Prohibitin Expression and Function in Ethanol Treated Pancreatic β-Cells

by

Jong Han Lee

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Department of Physiology

University of Manitoba

Winnipeg, Manitoba

ABSTRACT

Diabetes is the most common metabolic disease in the world. Over 90% of diabetic patients are diagnosed with type 2 diabetes. Although the primary cause of diabetes is not known, insulin resistance and pancreatic β-cell failure are considered important factors in the development of type 2 diabetes. Both characteristics of type 2 diabetes may be triggered by mitochondrial dysfunction. Alcoholism is known as a risk factor for type 2 diabetes. Excessive or chronic alcohol consumption leads to increased oxidative stress and mitochondrial dysfunction in β-cells. Prohibitin (PHB) is an evolutionarily conserved protein that plays a role as a mitochondrial chaperone. Although it has anti-oxidant effects in several cell types, its role in pancreatic β-cells is not known.

In this thesis, we have investigated the role of prohibitin in ethanol (EtOH) treated pancreatic β-cells. The specific aims were to determine:

(a) the effects of ethanol on prohibitin expression in β-cells,

(b) the distribution of prohibitin in β-cells,

(c) the effect of prohibitin on alcohol dehydrogenase (ADH) expression in β-cells,

(d) the effects of prohibitin on mitochondrial function in β-cells,

(e) the role of prohibitin in β-cell survival,

(f) the effect of prohibitin on insulin secretion.

To address these aims, mitochondrial dysfunction was induced in RINm5F and INS-1E pancreatic β-cell lines by treatment with ethanol, which has been shown in other cell types to cause oxidative stress and apoptosis. First, endogenous PHB was determined by western blot and real time polymerase chain reaction (PCR) in RINm5F rat insulinoma cells. The cellular distribution of PHB was visualized using immunocytochemistry and further confirmed using western blot of different cellular fractions. Alcohol dehydrogenase (ADH) expression was detected by western blot and real time PCR. As well, the metabolism of ethanol was determined by assaying ethanol-induced ADH activity as well as by measuring ethanol concentration left in cell culture media after 24 h incubation. Mitochondrial function was determined using various methods including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction, reactive oxygen species (ROS) production, uncoupling protein 2 (UCP2) expression, adenosine triphosphate (ATP) production, mitochondrial respiratory complex activity, and mitochondrial membrane potential. The interaction of PHB to mitochondrial proteins was examined by immunoprecipitation (IP). Cell death was monitored by flow cytometry analysis with fluorescein isothiocyanate (FITC)-annexin V staining and Hoechst 33342 nuclear staining. The pathway involved in apoptosis was identified by immunoblot for activation of c-Jun N-terminal kinase (JNK), expression of the proapoptotic protein Bax, and the cleaved caspase-3 assay. The effect of prohibitin on insulin secretion in β-cells was determined using a rat insulin enzyme linked immunoassay (ELISA).

PHB was expressed in β-cells under normal culture conditions and co-localized with the nuclear probe Hoechst 33342 in the nucleus and with the mitochondrial probe Mitofluor in the perinuclear area. Ethanol treatment increased PHB expression in β-cells

and induced PHB translocation from the nucleus to the mitochondria. Alcohol dehydrogenase 5 (ADH5) was expressed in pancreatic β-cells and increased with ethanol incubation. In addition, the total ADH activity was increased. In ethanol treated β-cells, MTT reduction and ATP production decreased, whereas ROS, UCP2, p-JNK, Bax and cleaved caspase-3 levels increased. Ethanol treatment decreased the interaction of PHB to mitochondrial proteins, impaired the activity of mitochondrial respiratory complexes I and IV, and resulted in a reduction of mitochondrial membrane potential. In addition, flow cytometry analysis of RINm5F cells showed increased apoptosis, while Hoechst 33342 nuclear staining showed small and condensed nuclei after ethanol treatment. In INS-1E rat insulinoma cells, ethanol decreased glucose induced insulin secretion. Exogenously applied PHB or PHB overexpression attenuated ADH activity, prevented the deleterious effects of ethanol on mitochondria and protected from the apoptotic effects of ethanol, whereas PHB knockdown using small interfering RNA (siRNA) of PHB enhanced ethanol induced apoptotic effects on both pancreatic β-cells. In addition, PHB increased the levels of the transcription factors: pancreatic and duodenal homeobox 1(PDX-1) and v-maf musculoaponeurotic fibrosacoma oncogene homolog A (MafA). The aggregate result was a restoration of glucose induced insulin secretion by PHB in ethanol exposed pancreatic β-cells.

In conclusion, ethanol causes mitochondrial dysfunction in pancreatic β-cells by impairing mitochondrial complexes I and IV, and induces apoptosis via the JNK pathway. These harmful effects of ethanol result in a reduction of insulin secretion. PHB prevents ethanol-induced mitochondrial dysfunction, apoptosis, and β-cell failure by stabilizing

mitochondrial complexes I and IV and partially inhibiting ADH activity during ethanol metabolism. PHB in itself increases the levels of β-cell transcription factors. As a result, PHB restores insulin secretion in ethanol exposed pancreatic β-cells.

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ABBREVIATIONS

ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	basepair(s)
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CM-H_2DCFDA	5-(and-6)-chloromethyl 2',7'dichlorodihydrofluorescein diacetate
°C	degree Celsius
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
EtOH	ethanol
EDTA	ethylene diaminetetraacetic acid
FBS	fetal bovine serum
$FADH_2$	flavin adenine dinucleotide, reduced
g	gram
GPx	glutathione peroxidase
GSH	glutathione, reduced
GSHR	glutathione reductase
GSSG	glutathione disulfide
HCl	hydrochloric acid

H_2O	water
KCN	potassium cyanide
KRBH	Krebs-Ringer bicarbonate HEPES buffer
kg	kilogram
KCl	potassium chloride
kDa	kilo Daltons
mtDNA	mitochondrial deoxyribonucleic acid
M	molar
$MgCl_2$	magnesium chloride
mM	millimolar
min (s)	minute (s)
ml	millilitre
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMS	phenazine methosulfate

PHB	prohibitin
PARP	poly-ADP-ribose polymerase
RPMI	Roswell Park Memorial Institute culture medium
RT	room temperature
g	gravitational force
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
s	second
siRNA	small interfering RNA
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBS	tris buffered saline
TBS-T	tris buffered saline with 0.1% Tween 20
TEMED	N,N,N',N-tetramethylethylenediamine
Tris	tris (hydroxymethyl) amino methane
Tween-20	polyxyethylene (20) sorbitan monolaurate
TCA	tricarboxylic acid cycle
µl	microliter
µm	micrometer
µg	microgram

UCP2	uncoupling protein 2
3’-UTR	3’ untranslated region
%	percent

I. INTRODUCTION

Research rationale

Diabetes is a very common metabolic disease characterized by insulin resistance and hyperglycemia. In type 2 diabetes, high blood glucose occurs when pancreatic β-cells fail to compensate for insulin resistance. Pancreatic β-cell dysfunction is recognized as an early event in the development of type 2 diabetes [1]. The increasing prevalence of type 2 diabetes is attributed to unhealthy lifestyle choices, which include taking high calorie diets, lack of physical activity, and smoking. These factors are also associated with oxidative stress and mitochondrial dysfunction.

Although moderate alcohol consumption may have health benefits [2, 3], excessive alcohol consumption has been reported as a risk factor for type 2 diabetes [3]. The diabetogenic effects of ethanol may be due to excessive caloric intake and obesity, induction of pancreatitis, and impairment of liver function [4]. Recent animal studies have shown that ethanol increases insulin resistance in peripheral tissues such as liver and skeletal muscle [5-7]. In humans, insulin secretion was reported to be decreased in association with abnormal glucose tolerance among patients with chronic ethanol-induced pancreatitis [8]. In addition, several studies have proposed that ethanol directly inhibits insulin secretion in hamster insulinoma tumor (HIT) β-cells, isolated rat islets, and perfused rat pancreas [9-12]. On the other hand, there have been contradictory reports, sometimes from the same groups, of ethanol augmenting [13, 14], inhibiting [15] glucose-induced insulin secretion, and priming β-cells to glucose stimulation [16]. These

contradictory reports may be explained by methodological variations such as different cell lines, different ethanol concentrations and exposure time. One recent study reported that ethanol inhibits β-cell metabolic activity, as judged by the MTT assay, without increasing cell death [12]. Presumably, the effect of ethanol on β-cells is mediated by oxidative stress resulting from ethanol metabolism. In RINm5F cells, antioxidant treatment increased β-cell survival against hydrogen peroxide induced oxidative stress [17]. However, although both oxidative and non oxidative pathways of ethanol metabolism are present in the exocrine pancreas [18], pathways of ethanol metabolism have been poorly characterized in pancreatic β-cells. An earlier report documented the presence of alcohol dehydrogenase (ADH) in human endocrine pancreas using immunohistochemistry, but did not determine the cell type involved [19]. Similarly, some ADH isoforms have been detected in murine islets [20] and human pancreatic islets [21], but their function has not been determined.

Even though the mechanisms are not clear, the adverse effects of ethanol could be attributed either to direct toxicity of ethanol or to indirect effects involving its metabolites and generation of reactive oxygen species (ROS) [22, 23]. Epidemiological studies have suggested that ADH polymorphism may influence the risk for type 2 diabetes through metabolite toxicity [24-26]. Excessive alcohol consumption increases the production of ROS as well as mitochondrial dysfunction in liver and brain tissues, which in turn leads to cell injury [27-29]. Mitochondrial glutathione (GSH) plays a critical role in the protection of mitochondria from ROS attack in mitochondrial complexes. In chronic ethanol fed rats, the level of liver mitochondrial GSH was significantly decreased [30].

Although ROS can be useful as signaling molecules in coordinating and regulating some cellular signaling pathways under normal physiological conditions [31, 32], they become toxic oxidant molecules if produced excessively and overwhelm the cellular anti-oxidant systems [33]. Accumulated ROS in the mitochondria may be released to the cytoplasm, and may lead to secondary damages such as the impairment of transport mechanisms, ion channel modification, lipid peroxidation, modification of essential proteins and DNA damage; these all happen in different cellular compartments. Furthermore, the damage occurring to mitochondrial metabolism may accentuate oxidative damage so that it activates cellular death pathways resulting in apoptosis. Increased ROS production is one of the earliest events in glucose intolerance and it may be a mechanism of pancreatic β-cell dysfunction in type 2 diabetes, as β-cells are particularly vulnerable to oxidative stress due to their insufficient anti-oxidant capacity [34-36].

Prohibitin (PHB) is a 30 kDa evolutionarily conserved multifunctional protein and is present in multiple cellular compartments [37], which include the nucleus [38], plasma membrane, and mitochondria, as well as in lipid droplets shed from adipocytes [39] and in the circulation [40, 41]. Originally, PHB was identified as a tumor suppressor in liver tissue [42]. In following reports, the tumor suppressor role of PHB was attributed to the 3' untranslated region (3'UTR) of the PHB mRNA rather than the PHB protein itself [43, 44]. Later on, PHB was also reported as a negative regulator of cell cycle [43, 45]. Recently, PHB has also been shown to be implicated in the regulation of transcription [38], apoptosis, and signal transduction in the Ras-Raf pathway [46, 47]. As well, PHB has been reported to function as an anti-inflammatory molecule [48] and a ligand binding site at the

plasma membrane [49, 50]. However, the best characterized PHB role in the cell is that of a chaperone involved in the assembly of subunits of respiratory complexes in the mitochondria [51, 52].

Thererefore, it is possible that PHB, in its role as a mitochondrial chaperone, protects cells against oxidative stress as reported in yeast [52]. It is not known, however, if PHB is expressed or plays a role in pancreatic β-cells. Thus, the overall goal of this thesis was to investigate the effect of prohibitin on β-cell dysfunction caused by oxidative stress, using as a paradigm the adverse effects of ethanol. The first objective was to investigate the expression and anti-oxidant effects of PHB in pancreatic β-cells exposed to ethanol. The second objective was to investigate the mechanisms by which PHB protects pancreatic β-cells against ethanol.

Literature review

1. Pancreas: Structure and Physiology

The pancreas is one of the major endocrine organs in mammals. It was first described by the Greek anatomist Herophilus. It is an elongated, tapering retroperitoneal organ of about 12 - 15 cm in length, lying in the abdominal cavity behind the stomach [53]. It is divided into three regions, which are called the head, body and tail. The head is an expanded portion lying close to the c-shaped region of the duodenum. The body and tail extend across the midline of the body toward the hilum of the spleen. The pancreatic duct is connected to the duodenum through which pancreatic juice is secreted into the duodenum. The pancreas contains both endocrine and exocrine tissues. Primarily, it participates in digestion and the hormonal regulation of blood glucose levels [53, 54].

The exocrine tissue of the pancreas is known as the tubuloacinar gland, which is organised like bunches of grapes. It consists of both acinar cells and duct cells responsible for food digestion. The exocrine tissue is sprinkled with the smaller endocrine glands, termed the islets of Langerhans [53, 54]. The acinar cells of exocrine tissue produce digestive enzymes and alkaline fluid, which is named pancreatic juice, and secretes pancreatic juice into the small intestine through exocrine ducts in response to small intestine hormones (such as cholecystokinin and secretin). Digestive enzymes contain trypsin, chymotrypsin, pancreatic amylase and lipase for the digestion of carbohydrates, proteins and fat. Thus, the pancreatic juice can digest macromolecules into smaller

molecules and neutralize the acid chyme arriving from the stomach. The digested small molecules are mostly absorbed along the small intestine [53, 54].

In contrast to the exocrine pancreas, the endocrine pancreas is made up of approximately one million cell clusters called islets of Langerhans. The islets of Langerhans were first discovered in 1869 by the German anatomist Paul Langerhans [55]. Islets are scattered throughout the exocrine part of the pancreas and comprise only 2-3% of the whole pancreatic mass [54]. Islets are composed of five main cell types, i.e. α-cells (15-20% of total islet cells), β-cells (65-80%), δ-cells (3-10%), ε-cells (1%), and PP-cells (3-5%). α-cells produce glucagon, which increases blood glucose level; β- cells produce insulin, which decreases blood glucose level; δ-cells produce somatostatin, which inhibits α and β cell secretion as well as exocrine secretion from acinar and duct cells; PP cells produce pancreatic polypeptide, which essentially inhibits exocrine pancreatic secretion; and ε-cells produce ghrelin, which stimulates appetite and growth hormone release [56-58]. The endocrine tissue mainly participates in the regulation of glucose homeostasis and secretes its hormones into the blood stream via exocytosis in response to nutrients, among which glucose has a predominant role [54].

2. Diabetes

2.1 General

Diabetes is a very common metabolic disease characterized by hyperglycemia. At the beginning of the year 2000, the prevalence of diabetes had been estimated to be around 2.8% of the total world population [59]. Unlike in the past several decades [60], the

prevalence of diabetes has been rapidly increasing regardless of age, and striking both the developed and developing countries [61]. Thus, diabetes is a major public health problem at this point in time. The rapidly increasing prevalence brings serious concerns to health care systems because of increasing risk for premature death and serious complications resulting from severe damage to blood vessels in the eyes, kidneys, the heart and nervous system.

Recently, the World Health Organization predicted that the worldwide number of patients with diabetes will increase from 171 million to 300 million by 2025 [61-63]. Moreover, diabetes constitutes 5-10% of all health care costs in the western world and the majority of these costs are due to diabetic complications [64]. For example, the total estimated cost of diabetes in the Unites States amounted to $ 174 billion in 2007, accounting for about one-tenth of all healthcare expenditures [65]. These costs will likely increase in the near future not only in the Unites States but also in other countries. Diabetes is a heterogenous and complex disease, primarily falling into the two broad categories of type 1 and type 2 diabetes [59].

2. 2 Type 1 Diabetes

Type 1 diabetes accounts for 5-10% of all diabetes cases. Although the real cause of type 1 diabetes is still elusive, this condition is known as an autoimmune-mediated disease in which the immune system considers pancreatic β-cells as foreign and destroys them, leading to a reduction of β-cell mass. As a consequence, type 1 diabetes in its overt form is characterized by absolute insulin deficiency [66]. The lack of insulin leads to increased

blood and urine glucose levels. The classical symptoms of type 1 diabetes are polyuria, polydipsia, polyphagia, and weight loss. Patients with type 1 diabetes have to take exogenous insulin for survival and to prevent the development of ketoacidosis [59]. Another feature of type 1 diabetes is the typical, but not invariable, early onset (before 25 age) in comparison to type 2 diabetes.

2.3 Type 2 Diabetes

Type 2 diabetes is the most common form of diabetes and accounts for more than 90% of all patients with diabetes. It is also known as adult onset diabetes because of its classically late onset compared to type 1 diabetes. However, this term is not correct any more due to the increased incidence of type 2 diabetes among young individuals often in association with obesity. It was reported in the mid-2000's that 17% of children in the United States between ages 2 and 19 years were overweight and at risk for developing type 2 diabetes in the future [67]. This change may be caused by pronounced changes in modern human lifestyles [59]. The increasing prevalence of type 2 diabetes may also be associated with increasing prevalence of metabolic syndrome, a condition characterized by high blood pressure, overweight, insulin resistance, elevated serum triglycerides, and reduced serum high density lipoprotein cholesterol [60]. Although metabolic syndrome is not a direct cause for diabetes, it has been known to be an indicator for future development of type 2 diabetes. Type 2 diabetes begins with insulin resistance in pheripheral tissues such as the liver and skeletal muscle, which show a reduced response to insulin. The disease gradually progresses to the point where the pancreatic β-cells do not produce enough insulin required for the regulation of glucose homeostasis. Presently, the cause of insulin resistance and

diabetes progression is not clear. Recently, however, a number of studies have suggested potential mechanisms including impaired mitochondrial function, altered insulin signalling caused by cellular lipid accumulation, pro-inflammatory signals, endoplasmic reticulum stress, and reduced incretin dependent and independent β-cell insulin secretion [68]. Type 2 diabetes is potentially more controllable than type 1 diabetes, if it is recognized early. Its onset is often insidious, but symptoms such as polyuria, polydipsia, polyphagia, and weight loss may occur. Table 1 provides a comparison between type 1 and type 2 diabetes. Apart from these two classical types of diabetes, several monogenic defects have been identified wihich lead to the so-called genetic diabetes. Among genetic diabetes forms of diabetes, the best known is a group of diseases known as maturity–onset diabetes of the young (MODY) , where the genes involved include among others hepatocyte nuclear factor 4 α, (MODY1), Glukokinase gene (MODY 2) , hepatocyte nuclear factor 1 α (MODY 3) [69].

Table 1: Comparison of type 1 and type 2 diabetes

Type 1 diabetes	**Type 2 diabetes**
- Autoimmune β-cell death	- Insulin resistance and obesity
- Absolute insulin deficiency	- Relative insulin deficiency
- Generally diagnosed before age 25	- Usually diagnosed after age 35
- Accounts for 5-10% of diabetes	- Accounts for ~90% of diabetes
- Insulin required for survival	- Insulin may be required for control

3. Oxidative stress

3.1 General

Oxidative stress is defined as the imbalance between the generation of free radicals and the body's antioxidant defense systems [70]. Generally, free radicals such as reactive oxygen species are generated through a variety of cellular biochemical reactions during cellular metabolism. By definition, a free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outer orbital. As the name implies, these free radicals are highly unstable and have higher molecular reactivity [70, 71]. As well, these molecules may diffuse away from their site of generation to other target sites. Thus, increased free radical production can increase cellular injury through reacting indiscriminately with organic molecules such as proteins, lipids, carbohydrates and nucleic acids [72, 73]. As a consequence, these reactions lead to DNA damage, mitochondrial dysfunction and cell membrane damage. For this reason, free radicals have been implicated in the development and progression of various diseases including diabetes, cancer, cardiovascular disease, Parkinson's disease and Alzheimer's disease [31].

3. 2 Production of free radicals

ROS generated through cellular metabolic or chemical reactions include superoxide (O_2^-; the precursor of most ROS and a mediator in oxidative chain reactions), hydroxyl radical (OH^-; one of the strongest oxidants in nature), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$) and hypochlorite [31, 70]. ROS are mainly produced in three ways. Within mammalian cells, NADPH oxidases are the only enzymes able to generate ROS without co-enzymes. NADPH oxidase is present on the cell membrane of

polymorphonuclear cells, endothelial cells and macrophages [74]. In addition, there are several enzyme systems, which transfer electrons to molecular oxygen leading to production of superoxide. These enzyme systems include cytochrome P-450 reductases, nitric oxide synthase, cyclooxygenases, lipoxygenases and xanthine oxidases [31, 75]. In the mitochondrial electron transport chain, ROS is also produced through leaking of electrons to oxygen during ATP production. Mitochondria are an important site of ROS generation as well as major targets of ROS. Accumulation of ROS induces an imbalance or weakness of cellular antioxidant systems, which in turn accelerate ROS generation [76-78]. ROS production is also induced in response to exogenous chemicals such as radiation, pollution, smoke, drug consumption, hormones and other xenobiotic chemicals [79]. Recently, new genetic pathways were discovered in the aged brain. These pathways produce ROS through alterations in the methylation or oxidation of cytosine-phosphate-guanine (CpG) dinucleotide [80].

3. 3 DNA modification

Oxidative modification of DNA, including DNA strand breaks and tandem lesions, frequently occurs at either the bases or the sugar-phosphate backbone [81]. For example, hydroxyl radicals can be inserted into the double bonds of heterocyclic DNA bases, inducing modification of bases such as ring saturation, ring opening, ring contraction, and hydroxylation, which can result in severe damage of DNA molecules [82].

Besides DNA modifications caused by ROS, exocyclic adducts also induce DNA damage. For instance, malondialdehyde (MDA) and 4- hydroxynonenal (4-HNE) produced by lipid peroxidation can react with DNA bases and form exocyclic DNA adducts [83, 84].

As a consequence, oxidative stress induced DNA damage can lead to disruption in transcription, translation, and DNA replication. In addition, the capacity of DNA repair is lost, resulting in mutations in DNA base pairs [85]. DNA mutations can lead to cancer, cell senescence or cell death [86]. DNA impairment can also disturb cell signalling pathways which are important in terms of cellular response [87]. For these reasons, DNA damage has been considered as a major mechanism leading to numerous human diseases.

3. 4 Protein modification

Protein oxidation is generally defined as a covalent modification of a protein induced either directly or indirectly by ROS. Common protein modification modalities include protein carbonylation, sulfur oxidation (S-thiolation, cysteine disulfides, methionine sulfoxide), crosslinking at tyrosine residues**,** tryptophanyl modifications, and amino acid interconversions [88, 89].

Protein modification at critical sites leads to alteration of protein structure or induces protein selective degradation or aggregation. Loss or dysfunction of particular biochemical protein molecules can lead to loss or dysfunction of a particular biochemical function [88]. For example, protein carbonylation caused by ROS themselves or lipid peroxidation products such as MDA [90] can impair enzyme activity or increase the degradation of the protein [91]. Therefore, oxidation of proteins plays a key role in the pathogenesis of a number of diseases such as diabetes [35], atherosclerosis [92] and liver diseases [93, 94], as well as in aging [95].

3. 5 Lipid modification

A free radical prefers to steal electrons from cellular lipid molecules. For this reason, this process is sometimes referred to as the oxidative degradation of lipids. Although the nature of the oxidative injury that causes cell death is not clear, the mechanisms by which oxygen radicals damage membrane lipids are well accepted. Unsaturated fatty acids are abundant in cellular membranes and in low density lipoproteins [96]. The unsaturated fatty acids allow for fluidity of cellular membranes and provide binding sites for receptors. Free radicals formed by enzyme reactions (e.g; lipoxygenases, phospholipase A2, glucose-6-phosphatase and cytochrome P-450) during cellular metabolism can attack lipid membranes [31] and then react with unsaturated lipids resulting in lipid peroxidation. For example, oxidative damage is often exclusively associated with these peroxidation reactions in membrane lipids. Hydrogen peroxide and lipid hydroperoxides activate lipoxygenases [97]. As a consequence, membrane structures or compositions are altered or destroyed [98]. Phospholipase A2 also catalytically hydrolyzes lipids on the membranes [99] and generates free lysophospholipids and free fatty acids resulting in the disruption of membrane structure and function [100]. The plasma membrane plays very important roles in protecting a cell from the outside and is a mediator of extracellular signaling events. Therefore, alteration of membrane structure and function can change intracellular biological activities as follows:

a. by changing membrane fluidity, selective permeability and integrity;
b. through covalent crosslinking of lipid and proteins;
c. by inactivation or inhibition of membrane bound enzymes and receptors.

As a consequence, these alterations can change cellular functions and gene expression, and lead to changes in phenotype [101, 102].

In addition, increased lipid peroxidation products (ketoaldehydes, MDA, 4-HNE, glyoxal, and 4-oxo-2-nonenal) are involved in DNA damage, mutagenesis [103] and induction of diseases such as liver diseases [104] and diabetes [35].

4. Oxidative stress in diabetes mellitus

The development of type 2 diabetes is usually characterized by insulin resistance and hyperglycemia. Pancreatic β-cell function continuously and gradually deteriorates in type 2 diabetes in spite of treatment, and this has been attributed to glucose toxicity due to high blood glucose which results in overproduction of ROS.

Mitochondria are central regulators of cellular energy production as well as the main sites of ROS production. In pancreatic β-cells, mitochondria are pivotal to the control of insulin secretion, which consists of two phases known as the rapid or first phase and the slower or second phase. Both phases of insulin secretion are dependent on glucose metabolism and mitochondrial oxidative phosphorylation, which produces adenosine triphosphate (ATP). Cellular ATP increases through glucose metabolism and then blocks plasma membrane located ATP-sensitive potassium channels, allowing voltage-gated calcium channels to open. The resulting calcium influx triggers the release of insulin-containing granules by exocytosis [105]. The rapid, first phase is completed within 5 minutes. In this phase, insulin granules in the readily released pool are already docked to the plasma membrane. This phase of insulin secretion accounts for only about 5% of the

total insulin released after increasing blood glucose. In the slower, second phase, maximal quantities of insulin are released in about 60 minutes. Insulin granules are first moved from the reserve pool to the readily released pool of granules, and then docked onto the plasma membrane to be released. This phase is quantitatively the major contributor to insulin secretion [106]. These glucose induced insulin responses are impaired or lost in patients with diabetes.

ATP synthesis required for insulin secretion is reduced by mitochondrial dysfunction. Impaired mitochondrial function further leads to ROS production, and increased ROS production accentuates mitochondrial dysfunction or activates novel protein kinases in both peripheral tissues and pancreatic β-cells [1, 68]. Pancreatic β-cells are particularly vulnerable to oxidative stress due to their low levels of antioxidant enzymes (superoxide dismutases, catalase and glutathione peroxidase) compared to other organs [107, 108]. Several studies have suggested that oxidative stress is generated under diabetic conditions and is possibly involved in the ongoing pancreatic β-cell dysfunction characteristic of diabetes [109]. In experimental animals, antioxidant treatment suppresses apoptosis in β-cells, restores insulin synthesis, insulin content and insulin secretion under hyperglycemic conditions. This supports the hypothesis that oxidative stress induces apoptosis in β-cells and reduces β-cell mass [110, 111].

Taken together, these studies indicate that oxidative stress and mitochondrial dysfunction are implicated in β-cell dysfunction in diabetes and that antioxidant treatment can protect β-cells against glucose toxicity [35].

5. Antioxidants

5.1 Antioxidant System

Antioxidants are defined as any substance being capable of delaying or preventing the oxidation of cellular molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent resulting in production of free radicals. Antioxidants provide their own electrons to free radicals. After a free radical gets the electron from an antioxidant, it becomes stable and can no longer attack the cell [33]. Using antioxidants has been reported as a therapeutic strategy to counteract cellular injury in certain conditions including diabetes, aging, hypertension, atherosclerosis, metabolic syndrome, inflammation, liver disease and neurological disorders (Alzheimer's and Parkinson's diseases).

Antioxidants are produced within the cell or can be provided exogenously. Antioxidants have been catagorized as hydrophilic or hydrophobic. Hydrophilic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione, catalase, ascorbic acid (vitamin C) and uric acid. The list of hydrophobic antioxidants is shorter and includes tocopherols (vitamin E), flavonoids and coenzyme Q_{10}. According to their mechanisms of action, antioxidants can also be classified as enzymes (e.g. superoxide dismutases, catalase, glutathione peroxidase) and non-enzymatic systems (e.g. vitamin C, vitamin E, selenium, copper, zinc) [33]. In addition, there are compounds (e.g. free amino acids, peptides and proteins) which can act as ROS scavengers at high concentrations.

6. Mitochondria

6.1 Mitochondrial structure and role in oxidative repiratory chain

Mitochondria are double membrane-enclosed cellular organelles present in most eukaryotic cells with a size of about 0.5- 10 µm in diameter [112]. Mitochondria consist of several compartments including the outer membrane, the inner membrane, the inter-membrane space, cristae and matrix. The inner membrane contains mitochondrial respiratory complexes and the matrix contains the components of the TCA cycle and the β-oxidation pathway, which generate acetyl-CoA from pyruvate and acyl chains, and reducing cofactors such as NADH and $FADH_2$ [68, 113].

With the exception of the nucleus, the mitochondria are the only cellular organelles to possess their own genetic material, called mitochondrial DNA (mtDNA). The circular mtDNA consisting of 16,569 base pairs encodes twenty-two transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs) and thirteen polypeptides that are translated within the mitochondria, all of which are essential for electron transport systems and adenosine triphosphate generation, and consequently for normal cellular physiology [114, 115]. Cellular energy is produced by diverse enzymes within the electron transport chain, which is comprised of 5 complexes [113].

