GAD 65 AND ITS ROLE IN PANCREATIC TISSUE SURVIVAL

by

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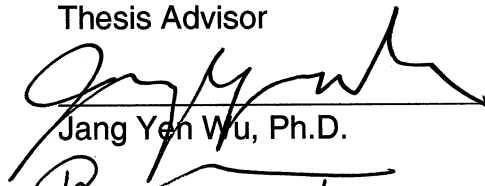
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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Howard Prentice, Department of Biomedical science, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

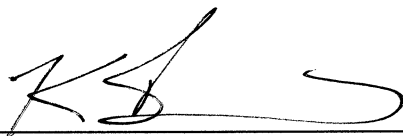SUPERVISORY COMMITTEE:

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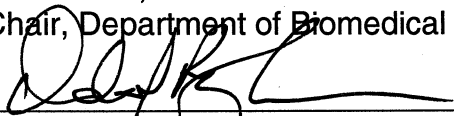Thesis Advisor

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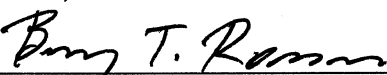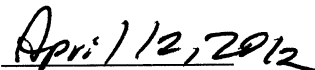
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ABSTRACT

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Title: GAD 65 and Its Role in Pancreatic Tissue Survival

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We employed three genotypes of GAD 65, wildtype (GAD 65 +/+), heterozygous (GAD 65 +/-) and knockout (GAD 65 -/-) to investigate the role of GAD 65 in survival of pancreatic islets. We analyzed the mRNA expression of pro-survival proteins including Bcl2 and Bax in pancreas of wildtype, heterozygous and knockout using Reverse Transcriptase Polymerase Chain Reaction (RTPCR). The level of expression of Bcl2 mRNA was down regulated in knockout mice pancreas and Bax to Bcl2 ratio was found higher in knockout mice pancreas suggesting higher cell death rate. However, further studies are required to recognize and understand the specific connections between apoptotic pathways and GAD 65 in pancreatic islets.

DEDICATION

This manuscript is dedicated to my late father who taught me the importance of education in life and I also want dedicate this to my Mom and brother who have been an unfailing support of my life.

GAD 65 AND ITS ROLE IN PANCREATIC TISSUE SURVIVAL

TABLES

FIGURES

INTRODUCTION

GAMMA-AMINOBUTYRIC ACID

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter and it is expressed in almost every part of the brain where it plays a major role in regulation of neuronal firing. GABA is also widely expressed in non-neuronal peripheral tissues including pancreas, gonads and spleen. GABA induced action in these tissues are not quite understood yet and should be further explored.Glutamic-acid decarboxylase (GAD) is the synthesis enzyme responsible for production of GABA from glutamate. Two different isoforms are present. GAD 65 and 67 which are named on the basis of their molecular weight (Bu *et al.* 1992). GAD 65 and GAD 67 are coded by two distinct genes GAD 65 and 67 are different from each other in several aspects such as amino acid sequence, antigenicity, cellular and sub-cellular location (Jin *et al.* 2003). It has been found that during early embryonic ages GAD 65 and GAD 67 both are present in cell bodies but later after birth they become localized to different part of the GABAnergic neuron (Tran *et al.* 2003). GAD 67 is widely distributed in the GABAnergic neuron where as GAD 65 is more localized to the axon terminals of these neurons. Many investigators have shown evidence indicating that GAD 65 and 67 both are involved with GABA synthesis but with different specific roles (Wei & Wu 2008). GAD 67 plays an important role during synaptogenensis. It is

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believed that it produces GABA which acts as a trophic factor whereas GAD 65 is more concentrated in axon terminals where it forms a complex with Vesicular GABA transporter (VGAT) which is itself important for efficient packaging and transportation of GABA through synaptic vesicles (Jin *et al.* 2003).Our lab has proposed a model explaining the role played by GAD 65 distinct from GAD 67 during the packaging and transportation of GABA into synaptic vesicles(Buddhala *et al.* 2009) (Fig. 1-1).
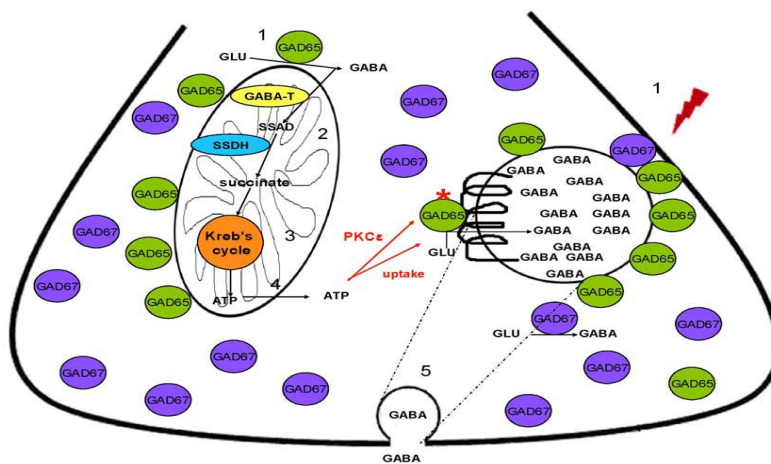
Figure 1-1 Proposed model of the role of Mitochondrial GAD 65 in providing energy to drive GABAnergic synaptic vesicles (Buddhala et al 2009)

GABA IS WIDELY DISTRIBUTED IN NON NEURONAL PERIPHERAL ORGANS

GABA distribution extends beyond just brain and neuronal tissues. It has been found that GABA is widely expressed in non-neuronal tissues of peripheral organs including pancreas, spleen, and gonads (Pleau *et al.* 1997). The functions performed by GABA in brain have been extensively investigated whereas there are still several questions that are unanswered in the case of the role of GABA in peripheral tissues. The presence of GABA in pancreas became an important

focus when it was shown that GAD 65 and 67 are expressed in different non neuronal peripheral tissues. It has been reported that in these peripheral tissues GABA is acting as a signaling molecule and affects several biochemical pathways in a regulatory manner. These findings have again put GAD and GABA system in spotlight and emphasized roles for GABA beyond neurotransmission.

GAD 65 AND 67 EXPRESSION VARIES IN HUMAN, RAT AND MOUSE

The GABAnergic neurons and pancreatic islets are two of the best known sites where GAD is highly expressed. Interestingly, the degree of expression of GAD 67 and 65 is quite variable between the different species like human, rat and mouse. Mouse Brain and pancreatic islets both express GAD 65 and GAD 67(Kim *et al.* 2004).If we take a look at the levels of expression of GAD 65 in pancreatic islets in Mouse, Rat and Human, it has been observed that GAD 65 is highly expressed in Rat and Human pancreatic islets where as mouse islets show several fold lower expression of the GAD 65 in their pancreatic islets. On the other hand expression of GAD 67 in pancreatic islets of mice predominates and in comparison to Human pancreatic islets or Rat islets it is very high. Recent studies have shown that GAD 67 is expressed in fetal pancreas during development and in adult pancreas (Korpershoek *et al.* 2007). In case of diabetes mellitus I, presence of autoantibodies against GAD 65 and GAD 67 have been a puzzle from more than three decades.

PANCREATIC ISLETS AND GLUCOSE HOMEOSTASIS

Glucose homeostasis in blood is maintained by the balance of opposing actions of insulin and glucagon. Both insulin and glucagon are secreted by pancreatic

islets but by different cells of the islets. Alpha cells secreting glucagon are more prevalent in the mantel area of islets and the beta cells of the islets which are responsible for production of insulin are concentrated in the core region of the islets (Steiner *et al.* 2010). High glucose levels in blood serum initiates the insulin secretion and insulin in turn acts as an activator for glucose transporters at cell membranes leading to absorption of glucose from the blood to muscle and adipose tissues. By contrast, glucagon acts in the opposite way, being secreted when the glucose level in the blood is low. The secretion of glucagon causes activation of all the processes and pathways responsible for glucose production including glycogenolysis, gluconeogenesis, lipolysis. When glucagon is high insulin levels are low and vice versa. The up-regulation of one hormone and the down regulation of the other with variations in glucose levels in blood represent a complex and tightly regulated system.

