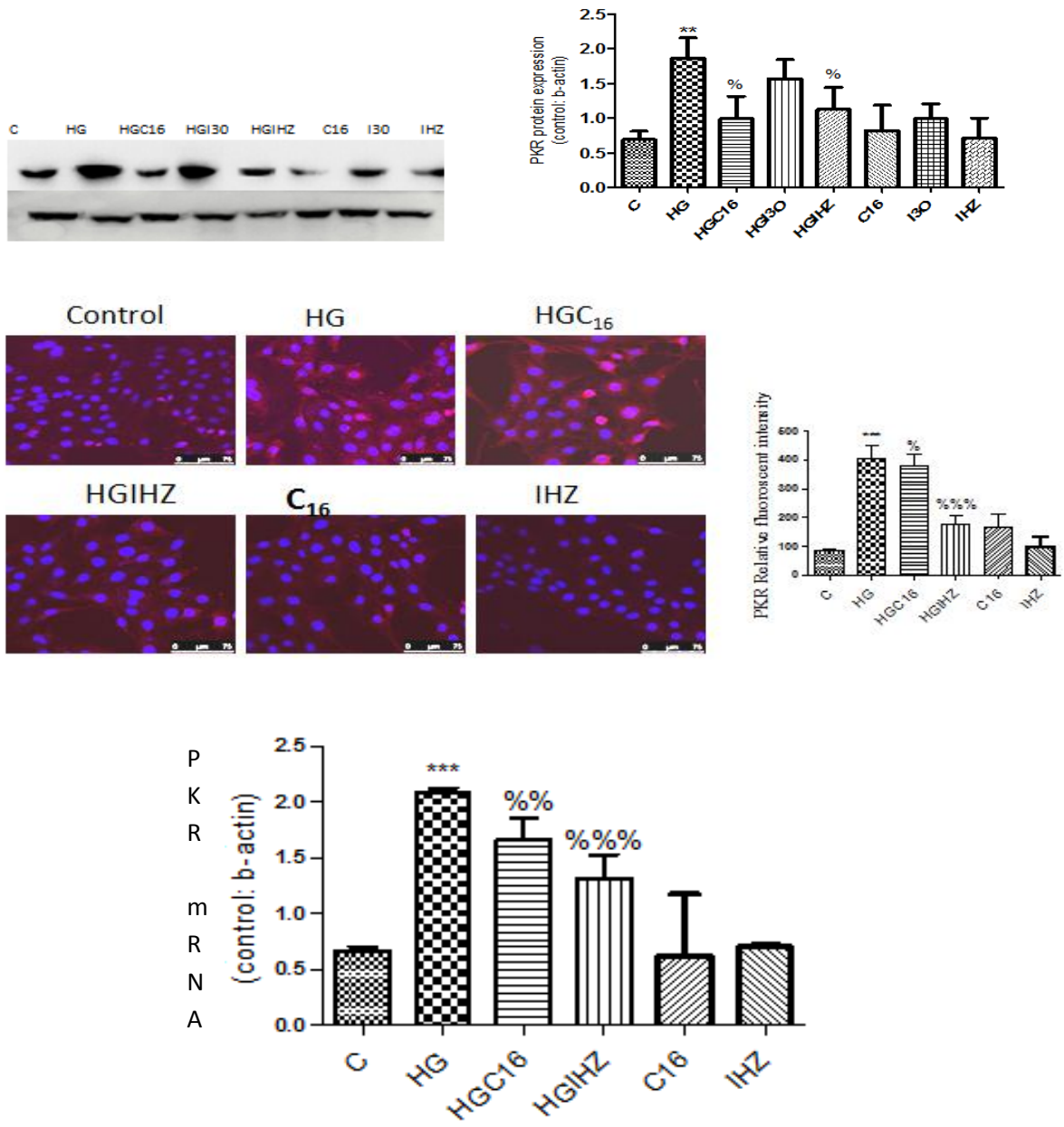
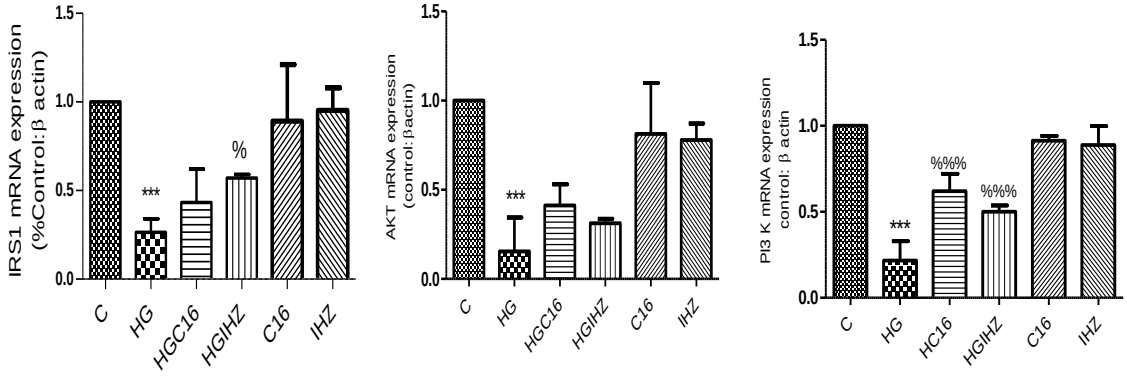


Figure 4.4 : Attenuation of HG induced PKR expression using specific PKR inhibitor C16 and novel PKR inhibitors.

Cultured L6 muscle cells were incubated with normal culture medium (control,Con) or medium containing glucose (25 mM) (A) for 24 h. Imoxin (5 µM), I30 and IHZ was incubated alone or with glucose (25 mM) (A) for 24 h. Protein was isolated from all groups and PKR expression was measured using western blot. n=3 for each treatment. ***P*<0.01*vs*. respective control (Con). % *P*<0.05 *vs*. respective glucose group.

4.3.3 Impaired insulin signaling and glucose uptake in high glucose treated L6 muscle cells, which was attenuated by imoxin:

High glucose treatment (25mM) for 24 h caused a significant reduction in mRNA expression of IRS-1, P13K and Akt (Fig.4.5). The effects of high glucose were attenuated by selective PKR inhibitor, imoxin . HGIHZ also attenuated significantly HG induced changes in insulin signaling. High glucose treatment (25mM) for 24 h caused a significant reduction in insulin stimulated glucose uptake in L6 muscle cells while no significant change in glucose uptake was observed at basal level . The effects of high glucose were attenuated by selective PKR inhibitor, imoxin and IHZ co-treated with HG (Fig. 4.5)



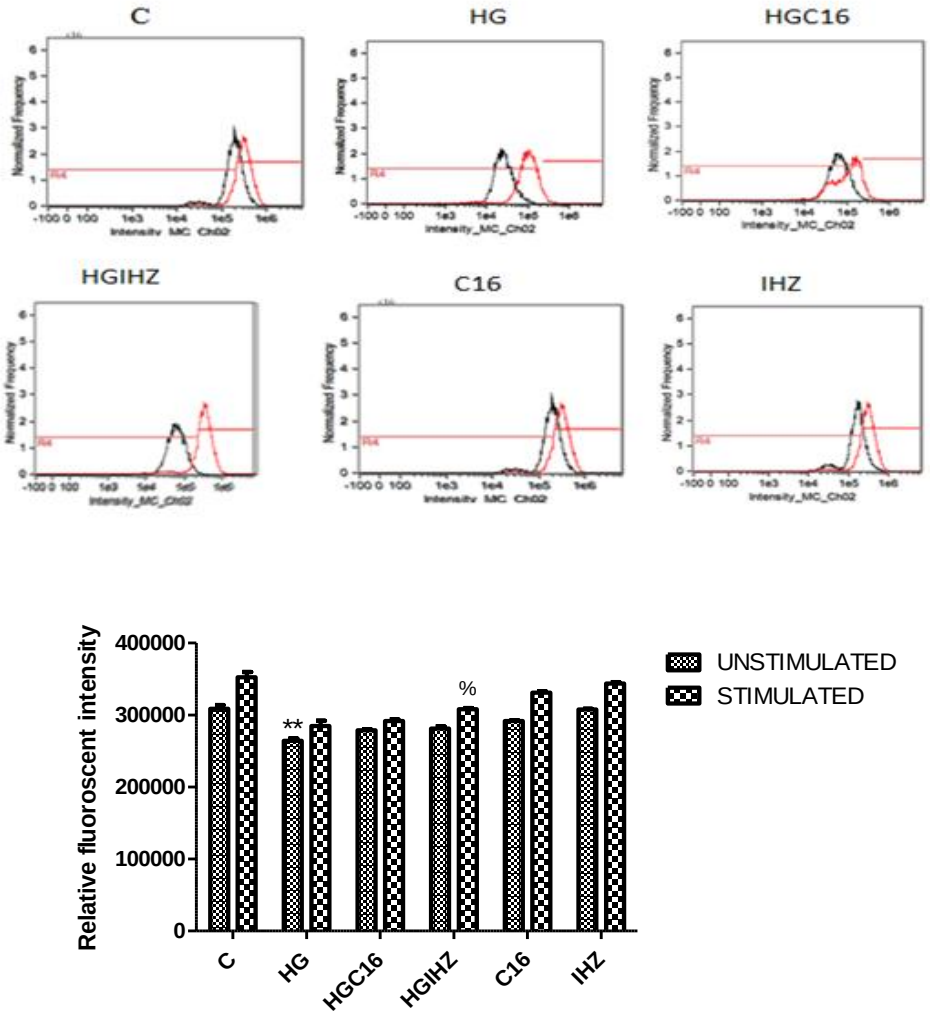
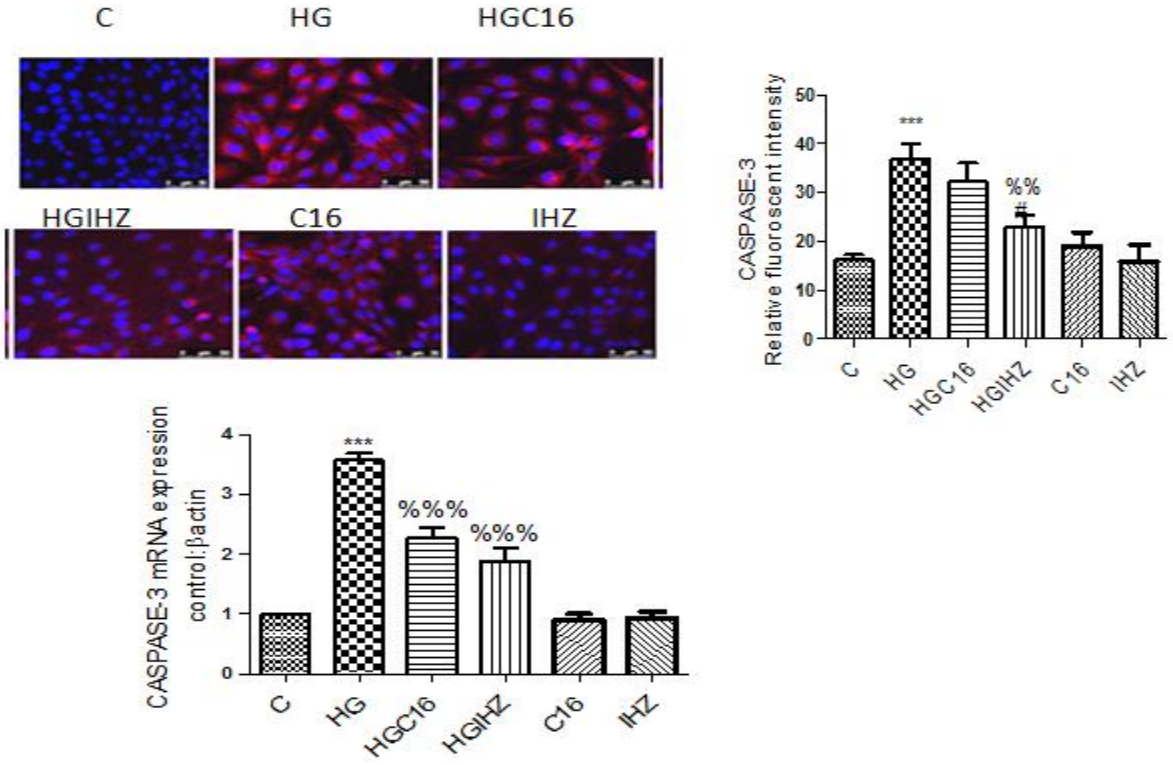


Figure 4.5: Imoxin attenuates high glucose induced impaired insulin signaling and glucose uptake: Cultured L6 muscle cells were incubated with normal culture medium (control,Con) or medium containing glucose (25 mM) (A) for 24 h. Imoxin (5 µM) and IHZ was incubated alone or with glucose (25 mM) (A) for 24 h. IRS-1, Akt and PI3K mRNA expression was measured by RT-PCR. Glucose uptake was measured by FACS analysis. n=4 for each treatment. ***P<0.01*vs*. respective control (Con). % P<0.05, %%% P<0.001 *vs*. respective glucose group.

4.3.4 Inhibition of PKR attenuates high glucose induced increases caspase-3 and reactive oxygen species production in cultured L6 muscle cells

Incubation of cultured L6 muscle cells with high glucose (25 mM) for 24 h significantly increased and caspase-3 (Fig. 4.6) expression as detected by immunofluorescence staining. The increased JNK caspase-3 expression induced by high glucose was attenuated by imoxin (5 μM) coincubated with high glucose, IHZ coincubated with HG (Fig. 4). Incubation of cultured L6 muscle cells with high glucose (25 mM) for 24 h significantly increased reactive oxygen species production, measured by FACS analysis, which was attenuated by imoxin (5 μM) and IHZ (10 μM) co-incubated with high glucose (Fig. 4.6)



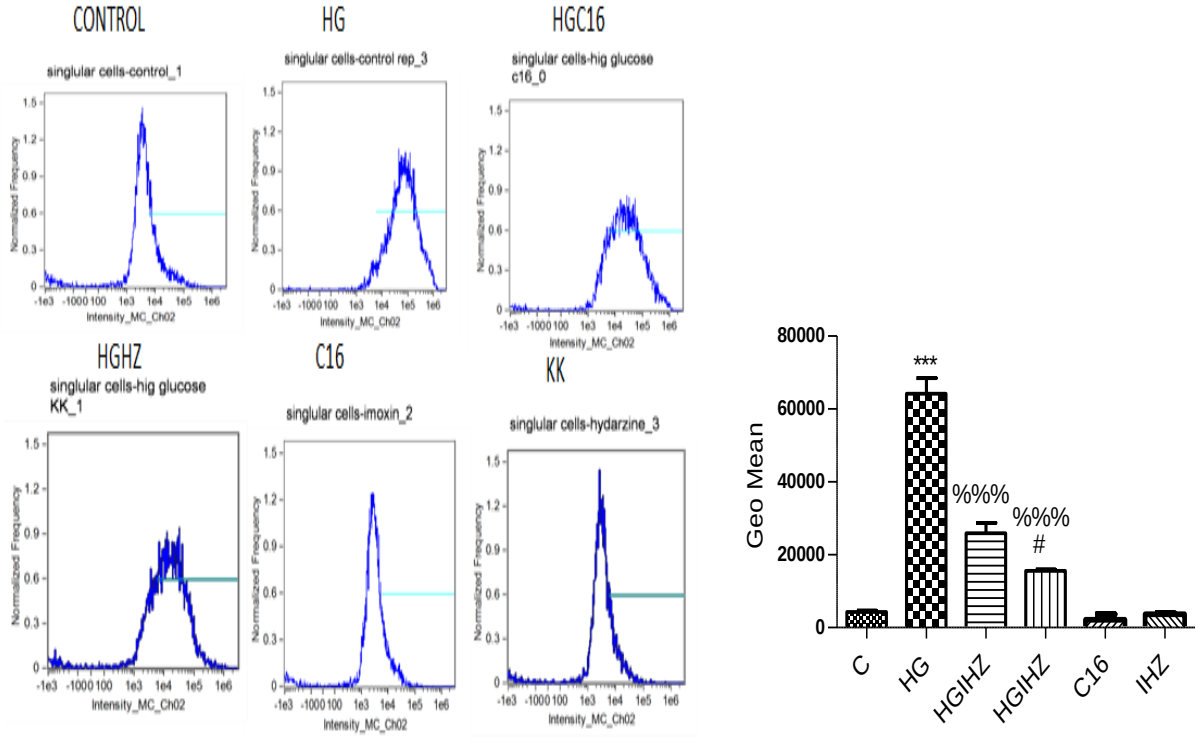
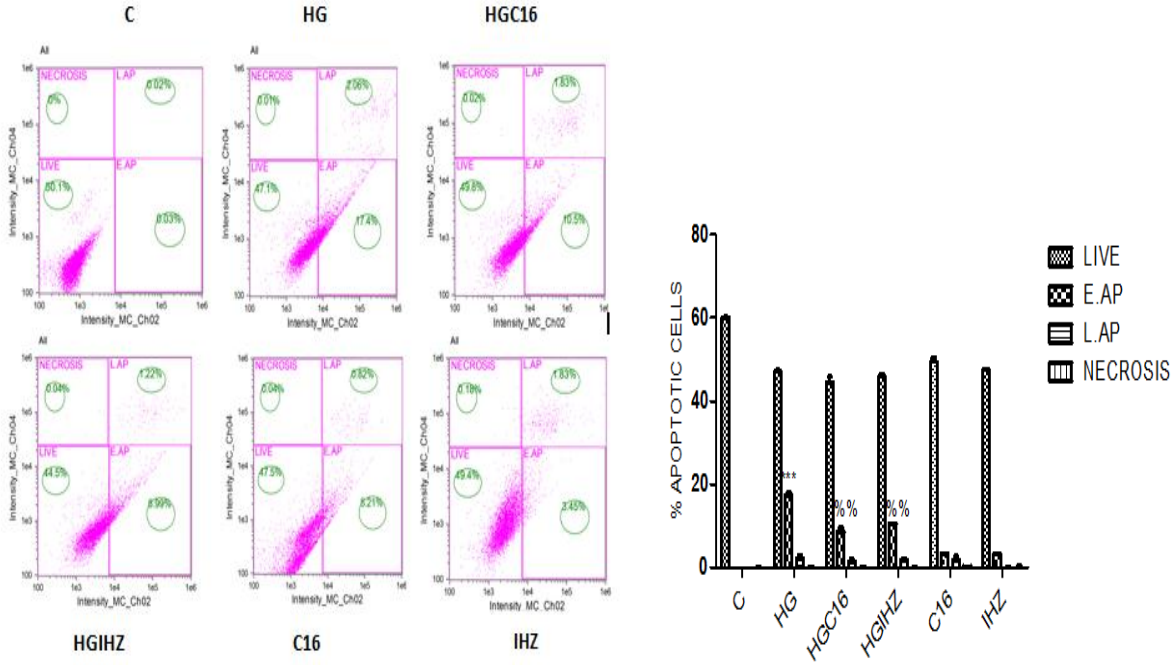


Figure 4.6: Exogenous high glucose induces caspase-3 expression and reactive oxygen species production in cultured L6 muscle cells, attenuation by imoxin: Cultured L6 muscle cells were incubated with normal culture medium (control,Con) or medium containing glucose (25 mM) (A) for 24 h. Imoxin (5 μM) and IHZ was incubated alone or with glucose (25 mM) (A) for 24 h. caspase-3 expression was measured by immunofluorescence. ROS generation was measured by FACS analysis. n=4 for each treatment. ***P<0.01*vs*. respective control (Con). %%P<0.01, %%% P<0.001 *vs*. respective glucose group. *#P< 0.5 vs* HGC16.

