HIGHER LEVELS OF ER STRESS PROTEINS OCCUR IN PANCREATIC ISLETS OF DIABETIC PRONE VERSUS RESISTANT RATS

A Major Qualifying Project Report

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ABSTRACT

Bio Breeding (BB) rats are a well known model for juvenile diabetes. Diabetic prone (DP) and resistant (DR) lines were created by in-breeding. DP rats spontaneously develop diabetes via pancreatic beta cell death, but the exact mechanism remains unknown. This project investigated cellular levels of several proteins thought to play a role in endoplasmic reticulum (ER) stress, which could lead to beta cell death. Levels were compared in pancreatic islets from DP and DR thapsigargin-stressed and non-stressed rats by western blot and real-time PCR assays. The results indicated that DP rats manifested higher levels of ER stress proteins.

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BACKGROUND

Diabetes Mellitus

History

The term diabetes mellitus originates from Latin and Greek roots. Diabetes is a Greek word, meaning to siphon, and Mellitus comes from the Latin word that means "sweet like honey" (Lundstrom and Rossini 2004). This is a fitting name for the condition, because the most obvious signs of the disease are excessive urination, and in 1679, an unnamed physician tasted the urine of a diabetic patient and commented on its sweet taste (Lundstrom and Rossini 2004).

In 1889, two scientists, named Von Mering and Minkowski observed that when an animal's pancreas is removed, the animal develops the condition known as diabetes (Ibid.). Naunyn, Minkowski, Opie, Schafer, also indicated that diabetes was brought about by lack of a protein hormone produced by the islets of Langerhans in the pancreas (Anonymous 1965). This protein was later named "insulin" by Schafer (Anonymous 1965). This lead many to believe that the insulin is the hormone responsible for the metabolism of sugars, and its absence leads to the accumulation of an excess of sugar glucose in the blood, which is later excreted into the urine (Anonymous 1965). Attempts were then made, logically, to provide diabetic patients with the protein insulin by ingesting fresh pancreas, but to no avail (Anonymous 1965). Assuming insulin might be absorbed by a patient's GI system if ingested, this raised the question, how to extract insulin before degradation by the proteolytic enzymes of the pancreas?

This problem caught the attention of Dr. Frederick Banting (Figure 1), whom had recently returned from the western front of the First World War, where he was wounded at the battle of Cambrai (Anonymous 1965). Upon reading an article by Moses Baron, Banting hypothesized that he could preserve the islets of Langerhans and the degradation of insulin by experimentally closing off the pancreatic duct that secretes trypsin. If the trypsin producing cells degenerate, and the islets of Langerhans remain, intact insulin could potentially be extracted (Anonymous 1965).

Fig 1. Nobel Prize Winners: Dr. Banting (Left) and Dr. McLeod (Right) (Anonymous 1965)

Upon discussing the possibility of isolating intact insulin, Banting convinced J.J.R. McLeod, Professor of physiology at the University of Toronto to grant Banting the facilities and the assistance needed, in the form of a medical student named Charles Best, to conduct the research (Anonymous 1965). The work they performed lead to the discovery and extraction of insulin. In 1921, Banting and Best injected insulin into a diabetic test animal with the result they expected, a drop in blood sugar (Lundstrom and Rossini 2004). This meant that they had successfully, positively identified insulin as the hormone responsible for the metabolism of sugar, and the following extraction of the protein hormone.

Banting later proceeded to study silicosis, cancer, and the physiology involved with powered flight. Unfortunately, Banting's outstanding career was cut short. He was

called back into service for World War II and was killed in an air disaster over

Newfoundland in 1941 (Anonymous 1965).

The Pancreas

The pancreas is a soft, triangular organ composed of both endocrine and exocrine

cells, and is located in the abdomen, partially behind the stomach (Marieb 2002). The

exocrine cells secrete their enzymes into a duct that delivers them to the small intestine

(Marieb 2002). Also in the pancreas, mixed with the exocrine (acinar) cells are about one

million pancreatic islets (previously referred to as the islets of Langerhans) (Marieb

2002) (Figure 2). These so called "islets" contain two types of endocrine cells, dubbed
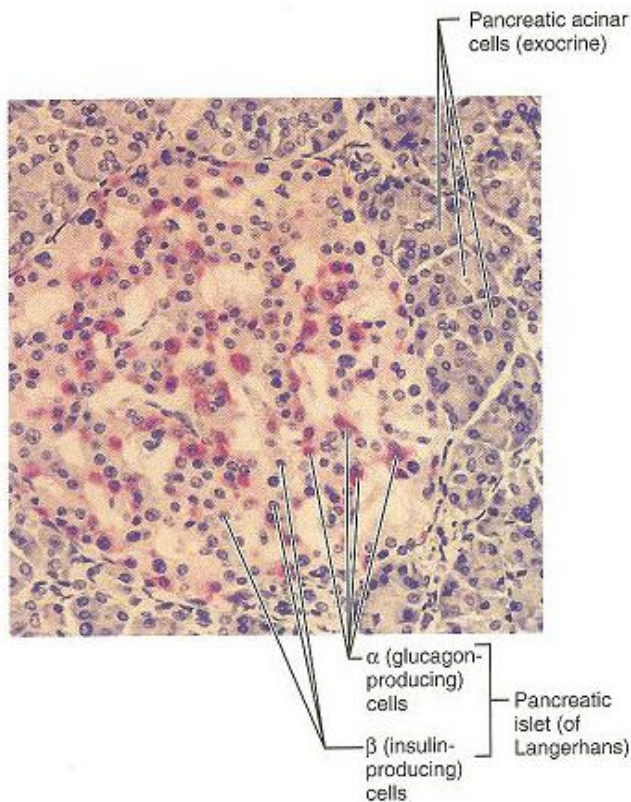
alpha and beta cells. These cells produce glucagon and insulin, respectively. Glucagon is a 29-amino acid poly-peptide that is so powerful, that one molecule can trigger the release of 100 million molecules of glucose into the blood (Marieb 2002). The target organ of glucagon is the liver, where it promotes such metabolic functions as, the breakdown of glycogen to

Fig 2. Pancreatic Morphology.
(Marieb, 2002)

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glucose, the synthesis of glucose from lactic acid and the release of glucose to the blood by the liver cells which cause glucose levels to rise in the blood (Marieb 2002). Insulin, on the other hand, is a 51-amino-acid protein that is comprised of two amino acid chains held together by disulfide bonds (Marieb 2002). Insulin is first produced as a larger polypeptide called proinsulin, which just prior to release by the beta cells is cleaved by enzymes to create the insulin in its functional form (Marieb 2002). The primary effect of insulin is to lower blood sugar levels by optimizing the membrane transport of simple sugars, such as glucose into body cells (Marieb 2002). In particular, insulin enhances the transport of glucose to muscle and fat cells, however, it does not assist or accelerate the entry of glucose into the kidneys, liver or brain (Marieb 2002).

Once interacting with target cells, insulin triggers the catalysis of the oxidation of glucose for ATP-manufacture, aids in the production of glycogen by joining glucose molecules, and also converts simple sugars to fat molecules (Marieb 2002).