INSULIN BIPHASIC SECRETION

The biphasic secretion of insulin is glucose dependent where the first phase is rapid and essentially instant and second phase arrives late and is sustained for longer (Rorsman *et al.* 2000). When glucose level is high in blood the uptake of glucose through glucose transporters increases and breakdown of glucose molecules in glycolysis and Kreb's cycle leads to high production of ATP which leads to an elevated ATP/ADP ratio (Minami *et al.* 2003, Wiederkehr & Wollheim 2012).This causes closing of ATP dependent K+ channels resulting in depolarization of the beta cell membrane. Depolarization leads to opening of Voltage dependent Ca ++ Channels, and increased inflow of Calcium through

these channels triggers the exocytosis of insulin containing secretory granules (Jensen *et al.* 2008). Above mentioned pathway is a KATP dependent pathway but in addition to this it is believed that there is another KATP independent pathway that regulates late phase secretion of insulin (Kasai *et al.* 2010, Newgard *et al.* 2002) . The first proposal mentioning the involvement of KATP independent stimulation of insulin secretion came in 1988 where with sulfonylureas treatment glucose evoked the KATP-independent stimulation of insulin secretion .Further, using a knockout mouse models with ablation of either two functional subunits of KATP dependent channel the mice showed reduction in glucose stimulated insulin secretion (Kawamori *et al.* 2009, Liberman *et al.* 2011).

INSULIN AND GABA HAVE REGULATING EFFECT ON GLUCAGON

Following high level glucose in the blood and a subsequent exocytosis of insulin, this release of insulin exerts negative regulation on neighboring alpha cells suppressing the release of glucagon (Cooperberg & Cryer 2010). The mechanism behind this regulation is not very clear, but there is evidence showing that insulin exocytosis is accompanied by release of micro vesicles carrying GABA which interacts with the GABA receptor chloride channels present on the alpha cell membrane (Bonaventura *et al.* 2012, Rorsman et al. 2000). On the other hand insulin causes activation of the potassium channels on alpha cells. All these channels opening works to prevent depolarization of the membrane of alpha cells that is required for glucagon release. Hence, glucagon secretion is suppressed (Fahien & Macdonald 2011, Kawamori et al. 2009).

VIABILITY OF PANCREATIC CELLS AND ROLE OF GABA

It is known that GABA enhances cell proliferation in the developing nervous system and supports the survival of the neurons. With respect to the role of GABA in survival and protection of pancreatic cells it has been seen that GABA has a distinctively different effect on beta cells in comparison to its effect on neuronal cells where it initiates hyperpolarization. In beta cells GABA causes depolarization which leads to activation of PI3K/ AKT dependent pathways (Soltani *et al.* 2011). And the fact that these pathways play an important role in growth and supports the concept that GABA has protective influences on beta cells in terms of growth and survival. It has been shown that GABA can rescue the beta cells in diabetic mice and can reverse the state of hyperglycemia (Froestl 2011).

There are studies demonstrating the up-regulation by GABA of the anti-apoptotic protein Bcl-xl. These investigators emphasize the ability of GABA to suppress apoptosis and also act as a mitogen enhancing beta cell proliferation (Ligon *et al.* 2007).)

One the basis of the above information we propose GABA is a crucial component of Beta cell and alpha cell signaling in the pancreas. GAD 65 is a GABA producing enzyme and performs the important role in GABA synthesis and facilitating GABA packaging and transportation into synaptic vesicle. It was found that reducing the autoimmune response towards GAD 65 could increase the rate of survival of Beta cells grafts in diabetes prone mice (Tian *et al.* 1996). On the basis of above given details we propose that GAD 65 activity determines the

level of protection provided by GABA to pancreatic islets. To investigate this topic we employed the three different genotypes of GAD 65 mice GAD 65 Wildtype, GAD 65 Heterozygous and GAD 65 Knockouts.

HYPOTHESIS

GABA is an important inhibitor neurotransmitter and its expression in pancreatic islets other than brain is well known where GABA plays regulatory role among the signaling of islets cells and their survival. GABA is produced by enzyme GAD 65 using glutamate as substrate. We propose that activity of GAD 65 determines the level of protection provided by GABA to Pancreatic islets.

OBJECTIVE

To investigate the impairment in level of protection provided by GABA to pancreatic tissue in absence of GAD 65 in knockout mice by measuring level of prosurvival proteins including Bcl2 and Bax.

MATERIALS AND METHODS

PROTEIN EXTRACTION

Tissues were excised from mice after euthanizing. After harvesting, tissues were added immediately to lysis buffer using a ratio of 10 ml for 1gm tissue and homogenized. 5% Beta mercaptoethanol and protease inhibitor (1:100) were added in advance. Samples were then transferred to 1.5ml centrifuge tubes and set for incubation at 4 degree for 30 min on a shaker. After shaking samples were centrifuged at high speed (25000 rpm) for 30 min at 4 C and supernatants were collected in separated tube.Protein estimation was done using Bradford Assay. Protein loading dye was added in the end. βeta-Mercaptoethanol was added in Loading dye right before its addition to the protein sample. Samples were stored at -20 degree.

IMMUNOBLOTTING

GEL PREPARATION FOR SDS PAGE

For a set of four Resolving Gels 8 ml of 40 % Bis Acrylamide, 5 ml of Resolving gel buffer, 6.8 ml of autoclaved DI water, 0.2 ml of 10% sodium dodecyle sulfate were dissolved in a 50 ml falcon tube and 80 ul of 10% of Ammonium persulfate and 30 ul of TEMED were added right before pouring the gels to set. Stacking gel was prepared using 1 ml of 40% Bis Acrylamide, 6 of dH2O , 2.5 of stacking gel buffer , 100ul of 10% SDS , 50ul of 10% Ammonium per sulfate, 10ul of TEMED.

ELECTROPHORESIS - SDS-PAGE

Gel electrophoresis apparatus was filled with SDS Glycine buffer and prepared gels were placed in gel holder apparatus. The inner compartment (between the gels) was filled with freshly made SDS glycine buffer. Each well was loaded with 35 ul protein samples using a 200P pipette and gel apparatus was run at 200 V to separate protein on the gel.

TRANSFER

In order to make the target proteins accessible to antibody binding they were transferred from gel to nitrocellulose membrane.The Gels were placed on Nitrocellulose Membrane and then sandwiched between two filter papers of same size and they were further padded with sponges from both sides. This arrangement was then transferred to a transfer tank filled with Transfer buffer and transfer was run for 1.5 hour at 30V.After transfer the nitrocellulose membranes were placed in separate box and rinsed with blocking buffer 3 to 4 times.

BLOCKING

To avoid non specific binding during antibody treatment the Nitrocellulose membrane was kept in the box with 4 ml of blocking buffer containing 5% of milk for 2 Hours. After 2 Hrs of blocking the blocking buffer was discarded and the membrane was washed twice with blocking buffer.

PRIMARY ANTIBODY TREATMENT

4 ml of blocking buffer containing the primary antibody was added to the box

containing the membrane. The ratio of Primary antibody to buffer was 1:1000. The membrane was incubated in primary antibody for 8 Hrs of incubation at 4 Celsius on a shaker. After 8 Hrs the primary antibody was discarded and the membrane was set to wash for 30 minutes, Then 15 minutes and then15 minutes using blocking buffer.

SECONDARY ANTIBODY TREATMENT

Secondary antibody was added with blocking buffer to the membrane. The ratio used was 1:50,000 and membranes were left for incubation on a shaker for 2 hrs. Membrane was wash for 15 minutes, then10 minutes and then10 minutes using blocking buffer.

DETECTION (ALL STEPS WERE PERFORMED IN DARK)

In a 5 ml tube 1 ml Substrate A was added with 1 ml of substrate B. Then this mixture was poured onto the membrane.After 2 minutes of incubation the membrane was dried and was then covered with plastic wrap.The membrane was then taped in a cassette against a photographic film and exposed for 30 seconds. The target protein was tracked by matching the film image to a ladder present on membrane.

SEMI-QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE REACTION CHAIN REACTION (RTPCR)

RNA ISOLATION

After harvesting, tissues were placed immediately into stabilization buffer. 10 ul of buffer was used for 1 mg of tissue. Tissues were then added to RLT buffer.