Complex I

Complex I is also called NADH dehydrogenase. It consists of more than 40 protein subunits, seven of which are encoded by mtDNA [113]. The function of complex I is to remove two electrons from the reduced cofactor, NADH, and then transfer them to a lipid-

soluble carrier, namely ubiquinone (Fig. 1). For this reason, it is also called NADH: ubiquinone oxidoreductase. The reduced ubiquinone molecules diffuse along the membrane. At the same time, four protons are translocated across the inner mitochondrial membrane, resulting in a proton gradient. Complex I has also been known as one of the main sites for production of the superoxide anion (or free radical) due to easy leakage of premature electrons to oxygen. The released electron produces superoxide anion when reacting with oxygen. The superoxide anion is considered as a major intracellular substrate for peroxynitrite, hydrogen peroxide and hydroxyl radicals [103, 116-118].

Complex II

Complex II, also named succinate dehydrogenase (SDH) or succinate-coenzyme Q reductase, is located at the matrix side of the mitochondrial inner membrane, unlike the other mitochondrial complexes. It consists of four protein subunits (SDHA, SDHB, SDHC, and SDHD) encoded by nuclear DNA [113]. It reduces succinate, fatty acids and glycerol 3-phosphate, and then transfers electrons to the quinine pool (Q) called ubiquinone, without pumping protons into the mitochondrial intermembrane space (Fig. 1). Complex II does not function as a proton pump unlike mitochondrial complexes I, III and IV [118-120].

Complex III

Complex III is also known as cytochrome b-c_1 complex or ubiquinol cytochrome c oxidoreductase. The complex contains 11 different subunits; one subunit (cytochrome b) is encoded by mitochondrial DNA, and the remaining subunits are encoded by nuclear DNA

[113]. Complex III removes electrons from ubiquinol (QH_2), which is the reduced form of ubiquinone, also known as coenzyme Q (CoQ). Subsequently, the removed electrons are transferred to cytochrome c (a water soluble electron carrier), which in turn donates them to complex IV (Fig. 1). At the same time, the complex builds a proton gradient in the mitochondrial intermembrane space. Hence, it may leak electrons to oxygen when electron transfer is altered or impaired, which in turn may result in the formation of free radicals such as superoxide. Therefore, this complex is considered to be a major site for production of the superoxide anion [118, 120].

Complex IV

Complex IV, also called cytochrome c oxidase or cytochrome c oxidoreductase, is a complex of 13 different subunits [113]. Three of these subunits (I, II, and III) are encoded by mitochondrial DNA and ten are encoded by nuclear DNA. The complex contains two heme groups and two copper atoms as prosthetic groups. It takes electrons from cytochrome c and transfers them to molecular oxygen to produce water (Fig. 1). At the same time, it generates a proton gradient by moving protons across the mitochondrial membrane. Genetic alterations of complex IV have been shown to be a major cause of mitochondrial abnormalities in diabetes and Alzheimer disease [118, 120].

Complex V

Complex V is an ATP synthase, which is responsible for cellular ATP production. It consists of approximately 16 protein subunits, among which two subunits are encoded by mtDNA [113]. This step is a terminal step in the mitochondrial respiratory chain. ATP

synthase is present in the membrane and produces ATP from ADP and phosphate (P_i) driven by a flux of protons using the previously formed proton gradient (Fig. 1). The flux goes from the side with high proton electrochemical potential (protochemically positive side) to the protochemically negative side [118, 120].

6.2 The functions of mitochondria

Mitochondria mainly participate in cellular energy production in which they convert energy from food into chemical energy in the form of ATP. In addition, mitochondria are involved in multiple essential cellular events or functions such as cell cycle, growth, differentiation, signaling, death (apoptosis); the regulation of cytoplasmic calcium homeostasis; the synthesis of all cellular iron-sulfur clusters, biosynthesis of steroids, metabolic detoxification, and generation of ROS [68, 121]. Recently accumulated evidence has indicated that mitochondria are much more important than previously thought and have been implicated in the development of several human diseases, including mitochondrial disorders, diabetes and cardiac dysfunction. It has even been suggested that mitochondria play a role in the aging process [1, 113, 114].

6.3 Effect of ROS on mitochondria

Over the last several decades, many studies have suggested that numerous human diseases are associated with the generation of cellular oxidative stress. Mitochondria are a major site of cellular ROS formation and at the same time a major target of oxidative stress. ROS are free radicals that react very easily with other cellular molecules due to the presence of unpaired electrons. Such molecules may damage mitochondrial

macromolecules. The macromolecules that are damaged include proteins, lipids and mitochondrial nucleic acids. Macromolecule damage may deplete mitochondrial antioxidant systems such as glutathione peroxidase and glutathione [29]. Moreover, mtDNA is more vulnerable to oxidative stress compared to nuclear DNA due to the absence of a local DNA repair system [86, 114].

Therefore, mtDNA is a critical cellular target for oxidative damage. As a consequence, it may also lead to increased susceptibility to lethal oxidative injury through the loss of function of the electron transport system, loss of mitochondrial membrane potential, and reduction of ATP generation [122]. These injuries increase the sensitivity of cells to other proapoptotic or damaging signals, ultimately resulting in cell death [116].

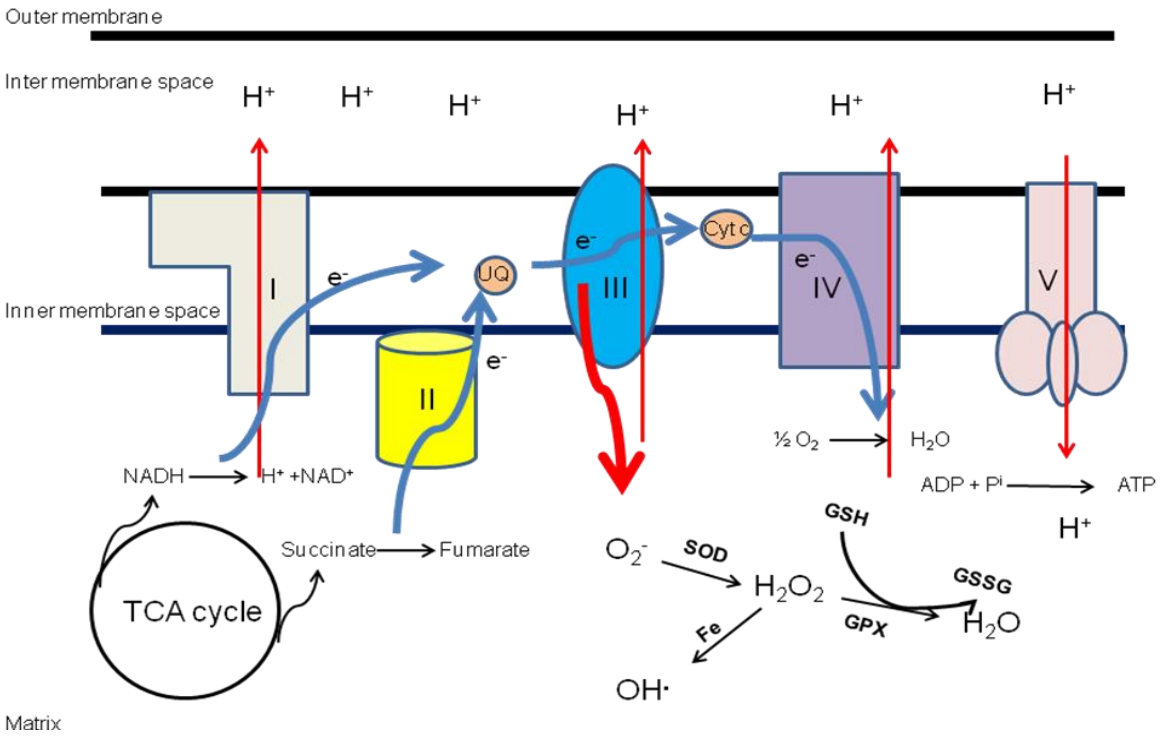


Figure 1: Schematic diagram of the oxidative respiratory chain in the mitochondria

TCA cycle produces reducing cofactors such as NADH. The accumulated reducing cofactors in the mitochondria are further oxidized in mitochondrial oxidative respiratory chain by complexes I and II. Overflow of electrons released from NADH or succinate sometimes combine with oxygen at the level of complexes I, III or IV, which leads to formation of superoxide as the main source of reactive oxygen species.
TCA cycle: Tricarboxylic acid cycle; e-: Electron; UQ: Ubiquinone; cyt: Cytochrome c; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; GSH: Reduced glutathione; GSSG: Glutathione disulfide (oxidized glutathione); NAD: Nicotinamide adenine dinucleotide; NADH: Reduced nicotinamide adenine dinucleotide
I: NADH dehydrogenase (mitochondrial complex I)
II: Succinate dehydrogenase (mitochondrial complex II)
III: Cytochrome b-c_1 complex (mitochondrial complex III)
IV: Cytochrome c oxidase complex (mitochondrial complex IV)
V: ATP synthase (mitochondrial complex V)

7. Cell death

7.1 General

Naturally, all living organisms must eventually die. However, cell death is not a simple event among organisms. It is an important cellular event involved in development, disease and the aging process. In multicellular organisms, cell death is classified as apoptosis, necrosis or autophagy, depending on the mode of death [121]. Apoptosis and autophagy are known as two fundamental types of programmed cell death whereas necrosis is commonly known as non-programmed cell death [123, 124]

7.2 Apoptosis

Apoptosis originated from an ancient Greek word that describes leaves falling from a tree, which is commonly thought of as a symbol of death. Cell undergoing apoptosis possesses specific morphological, biochemical and molecular characteristics [125]. The distinction between apoptosis and necrosis is due to the differences in plasma membrane integrity during the entire cell death process. In apoptosis, cellular plasma membrane integrity is preserved until late in the process ensuring that the event is regulated. In contrast to apoptosis, necrosis collapses the cell membrane at the beginning of the death event, and the cell cannot control the whole process in an orderly manner [126, 127]. The initiation of apoptosis begins with the degradation of cytoskeletal proteins by aspartate-specific proteases, thereby resulting in the collapse of subcellular components. The process involves chromatin condensation, nuclear fragmentation, and the formation of plasma-membrane blebs. In spite of cell death, apoptosis promotes the recycling of biological molecules and does not induce an immune response [121, 128].

7.2.1 Apoptotic pathways

Apoptosis is driven by two different pathways including the extrinsic pathway (death-receptor pathway) and the intrinsic pathway (mitochondrial pathway).

Extrinsic pathway

The extrinsic pathway is mediated by death receptors on the plasma membrane surface that respond to death signals such as Fas ligand, tumor necrosis factor (TNF)-α and tumor necrosis-related apoptosis-inducing ligand (TRAIL). These are key players in apoptosis and can activate the caspase cascade. For example, members of the TNF super-family bind to cell surface through TNF receptor 1 (TNFR1), which initiates the formation of the multi-protein death-inducing signaling complex with TNFR1-associated death domain protein (TRADD) [129]. Activation of this complex causes conformational changes in its components on the intracellular portion of the plasma membrane, which recruits TNF receptor-associated factor 2 (TRAF-2) leading to the activation of NF-κB and the c-Jun-N-terminal kinase (JNK) pathway, or triggers the catalytic activity of caspase 8, which is a central mediator in the apoptotic pathway. This extrinsic pathway can induce cell death directly without a mitochondrial amplifying step or indirectly through a mitochondrial amplifying step [130]. However, the ligand for Fas (FasL or CD95L) activates apoptosis through the death-inducting signaling complex (DISC) formation. This signal recruits Fas-associated protein with death domain (FADD) without TRADD involvement. Thus, this pathway is relatively fast and is not associated with classic signaling pathways. TRAIL initiates apoptosis through the death receptor 4 (DR4) or death receptor 5 (DR5) in a similar manner to FasL [131].

Intrinsic pathway

The intrinsic pathway is activated after intracellular sensors indicate overwhelming cell damage. Mostly, the activators of the pathway are increased intracellular reactive oxygen species, unfolded or misfolded proteins, DNA damage, nutrient deprivation and lack of growth factors. These activators ultimately lead to a loss in the membrane potential of mitochondria and increase mitochondrial permeability, thereby promoting the release of proapoptotic proteins such as cytochrome c into the cytosol [132, 133]. Once cytochrome c is released, it binds with apoptotic protease activating factor 1 (Apaf-1) and ATP, which then binds to caspase 9 creating an apoptosome. Another important protein in the intrinsic pathway is diablo homologue (SMAC/DIABLO), which antagonizes cytosolic inhibitors of proapoptotic proteins, thus allowing the activation of caspases and hence the progression to apoptosis. As a consequence, activated caspase 8 (death receptor pathway) and caspase 9 (mitochondrial pathway) activate caspases 3, 6, and 7. These activated proteases lead to cell death by cleaving numerous proteins and activating DNases [121, 128, 132]. Factors that determine which death pathway is activated include the stage of the cell cycle, and the type and magnitude of the apoptotic stimulus [121, 129].

7.3 Autophagy

In addition to apoptosis, autophagy is another form of programmed cell death. Autophagy derives from a Greek word meaning “to eat (phagy) oneself (auto)”, in reference to the formation of a cytoplasmic isolation membrane (phagophore) where cellular components are degraded by lysosomal hydrolases. Three types of autophagy

(macroautophagy, microautophagy, and chaperone-mediated autophagy) have been identified on the basis of how lysosomes receive material (cargo) for degradation. Macroautophagy envelops the cargo with a double-membrane structure forming the autophagosome. Phagophore formation is initiated by beclin-1, a regulator of kinase activity, which binds to vacuolar protein sorting 34 (a member of the phosphoinositide 3 kinase family) and triggers the nucleation of the phagophore from the endoplasmic reticulum. The phagophore eventually matures and becomes an autophagosome through a concerted action of several proteins including autophagy related genes, ubiquitins, and proteases [134]. The outer membrane of the autophagosome ultimately fuses with lysosomes and the cargo is degraded. In microautophagy, the lysosomal membrane invaginates and engulfs the cargo for degradation in the newly formed phagophore. The chaperone-mediated autophagy uses unfolded proteins which translocate across the lysosomal membrane under the influence of cytosolic chaperones such as heat-shock cognate protein70. The lysosome-associated membrane protein type 2A, which is a receptor in the lysosomal membrane, is a critical component of the chaperone-mediated autophagy. Importantly, this system is also stimulated by oxidative stress such as that induced by ethanol, and dysregulation of autophagy is associated with various diseases such as neurodegeneration, cardiomyopathy and type 2 diabetes [121, 135]. Recent evidence suggests that autophagy can be selective and targeting pertinent cargoes, or non-selective and directed towards big chunks of cytoplasmic material. Thus, autophagy is a cellular adaptive response to sublethal stress, such as nutrient deprivation, that supplies the cell with metabolites for fuel. Through this process, cells degrade their own nonessential, redundant, or damaged organelles and macromolecular components which are thereby

recycled for ATP production [121, 135, 136]. Therefore, autophagy may be considered as cell survival mechanism under conditions of reduction of cellular energy availability.

7.4 Necrosis

In contrast to both apoptosis and autophagy, necrosis is a form of non-programmed cell death that occurs in response to severe damage or acute cellular stress. These stresses include the depletion of ATP caused by metabolic failure, injury, infection and inflammation [121, 137]. In necrosis, cellular organelles first swell or their membrane is ruptured and then they release intracellular contents. On compromising or collapsing organellar membranes, proteolytic enzymes released from lysosomes enter the cytosol, and induce the degradation of essential proteins involved in cell survival [124, 138]. In addition, reactive oxygen species, poly-ADP-ribose polymerase (PARP), increased intracellular calcium ions, calcium-activated non-lysosomal proteases (calpains) and cathepsins have been known to act as mediators for necrosis [139, 140]. Due to the uncontrolled nature of this form of cell death, necrosis is accompanied by inflammatory responses through the release of intracellular contents after the collapse of the plasma membrane.

8. Ethanol

8.1 General

Ethanol is soluble both in water and lipids. Thus, it can diffuse rapidly through the plasma membrane unchanged along the whole length of the digestive tract [141]. Absorption of consumed ethanol takes place in the stomach (about 20%) and the small

intestine (about 80%). The majority of the absorbed ethanol is metabolized quickly by enzymes in the liver, which is a major site of ethanol metabolism under physiological conditions. Ethanol metabolism increases cellular reactive oxygen species, which may lead to oxidative processes including deterioration of the mitochondrial function. In turn, mitochondrial dysfunction may contribute to cellular ROS production in cells exposed to ethanol [22].

Ethanol induced ROS production can oxidatively modify and inactivate mitochondrial proteins, which further disrupt the structure and function of the mitochondria and further increase ROS production [142]. Mitochondrial DNA is also a critical target of increased ROS due to a lack of a DNA repair system. For example, mouse hepatic mtDNA decreased by 50% after acute exposure to ethanol [143]. Even in other tissues which express lower levels of ADH, mtDNA levels decrease after acute ethanol exposure [144]. The increased ROS production may activate cell death signaling pathways such as apoptosis signaling kinase-1 (ASK-1) which activates p38 mitogen-activated protein kinase (MAPK) and c-Jun-N-terminal kinase stress signaling cascades [145]. All of the above may be the cause of cell injury or death in cells exposed to ethanol.

8.2 Pathways of ethanol metabolism

Ethanol-induced oxidative stress pathways include metabolic pathways (direct effects) and non-metabolic pathways (indirect effects) [146]. In the metabolic pathway, ethanol is metabolized through three enzymes: alcohol dehydrogenase (ADH), microsomal ethanol-oxidizing system (MEOS) and catalase (Fig. 2). On the other hand, the non-metabolic

pathway leads to the formation of fatty acid ethyl esters (FAEEs) by enzymatic esterification of ethanol and fatty acids. Both pathways may produce free radicals, which influence cellular redox status and interfere with the cellular antioxidant system [141, 146].

8.2.1. Alcohol dehydrogenase

ADH, a zinc-containing enzyme, was discovered in the mid-1960s and is responsible for the majority of the alcohol metabolism in the liver and other tissues. Seven isoforms of ADH named ADH1-7 have been identified [147]. ADH converts alcohol into acetaldehyde, which is ten fold more toxic than ethanol. In this reaction (Fig. 2), which takes place in the cytosol, ADH transfers a hydride from ethanol to NAD^+ (nicotinamide adenine dinucleotide) which acts as the hydrogen acceptor and produces the reducing cofactor NADH [141, 148].

8.2.2. Microsomal Ethanol-Oxidizing System

The microsomal ethanol-oxidizing system (MEOS) also participates in ethanol metabolism. This pathway consists of several enzymes found in microsomes. The primary component is a cytochrome P450, which includes several isoenzymes such as the 2E1 (CYP2E1), 1A2 and 3A4 isoforms. Ethanol metabolism by the cytochrome P450 isoenzymes (Fig. 2) produces acetaldehyde and H_2O_2 as final products [149].

This pathway is activated as a second line reaction in response to excessive ethanol that overwhelms ADH capacity. Activation of this pathway has also been linked to increased generation of hydroxyl radicals [150].

8.2.3. Catalase

Catalase is a heme enzyme found in peroxisomes and is triggered by acute ethanol load in the presence of H_2O_2, which increases with peroxisomal fatty acid oxidation in the liver. Catalase uses H_2O_2 to peroxidize ethanol to acetaldehyde (Fig. 2) and this reaction also produces water [151].

8.2.4. Aldehyde dehydrogenase

Through the above three pathways ethanol oxidation gives rise to acetaldehyde, which is further oxidized to acetate by aldehyde dehydrogenases (ALDH): $CH_3CHO + NAD^+ \rightarrow CH_3COOH + NADH + H^+$

Acetate is then transformed into acetyl CoA by acetyl CoA synthase in the mitochondria [148]. In additon, ethanol oxidation increases cytosolic $NADH/NAD^+$ ratio. The accumulated NADH in the cytosol then is indirectly conveyed to the mitochondria for oxidation. The electron is first transferred onto oxaloacetate to form malate, which then enters the mitochondria carried by the malate-aspartate shuttle and passes the electron onto NAD^+ to form NADH [152]. This regenerated NADH is subsequently reoxidized within the mitochondrial electron transport system, which contributes to the proton gradient in the inner mitochondrial membrane. This is a major source of energy required for ATP production by the ATP synthase complex. On the other hand, the enhanced reducing pressure on the mitochondrial electron transport chain due to increased $NADH/NAD^+$ ratio leads to increased ROS production in both acute and chronic ethanol exposure [116, 141]. In addition, ethanol metabolism reduces the levels of mitochondrial gluthathione resulting in a reduction of cellular antioxidant capacity.

8. 3 The role of ethanol in oxidative stress

Ethanol-induced oxidative stress is associated with the metabolism and non-metabolism of alcohol. Since the involvement of oxidative stress in ethanol toxicity was first proposed in the early 1960s [153], many studies have shown that ethanol promotes the formation of free radical intermediates in several cell types, such as hepatocytes and endothelial cells [154, 155]. Ethanol metabolites are generated in the cytosol and are further metabolized in the cytosol and mitochondria, which in turn increases redox pressure and promotes ROS production [116]. As well, alcohol may alter the levels of certain metals such as iron, known to alter cellular redox. Ethanol abuse in human subjects leads to impaired utilization or increased abnormal deposition of iron in the liver [156]. Ethanol also increases the expression of the transferrin receptor, resulting in an increase of iron uptake in rat hepatocytes [157]. In addition, ethanol reduces cellular antioxidant systems, which further facilitates ROS formation. GSH homeostasis is important in preventing ethanol-mediated oxidative injury. Chronic ethanol intake lowers the level of mitochondrial GSH, which precedes the development of mitochondrial dysfunction and lipid peroxidation [158-160]. Moreover, ethanol decreases the enzymatic activity of superoxide dismutase, catalase and GSH peroxidase in rat liver [161]. Decreased antioxidant systems and increased lipid peroxidation have been linked to an impairment of mitochondrial oxidative phosphorylation and mitochondrial dysfunction. As a consequence, the induction of oxidative stress is associated with a collapse of the mitochondrial membrane potential, which results in apoptosis [162]. Brain tissue is more susceptible to oxidative stress due to relatively low levels of antioxidant enzymes compared with its high consumption of oxygen. Ethanol impaires mitochondrial membrane integrity and induces apoptosis through

the activation of caspases in cerebellar granule neurons [27]. In contrast, the use of antioxidants or free radical scavengers reduces cellular injury in ethanol fed rodents [163-167]. Collectively, these studies indicate that ethanol, either directly or indirectly through its metabolism, induces cellular oxidative stress and mitochondrial dysfunction, which may result in cell death.

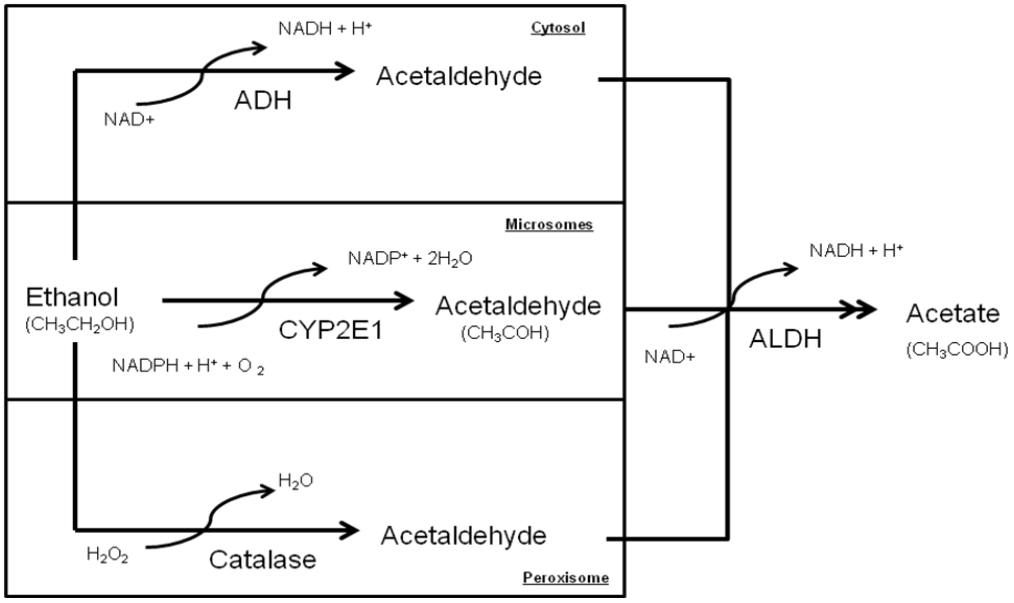


Figure 2: Three pathways involved in ethanol metabolism

In the metabolic pathway, ethanol is metabolized through three enzymes (alcohol dehydrogenase, CYP2E1 and catalase) in different cellular compartments (cytosol, microsomes and peroxisomes, respectively), and all these reactions generate acetaldehyde, a highly reactive product.

9. Prohibitin

9.1 General

Prohibitin (PHB) is a 30 kDa multifunctional protein. It has been found in multiple cellular compartments including the cell nucleus in breast and prostate cancer cells [38, 168], mitochondria in human fibroblasts and prostate cancer cells [51, 52, 168], and plasma membrane in intestinal epithelial cells [37, 48]. Originally, PHB was identified as a tumour suppressor in liver tissue [42, 43] and was hence called prohibitin. Later on, the reason for this activity was attributed to the 3'-UTR (untranslated region) of the prohibitin mRNA [44]. PHB belongs to a family of proteins known as SPFH (Stomatin, prohibitin, Flotilin, HflC and HflK) family of proteins which are associated with lipid rafts in the plasma membrane [169]. Although it was originally identified as a tumour suppressor protein, with mutations occurring in various cancers, many studies have suggested that PHB has diverse cellular roles such as a regulator of the cell cycle [43, 45, 170], inhibitor of cell growth and an anti-inflammatory protein [48]. Recently, studies have suggested that PHB may be a regulator of transcription [38], a ligand binding site at the plasma membrane protein participating in signal transduction [46, 47, 171] and a secreted protein [172] found in the circulation [40, 41].

9.2 The roles of PHB in the mitochondria

PHB is best known as a mitochondrial chaperone. PHB forms a complex with PHB2 in the mitochondria where it prevents membrane protein degradation by the mitochondrial m-AAA protease [173]. The PHB complex has also been reported to hold and stabilize unassembled mitochondrial proteins related to respiratory complex IV in yeast [52] as well

as to stabilize the mitochondrial genome [174, 175]. The PHB complex is also associated with mitochondrial morphogenesis. Loss of the PHB complex leads to severe changes in mitochondrial morphology in *C. elegans*, mouse embryo fibroblasts (MEFs) and HeLa cells [176-178], and deletion of PHB or PHB2 results in destabilization of Optic atrophy 1 protein (OPA1) [176, 178], which is required for mitochondrial fusion. Moreover, there are several reports on the effects of PHB on mitochondrial function in yeast and plants, where loss of PHB genes reduced the mitochondrial membrane potential [179, 180] or mitochondrial cristae [181]. Recently, PHB-knockdown in endothelial cells was shown to induce mitochondrial depolarization due to a reduction of complex I activity [182], whereas mitochondrial PHB expression in cardiomyocytes increased with injury in chronically stressed rats and protected against oxidative stress-induced cell death [183, 184]. Bailey and colleagues showed that chronic ethanol exposure increases PHB expression in rat liver. However, PHB expression decreased with S-adenosylmethionine, a glutathione precursor, which prevented ethanol-induced mitochondrial dysfunction [185]. A recent study showed that the interaction of prohibitin with mitochondrial complex IV was reduced in liver cells transfected with hepatitis C virus core protein and that this lack of association resulted in activity reduction for this complex [186]. To our knowledge, there have been no reports of interaction of PHB with mitochondrial complexes II or III. Taken together, accumulating evidence shows that depletion of PHB result in increased sensitivity to oxidative stress [177, 181]. Figure 3 provides a summary of roles of PHB in the mitochondria.

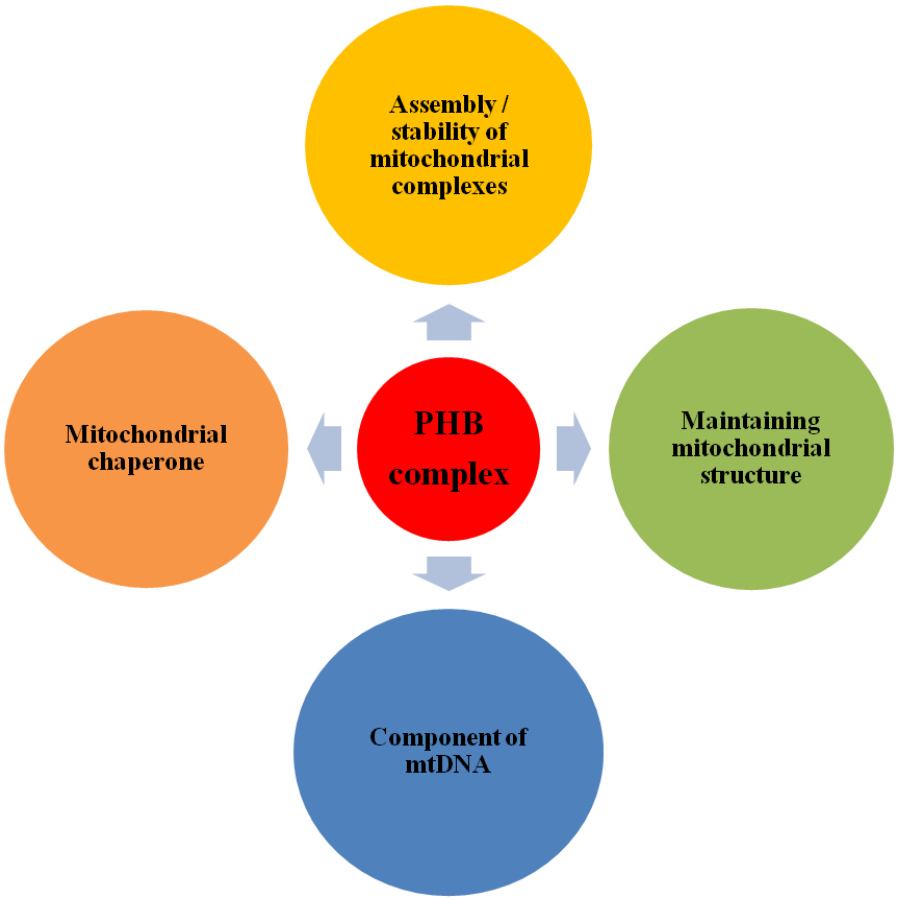


Figure 3: A summary of roles of PHB in the mitochondria

The PHB complex has been proposed as playing diverse roles in the mitochondria. This diagram is showing the main functions reported in the literature.