Diabetes General Description

Diabetes Mellitus is in essence, a failure to maintain homeostasis, which results from an absence or deficiency of insulin, leading to high blood sugar levels. Since insulin is absence or deficient, glucose in unable to enter most target tissues. When blood sugar is too high, the person begins to feel nausea and jittery due to a fight or flight response (Marieb 2002). In people without diabetes, blood glucose levels are typically maintained around 60-110mg/dL (Lundstrom and Rossini 2004). Due to this fight or flight response, the body prepares itself for survival by making available more glucose. Ergo, blood glucose levels achieve even higher levels, to the point that excesses of

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glucose are expelled from the body by urination; which is called glycosuria (Marieb 2002).

If sugars are not available for metabolism due to lack or absence of insulin, fats are activated for energy acquisition, and fatty acid levels in the blood rise; such a state of high fatty acid levels is termed lipidemia (Marieb 2002). In severe cases of diabetes mellitus, the level of ketone bodies (the metabolites of fatty acids) and fatty acids in the blood reach dangerous levels (Marieb 2002). Ketone bodies, such as acetone, are strong organic acids (Marieb 2002). When such compounds accumulate faster than they can be excreted into the urine (large volumes of ketone bodies in the urine is called ketonuria), blood pH drops leading to ketoacidosis (Marieb 2002).

Severe and prolonged ketoacidosis is life-threatening. In an effort to expel carbon dioxide byproducts of balancing the low blood pH, the nervous system induces hyperventilation (Marieb 2002). If untreated, ketoacidosis results in death by disturbing heart activity, oxygen transport, nervous system function leading to eventual coma and death (Marieb 2002).

Polyuria, polydipsia, and polyphagia are the three main signs of diabetes. Polyuria, large volumetric urine output, is due to an excess of glucose filtrate in the kidneys, which inhibits water reabsorbtion by the kidneys (Marieb 2002). Because the body is also expelling a large volume of ketones, electrolytes are also lost, resulting in electrolytic imbalance. This can lead to severe abdominal pains, vomiting, but also dehydration in the process (Marieb 2002). This stimulates the hypothalamic thirst centers to manifest excessive thirst, called polydipsia (Marieb 2002). While plenty of glucose is

available, it is not usable due to the lack of insulin, so the body is compelled to

metabolize proteins and fat stores for energy (Marieb 2002).

According to the American Diabetes Association 18.2 million people have

diabetes (2002) (Lidstone 2005), 10% of whom have Type I diabetes (Lundstrom and

Rossini 2004; Lidstone 2005).

> In 2002 alone, direct and indirect expenses of the disease totaled $132 billion
> spent on diabetes and its complications. Direct expenditures include health care
> costs, treatment of diabetes and its complications, as well. $92 billion was
> directly attributable to diabetes (not including cost of family caregivers loss of
> income and similar expenses). In addition to its tremendous cost to healthcare,
> diabetes increases mortality, which in turn, results in lost income since effected
> individuals may not be able to work. This phenomenon of lost income is
> accounted for under "indirect expenses", which total about $40 billion (Lidstone
> 2005).

In 2000, Louisiana was the worst state in the nation, with regards to diabetes

mortality; 41 in 100,000 people, costing about 2.2 billion dollars (Webster 2005). Some

sources have reported that diabetes complications consume 15% of the United State's

health care budget (Qin et al 2004).

In the United States, diabetes is the leading cause of non-traumatic amputation,

end-stage renal disease, and is responsible for increased morbidity, via cardiovascular

disease (Cavellerano 2005). In 2002 alone, there were over 50,000 lower body

amputations due to the complications from diabetes (Webster 2005). In 2004, four

people suffered diabetic amputations each day in Louisiana alone (Webster 2005).

According to the NKUDIC (National Kidney and Urologic Disease Information Clearing

House) website, about 392,000 U.S. Residents had end-stage renal disease, and 138,483

had the disease as a complication of diabetes (Anonymous 2006)). Diabetes is also the

leading cause of blindness. Diabetic patients are twenty-five times more likely to become

legally blind than their non-diabetic counterparts, further, only about 40% of diabetic

patients receive regular eye examinations (Cavallerano 2005). There is also an

association between diabetes and peripheral artery disease (American Diabetes

Association 2004).

> Diabetes affects nearly every vascular bed; however, the pervasive
> influence of diabetes on the atherothrombotic milieu of the peripheral
> vasculature is unique. The abnormal metabolic state accompanying
> diabetes results in changes in the state of arterial structure and
> function. The onset of these changes may even predate the clinical
> diagnosis of diabetes. Relatively little is known about the biology of
> peripheral artery diseas (PAD) in individuals with diabetes in particular.
> However, it is felt that the atherogenic changes observed with other
> manifestations of atherosclerotic disease, such as coronary and carotid artery
> disease, are generally applicable to patients with both PAD and diabetes
> (American Diabetes Association 2004).

While diabetes is indeed, a growing problem in America, it is also a worldwide

issue. The UK has estimated that there are about two million people with diabetes (3% of

the population), and many more may be undiagnosed (O'Neil 2006). Also, the UK

department of health also estimates that 85% of all children with diabetes are not

reaching or maintaining optimum glucose levels, which means, they are at a greater risk

for heart disease, blindness and stroke later in life (O'Neil 2006).

Type I and II Diabetes

There are two major forms of diabetes mellitus; type I and type II. Type I

diabetes, also known as insulin dependent diabetes mellitus (IDDM), was known as

juvenile diabetes for quite some time because the generation of the disease was quite

sudden, and typically before the age of fifteen (Marieb 2002). However, the term

"juvenile diabetes" became antiquated since pediatricians have seen a rise in the prevalence of children as young as two years of age developing type II diabetes in families with a history of the disease (Cavallerano 2005).

Type II diabetes, also known as NIDM (non-insulin dependent diabetes mellitus), usually occurs after the age of 40, thus it was formerly known as "adult-onset diabetes" (Marieb 2002). NIDM is caused by a resistance to insulin; their beta-cells produce insulin, but their insulin receptors are non-responsive (Marieb 2002). Without an appropriate sensitivity to insulin, glucose builds up in the blood (Lundstrom and Rossini 2004). NIDM is a slow, gradual onset condition that is frequently uncovered in a routine physical examination (Lundstrom and Rossini 2004). NIDM is typically unmasked by some sort of stressor, such as weight gain, pregnancy, certain prescription drugs, or other illness, however certain factors leading to insulin resistance are hereditary (Lundstrom and Rossini 2004). Fortunately, patients with NIDM do not usually manifest ketoacidosis, and in many cases exercise, diet and oral medications in conjunction with each other form an adequate management technique (Marieb 2002).

The focus of this MQP is Type I diabetes. While many scientists have tried to figure out for many years exactly what causes Type I diabetes, or, insulin-dependent diabetes mellitus (IDDM), the specific, exact one mechanism is unknown. However, some factors that lead to the susceptibility of Type I diabetes are known, including genetics, immune system and environment (Lundstrom and Rossini 2004). While 80% of new cases of IDDM have no family history, there are some familial correlations (Lundstrom and Rossini 2004). If one parent has IDDM, the risk of the child contracting the disease is 7%, however, the risk is 30-50% if an identical twin of the child has the

condition (Lundstrom and Rossini 2004). There are also environmental factors that can lead to IDDM, such as viral infection. Mumps, rubella and Coxsackie viruses have all been associated with the development of IDDM (Lundstrom 2004; Rossini et al 1993). IDDM is an autoimmune disorder, where the immune system is misdirected to attack the body's own cells; in the case of IDDM, the essential beta-cells of the pancreas are destroyed. Consequently, the body can no longer make insulin, and IDDM is presented (Lundstrom and Rossini 2004).