1000 ul of RLT was added for each brain sample and 250 ul for each pancreas sample. Tissues were homogenized and transferred into RNAase safe centrifuge tubes. After homogenization samples were centrifuged for 30 minutes at a high speed (25000 rpm) at 4 degree. Supernatants were pipette into separate tubes and an equal amount of 70 percent alcohol was added. 700 ul of each sample were then transferred into separate RNAeasy mini columns with collecting tubes.Samples were centrifuged for 30 seconds at 10,000 rpm speed and the flow through was discarded and collecting tubes were put in place. 700 ul of RW1 buffer were added to each RNA easy mini columns and columns were centrifuged for 30 seconds on 10,000 rpm. The flow through were discarded and collecting tubes were put in place. 500 ul of RPE buffer were added to each RNA easy mini columns and columns were centrifuged for 30 seconds on 10,000 rpm. The flow through were discarded and collecting tubes were put in place and last Step was repeated. Again flow through were discarded and collecting tubes were replaced by new collecting tubes.To each RNA easy mini column 30 ul of DNAase RNA ase free water was added and columns were centrifuged at 10,000 rpm for 1 minute. Flow through was collected in PCR safe tubes. Samples were always kept on ice. RNA quantification was carried out using spectrophotometer.

REVERSE TRANSCRIPTION (RT)

Samples were normalized on the basis of measurements results from spectrophotometer readings and diluted with RNAase DNAase free water as needed. In a 0.2 ml PCR tube 5 ul of diluted RNA sample was taken and combined with: 1 ul of Oligo(dT)20 Primer,1 ul of annealing buffer and 1 ul of

RNAase/ DNAase water. Samples were incubated in thermal cycler for 5 minutes at 65 degree Celsius. After incubation samples were placed immediately on ice. 10 ul of 2X First Strand Reaction Mix were added to each tube and 2 ul of Superscript III/RNAase OUT Enzyme mix. Samples were incubated in thermal cycler as follows: 50 mins at 50 degree Celsius, 85 degree Celsius for 5 minutes. Terminate the reactions at 4 degree Celsius.RT products (cDNA) were stored at - 20 C.

Gene Name	Primer Sequence (5'-3')	PCR Product Length (bp)
GAD 65	F: GCTCATCGCGTTCACATCAG R: AGTAACCCTCCACCCCAAGC	300
GAD 67	F: GGCTGATTACCTCTACGCCAAG R: CCTGACCCAACCTCTCTATCTCC	320
BCL2	F: TGGGATGCCTTTGTGGAACT R: GAGACAGCCAGGAGAAATCA	67
BAX	F: CCA CCAGCTCTGAACAGATC R: CAGCTTCTTGGTGGAGGCAT	140
GD3PH	F: TCCATGACAACTTTGGCATCGTGG R: GTTGCTGTTGAAGTCACAGGAGAC	366

Table 2.1 List of primers with sequences and product size

POLYMERASE CHAIN REACTION (PCR)

In a PCR tubes (0.5 ml) 3ul of a sample (cDNA) was added and combined with 1 ul of Forward Primer, 1 ul of Reverse Primer and 45 ul of PCR supermix PCR reaction was run as described later.

AGAROSE GEL ELECTROPHORESIS

All PCR samples were run using agarose gel. 2% of agarose was added in tris/borate/EDTA buffer and was kept in water bath till completely dissolved. 2 ul Ethidium bromide was added in 100ml of gel. 10 ul of a PCR sample was added in each well and gel was run at 95 volt.

GENOTYPING

DNA EXTRACTION

The mouse tail was snipped under the hood. Hood, gloves and scissors were wiped with ethanol. The mouse tail was snipped using autoclaved scissors and after every tails snipping scissors were wiped using 70% ethanol. Tail piece was kept in autoclaved 1.5 ml centrifuge tube on ice and was then chopped with help of autoclaved scissors and 0.5 ml of DNA extraction buffer was added. Tubes were vortexed for 15 seconds. All tubes containing the chopped tail in DNA extraction buffer were then placed in water bath at 65 C for 30 minute. After 30 minutes tubes were vortexed for 15 seconds and transferred to boiling water bath for 8 minutes. Tubes were vortexed for 15 seconds. Tubes were immediately transferred on ice. Tubes were centrifuged for 3 minutes on 13,000 rpm and supernatant was collected which was the containing the genomic DNA. Tubes were stored at -20 C. To precede Polymerase chain reaction PCR master mix was prepared using following combination for each sample. 10 uM G65S3- 2 ul , 10 uM G65A3- 4ul ,10 uM RneO3- 2 ul, Fail safe PCR mix- 25 ul and Autoclave Nano water- 11.5.Total 44.5 ul of Master Mix.The PCR reaction mix was prepared adding 5 ul of sample to 44.5 ul of Master Mix. 0.5 ul of Polymerase enzyme was added to each tube. All steps were performed on ice. PCR was run

using following using preset program.

GLUCOSE ASSAY

Blood was collected after 8 hrs fasting. Collected blood samples were kept for clotting at ambient temperature of 24 C in an incubator and then centrifuged for 10 minutes at speed of 13,000 RPM. Supernatant (serum) was collected using pipette and was stored at – 80 degree Celsius.

Blood was collected after 8 hrs fasting. Collected blood samples were kept for clotting at ambient temperature of 24 C in an incubator and then centrifuged for 10 minutes at speed of 13,000 RPM. Supernatant (serum) was collected using pipette and was stored at – 80 degree Celsius

STANDARD CURVE PREPARATIONS

Standard was diluted to 1 nmol/μl by adding 10 μl of the Glucose Standard to 990 μl of Glucose Assay Buffer mixed well. 0, 2, 4, 6, 8, 10 μl was added into a series of wells of a 96 well plate. Volume was adjusted of all wells to 50 μl with Glucose Assay Buffer to generate 0, 2,4, 6, 8, 10 nmol/well of Glucose Standard.

SAMPLE PREPARATIONS

Serum samples were prepared in 50 μl/well with Glucose Assay Buffer in a

96-well plate. Serum 0.5 μl/assay was used.

GLUCOSE REACTION MIXTURE

 A total 50 μl Reaction Mixture was prepared for each well containing:

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46 µl Glucose Assay Buffer

2 µl Glucose Enzyme Mix

2 µl Glucose Substrate Mix

Reaction was incubated for 30 minute with protection from light. Absorbance was measured at 450 nm in a micro-plate reader.

CALCULATIONS

To correct for background all readings were subtracted by the value derived from the 0 glucose control . Standard curve was plotted using readings of standard glucose samples. Sample readings were applied to the standard curve. Glucose concentrations of the test samples were calculated using following formula:

C = Sa/Sv   (nmol/µl or µmol/ml, or mM)

Where: Sa is sample amount (in nmol) calculated from standard curve.

Sv is sample volume (in µl) added into the sample wells.Glucose Molecular Weight 180.16.

REAGENTS

5X Glycine Running Buffer

108 g of Tris base , 94 g of Glycine, 50 ml of 10% Sodium dodecyle Sulfate was addedto 700 ml of water and after solid was dissolved , volume was adjusted to 1000 ml.

1X Transfer Buffer

1.5 gm of Tris, 7.21 g of Glycine and 200ml of 20% of Methanol was dissolved in DI water and volume was adjusted to 1000 ml with DI water.

Blocking Buffer

2.42g Tris, 8g NaCl was dissolved in 1000 ml of DI water and pH was adjusted to 7.6. And 0.1 % Tween was added right before use in an aliquot.

TBE 10 X Buffer

In 700 ml DI water 40 ml of 0.5M EDTA (pH 8.0), 55 g of Boric acid and 108 g of Tris Base was dissolved and then volume was adjusted to 1000 ml. An aliquot was diluted to 1X just before use.

Stacking gel Buffer

0.5 M Tris HCl buffer was prepared by adding 6.06 g Tris Base in 100 ml DI water and pH was adjusted to 6.8.

Resolving Buffer

M Tris HCl buffer was prepared by adding 18.16 g in 100 ml of DI water and pH was adjusted to 8.8.

DATA ANALYSIS

All results are presented as means ± SE. Statistical analysis of all the data was performed using PRISM 5 and SPSS (IBM) Statistical software. For determining the statistic significance one way ANOVA, posthoc tukey and dunnett were used.