II. HYPOTHESIS & OBJECTIVES

Hypothesis

Recent studies have suggested that prohibitin may play an important role in the cellular antioxidant defence system due to its role as a mitochondrial chaperone. This function, however, has not been demonstrated in pancreatic β-cells and prohibitin was not known to be expressed in these cells. The hypothesis of this study is that prohibitin can prevent β-cell failure characteristic of diabetes by reducing oxidative stress, protecting mitochondrial function, and promoting β-cell survival.

Objectives

The overall objective of this thesis is to examine the role of prohibitin in ethanol treated pancreatic β-cell function. To clarify this, the specific aims are to determine:

(a) the effects of ethanol on prohibitin expression in β-cells,

(b) the distribution of prohibitin in β-cells,

(c) the effect of prohibitin on alcohol dehydrogenase (ADH) expression in β-cells,

(d) the effects of prohibitin on mitochondrial function in β-cells,

(e) the role of prohibitin in β-cells survival,

(f) the effect of prohibitin on insulin secretion.

III. MATERIALS AND METHODS

A. Materials

Chemicals used in this thesis were purchased from the companies listed in Table 2.

Table 2: Materials used in the experiments

Product	Company
(Monoclonal)-anti-JNK antibody	Santa Cruz (Santa Cruz, CA, USA)
(Monoclonal)-anti-p-JNK antibody	Santa Cruz (Santa Cruz, CA, USA)
(Polyclonal)-anti-ADH5 antibody	Santa Cruz (Santa Cruz, CA, USA)
(Polyclonal)-anti-cytochrome c oxidase subunit III antibody	Santa Cruz (Santa Cruz, CA, USA)
(Polyclonal)-anti-Bax antibody	Santa Cruz (Santa Cruz, CA, USA)
(Monoclonal)-anti-actin antibody	Sigma-Aldrich (Mississauga, CA)
(Polyclonal)-anti-PHB antibody	Santa Cruz (Santa Cruz, CA, USA)
(Polyclonal)-anti-histone H1 antibody	Santa Cruz (Santa Cruz, CA, USA)
(Polyclonal)-anti-cleaved caspase-3 antibody	Cell Signalling Technology (Denvers, MA,USA)
(Monoclonal)-anti-caspase-3 antibody	Cell Signalling Technology (Denvers, MA,USA)
(Polyclonal)-anti-uncoupling protein-2 antibody	Alpha Diagnostic International (San Antonio,TX, USA)
Anti-rabbit-FITC antibody	Santa Cruz (Santa Cruz, CA, USA)
Anti-His-FITC antibody	Molecular probes (Burlington, CA)
(Polyclonal)-anti-MafA antibody	Santa Cruz (Santa Cruz, CA, USA)

(Polyclonal)-anti-PDX-1 antibody	Santa Cruz (Santa Cruz, CA, USA)
(Monoclonal)-anti-threonine antibody	Sigma-Aldrich (Mississauga, CA)
ATP bioluminescent assay kit	Sigma-Aldrich (Mississauga, CA)
Control (scramble) siRNA-A	Santa Cruz (Santa Cruz, CA, USA)
Cell culture flasks	Corning Incorporated (Corning , NY, USA)
CM-H_2DCFDA	Molecular Probes (Burlington, CA)
Complex I Enzyme Activity Microplate assay kit	Mitosciences (Eugene, OR, USA)
Complex II Enzyme Activity Microplate assay kit	Mitosciences (Eugene, OR, USA)
Cytochrome c	Sigma-Aldrich (Mississauga, CA)
Chamber slide	Nalge Nunc International (Tokyo, Japan)
DMSO	Sigma-Aldrich (Mississauga, CA)
100 mM dNTP	Invitrogen (Burlington, CA)
EGTA	Sigma-Aldrich (Mississauga, CA)
Electrophoresis/electroblotting materials	Bio-Rad (Hercules,CA, USA)
EDTA	Sigma-Aldrich (Mississauga, CA)
Enhanced chemiluminescence kit	Amersham (Manassas, NJ, USA)
Ethanol	Health Sciences Centre (Winnipeg, CA)
Fetal bovine serum	Invitrogen (Burlington, CA)
FuGENE HD transfection reagent	Roche (Penzberg, Germany)
Glucose	Sigma-Aldrich (Mississauga, CA)
His-tagged recombinant human prohibitin	AmProx American Proteomics

	(Carlsbad, CA, USA)
Hoechst 33342	Molecular Probes (Burlington, CA)
Heat shock protein-polyclonal 60 antibody	Santa Cruz (Santa Cruz, CA, USA)
Horseradish peroxidase-conjugated secondary antibody	Santa Cruz (Santa Cruz, CA, USA)
Hydrogen peroxide	Sigma-Aldrich (Mississauga, CA)
HEPES	Sigma-Aldrich (Mississauga, CA)
INS-1E cells (Rat)	Provided by Universities of Geneva & Toronto
KCN	Sigma-Aldrich (Mississauga, CA)
4 methyl pyrazole	Sigma-Aldrich (Mississauga, CA)
MitoPT^{TM} JC-1 Assay kit	ImmunoChemistry Technologies (Bloomington, MN, USA)
2-mercaptoethanol	Fisher Scientific (Ottawa, CA)
MTT	Sigma-Aldrich (Mississauga, CA)
Mitochondrial dye Mitofluor ^{TM} Red 589	Molecular Probes (Burlington, CA)
Microplates	Corning Incorporated (Corning, NY,USA)
Mouse prohibitin siRNA	Santa Cruz (Santa Cruz, CA, USA)
Opti-MEMI reduced-serum medium(1×), liquid	Invitrogen (Burlington, CA)
Optical adhesive film	Applied Biosystems (Foster city, CA, USA)
Optical 96-well reaction plate	Applied Biosystems (Foster city, CA, USA)
pCMV6-XL5 vector containing human PHB	Origene Technologies (Rockville, MD,USA)

Penicillin	Invitrogen (Burlington, CA)
Protease inhibitor cocktail tablets	Roche (Penzberg, Germany)
Protein Assay Kit	Bio-Rad (Hercules,CA, USA)
Protein A agarose	Upstate (Billerica, MA, USA)
PBS	Invitrogen (Burlington, CA)
5 ml polystyrene round-bottom tube (BD Falcon)	Fisher Scientific (Ottawa, CA)
PMSF	Fisher Scientific (Ottawa, CA)
Paraformaldehyde	Sigma-Aldrich (Mississauga, CA)
RNase free water	Sigma-Aldrich (Mississauga, CA)
Rotenone	Sigma-Aldrich (Mississauga, CA)
RPMI 1640 medium without glucose	Invitrogen (Burlington, CA)
Random primer	Invitrogen (Burlington, CA)
RINm5F rat insulinoma cells	American Type Culture Collection (Manassas, VA, USA)
RPMI 1640 medium	American Type Culture Colletion (Manassas, VA, USA)
SDS	Sigma-Aldrich (Mississauga, CA)
Sodium pyruvate	Sigma-Aldrich (Mississauga, CA)
SYBR green PCR master mix	Applied Biosystems (Foster city, CA, USA)
Sodium bicarbonate	Sigma-Aldrich (Mississauga, CA)
Streptomycin	Invitrogen (Burlington, CA)
SuperScriptII RNaseH reverse transcriptase	Invitrogen (Burlington, CA)

siRNA transfection reagent	Santa Cruz (Santa Cruz, CA, USA)
siRNA transfection medium	Santa Cruz (Santa Cruz, CA, USA)
Trypsin-EDTA	Invitrogen (Burlington, CA)
Tween-20	Sigma-Aldrich (Mississauga, CA)
Trizol reagent	Invitrogen (Burlington, CA)
Ubiquinone-2	Sigma-Aldrich (Mississauga, CA)
Ultra sensitive rat insulin ELISA kit	Crystal Chem Incorporation (Crystal Chem, Downers Grove, IL, USA)
Vybrant apoptosis assay kit	Invitrogen (Burlington, CA)
X-ray films	Sigma-Aldrich (Mississauga, CA)

B. Methods

1. Cell lines and culture conditions

Both rat insulinoma RINm5F and INS-1E β-cells used in this study were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin at 37 ^{o}C in an incubator with a humidified atmosphere of 5% CO_2 and 95% O_2. For INS-1E cells, the medium also contained 50 µM 2-mercaptoethanol and 1 mM sodium pyruvate. In all the experiments where ethanol was used in this thesis, the ethanol concentration was 80 mM. This ethanol dosage was determined using a dose response curve (Fig. 4), where this dosage, when compared to 0 mM ethanol, showed a statistically significant adverse effect on MTT reduction in both RINm5F and INS-1E β-cells. For PHB exposure, cells were either incubated with recombinant His-tagged PHB or transfected with human PHB gene (see #5 below). Recombinant PHB concentration (10 nM) was chosen according to a previous study in our group, this concentration corresponding to the half-maximal concentration shown to inhibit insulin-stimulated glucose oxidation and pyruvate carboxylase in adipocytes [187]. Table 3 shows the treatment conditions used in the experiments.

Table 3: Treatment conditions used in the experiments

Components	G1	G2	G2E	G2P	G2EP
Glucose (mM)	5.5	25	25	25	25
Ethanol (mM)	0	0	80	0	80
Prohibitin (nM)	0	0	0	10	10

2. Determination of mRNA expression

Total RNA was prepared from INS-1E and RINm5F cells using the TRIZOL reagent as described in the manufacturer's manual (Invitrogen, Carisbad, CA, USA). Briefly, to lyse the cells, 1 ml of TRIZOL reagent was directly added to the culture flask, and subsequently the cell lysate was passaged several times through a 1 ml of pipette. The homogenized samples were incubated for 3 minutes (min) at room temperature (RT) to complete dissociation of nucleoprotein complexes, and then 0.2 ml of chloroform was added. Samples were vortexed vigorously for 15 seconds (s) and incubated at RT for 10 min. Sample tubes were then centrifuged at 13,000 x g for 15 min at 4℃. After centrifugation, the aqueous phase containing RNA was carefully transferred to a new clean tube. To precipitate the RNA, 0.5 ml of isopropyl alcohol was added to the tube and then incubated at RT for 10 min. The RNA precipitation was completed by centrifugation at 13,000 × g for 15 min at 4 ℃. The pellet was washed with 1 ml of ice cold 75% ethanol and centrifuged at 13,000 x g for 15 min at 4 ℃. The RNA pellet was air-dried on the bench and resuspended in RNase-free water. Before making a cDNA, the concentration of RNA was determined using a spectrophotometer at 260 and 280 nm, followed by electrophoresis on a 1% agarose gel to monitor the integrity of RNA. RNA samples were stored at -80 ℃ until use.

Real time PCR was performed in an ABI 7500 thermocycler. The cDNA was synthesized with 1 μg of total RNA using SuperScriptII RNaseH reverse transcriptase and random primers (Invitrogen). The primers used in real time PCR (Table 4) were designed using Primer Express software (version 3.0) provided by Applied Biosystems. The

reactions were performed in triplicate with reaction components (Table 5) under the following conditions: 5 min at 94 ^{o}C, 15 s at 94 ^{o}C, 20 s at 59-60 ^{o}C, 40 s at 72 ^{o}C for 40 cycles. Data were analyzed by the ΔΔCt (threshold cycle) method [188] using an ABI 7500 system software, and mRNA levels were normalized to actin mRNA.

Table 4: List of primers used in the experiments

Primers	**Sequences(5'→3')**
PHB	
Forward	GATTTACAGACAGTGGTGCACACA
Reverse	GGGTTCGTATGGCTGGAAAA
ADH 5	
Forward	CCGCCCCTTTGGATAAAGTC
Reverse	GCCCCGTAGCCAGTTGAA
MafA	
Forward	AGGCCAACCGTGAAAAGATG
Reverse	CCAGAGGCATACAGGGACAAC
PDX-1	
Forward	CCCAGCCGCGTTCATCT
Reverse	CTCCTGCCCACTGGCTTTT
Insulin 1	
Forward	CTGCCCAGGCTTTTGTCAA
Reverse	TCCCCACACACCAGGTACAGA
Insulin 2	
Forward	AGCACCTTTGTGGTTCTCACTTG
Reverse	CGATGCCGCGCTTCTG
Actin	
Forward	AGGGAAATCGTGCGTGACAT
Reverse	GAACCGCTCATTGCCGATAG

Table 5: Real time PCR reaction components

Components	Volumes (µl)
cDNA template	5
2 × Cyber green master mixture	12.5
Forward primer (5 µM)	0.5
Reverse primer (5 µM)	0.5
RNase-free water	6.5
Total volume	25

3. Small interfering RNA (siRNA) transfection

Small interfering RNA (siRNA) is known as a double-stranded RNA (dsRNA) molecule having 20-25 nucleotides in length. It interferes with the expression of a specific gene sequencing homologically. The process begins with dsRNA being broken down by Dicer, which is an enzyme for converting either long dsRNAs or small hairpin RNAs into siRNAs (approximately 21 nucleotides in length). Each siRNA fragment incoporates into RISC (RNA-induced silencing complex). The RISC then binds to target mRNA and degradates it, resulting in silencing of the target gene [189].

PHB siRNA was used to examine PHB function in ethanol treated pancreatic β-cells. According to the manufacturer, the PHB siRNA is a pool of three target-specific 19-25 nucleotide siRNAs with the following sequences:

360

CAGCTTCCTCGTATCTACATTCAAGAGATGTAGATACGAGGAAGCTGTTTTT;

1179

CCATTCTGCCGTATATTGATTCAAGAGATCAATATACGGCAGAATGGTTTTT;

1624

CTCAGAGATTGCCCTTTCTTTCAAGAGAAGAAAGGGCAATCTCTGAGTTTTT.

A scrambled control siRNA, with no sequence homology to any known rat gene, was provided by the manufacturer (Santa Cruz).

To transfect with siRNA, RINm5F cells (~ 9.0×10^4 cells/well) and INS-1E cells (~ 2.0×10^5 cells/well) were cultured in antibiotic-free RPMI 1640 medium for 24 h. The transfection was performed at ~70% of cell confluency to increase the transfection efficiency. siRNAs were resuspended in 330 μl of the RNase-free water according to the manufacturer's instructions to make a stock concentration of 10 μM. Subsequently, PHB siRNA and siRNA transfection reagent were carefully mixed in a 1:50 ratio and incubated at RT for 15 min. The transfection mixture containing either PHB siRNA or control siRNA was then added dropwise to cells cultured in 6 well plates. The transfection was completed by incubating the cells for 7 h in siRNA transfection medium without serum and antibiotics. The cells were then washed with siRNA transfection medium and replaced in

fresh normal growth medium. After 24 h, the cells were further incubated in the medium with or without 80 mM ethanol for 24 h.

4. Overexpression of PHB

For overexpression of PHB, we used the pCMV6 XL5 vector, which is a mammalian cell protein expression vector, containing the human PHB gene (Origene Technologies). RINm5F and INS-1E cells were cultured for 24 h. The transfection was performed at ~70% of cell confluency. Two micrograms of PHB vector or control vector and 3 µl of a FuGENE HD transfection reagent (Roche Applied Science) were mixed carefully in 100 µl of Opti-MEMI Reduced serum medium. After mixing well with pipetting, the mixture was incubated at RT for 15 min. The transfection mixture containing either PHB vector or control vector was then added to cells cultured on 6 well plates with gentle shaking. The cells were then washed at 24 h with 1 ml of normal growth medium. The cells were replaced in fresh normal growth medium with or without 80 mM ethanol for 24 h.

5. Determination of ethanol concentration

Ethanol assays were performed enzymatically using the EnzyChrom ethanol assay kit according to the manufacturer's instructions (Bioassay systems). The kit uses alcohol dehydrogenase, which oxidizes ethanol into acetaldehyde and leads to the formation of NADH. The NADH produced is subsequently coupled to a formazan/phenazine methosulfate (PMS) reagent, yielding a chromophore, the intensity of which increases proportionate to the ethanol concentration in the sample.

Briefly, cell culture media were collected at 24 h after incubation with 80 mM ethanol in a 96 well plate. Immediately, the media were diluted 1:4 with water and 10 µl of the diluted media were transferred into each well. Subsequently, 90 µl of the working reagent provided with the kit was added into each well and the OD change at 565 nm was measured at 0 min and 5 min.

6. Measurement of ADH activity

ADH activity was assessed using MTT and PMS as substrates for NAD/NADH produced by ADH conversion of ethanol into acetaldehyde. Cells were collected and then washed twice using cold PBS. Subsequently, the cells in lysis buffer were sonicated twice for 15 s followed by vortexing. The supernatants were collected at 9,000 g, 4 ^{o}C for 10 min. Protein concentration in each fraction was determined by the Bio-Rad assay using BSA as standard. 200 µg protein per sample were resuspended in 860 µl of reaction mixture containing MTT (0.03 mg/ml), PMS (1.11 µM), NAD^{+} (2.78 mg / ml), and EtOH (0.4%) in Tris-HCl and 1% Triton, pH 8.2. ADH activity was measured at 570 nm spectrophotometrically after adding 40 µl of samples. Yeast ADH was used as standard. Changes in OD were measured at 0 min and 45 min at 25 ^{o}C. The ADH activity was normalized to protein concentrations and expressed as % of ethanol untreated control.

7. Determination of mitochondrial membrane potential

Mitochondrial membrane potential was determined with MitoPT^{TM} JC-1, which detects the mitochondrial permeability transition, an early indicator of the initiation of apoptosis. JC-1 allows easy distinction between healthy cells (stained in red fluorescence),

and apoptotic or mitochondrial membrane compromised cells (stained in dark green fluorescence) [190]. Cells were incubated with 80 mM ethanol for 24 h after transfection with a pCMV6-XL5 vector containing the human PHB clone. According to the manufacturer's instructions, the media were replaced with 50 µl of media containing 1 × MitoPT^{TM} JC-1 dye reagent and incubated at 37 ℃ for 15 min. After washing, the media were replaced with 100 µl of normal media. The accumulated fluorescent compound in mitochondria was observed under a Nikon Eclipse TE2000-E fluorescence microscope.

As an alternative method, we employed tetramethylrhodamine methyl ester (TMRM), which specifically accumulates within negatively charged mitochondria. We used 1 µM of carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) as a positive control. Cells were incubated and treated with 80 mM ethanol as described above. At 24 h, culture media were replaced with 100 µl of media containing 5 µM TMRM and maintained for 20 min at 37 ^{o}C. The media were then replaced with 100 µl of PBS, and the accumulated fluorescent compound was measured at 544 nm excitation wavelength and 590 nm emission wavelength using a SpectraMax Gemini XS Fluorescence Microplate Reader with the SoftMax Pro software (Molecular Devices).

8. MTT assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay which tests the ability of living cells to reduce a soluble yellow tetrazolium salt to blue formazan crystals by mitochondrial dehydrogenases. For the MTT assay, equal numbers of cells (1×10^4/well for RINm5F cells and 2.3×10^4/well for INS-1E cells) were plated onto each well of a 96 well plate and

cultured for 1-2 days until ~70% confluence. After ethanol treatment for 24 h, culture media were replaced by 100 µl of PBS containing 0.5 mg/ml MTT, and the incubation was continued for 3 h at 37 ^{o}C. The MTT-containing media were removed after 3 h and replaced with 200 µl of dimethyl sulfoxide (DMSO) to dissolve the formazan. The cells were left under the dark for 30 min at RT. The reduction of MTT to formazan was quantified by measuring the absorbance at 540 nm and 630 nm using a Spectra Max 340 plate reader (Molecular Devices, Sunnyvale, CA).

9. ROS assay

Cellular ROS production was determined using the fluorescent probe 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). Due to its lipophilic characteristics, the CM-H_2DCFDA probe passes freely through the cell membrane and is cleaved by intracellular esterase into its nonfluorescent form, CM-H_2DCF (5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein). The nonfluorescent form of CM-H_2DCF is oxidized into the highly fluorescent compound CM-DCF in the presence of ROS [191]. Briefly, the media were replaced by 100 µl of RPMI 1640 containing 5 µM of CM-H_2DCFDA after ethanol treatment for 24 h and then the plates were further incubated at 37 ^{o}C for 2 h. After washing, the media containing CM-H_2DCFDA were replaced with 100 µl of PBS. Immediately, the oxidized fluorescent signal was measured at the excitation length 488 nm and emission length 505 nm using the SpectraMax Gemini XS Fluorescence Microplate Reader with the SoftMax Pro software (Molecular Devices).

10. Flow cytometry

Annexin V is a cellular protein in the family of annexins, a group of proteins known to interact with cell membrane components. Annexin V preferentially binds to negatively charged phospholipids like phosphatidylserine (PS), which under normal conditions is predominantly present on the inner surface of the plasma membrane facing the cytosol. Once apoptosis is triggered, the phospholipid asymmetry of the plasma membrane is broken up and PS is released to the outer layer of the membrane. Thus, annexin V binding to PS is a hallmark of early apoptosis during which the cell membrane remains intact.

For these experiments, cells were incubated with 80 mM ethanol for 24 h, and then were harvested by mild trypsinization and washed with cold PBS to remove background signal caused by the color of medium. The cells were resuspended in 100 µl of annexin-binding buffer. Subsequently, the cells were stained with 1 µl of propidium iodide and 5 µl of FITC annexin V for 15 min at RT according to the manufacturer's instructions. The reaction was stopped by adding 400 µl of cold annexin-binding buffer after incubation for 15 min. The stained cells were immediately analyzed up to 2×10^4 cells by flow cytometry using a high speed Beckman Coulter EPICS ALTRA flow cytometer (Beckman Coulter Canada Inc., Mississauga, ON). Histograms were acquired and analyzed using the EXPO 32 Multi COMP MFA software, Version 1.2B supplied with the instrument.

Floating (apoptotic) cells were resuspended by gently swirling the culture medium and harvested by mild centrifugation at 100 g for 3 min, while attached cells were collected after trypsinization [192, 193]. Cell counting was performed using a Beckman Coulter Z2

Particule Count and Size Analyzer. The ratio of floating to the attached cells was used as an index of apoptosis.

11. ATP measurement

The cellular ATP concentration was determined using an ATP bioluminescent assay kit according to the manufacturer's instructions. The kit contains D-luciferin, which is oxidized by luciferase in an-ATP dependent manner and generates chemiluminescence. A standard calibration curve was generated using serial dilutions of an ATP standard from 2×10^{-7} to 2×10^{-3} M. The same amount of cell extract was mixed with 100 µl of the luciferase assay reagent in disposable polystyrene tubes. After mixing several times, the tube was incubated in the dark at RT for 3 min. The light produced was immediately measured for 30 s at 560 nm with an LB 9507 Lumat luminometer (EG & G Berthold, Germany).

12. Protein extraction

For the total protein fraction, cells were collected using 0.05% trypsin-0.02% EDTA. The cells were washed twice with ice cold phosphate-buffered saline (PBS) and then resuspended in a 30 µl of lysis buffer (1% Igepal, 0.1% SDS, 0.5% deoxycholic acid, 1 mM PMSF in PBS, pH 7.2 and protease inhibitors). After vigorous vortexing for 1 min, the cells were placed on ice for 30 min, and then the homogenates were centrifuged at 13,000 g, 4 ^{o}C, for 20 min. The supernatants were carefully collected and stored at –25 ^{o}C until use.

Nuclear fractions were prepared as described [194]. After collecting with 0.05% trypsin-0.02% EDTA, the cell pellets were resuspended in 400 µl of cold buffer A (10 mM KCl, 0.1 mM EGTA , 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM HEPES, pH 7.9 and protease inhibitors) by gently mixing. The cells were placed to swell on ice for 15 min, and then 10 µl of 1% Igepal were added into each tube. After vigorous vortexing for 10 s, the homogenates were centrifuged at 13,000 g, 4 ^{o}C, for 30 s. The supernatants, representing cytosolic fractions, were carefully collected and stored at –25 ^{o}C until use. The pellets were resuspended in 50 µl of ice cold buffer B (0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 20 mM HEPES, pH 7.9 and protease inhibitors) by gently mixing. After further centrifugation at 13,000 g, 4 ^{o}C, for 5 min, the supernatants, representing nuclear fractions, were collected and stored at –25 ^{o}C until use.

Mitochondrial fractions were prepared using a cytosol / mitochondria fractionation kit from Calbiochem (Germany). Briefly, cells were collected with 0.05% trypsin-0.02% EDTA. After washing twice with ice cold phosphate-buffered saline and centrifugation at 2500 g for 5 min 4 ^{o}C , the cells were resuspended in a 300 µl of Cytosol Extract Buffer provided with the kit and then incubated on ice for 10 min. The cells were then homogenized and centrifuged at 850 g for 10 min at 4 ^{o}C. The supernatant was transferred to a clean tube and centrifuged at 9,000 g for 30 min at 4 ^{o}C. After centrifugation, the supernatant was discarded and the pellet, representing the mitochondrial fraction, was resuspended in 50 µl of Mitochondria Extract Buffer provided with the kit and vortexed for 10 s. The mitochondrial fraction was stored at –80 ^{o}C until use. Protein concentration in each fraction was determined by the Bio-Rad assay using BSA as the standard.

13. Western blot analysis

Equal amounts of proteins were boiled for 5 min to denature and unfold the proteins and then separated based on the molecular weight by SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose membrane using a semi-dry blot apparatus (Trans-Blot SD Cell; Bio-Rad). After transfer, the membrane was blocked for 1 h at RT with 5% non-fat dry milk in Tris-buffered saline-0.1% Tween 20 (TBS-T), to avoid nonspecific antibody binding. After blocking, the membrane was rinsed twice with TBS-T and incubated with a primary antibody at RT for 1 h. After further washing twice for 7 min with TBS-T to remove unbound primary antibody, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed two times for 7 min respectively. The protein-antibody complexes were detected using the enhanced chemiluminescence (ECL) detection kit. The same membrane was subsequently stripped with 15 ml of strip buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50 ^{o}C for 30 min and washed three times for 10 min to completely remove 2-mercaptoethanol. The stripped membrane was reprobed with antibody as appropriate listed in Table 6. Quantitative image analysis of each target protien was performed using NIH Image software (*Image J*).

Table 6 List of antibodies used in the experiments

Antibodies	Dilution rate
Actin	1: 5000
ADH5	1: 1000
Bax	1: 1000
Cleaved caspase 3	1: 1000
Caspase 3	1: 1000
Histone 1	1: 1000
Heat shock protein 60	1: 1000
JNK	1: 1000
MafA	1: 1000
p-JNK	1: 1000
PDX-1	1: 1000
PHB	1: 650
UCP2	1: 1000

14. Immunocytochemistry

Immunostaining was completed using chamber slides (Nalge Nunc International, Tokyo, Japan). Equal number of RINm5F cells (1 x 10^5/well) were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin until 70-80% confluence. After rinsing with PBS, the cells were incubated for 24 h as mentioned in Table 3. To investigate whether exogenous His-tagged PHB added to the culture media entered the cells following incubation, the media were removed, and the cells were

washed, and then fixed with 4% paraformaldehyde for 30 min. The cells were then incubated with PBS-BT (1% Bovine Serum Albumin, 0.1% Tween 20) at RT for 1 h to prevent nonspecific antibody binding. The cells were subsequently incubated with the mitochondrial dye Mitofluor^{TM} Red 589 (final concentration: 5 µg/ml) for 20 min to stain mitochondria, and then serially incubated with anti-His-FITC (1:650) for 1 h for exogenous His-tagged PHB, and Hoechst 33342 (final concentration: 2.5 µg/ml) for 5 min for nucleus staining. To detect the distribution of endogenous PHB, the cells were fixed with 4% paraformaldehyde at RT for 30 min and then nonspecific antibody binding sites were blocked with PBS-BT at RT for 1 h. The cells were then serially incubated with a rabbit anti-PHB antibody (1:650) for 1 h, Mitofluor^{TM} Red 589 for 20 min, anti-rabbit FITC (1:650) for 1 h, and Hoechst 33342 for 5 min at RT. The stained images on the slide were captured with a Nikon Eclipse TE2000-E fluorescence microscope.

15. Immunoprecipitation (IP)

Equal amounts of mitochondrial extracts were incubated overnight at 4 °C and constant rotation with 1 µg of cytochrome c oxidase subunit III antibody in 500 µl of PBS buffer, pH 7.2, containing 1% Igepal, 0.1% SDS, 0.5% deoxycholic acid, 1 mM PMSF, 1 mM NaF, 2 mM Na_3VO_4 and protease inhibitors. After overnight incubation, 50 µl of protein A agarose were added to each tube followed by incubation at 4 °C for an additional 3 h. The beads were washed three times with 600 µl of the same cold buffer. The pellets were resuspended in 50 µl of SDS sample buffer and stored at -25 °C until use. The immune complexes were separated by electrophoresis on 12 % polyacrylamide gels. After transfer onto nitrocellulose membranes, the blots were probed with appropriate antibodies

(cytochrome c oxidase subunit III and PHB). Subsequently, the same membrane was reprobed, after stripping, with the appropriate antibody (cytochrome c oxidase subunit III and PHB).