4.3.5 Inhibition of PKR attenuates high glucose induced increases JNK expression and apoptosis in cultured L6 muscle cells

Incubation of cultured L6 muscle cells with high glucose (25 mM) for 24 h significantly increased and JNK (Fig. 5 A) expression as detected by immunofluorescence staining. The increased JNK expression induced by high glucose was attenuated by imoxin (5 μM) and IHZ co-incubated with high glucose. Incubation of cultured L6 muscle cells with high glucose (25 mM) for 24 h significantly increased the apoptosis quantified by flow cytometry analysis (Fig.4.7). IHZ co-incubated with HG for 24 h showed significant attenuation of apoptotis compared to HG with imoxin.



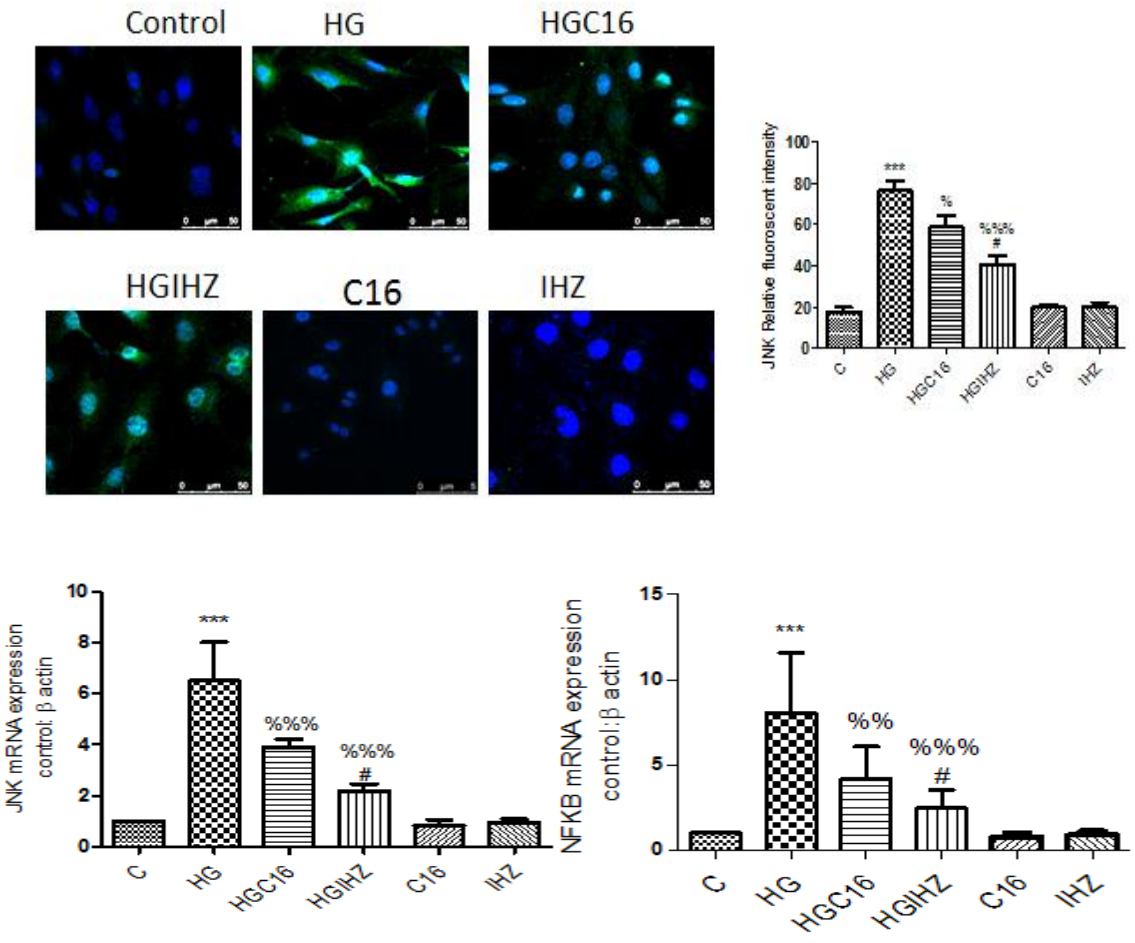


Figure 4.7: Exogenous high glucose induces JNK expression and apoptosis in cultured L6 muscle cells, attenuation by imoxin: Cultured L6 muscle cells were incubated with normal culture medium (control,Con) or medium containing glucose (25 mM) (A) for 24 h. Imoxin (5 μM)and IHZ was incubated alone or with glucose (25 mM) **(A)** for 24 h. Apoptosis was measured by FACS analysis using Annexin-IV assay kit. n=4 for each treatment. ***P<0.001*vs*. respective control (Con). %P<0.5, %%P<0.01, %%%P<0.001 *vs*. respective glucose group. *# P<0.5 vs*. HGC16 **(B).**JNK expression was measured by immunofluorescence.

4.3.6 Summary

We studied the effect of HG on PKR protein/mRNA expression, reactive oxygen species (ROS) production and gene markers of inflammation, oxidative stress and apoptosis (JNK, NF-κB, caspase-3) in high glucose-treated L6 myotubes. L6 cells exposed to HG showed impair insulin signaling this was measured using Q-PCR and flowcytometry. Incubation of L6 cells with high glucose for 24 h significantly increased mRNA expression of JNK, an inflammatory and apoptotic transcription factor and NF-κB, an oxidative stress marker gene were significantly increased in high glucose-treated cells. High glucose treatment for 24 h also significantly increased caspase-3 mRNA. The effects of high glucose on PKR mRNA/protein expression, ROS production and proinflammatory and apoptotic gene markers were attenuated by IHZ (10 μmol/l) co-incubated with high glucose.

4.4 In-vivo screening of novel PKR inhibitor IHZ in High Fructose induced Diabetes via GLUT-4/PdX in wistar rats

Diet sources and brewed beverages rich in fat, sugar and energy are now commonly consumed in many societies in today's world (Nolan CJ and Damm P, 2011). In addition to physical activity, genetic predisposition (Schwenk RW and Vogel H , 2013) and during birth environment (Nolan CJ and Damm P, 2011, Dabelea D and Pettitt DJ, 2001) these foods are considered as major causes of the obese humans in environment . In the middle of 19th century, all these sweeteners and food became widely available for consumption. This was more because these were known for its processed and low calorie food (Tappy L and Le KA, 2010). Fructose is widely used for its commercial purpose as an alternative sweetening substitute especially (fructose corn syrup) for glucose or sucrose , in the preparation of condiments, carbonated beverage and desserts (Elliott SS et al, 2002). Studies recently reported and confirmed that intake of high quantities of carbohydrates in food and beverage increases the risk leading to obesity, dyslipidaemia (Welsh JA et al, 2010; Dabelea D and Pettitt DJ, 2001 ; Elliott SS et al, 2002), insulin resistance and finally vascular complications.(Dekker MJ, 2010; Vasdev S and Longerich L, 2004). A recent epidemiological analysis also reported diabetes include many stress and inflammatory pathways provoked such as JNK, Ikb (Basu S, 2013) .

International diabetes federation report 2017 reported india stands in second place in type 2 diabetes with a population of 90.2 million and this may increase to 130.4 with first place in the world (International Diabetes Federation Reports, 2017). Type 2 diabetes occurs due to less or no response of peripheral organs to insulin which is known as insulin resistance. Insulin released from pancreas was decreased due to apoptosis and decrease in ß cell number (American Diabetes Association, 2017). Liver, skeletal muscle and adipose tissue are insulin signaling organs. In metabolic disorders, it is not known how these and other inflammatory signaling

molecules are regulated to disrupt metabolism. However the translation of these studies into the clinic is halted due to the lack of effective pharmacological agents.

Double stranded Protein kinase R is a serine threonine kinase which is activated in many stress conditions, infections by pathogens, toll like receptors (TLR'S) and nutrient factors (Clemens MJ *et al*, 1975; Farrell PJ *et al*, 1977). Activation of PKR occurs due to heterodimerisation and homodimerisation and this leads to phosphorylation of eif2alpha which helps in inhibition of protein synthesis (Farrell *et al*,1977).

Studies reported that PKR-/- in rats and mice show impaired or no response to TLR (Hsu L *et al*, 2004). PKR was reported to be an important signaling molecule for many down stream molecules such as NFKB, JNK, IkB . Studies reported pkr gene deficit mice showed less expression of NFKB (Kumar A *et al*, 1994). Increased PKR expression was reported in obese liver and white adipose tissue. PKR knock out has shown protection to insulin resistance (Nakamura ,2010) .

Thus the purpose of our study is to investigate whether PKR, plays a role in high carbohydrate induced type 2 diabetes and diabetic cardiomyopthay, the mechanisms involved and its prevention by using novel inhibitors of PKR. Till date PKR selective inhibitors reported are C16/ Imoxin and 2-Aminopurine. Recent studies reported Indirubin 3 oxime and indirubin 3 hydrazone as PKR inhibitors through docking studies (Bryk *et al*, 2011). Indirubin , is one of the main active ingredients of Chinese herb drug, has been known for its anti-cancer activity. Earlier our lab reported that, I30 has attenuated the increased PKR expression after exposure to high glucose in cardiomyocytes (Udumula MP et al, 2016). Indirubin hydrazone (IHZ) a novel derivative of indirubin was reported as cyclin dependent kinase (CDK) inhibitor and our lab has reported C16 action on PKR expression after exposing to high glucose in l6 muscle cells

(Udumula MP et al, 2017), so we further investigated its role in-vivo in high fructose diet supplies rats.

4.4.1 Animals

5-6-week-old male wistar rats with a mean body weight (b.w.) of 180-220g were randomly divided into 4 groups of 6 animals in each group as follows: Normal control (NC), Diabetic control (DBC) with Fructose-20 (FR20), Fructose-20 and IHZ, IHZ alone. Animals were housed as 3 rats per polycarbonated cage in a temperature and humidity controlled room (22 ± 1°C, 45–60% humidity) with a set of 12 h light-dark cycle. The rats were fed a commercially available rat pellet diet ad libitum throughout the 6 weeks experimental period. Control groups were supplied with normal drinking water ad libitum. Diabetes control group were fed with FR20 were supplied with 20% fructose solution. The animals were maintained according to the rules and regulations of the Institutional ethical committee (Ethical approval number: **BITS-HYD/IAEC/2016/06)**

4.4.2 Chemicals and kits

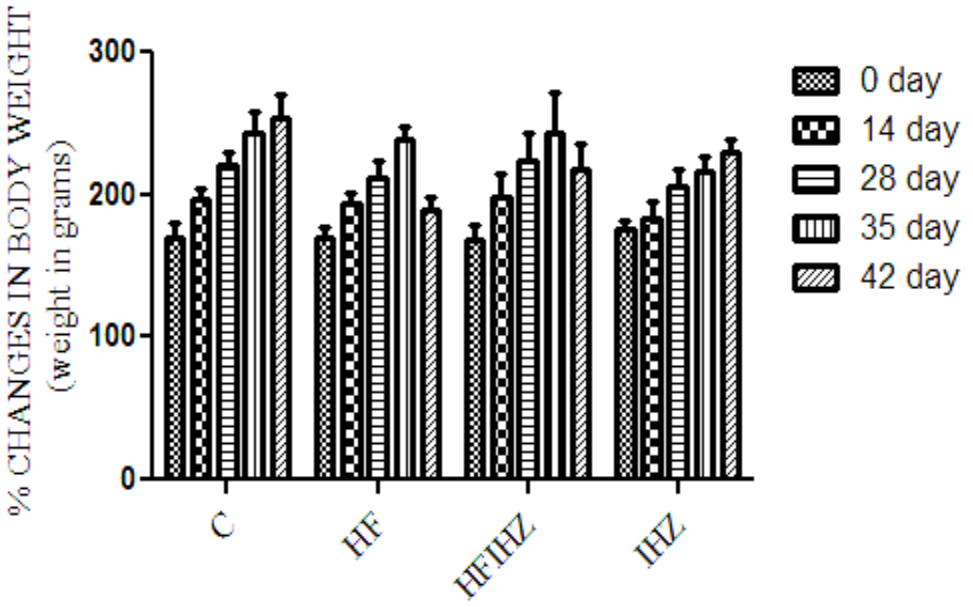
Commercial kits for HDL, LDL, SGOT, TG'S , Total cholesterol were purchased from tulip diagnostics and autospan bio systems. DCFDA, MDA, Streptozotocin, Primers were purchased from Sigma Aldrich. Haematoxylin, Eosin, Sirius red were purchased from Thermofischer scientific. Antibodies were purchased from Santacruz and Abcam.

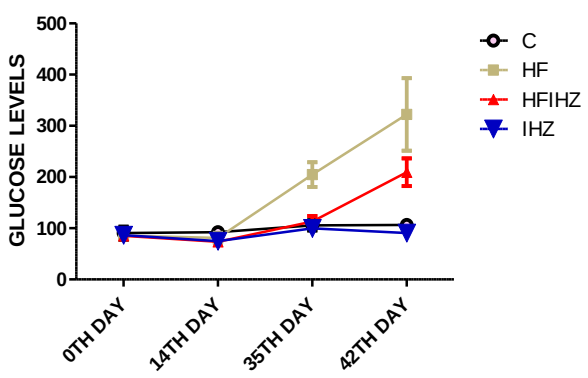
4.4.3 Induction of Hyperglycemia

Animals were feeded with fructose -20% in drinking water for 5 weeks , Low dose of STZ (35 mg/kg b.w.) dissolved in citrate buffer (pH4.4) was given in i.p route to diabetic control and FR-20 and IHZ group while the animals in NC group were injected with vehicle buffer only.

4.4.4 Effect of IHZ on body weight, blood glucose and aspartate transaminase levels

To investigate the role of IHZ on body weight we weighed the animals regularly at end of each week. HF treated group showed increase in body weight. Blood was collected and plasma samples were isolated on 0, 14th and 42nd day. Blood glucose levels and AST levels were analysed using commercial kits. HF treated group showed more glucose and AST levels which was significantly attenuated by IHZ (10µM) on co-administered with HF.





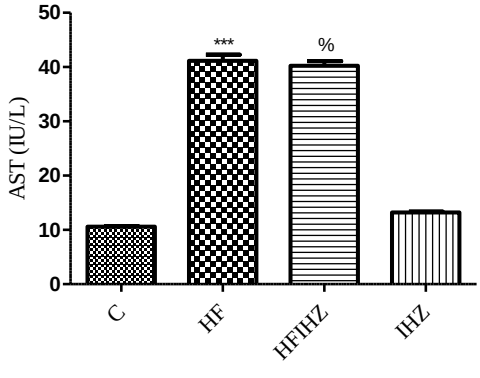


Figure 4.8 IHZ ATTENUATES HF INDUCED BODY WEIGHT CHANGES AND GLUCOSE LEVELS

Animals were (A) weighed at the end of each week after giving treatment. Plasma was isolated on 0,12 and 42days from C, HF (20%) , HF and IHZ (10M) and IHZ alone treated animals and following parameters were (B) Glucose levels were measured using glucometer and (C) Aspartate transaminase levels were measured, n=6 for each treatment *** *P<0.001 Vs C, % P<0.05 Vs HF.*

4.4.5 **Effect of IHZ on lipid profile**

We investigated role of IHZ on lipid profile, we performed TC,TG'S, LDL and HDL assays in plasma samples on 0,14th and 42nd day. HF treated group showed more TC,TG'S and LDL which was significantly attenuated with IHZ co-administered with HF. HDL levels were significantly decreased in HF treated animals which was attenuated with IHZ co-administered with HF.

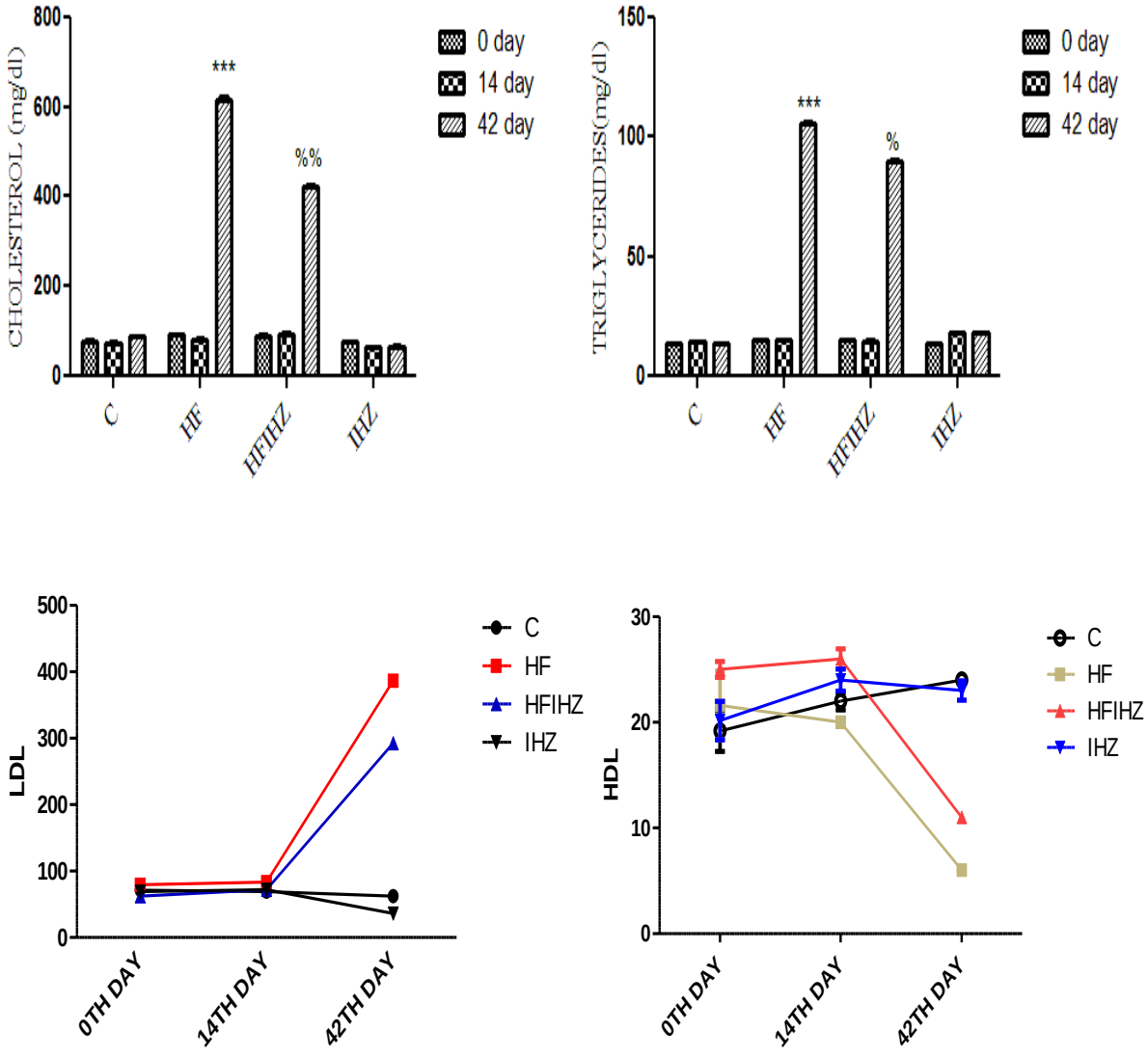


FIGURE 4.9 IHZ ATTENUATES HF INDUCED CHANGES IN LIPID PROFILE

Plasma was isolated on 0,12 and 42days from C, HF (20%) , HF and IHZ (10M) and IHZ alone treated animals and following parameters were (A) Total cholesterol (B) Triglycerides (C) LDL (D) HDL levels were measured using commercial kits, n=6 for each treatment *** *P<0.001 Vs C, %% P<0.01 Vs HF*.