Statistical significance was set at P < 0.05.

RESULTS

GENOTYPING

We performed PCR using extracted DNA samples from different mice after they were weaned at the age of two weeks. The genotype of each mouse was known on the basis of results from DNA gel analysis. The samples with two bands were heterozygous, the samples with upper band only were wildtype and the samples with lower band only were the knockouts (Fig 3-1). The previous samples with known genotypes were used as controls (fig. 3-2).

EXPRESSION OF GAD 67 MRNA IN PANCREATIC TISSUE OF MICE

We investigated the expression of GAD 67 protein in pancreas of our different genotype. Total mRNA was extracted from pancreas of each genotype and reverse transcribed to cDNA. PCR was run probing GAD 67 with specific primer. We found that GAD 67 expression was normal in pancreas of wildtype, hetrozygous and knockout mice (fig. 3-3).

EXPRESSION OF GAD 65 MRNA IN PANCREATIC TISSUE OF MICE

We targeted to look for the expression of GAD 65 in pancreas of all the three genotypes. Our results showed normal expression of GAD 65 in pancreas of our wildtype where as expression GAD 65 was lower in pancreas of hetrozygous mice (fig 3-4). The Gad 65 expression was absent in knockout mice and if there was a vague band present that may be non functional mRNA as our western

result have shown that there is no GAD 65 protein is present in knockout mice.

WESTERN BLOTTING TARGETING GAD 65 AND 67 RESULTS

Brain tissues were harvested from the mice and immediately protein was isolated using the method explained in method section. After isolation the protein was estimated and samples were normalized for loading the same amount of protein in each. After performing westerns the results showed that expression of GAD 65 was normal in wildtype mice and was suppressed in heterozygous mice. In knockout mice GAD 65 expression was completely absent. On the other side GAD 67 expression was unaltered in the three different genotypes showing same level (fig. 3-6).

PRESENCE OF GAD 65 IN PERIPHERAL TISSUES

We isolated the protein from different organ tissues like pancreas, adrenal, testis including brain and Western blotting was performed using these samples to investigate the presence of GAD 65 in these tissues and our results showed that GAD 65 is expressed in these tissues with different levels of expression. Brain tissues were harvested from the mice and immediately protein was isolated using the method explained in method section. After isolation the protein was estimated and samples were normalized for loading the same amount of protein in each. After performing westerns the results showed that expression of GAD 65 was normal in wildtype mice and was suppressed in heterozygous mice. In knockout mice GAD 65 expression was completely absent. On the other side GAD 67 expression was unaltered in the three different genotypes showing same level (fig. 3-5).

The mice were divided in two groups on the basis of age where group 1 was including mice aged 8 to 10 weeks and group 2 was including mice aged 10 months.

BCL2 MRNA EXPRESSION IN BRAIN OF GROUP 1

We extracted total mRNA from brain of all the three genotypes and carried out RTPCR using specific primers probing Bcl2. PCR products were detected using agarose gel electrophoresis. The mRNA expression of Bcl2 was not greatly altered in the brains of all the three genotypes (fig 3-7).

EXPRESSION OF BCL2 MRNA IN PANCREAS OF GROUP 1

We also performed RT-PCR to investigate the expression of Bcl2 in the pancreas of the different genotypes of mice at the age of 8 to10 weeks (group 1). Our results show that the expression Bcl2 is significantly lower in knockout mice in comparison to wildtype (fig 3-8). The expression in heterozygous mice is also lower than wildtype but the difference was not significant.

EXPRESSION OF BAX OF GROUP 1

Bax mRNA expression in brain of group 1 mice was found unaltered in all there genotypes (Fig. 3-9). Bax mRNA expression level was also investigated in the pancreas of all three genotypes in group 1 and we found that the expression of Bax was similar among the three genotypes (fig. 3-10).

EXPRESSION OF BAX MRNA IN PANCREAS OF GROUP 1

RT was performed using the total mRNA extracted from pancreas of all the three genotypes and the products were used to run PCR probing for Bax using specific

primers. The results showed not any significant difference in the mRNA expression of Bax in all the three genotypes of Group 1 mice (fig.3-12).

RATIO OF BAX/BCL2 IN PANCREAS OF GROUP 1

Bcl2 mRNA expression levels in pancreas of all the three genotypes were densitometric analyzed and ratio of Bax expression to Bcl2 expression was calculated for each genotype (fig 3-11). The result shows that Bax/Bcl2 ratio is significantly higher in knockout mice than Bax/Bcl2 ratio of wildtype.

BCL2 EXPRESSION IN GROUP 2

Bcl2 expression in group 2 did not follow the same pattern as group 1 which raises questions regarding the effect of age on levels of expression of pro-survival proteins. The brain of all the different genotype mice again was showed no significant difference (fig.3-12). It was shown that the there was significant lower expression of Bcl2 in pancreas of Wildtype mice in comparison to both heterozygous and Wildtype mice. If we compare just heterozygous to knockout there was increased expression of Bcl2 in heterozygous mice (fig 3-13). It suggests that brain has a rescue mechanism to enable it to function in the absence of GAD 65.

LEVEL OF GLUCOSE IN SERUM OF THE THREE GENOTYPES

The level of glucose was measured in the three different genotypes of GAD 65 wildtype, heterozygous and knockout after 8 hrs fasting (fig 3-14). The results of the glucose assay performed using the collected serum from these genotypes showed no difference in the levels of glucose in the serum of all three genotypes
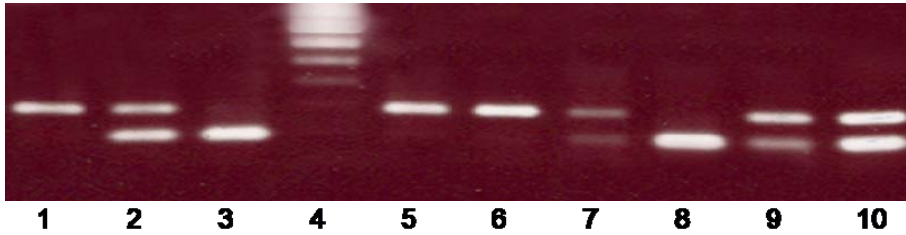
fell in the normal range of glucose levels.

Figure 3-1 Genotyping results 1. PCR products detected on an agarose gel. Lane 1, 2 and 3 are controls for wildtype, heterozygous and knockout for GAD 65. Lane 4 is a 50 bp ladder. Lane 5 and 6 are representing wildtype mice. Lane 7, 9 and 10 are showing hetrozygous mice. Lane 8 is showing a knockout mouse.
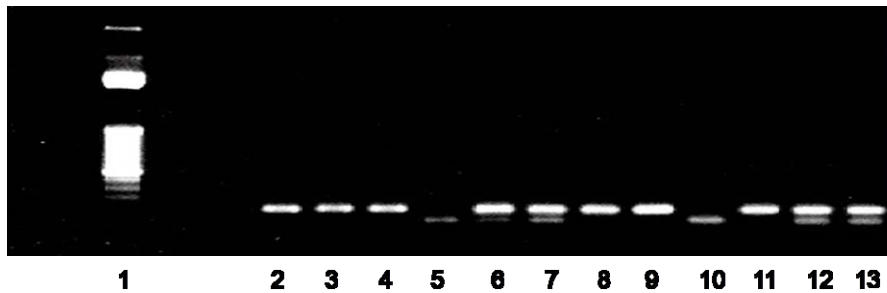

Figure 3-2 Genotyping results 2. PCR products detected on an agarose gel. Lane 1 is representing the 50 bp ladder. Lane 2, 3,4,8,9 and 11 are showing wildtype mice. Lane 5 and 10 are showing knockouts and 6,7,12 and 13 are showing heterozygous mice.