16. Determination of insulin secretion

Glucose induced insulin secretion (GIIS) was investigated using INS-1E cells, which are known to respond to glucose [195], but not in RINm5F cells as these cells show poor glucose response [196]. INS-1E cells were further cultured for 24 h following treatment conditions described in Table 3. After PHB transfection, the cells were washed twice with Krebs-Ringer Bicarbonate Hepes buffer (KRBH). The cells were then preincubated at 37 ℃ for 30 min in 750 µl of Krebs-Ringer Bicarbonate Hepes buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, and 10 mM Hepes, pH 7.4) containing 0.1% bovine serum albumin and 2.2 mM glucose. After washing, cells were stimulated at 37 ℃ for 30 min with 2.2 mM or 16.7 mM glucose in 750 µl of KRBH. The media were harvested and stored at -80 ℃ until use. Insulin secretion was determined by a rat insulin enzyme linked immunoassay kit according to instructions provided by the manufacturer. The secreted level of insulin was normalized to total protein.

17. Measurement of NADH dehydrogenase activity

Mitochondrial NADH dehydrogenase **(**complex I) activity was determined using the Complex I Enzyme Activity Microplate Assay kit (Mitosciences) according to the manufacturer's instructions. Briefly, cells were collected with 0.05% trypsin-0.02%

EDTA, washed twice with cold PBS and lysed with the detergent provided with the kit. Equal protein amounts were diluted with the incubation buffer to 400 µl, half of which was transferred in duplicates into two wells of a 96 well plate, and the plate was incubated at RT with rotation for 3 h. After washing twice with 300 µl of buffer, the plate was faced down on paper towel to remove buffer. Two hundred microliters of reaction buffer was then added to each well and OD measurement was performed automatically every min for 30 min at 450 nm in an ELx 808 Ultra Microplate Reader (Bio-TEK Instruments, Winooski, VT).

18. Measurement of succinate dehydrogenase activity

Succinate dehydrogenase (complex II) activity was determined using Complex II Enzyme Activity Microplate Assay kit (Mitosciences) according to the manufacturer's instructions. Briefly, equal amounts of proteins were diluted with the incubation buffer to 100 µl, half of which was transferred in duplicates to a 96 well plate as described for complex I. The plates were incubated at RT with rotation for 2 h. After washing twice with 300 µl of buffer, the plate was faced down on paper to dry out. The plate was further incubated at RT for 30 min in 40 µl of a lipid solution provided with the kit before adding 200 µl of reaction buffer. The change in OD values at 630 nm was measured automatically every min for 1 h in an ELx 808 Ultra Microplate Reader (Bio-TEK Instruments).

19. Measurement of cytochrome b-c_1 complex activity

Cytochrome b-c_1 (complex III) activity was measured as described [197]. In brief, cells were collected with 0.05% trypsin-0.02% EDTA, and washed twice with cold PBS at 350

g for 5 min. The cells were homogenized by sonication for 2× 10 s prior to protein quantification. Equal amounts of proteins were mixed in reaction buffer containing 25 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 2 mM KCN, 2 µg/ml rotenone, 2.5 mg/ml bovine serum albumin and 50 µM cytochrome c, and then incubated at RT for 2 min. Right after adding 50 µM ubiquinol-2, OD was measured every minute for 5 min at 550 nm using a spectrophotometer (Pharmacia Biotech Ltd., Cambridge, UK).

20. Measurement of cytochrome c oxidase complex activity

Cytochrome c oxidase (complex IV) activity was quantified by measuring the rate of oxidation of reduced cytochrome c at 550 nm [197]. The cells were washed with cold PBS twice using centrifugation at 350 g for 5 min. The cells were then homogenized and equal amounts of proteins were mixed with Lauryl Maltoside (LM) buffer (40 mg / ml) and incubated at RT for 1 min, and then with 20 mM of potassium phosphate solution (Kpi solution containing KH_2PO_4 and K_2HPO_4, pH 7.4). Immediately, 40 µM of reduced cytochrome c were added into and mixed, and change in OD value at 550 nm was monitored using a spectrophotometer (Pharmacia Biotech Ltd., Cambridge, UK).

Statistical analysis

The data were analyzed by one way ANOVA with Tukey multiple comparisons or Student's *t*-test, and are presented as the mean ± SEM and $P < 0.05$ was considered significant. Treatment results were compared with control. For RINm5F cells, control is defined as cells incubated with 25 mM glucose concentration alone, as this is the

recommended glucose concentration for RINm5F cell culture (ATCC). For INS-1E cells, control refers to cells not treated with ethanol.

IV. RESULTS

1. PHB is expressed in β-cells and increased by ethanol.

To determine the expression of PHB in β-cells, we first performed immunocytochemistry using RINm5F cells and found that endogenous PHB is present in the nucleus as well as in the perinuclear area (Fig. 4), the latter suggesting mitochondrial localization. We also analyzed PHB protein level by western blot and mRNA expression by real time PCR. Using these techniques we confirmed that PHB protein is present in pancreatic β-cells, has a tendency to increase at the low (G1: 5.5 mM) compared with the high (G2: 25 mM) glucose concentrations, and clearly increases by 92% in cells treated with ethanol (Fig. 5). PHB mRNA expression showed a similar expression pattern as the protein level (Fig. 6).

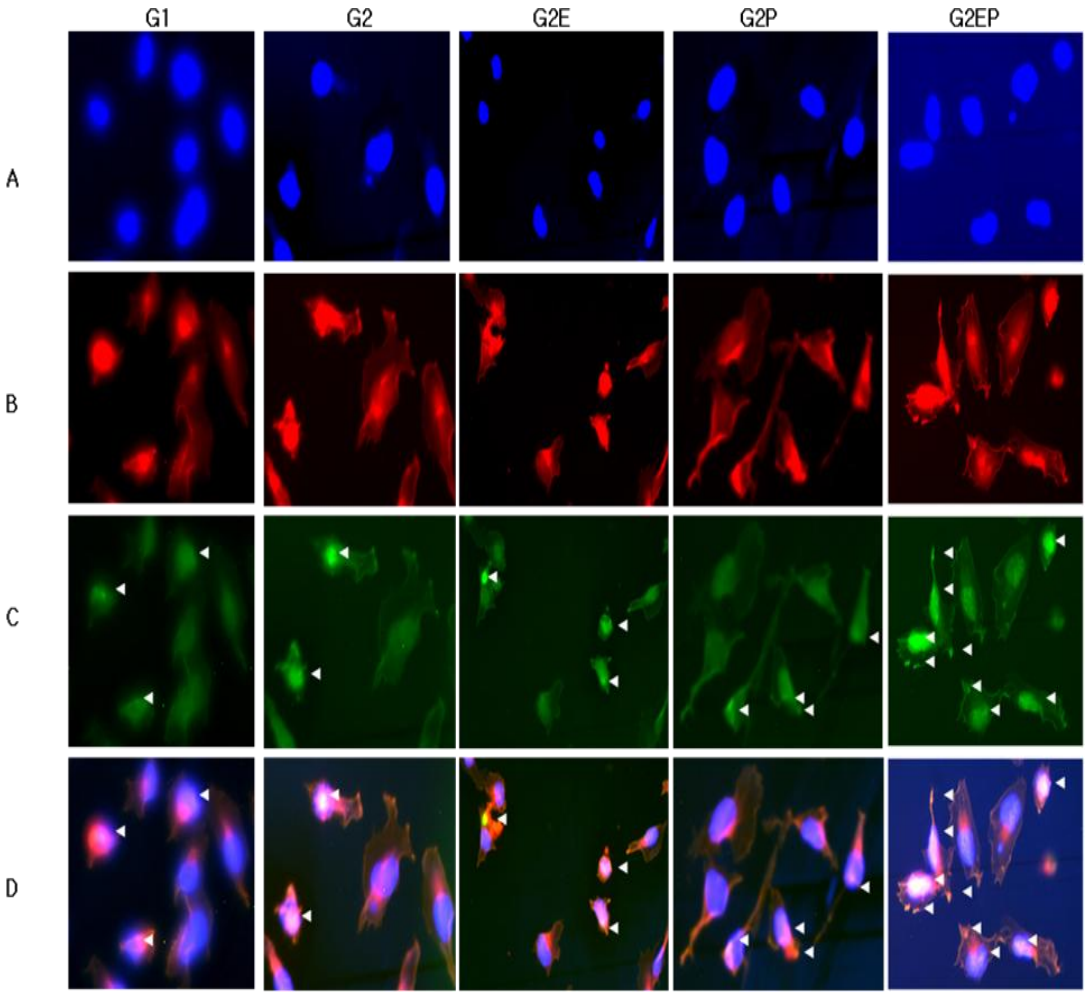


Figure 4: Cellular distribution of endogenous PHB in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). At 24 h, cells were stained with Hoechst 33342 (blue), Mitofluor Red 589 (red) and anti-PHB/anti-rabbit-FITC (green). n = 3 experiments
A: Hoechst 33342 staining for nuclei; B: Mitofluor Red 589 staining for mitochondria;
C: anti-PHB/anti-rabbit-FITC staining for PHB; D: merge. Arrows indicate staining of PHB.

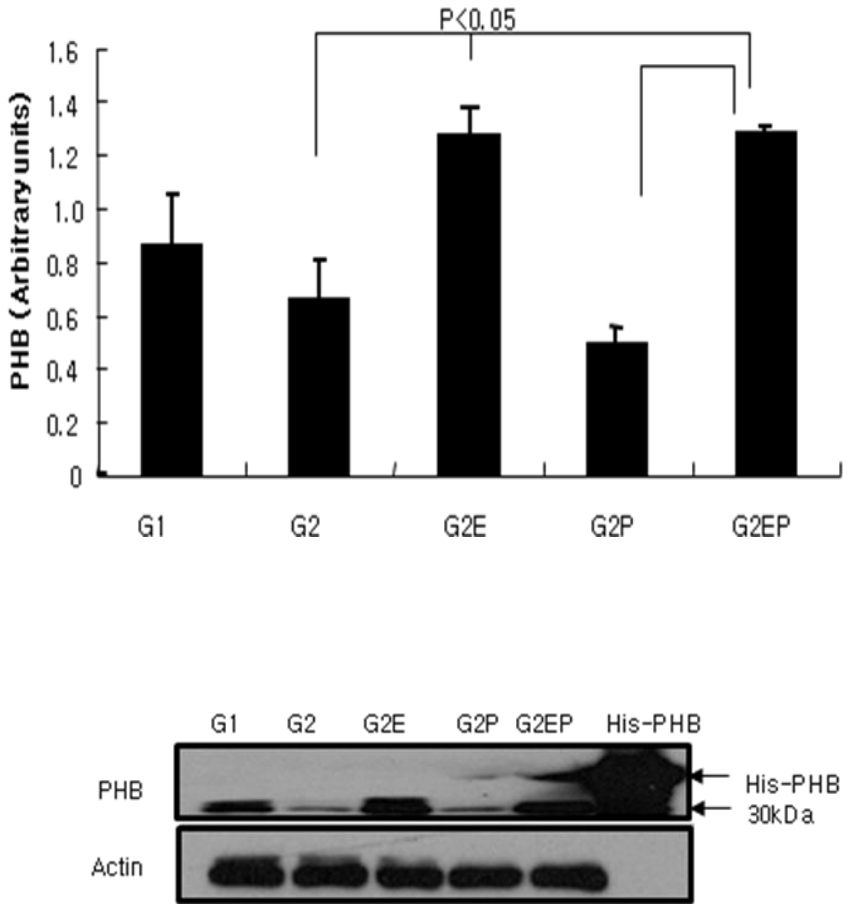


Figure 5: Effect of ethanol and exogenous PHB on PHB protein expression in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). Cell extracts were immunoblotted with anti-PHB. A representative western blot is shown where recombinant human PHB was run in parallel with cell extracts, and the 30 kDa endogenous PHB band is seen below the His-tagged recombinant PHB (exogenous PHB). Bars show endogenous PHB protein (30kDa) expressed as the mean ± SEM arbitrary units relative to actin (n = 3 experiments).

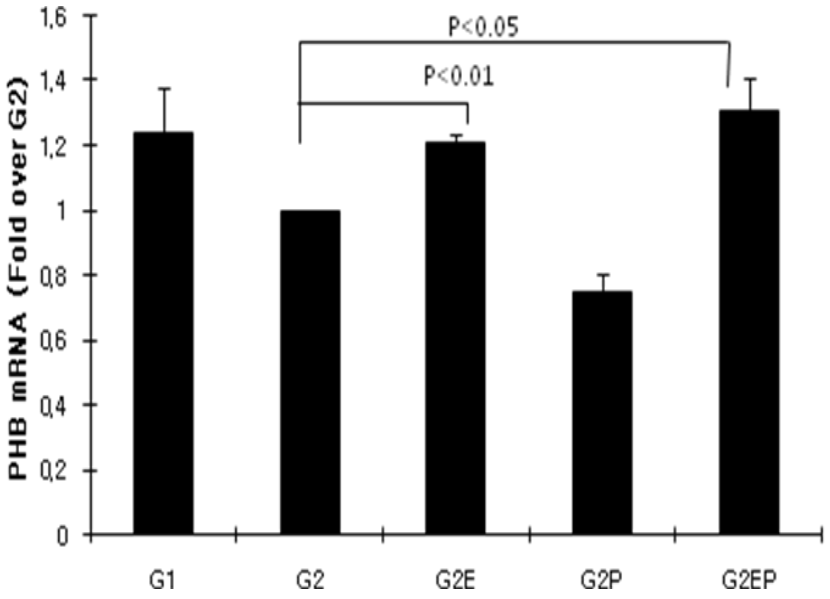


Figure 6: Effect of ethanol on PHB mRNA expression in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). PHB mRNA levels were determined using real time PCR. PHB mRNA levels were expressed as the mean ± SEM fold of G2 (n = 3 experiments) after being normalized to actin mRNA.

2. PHB localizes to the mitochondria.

To confirm PHB mitochondrial localization, cellular fractions were prepared and analyzed by western blot. In a western blot analysis with the nuclear fraction, the level of PHB protein decreased in the cells exposed to ethanol (Fig. 7A), whereas PHB increased in the cytoplasmic fraction (Fig. 7B), suggesting PHB protein exclusion from the nucleus with ethanol treatment. Subsequently, in western blot analysis of mitochondrial fractions,

we found that PHB colocalizes with the mitochondrial marker heat shock protein 60, indicating PHB is preferentially localized to the mitochondria in cells exposed to ethanol (Fig. 7C).

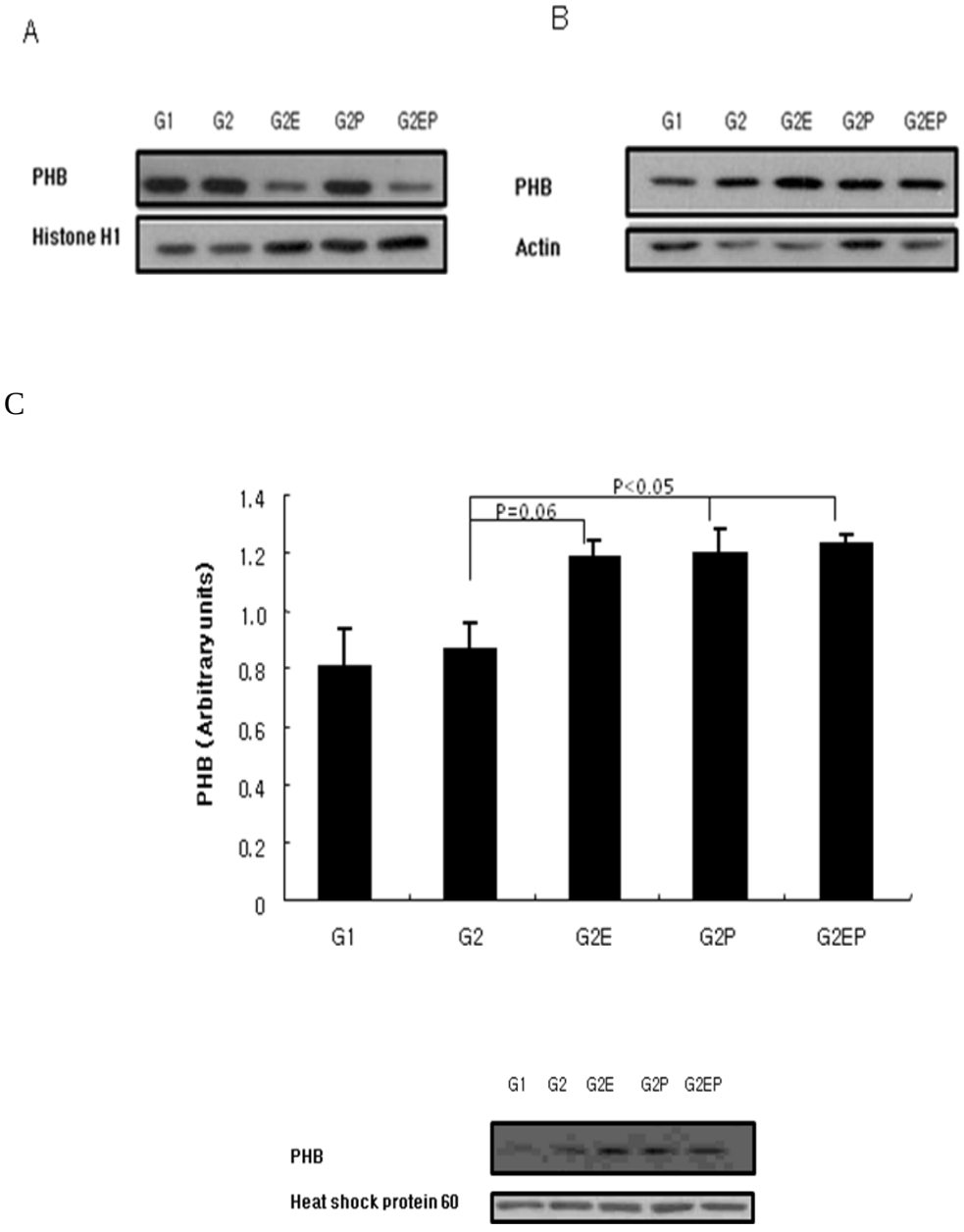


Figure 7: Effect of ethanol and exogenous PHB on endogenous PHB localization in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM).
A. Representative western blot of PHB in nuclear fraction with histone H1 as a nuclear marker showing decreased PHB level in RINm5F cells exposed to ethanol (n = 3 experiments). B. Representative western blot of PHB in cytoplasmic fraction with actin as cytoplasm marker (n = 3 experiments). C. Endogenous PHB protein in the mitochondrial fraction, expressed as the mean ± SEM arbitrary units relative to the mitochondrial marker heat shock protein 60 (n = 3 experiments), with a representative western blot.

3. PHB is phosphorylated on threonine residues by ethanol.

We were intrigued by changes in the cellular distribution of PHB under conditions of oxidative stress induced by ethanol and sought to investigate whether endogenous PHB is post-translationally modified. It has, indeed, been suggested that post-translational modification of proteins may be implicated in their cellular distribution [198, 199]. Cellular extracts were subjected to western blot analysis using a combination of anti-PHB and anti-phospho-aminoacid antibodies. Image substraction showed that, in cells exposed to ethanol, anti-prohibitin antibody detected a protein band of 43kDa also recognized by anti-phospho-threonine antibodies (Fig. 8).

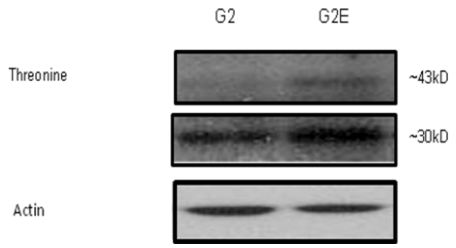


Figure 8: Ethanol increases the phosphorylation of PHB

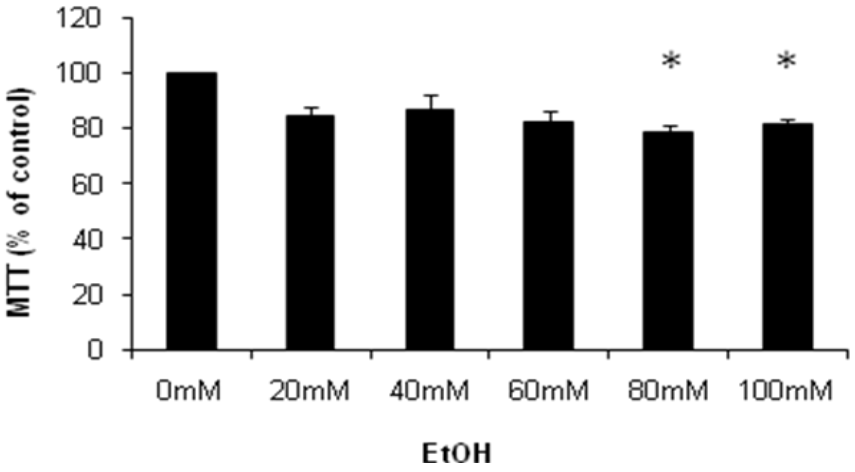
RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM). Representative western blot of cellular extracts blotted with anti-phosphothreonine antibodies, showing PHB phosphorylation on threonine residue is increased with ethanol treatment (n = 3 experiments).

4. PHB restores mitochondrial function in ethanol exposed pancreatic β-cells

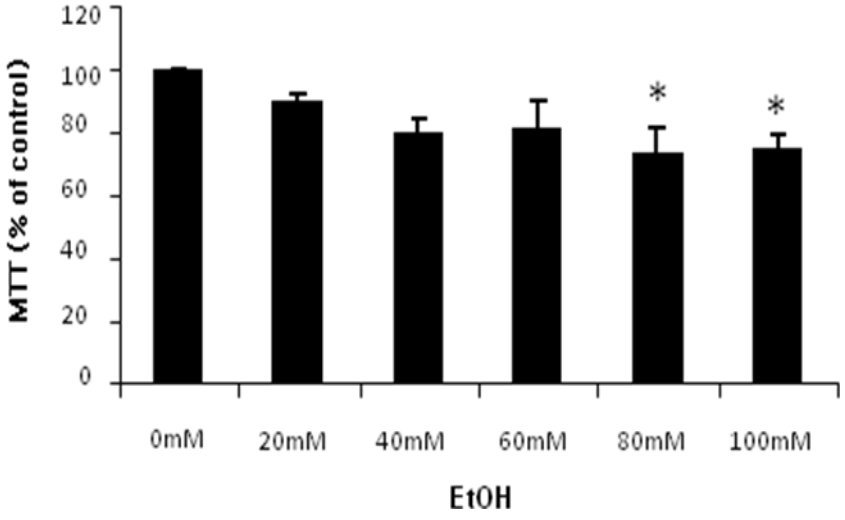
The toxic effects of ethanol on pancreatic β-cells were first determined using the MTT assay in both RINm5F and INS-1E cells incubated with various concentrations of ethanol for 24 - 48 h (Fig. 9). MTT in both cell lines at 24 h showed a dose dependent reduction, which was statistically significant at 80 mM ethanol. At this concentration, MTT reduction was decreased by approximately 30% compared to controls in both RINm5F (Fig. 9A) and INS-1E cells (Fig. 9B). As well, MTT reduction was comparable with that observed at low glucose concentration of 5.5 mM (labelled G1, Fig. 10), which is much lower than the 25 mM glucose concentration (labelled G2) recommended for maintenance of RINm5F cells in culture. As shown for INS-1E cells (Fig. 9C), there was little difference in this toxic

effect of ethanol between 24 h and 48 h, where the effect at 40 mM became statistically significant.

A (RINm5F)



B (INS-1E)



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C (INS-1E)

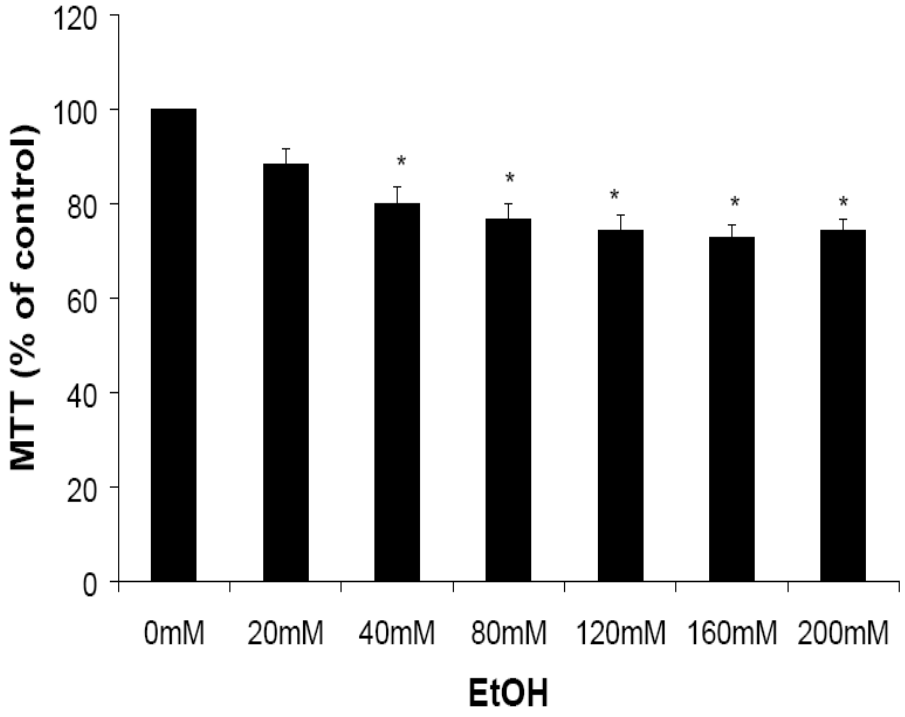


Figure 9: Effect of ethanol on MTT reduction in RINm5F and INS-1E pancreatic β-cells

RINm5F cells (A) and INS-1E cells (B) were incubated for 24 h in RPMI 1640 medium containing various concentrations of EtOH. INS-1E cells were further incubated for 48 h (C). The results are expressed as percentage of control without EtOH and shown as the mean ± SEM, n = 4 experiments. * $P < 0.05$ versus 0 mM control.

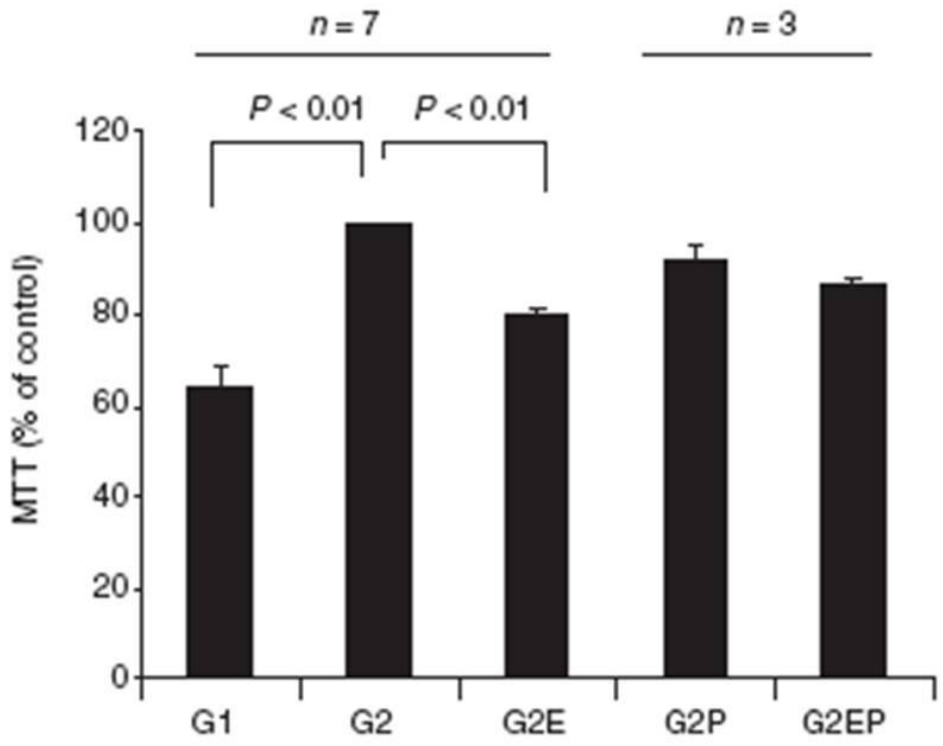


Figure 10: Effect of ethanol and PHB on MTT reduction in RINm5F cells

RINm5F cells were incubated for 24 h with or without EtOH (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). The results are expressed as the mean ± SEM percentage of G2. * $P < 0.05$ versus control.

Because studies in several tissues have shown that excessive alcohol consumption leads to cell injury through production of ROS and mitochondrial dysfunction [116, 141, 200], ROS production was determined in RINm5F cells using the fluorescent probe CM-H_2DCFDA. Ethanol at 24 h increased ROS production by 43% compared with the cells incubated without ethanol at a high glucose concentration (Fig. 11). In contrast, incubation of cells with recombinant PHB decreased ROS production to control level. Of note, ROS

production also decreased by ~80% under low glucose condition, suggesting that ROS production was related to oxidative phosphorylation.

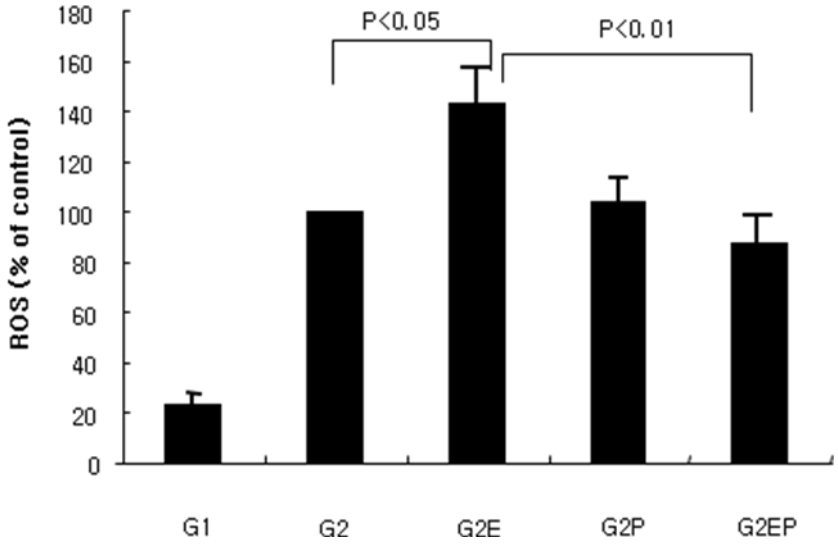


Figure 11: Effect of ethanol and PHB on ROS production in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). ROS production was determined using the fluorescent probe CM-H_2DCFDA. The results are expressed as the mean ± SEM percentage of G2, n = 3 experiments.