4.4.6 Effect of IHZ on vascular parameters

HF is reported for its induction of oxidative stress , here we examined whether IHZ can attenuate HG induced superoxides. Plasma was incubated with TCA and TBA for TBARS assay. Tissue was homogenized and incubated with griess reagent for nitrite assay pancreas was sectioned for DCFDA staining. HF treated animals for 42 days in drinking water showed significantly increased TBARS, nitrite and ROS production which was attenuated by IHZ (10 µM) co-treated with HF (Fig. 4.10)

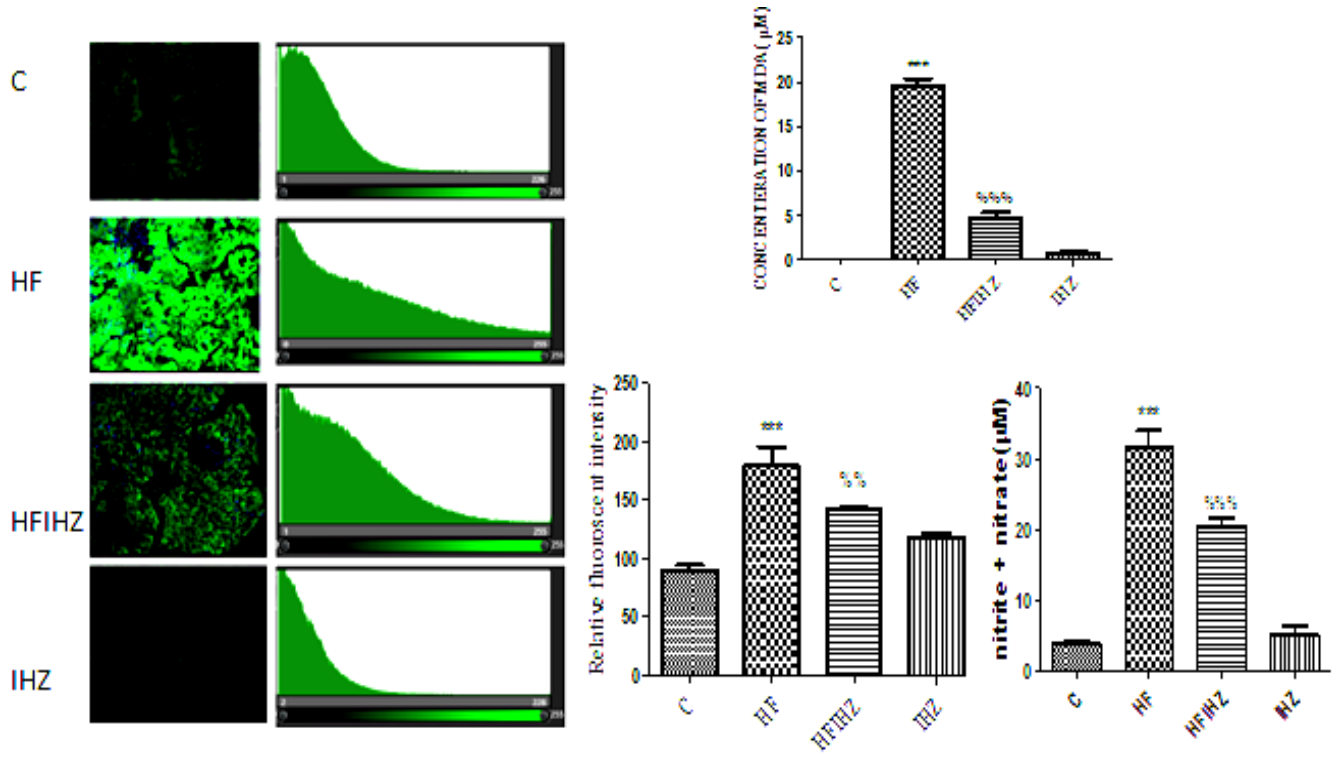
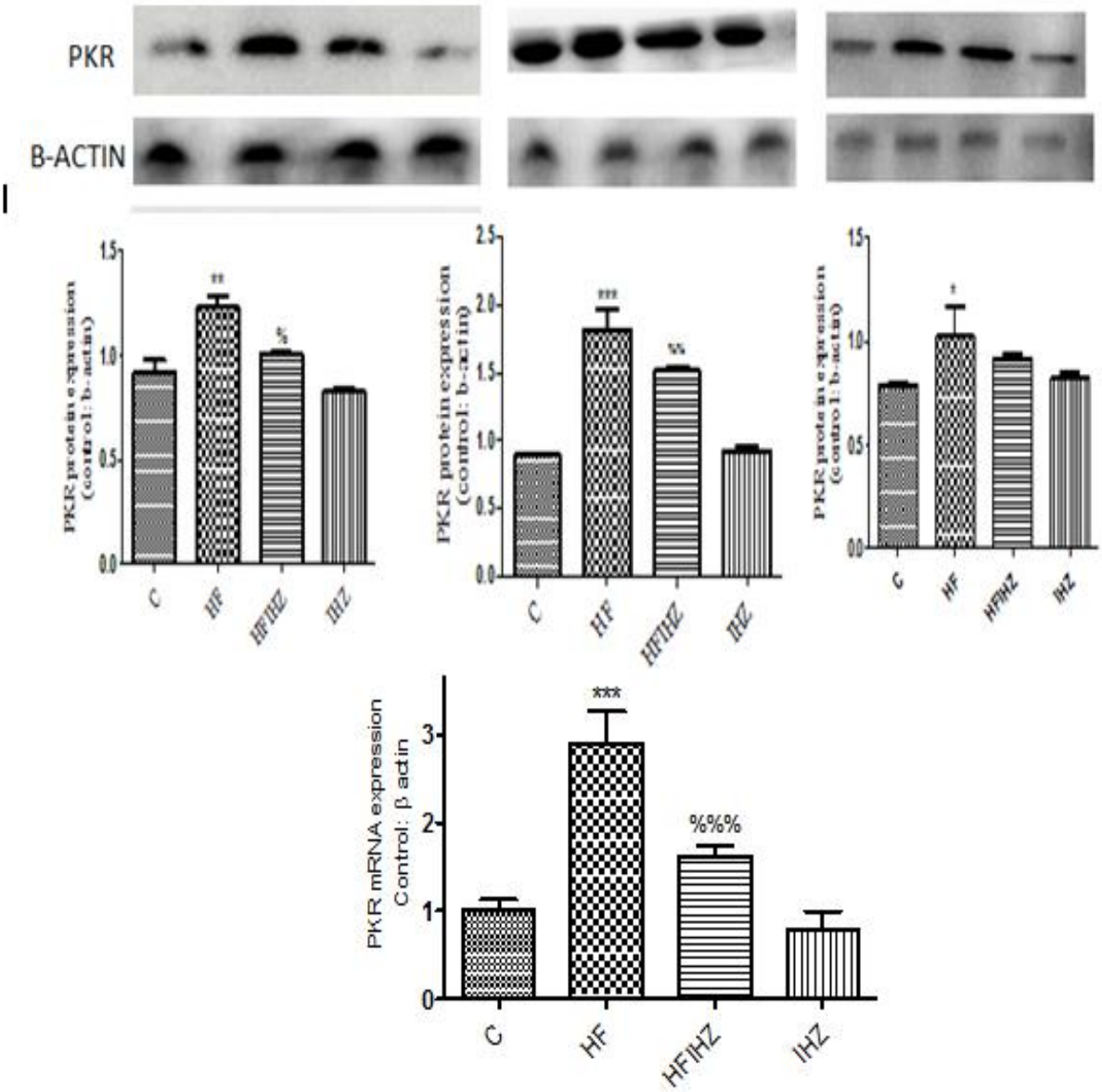


FIGURE 4.10 IHZ ATTENUATES HF INDUCED CHANGES IN VASCULAR REACTIVITY IN PANCREAS

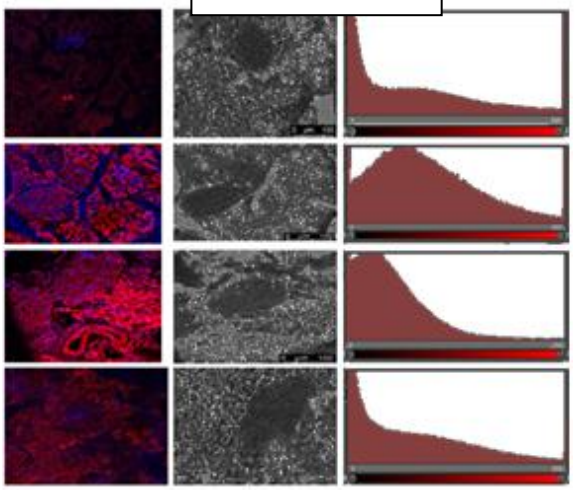
Animals were sacrificed and pancreas was isolated from all treatment groups. Part of pancreas was homogenized and used for estimation of (A) Inos (nitrite and nitrate) (B) MDA levels (C) Reactive oxygen species. n=3 for each treatment group*** *P<0.001 Vs C, %% P<0.01,%%% P<0.001 Vs HF*.

4.4.7 Effect of IHZ on PKR expression

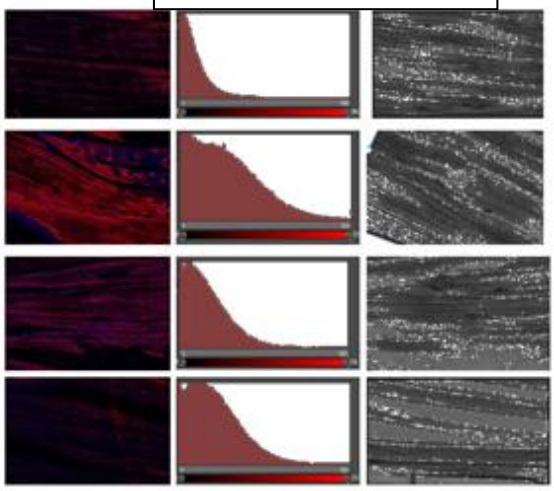
To investigate the effect of IHZ on PKR expression we performed protein expression studies using western blot and immunohistochemistry. We also did mRNA studies using RT-PCR. HF group treated animals showed significant increase in PKR expression which was attenuated with IHZ(10µM) on c0-administration with HF group.


PKR
B-ACTIN
PKR protein expression (control: b-actin)
C
HF
HFIHZ
IHZ
PKR mRNA expression Control: β actin
***
%%%

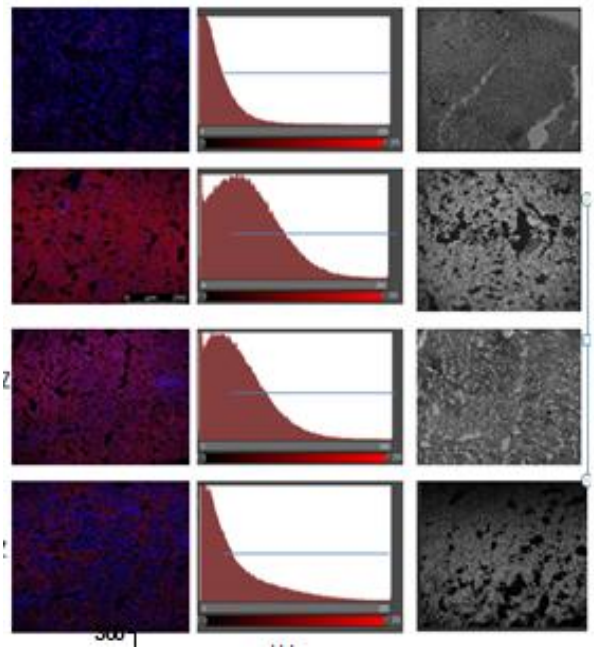
PANCREAS (40X)



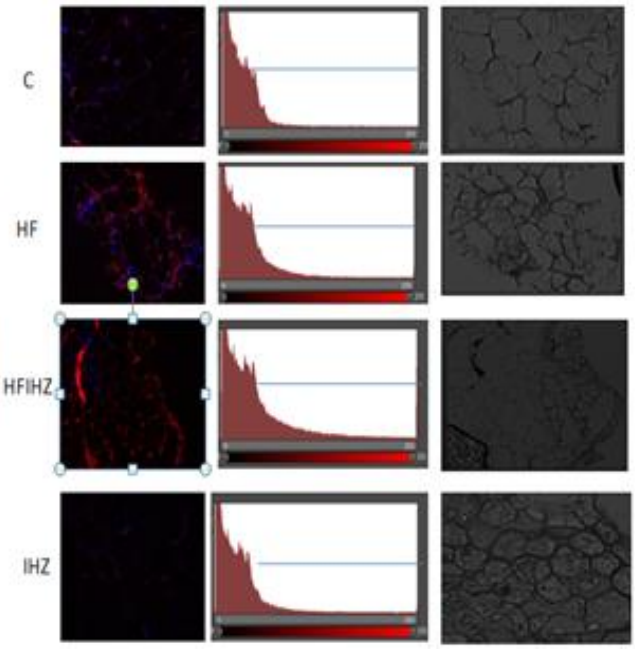
SKELETAL MUSCLE(40X)



LIVER (40X)



ADIPOSE(40X)



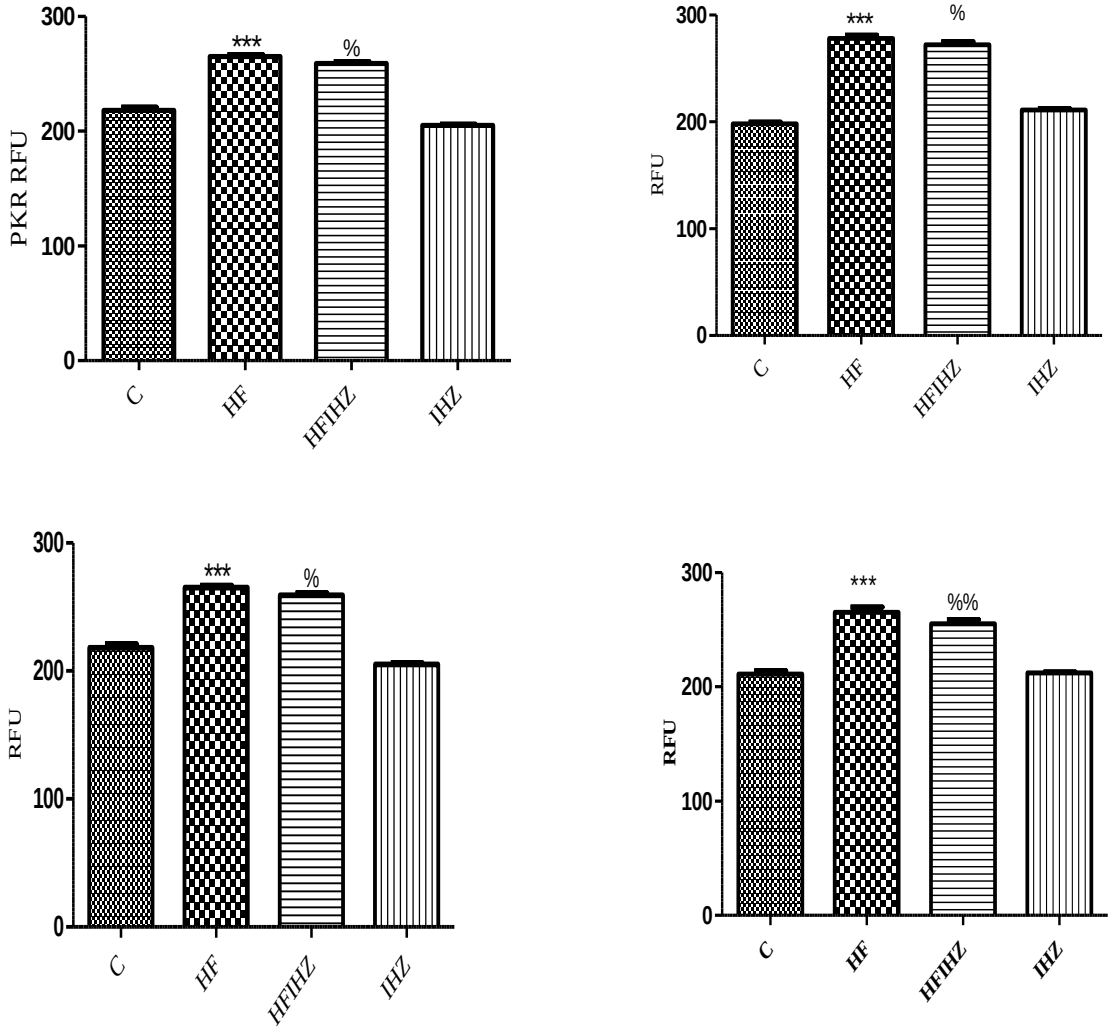
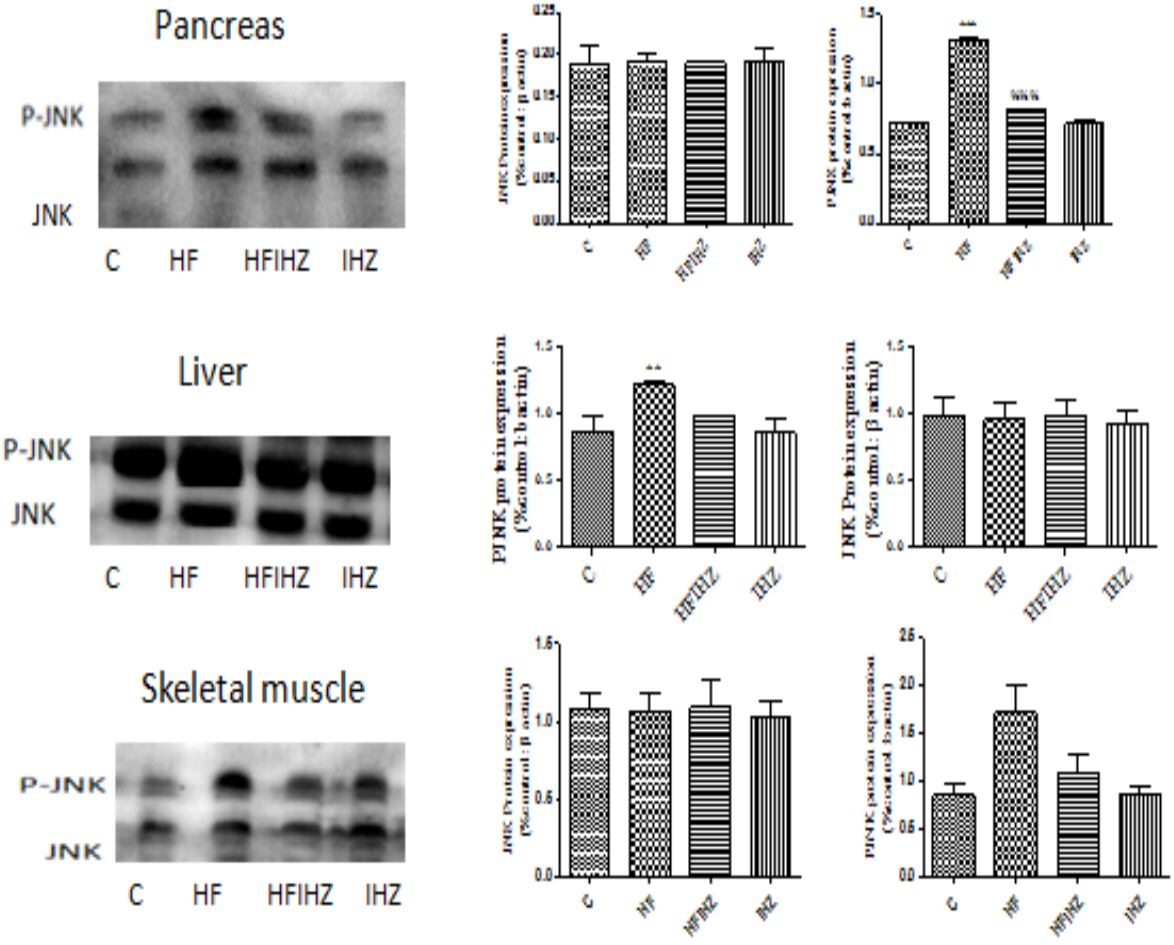