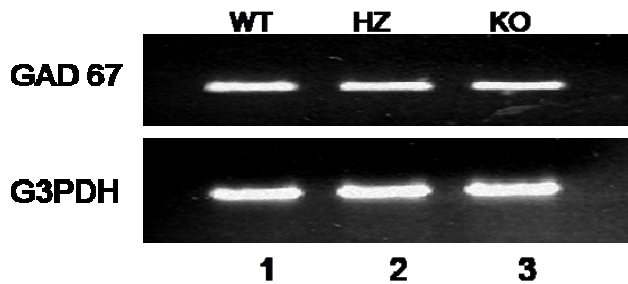**GAD 67 mRNA expression in pancreas of Group 1**

Figure 3-3 mRNA expression of GAD 67 in pancreas of group 1. Total mRNA was isolated from pancreas each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. Lane 1 is showing the expression of GAD 67 in pancreas of wildtype mice. Lane 2 and 3 are showing expression of GAD 67 in pancreas of heterozygous and knockout mice respectively. Lane 4, 5 and 6 represents control GD3PH expression in each genotype wildtype , hetrozygous and knockout respectively.
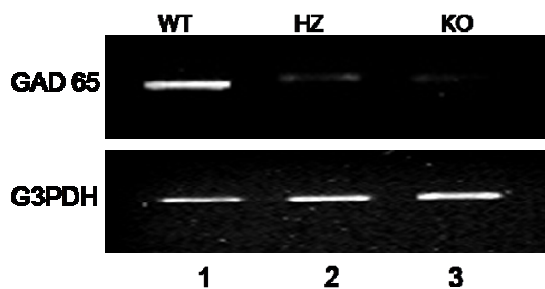

Figure 3-4 mRNA expression profiles of GAD 65 in pancreas of group1. Total mRNA was isolated from pancreas each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. Lane 1 represents

expression of GAD 65 in wildtype mice pancreas lane 2 and 3 represents expression of GAD 65 in heterozygous and knockout respectively. Lane 4, 5 and 6 are representing controls (GD3PH) for wildtype , hetrozygous and knockout respectively.
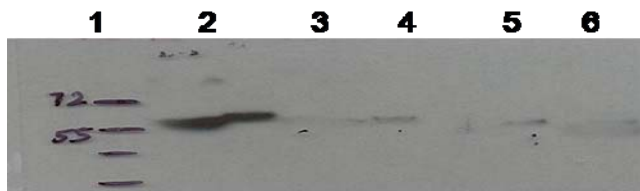
Figure 3-5 Expression of GAD 65 in peripheral organs. This western blot analysis shows Lane 1 is protein ladder and 2nd lane is showing expression of GAD 65 in brain tissue. 3rd lane, 4th and 5th are showing expression GAD 65 in pancreas, testis and adrenal respectively.

Figure 3-6 Expression of GAD 65 and 67 in brain of group 1. Western Blotting analysis of GAD 65 and 67 showed Lane 1Wildtype where expression of GAD 65 and 67 was present. Lane 2 shows heterozygous mouse and it was found that the expression of GAD 65 was reduced in comparison to Wildtype. Lane 3 we used Knockout which showed no expression of GAD 65. Expression GAD 67 in all the three genotypes was same.

(a)

WT   HZ   KO

Bcl2

GD3PH

1   2   3

(b)

Bcl2 mRNA expression in brain of Group 1

N=3

% Expression (Relative to Control)

Genotypes of GAD 65

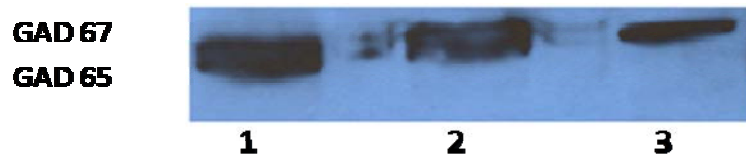Figure 3-7 Bcl2 mRNA expression in brain of group 1(a) mRNA expression profiles of Bcl2 and GD3PH.Total mRNA was isolated from brain of each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. (b) 1, 2 and 3 are densitometric analysis of Bcl2 mRNA expression in brain of wildtype , heterozygous and knockout. The data shows the expression of Bcl2 is unaltered in the absence of GAD 65.

(a)

WT HZ KO

Bcl2

GD3PH

1 2 3

(b)

Bcl2 mRNA expression in pancreas of Group 1

N=8

P= 0.03

Genotypes of GAD 65

Figure 3-8 mRNA expression profiles of Bcl2 and GD3PH in pancreas.(a) Total mRNA was isolated from pancreas each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. Lane 3 shows mRNA expression of Bcl2 in pancreas of knockout Lane 1and 2 shows mRNA expression of Bcl2 in pancreas of wildtpye and heterozygous. (b) 1, 2 and 3 shows densitometric analysis of Bcl2 mRNA expression in pancreas of wildtype, heterozygous and knockout, respectively. *P < 0.03 (N=8)
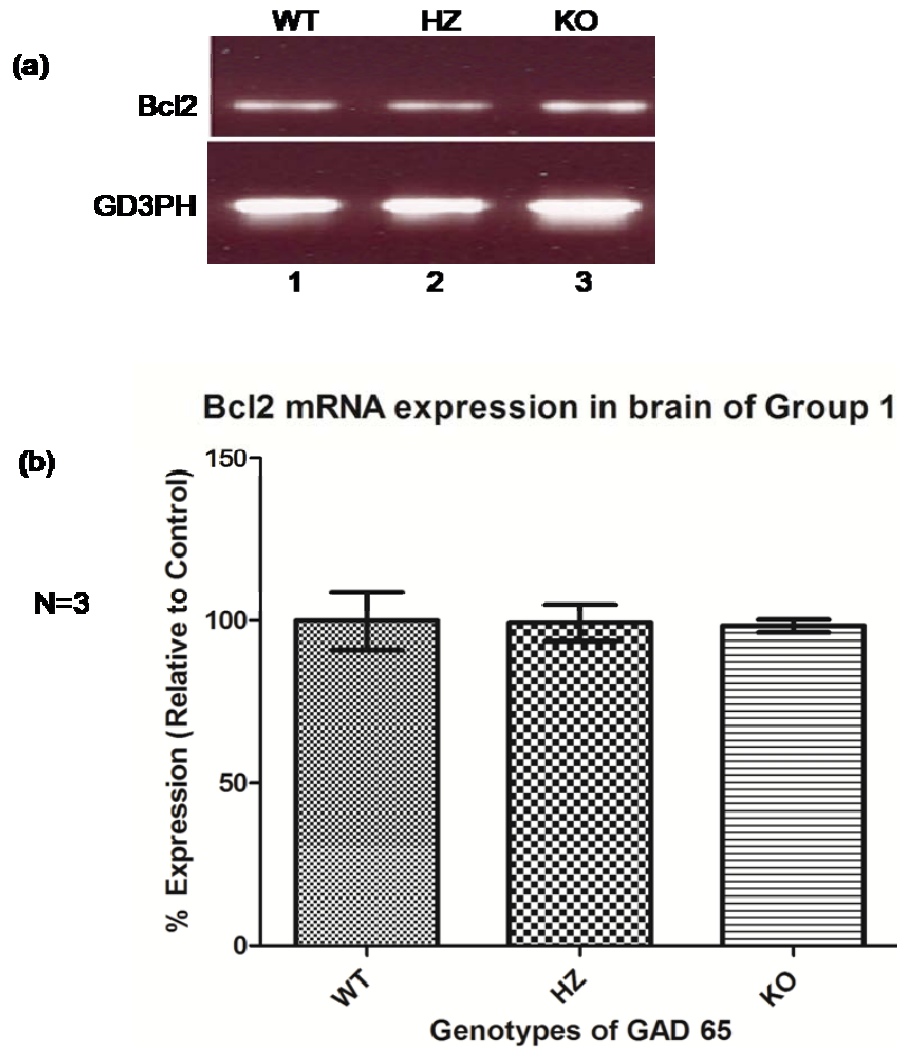
26