Management of IDDM

Through careful monitoring of blood sugar levels, patients with IDDM can develop a plan with their health care provider to maintain an optimal glucose level. First, a regular regiment of a nutritious low sugar meal plan, physical activity and exercise are crucial. Careful meal planning can greatly aid maintaining blood sugar at an optimal level, provide enough calories for ideal body composition, lower cholesterol and ultimately postpone diabetic complications (Lundstrom and Rossini 2004). Unfortunately, while measures may be taken to manage glucose levels and delay complications of IDDM, IDDM patients' lives are cut short up to one-third due to complications such as kidney malfunction, nerve impairment and cardiovascular disease (Voet and Voet 2004).

However, patients with IDDM must also undergo insulin injection therapy, which, to date, is the most effective, practical treatment. This treatment first utilized injecting isolated insulin from pig and cow pancreas, however more recent breakthroughs in

recombinant DNA technology has rendered the large scale biomanufacture of insulin from *E. coli* bacteria possible (Lidstone 2005).

The main objective of the biomedical endeavor to combat diabetes is to create some way to mimic the proper function of a non-diabetic pancreas in patients with IDDM. One advance towards this goal is development of an insulin pump. The insulin pump administers a basal amount of insulin via a catheter. However, there are many drawbacks including, constantly wearing a bulky pump, infection at the site of catheter infiltration in immune compromised patients, and also periodic refilling the insulin reservoir (Lidstone 2005). Furthermore, pumps are not only still not approved for wide usage yet, but, they are also expensive and not yet available to everyone, especially those in economically disadvantaged regions (Pollock 2006).

Another major step towards nullifying the effects of IDDM, is islet transplantation. Islet transplantation involves implanting live pancreatic islets from either an animal such as a pig, or a cadaver, thus producing insulin in a manner similar to the patient of a non-diabetic patient. However, there are also drawbacks to islet transplantation therapy including, toxicity associated with the immuno-supression associated with the islet transplantation, and transplant rejection. However, trans-gene therapies are being researched in order to prolong beta-cells (either transplanted or pre-diabetic) by up-regulating the expression of anti-apoptotic genes (McKeon 2002) But right now, there are simply not enough cadaver islets to meet the needs of all the IDDM patients that could benefit from such a treatment (Fradkin 2004).

Beta Cell Death and IDDM

But what specifically triggers cell death in the insulin producing pancreatic beta cells? Apoptosis, or programmed cell death, seems to lie at the root of many autoimmune disorders, including IDDM. While it is well understood that a wide array of factors in concert lead to the IDDM condition, the one, single trigger to the disorder remains unknown. However, there is evidence that premature apoptosis of beta-cells is a paramount contributer to the disease. This poses the question, "Why does the immune system attack beta-cells?". One answer may be Class II major histocompatibility complexes (MHC's). Studies have shown that certain genetic variations (alleles) of the Class II MHC have been conserved in patients with IDDM. MHC's are highly polymorphic proteins vital to proper immune system function, to which foreign antigens must bind to, in order to be effectively identified as foreign. It is believed that autoimmunity against beta-cells is triggered in an organism susceptible to IDDM, by a foreign antigen, perhaps a virus or other environmental factor, which closely resembles a beta-cell component. In a patient with IDDM, the Class II MHC has trouble discerning "self" from foreign identifying antigens, and binds to the antigen with such a fury that it stimulates the immune system to launch a full fledged, and unusually vigorous attack on the antigen identified as "foreign". Unfortunately, in patients susceptible to IDDM, activated immune cells (autoreactive T-Cells) eventually infiltrate the pancreas, successfully attack and kill beta-cells due to the mistaken identity between beta-cell antigens and the original foreign antigen (Voet and Voet 2004). But, this is just one of the many factors that act in conjunction with each other to lead to beta-cell death and IDDM.

**Ian4/GIMAP-5 and ER Stress**

Ian4/GIMAP-5

Recent research has shifted focus of what causes the diabetic autoimmune cascade to genetic defects of the beta-cell itself. The focus of research on this MQP was the role of Ian4/GIMAP-5 and the endoplasmic reticulum (ER) stress response in IDDM. GIMAP proteins are a family of mitochondrial outer-membrane proteins with GTP-binding activity that are found in vertebrates and also higher-order plants, and have been named according to a variety of nomenclatures including IAN, IMAP and IAP (Pandarpurkar et al 2003; Bortell 2005). A frameshift mutation of the GIMAP-5 protein has been found to increase T-cell apoptosis and T-lymphopenia. While apoptosis is regulated by extrinsic and intrinsic signaling pathways, it is believed that GIMAP-5 is responsible for signaling functions in the intrinsic pathway by instigating permeabilization of mitochondria and release of apoptogenic factors (Pandarpurkar et al 2003). The absence of GIMAP-5 in T-cells has shown mitochondrial dysfunction, and increased levels of mitochondrial stress which is marked by raised levels of stress inducible chaperones. This T-cell absence has also been associated with the expression of diabetes; transfection of T-cells with GIMAP-5 specific small-interfering RNA also recapitulates the diabetic phenotype (Pandarpurkar et al 2003). It is believed that the loss of regulatory T-cells due to a GIMAP-5 mutation in BBDP (BioBreeding Diabetic Prone rats) leads to an unhealthy imbalance of regulatory T-cells to autoreactive "killer" T-cells that attack and kill the insulin producing beta-cells (Bortell 2005).

However, perhaps the beta-cells exposed to beta-cell stressors such as viruses or toxins like thapsigargin, are the initiators of their own death, and the T-cells are not solely responsible. GIMAP-5 could be a cellular protector from apoptosis. While GIMAP-5 knockdown in T leukemia cells leads to the diabetic phenotype, transfection of Jurkat T-cells with full length human GIMAP-5 cDNA template also creates a resistance to cell death by exposure to gamma-radiation and okadaic acid (Bortell 2005).

Further, high levels of GIMAP-5 have been seen in transformed cell lines, as expected for a protein that blocks apoptosis (Figure 3).
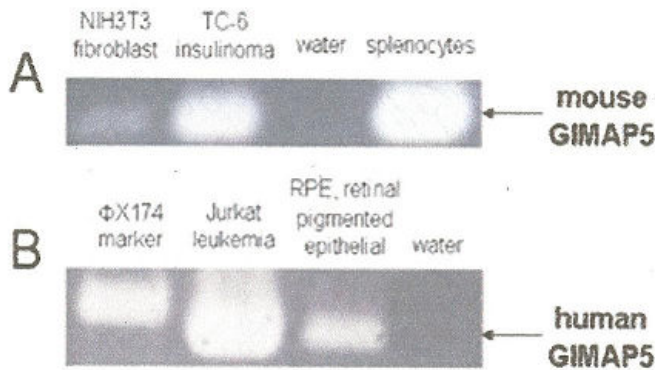
Fig 3. GIMAP-5 in Various Tissues. GIMAP-5 detected in Human (B) and mice (A) by RT-PCR (Bortell 2005)

In addition to that, high levels of GIMAP-5 mRNA have been observed in many other tissues such as cardiac tissue, digestive tract tissue and but most notable, mouse insulinoma; leading to the hypothesis that GIMAP-5 serves as a cellular protector by inhibiting apoptosis and beta-cell sensitivity in response to endogenous and environmental stress factors (Bortell 2005).