FIGURE 4.11 IHZ ATTENUATES HF INDUCED PKR EXPRESSION IN PANCREAS, SKELETAL MUSCLE, LIVER AND ADIPOSE TISSUE

Animals were sacrificed pancreas,skeletal muscle, liver and adipose was isolated from all treatment and normal groups. Part of tissues were homogenized and used for estimation of (A) protein expression (B) mRNA and remaining section was used for (C)Immunohistochemisrty. n=3 for each treatment group* *P<0.5,** P<0.01,*** P<0.001 Vs C,% P<0.5, %% P<0.01,%%% P<0.001 Vs HF.*

4.4.8 Effect of IHZ on PKR downstream markers

PKR was upregulated in HF treated group, as PKR is linked with inflammation next we saw the expression of inflammatory markers such as JNK and p-JNK using western blot and immunohistochemistry. HF treated group showed more JNK expression which was significantly attenuated by IHZ co-administered with HF group.



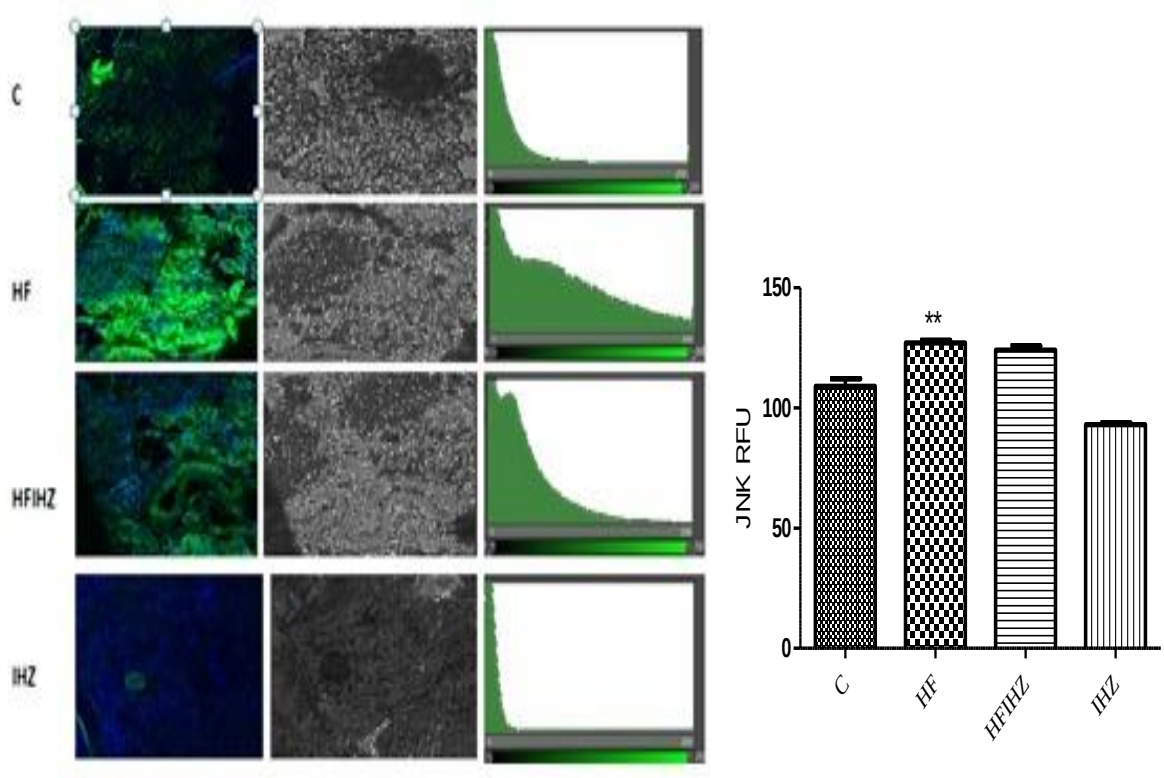


FIGURE 4.12 IHZ ATTENUATES HF INDUCED PKR DOWNSTREAM MARKERS(JNK and p-JNK)

Animals were sacrificed pancreas was isolated from all treatment and normal groups. Part of pancreas was homogenized and used for estimation of (A) protein expression, remaining section was used for (B)Immunohistochemisrty. n=3 for each treatment group,**P<0.01Vs C*

4.4.9 **Effect of IHZ on insulin signaling**

As pancreas and skeletal muscle are insulin signaling organs, we investigated IHZ role on gene markers of insulin signaling. RT-PCR studies were done for IRS-1 and GLUT-4 in skeletal muscle, PDX was done in pancreas. HF treated group showed impaired insulin signaling which was significantly attenuated by IHZ on co-administered with HF group.

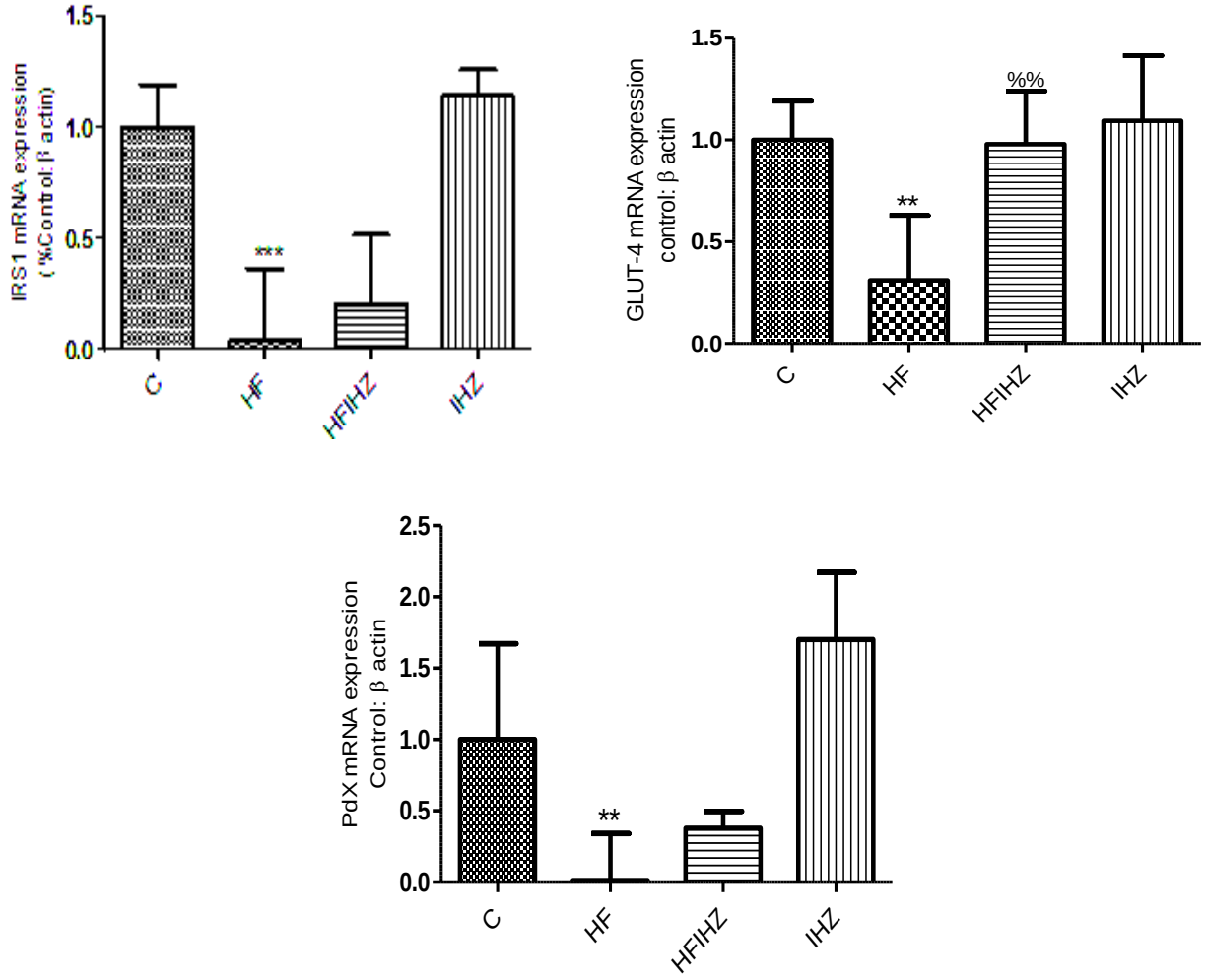
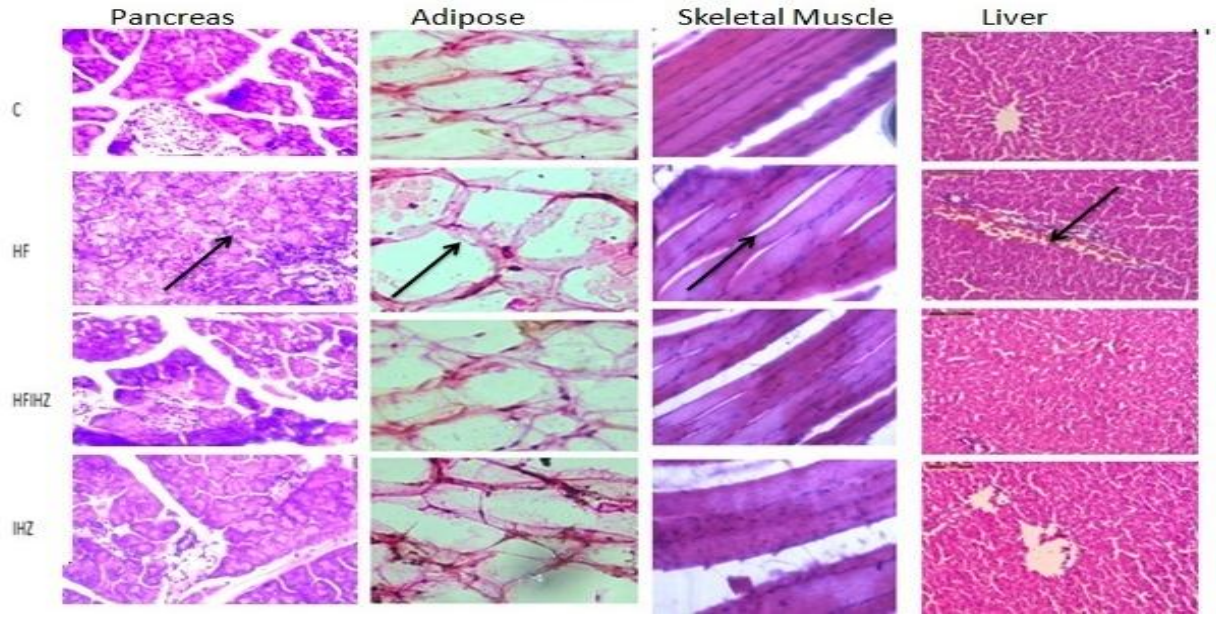


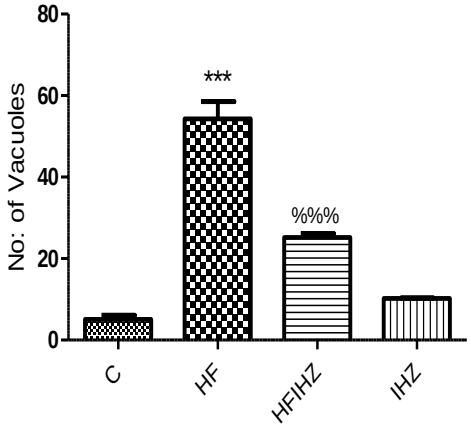
FIGURE 4.13 IHZ ATTENUATES HF INDUCED IMPAIRED INSULIN SIGNALLING IN SKELETAL MUSCLE AND PANCREAS

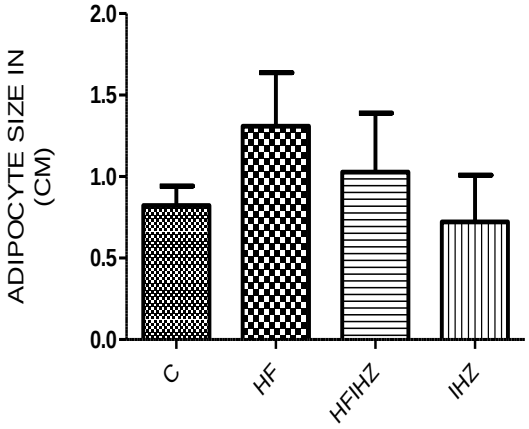
Animals were sacrificed pancreas and skeletal muscle was isolated from all treatment and normal groups. Part of tissues were homogenized and used for estimation of mRNA expression(A) GLUT-4 (B) IRS-1 (C) PdX, n=3 for each treatment group,** *P<0.01*,*** *P<0.001 Vs C %% P<0.01 Vs HF*.

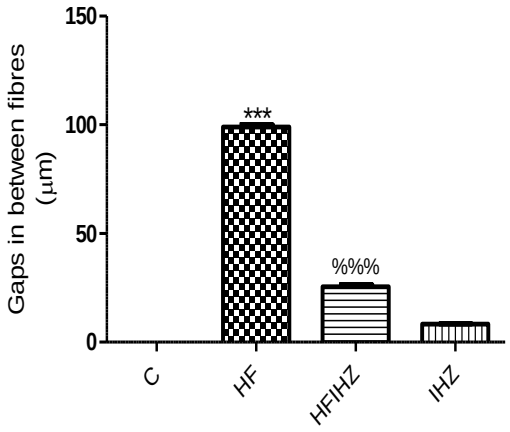
4.4.10 **Effect of IHZ on histo parameters**

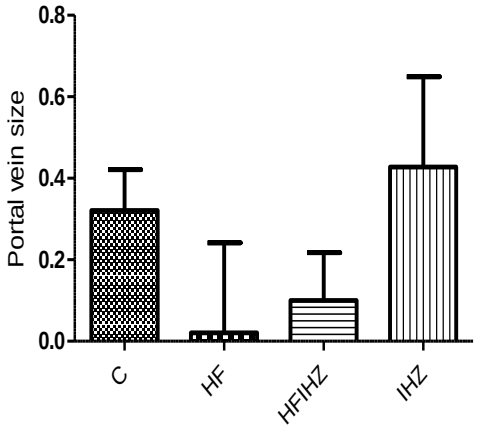
To investigate the role of IHZ on HE staining and fibrosis. Light microscopic investigations of H and E depicted that control animals resulted in regular histological architecture of pancreas, skeletal muscle, liver and adipose. However HF treated rats showed the irregular histological changes such as cell atrophy and significant vacuole formation in pancreas (A), irregualar muscle fiber arrangement (B)portal vein destruction in liver (C) increase in adipocyte area (D) which was significantly attenuated by IHZ(10µM) on co-administration with HF for 42 days. Sirius red staining photographs depicted that more fibrosis was seen in HF treated animals which was significantly attenuated in IHZ group co-administered with HF.