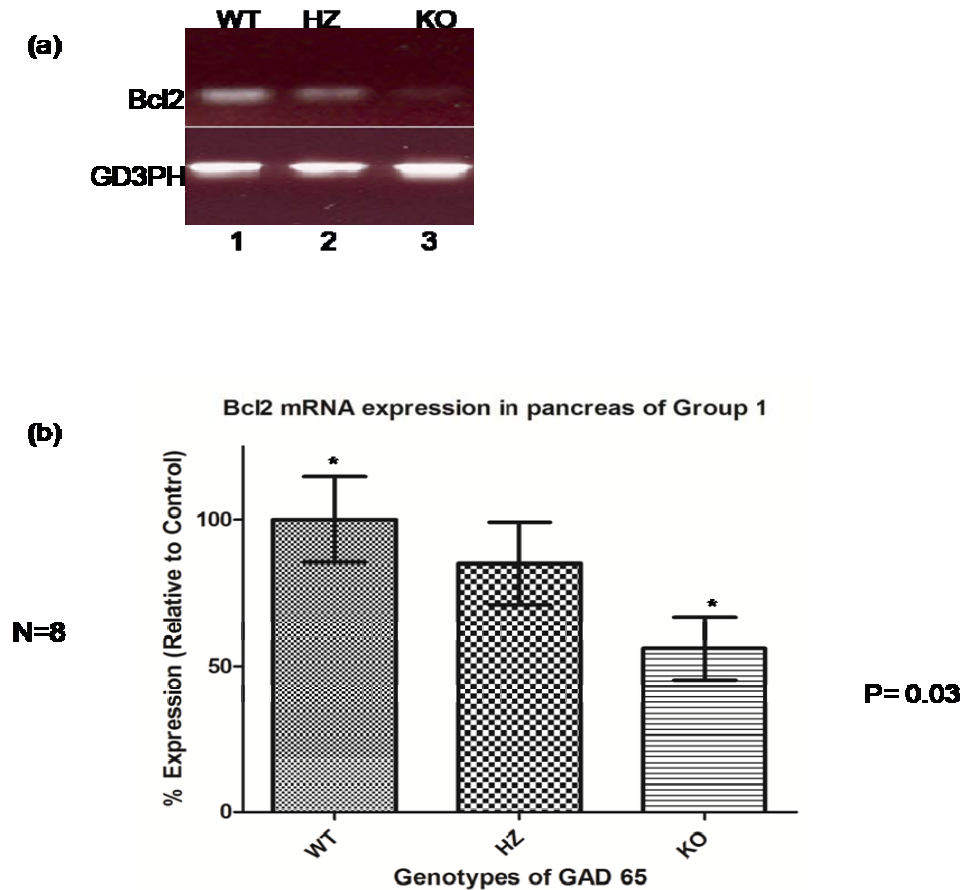Figure 3-9 Bax mRNA expression in brain of group 1 (a) mRNA expression profiles of Bax and GD3PH in brain. Total mRNA was isolated from brain of each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. Lane 1, 2 and 3 shows mRNA expression of Bax in brain of wildtype, heterozygous and knockout, respectively.(b) 1, 2 and 3 shows densitometric analysis of Bax mRNA expression in brain of wildtype , heterozygous and knockout , respectively.

(a)

WT   HZ   KO

Bax

G3DPH

1   2   3

(b)

Bax mRNA expression in pancreas of Group 1

N=8

% Expression (Relative to Control)
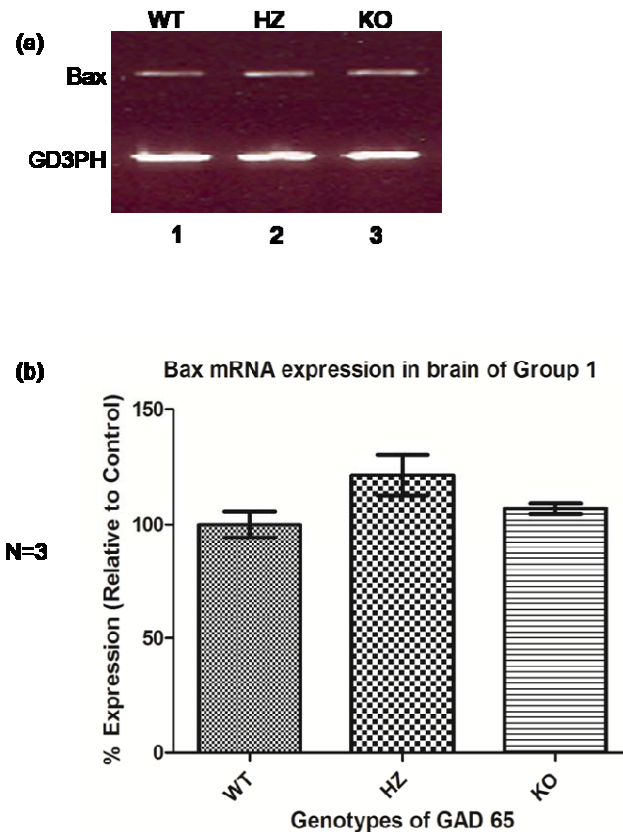
100

50

0

WT   HZ   KO

Genotypes of GAD 65

Figure 3-10 Bax mRNA expression in pancreas of group 1 (a) mRNA expression profiles of Bax and GD3PH in pancreas. Total mRNA was isolated from brain of each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. Lane 1, 2 and 3 shows mRNA expression of Bax in brain of wildtype, heterozygous and knockout, respectively. (b) 1, 2 and 3 shows densitometric analysis of Bax mRNA expression in brain of wildtype , heterozygous and knockout , respectively.
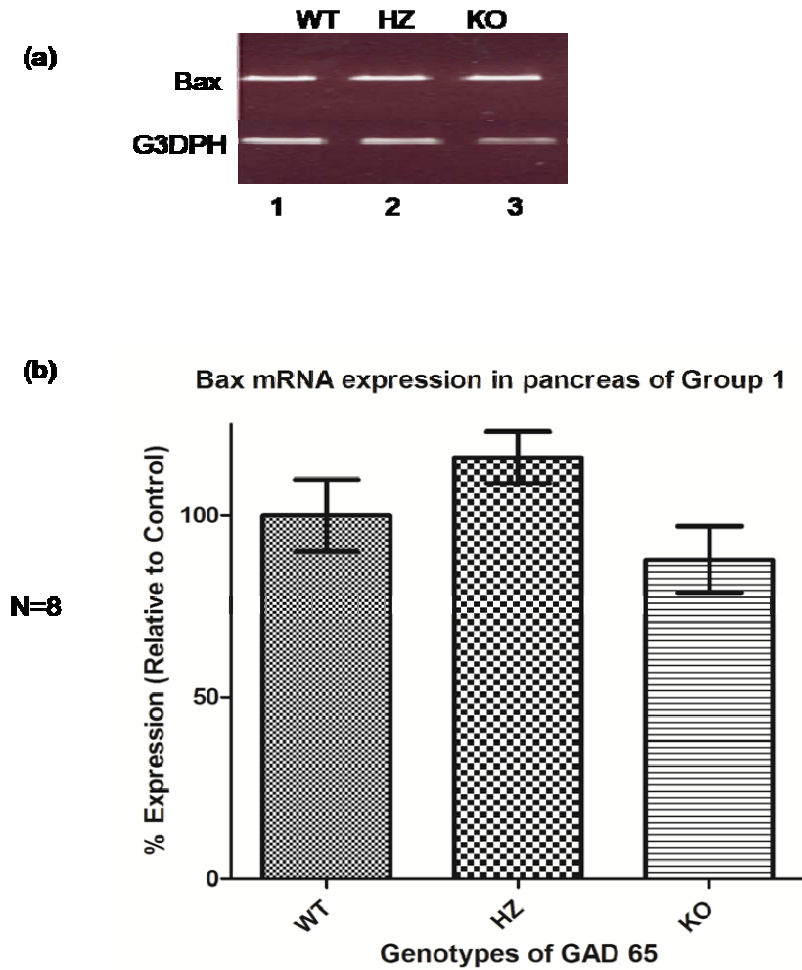
Bax to Bcl2 mRNA expression ratio in pancreas of Group 1

N=5

P= 0.002

Figure 3-11Ratio of Bax to Bcl2 mRNA expression in pancreas of group 1. 1, 2 and 3 are representing the densitometric analysis of ratio in pancreas of level of mRNA expression of Bax to Bcl2 in group 1 mice. The lane 3 is representing the Bax/Bcl2 ratio in knockout mice and it can be seen that the ratio is much higher in comparison to lane 1 showing the ratio of Bax/Bcl2 in Wildtype. Lane 2 is for heterozygous which is lower than the lane 3.*P< 0.002 (N=5)

(a)

(b)