Because oxidative stress has been suggested to induce uncoupling protein 2 (UCP2) expression at the expense of ATP synthesis [201], the levels of UCP2 protein and ATP production were determined in RINm5F cells. UCP2 protein level determined by western blot with an anti-UCP2 antibody was increased by 42% in cells exposed to ethanol, in parallel with increased ROS production (Fig. 12). Remarkably, the level of UCP2 was lower at low (G1) than at high (G2) glucose concentration, as observed for MTT reduction

and ROS levels (Fig. 12). As would be expected, ATP production decreased by ~40% (Fig. 13) with ethanol exposure compared to G2, in an inverse proportion with the UCP2 protein level.

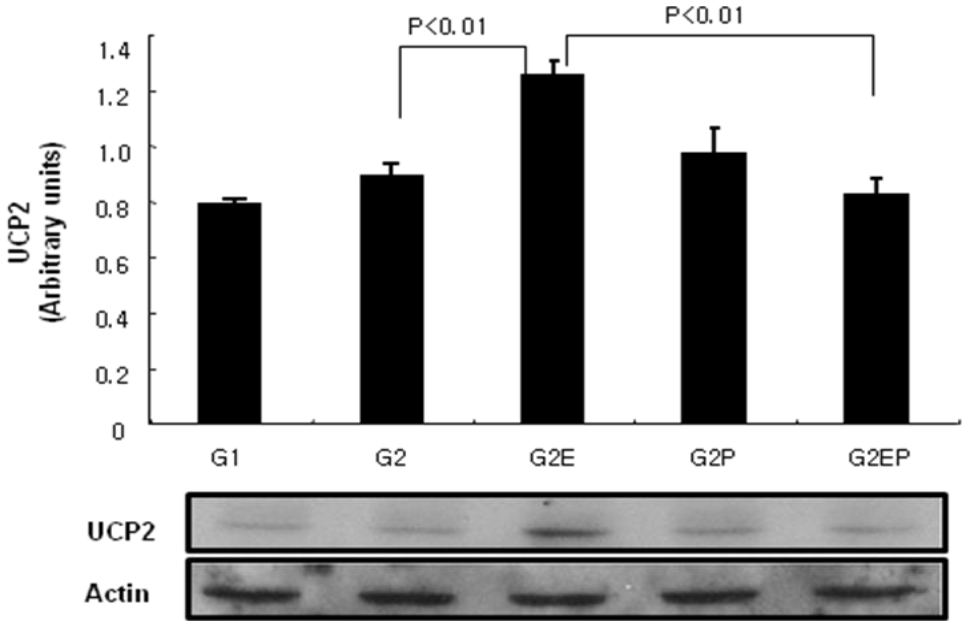


Figure 12: Effect of ethanol and PHB on UCP2 protein expression in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). UCP2 protein level was determined by immunoblotting with an anti-UCP2 antibody and the same membrane was stripped and reprobed with an anti-actin antibody. The results are expressed as the mean ± SEM arbitrary units relative to actin, n = 3 experiments.

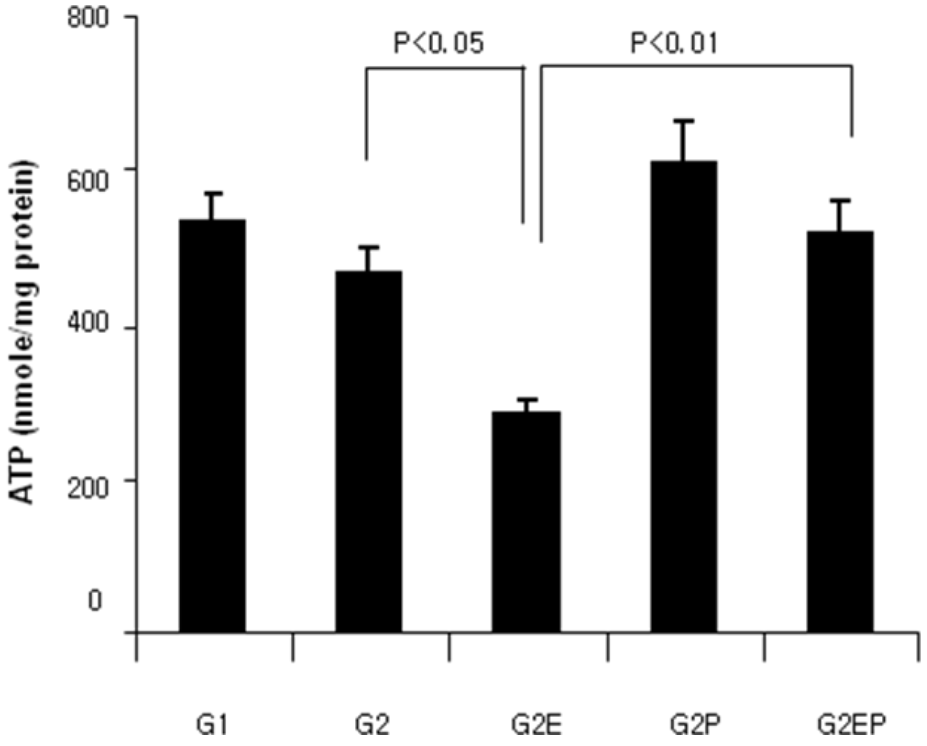
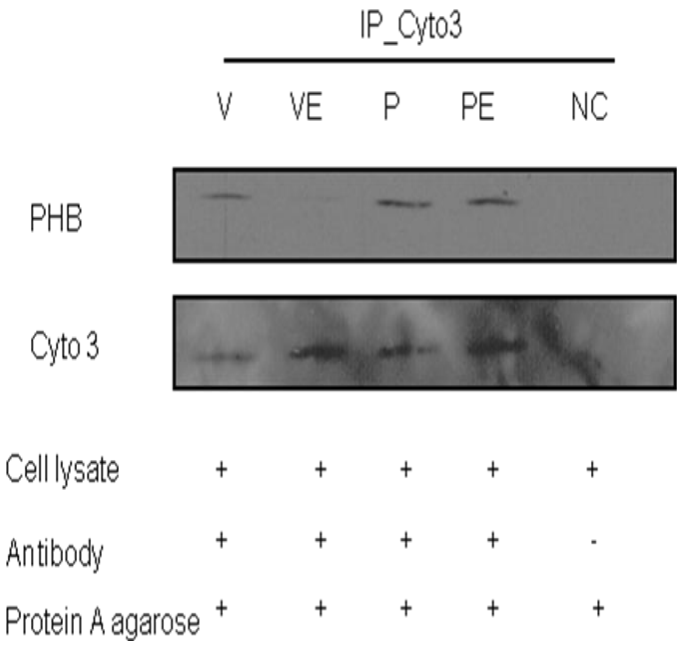


Figure 13: Effect of ethanol and PHB on ATP production in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). The cellular ATP production was measured using an ATP bioluminescent assay kit. The results are presented as the mean ± SEM, n = 4 experiments.

In contrast, treatment of ethanol exposed RINm5F cells with recombinant PHB reduced UCP2 protein levels (Fig. 12) in direct proportion to ROS production (Fig. 11), whereas this increased ATP production (Fig. 13) and cell viability determined with MTT, to control levels. These effects indicate that PHB restored mitochondrial function that was altered by ethanol. In particular, the effect of PHB on ATP levels suggested that PHB rescued mitochondrial complex V (ATP synthase). We therefore probed the effect of PHB on the upstream mitochondrial complex IV in RINm5F cells. We found that PHB treatment restored PHB interaction with cytochrome c oxidase subunit III, which had been disrupted by ethanol treatment (Fig. 14).

A



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B

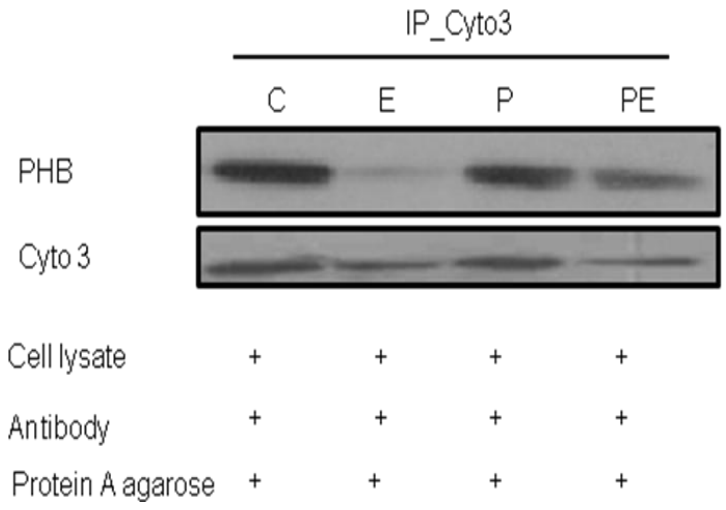
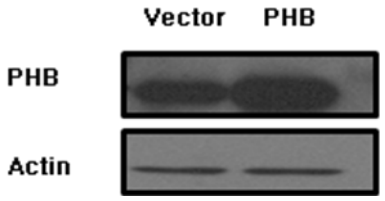


Figure 14: Effect of ethanol and PHB on cytochrome c oxidase

Cell lysates were immunoprecipitated with anti-cytochrome c oxidase subunit III (cyto 3) and then immunoblotted with anti-PHB or anti-cyto 3 using the same stripped membrane. Results show representative blots (n = 3 experiments).
A. ***INS-1E cells*** incubated with 80 mM ethanol for 24 h after transfection with PHB. V; vector, VE; vector_EtOH, P; prohibitin, PE; prohibitin_EtOH, NC; negative control without primary antibody
B. ***RINm5F cells*** incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM). C; control, E; EtOH, P; prohibitin, PE; prohibitin_EtOH

Similarily, PHB protein was abundantly expressed in both RINm5F (Fig. 15A) and INS-1E (Fig. 15B) cells transfected with the human PHB clone. PHB over-expression prevented alterations of mitochondrial function caused by ethanol. Indeed, PHB prevented MTT reduction in ethanol treated cells, and this effect was also evident in INS-E1 cells treated with 10 µM H_2O_2 (Fig. 16), supporting that the effects of PHB on MTT in ethanol exposed cells were mediated by its antioxidant properties.

A



B

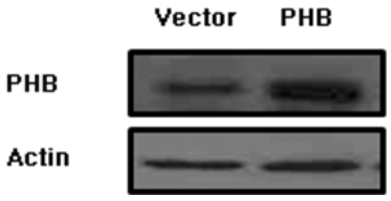


Figure 15: Over-expression of PHB in RINm5F and INS-1E cells

RINm5F (A) and INS-1E cells (B) were transfected with pCMV6-XL5 vector containing the human PHB gene and over-expressed for 24 h. Representative western blot showing PHB protein expression levels after transfection (n = 3 experiments)

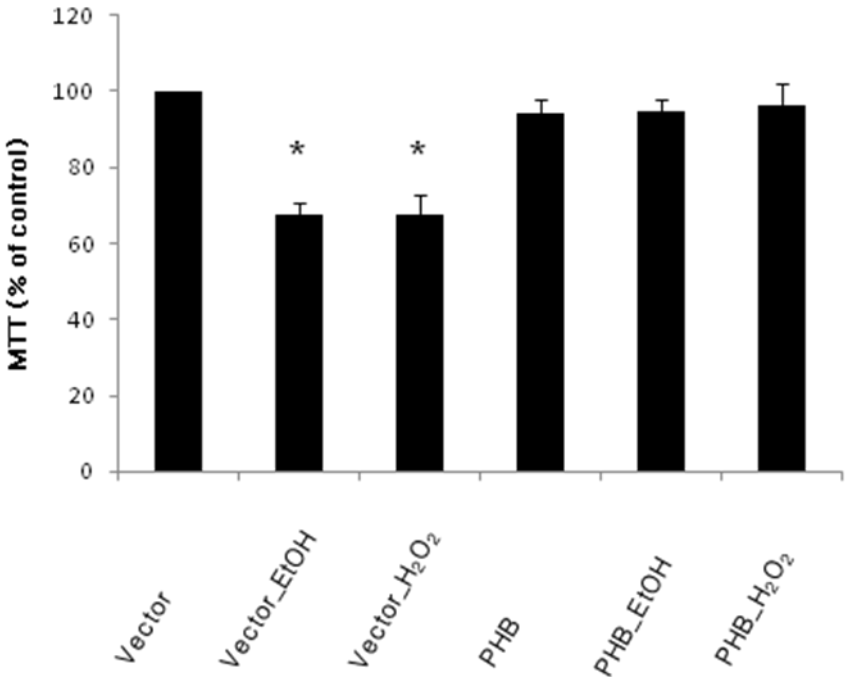


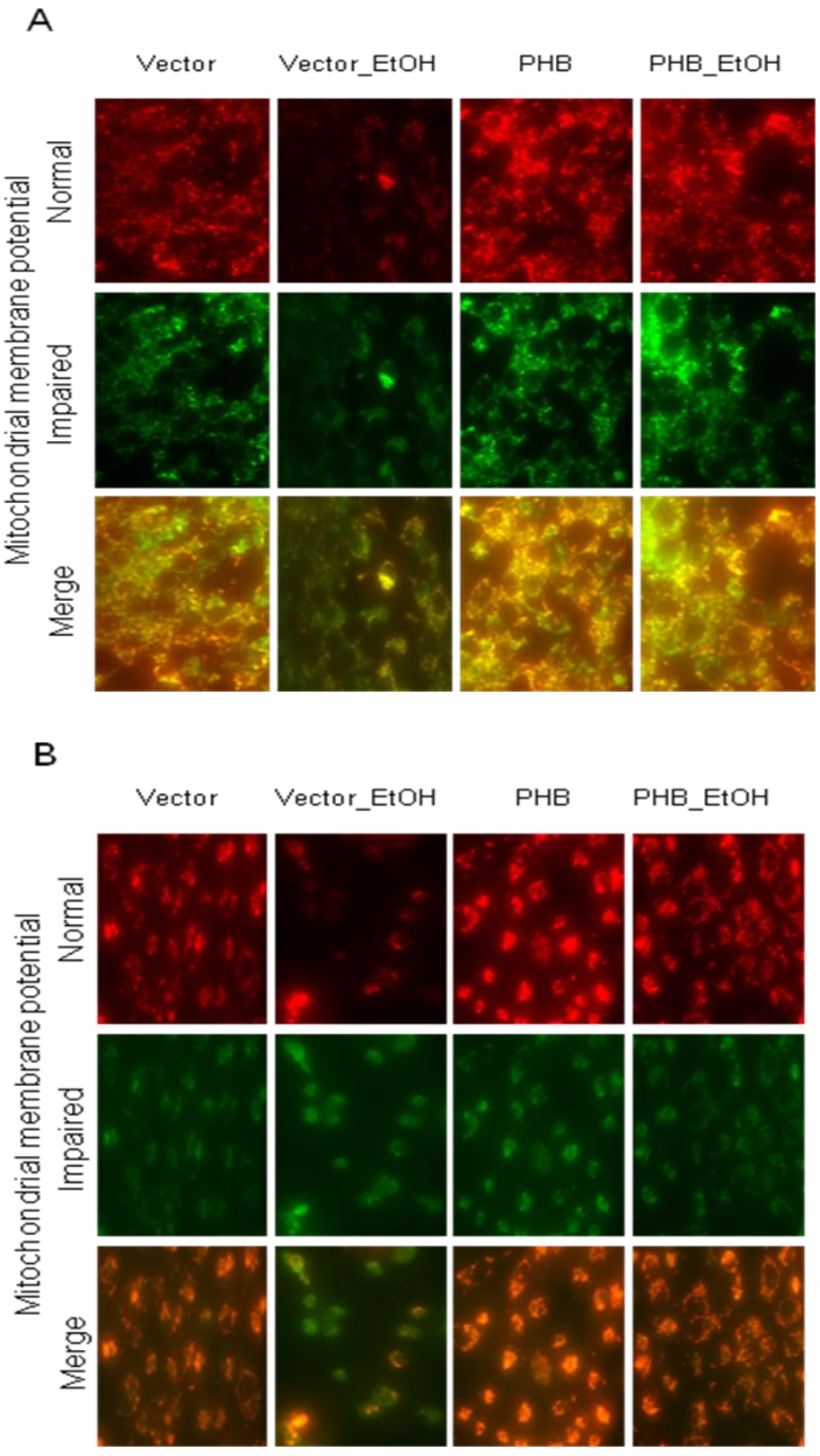
Figure 16: Effect of over-expression of PHB on MTT reduction in INS-1E cells

INS-1E cells were transfected with pCMV6-XL5 vector containing the human PHB gene. MTT reduction was measured after incubation of EtOH or 10 µM H_2O_2 for 24 h. The results were expressed as the mean ± SEM percentage of vector control. * $P < 0.05$ versus vector.

We next determined the interaction of PHB with cytochrome c oxidase subunits in PHB over-expressing INS-1E cells exposed to ethanol. PHB restored the interaction of cytochrome c oxidase subunits in INS-1E (Fig. 14), as already shown in RINm5F cells. This restoration of PHB-cytochrome c interaction may explain the associated recovery of cytochrome c oxidase activity (Fig. 22). These results are in agreement with the reports that PHB binds to cytochrome c oxidase subunit III in yeast [51, 52].

Because increased ROS levels and decreased ATP production indicate an impairment of mitochondrial function, we determined the activity of mitochondrial electron transport chain and mitochondrial membrane potential. This was carried out in both RINm5F and INS-1E cells.

We determined the mitochondrial membrane potential using JC-1 and found it to be reduced in both RINm5F and INS-1E cell lines after exposure to ethanol (Fig. 17). In a different approach, the mitochondrial membrane potential was measured by TMRM in RINm5F cells and also found to be significantly decreased by ethanol exposure (Fig. 18). In contrast, the mitochondrial membrane potential did not collapse after ethanol treatment of PHB over-expressing β-cells (Fig. 17, 18).


A
Vector
Vector_EtOH
PHB
PHB_EtOH
Mitochondrial membrane potential
Normal
Impaired
Merge
B
Vector
Vector_EtOH
PHB
PHB_EtOH
Mitochondrial membrane potential
Normal
Impaired
Merge

Continued

Figure 17: Effect of ethanol and PHB on mitochondrial membrane potential determined using JC-1 in INS-1E and RINm5F cells

INS-1E and RINm5F cells were incubated with 80 mM ethanol for 24 h after transfection with pCMV6-XL5 vector containing the human PHB clone and then the cells were stained with MitoPT^{TM} JC-1 dye for 15 min to detect the mitochondrial membrane potential transition (n=3 experiments). A. INS-1E, B. RINm5F. Normal: red filter showing healthy cells; Impaired: green filter showing apoptotic cells or cells with compromised mitochondrial membrane.

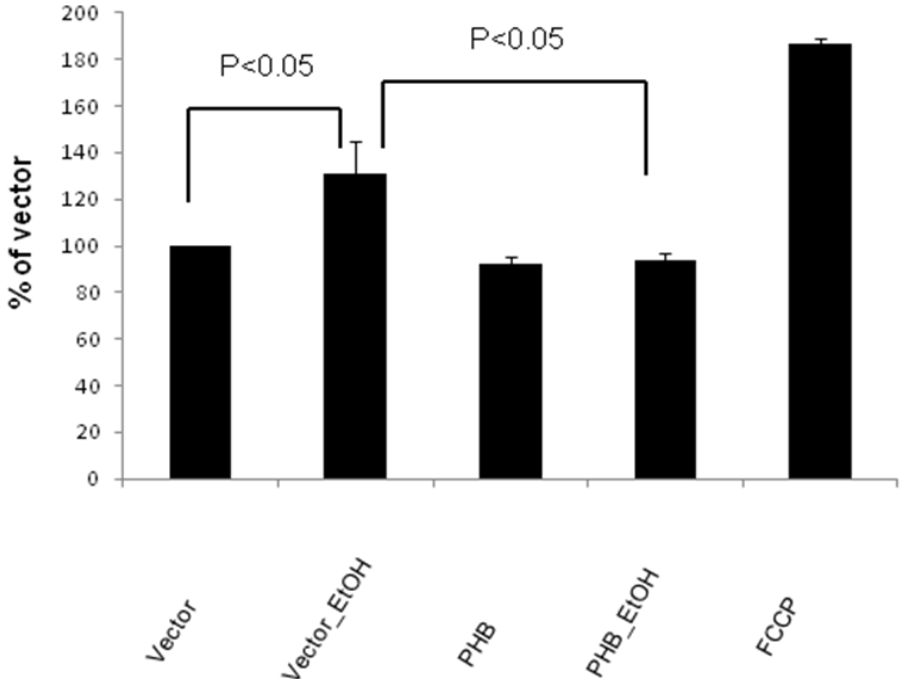


Figure 18: Quantification of the effect of ethanol and PHB on mitochondrial membrane potential determined using TMRM in RINm5F cells

RINm5F cells were incubated with 80 mM ethanol for 24 h after transfection with pCMV6-XL5 vector containing the human PHB clone and then the cells were incubated with 5 µM TMRM for 20 min. Fluorescence intensity was measured at 544 nm excitation and 590 nm emission using a fluorescence microplate reader (n = 3 experiments). FCCP: carbonylcyanide p-trifluoromethoxyphenylhydrazone was used as a positive control. The results are expressed as a percentage of the vector and shown as the mean ± SEM

The membrane potential is a reflection of overall electron transport chain function. To further explore the contribution of each mitochondrial complex to this impairment in membrane potential, we determined the activity of complexes I-IV. INS-1E cell incubation with ethanol decreased the activity of NADH dehydrogenase (complex I) by ~ 46% (Fig. 19) and that of cytochrome c oxidase complex (complex IV) by ~ 33% (Fig. 22). However, PHB over-expression prevented alterations of NADH dehydrogenase and cytochrome c oxidase activities in ethanol exposed cells (Fig. 19, 22). Interestingly, cytochrome c oxidase activity paralleled PHB interaction to subunit III (cyto 3) of cytochrome c oxidase complex as well as ATP production, as shown in both INS-1E cells (Fig. 14A) and RINm5F cells (Fig. 14B). Remarkably, however, either ethanol or PHB had no effect on the activity of succinate dehydrogenase (complex II, Fig. 20) or cytochrome b-c_1 complex (complex III, Fig. 21).

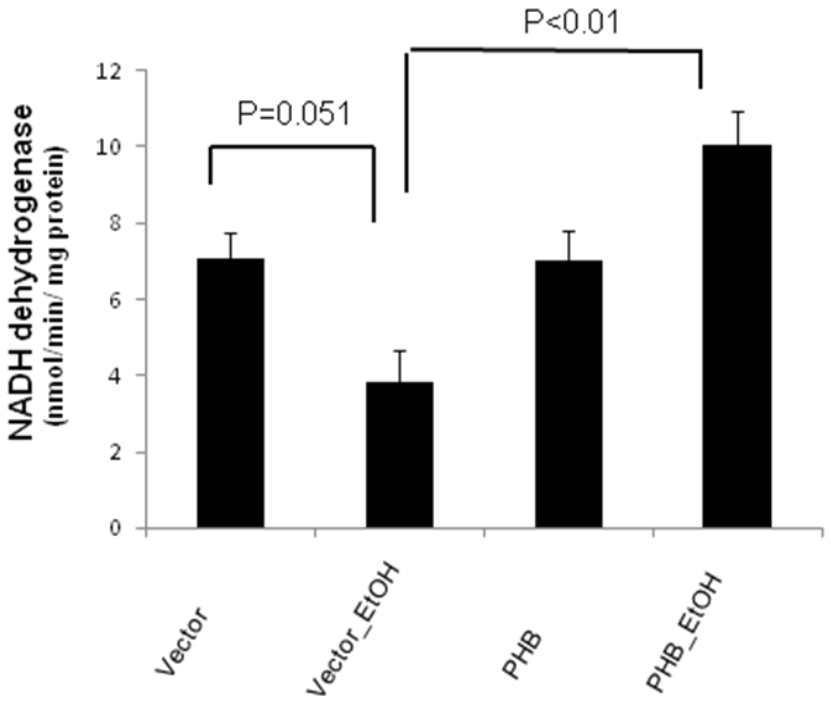


Figure 19: Effect of ethanol and PHB on NADH dehydrogenase activity (complex I) in INS-1E cells

INS-1E cells were transfected with a pCMV6-XL5 vector containing the human PHB gene and then incubated with 80 mM ethanol for 24 h. NADH dehydrogenase activity was determined by measurement of OD values every minute at 450 nm for 30 min. The results are expressed in nmol/min/mg protein and shown as the mean ± SEM, n = 5 experiments.

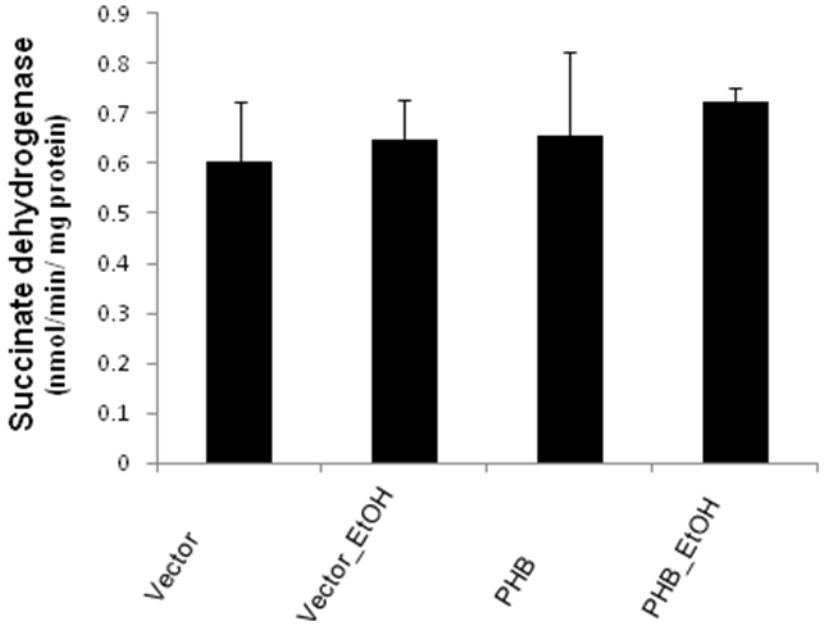


Figure 20: Effect of ethanol and PHB on succinate dehydrogenase activity (complex II) in INS-1E cells

PHB was over-expressed in INS-1E cells and the cells were further incubated with 80 mM ethanol for 24 h. Change in OD values was assessed every minute for 1 h at 630 nm. Results are expressed in nmol/min/mg protein and shown as the mean ± SEM,
n = 4 experiments.

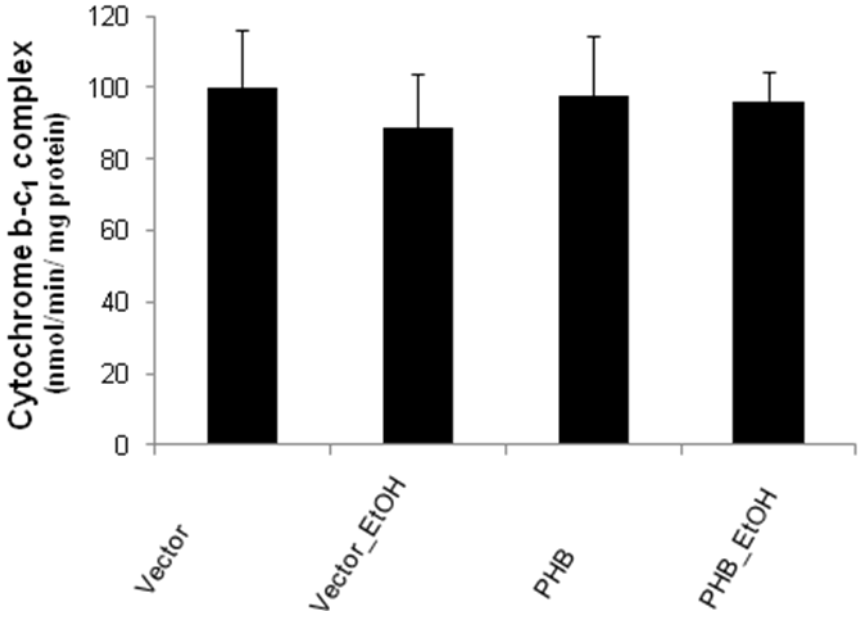


Figure 21: Effect of ethanol and PHB on cytochrome b-c_1 complex activity (complex III) in INS-1E cells

PHB over-expressing INS-1E cells were treated with ethanol for 24 h. Cytochrome b-c_1 complex activity was determined by measurement of OD values at 550 nm for 5 min. The results are expressed in nmol/min/mg protein and shown as the mean ± SEM,
n = 4 experiments.