In a comparison between BBDP (GIMAP-5 -/-) rats and GIMAP-5 +/+ Wistar rats, there is a decreased level of glucose stimulated first phase insulin release in BBDP rats. However, the most striking aspect of this phenomenon is that this difference in

insulin release was pre-diabetes/pre-insulitis, and did not change after the onset of

diabetes. This lead to the hypothesis that the GIMAP-5 -/- mutation was the underlying

defect in the BB DP rats that may have resulted in the diabetic state. Further, this also

shows that GIMAP-5 encompasses more functions than previously believed, including

insulin secretion (Bortell 2005).

Collectively, it can be ascertained that GIMAP-5 is essential for proper beta cell

function. The absence of GIMAP-5 is believed to lead to regulatory T-cell death, leading

to an imbalance of regulatory T-cells and cytotoxic "killer" T-cells, allowing T-cells to

destroy beta-cells. This hypothesis is further supported by evidence showing that

insulinoma cells (which grow prolifically, and are too resilient) show high levels of

GIMAP-5 but can be recapitulated to regular cell death by GIMAP-5 knockdown (Bortell

2005). In beta-cells, GIMAP-5 is shown to perform a role as a protector. It has been

hypothesized that GIMAP-5 helps mount a resistance to environmental stress, as shown

in Jurkat T-cell lines. Further, even in a pre-diabetic state, diabetic prone rat models have

notably less GIMAP-5 expression than their healthy Wistar counterparts (Bortell 2005).

ER Stress

Another purpose of this project was to observe the ER stress response to exposure

to toxin, in the BBDP and BBDR rats in terms of expression of stress related proteins

Bip/GRP78, GRP94 and CHOP/GADD153.

The ER (endoplasmic reticulum) is a multifunctional organelle that is used for the

production of steroids, cholesterol and other lipids, along with biosynthetic enzymes and

other proteins. The total concentration of such proteins in the ER can reach 100 mg/ml

(Kaufman 2002). Fortunately, the ER has many, very intricate pathways to ensure that the protein-folding capacity of the ER is not overwhelmed. This conglomeration of pathways is termed the unfolded protein response (UPR). UPR is an exceedingly important requirement for cells to survive ER stress that can result from a host of origins including, but not limited to, agitation of calcium homeostasis, elevated demand for secretory protein synthesis, and glucose deprivation (Kaufman 2002).

This UPR is dependent upon highly evolved chaperone proteins that prevent the accumulation of unfolded or misfolded proteins caught in a kinetic energy trap (Kaufman 2002). For the sake of this MQP, the chaperones focused upon are, Bip/GRP78, GRP94, and GADD153/Chop. Glucose deprivation induces expression of GRP's (glucose-regulated proteins). GRP94, is a part of this family of chaperones that are expressed when available energy cannot meet the energy demands of proper protein folding. In this scenario, GRP's prevent mis-folded proteins from entering the golgi (Kaufman 2002). Bip/GRP78 is also a similar chaperone. Bip uses the energy from ATP-hydrolysis to promote folding and prevent the accumulation of such proteins in the ER. Rodent studies have shown that perturbation of the redox state of the ER, (for example, ascorbate deprivation) results with a raised expression of Bip (immunoglobulin-binding protein) and GRP94 (Margittai et al 2005). GADD-153/Chop (growth arrest and DNA damage) is the last of the ER stress proteins that were of specific interest to this project. Chop expression has been seen to precede beta-cell death in some studies, but also, Chop expression has been seen to increase in nutrient deprivation (Hajar and Jonas 2005; Rees et al 1999).

In this MQP, thapsigargin was used to create ER stress. Thapsigargin is an inhibitor of sarcoendoplasmic reticulum Ca++-ATPases (Zhou et al 1998). Time and concentration dependent exposure to thapsigargin creates a response where DNA fragmentation increases, cellular viability decreases, and nuclear chromatin staining changes, characteristic of apoptosis (Zhou et al 1998). Studies by Zhou et al show that thapsigargin causes apoptosis by depleting intracellular stores of Ca++ and leading to the release of metabolites from arachidonic acid metabolism (Zhou et al 1998). Thapsigargin treatment also has similar results to cyclopiazonic acid and caffeine, which are other known toxins that contribute to apoptosis (Zhou et al). Thus, thapsigargin was used because it induces ER stress by agitating the balance of Ca++ in the ER, and results in an inevitable apoptosis, preceded by in increase of chaperones including, Bip/GRP78, GRP94 and GADD-153/Chop expression.

Animal Modeling

Animal models have been used for many years in the field in biotech research in order to better understand the mechanisms that cause diseases and also, they help us understand the mechanisms by which we may treat and cure given conditions. While many animal models have been used throughout history, rodents are particularly useful because much is known about the genomes of rodents such as rats, hamsters and mice, and their life spans are far shorter than their primate counterparts. The relatively inexpensive cost and ease of handling of rodents, compared to primates are particularly noteworthy. Chimpanzees are fairly rare, and expensive, costing about $50,000 (Adams 2004). Indian macaques have been used for HIV research, however, they are now in

short supply, their cost tallies about $5,000 each, the breeding is time consuming, and the gestation period is fairly long. Instead of using primates, rodents can be used to collect data for about 1% the cost and breeding takes only about a quarter the time, about five to nine months (Adams 2004).

This MQP extensively used the BioBreeding/Wor diabetic prone (BBDP) and BioBreeding diabetic resistant rat (BBDR) models. This rodent diabetes model was exceptionally useful because their expression of the diabetic phenotype closely models that of humans. The BBDP rat strongly resembles IDDM in humans from the spontaneous onset via autoimmunity to inflammation of the beta-cells and pancreatic islets to the eventual cell death. The classic method to ameliorate type I diabetes is the use of cyclosporine to induce immunosupression. This tactic has been shown to clearly preserve the insulin secretory ability in both human trials and trials with the BBDP rat (Mordes).

Through in-breeding of the DP rats, the diabetic resistant (DR) phenotype was created. This model is of particular interest to this MQP, because it serves as a great comparison to the DP rat. Furthermore, in essence, the allelic difference between the DR and DP rat may hold answers to many questions regarding the genetic factors that lead to the diabetic phenotype in humans.

PROJECT PURPOSE

The purpose of this MQP was to investigate the cellular mRNA and protein levels of GIMAP-5 and the chaperone proteins such as Bip/GRP78, GRP94, and GADD-153/Chop in beta-cells in DP and DR rats. In some experiments, the environmental stressor thapsigargin was used to challenge the ER as a comparison to non-stressed animals. The experimental approach utilized LightCycler RT-PCR and western-blot/SDS-PAGE assays. This will provide valuable information regarding the factors that lead to beta-cell apoptosis and therefore, IDDM. The hypothesis is that in cells under ER stress, such as the beta-cells of DP rodents, there will be a higher level of ER stress associated chaperones, such as Bip, GRP94 and CHOP, and lower levels of therapeutic GIMAP-5.