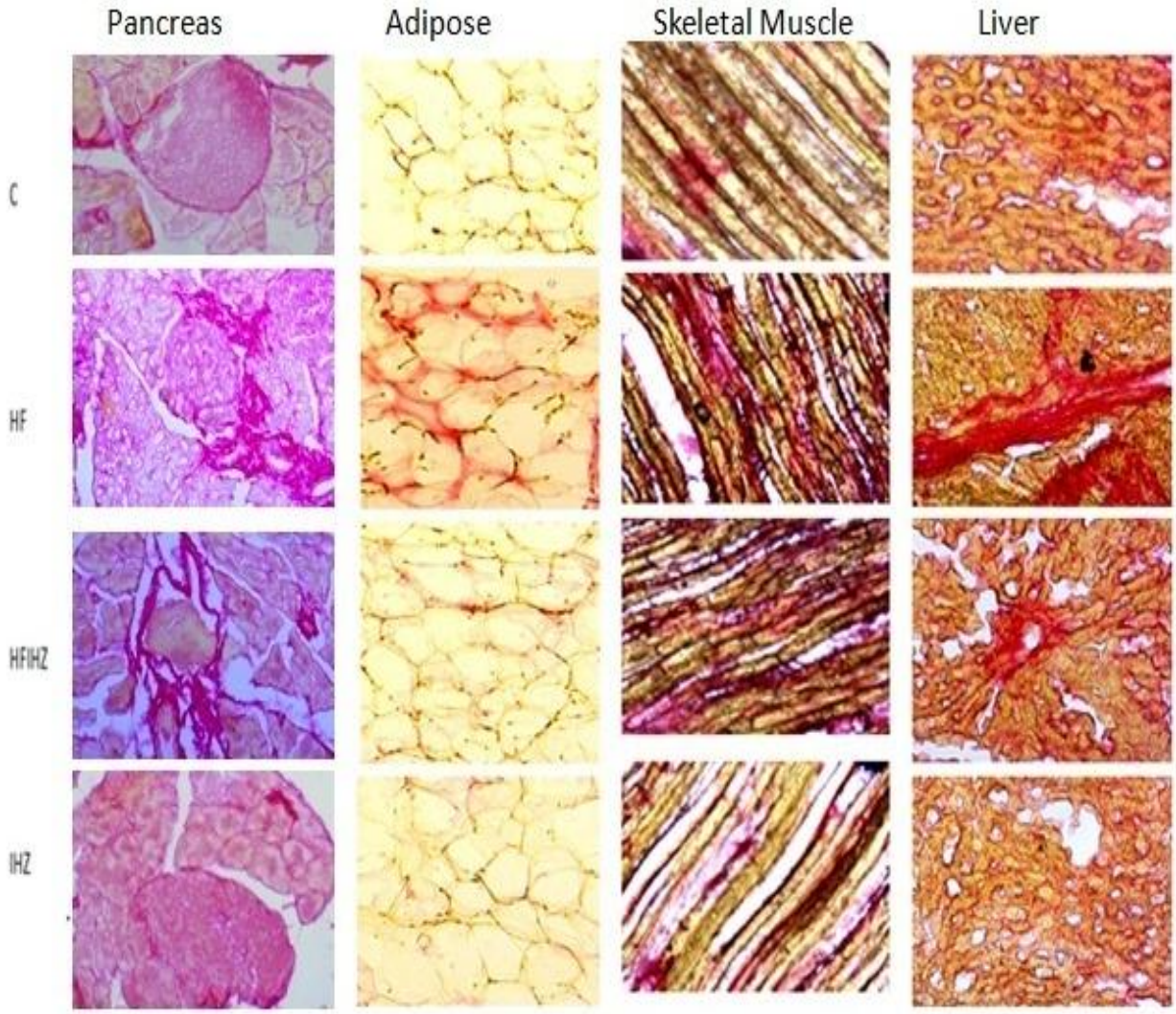



No: of Vacuoles
80
60
40
20
0
***
%%%
C
HF
HFIHZ
IHZ


ADIPOCYTE SIZE IN (CM)
2.0
1.5
1.0
0.5
0.0
C
HF
HFIHZ
IHZ


Gaps in between fibres (µm)
150
100
50
0
***
%%%
C
HF
HFIHZ
IHZ


Portal vein size
0.8
0.6
0.4
0.2
0.0
C
HF
HFIHZ
IHZ


Pancreas
Adipose
Skeletal Muscle
Liver
C
HF
HFIHZ
IHZ

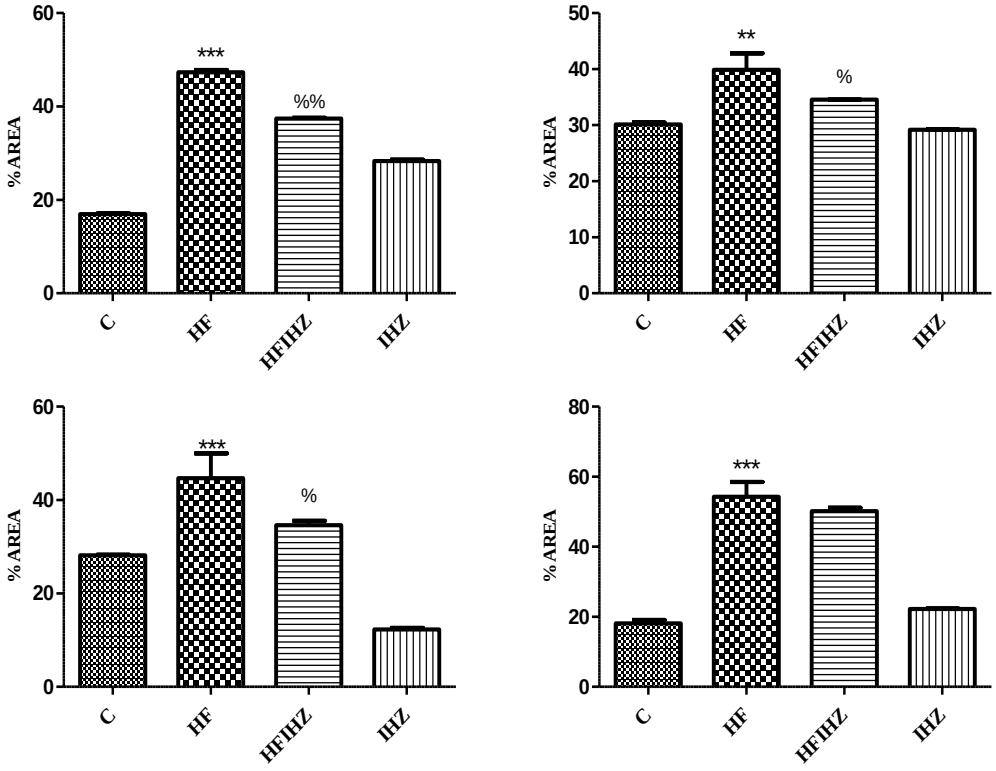


FIGURE 4.14 IHZ ATTENUATES HF INDUCED MORPHOLOGICAL CHANGES IN PANCREAS, SKELETAL MUSCLE, LIVER AND ADIPOSE

Animals were sacrificed pancreas, skeletal muscle, liver and adipose was isolated from all treatment and normal groups. Tissues were sectioned of 4-5µm and (A) H and E staining was done to see any morphological changes that occurred (B) Sirius red staining was done to see the collagen deposition. n=3 for each treatment group,** *P<0.01,*** P<0.001 Vs C %% P<0.01,% P<0.5 Vs HF.*

4.4.11 Effect of IHZ on HF induced fibrosis

To investigate the effect of IHZ on fibrosis we performed massions trichome staining. Pancreas was sectioned of 5µM thickness. Staining was performed and counterstained using aniline blue solution. Fibrotic sections were stained in blue colour, while normal areas were stained in pink colour.

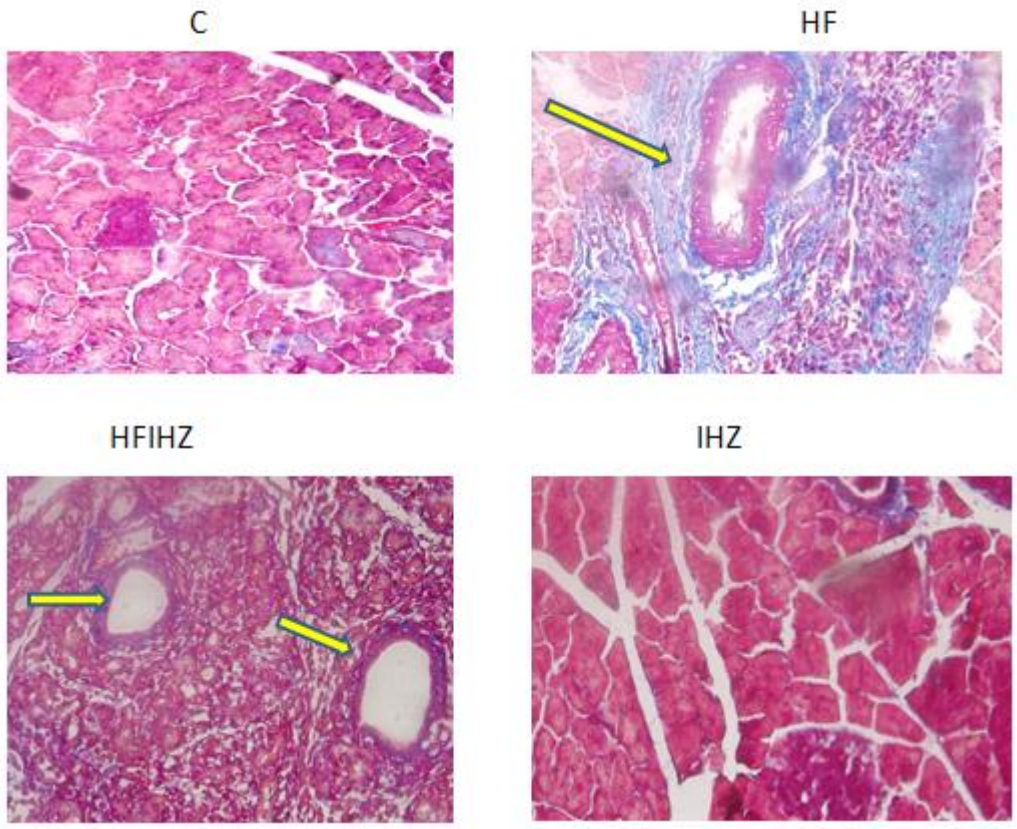


Fig 4.15 Representative photomicrographs showing the fibrosis evaluated by Masson Trichrome staining in the pancreas of control (A), HF treated animals (B), HF treated and IHZ treated animals (C),IHZ alone treated. Yellow color arrow indicates fibrosis. Histogram shows quantitative analysis of % fibrotic area. All the values were expressed as mean ± SEM, ***p<0.001 as compared to control group ****p<0.0001 as compared to control group (n=6).

4.4.12 **Effect of IHZ on programmed cell death.**

To investigate the effect of IHZ on apoptosis we performed insitu apoptosis. Pancreas was sectioned of 5µM. In situ apoptosis was performed and counterstained at last with methyl green. Apoptotic areas were stained in brown colour, while normal areas stained in green colour.

HF treated animals significantly showed increased apoptosis, which was attenuated with IHZ(10µM) on co-incubation with HF.

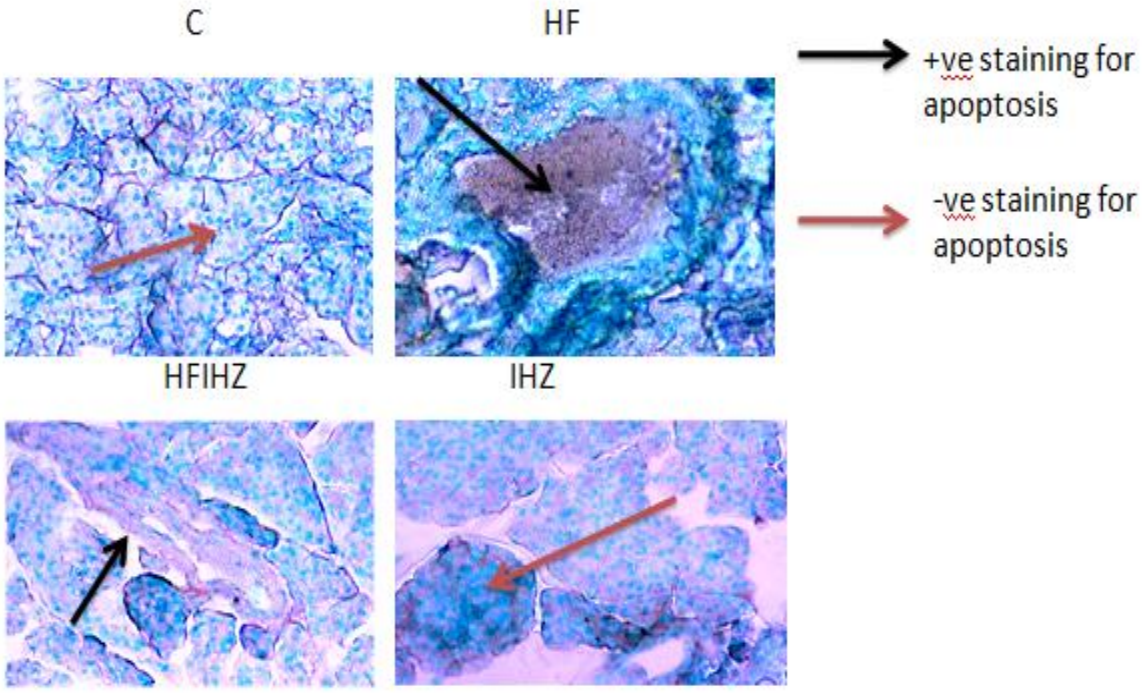


FIGURE 4.16 IHZ ATTENUATES HF INDUCED APOPTOSIS IN PANCREAS

Pancreas was sectioned of 4-5µm, In-situ apoptosis was done using the abcam commercial kit

4.4.12 Summary

HF 20% along with STZ was used as a model to induced diabetes. Biochemical parameters were measured. PKR expression was measured in pancreas, liver, skeletal muscle and adipose tissue using western blot and immunohistochemistry. HF has induced PKR expression this was attenuated by IHZ in presence of HF. Insulin markers such as IRS-1 and PDX were measured using Q-PCR. HF has impaired these markers, where as IHZ has corrected it. Histoloy studies were performed damaged islets, increased adipocyte size, irregular fibres and portal vein destruction were seen in pancreas, adipocytes, skeletal muscle and liver respectively. Tunnel staining was performed in pancreas apoptosis was seen in HF treated group this was attenuated by IHZ.

4.5 In-vitro screening of novel PKR inhibitor Indirubin-3-hydrazone in H9C2 cardiomyocytes

Diabetes and cardiovascular disorders constitute the most significant health burden to worldwide population (Carvalho M, 2012). Type I and type II diabetic patients are at increased risk of developing cardiovascular diseases (Grundy SM, 1999). Under obese and metabolic stress conditions several inflammatory pathways get activated which in turn lead to activation of downstream signaling molecules such as Jun NH2-terminal kinase (JNK) and NFKB. These pathways play a significant role in development of insulin resistance and cardiovascular diseases by controlling the inflammatory responses in metabolic tissues, inhibition of insulin receptor signaling and disruption of systemic glucose and lipid homeostasis (Baker RG and Hayden MS, 2011; Arkan MC, 2005; Frantz P , 2005; Hirosumi J, 2002; Kaneto H *et al*, 2004; Osborn O and Olefsky JM, 2012). Double stranded protein kinase R (PKR) is one such molecule that is integrated with both nutrient and pathogen response system, is activated by DsRNA, several stress signals, nutrients and pathogens (Shi H *et al*, 2006). PKR plays an important role in signal transduction and transcriptional control of several inflammatory pathways such as IkB/NFKB and JNK (Lee SB, Bablanian R and Esteban M,1996; Lee SB and Esteban M, 1994). It has been reported that PKR activation is triggered by various factors such as oxidative stress, metabolic stress and inflammation, and surprisingly all of these factors have been found to be upregulated in vascular complications and diabetic conditions (Gil J, Alcami J and Esteban M, 1999). Previous studies have reported that PKR gene silencing (PKR-/-) in mice relates with improvement in insulin resistance (Gil J, Alcami J and Esteban M, 1999; Williams B, 1997). Increased translocation and myocardial expression of PKR has been demonstrated in human subjects suffering from congestive heart failure (CHF) (Nakamura T *et al*, 2010). Congestive heart failure (CHF) is associated with cardiomyocyte hypertrophy, apoptosis and inflammation. PKR plays a significant role in development of CHF by intensifying apoptosis and inflammation

of cardiomyocytes (Wang H *et al*, 2014). Based on this research pharmacologically targeting PKR may be an effective therapeutic strategy for treatment of diabetes and vascular complications.

Indirubin is one of the main active ingredients of Chinese herb drug and has been known for its anti-proliferative effect in inhibition of cyclin dependent kinases (CDK) and glycogen synthase kinase-3ß (GSK-3 ß) (Wang J and Song Y, 2006). We have also reported recently that indirubin-3-oxime (I3O) has beneficial effects against high glucose induced oxidative stress and apoptosis in cultured rat cardiomyocytes. Further, we found that I3O is also capable of attenuating high glucose induced increased PKR expression (Udumula MP *et al*, 2016). Indirubin- 3-hydrazone (IHZ), a novel derivative of indirubin is a reported cyclin dependent kinase (CDK) inhibitor (Kim YC *et al*, 2012). . However the effect of IHZ on PKR signaling is not yet reported, so the aim of the present study was to investigate the effect of IHZ on PKR signaling pathway and underlying molecular mechanism.

4.5.1 Effect of Novel PKR inhibitor (IHZ) on HG induced PKR activation

Novel PKR inhibitor at different concentrations (3, 10, 30µm) was incubated with HG in-vitro in H9C2 cells. C16 was used as a standard for comparison of PKR inhibition levels. Incubation of HG with novel inhibitor at 3µm for 24 hours did not showed any significant reduction, where as incubation of HG with 10 and 30µm showed almost 75% of the PKR inhibition (Fig 4.16). So based on this observation we have selected 10µm concentration for our further investigation.

Time dependent effect of IHZ in HG treated H9C2 cells

Time dependent effect of IHZ was examined in cultured H9C2 cells. Incubation of H9C2 with HG (25mM) and IHZ (10µm) alone and or in combination was incubated for a period of 3, 6,

12 and 24 hours. Following this time dependent studies we examined that there was no significant decrease in PKR expression at a time point of 3,6 hours, however significant reduction in PKR expression was observed at 12 and 24 hr time point (Fig 4.16) .