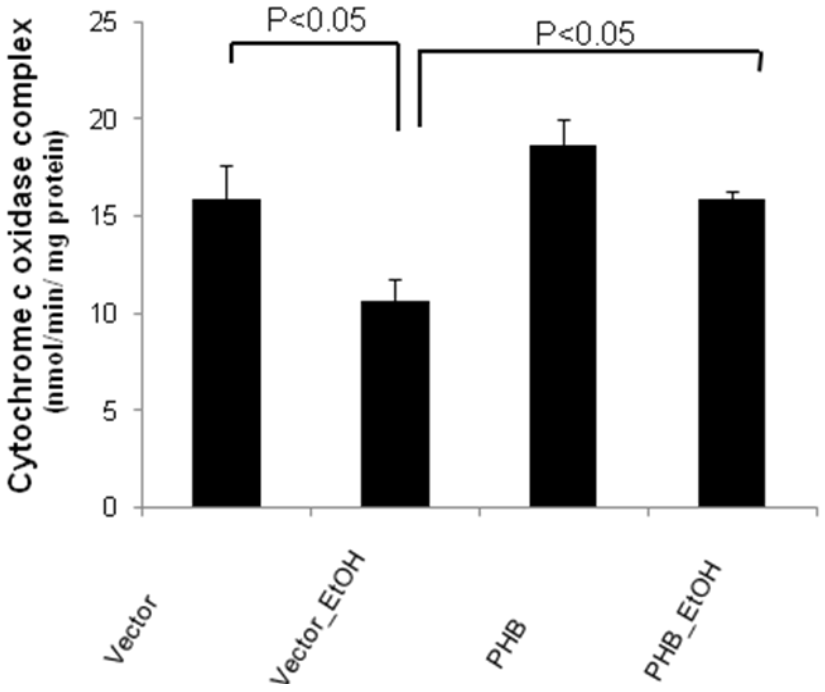


Figure 22: Effect of ethanol and PHB on cytochrome c oxidase complex activity (complex IV) in INS-1E cells

INS-1E cells were incubated with 80 mM ethanol for 24 h after transfection with PHB. Cytochrome c oxidase activity was determined by measuring the rate of oxidation of reduced cytochrome c at 550 nm for 1 min using a spectrophotometer. The results are shown in nmol/min/mg protein and as the mean ± SEM, n = 4 experiments.

5. PHB prevents apoptosis in ethanol exposed pancreatic β-cells

The above results showed that ethanol increases ROS production resulting in mitochondrial dysfunction, which may cause apoptosis. These results led us to investigate whether β-cells undergo increased apoptosis as a result of ethanol exposure. Mechanistically, mitochondrial dysfunction could induce activation of the c-Jun-N-terminal kinase pathway, which would increase the expression of the proapoptotic protein Bax and activation of the executioner caspase-3, resulting in cell death [202, 203].

To determine whether ethanol increases apoptosis, we studied morphological changes characteristic of apoptosis. We stained the cells exposed to ethanol for 24 h with Annexin V and Hoechst 33342. Annexin V translocation to the exterior aspect of the plasma membrane is an early marker of apoptosis. Using flow cytometry of cells stained with FITC-annexin V, apoptotic cell number was shown to increase ~two fold in cells exposed to ethanol (Fig. 23). In addition, ethanol treated cells had small and condensed nuclei (Fig. 4), consistent with apoptosis.

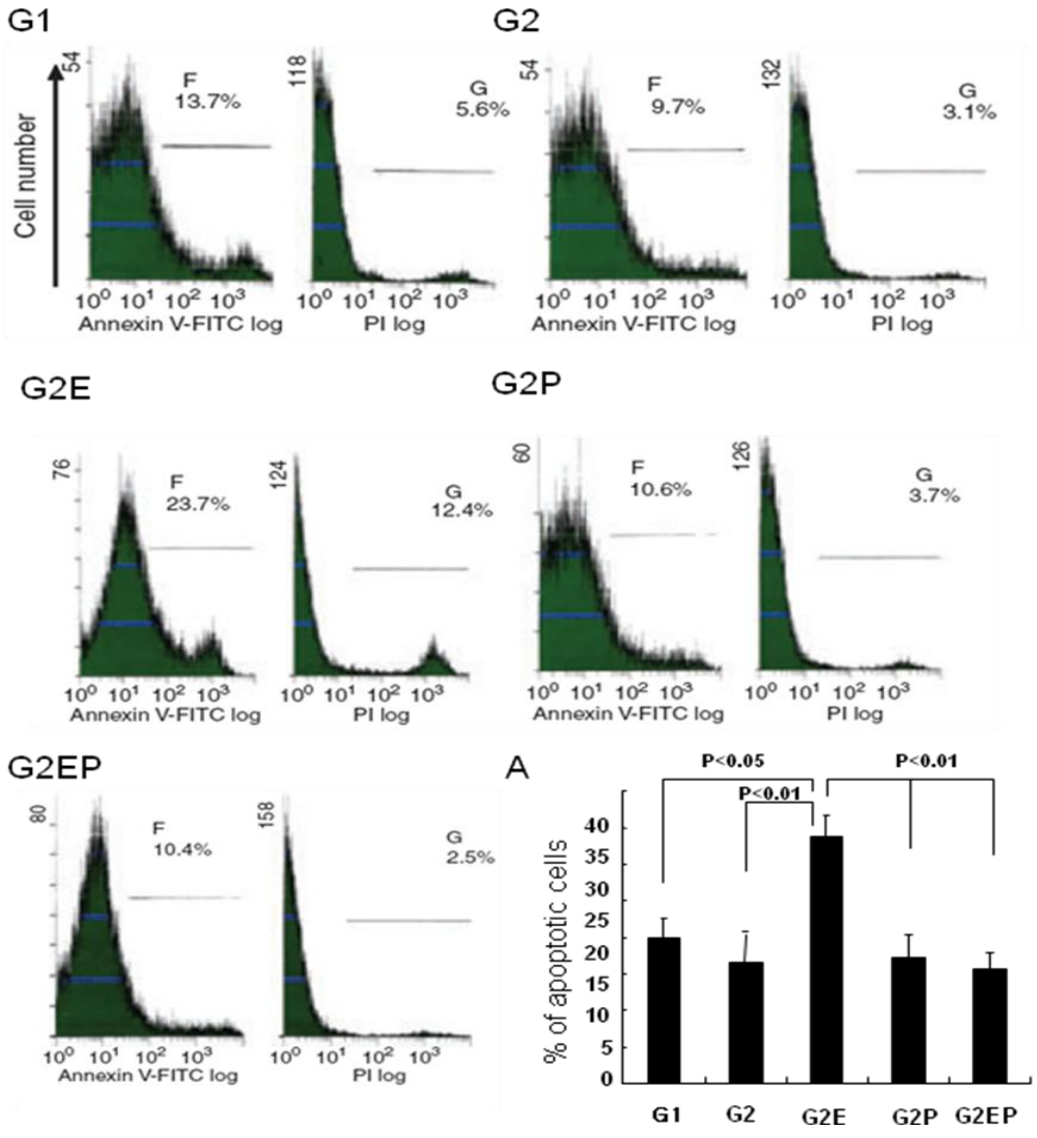


Figure 23: Effect of ethanol and PHB on apoptosis in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). Apoptosis was then determined by flow cytometry. Histograms from representative flow cytometry experiments are shown in G1, G2, G2E, G2P, G2EP. The % apoptotic cells (% of FITC-annexin V versus propidium iodide positive cells) obtained from 4 independent experiments using each treatment are shown as the mean ± SEM in A.

Because endogenous PHB has been reported to have anti-apoptotic effects in other cell systems [38, 204], we sought to investigate whether PHB protects β-cells against ethanol toxicity. To explore PHB effects on ethanol toxicity, we first used RINm5F cells. We determined the cellular distribution of exogenously applied PHB. Western blot analysis of total cellular protein extracts using anti-PHB antibodies showed two bands in cells incubated with recombinant PHB, one band migrating at the expected endogenous PHB molecular weight (30 kDa) and the other, slightly larger corresponding to His-tagged recombinant PHB (Fig. 5, top panel showing His-PHB). Using a fluorescence microscope, the His-tagged PHB did not localize to the nucleus, but showed a perinuclear distribution and colocalized with the mitochondrial dye Mitofluor^{TM} Red 589. These data suggest that exogenous PHB enters the cells and translocates to the mitochondria (Fig. 24B, C). PHB treatment reduced ethanol-apoptotic cell number to control level. In addition, Hoechst staining showed nuclei with restored normal appearance after PHB treatment (Fig. 4, 24).

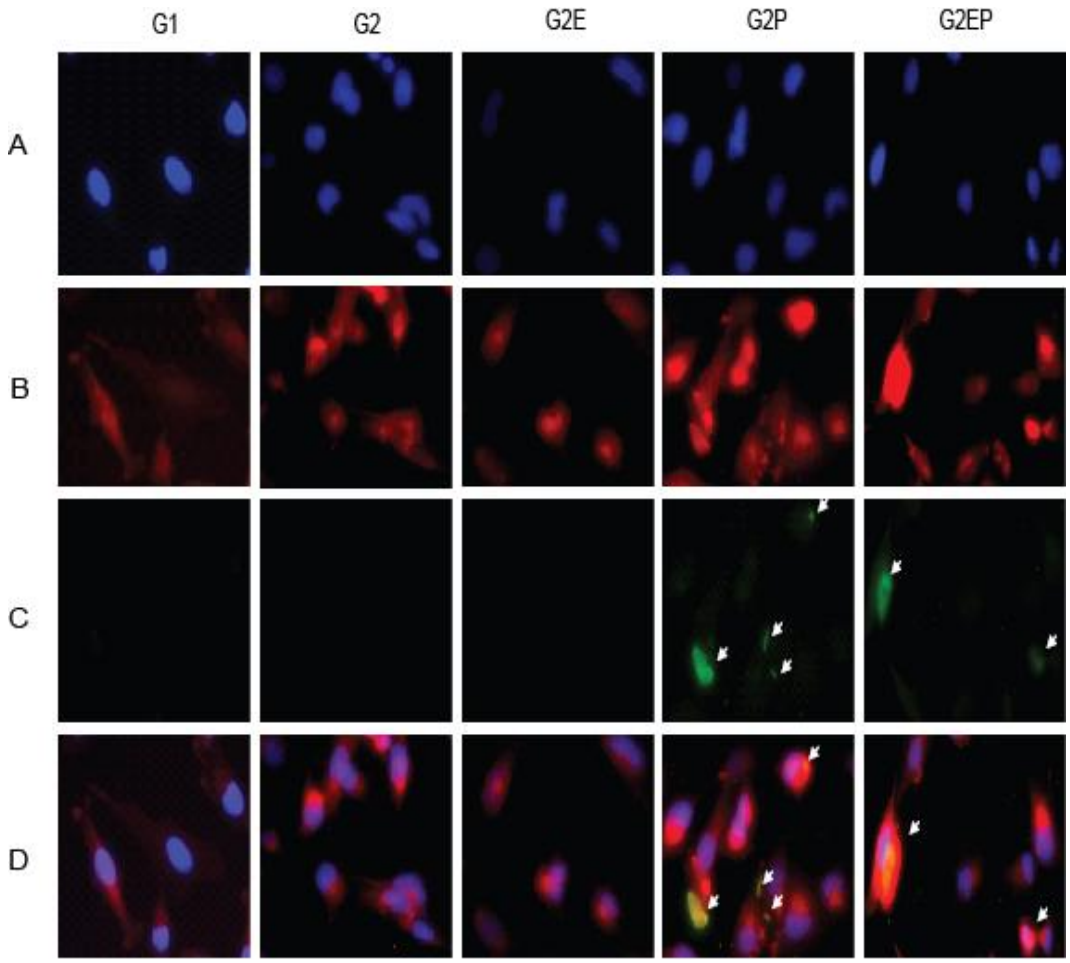


Figure 24: Localization of exogenous PHB in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). Cells were stained at 24 h (n = 3 experiments). A: Hoechst 33342 staining for nuclei; B: Mitofluor Red 589 staining for mitochondria; C: anti-His-FITC staining for exogenous PHB; D: merge. Arrows indicate staining of His-tagged PHB in C or both PHB and Mitofluor in D.

We next determined whether JNK is activated by determining its phosphorylation status using western blot with anti-JNK and p-JNK antibodies. Treatment of both RINm5F and INS-1E cells with ethanol increased p-JNK level by approximately two fold compared to control (Fig. 25). As a further link between JNK and caspase-3 in the apoptotic pathway, we determined the levels of the proapoptotic protein Bax, which has also been implicated in alterations of the mitochondrial membrane potential. We found Bax levels to be increased by ~ 40% in both RINm5F (Fig. 26A) and INS-1E (Fig. 26B) β-cells. In contrast, PHB treatment of RINm5F cells or PHB over-expression in INS-1E cells reduced JNK activation (Fig. 25) and Bax expression (Fig. 26) to normal levels.

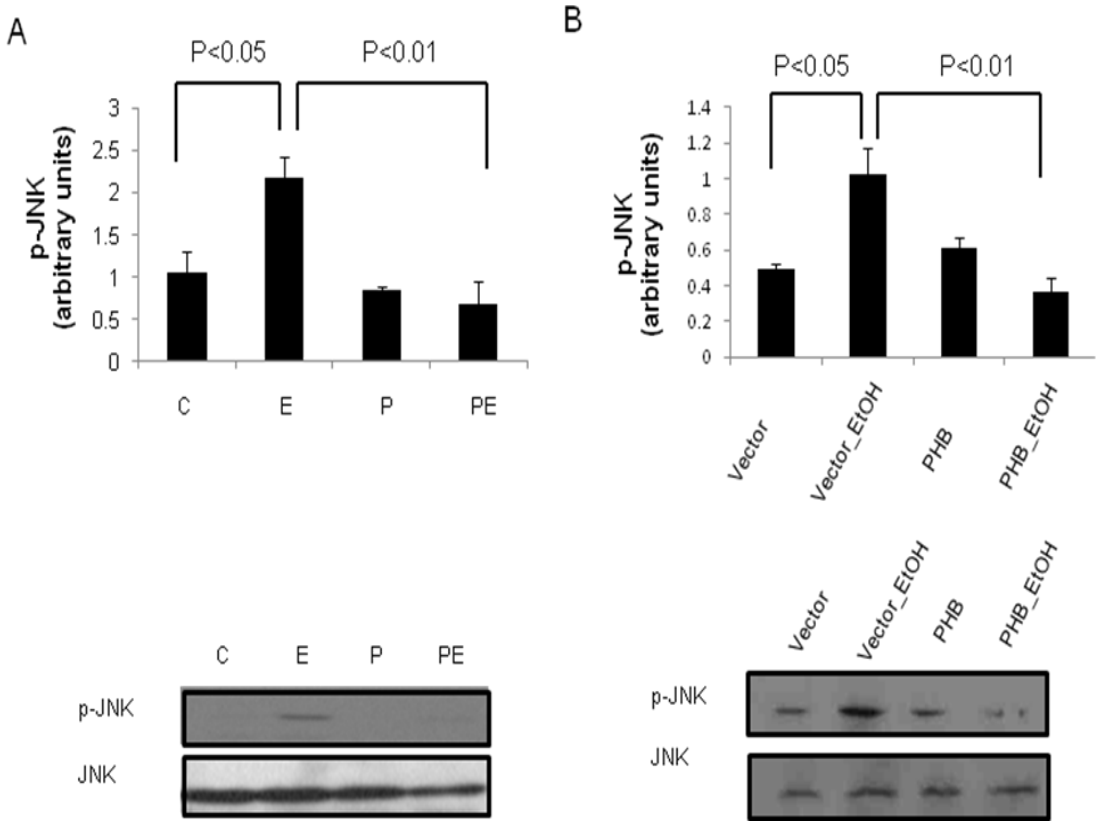


Figure 25: Effect of ethanol and PHB on JNK protein in RINm5F and INS-1E cells

Cell extracts were immunoblotted with anti-p-JNK antibody, and re-probed for JNK with the same stripped membrane. A. ***RINm5F cells*** incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM). C; control, E; EtOH, P; prohibitin, PE; prohibitin_EtOH B. ***INS-1E cells*** incubated with 80 mM ethanol for 24 h after transfection with PHB. p-JNK protein level expressed as the mean ± SEM arbitrary units relative to JNK (n = 3 experiments). The results also show a representative blot.

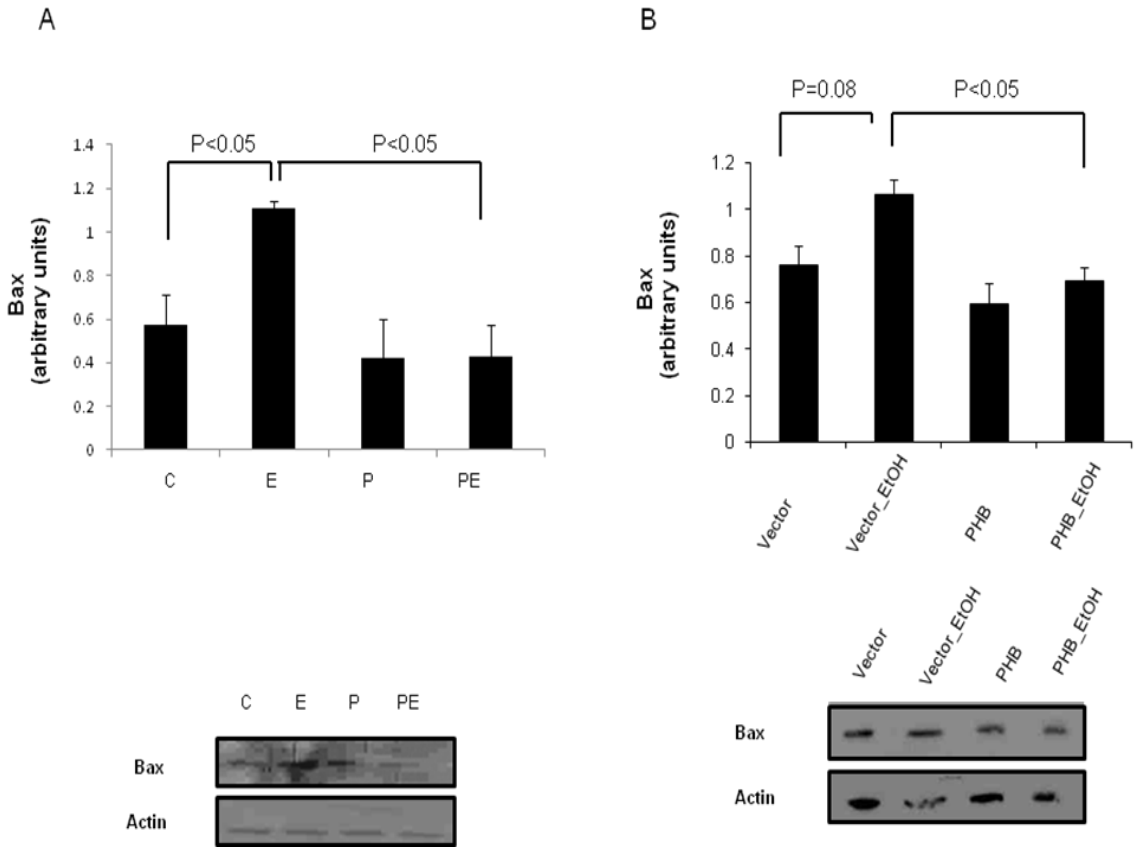


Figure 26: Effect of ethanol and PHB on proapoptotic protein Bax in RINm5F and INS-1E cells

Cell extracts were immunoblotted with anti-Bax antibody, and re-probed for actin with the same stripped membrane. A. ***RINm5F cells*** incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM). C; control, E; EtOH, P; prohibitin, PE; prohibitin_EtOH B. ***INS-1E cells*** incubated with 80 mM ethanol for 24 h after transfection with PHB. Bax protein level expressed as the mean ± SEM arbitrary units relative to actin (n = 3 experiments). The results also show a representative blot.

To determine changes in caspase 3, we used western blot with antibodies to total caspase-3 and cleaved caspase-3. Whereas total caspase-3 (35 kDa) was unchanged by ethanol treatment, we found cleaved caspase-3 level (17 kDa or 19 kDa) to be increased by ~ 40% in cells treated with ethanol (Fig. 27). In constrast, the cleaved caspase-3 level significantly decreased following incubation with recombinant PHB (Fig. 27). As well, the cleaved caspase-3 disappeared after PHB over-expression in both RINm5F and INS-1E cells, reflecting the reduction of JNK activation and Bax expression (Fig. 28)

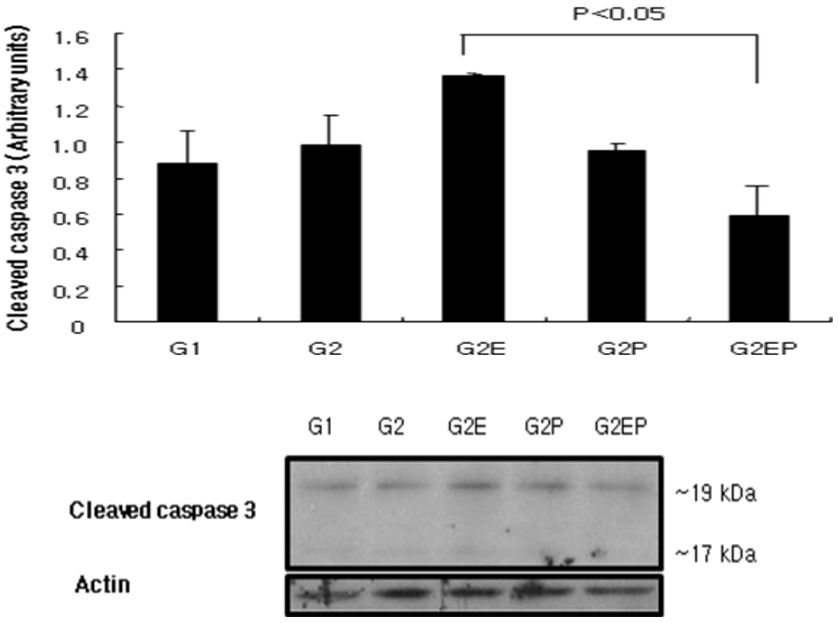
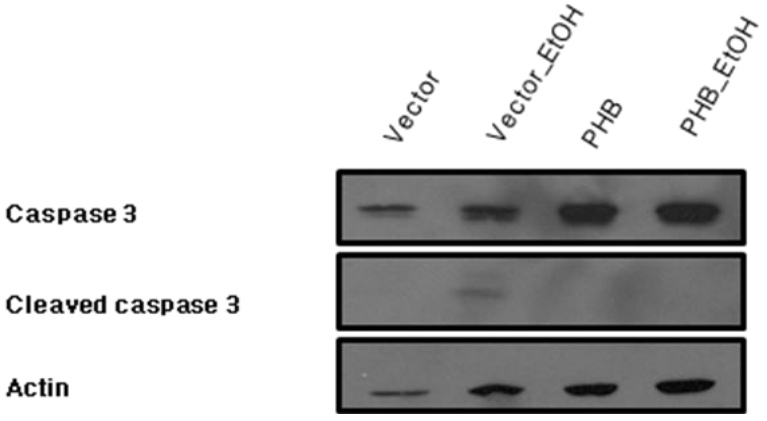


Figure 27: Effect of ethanol and exogenous PHB on cleaved caspase-3 in RINm5F cells

RINm5F cells were incubated for 24 h as described in material and methods. Cleaved caspase-3 was determined in cell extracts by western blot. A representative blot of n = 3 experiments is depicted showing 17 kDa and 19 kDa caspase-3 cleavage bands, and the levels of 19 kDa cleaved caspase-3 are shown expressed as the mean ± SEM arbitrary units relative to actin.

A



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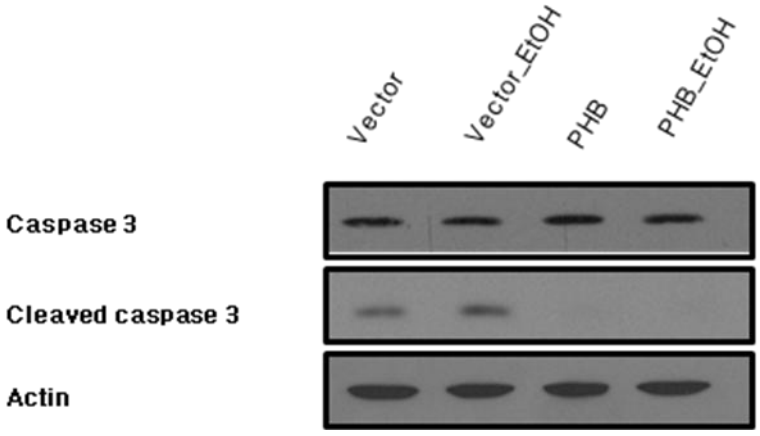
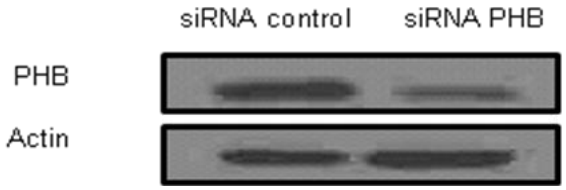


Figure 28: Effect of over-expression of PHB on cleaved caspase-3 in RINm5F and INS-1E cells

RINm5F and INS-1E cells were transfected with pCMV6-XL5 vector containing human PHB clone and subsequently incubated with 80 mM ethanol for 24 h. A representative western blot of caspase 3 and cleaved caspase 3 in cell extracts is shown (A; RINm5F, B; INS-1E; each n = 3 experiments)

To gain more insight into the antagonistic effects of PHB and ethanol, we knocked down PHB in RINm5F (Fig. 29A) and INS-1E (Fig. 29B) cells through transfection with PHB siRNA. To detect apoptosis under these conditions, we counted free floating RINm5F cells exposed to ethanol. We confirmed that ethanol induced apoptosis, expressed as the ratio of free floating to attached cells, is enhanced in the absence of PHB (Fig. 30).This ratio correlated with cleaved caspase-3 levels (Fig. 31).

A



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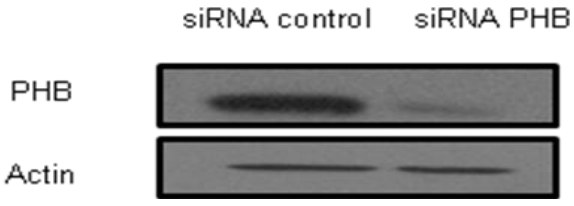


Figure 29: PHB siRNA in β-cells

RINm5F and INS-1E cells were transfected with PHB gene siRNA or control siRNA for 24 h. A. A representative western blot showing PHB protein expression level after transfection in RINm5F cells (n = 3 experiments). B. A representative western blot showing PHB protein expression level after transfection in INS-1E cells (n = 3 experiments)

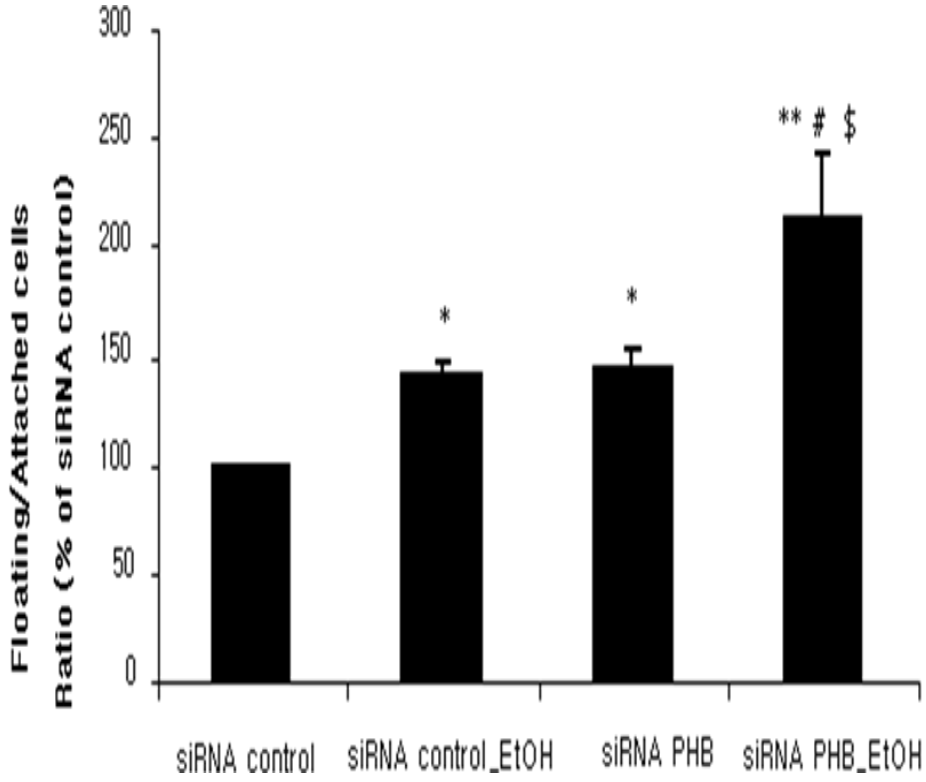
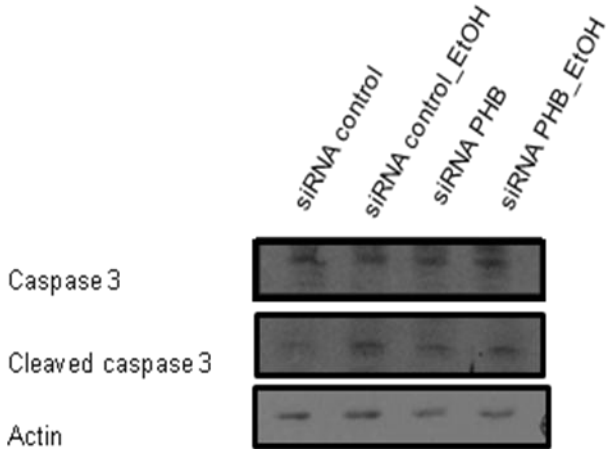


Figure 30: Effect of PHB siRNA on ethanol induced apoptosis in RINm5F cells.