METHODS

RNA Isolation (Qiagen 2001)

The isolation of RNA was a first step in this MQP to prepare template for RT-PCR reactions. The protocol was as follows:

1) Approximately 700-800 islets were harvested per sample by Linda Leehy.
2) Cell suspension was centrifuged for 5 minutes at 1,500 rpm.
3) All supernatant was carefully aspirated from the islet pellet.
4) Buffer RLT was added to the pellet to produce a total volume of approximately 600 µl.
5) The sample was homogenized with a pipet.
6) Transferred sample to directly to QIAshredder spin column with 2ml collection tube, and centrifuged for 2 min at 3,000 rpm.
7) Added 500 µl of 70% ethanol to the homogenized lysate, and mixed with pipetting.
8) Transferred sample to RNeasy column placed in a 2 ml collection tube. Centrifuged for 15 seconds at approximately 3,000 rpm. The resulting flow through was discarded.
9) Buffer RW1 (700 µl) was added to RNeasy Column and centrifuged for 15 seconds at approximately 3,000 rpm. The resulting flow through discarded.
10) Transfered RNeasy column to another 2ml collection tube. Add 500 µl of RPE buffer to RNeasy column. Centrifuged for 15 seconds at approximately 3,000 rpm. Discarded the flow through.
11) Another 500 µl of Buffer RPE added to the RNeasy column, and centrifuged for 2 minutes at approximately 3,000 rpm. Flow through was discarded with 2ml collection tube.
12) RNeasy column placed into another 2ml collection tube and spun at full speed for 1 minute. Flow through was discarded.

13) RNeasy column was transferred to another 1.5ml collection tube. 30-50 µl of RNase free water was transferred directly to silica gel membrane via pipet. Centrifuged for 1 minute at 3,000 rpm.

14) Repeated step 13.

15) All eppendorf tubes were briefly spun to ensure all droplets collected.

16) RNA was tested for concentration using spectrophotometer (see below).

Quantitation of RNA Yield:

1) Spectrophotometer was switched on and warmed up.

2) Spectrophotometer was zeroed with a blank cuvette containing 60 µl of milli-Q water.

3) RNA sample was combined with an appropriate volume of milli-Q water for a given dilution (see table).

4) Absorbance was read at 260 nm and recorded.

5) Absorbance was read at 280 nm and recorded.

6)   Calculation of RNA concentration: $[\text{RNA } (\mu g/\mu l)] = A_{260} \times (\text{dilution factor}) \times (0.040)$.

cDNA Synthesis for RT-PCR

cDNA synthesis was the next step before RT-PCR. RNA was added to eppendorf tubes with other reagents and was annealed by heating. After annealing RNA and primers specific for the sequences of Bip, GRP94 and CHOP were added to the mix RNA in the eppendorf tubes.

1) All reagents were briefly centrifuged before opening.

2) Annealed 1 µg RNA (H_2O), to 1 µl of oligo-dT primer and brought to a final volume of 12.5 µl d H_2O. The DNA engine was programmed as follows:

RT:Melt. (13 min), 65° C 10 min, end at 25° C (0.3°/sec) over 3 minutes
programmed onto DNA engine.

3) EXTEND: added AMV master mix (7.5 ul per reaction). 0.75 µl dH$_2$0, 4 µl
5xRT buffer, 0.25 µl Rnasin (40-50 U/µl), 1.5 µl dNTP 25 mM, 1 µl AMV 20-25
U/µl.

4) Gently vortexed and quick spun mix before aliquoting. Mixed with a finger flick
and quick spun tubes.

5) Programed DNA engine: 42°C for 60 min, 95° C for 2 min, 4° C forever.

6) Quick spun all reagents before opening.

7) Stored cDNA aliquots at -70°C.

LightCycler RT-PCR

cDNA samples were sent to authorized LightCycler RT-PCR technician, Mary
Lively for processing. The use of an Instrument such as the LightCylcer was indeed, a
privilege. This instrument uses glass capillary tubes as a reaction vessel (Figure 4).
Because the design of the capillary tubes allows for optimum heat transfer, the
LightCycler is one of the fastest thermal cyclers in the world, allowing for 30 PCR cycles
in under 20 minutes, a fraction of the time needed for conventional RT-PCR (Boehringer
Mannheim). Furthermore, samples are centrifuged down to the tips of the reaction vessel
capillary, where SYBER Green I dye preferentially binds to dsDNA. While ethidium
bromide also binds to dsDNA, the signal propagated by SYBER Green I is much stronger
(Rasmussen et al).

Another asset rendered by use of LightCylcer is that the collection of data is continuous. So, rather than analyzing the end point analysis of a conventional RT-PCR, the LightCycler shines a "maintenance free" LED to shine light though the capillary containing the PCR reaction, where the absorbance is read at the glass/composite tip of the vessel, constantly (Figure 5). The absorbances are calculated over time, and are used to construct a melting curve. This yields a melting curve that is used by LightCycler software later to calculate the relative concentrations of targeted molecules. In the case of this MQP, message signals for Bip, GRP94, and CHOP in both DP and DR rodents were the molecules targeted for analysis.
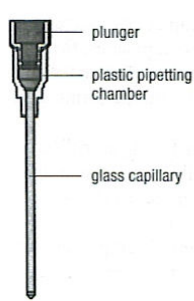
Fig 4. LightCycler reactor capillary vessel depicted to the left (Boehringer Mannheim).
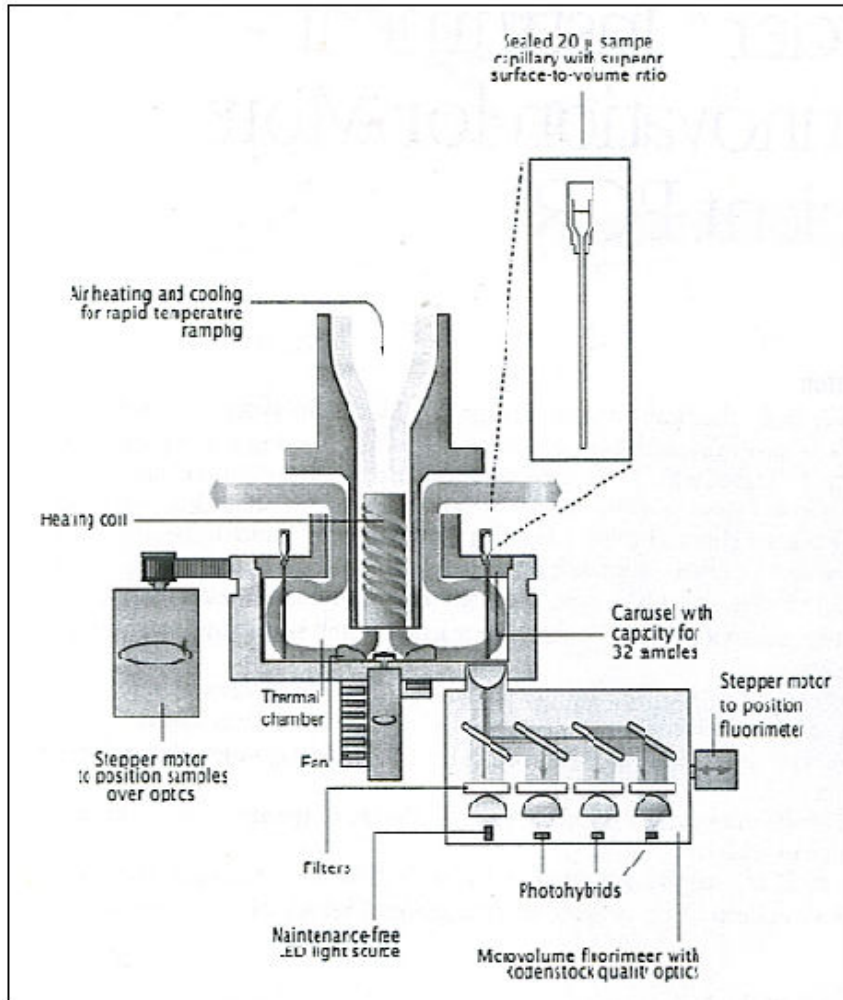
Fig 5. A schematic diagram of the Light Cycler (Boehringer Mannheim).