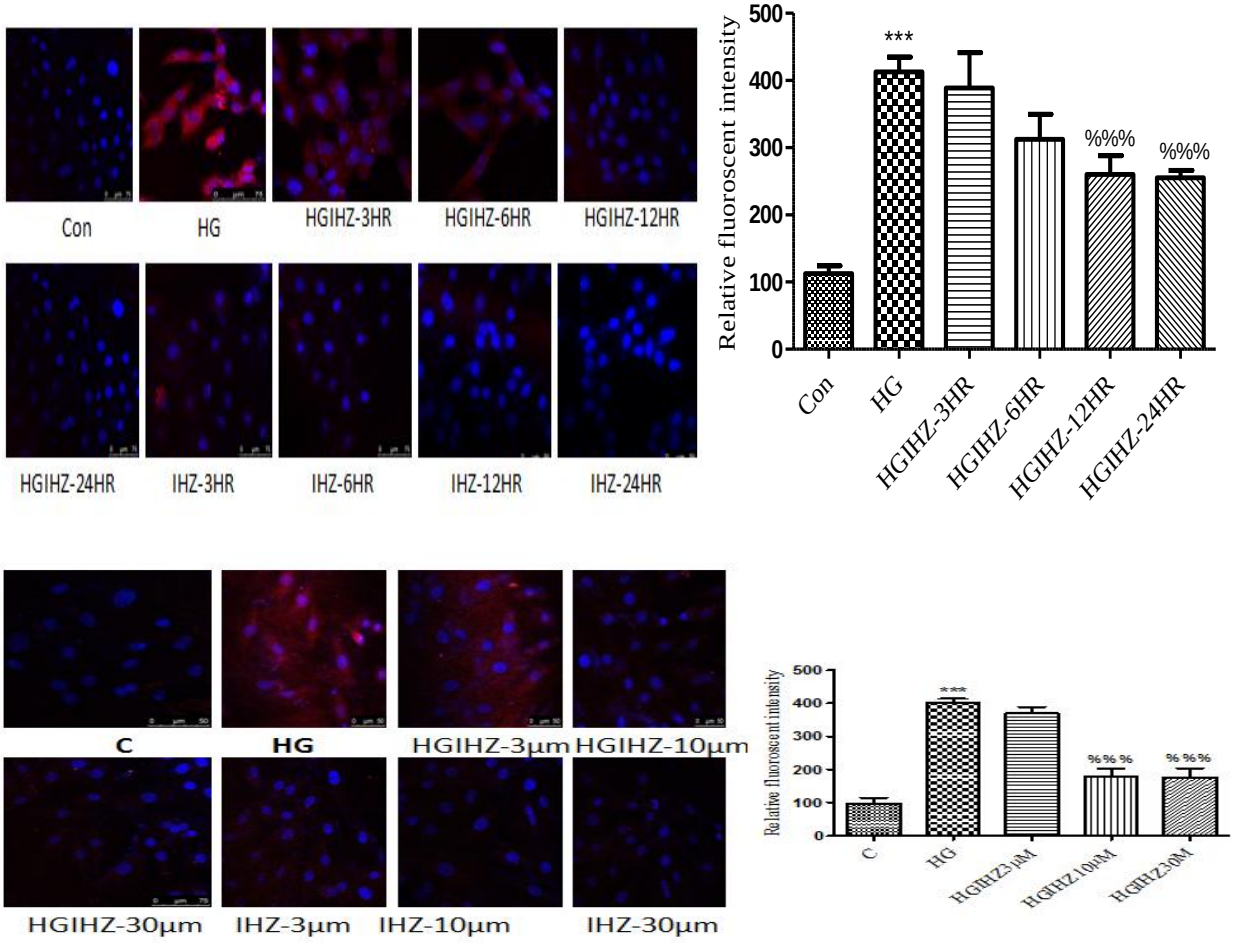
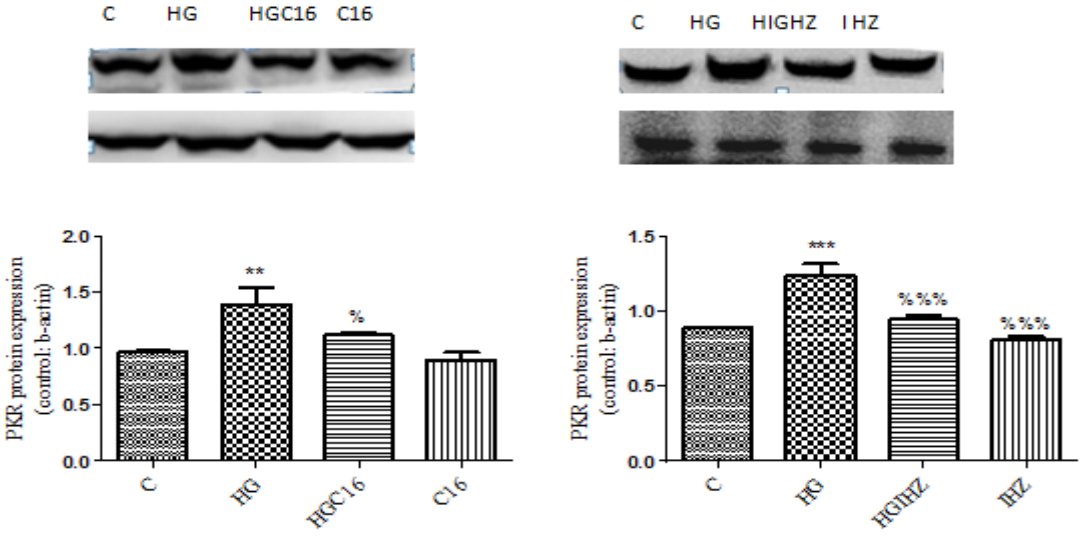


Figure 4.17: Concentration and time dependent effect of IHZ on PKR levels:

Cultured cells were incubated with different treatment groups Control (C), HG (25mM), C16(5µM), IHZ (3,10,30µM) alone or co-incubated with HG for 3,6,12 and 24 h(a). IHZ (10µM) for 24h showed increased PKR expression which was significantly attenuated by IHZ on co-incubation with HG using Immunohistochemistry (specific PKR antibody and texas red conjugated secondary antibody, n=4 for each group ***$p< 0.001$ *vs.* control (Con). $^{\%\%\%}p< 0.001$ *vs.* HG treated group.

4.5.2 IHZ inhibits HG induced PKR activity *in vitro*

We previously reported that HG induced PKR expression was attenuated by Indirubin 3-oxime, recently from docking analysis we found novel analogue of indirubin , indirubin hydrazone (IHZ) has inhibitory activity on PKR. In this study we investigated the in-vitro potential of IHZ in H9C2 cells on PKR by RT-PCR, immunohistochemistry and western blotting. So H9C2 cells were incubated with HG alone and in combination with novel inhibitor for 24 hours. Results of these experiments have shown significant changes in both protein and mRNA expression. The mRNA expression of PKR, was significantly increased in HG incubated cells and was significantly attenuated by IHZ (10µM) co-incubated with HG. There was modest decrease in PKR expression with HGIHZ compared to HGC16. In immunohistochemistry and western blotting (Fig.4.17). we observed increased fluorescence and band intensity for PKR in HG (25mM) incubated H9C2 cells compared to untreated cells. This was significantly decreased by IHZ co-incubated with HG.



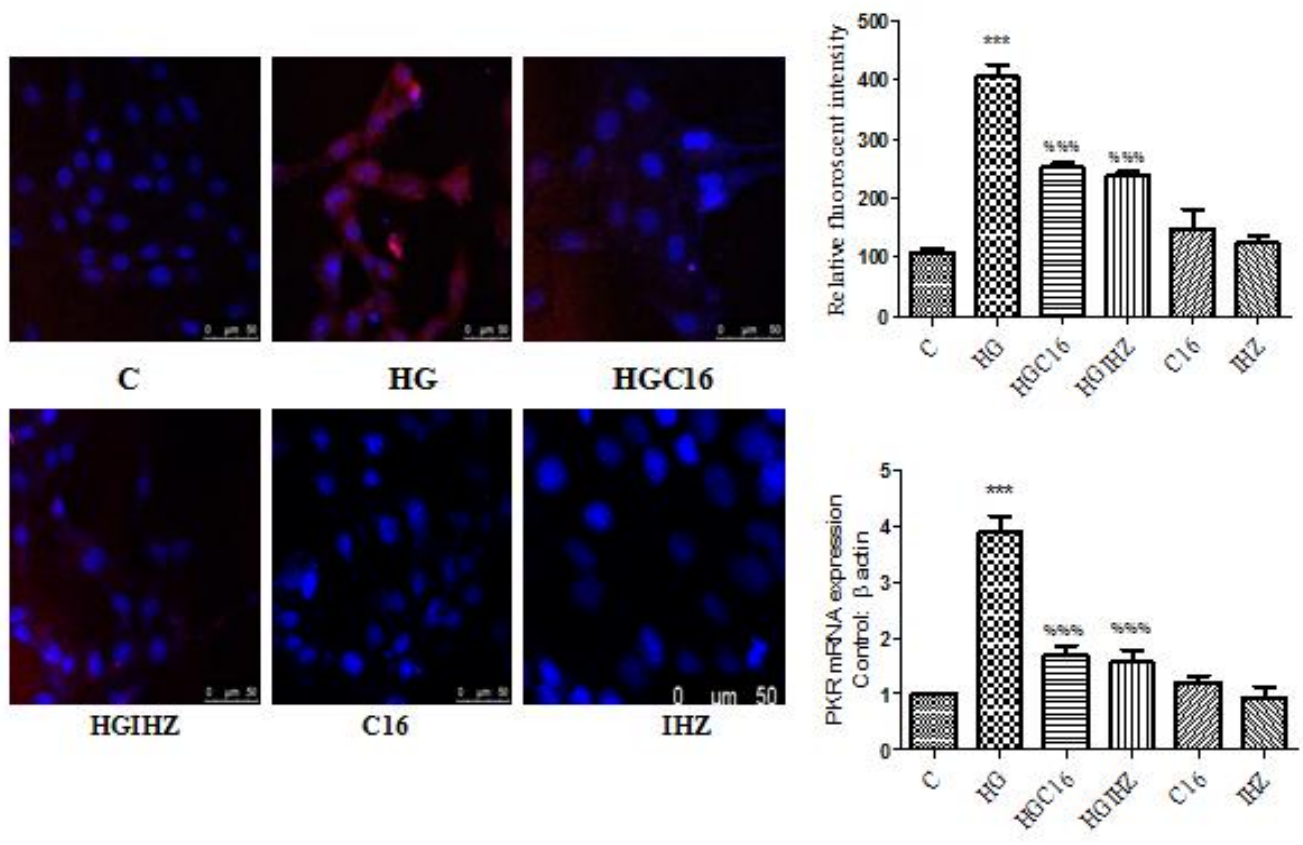
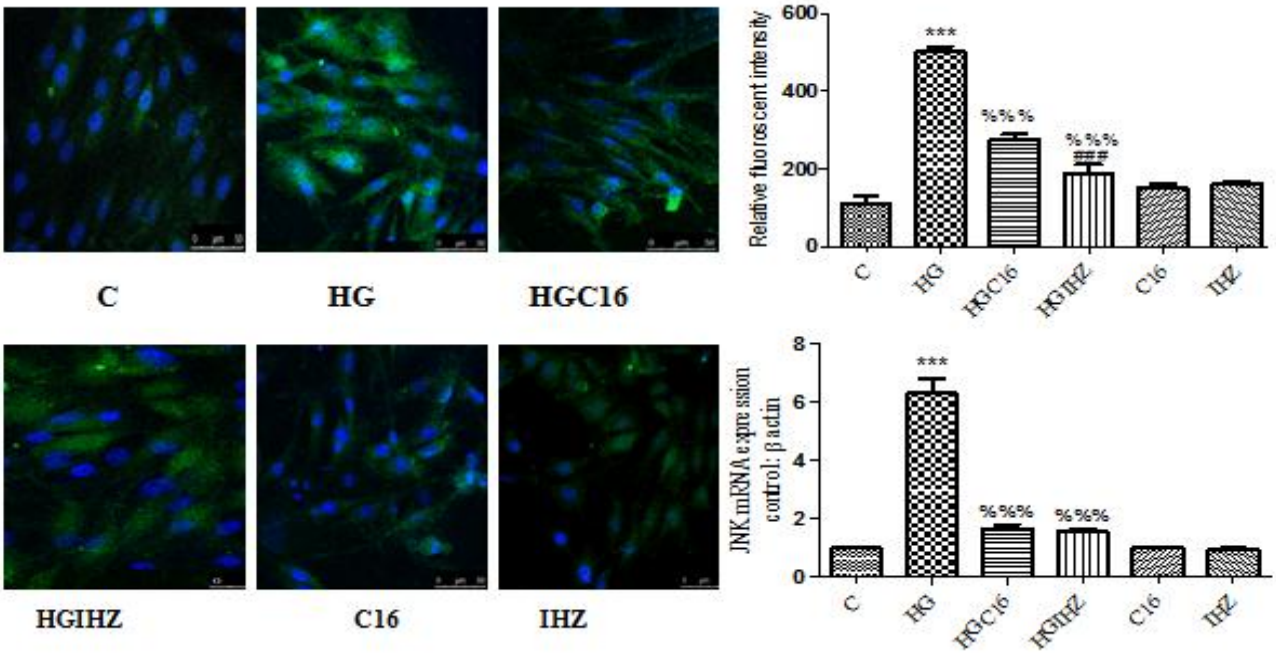


Figure 4.18: IHZ attenuates HG induced PKR expression:

Incubation of cultured cells with HG (25mM) significantly increased mRNA expression of PKR expression which was significantly attenuated by IHZ(10µM) on co-incubation with HG (A). Immunohistochemistry (B) Western blot (C) was performed , n=6 for each group ***$p<0.001$,**$p<0.01$ *vs*. control. $^{\%\%\%}p< 0.001$,$^{\%}p<0.5$ *vs*. HG, $^{\#}p<0.5$*vs* HGC16

4.5.3 Effect of IHZ on gene markers of apoptosis

We examined the effect of IHZ on gene markers associated with apoptosis. H9C2 cells were incubated with HG alone and in combination with C16 and IHZ for 24 h and analyzed for gene expression. The mRNA expression of NFKB an oxidative stress marker, JNK an apoptotic and inflammatory transcription factor was significantly increased in HG incubated H9C2 cells which was significantly decreased by IHZ (10µM) co-incubated with HG. Protein expression studies using western blot was performed for JNK and phospho-JNK. There was no change in protein expression of JNK but increased expression of phospho-JNK was observed when incubated with HG for 24 h compared to untreated cells this was significantly attenuated by IHZ with HG . Immunofluoroscence studies for JNK was performed and HG has increased the JNK expression this was significantly attenuated by IHZ co-incubated with HG (Fig 4.18).



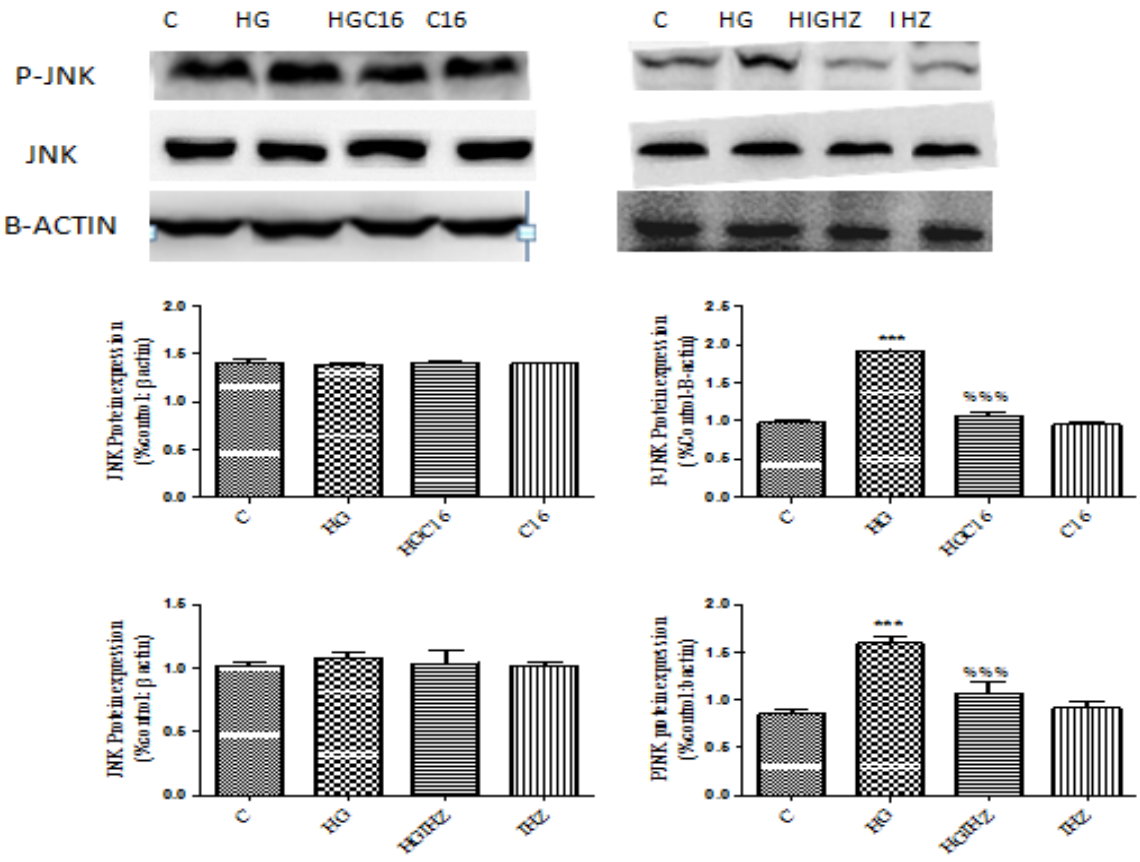


Figure 4.19: IHZ attenuates HG induced gene expression of JNK:

Incubation of cultured cells with HG (25mM) significantly increased mRNA expression of JNK, NFKB which was significantly attenuated by IHZ(10µM) on co-incubation with HG (A). immunohistochemistry (B),Western blot (C) was performed with specific JNK and phospho-JNK antibody, n=6 for each group ***$p<0.001$,**$P<0.01$ *vs.* control. $^{\%\%\%}p< 0.001$,$^{\%\%}p<0.01$ *vs.* HG treated group.

4.5.4 Effect of IHZ on HG induced apoptosis

Previously, we have reported that HG induces apoptosis, so here we have examined whether IHZ can prevent HG induced apoptosis in H9C2 cells or not. Cells were stained with DAPI. The DAPI staining is used to detect nucleus in live and fixed cells. Control cells have shown more regular and normal nucleus (DAPI), HG treated cells have shown fragmented nucleus (Fig 4.19). HG treated cells have shown more early apoptotic cells which was significantly attenuated by C16 and IHZ co incubated with HG. IHZ along with HG show significant reduction in early apoptotic percentage of cells compared to HGC16 .

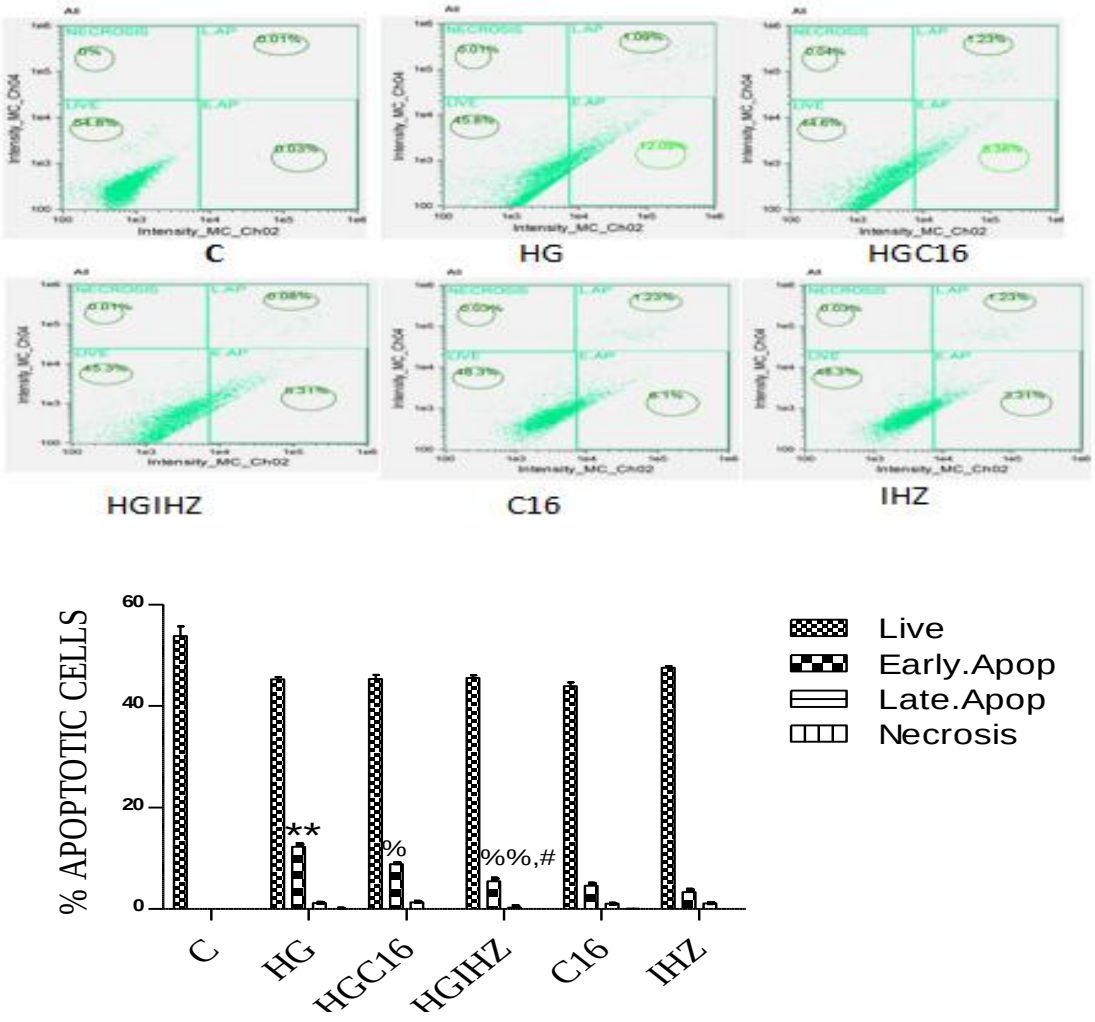


Figure 4.20: IHZ attenuates HG induced apoptosis in cultured cardiomyocytes:

Cells were incubated with HG for 24 h and then stained with DAPI. Regular nuclear morphology and uniformly distributed cytoplasm was observed in control. Condensed and fragmented nuclei was observed in HG treated cells which was attenuated by C16 and IHZ (10µM) along with HG (25Mm) (A). The FACS (B) analysis was performed using annexin V alexa fluor 488, the results explain percentage of live, early, late apoptotic and necrosis cells falling in that particular gating. HG (25Mm) has initiated more early apoptosis which was significantly attenuated by IHZ (10µM) when co-incubated with HG, n=5 for each group ***p<0.01 vs*. respective control early apoptotic cells. $^{\%\%}$ *p<0.01*, $^{\%}$ *p< 0.5 vs.* treated HG early apoptotic cells. $^{\#}$ *p<0.5 vs* HGC16 early apoptotic cells.