RINm5F cells were transfected with PHB gene siRNA or control siRNA and further incubated with 80 mM ethanol for 24 h. Cell number were determined by cell counter (n = 3 experiments). The results are expressed as the mean ± SEM percentage ratio of floating / attached cells. * $P < 0.05$ versus siRNA control, ** $P < 0.01$ versus siRNA control, # $P < 0.05$ versus siRNA control_EtOH, $ $P = 0.056$ versus PHB siRNA.

A



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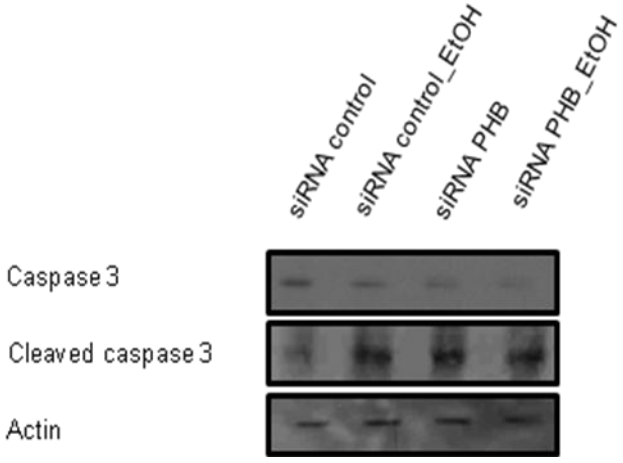


Figure 31: Effect of PHB siRNA on ethanol induced caspase-3 cleavage in RINm5F and INS-1E cells

PHB was knocked down in both RINm5F and INS-1E cells using transfection of PHB gene siRNA. The cells were subsequently incubated with 80 mM ethanol for 24 h. A representative western blot of caspase-3 and cleaved caspase-3 in cell extracts A. RINm5F cells (n = 3 experiments); B. INS-1E cells (n = 3 experiments).

6. PHB recovers glucose induced insulin secretion

Glucose induced insulin secretion (GIIS) was studied using INS-1E β-cells. Ethanol had no measurable effect on basal insulin secretion. However, GISS was decreased by about 26% in cells preincubated with ethanol compared to control (Fig. 32). In INS-1E over-expressing PHB, insulin secretion was comparable to control levels (Fig. 32).

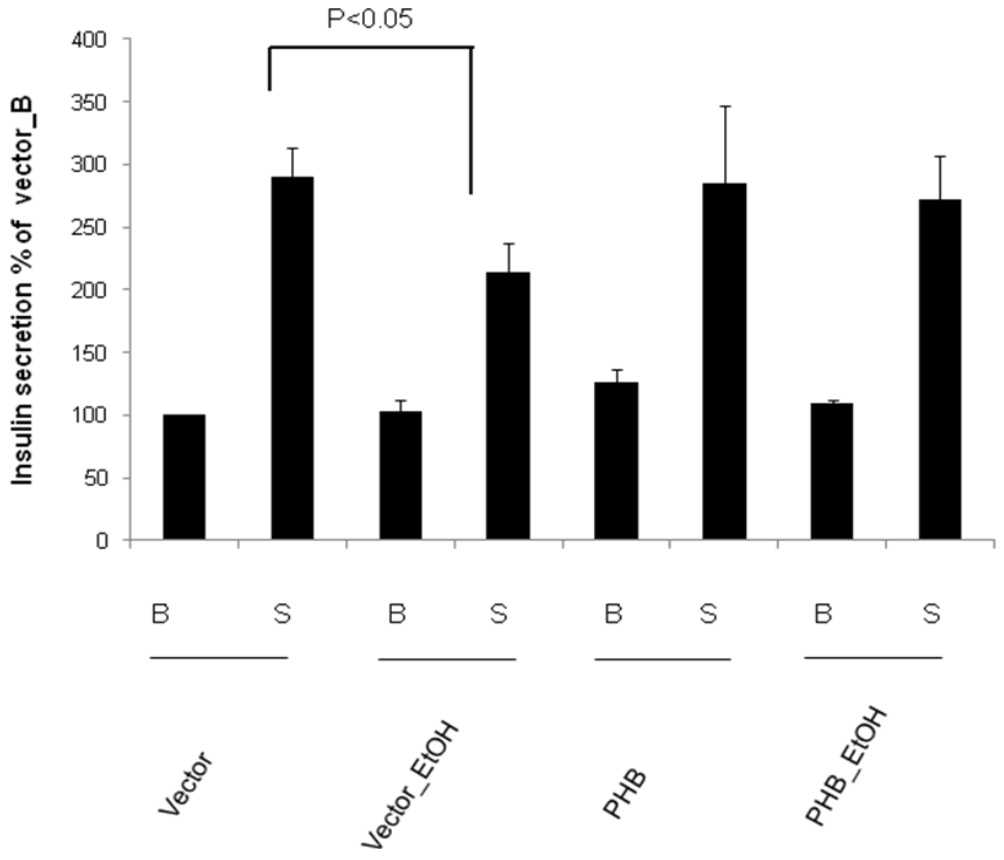
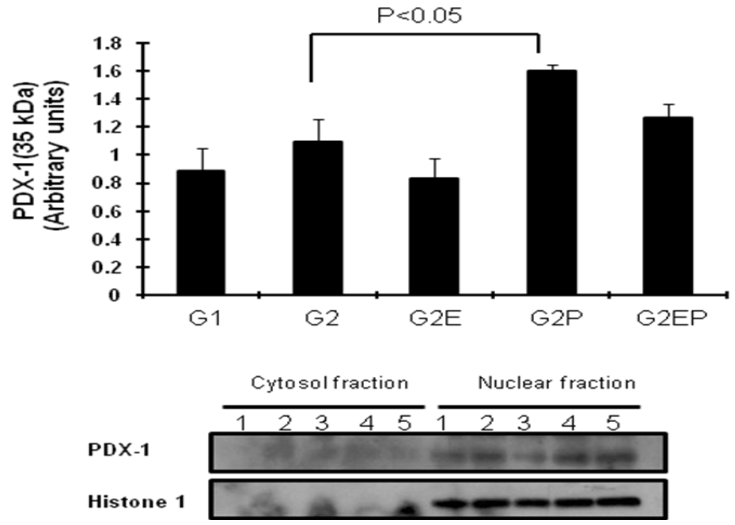


Figure 32: Effect of ethanol and PHB on glucose induced insulin secretion in INS-1E cells

Insulin secretion in INS-1E cells was measured after incubation at 37 °C for 30 min with 2.2 mM glucose (B, basal) and 16.7 mM glucose (S, stimulation). The results are expressed as percentage of control (vector at 2.2 mM glucose) and shown as the mean ± SEM, n = 5 experiments.

In subsequent studies, we investigated whether the effects of PHB on insulin secretion are mediated at the transcriptional level because PHB is found in the nucleus and has been shown to influence gene expression [38]. We first determined protein level of the transcription factors: pancreatic and duodenal homeobox 1 (PDX-1) and v-maf musculoaponeurotic fibrosacoma oncogene homolog A (MafA), in RINm5F cells exposed to ethanol and/or exogenously applied PHB (Fig. 33, 34). The levels of both the 35 kDa and the 43 kDa PDX-1 protein isoforms in whole cell extracts were not different among cell treatment groups. In a western blot of the nuclear fraction, ethanol insignificantly reduced the levels of both PDX-1 isoforms by ~20%. However, the levels of both PDX-1 isoforms were significantly increased by PHB (Fig. 33, $P < 0.05$). The transcription factor MafA also increased markedly in nuclei of cells exposed to PHB (Fig. 34, $P < 0.05$), but it was not altered by ethanol.

A



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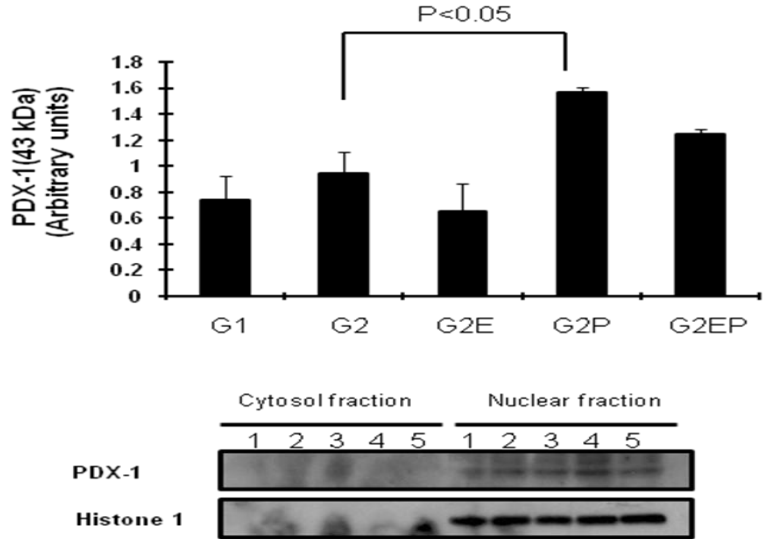


Figure 33: Effect of ethanol and exogenous PHB on PDX-1 protein in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). Nuclear extracts were then separated by SDS-PAGE and immunoblotted with anti-PDX-1 antibody. A. 35 kDa PDX-1, B. 43 kDa PDX-1. PDX-1 protein expressed as the mean ± SEM arbitrary units relative to actin (n = 3 experiments). 1: G1; 2: G2; 3: G2E; 4: G2P; 5: G2EP

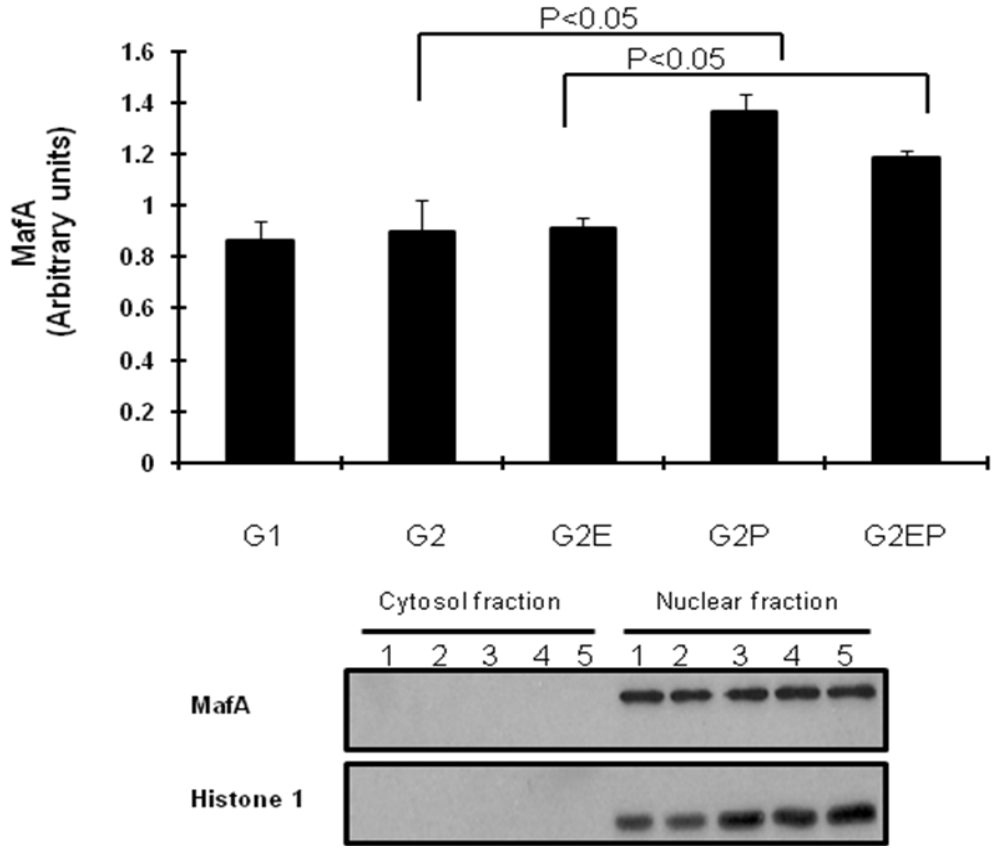


Figure 34: Effect of ethanol and exogenous PHB on MafA protein in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). Nuclear extracts were then separated by SDS-PAGE and immunoblotted with anti-MafA antibody. The figure shows a representative blot of MafA, and MafA protein expressed as the mean ± SEM arbitrary units relative to actin (n = 3 experiments). 1: G1; 2: G2; 3: G2E; 4: G2P; 5: G2EP

On the other hand, neither PDX-1 nor MafA mRNA levels were affected by ethanol or PHB (Fig. 35). Similarly, the mRNA levels of Insulin 1 and Insulin 2 genes remained unchanged following ethanol or PHB treatment (Fig. 35). Thus, PHB had no effect on the transcription of insulin genes or the transcription factors studied, which suggests that the effects of PHB on insulin secretion were more likely due to posttranscriptional influences such as recovery of mitochondrial function.

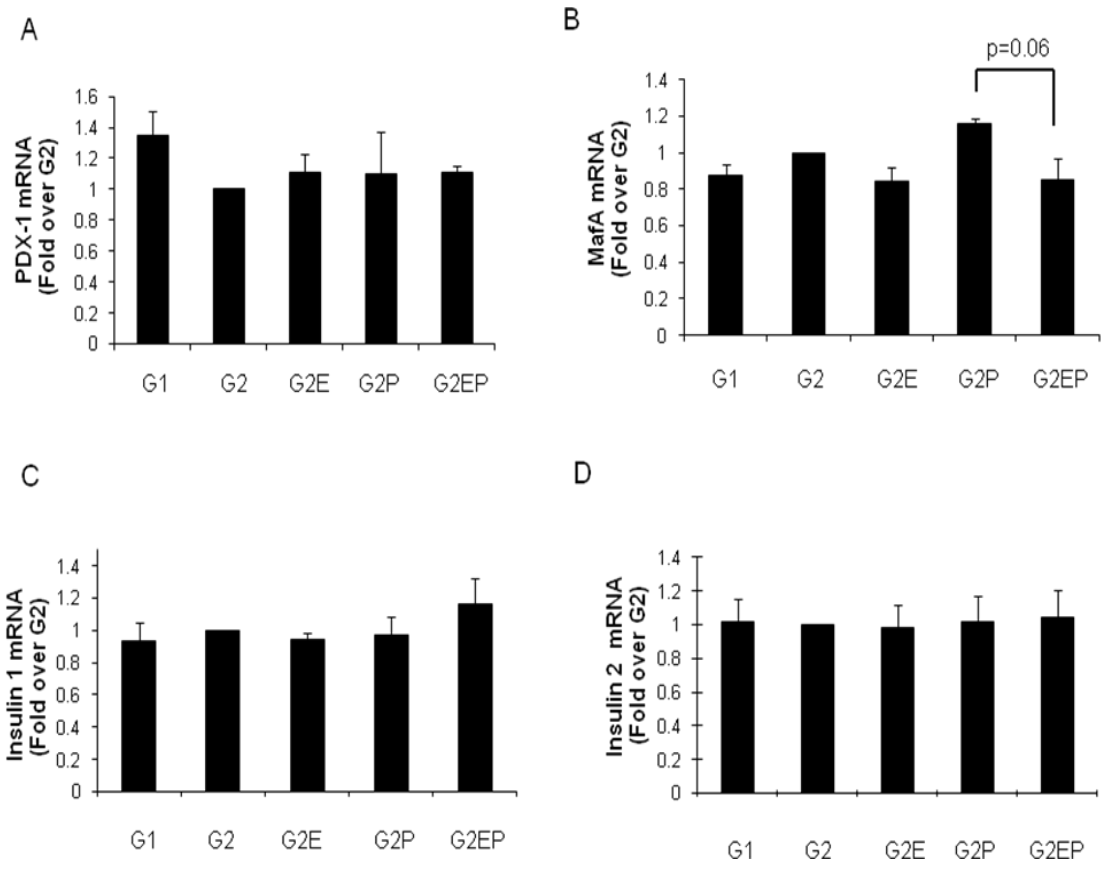


Figure 35: Effect of ethanol and exogenous PHB on mRNA expression of PDX-1, MafA and insulin in RINm5F cells

RINm5F cells were incubated for 24 h with or without ethanol (E, 80 mM) and with or without PHB (P, 10 nM) in the presence of glucose (G1: 5.5 mM, G2: 25 mM). A: PDX-1 mRNA (n = 4); B: MafA mRNA (n = 5); C: Insulin 1 mRNA (n = 4); D: Insulin 2 mRNA (n = 5). mRNA levels are expressed as the mean ± SEM fold over G2.

7. PHB inhibited ADH activity through regulation of ADH mRNA.

To investigate whether ethanol is metabolized in pancreatic β-cell, we first determined ethanol concentrations in the culture media after 24 h incubation with 80 mM of ethanol. The concentration of ethanol significantly decreased in β-cell containing media, as opposed to acellular media (Fig. 36), suggesting that these cells take up ethanol.

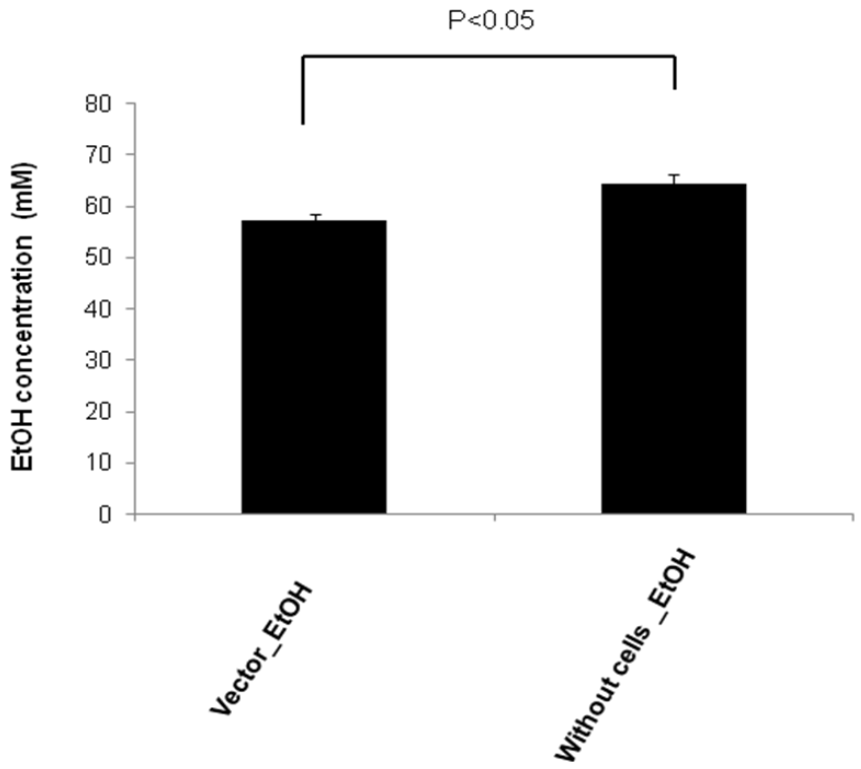


Figure 36: Effect of PHB on ethanol metabolism

Ethanol concentration in the media was determined at 24 h after incubation with 80 mM ethanol. The results are shown as the mean ± SEM. n = 4 experiments

Since ADH is a major enzyme of ethanol metabolism, at least in the liver [205, 206], we determined its activity in β-cell extracts from INS-1E cells (Fig. 37). Cellular ADH activity was increased with ethanol incubation compared to control cells (Fig. 37). This increase in ADH activity was attenuated by prohibitin (Fig. 37) as well as by simultaneous incubation with 4-methyl-pyrazole, which is a specific inhibitor of ADH (Fig. 37).

We next determined the protein and mRNA expression of ADH isoenzymes by western blot with different antibodies and by real time PCR using different primers respectively. We found that ADH5 protein level increased by ~ 90% of control in cells incubated with ethanol (Fig. 38) and ADH5 mRNA expression showed a similar level of expression as the protein (Fig. 38C). The expression of ADH1, which is abundant in hepatocytes, was faint in pancreatic β-cells.

In the presence of prohibitin, the ethanol concentration remaining in the cell culture media was statistically greater than in control cells, but smaller than that of acellular media. Western blot analysis showed that prohibitin decreased ADH5 protein (Fig. 38) and mRNA (Fig. 38) in ethanol treated β-cells when compared to control levels.

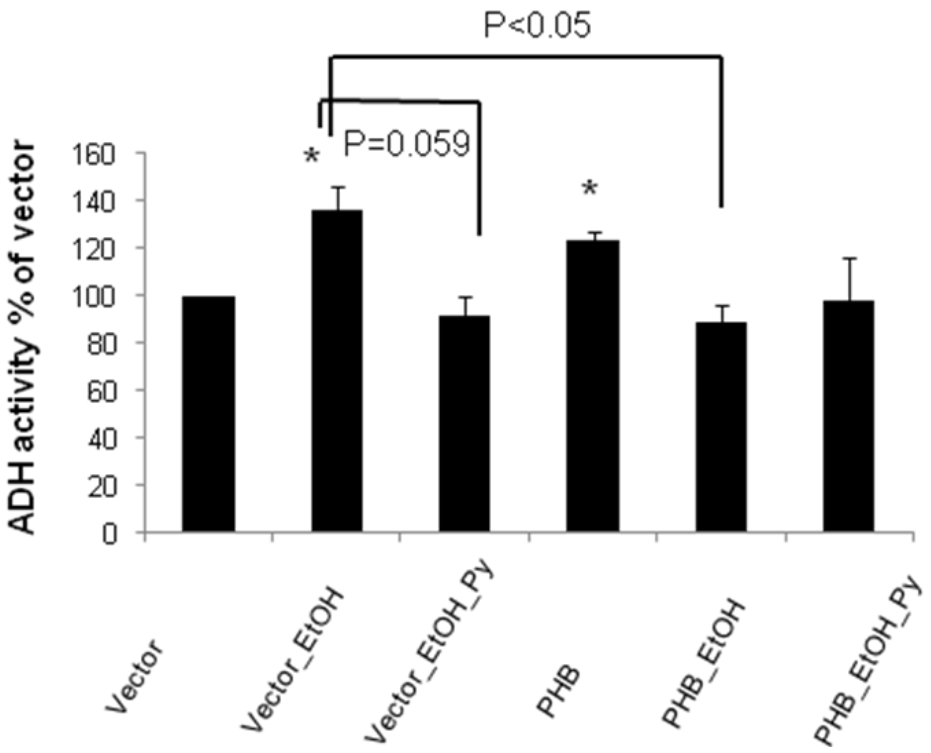
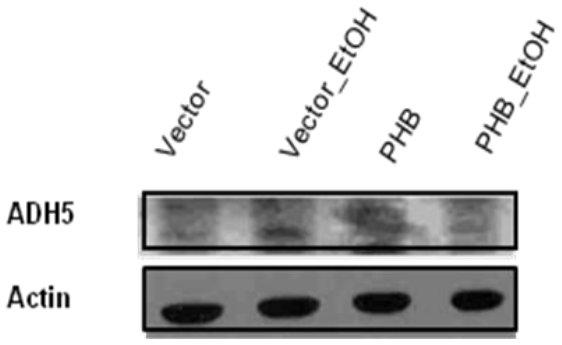


Figure 37: Effect of ethanol and PHB on total ADH activity

INS-1E cells were incubated with 80 mM ethanol for 24 h after transfection with pCMV6-XL5 vector containing human PHB clone. The total ADH activity was measured in equal amounts of cell extracts using a spectrophotometer at 570 nm. The activity is shown as the mean ± SEM fold of vector (n = 4 experiments). Py: 100 µM 4-methyl-pyrazole. * $P < 0.05$ versus vector

A



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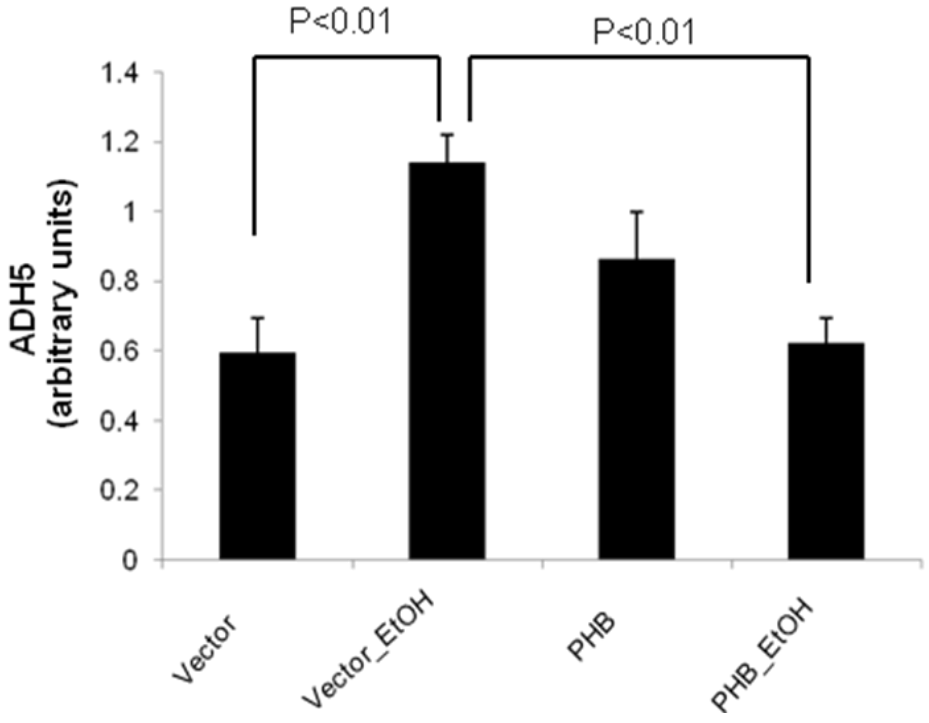

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C

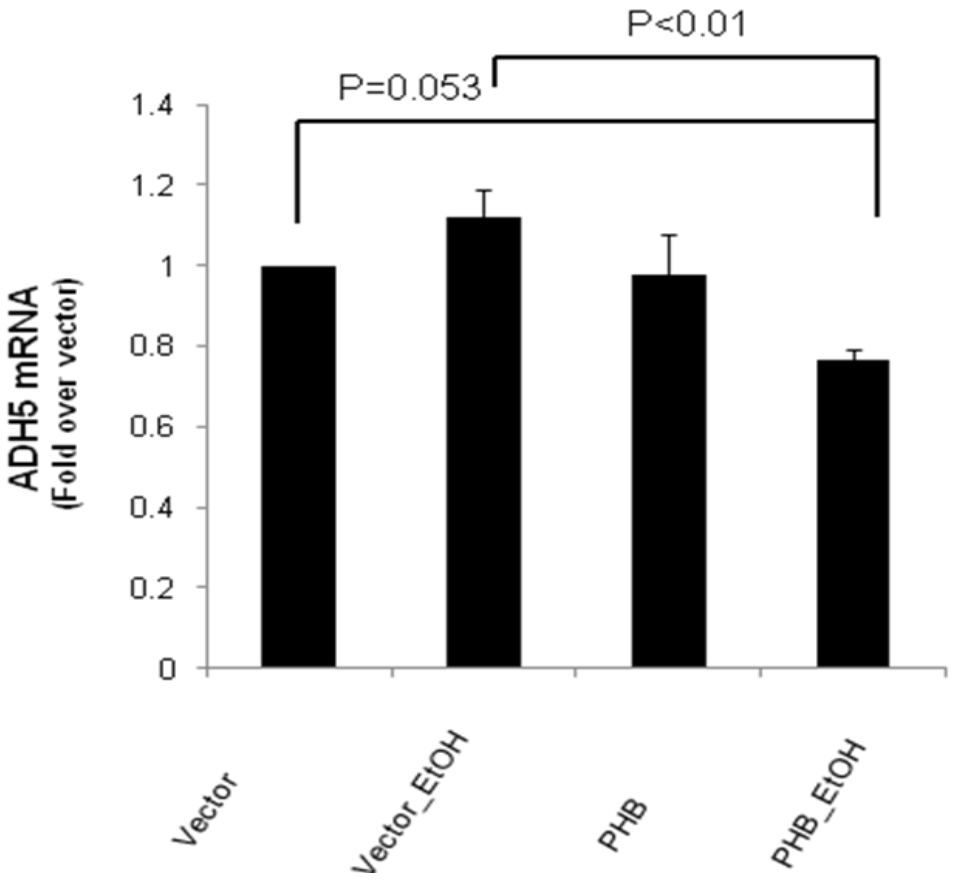

Figure 38: ADH5 is expressed in INS-1E cells and increased with ethanol treatment.

INS-1E cells were incubated with 80 mM ethanol for 24 h after transfection with pCMV6-XL5 vector containing the human PHB clone. Cell extracts were immunoblotted with anti-ADH5 and reprobed with anti-actin antibody with the same stripped membrane. A: A representative western blot of ADH5 protein; B: ADH5 protein represented as the mean ± SEM arbitrary units relative to actin (n = 4 experiments); C: ADH5 mRNA expressed as the mean ± SEM fold of vector (n = 4 experiments).

V. DISCUSSION

The purpose of this thesis was to investigate the effects of prohibitin on pancreatic β-cell dysfunction caused by oxidative stress using as a paradigm the deleterious effects of ethanol. We found that ethanol is metabolized in β-cells and confirmed that it induces oxidative stress and apoptosis in these cells, as has been shown in other cell types [207, 208]. We then examined whether PHB is expressed in β-cells and what role PHB plays in these cells. While ethanol induced β-cell dysfunction by causing mitochondrial dysfunction, PHB protected these cells from ethanol toxicity by preventing mitochondrial dysfunction and apoptosis, and by rescuing the insulin secretory response to glucose.