N=3
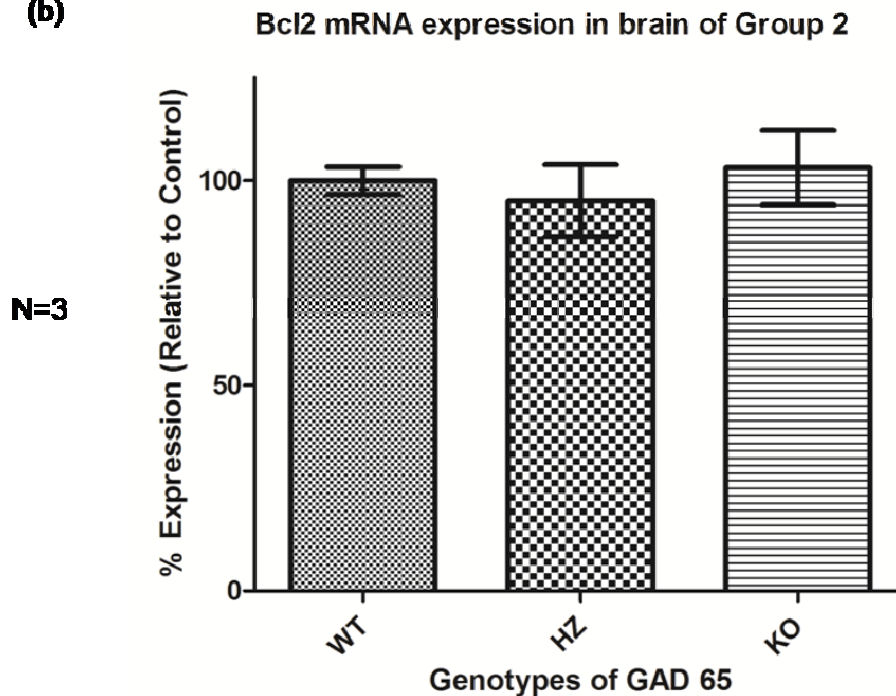
Figure 3-12 Bcl2 mRNA expression of brain of group 2 (a) mRNA expression profiles of Bcl2 and GD3PH in brain of Group 2. Total mRNA was isolated from brain of each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. Lane 1, 2 and 3 shows Bcl2 mRNA expression in brain of wildtype, heterozygous and knockout mice respectively. (b) 1, 2 and 3 shows densitometric analysis of mRNA expression of Bcl2 in brain of wildtype,

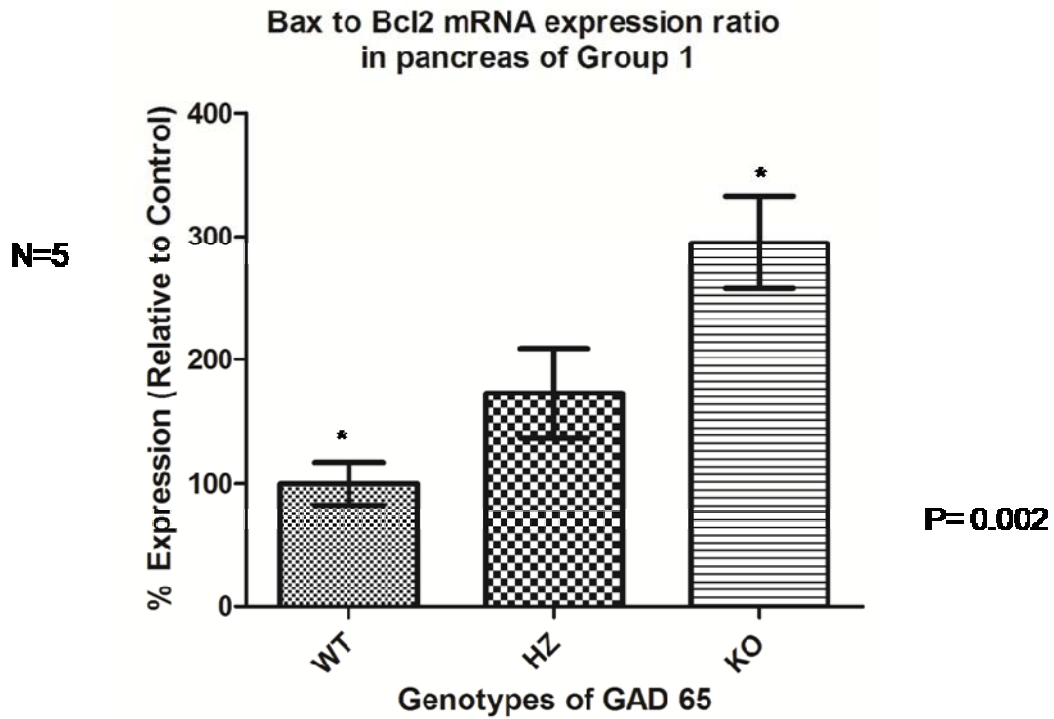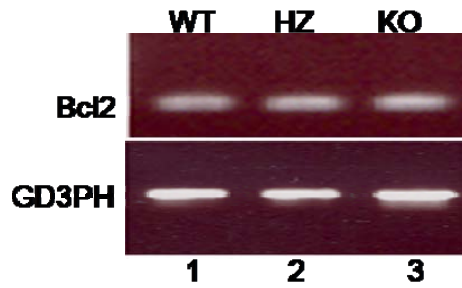heterozygous and knockout mice respectively.
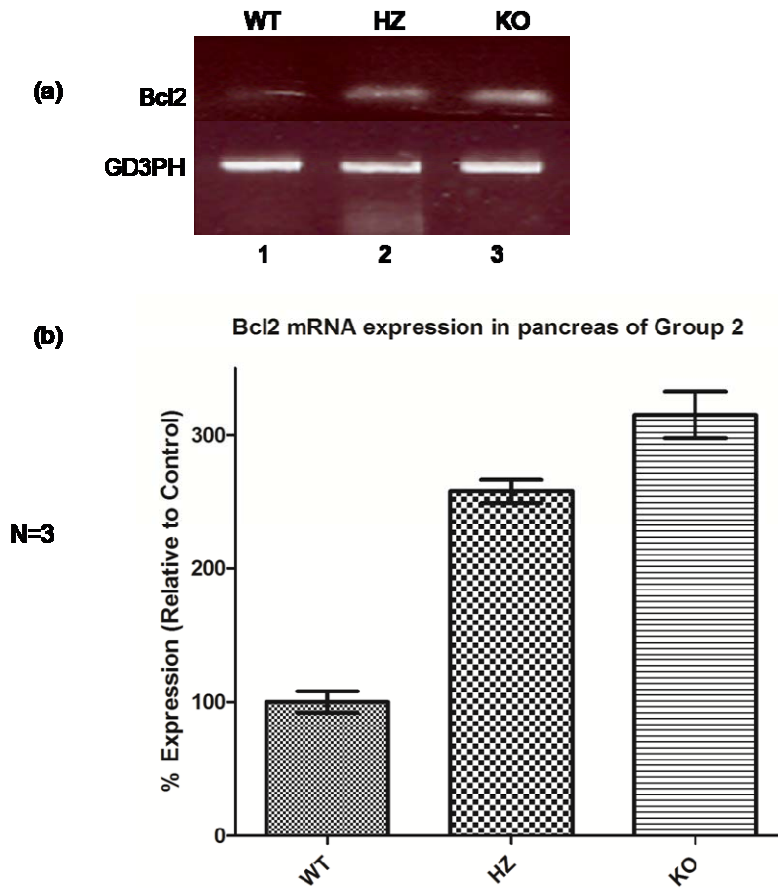
Figure 3-13 Bcl2 mRNA expression in pancreas of group 22 (a) mRNA expression profiles of Bcl2 and GD3PH in pancreas .Total mRNA was isolated from pancreas each genotype and was subjected to RT-PCR with gene specific primers. PCR products were analyzed by DNA gel electrophoresis and stained with Ethidium Bromide. Lane 1, 2 and 3 shows expression of Bcl2 mRNA in pancrease of Wildtype, heterozygous and knockout respectively. (b) 1, 2 and 3 shows densitometric analysis of Bcl2 mRNA expression in pancreas of wildtype, heterozygous and knockout respectively.