Standard Western Blot/SDS-PAGE Protocol

Western Blots/SDS-PAGE assays have been used for quite some time in the field of biochemistry and are still some of the most useful assays today to detect the presence of a specific protein in cell lysates. In this MQP, proteins were electrophoresed down a polyacrylamide gel, so they were arranged by molecular weight. A primary antibody was then used to preferentially bind to selected proteins that were transferred to the membrane. A secondary antibody that is conjugated with horseradish peroxidase (HRP)

was then preferentially bound to the primary antibody that was previously attached to the targeted protein. The membrane was then treated with ECL, a developing reagent used to induce the luminescence of HPR present upon the protein in question. When film was exposed to the light rendered by the glowing HRP in a dark room, a negative image is created. The film was later developed, and if properly done under the right conditions, there will be dark bands presented at the expected molecular weight position of the targeted protein. Bands indicating the presence of Bip, GRP94, CHOP and GIMAP-5/IAN4 should appear at 78 kDa, 94 kDa, 31 kDa and 35 kDa on the film, respectively.

Preparation of Islet Whole Cell Lysates

1) Approximately 800 islets were isolated from DR or DP rats for each experimental sample.

2) Islets were placed into 10 ml of RPMI containing 5% horse serum, and 1% penicillin/streptomycin/gentimycin antibiotic.

3) Islets were plated in 10cm petri dishes. DP and DR thapsigargin-treated samples were given 50 µl of 1 mM thapsigargin solution, each. Untreated samples were left untouched.

4) DP treated, DR treated, DP untreated, and DR untreated samples were all incubated at 37˚C in the tissue culture room for 2 hours.

5) Islets were then transferred from petri dishes to 15ml Falcon tubes and centrifuged for five minutes at 1,500 rpm, 4˚C.

6) The supernatant was aspirated from the islet pellet and removed.

7) 125 µl of Brij protein lysis buffer was added to each of the four samples. All samples were then left to incubate on ice in the 4°C cold room for >30 minutes.

8) Protein lysate concentrations were determined by comparison to a BSA standard curve, measuring optical density (A_{590}).

Immunoblot Protocol

1) 30 µg of protein was loaded into the wells of a ten to fifteen well, 8 cm x 10 cm x 1 mm poly-acrylamide gel. All western blotswere conducted with the same composition for each gel:

- 10% Lower Gel Mixture:
  - 2.45 ml Milli-Q water
  - 1.25 ml 40% acrylamide (Bis)
  - 1.3 ml 4x lower tris buffer.
  - 2.5 µl TEMED
  - 25 µl 10% APS
- 4% Upper Gel Mixture:
  - 1.95 ml Milli-Q water
  - 0.3 ml 40% acrylamide/(Bis)
  - 0.75 ml 4x upper tris buffer
  - 3.0 µl TEMED
  - 15 µl APS

2) The loaded gel with 30 µg protein in each laden well was then run for 45 minutes at 180 V.

3) After the gel was run, proteins were transferred under 100 v for 2 hours to a Millipore PVD membrane and allowed to stand in 50 ml of 5% blotto buffer overnight (2.5 g blotto added to 50 ml TBS).

4) The next day, the membrane was probed for the targeted protein by immersion in antibody solution for 45 minutes with the respective primary antibody solution:

- Bip: 1:500 mouse Ab to 1% blotto buffer (1g blotto to 100 ml TBS).
- CHOP: 1:500 mouse Ab to 1% blotto buffer.
- GIMAP5/IAN4: 1:500 mouse Ab to 1% blotto buffer (custom constructed by Sigma-Genosys).
- GRP94: 1:2000 rabbit Ab to 1% blotto buffer.

5) After probing with primary antibody and rinsing, the membrane was probed with a secondary antibody conjugated with horseradish peroxidase (HRP) by immersion in secondary antibody solution for 30 minutes:

- Bip, CHOP and GIMAP5/IAN4: 1:3000 goat anti-mouse Ab to 1% blotto buffer.
- GRP94: 1:3000 goat anti-rabbit Ab to 1% blotto buffer.

6. After the secondary probe incubation and rinsing off of excess secondary antibody, the membrane was developed in a dark room with enhanced chemiluminescence (ECL) Pierce treatment.

RESULTS

This MQP was performed to quantitate the levels of ER stress in DP and DR rodent islets by measuring the levels of ER stress mRNAs and proteins. By culturing the islets in the presence of thapsigargin (a known inducer of apoptosis and ER stress) both DP and DR islets should show the expression of ER stress chaperone proteins such as Bip, GRP94 and Chop at their maximum inducible levels. Those levels will be compared to untreated islets (no thapsigargin) representing basal levels of ER stress chaperone expression.   Thus we can learn how high the basal levels of ER stress proteins are in DP and DR rats, and their ability to adapt to an environmental stressor.

Light Cycler RT-PCR

In the initial set of experiments, total cellular RNA was isolated from non-stressed (non-thapsigarin-treated) DP and DR rats, then real-time RT-PCR was used to quantitate the levels of ER stress mRNAs GRP94, Bip, and CHOP. The levels are shown in Table I. Higher levels of all three mRNAs tested were observed in the DP rats.

Table I. Levels of GRP-94, Bip, and CHOP mRNAs in Islet RNA
Quantitated by Light Cycler RT-PCR

mRNA	Sample Origin/Targeted Message Signal	Calculated Concentration (LightCylcer Units)
GRP94	DP1/GRP94	28.99
	DP2/GRP94	28.57
	DR1/GRP94	19.57
	DR2/GRP94	18.84
Bip	DP1/Bip	154.2

| mRNA	Sample Origin/Targeted Message Signal	Calculated Concentration (LightCylcer Units)
	DP2/Bip	153.1
	DR1/Bip	120.0
	DR2/Bip	117.6
CHOP	DP1/CHOP	48.55
	DP2/CHOP	41.79
	DR1/CHOP	30.10
	DR2/CHOP	27.81

The mRNA levels from Table I are shown in histobar plots in Figures 6-8 below. The following p values were obtained for the differences between DP and DR rats for each gene: Bip $p = 0.005$, GRP94 $p = 0.006$, and CHOP $p = 0.04$. All p values are under 0.05, so are statistically significant.

Message Signal for GRP94 in Islets
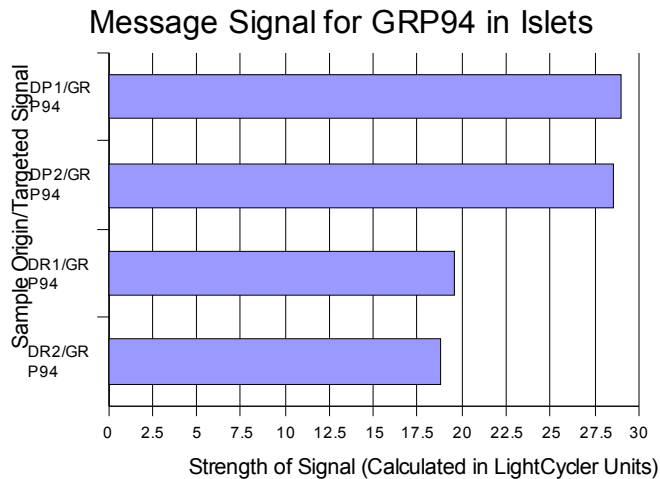
Fig 6. Histogram illustrating the mRNA signal for GRP94 expression in islets, quantitated by LightCycler RT-PCR.