4.5.5 Effect of IHZ on HG induced reactive oxygen species production

HG is a known inducer of oxidative stress, we investigated whether IHZ can prevent the HG induced reactive oxygen species generation. Incubation of cultured H9C2 cells with HG (25 mM) for 24 h significantly increased reactive oxygen species production where the shift was towards left (Fig.7a), which was attenuated by IHZ (10µM) co-incubated with HG (25mM). we also examined that IHZ co-incubated with HG showed significant reduction in ROS generation when compared to C16 with HG (Fig 4.20) .

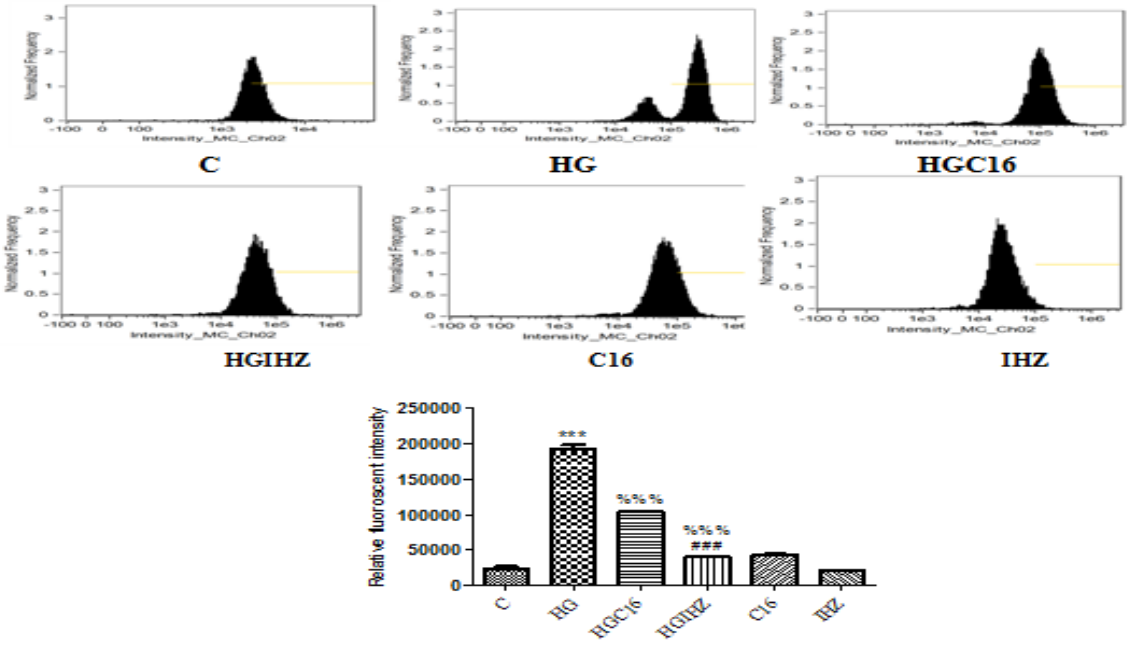


Figure 4.21: IHZ attenuates HG induced increase reactive oxygen species production: Incubation of cultured cardiomyocytes with HG for 24 h induced increase in ROS generation which was significantly attenuated by HZ when co-incubated with HG which was analysed by FACS, n=3 for each group ***$p<0.001$ *vs.* control. $^{\%\%\%}p < 0.001$ *vs*. HG . $^{\#\#\#}$ $p<0.001$ *vs* HGC16

4.5.6 Effect of IHZ on Capase-3 activation

Caspases are important markers of apoptosis especially caspase 3 which is activated by extrinsic and intrinsic pathway. Activity of caspase 3 was significantly increased after incubating cardiomyocytes with HG which was significantly attenuated by IHZ (10µM) co-incubated with HG which was observed by immunohistochemistry and also by Q-PCR (Fig.4.21).

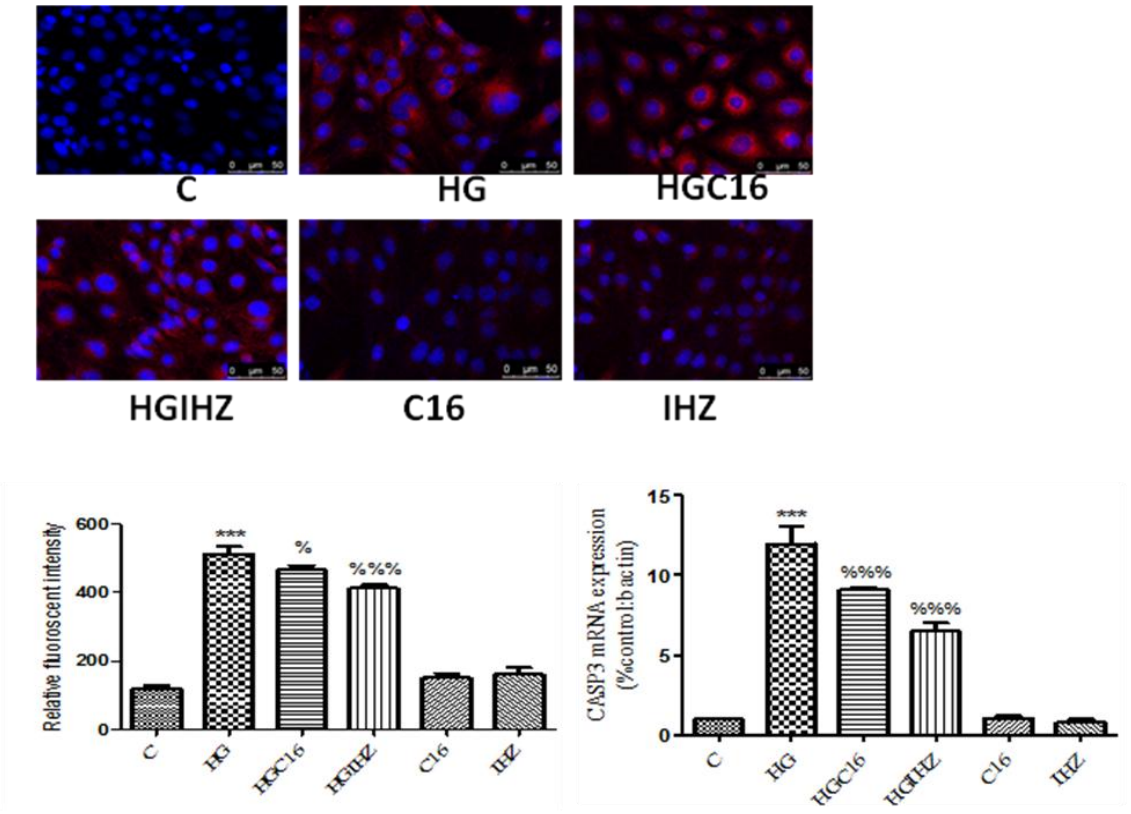


Figure 4.22: IHZ attenuates HG induced Caspase 3 expression

Cultured cardiomyocytes incubated with HG (25mM) increased caspase 3 expression and was significantly attenuated by IHZ(10µM) in combination with HG this was performed by immunohistochemistry (A) and also Q-PCR (B), n=6 for each group ***$p< 0.001$ *vs*. control.$^{\%\%\%}$ $p< 0.001$, $^{\%}p<0.5$ *vs*. treated HG.$^{\#}$ $p<0.5$*vs* HGC16.

4.5.7 Effect of IHZ on iNOS production

Inducible nitric oxide (iNOS) production is a key mediator to promote apoptosis, so we examined whether HG can induce iNOS so in H9C2 cells incubated with HG (25mM) there was significant increase in iNOS production when compared with untreated cells which was significantly decreased by IHZ (10µM) co-incubated with HG (Fig.4.22). However, it was not significant when compared to HGC16.

4.5.8 Effect of IHZ on HG induced LDH Production

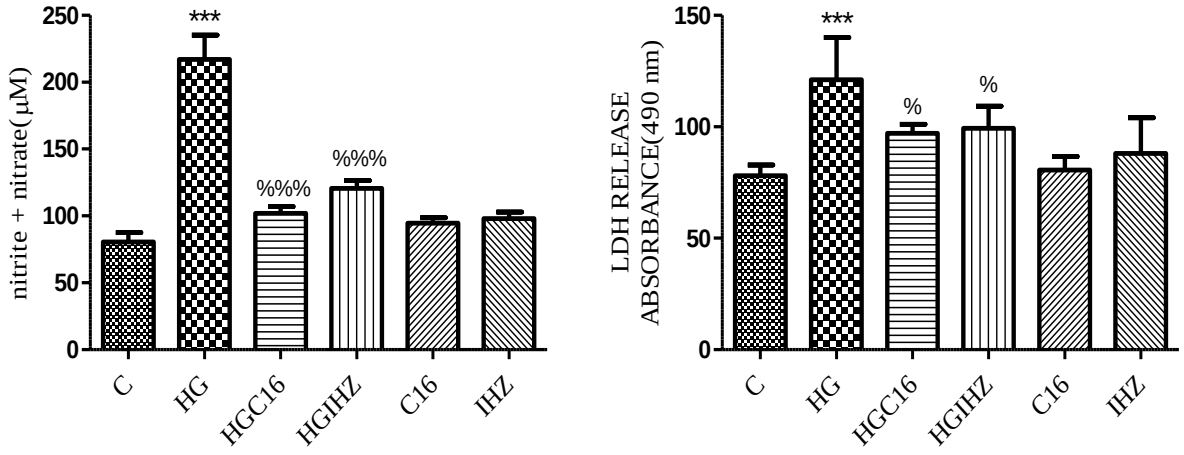
Plasma membrane integrity was determined by monitoring the cytoplasmic levels of LDH enzyme in the extracellular medium. As expected HG has induced more LDH production which was significantly attenuated by IHZ (10µM) co-incubated with HG .

4.5.9 Effect of IHZ on HG elicited autophagy in cardiomyocytes

Autophagy is followed by oxidative stress, in the present study we have examined the reduced formation in HG treated cultured cells which may be due to autophagy using rhodamine staining. Moreover when cells were exposed to HG along with IHZ vacuole formation was recovered when compared to HG alone treated cells. (Fig 4.22).

4.5.10 Effect of IHZ on Cellular integrity

Prominent nuclei and irregular shaped cell structure was found in cells treated with HG, this was significantly attenuated on treating with C16 and IHZ (10µM) co incubated with HG (Fig 4.22).



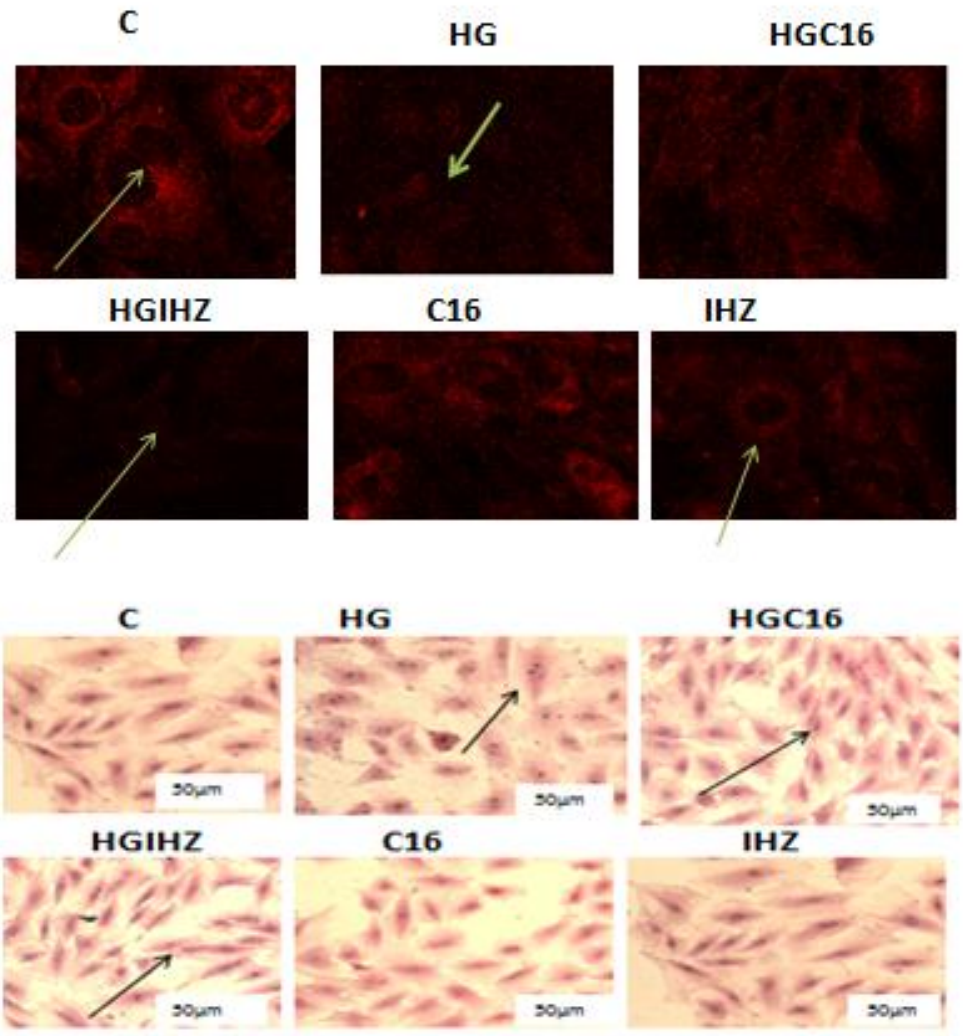


Figure 4.23: IHZ attenuates HG induced iNOS, LDH and no vacuole formation:

H9C2 cells were incubated with HG (25mM) for 24 h and iNOS production was significantly increased which was significantly attenuated by IHZ(10µM) incubated with HG , n=8 for each group ***$p< 0.001$ *vs.* control.$^{\%}$ $p< 0.5$ *vs*. HG. H9C2 cells were incubated with HG (25mM) for 24 h and LDH production was significantly increased which was significantly attenuated by IHZ (10µM) incubated with HG , n=8 for each group ***$p< 0.001$ *vs.* control.$^{\%\%\%}$ $p< 0.001$ *vs*. HG. Cells after incubating with HG has shown vacuole formation which was attenuated by IHZ (10µM) , n=6 for each group. H9C2 cells were incubated with HG for 24 h and prominent nuclei and irregular structure was observed which was significantly attenuated by IHZ (10µM) , n=6 for each group.

4.5.11 Summary

We studied the effect of HG on PKR protein/mRNA expression, reactive oxygen species (ROS) production and gene markers of inflammation, oxidative stress and apoptosis (JNK, NF-κB, Bcl-2, caspase-3) in high glucose-treated cardiomyocytes. Incubation of H9C2 cells with high glucose for 24 h significantly increased mRNA expression of JNK, an inflammatory and apoptotic transcription factor and NF-κB, an oxidative stress marker gene were significantly increased in high glucose-treated H9C2 cells. High glucose treatment for 24 h also significantly increased caspase-3 mRNA. HG induced autophagy in cells this was inhibited by IHZ. The effects of high glucose on PKR mRNA/protein expression, ROS production and proinflammatory and apoptotic gene markers were attenuated by IHZ (10 μmol/l) co-incubated with high glucose.

CHAPTER 5

GENERAL DISCUSSION

Metabolic syndrome (MS) is a cluster of risk factors containing metabolic disorders such as obesity, hypertension and insulin resistance which occur repeatedly. All these synergistically increase the risk of cardiovascular complications (Nisoli et al., 2007). Cardiovascular complications represent major reason for mortality and morbidity in diabetic patients (Marks & Raskin, 2000). Numerous studies have been reported supporting a strong link between insulin resistance and endothelial dysfunction (Steinberg et al., 1996). In addition, MS is also associated with changes in vascular responsiveness to vasoconstrictors and vasodilators (De Vriese et al., 2000). Moreover, studies have reported that high glucose induces diabetes by causing reactive oxygen species generation and apoptosis, whereas in case of high fat diet (HFD), induction of diabetes is via fibrosis, oxidative stress and inflammation (Dal S,2015). However, the molecular mechanism underlying this process is not fully understood. When excess of these carbohydrates are carried into liver this hepatic metabolism gets disturbed and this leads mainly to two consequences: One of them is metabolism of glucose and uptake of glucose, the second major consequence is *de novo* lipogenesis and triglyceride synthesis. Fructose in presence of fructokinase converts to fructose-1 phosphate later to glyceraldehyde then a series of process finally metabolized to ATP and carbondioxide. On the other hand excess fructose intake results in more production of hepatic triglycerides which reduces insulin sensitivity and finally leads to insulin resistance (Romaguera D, 2013).

Insulin resistance is caused mainly when there is reduced or negligible response from insulin sensitive organs such as skeletal muscle, liver and adipose to insulin levels. Impaired glucose uptake has been observed in these tissues under insulin resistance state. Glucose transporters (GLUTS) play a crucial role in facilitative transport of glucose present in the body. GLUT4, an abundant glucose facilitative transporter isoform is present in skeletal muscle and

adipocytes, it sis a major transporter responsible for glucose transport in response to insulin (Cushman SW, 1998; Suzuki I, 1980; Birnbaum MJ,1989; Charron MJ). GLUT 2 is another important glucose transporter expressed predominantly in pancreaticß cells and involved in insulin secretion (Miyazaki J, 1990). Type 2 diabetes is caused by activation of multiple pathways including oxidative stress, high calorie diet as well as inflammation (Der S,1995).