Glucose level in serum after 8 hrs fasting
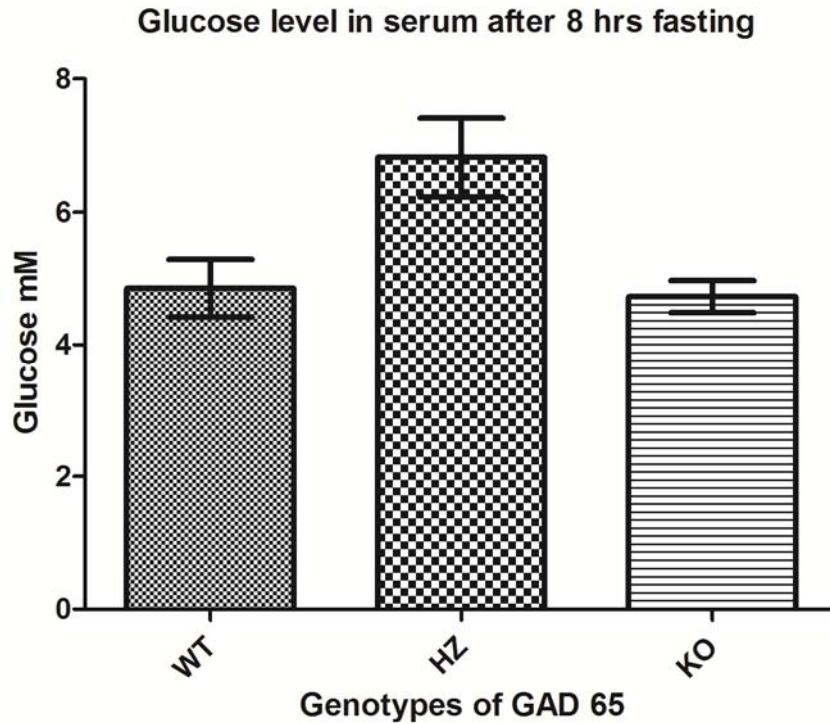
Figure 3-14 Glucose level in serum of group 1 after 8 hours of fasting Representing glucose assays performed using the serum collected from all three different genotypes after 8 hrs of fasting. The results from the glucose assays suggests that the level of glucose among the three genotypes is not different as shown lane 1 (wildtype mice), lane 2 (heterozygous) and lane 3 (knockout mice). The glucose level of all the three genotypes wildtype, heterozygous and knockout fell within the normal range of serum glucose levels which are from 4 to 7 mM. (N=8)

DISCUSSION

We investigated the role of GAD 65 in protection provided by GABA to pancreatic islets. It was known that glucose stimulates insulin secretion in beta cells but blocking GABA A receptors leads to reduced secretion of insulin from beta cells (Wendt *et al.* 2004). Interestingly, in diabetic mice the treatment with GABA increased cell replication in beta cells and decreased apoptosis, resulting increased mass of beta cells and reversal of hyperglycemia (Froestl 2011). Therapy with GABA causes longer survival of transplanted beta cells in diabetic mice by decreasing inflammatory responses and enhancing regulatory T cells.

 We are investigating whether ablation of GAD 65 gene in knockout mice may lead to an impaired GABA system resulting in less protection for pancreatic islets. Several previous studies have shown the presence of the anti gad 65 antibodies in patients with Diabetes mellitus type 1(Davison *et al.* 2008, Wherrett *et al.* 2011, Yamamoto *et al.* 2004). It was recently shown that GAD 65 gene therapy can preserve the functional ability of Beta cells in the patients with recent onset of Diabetes mellitus type 1(Agardh *et al.* 2005, Axelsson *et al.* 2011, Tilz *et al.* 2011). The complete role of GAD 65 in pancreatic islets has been a mystery for some time but several studies point towards its importance in survival of beta cells of pancreatic islets (Pleau *et al.* 1997, Tian *et al.* 2011).

 The glucose homeostasis in human body is based on the regularity and

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coordination of the two hormones- insulin produced by beta cells and glucagon produced by alpha cells in pancreatic islets. Beta cell destruction leads to high levels of glucose in the circulation which further causes more injury to beta cells (Cnop *et al.* 2005, Holst *et al.* 2008). Glucose contributes proactively for further beta cells destruction. Hyperglycemic conditions stimulate iNOS and ncNOS activity leading to NO mediated beta cell dysfunction and destruction (Kadri *et al.* 2012).

CD8+ cytotoxic T cells are dominant contributors to beta cell destruction. It has been reported that CD8+ Cytotoxic T cell clones isolated from NOD mice can lead to development of Diabetes Mellitus type 1(Kim *et al.* 2004, Lee *et al.* 2004). The perforins and granzymes secreted by CD8+ T cells play important roles in beta cell destruction and in the absence of these molecules Beta cell destruction is reduced significantly (Thomas & Kay 2011). Fas ligand receptor is not normally expressed in beta cells but its expression becomes at the time of appearance of hyperglycemia in system.

 Cellular stress is another factor which can contribute to beta cells death (Soltani et al. 2011). Hyperglycemia is responsible for stimulation of several stress pathways in islets including those associated with oxidative stress. Beta cells express fewer antioxidant enzymes by comparison to other peripheral tissues in the human body which makes them a vulnerable target for oxidative stress. Our findings show that knockout mice of Group 1 (Age 8 to 10 weeks) have higher level of Bax/Bcl2 ratio by comparison to Wildtype mice which show normal levels of expression of GAD 65. The increase of the Bax/Bcl2 ratio is known for up-

regulation of caspase 3 eliciting leads to enhanced apoptosis (Ligon *et al.* 2007). The present study suggests that GAD 65 is an important factor in determining the level of protection provided by GABA to pancreatic islets.

The current study was done on intact pancreatic tissue but isolating islets from pancreatic tissues could provide a more precise analysis of involvement of GAD 65 in pathways of beta cells survival. However, the isolation of pancreatic islets results in only a moderate recovery of viable cells. Also, with time isolated islets have shown enhanced activation of inflammatory pathways. Hence, the effect of isolation and culture on beta cells is only partially understood and data on this system may be difficult to interpret.

Glucose homeostasis in blood is maintained by the coordination of two hormones glucagon and insulin. Insulin is released by beta cells when the level glucose is high in blood. Insulin causes activation of glucose transporters which in turn enhance the absorption of glucose from the blood to muscles and adipose tissues. Glucagon is released by alpha cells of islets when the glucose level is lower in blood and results in activation of the glucose producing pathways. Substantial evidence suggests that loss of beta cells could lead to elevated blood glucose.

We have also investigated the level of glucose in serum of different GAD 65 genotypes of mice Wildtype, heterozygous and knockout. We found that there was no significant difference in glucose level for these different genotypes and their glucose levels fell within the normal range of blood glucose. However, a

glucose tolerance test may be needed to study the ability of knockout mice to maintain physiological blood glucose regulation. This test may further provide details of efficiency of insulin sensitivity and regulation in the absence of GAD 65.

We have also studied the expression of Bcl2 in brain in the three genotypes and we found that there was no difference in level of expression of Bcl2. This data suggests that brain has more complex mechanisms regulation for survival of its cells and implies that other factors that could regulate survival even when GAD 65 is not present.

The expression of Bcl2 in pancreas of 10 month old mice of group 2 showed a different pattern and we did not see the same reduced level of expression in knockout mice. Age factor could be the possible reason behind the differential pattern of Bcl2 expression in pancreas. The expression level of Bax in pancreas was not influence by changes in GAD 65 as we found a similar pattern of expression in all the three genotypes. In both group 1 and 2 there was no reduction in levels of expression of Bax in any of the GAD 65 Genotypes.

Beta cells release large amounts of GABA with the release of insulin and some studies suggest that GABA exerts a suppressive effect on glucagon secretion from alpha cells and also that the alpha cells posses receptors for GABA (Xu E *et al*). This raises questions regarding roles performed by GABA as a regulatory molecule in pancreatic islets. It was shown in previous studies that with secretion of insulin in pancreatic islets there is enhanced release of GABA and that the GABA receptors present on alpha cells of islets are activated and in turn exert an

inhibitory effect on glucagon release from the alpha cells. As discussed above, GAD 65 could be an important factor in affecting the regulatory role of GABA on glucagon secretion.

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