Message Signal for Bip in Islets

Fig 7. Histogram illustrating the mRNA signal for Bip expression in islets, quantitated by LightCycler RT-PCR.

Message Signal for CHOP in Islets
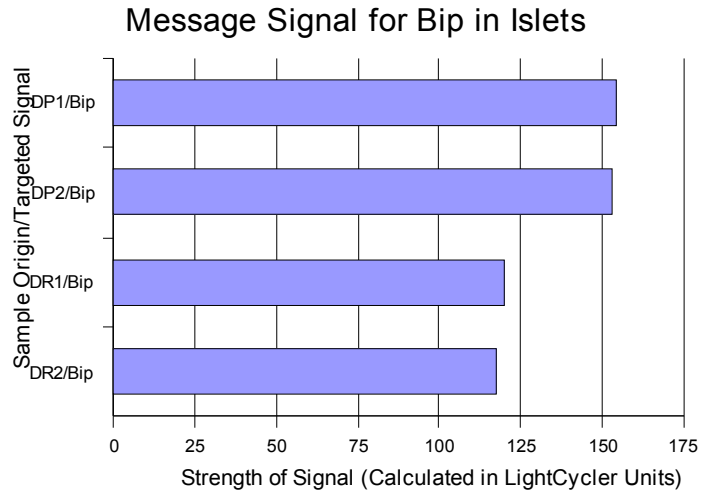
Fig 8. Histogram illustrating the mRNA signal for CHOP expression in islets, quantitated by LightCycler RT-PCR.
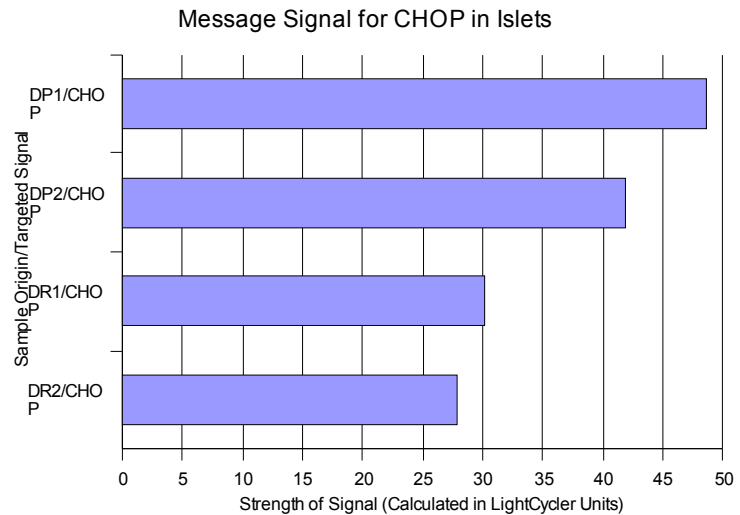
This set of data generated by the light cycler shows clearly that there is indeed a greater signal for ER stress associated chaperone proteins (Bip, GRP94 and CHOP) in the BBDP rats than the BBDR rats. HPRT was used as a housekeeper control (data not shown). The data indicates that even in the absence of environmental stressors, DP rats

express higher levels of mRNAs for Bip, GRP94 and CHOP chaperones, which indicates their islets may be under a higher level of ER stress.

Western Blots

Immunoblots were used to compare the cellular levels of chaperone proteins in DP and DR islet extracts (Figures 9-12).
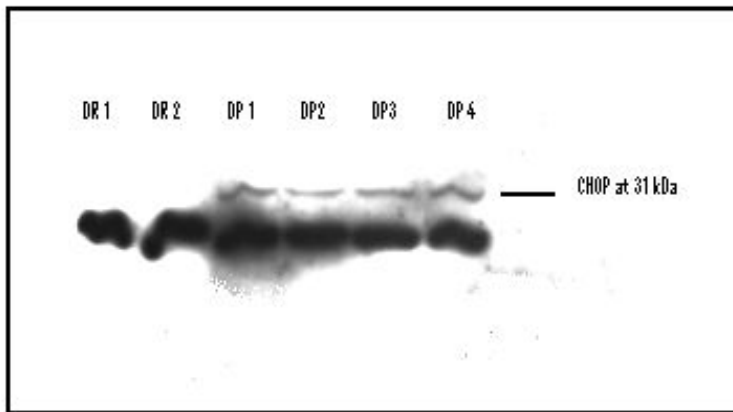
Fig 9. CHOP Western Blot. The CHOP protein is observed at 31 kDa in the DP rats (right 4 lanes, see arrow), but not the DR rats (left 2 lanes).

The Figure 9 western data shows that DP rodents express higher levels of CHOP in the islets, even with no exposure to environmental stress. This is a strong indication that BBDP rodents are under a higher level of ER stress at rest compared to their BBDR counterparts. Figure 10 shows the levels of CHOP in stressed and non-stressed rats, and the data is quantitated in Figure 11.
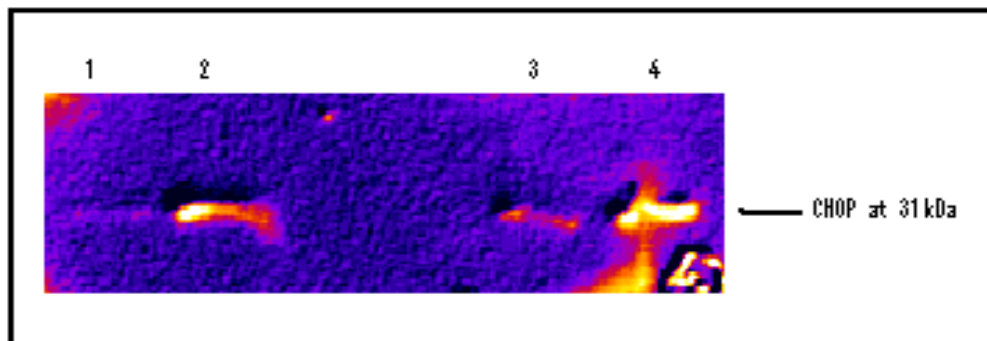
Fig 10. CHOP Expression Induced by Thapsigargin in Islets. The western blot shows the bands presented upon probing for CHOP at 31 kDa. Lane-1, DP untreated; lane-2, DP treated with thapsigargin; lane-3, DR untreated; lane-4 DR treated. Image enhanced using Scion Image (NIH).