Our study started with hypotheses of exposing cultured cells as well as animals to high carbohydrates levels and correlating it to PKR expression. We treated H9C2 rat cultured cardiomyocytes for 3,6, 12 and 24 hours with high glucose (25mM) and measured PKR expression. Elevated PKR levels were found at 24h (Udumula MP,2016). We also measured downstream markers of PKR pathway, JNK and NFkB, and observed upregulation of these markers as well. As ROS plays an important role in many metabolic diseases, so we measured ROS levels using DCFDA assay. ROS levels were signigicantly increased in HG treated cellsTo understand the molecular mechanism for increased PKR expression, novel (synthesized in lab) and selective PKR inhibitor, C16 (imoxin) was used in subsequent studies. We have selected H9C2 cardiomyocytes and L6 skeletal muscle as these are insulin signaling cells (Udumula MP, 2017). We incubated L6 skeletal muscle cell myotubes with HG or in combination with C16, a specific PKR inhibitor and novel PKR inhibitors, I30 and IHZ for 24h. HG induced PKR expression which was significantly attenuated by C16. I30 also decreased HG induced PKR expression but this was was comparatively less significant compared to C16. IHZ also significantly attenuated HG induced PKR expression and inhibition was significant even compared to C16. So we selected IHZ for further screening. As diabetes is characterized by impaired insulin signaling, we next measured expression of insulin signaling pathway markers such as IRS-1, PI3-K and AKT. HG impaired the expression of downstream markers and this

was significantly attenuated by C16 and IHZ. As HG induces ROS generation, we next measured ROS levels using flowcytometry, HG induced ROS production was significantly attenuated by C16 and IHZ. IHZ attenuation was more significant even compared to C16. Apoptosis was measured using flowcytometry and immunofluorescence staining, IHZ significantly attenuated HG induced apoptosis even compared to C16. Previous studies have reported that in PKR knock mice and cells, little to no expression of apoptotic and inflammatory markers, NFKB and JNK. So our study was to investigate further mechanism underlying between PKR activation and JNK upregulation leading to diabetes. JNK and NFkB expression was measured using western blot, Q-PCR and immunocytochemistry. HG nduced JNK and NFkB expression which was significantly attenuated by C16 co-incubated with HG. JNK expression was even more significantly attenuated by novel PKR inhibitor IHZ. So all these findings gave us a supportive evidence to further find the exact mechanism underlying this phenomenon. So we further evaluated the effect of IHZ in high fructose (HF) treated rats. HF treated rats showed increase in biochemical parameters such as body weight, total cholesterol, triglycerides, aspartate transaminase and low density lipoprotein. HF treated rats also showed decrease in HDL levels. Increase level of these biochemical markers were attenuated by IHZ along with HF group. Vascular parameters such as reactive oxygen species, nitrite and nitrate and malonaldehyde levels were increased in HF group. These parameters were attenuated in IHZ group in presence of high fructose. Activated PKR expression was reported in white adipose tissue and liver (24). As pancreas, liver , adipose tissue and skeletal muscle are insulin signaling organs, we measured PKR expression in these organs. PKR expression was determined using immunohistochemistry, western blot and Q-PCR. In HF group animals, there was increased PKR expression compared to control, where as in IHZ co-administered along with HF group attenuated the increased PKR

expression compared to HF. Studies have reported that JNK and NFKB are the downstream markers that are activated by PKR (Takahisa Nakamura ,2014; Hsu, L. **C** 2004). So we next evaluated JNK and p-JNK expression using western blot and immunohistochemsitry. HF treated rats showed increase JNK expression compared to control, where as IHZ co-administered along with HF group attenuated the HF effects.

Our lab and other studies reported that HG impaired regular insulin signaling (24), so we next performed Q-PCR studies for various mRNA expression. IRS-1 which helps in activating further downstream markers such as PI3-K, PDK, PKB and GSK3 helps in translocation of GLUT4 for further glucose uptake (Krook, 1998). GLUT4 is an important transporter present in skeletal muscle which helps in glucose uptake. So we measured the expression of GLUT-4 and IRS-1, HF treated rats showed decrease in GLUT4 and IRS-1 expression where as IHZ in presence of HF group significantly attenuated this effect. PDX 1 an important marker present in pancreas activates insulin. It is also an insulin promoter factor and helps in pancreas development and ß cell maturation (Kei Fujimoto, 2009). So we next evaluated the expression of PDX1 in pancreas. HF treated rats showed impaired PDX1 expression compared to control where as in IHZ co-treated with HF group attenuated the HF effects.

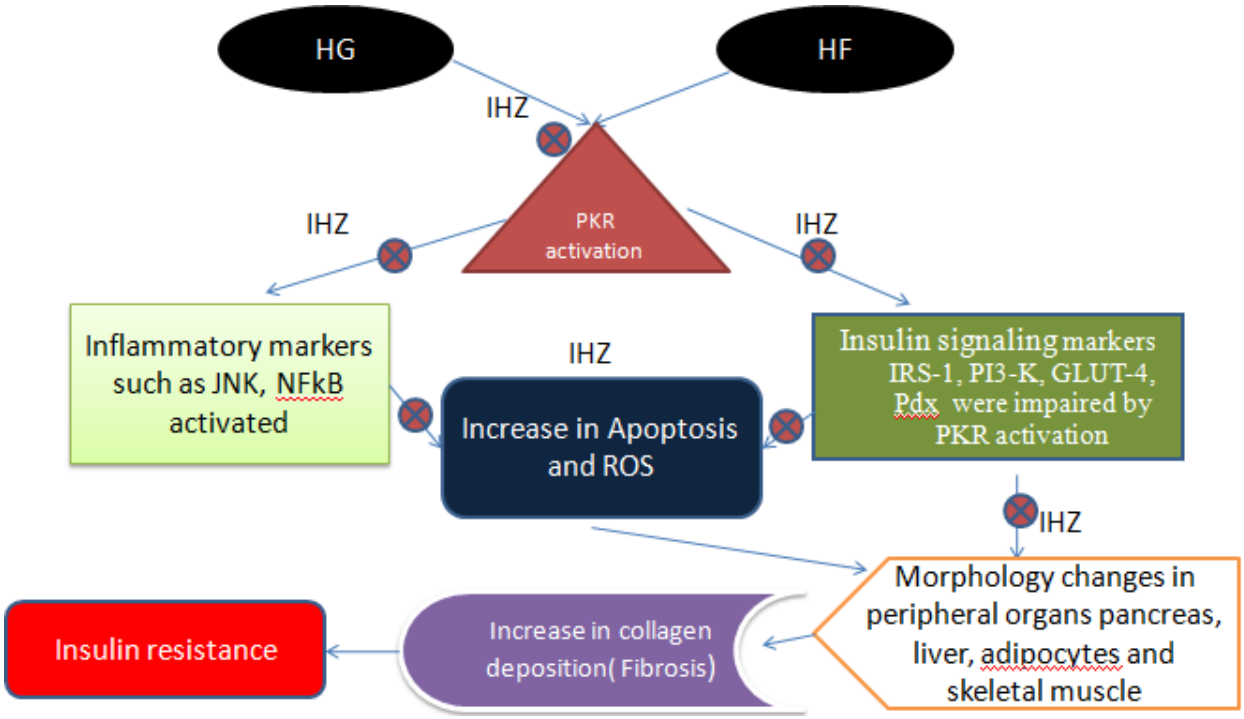
As HG impairs cell integrity (Delafield F, 1984), we next performed H and E staining to see the morphological changes in pancreas, liver, adipose tissue and skeletal muscle. We observed islet destruction (pancreas), irregular muscle fiber (skeletal muscle) orientation, portal vein destruction (liver) and adipocyte hypertrophy (adipocytes) in HF treated rats which was significantly decreased in IHZ in presence of HF group. Studies have reported the formation of fibrosis or collagen deposition in fructose treated rats (Whittaker P, 1994), so we measured the collagen deposition using Sirius red staining in pancreas, skeletal muscle, liver and adipose

tissue. HF treated group showed increased collagen deposition which was attenuated by IHZ in HF group. Apoptosis is an important characteristic in diabetes, studies reported that beta cell destruction causes diabetes (Wang C, 2011). So we next measured apoptosis using tunnel staining, HF treated rats showed increase in apoptosis, Has well as damaged islets in brown staining. This was decreased by IHZ in presence of HF group.

Taken together, these findings suggest PKR plays a pivotal role in the progression of beta-cell dysfunction and insulin resistance, thus targeting PKR could be a effective therapeutic target for diabetes. So targeting PKR by safe and selective inhibitors can be an important strategy in combating metabolic disorders.

Chapter 6
Recapitulation and Future perspectives

Summary


HG
HF
IHZ
PKR activation
IHZ
IHZ
Inflammatory markers such as JNK, NFkB activated
IHZ
Increase in Apoptosis and ROS
Insulin signaling markers IRS-1, PI3-K, GLUT-4, Pdx were impaired by PKR activation
IHZ
Morphology changes in peripheral organs pancreas, liver, adipocytes and skeletal muscle
Increase in collagen deposition(Fibrosis)
Insulin resistance

- High glucose (25mM) induced PKR expression was reported for the first time in Cardiomyocytes.
- I3O novel PKR inhibitor was screened for its anti-oxidant and anti-apoptotic activity in H9C2 cell line using DCFDA assay and Caspase-3 and Bcl2 mRNA expression.
- I3O and IHZ along with specific PKR inhibitor C16 were screened for its inhibitory action on PKR . IHZ attenuated PKR expression this was significant compared to C16 in presence of HG, the work was done in L6 myotubes.
- IHZ corrected HG impaired regular insulin signaling this work was done using flowcytometry.
- Apoptosis study was also performed using flowcytometry. HG induced early apoptotic population this was attenuated by IHZ significantly when compared to commercial C16.
- PKR downstream markers JNK and NFkB were also measured using Q-PCR. IHZ attenuated this HG induced JNK nad NFkB expression.
- IHZ was further screened by in-vivo studies. HF has induced PKR expression in pancreas, liver, skeletal muscle and adipose tissue. This was attenuated by IHZ.
- Lipid parameters such as total cholesterol, total triglycerides, LDL were increased in HF treated group where as HDL levels were decreased. All these were corrected by IHZ.
- Insulin markers such as IRS-1 in skeletal muscle and PdX in pancreas were measured using Q-PCR. Both these markers were impaired in HF treated group and corrected by IHZ along with HF.
- Histology studies were performed on pancreas, liver, skeletal muscle and adipose. HF treated group results showed damaged morphology and increased collagen deposition in all. This was decreased in IHZ in presence of HF group.

- Apoptosis was performed in pancreas. Islets were dead in HF treated group which we observed by brown staining.
- IHZ role was reported in Cardiomyocytes for the first time.
- HG induced autophagy was measured using rhodamine staining.
- PKR up-regulation using high carbohydrate diet was studied in the present work. Mechanism between PKR upregulation and Diabetes was reported.

Future Perspectives of This Study Include

- The demonstrated activity of these novel compounds on PKR expression, Insulin markers, ROS and apoptosis, could be extended to various other cell lines such as adipocytes and hepatocytes.

- Effect of potent compounds in-vivo can be further screened using Hyperinsulinemic-euglycemic clamp studies.

- Pharmacokinetics of these compounds in both healthy and disease rat/mouse models could be evaluated.

CHAPTER 7

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APPENDIX

LIST OF PUBLICATIONS

FROM THESIS WORK

Udumula MP, Babu MS, Kalra J, Bhat A, Dhar I, Sriram D, Dhar A. Novel PKR inhibitor IHZ attenuates high fructose induced diabetes via IRS-1,PdX/JNK pathway in wistar rats (Under Communication).

Udumula MP, Babu MS, Kalra J, Bhat A, Dhar I, Sriram D, Dhar A. Pharmacological evaluation of novel PKR inhibitor Indirubin-3-hydrazone in Cardiomyocytes. Life Sciences (Accepted).

Udumula MP, Babu MS, Bhat A, Dhar I, Sriram D, Dhar A High glucose impairs insulin signaling via activation of PKR pathway in L6 muscle cells. Biochem Biophys Res Commun. 2017 May 6;486(3):645-651. (Accepted)

Udumula MP, Medapi B, Dhar I, Bhat A, Desai K, Sriram D, Dhar A. The Small Molecule Indirubin-3'-Oxime Inhibits Protein Kinase R: Antiapoptotic and Antioxidant Effect in Rat Cardiac Myocytes. Pharmacology. 2016;97(1-2):25-30. (Accepted)

OTHER PUBLICATION

Babu MS, Bhat A **, Udumula MP**, , Dhar I, Dhar A. Activation of double-stranded RNA-dependent protein kinase R mediates high carbohydrate and high lipid mediated oxidative stress and apoptosis in cultured H9C2 cardiomyocytes. Pharmacological Reports (Accepted).

Kalra J, Babu MS, Bhat A, Dhar I, **Udumula MP**, Dhar A "Imoxin attenuates high fructose induced oxidative stress and apoptosis in renal epithelial cell via down regulation of PKR Pathway". Fundamental and Clinical Pharmacology. (Accepted)

Dhar A, **Udumula MP**, Medapi B, Bhat A, Dhar I ,Malapati P, Babu MS, Kalra J, Sriram D, Desai KM Pharmacological evaluation of novel alagebrium analogs as methylglyoxal scavengers in vitro in cardiacmyocytes and in vivo in SD rats.. Int J Cardiol. 2016 Nov 15;223:581-589. (Accepted)

Dhar A, **Reddy P**, Bhat A, Desai K, Dhar I. Double Stranded RNA Dependent Protein Kinase (PKR) and Type 2 Diabetes.. Pharm Pharmacol Int J 2(2): 00015. (Accepted)

PAPERS PRESENTED AT NATIONAL/INTERNATIONAL CONFERENCES

1. **Mary Priyanka Udumula**, Mangali Suresh Babu, Audesh Bhat, Indu Dhar, Dharmarajan Sriram, Arti Dhar*. ACTIVATION OF DOUBLE-STRANDED RNA-DEPENDENT PROTEIN KINASE PATHWAY IMPAIRS INSULIN SIGNALING IN L6 MUSCLE CELLS. Presented at Drug Discovery and Development, 16th-17thMarch, 2017 at National Institute of Pharmaceutical Education and Research, Hyderabad.
2. **Mary Priyanka Udumula**, Mangali Suresh Babu, Audesh Bhat, Indu Dhar, Dharmarajan Sriram, Arti Dhar*. ACTIVATION OF DOUBLE-STRANDED RNA-DEPENDENT PROTEIN KINASE PATHWAY IMPAIRS INSULIN SIGNALING IN L6 MUSCLE CELLS. Presented at Ramanbhai Foundation 8th International Symposium on Current Trends in Health care, Feb 2-4th, 2017 at Ahmedabad, Gujarat.
3. **Mary Priyanka Udumula**, Brahmam Medapi, Audesh Bhat, Indu Dhar, Kaushik Desai, Dharmarajan Sriram, Arti Dhar. INDIRUBIN-3'-OXIME INHIBITS PROTEIN KINASE R: ANTIAPOPTOTIC AND ANTIOXIDANT EFFECT IN RAT CARDIAC MYOCYTES. Presented at Indo Global Diabetes Summit, Nov 23rd-25th, 2015 at Electronic City, Bangalore.

Other Conferences

1. Kalra J, **Udumula MP** ,Babu MS, Bhat A, Dhar I, Dhar A. "IMOXIN ATTENUATES HIGH FRUCTOSE INDUCED OXIDATIVE STRESS AND APOPTOSIS IN RENAL EPITHELIAL CELL VIA DOWNREGULATION OF PKR PATHWAY". Presented at International Conference on Challenges in Drug Discovery and Development, 2-4thMarch, 2017, BITS-Pilani campus.
2. Mangali SB**, Udumula MP**, Kalra J, Bhat A, Dhar I, Dhar A. EFFECT OF GLUCOTOXICITY AND LIPOTOXICIY ON PKR MEDIATED CARDIOMYOPATHY-PREVENTION AND MECHANISM. Presented at International Conference on Challenges in Drug Discovery and Development, 2-4th March, 2017, BITS-Pilani campus.

3. Arti Dhar ,**Mary Priyanka Udumula** , Brahmam Medapi , Audesh Bhat , Indu Dhar, Mangali Suresh Babu , Jaspreet Kalra , Prasanthi Malapati , Dharmarajan Sriram, Kaushik Desai. CARDIOPROTECTIVE EFFECT OF NOVEL ALAGEBRIUM ANALOGS IN VITRO IN CULTURED CARDIAC MYOCYTES AND IN VIVO IN SD RATS VIA INHIBITION OF METHYLGLYOXAL PATHWAY. Presented at Sipra Innovative Research Awards, 2nd July, 2016 Sipra labs, Hyderabad.

BIOGRAPHY OF MARY PRIYANKA UDUMULA

Mary Priyanka Udumula completed her Bachelor of Pharmacy from Chaitanya Institute of Pharmaceutical Sciences in year 2011and M.Pharm (Pharmacology) from Vaagdevi college of Pharmacy, Kakatiya University, Warangal, Telangana in year 2014. In 2014, she got admitted for PhD program at Department of Pharmacy, Birla Institute of Technology and sciences (BITS-Pilani), Hyderabad campus, under the supervision of Dr. Arti Dhar. She was awarded with DST-Inspire fellowship in 2014 December for securing University first rank. She has published 4scientific papers in International journals. She had presented papers at various National and International Conferences. She has won 2 best poster awards for presenting her work with a title Activation of Double-Stranded RNA-Dependent Protein Kinase Pathway Impairs Insulin Signaling in L6 Muscle Cells. Presented at Drug Discovery and Development, 16th-17thMarch, 2017 at National Institute of Pharmaceutical Education and Research, Hyderabad. Other award won at Indo Global Diabetes Summit, Nov 23rd-25^{th,} 2015 at Electronic City, Bangalore with a title Indirubin-3'-Oxime Inhibits Protein Kinase R: Antiapoptotic and Antioxidant Effect in Rat Cardiac Myocytes.

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BIOGRAPHY OF DR. ARTI DHAR

Dr Arti Dhar is currently working as an Assistant Professor in the Department of Pharmacy at Birla Institute of Technology and Science (BITS), Pilani, Hyderabad campus. Dr. Dhar received her PhD from University of Saskatchewan, Canada in the year 2010. During her PhD she received scholarships from Heart and Stroke Foundation of Canada (HSFC) and Arthur Smith Memorial Scholarship from University of Saskatchewan, Canada. Dr. Dhar also won numerous travel awards from Canadian Physiological Society and Canadian Hypertension Society. Her PhD thesis was nominated for Governor General's Gold medal and her thesis work was presented on CBC channel Canada and on campus news channel in March 2011. Dr. Dhar did her postdoctoral trainings from Lake head University, Ontario, Canada and University of Saskatchewan, Canada from the year 2010 to 2013. After joining BITS in 2014, she has received research funding from DST under Young scientist scheme, CSIR extramural research funding, UGC under early career award and from BITS under additional competitive grant. Her prime research interests are centered on novel therapeutic targets for cardiovascular and metabolic disorders. Currently 3 students are pursuing PhD under her guidance. She has several National and International publications to her credit.