## CHOP Expression Induced by Thapsigargin
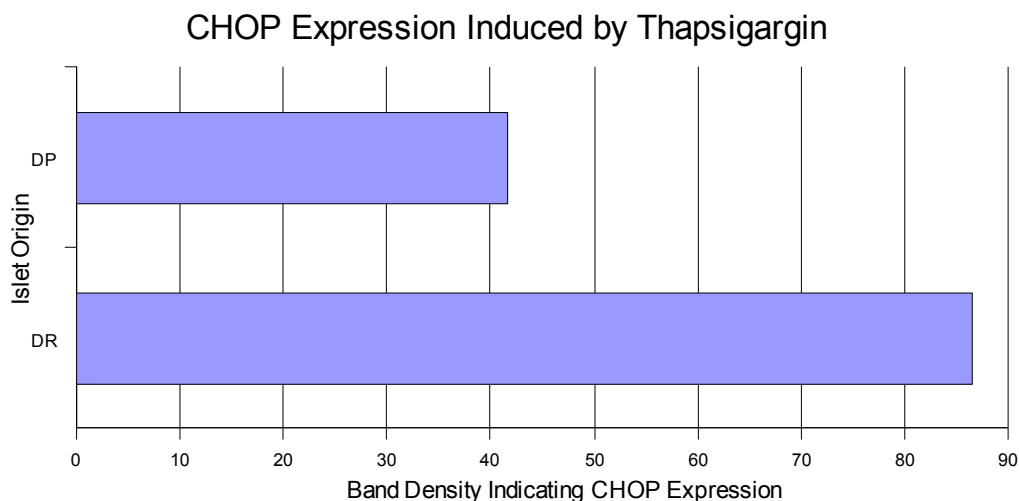
Fig 11. Quantitation of the Levels of CHOP Protein Induced by Thapsigargin Exposure. This histogram illustrates the difference of band densities (quantitated using Scion Image [NIH]) between the untreated and treated islets in the above western blot (Fig 10), representing the CHOP expression in response to the ER stress created by thapsigargin exposure in islet culture.

As expected, the stressor thapsigargin increased CHOP protein levels in both DP and DR animals (approximately 2.0 fold) and the DR rats showed higher levels overall, indicating the DR rats are capable of upregulating this protein more than the DP rats.

Western blots probing for GIMAP-5 rendered inconclusive results (data not shown). GIMAP-5 trials repeatedly yielded very weak bands inconsistently presented in both BBDR and BBDR islets. This could be attributed to poor antibody binding affinity leading to weak, non-specific binding.

Probing for Bip (Figure 12) presented consistent, yet, inconclusive results as well.

Bands were observed in the vicinity of 78 kDa (the expected size), however, they were

approximately equal in intensity in both DP and DR islets. This could be due to the
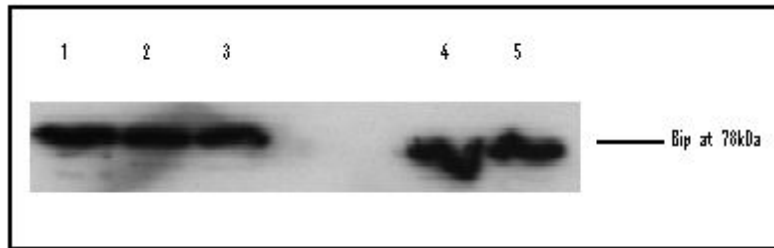
Figure 12. Levels of Bip Protein in DP and DR Rat Islets. Lane-1 and 2, lysates from BBDP islets; Lane -3, lysate from thapsigargin treated BBDP islets; Lane-4, lysate from untreated BBDR islets; Lane-5, lysate from thapsigargin treated BBDR islets. As shown above, Bip is expressed at approximately the same levels regardless of the environmental stressors.

overall very high levels of this very common chaperones' message signal in both BBDR

and BBDP islets (Fig. 7). There was a similar conflict between the LightCycler results

and the results expressed via western blot with GRP94. The immunblot analysis

indicated approximately equal levels of GRP94 between BBDP and BBDR islet lysates

(data not shown), while the LightCycler RT-PCR data showed a distinct difference in the

message signal for GRP94.

DISCUSSION

After analyzing the results of the studies conducted for this MQP a few conclusions may be drawn. The first conclusion is that the islets from BBDP rats do, in fact, have many indicators of elevated ER stress which may be a contributing factor to the apoptosis of pancreatic beta-cells. LightCycler RT-PCR showed consistently higher levels of the mRNA signals for the chaperone proteins Bip, GRP94 and CHOP, which are associated with high ER stress. CHOP mRNA levels showed the greatest difference between the two rodent phenotypes.

The immunoblot data showed somewhat less consistent results than the RT-PCR. Again, data gathered on the CHOP protein expression was the clearest. Strong bands presented in the BBDP lanes at the expected size of 31 kDa, which were undetectable in the BBDR lanes (Figure 9). This is consistent with the islets of BBDP rodent models maintaining a higher level of ER stress than BBDR rodents, perhaps placing them at a higher risk for spontaneously developing IDDM via beta-cell apoptosis. CHOP also proved to be the most successful target protein in the thapsigargin stress experiment (Figure 10). While some of the bands were indeed faint, they were able to be enhanced using Scion Image (NIH) to produce very exciting observations. As expected, the thapsigargin-stressed islets expressed more CHOP than their unstressed counterparts. But unexpectedly, CHOP levels were 2.0 fold higher in stressed DR islets than in stressed DP islets. If CHOP is a marker of ER stress, we would expect it to be higher in the DP animals. But if CHOP is a protein induced by stress to help alleviate it, perhaps its expression would be higher in DR animals that maintain stress levels low enough to avoid diabetes. Since thapsigargin can only induce stress to the maximum level of ER

stress marked by CHOP expression, a lower level of CHOP being induced by exposure to an environmental stress factor, such as thapsigargin, shows that the BBDP rodents have an elevated level of stress to begin with. Conversely, a high level of induced CHOP expression would display low level of ER stress without the agitation by environmental factors. Further, since BBDR rodents have a greater ability to induce an ER stress response, this could also mean that they can buffer against resulting apoptosis from toxins, or other environmental stress upon the islet system.

The Bip and GRP94 trials were less clear. While tested numerous times, the data yielded approximately equal levels of expression of the protein at 78 and 94 kDa respectively, regardless of the BBDP or BBDP phenotype. But this finding is not without merit. The presentation of bands from GRP94 and Bip in both phenotypes could be simply due to the sensitivity/insensitivity of the western blot protocol. Perhaps the ER stress generated by the BBDP rats perturbs the GADD-153/CHOP pathway and not the glucose regulated protein pathways associated with Bip and GRP94.

Another recommendation for further research would be to develop and manufacture a more effective GIMAP-5/IAN4 antibody than the one purchased from Sigma for this MQP. In the course of this MQP, many attempts were made to successfully probe for GIMAP-5 on a western blot. However, the GIMAP-5 antibody used in this study (Sigma-Genosys) was lack-luster at best, and produced no striking GIMAP-5 signals on the western blot to complement the excellent LightCycler data, and prior research linking GIMAP-5 mutations in T-cells to the BBDP phenotype. There was far too weak of a signal and far too much non-specific binding in numerous trials. If a high performance GIMAP-5 antibody could be developed for western blot protocols, then

a link between the GIMAP-5 and ER stress could be inexpensively investigated. If therapeutic GIMAP-5 is found to be present in BBDR islets as originally expected, studies should be conducted to knockdown the expression of GIMAP-5 in the islets of BBDR rodents and see if the diabetic phenotype is recapitulated as it is in similar T-cell trials. Also, in conjunction with the possible knockdown study, ER stress chaperone levels and their respective message signals should be observed in islets before and after administering siRNA.

Although this study has not soundly refuted or proven any specific theories about the ER stress responses in islets, it has made observations that are interesting, has sparked thought, and has pointed out areas with room for improvement. Nonetheless, this MQP has achieved its overall goal of making an intellectual contribution to the field of diabetes research.

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