1. Ethanol is metabolized in β-cells.

Ethanol induces cell injury via direct effects or indirectly through its metabolites, as has been shown in tissues such as liver and brain [28, 209, 210]. Acetaldehyde is the primary metabolic product of ethanol. It is more toxic than ethanol [211] and known to trigger both ROS production and apoptosis [148]. Chronic ethanol exposure enhances free radical generation leading to accumulation of superoxide anions [212]. ADH is a major ethanol metabolizing enzyme and its expression in cardiomyocytes generates elevated amounts of acetaldehyde, resulting in increased lipid peroxidation and protein carbonyl formation in the heart and liver as well as exacerbation of cardiac contractile dysfunction in mice consuming ethanol [213]. Ethanol abuse is a major cause of liver cirrhosis and cardiomyopathy. Ethanol is also known to cause pancreatitis and recent studies indicate

that ethanol is metabolized in the exocrine pancreas at a rate similar to that of the liver [18]. Whether ethanol is metabolized in β-cells, however, has remained unknown.

In order to investigate whether ethanol is metabolized in pancreatic β-cells, we first determined the ethanol concentration remaining in β-cell culture media after 24 h of incubation with 80 mM ethanol. In β-cell containing media, ethanol concentration decreased significantly during this period compared to acellular media. Since ADH is the main enzyme implicated in ethanol metabolism [18, 205, 206], we measured total ADH activity in β-cell lysates in the presence of 4-methyl pyrazole, which is a specific inhibitor of ADH [214]. Total ADH activity increased with ethanol incubation and decreased to control levels in the presence of 4-methyl pyrazole, indicating that ADH is involved in ethanol metabolism in these cells. A search for ADH isoenzymes using western blot analysis and real time PCR identified ADH5 as the isozyme predominantly present in β-cells. These results are in line with earlier reports of the presence of ADH in human endocrine pancreas [19] and more recent localization of some ADH isoforms in murine and human islets, where the cell type involved, however, was not defined [20, 21]. Thus, ethanol is metabolized in pancreatic β-cells by alcohol dehydrogenase. This conclusion implies that acetaldehyde, the main product of ethanol metabolism, may cause oxidative damage in β-cells, and the data is consistent with epidemiological studies suggesting that ADH polymorphism influences the risk for type 2 diabetes through metabolite toxicity [24-26].

2. Ethanol alters mitochondrial function in β-cells.

It is accepted that alcohol consumption leads to cell injury through production of ROS and mitochondrial dysfunction and such studies have generally been performed in

liver, brain and exocrine pancreas [116, 141, 200]. For example, ethanol decreased the pancreatic pool of ATP and glutathione (GSH), and increased post-translational protein modifications through oxidative stress in rat pancreas [215] and liver tissue [28, 185]. ROS have been implicated in β-cell dysfunction and apoptosis in rodent models of diabetes [110, 216-218], and changes in mitochondrial function, including increased ROS and UCP2 expression, lower ATP, and decreased ATP/ADP ratio have also been documented in β-cells from patients with type 2 diabetes [219]. It is therefore plausible to consider that ROS produced by ethanol exposure may also adversely affect β-cells.

Ethanol metabolism onto acetaldehyde in pancreatic β-cells could be particularly harmful because these cells have very low expression of antioxidant enzymes and are very sensitive to oxidative stress [36, 220]. Humans with chronic ethanol-induced pancreatitis have abnormal glucose tolerance in association with decreased insulin secretion [8], and ethanol inhibited glucose-stimulated insulin secretion in isolated rat islets [9] and perfused rat pancreas [220]. Ethanol has also been found to inhibit basal, but not glucose-stimulated insulin secretion, in hamster insulinoma tumor (HIT) β-cells [12]. On the other hand, there have been reports of ethanol augmenting glucose-induced insulin secretion [13, 14], and priming β-cells to glucose stimulation [16]. These contradictory reports may be explained by methodological variations such as different cell lines, different ethanol concentrations and exposure time. One recent study suggested that ethanol acutely amplifies insulin secretion in vivo by stimulating islet blood flow [221], while another study reported that ethanol acutely increases insulin secretion by triggering β-cell swelling due to secondary water transport [222]. Ethanol exposure in these experiments was acute (less than 1h) as

opposed to chronic (lasting 24 h) in studies (including this thesis) that showed inhibition of insulin secretion by ethanol. In addition, the stimulatory effects of ethanol were identified through glucose-independent mechanisms, contrary to this thesis and the majority of reports.

The literature is particularly poor in regard to the effects of ethanol on β-cell mitochondrial function, except for a suggestion in hamster insulinoma tumor (HIT) β-cells, where ethanol inhibited β-cell mitochondrial activity as judged by the MTT assay [12]. Several lines of evidence in this thesis indicate that ethanol alters mitochondrial function in β-cells. First, MTT reduction was altered by ethanol in both cell lines used in this study. These adverse effects of ethanol were dose dependent and, at 24 h, were statistically significant starting at 80 mM (368 mg/dl) of ethanol. This ethanol concentration is physiologically relevant as it is within levels found in the blood of clinically non-intoxicated alcohol drinkers [26, 223]. Second, ROS production was increased in ethanol exposed β-cells (Fig. 11), and this increase was associated with proportionally increased UCP2 (Fig. 12) and decreased ATP levels (Fig. 13). The results agree with the notion that oxidative stress induces UCP2 expression, which is implicated in the dissipation of the proton gradient in the mitochondrial inner membrane and reduces ATP synthesis [201]. Third, we found a reduction of the electron transport activity as manifested by a reduction of the mitochondrial membrane potential and mitochondrial NADH dehydrogenase (complex I), cytochrome c oxidase (complex IV) and ATP synthase (complex V).

These functional effects of ethanol are in line with those found in other tissues and are likely consequent to alterations of mitochondrial proteins [224, 225]. The toxicity of

ethanol on mitochondrial complexes varied among studies and affected one or several of these complexes. The electron transport and proton translocation rate at the NADH dehydrogenase complex decreased significantly in liver mitochondria isolated from ethanol-fed animals [226, 227]. Other studies found impairment of only cytochrome b-c1 oxidase [226, 228], both cytochrome b-c1 oxidase (complex III) and cytochrome c oxidase [185], or ATP synthase [229, 230] in rat liver after chronic ethanol exposure. In cerebella from neonatal rats exposed to ethanol during gestation, complex II synthesis was reduced only at high ethanol dosages, whereas complexes IV and V were already affected at low ethanol doses [231]. Similarly, chronic ethanol exposure in rat liver primary cells depressed the activity of complexes I, III, IV and V, but not of complex II [116, 142]. These complexes appear to be particularly sensitive to ethanol because, except for ATP synthase, they are the main sites of ROS production [232-235]. Thus, the overall activity of mitochondria would be expected to be impaired with ethanol exposure. However, because there are no prior reports on mitochondrial complex activity in β-cells exposed to ethanol, these results bring a significant contribution to our knowledge in this area.

Our studies in INS-1E cells showed that ethanol decreased mitochondrial activity at NADH dehydrogenase (complex I) by ~ 46% and cytochrome c oxidase complex (complex IV) by ~ 33%, but ethanol had no effect on succinate dehydrogenase or cytochrome b-c_1 complex. Several studies have shown that PHB binds to mitochondrial proteins resulting in their stabilization or protecting them from degradation [51, 52, 174, 175]. In ethanol exposed β-cells, the interaction of PHB to subcomponents of the mitochondrial cytochrome c oxidase complex was reduced, providing a possible explanation for the reduction in

activity of cytochorome c oxidase complex in β-cells. This, in turn, may explain the reduced activity of complex I, which has been suggested to be dependent on the integrity of complex IV [236, 237]. Reduced activity of complexes I and IV may explain the collapse of mitochondrial membrane potential as well as other markers of mitochondrial dysfunction associated with ethanol exposure such as increased UCP2 and decreased ATP levels.

3. Ethanol induces apoptosis in β-cells.

Using several approaches, we demonstrated that ethanol induces apoptosis in β-cells. First, ethanol increased JNK activation in RINm5F cells by approximately two fold compared to control, as shown by p-JNK levels. Second, the pro-apoptotic protein Bax level increased by ~ 40% in both RINm5F and INS-1E cell lines, which may result in the collapse of the mitochondrial membrane potential and in caspase-3 activation, ultimately causing cell death. Third, flow cytometry analysis using FITC-annexin V staining revealed a ~ two fold increase in the apoptotic cell number among cells exposed to ethanol, at the same time as cleaved caspase-3 level increased by ~ 40%. Annexin V staining and caspase-3 cleavage are well known markers of apoptosis. Finally, Hoechst 33342 staining showed small and condensed nuclei in the ethanol treated cells, which is also consistent with increased apoptosis. These observations indicate that ethanol induces apoptosis via JNK activation caused by ethanol-induced ROS production and mitochondrial dysfunction in β-cells, in agreement with the reduction of β-cell mass reported in rats chronically fed ethanol [238, 239].

These results are also consistant with findings in other cell types. In isolated rat hepatocytes, ethanol triggered both oxidative stress [240] and apoptosis via activation of stress signaling pathways involving c-Jun phosphorylation by JNK [241]. JNK belongs to the mitogen-activated protein kinase (MAPK) family, which is a well known stress signaling family. The MAPK family also includes extracellular signal-regulated kinase (ERK) and p38 MAP kinase. The ERK pathway is mostly coupled to the regulation of cell proliferation, differentiation and survival, while the JNK and p38 MAPK pathways are strongly associated with oxidative stress. Activation of JNK by ROS in hepatocytes is considered to be a cause of pro-apoptotic events such as increasing Bax level, which may alter mitochondrial membrane potential [28, 202] and insulin signaling [242, 243], whereas JNK activation in β-cells may lead to apoptosis and disturbances in insulin secretion [35].

4. Ethanol alters prohibitin expression and cellular distribution

Although prohibitin is known to be expressed in many tissues, we were the first to report on its expression in pancreatic β-cells. Under normal conditions, PHB is found in cell nuclei and the perinuclear area corresponding to the mitochondria, as has been reported in breast cancer cells [38].

In normal β-cells, endogenous PHB was found to be present in the nucleus and in the perinuclear area. After cell treatment with ethanol, PHB protein increased dramatically by 92%, and PHB mRNA expression showed similar expression levels. At the same time, the level of PHB protein decreased in the nucleus, while increasing in the cytoplasm, suggesting that PHB protein is excluded from the nucleus as a consequence of ethanol treatment. This redistribution of PHB was vizualized using fluorescence microscopy and

cellular fractionation, where western blot analysis of mitochondrial and nuclear fractions showed that PHB is preferentially localized to the mitochondria in cells exposed to ethanol.

Similar results have recently been reported in rat liver, in which chronic ethanol feeding increased PHB expression [185]. However, PHB expression decreased when ethanol-induced mitochondrial dysfunction was prevented by increasing the anti-oxidant capacity through feeding with S-adenosylmethionine, a precursor of glutathione [185]. Mitochondrial PHB expression also increased in cardiomyocytes with oxidative injury in chronically stressed rats [183, 184]. When rat granulosa cells were transfected with a PHB-green fluorescence protein fusion construct, this fusion protein moved from the cytoplasm into the mitochondria [244]. In breast cancer cells, PHB was also found to be exported from the nucleus upon apoptotic signaling [38]. Rastogi and colleagues recently reported that PHB contains a leucine-rich nuclear export signal, which facilitates its cytoplasmic translocation [245].

5. Exogenous PHB enters inside the cells

Following an initial report by Mengwasser and colleagues that PHB is found in the circulation [40], recently confirmed by others [41], we hypothesized that PHB may enter or re-enter the cells. We first incubated RINm5F cells with recombinant His-tagged PHB added to cell culture media. Western blot analysis of total cellular protein extracts using anti-PHB antibodies showed 2 bands of PHB immunoreactivity, one of which had the molecular size of the His-tagged PHB, suggesting that exogenous PHB enters inside the cells. Using fluorescence microscopy, the His-tagged PHB showed a perinuclear distribution and co-localized with the mitochondrial dye Mitofluor^{TM} Red 589, confirming

translocation of exogenous PHB to the mitochondria. For unspecifical reasons, however, exogenous PHB did not translocate to the nucleus.

PHB internalization has not been previously reported and mechanisms to explain this internalization are still unknown. However, our group has previously reported that exogenous PHB elicits metabolic changes in isolated adipocytes and binds to EH domain 2 (EHD2) [187]. Both EHD2 and PHB have been identified in lipid droplets released from 3T3L1 cells [39, 187], and EHD proteins have been shown to be involved in endocytosis and vesicle recycling [246]. PHB transport inside the cells could also involve a lipid raft or caveolin-dependent process, as caveolins and lipid rafts are involved in internalization of various molecules [247, 248], and PHB is present in lipid rafts on the cell membrane [247, 248] and abundant in the caveolin-1-rich fractions in some cells [47].

6. PHB protects β-cells from oxidative stress and apoptosis

To demonstrate the beneficial effects of PHB on mitochondrial function and cell survival, we conducted tests on RINm5F cells after incubation with recombinant PHB, as well as on RINm5F and INS-1E cells after transfection with human PHB gene. In some experiments, we knocked down endogenous PHB using the siRNA technique. In each set of these experiments, cells were exposed to ethanol to induce mitochondrial dysfunction.

In RINm5F cells simultaneously treated with ethanol and recombinant PHB, ROS production and UCP2 levels were both significantly reduced, whereas MTT reduction and ATP production increased, when compared to control levels. In cells over-expressing human PHB and treated with ethanol, MTT reduction was also normal. Similar results

were obtained using hydrogen peroxide (H_2O_2) instead of ethanol, bolstering the hypothesis that PHB protection of β-cells involved anti-oxidant mechanisms.

Other investigators have reported on antioxidant effects of PHB. Theiss and colleagues reported that in inflammatory bowel diseases PHB localizes primarily to the mitochondria and that PHB over-expression in CaCo-2 intestinal epithelial cells decreases ROS accumulation and protects these cells from oxidant-induced depletion of glutathione [249]. In a more recent study of cultured neonatal cardiomyocytes, PHB over-expression protected the cells against oxidative stress injury induced by hydrogen peroxide, as assessed using MTT reduction [183]. The authors also indicated in this study that the mitochondria-mediated apoptotic pathway was suppressed in PHB over-expressing cardiomyocytes, in agreement with our findings in β-cells.

While ethanol increased apoptosis in β-cells, PHB treatment prevented β-cell apoptosis as shown by normal appearance of cell nuclei, reduction of the proapoptotic protein Bax and cleaved caspase-3 levels, and decreased p-JNK protein level. Flow cytometry with annexin V staining also showed that the number of apoptotic cells was normal in cells exposed to both ethanol and PHB. In a different approach, PHB deletion by siRNA enhanced ethanol toxicity by increasing apoptotic cell number in RINm5F. These results also agree with findings in granulosa cells [244] and breast cancer cells [38], where over-expression of PHB attenuated the ability of staurosporine, camptothecin and serum withdrawal to induce apoptosis [38, 244].

7. PHB protects mitochondrial respiratory chain in β-cells

In addition to preventing ROS formation and its adverse effects on ATP synthase and apoptosis, PHB in β-cells was also found to rescue ethanol-induced injury of the respiratory chain. The activities of NADH dehydrogenase (complex I) and cytochrome c oxidase (complex IV), which were impaired by ethanol, were recovered by PHB exposure, in association with normalization of PHB interaction to cytochrome c oxidase, mitochondrial membrane potential, and ATP synthase. It was surprising that succinate dehydrogenase (complex II) and cytochrome b-c_1 complex (complex III) activities did not change in the presence of PHB. Our results are in line with those reported in other cell types. To our knowledge, there have been no reports of interaction of PHB with mitochondrial complexes II or III. Recently, PHB-knockdown in endothelial cells was reported to induce mitochondrial depolarization due to a reduction of complex I activity [182], whereas mitochondrial PHB expression in cardiomyocytes protected against oxidative stress-induced cell death [183, 184]. In yeast and plants, loss of PHB genes reduced the mitochondrial membrane potential [179, 180]. It has been reported that PHB plays a chaperone role in the stabilization of newly synthesized subunits of mitochondrial respiratory enzymes [177] leading to a reduction of their proteolysis [52]. This has been shown only for mitochondrial complex IV [52, 186], which agrees with our results. PHB is essential for normal mitochondrial development and its deficiency in yeast and *C. elegans* is associated with deficient mitochondrial function [177] and reduced life span [180, 250].

Both exogenous PHB treatment of RINm5F cells and endogenous over-expression of PHB in INS-1E cells improved the interaction of PHB to cytochrome c oxidase in the

presence of ethanol, and this correlated with cytochrorme c oxidase activity. These observations suggest that, in the presence of prohibitin, complex IV was not disintegrated by ethanol, consistent with the notion that prohibitin stabilizes mitochondrial complexes [51]. The relationship between prohibitin and the activity of mitochondrial complexes is not well known. One study recently showed that the interaction of prohibitin with mitochondrial complex IV was reduced in liver cells transfected with hepatitis C virus core protein and that this lack of association resulted in activity reduction for this complex [186]. The fact that prohibitin also rescued complex I activity suggests that prohibitin prevented complex I degradation by ethanol. That prohibitin did not physically interact with complex I has also been observed by others [186]. It is possible that prohibitin protected complex I indirectly by stabilizing complex IV, since a functional complex IV is required for the stability of complex I [236, 237]. In agreement with our finding, one study has reported that reduced cytochrome c oxidase activity leads to a compromised mitochondrial membrane potential and a decreased ATP level, increasing the sensitivity to apoptosis in a mouse fibroblast cell line [233].

8. PHB may regulate ethanol metabolism

While it is clear that ethanol increases PHB expression in β-cells, we were also surprised by the finding that prohibitin decreases ADH5 expression in ethanol treated β-cells at the mRNA level. The decreased ADH5 protein expression decreased total ADH activity by ~ 47 % compared to ethanol treated cells. It is important to note that this is the first study showing that PHB may influence ethanol metabolism. We speculate that prohibitin has a dual effect on ADH. *First*, prohibitin might down-regulate ADH in an

attempt to delay mitochondrial access to ethanol metabolites. A recent study indicated that TGF-β down-regulates ADH1 mRNA expression in liver cells [251], while prohibitin has been reported to mediate the effects of TGF-β in prostate cancer cells [204]. *Second*, as a mitochondrial chaperone, prohibitin may facilitate electron flow through the respiratory chain, resulting in increased clearance of NADH from ethanol and acetaldehyde metabolism and a consequent increase in ADH activity [252]. Such an increase in mitochondrial electron transport has been shown to attenuate oxygen-mediated damage in HeLa cells [232]. In addition, enhanced reoxidation of NADH by mitochondrial complex I has been associated with reduced ethanol toxicity and increased voluntary ethanol consumption in rats with a mutation in mitochondrial aldehyde dehydrogenase; the mitochondria in these rats had increased capacity to oxidize substrates that generate NADH for complex I, leading to higher acetaldehyde metabolism and reduced aversion for ethanol [253]. This hypothesis is also in line with observations that inactive aldehyde dehydrogenase 2 worsens glycemic control in patients with type 2 diabetes who ingest low to moderate amounts of alcohol [25]. These two mechanisms, which would lead to a protection against ethanol toxicity by prohibitin, remain to be firmly established in pancreatic β-cells.

9. PHB plays a role in glucose-induced insulin secretion

Glucose induced insulin secretion (GIIS) was examined in INS-1E cells, which are differentiated and known to be responsive to glucose, unlike RINm5F cells. GIIS was decreased by ~ 26% by ethanol and restored by PHB over-expression. The restoration of insulin secretion by PHB was associated with an increase of β-cell transcription factors

PDX-1 and MafA, in addition to a recovery of mitochondrial function discussed above. The improvement in mitochondrial function may be an explanation for the restoration of GIIS. It has been shown that reduced expression of the transcription factors PDX-1 and MafA is associated with β-cell dysfunction [254-256]. In the current study, we found that the nuclear content of PDX-1 and MafA were markedly increased by prohibitin and mildly reduced by ethanol, although their mRNA levels remained unchanged. The abundance of these proteins is regulated not only at the transcriptional, but also at the post-transcriptional level. Indeed, although acute exposure to glucose increases the mRNA for these proteins, oxidative stress induced by chronic high glucose results in a reduction of these proteins without parallel changes in their mRNA expression [254, 255]. It is possible that changes in mitochondrial redox caused by ethanol and prohibitin explain corresponding changes in the nuclear distribution of these transcription factors. These proteins, together with other transcription factors such as NeuroD/β2, PAX6, and the recently described Glis3 synergistically activate the insulin gene promoter and regulate β-cell differentiation [254-257]. Surprisingly, there was no parallel increase of Insulin 1 and Insulin 2 mRNAs with the transcription factors. As insulin secretion increased, however, it is possible that due to their long half-life of >30 h the increase in insulin mRNAs were not measurable during our experimental conditions, or that these mRNAs were more stable without quantitative changes, allowing increased insulin biosynthesis after exposure to prohibitin [258]. It has also been shown that PDX-1 and MafA not only stimulate insulin gene transcription, but also regulate other β-cell specific genes such as Glut2, glucokinase, K-ATP channel subunit Kir6.2 and the sulfonylurea receptor, which play instrumental roles in glucose stimulated insulin secretion [255, 259]. Increased levels of these transcription factors by

prohibitin, therefore, could attenuate alterations of insulin secretion caused by ethanol without having to activate insulin gene transcription. Additionally, PHB on its own could promote insulin secretion by increasing the levels of transcription factors.

In summary, we found that ethanol is metabolized mainly by ADH5 in INS-1E cells. The main targets of ethanol in mitochondria are NADH dehydrogenase and cytochrome c oxidase complex, alterations of which increase ROS production and induce overall mitochondrial dysfunction. These adverse effects in turn induce pro-apoptotic signaling via the JNK pathway. On the other hand, PHB inhibited total ADH activity through regulation of the ADH5 mRNA level and recovered NADH dehydrogenase and cytochrome c oxidase complex through its chaperone role in the mitochondria. Prohibitin improved mitochondrial membrane potential and mitochondrial function leading to increased insulin secretion in pancreatic β-cells exposed to ethanol. These effects of PHB on the mitochondrial electron transport chain in turn protect these cells from apoptosis induced by increasing ROS production as shown in the schematic diagram (Fig. 42). These are important findings with potential implications for diabetes prevention and treatment.

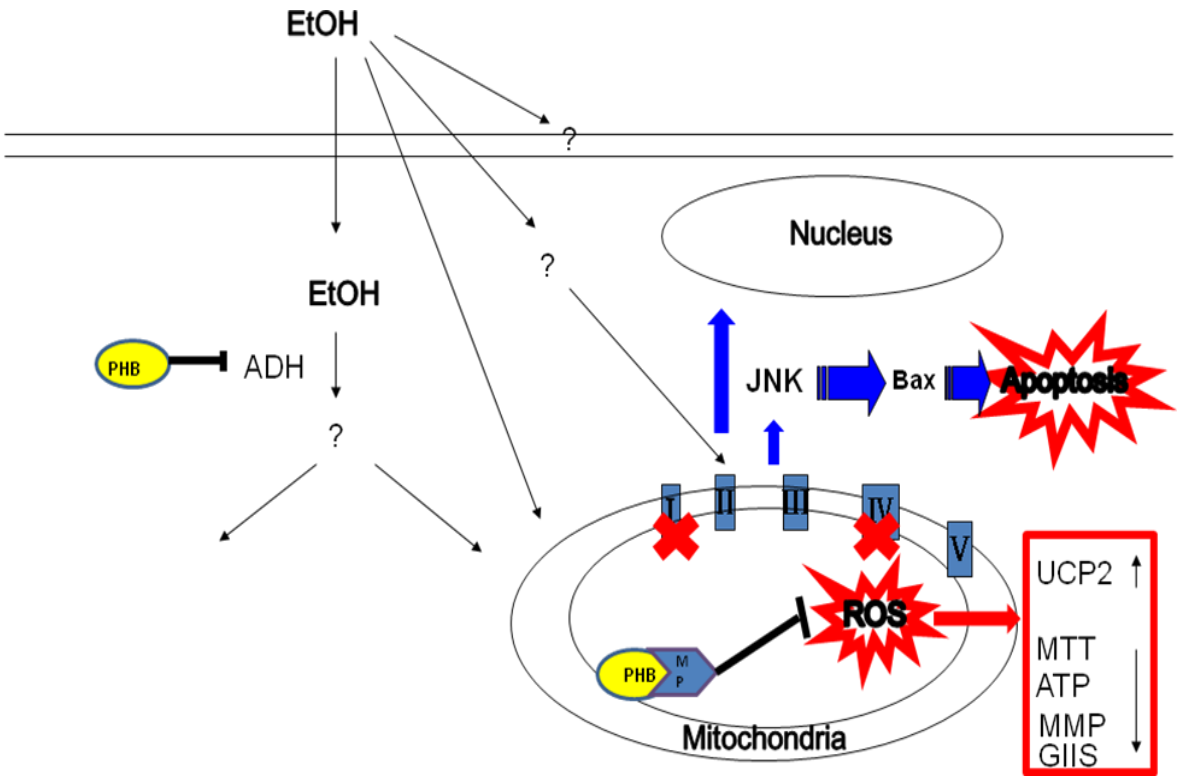

Figure 39: A diagram of ethanol and PHB effects on β-cells

Ethanol is metabolized mainly by ADH5 and decreases the activity of NADH dehydrogenase and cytochrome c oxidase complex in β-cells, which in turn increases cellular ROS production and mitochondrial dysfunction manifested by alterations of MTT, UCP2, ATP, mitochondrial membrane potential (MMP), and glucose induced insulin secretion (GIIS). Finally, these adverse effects of ethanol induce apoptosis via the JNK pathway. In contrast, PHB inhibits these deleterious effects by decreasing ROS production through stabilizing mitochondrial complexes or by inhibiting ADH activity through regulation of ADH5 mRNA level.
EtOH: ethanol; PHB: prohibitin; ADH: alcohol dehydrogenase; MP: mitochondrial proteins; ROS: reactive oxygen species; I, II, III, IV, V: mitochondrial complexes; JNK: c-Jun N-terminal kinas

VI. CONCLUSIONS

In this thesis, we found that ethanol is metabolized mainly by ADH5 and decreases the activity of NADH dehydrogenase and cytochrome c oxidase complex in the mitochondrial respiratory chain, which in turn increases cellular ROS production and mitochondrial dysfunction manifested by alterations in MTT, UCP2, ATP, mitochondrial membrane potential and insulin secretion. Consequently, these deleterious effects of ethanol in pancreatic β-cells induce apoptosis via the JNK pathway resulting in the reduction of insulin secretion.

However, exogenously applied PHB or endogenously over-expressed PHB inhibit these deleterious effects by decreasing ROS production through stabilizing mitochondrial complexes I and IV, or by inhibiting ADH activity through regulation of ADH5 mRNA level. As a consequence, the apoptosis induced in ethanol exposed β-cells is decreased resulting in a rescue of insulin secretion. The role of PHB in insulin secretion could also be due to its positive effects on the transcription factors MafA and PDX-1.

VII. FUTURE DIRECTIONS

The current study focused on the effects of PHB on β-cell survival and function using two rat insulinoma cells. Future experiments should search to clarify some of the questions left unaswered by this thesis. While INS-1E cells are quite well differentiated and responsive to glucose, RINm5F cells are not well differentiated and display limited glucose responsiveness. In addition, INS-1E cells require mercaptoethanol in culture media for survival.The fact that mercaptoethanol has anti-oxidant properties may have confounded the anti-oxidant effects of PHB in INS-1E cells. More insights might be gained by extending the studies of β-cell survival, mitochondrial function, and insulin secretion to pancreatic islets or primary β-cells from different species. An interesting finding in this respect was the observation that PHB enhanced the expression of β-cell transcription factors, independent of the oxidative effect of ethanol. The mechanisms leading to this enhancement and the link between PHB and insulin secretion through these factors remain entirely to be elucidated. It would definitely be interesting to determine why PHB preferentially targets the mitochondria during ethanol induced cellular stress. Presumably, PHB undergoes post-translational modifications allowing mitochondrial localization. The interaction of PHB with cytochrome c oxidase is an interesting observation which adds to the notion that PHB chaperones mitochondrial proteins. However, each of the mitochondrial respiratory complexes comprises several protein subunits and their specific interactions with PHB should be tested. Interestingly, Ande and Mishra [260] very recently reported that PHB is an iron regulated iron binding protein and that iron binding contributes to anti-oxidant properties of PHB in the mitochondria. Therefore, the

association of PHB with other factors and proteins in the mitochondria remains widely unknown and deserves further investigations.

Rastogi et al [245] recently reported that PHB contains a leucine-rich nuclear export signal which facilitates its cytoplasmic translocation, whereas Vessal et al[187] reported that PHB internalization involves lipid raft or caveolin-dependent processes. Using site–directed mutagenesis techniques and confocal microscopy analysis could reveal PHB domains responsible for its transcellular translocation. In addition, time course of PHB translocation across the plasma membrane combined with mass spectrometry could elucidate the kinetics of the internalization process and PHB binding partners in the plasma membrane. Internalization studies could also include mutant caveolin overexpression or inhibitors of endocytosis to address the underlying mechanisms of caveolin mediated PHB internalization.

A second major issue would be to elucidate mechanisms whereby PHB regulates ethanol metabolism through modulation of ADH gene expression. There is a need to determine if this effect of PHB is direct or indirect, and whether PHB binds to ADH promoter. ADH expression and activity need to be determined in β- cells where PHB has been overexpressed or knocked down.

Finally, the effects of PHB on gene expression need to be investigated in beta-cells. The present study showed that prohibitin decreases JNK phosphorylation and Bax expression in beta-cells, preventing apoptosis. This also suggests that PHB is implicated in the regulation of gene expression through this pathway. JNK pathway is activated by increased ROS production and the results are connected with diverse cellular responses such as apoptosis, insulin gene expression, and insulin signaling. Therefore, studies of gene expression

related to β cell function (e.g. PDX-1, MafA, Glut 2 and insulin) should be performed using several ethanol concentrations. In addition and perhaps most importantly, the effects of PHB on beta-cells should be studied under various concentrations of nutrients such as glucose and fatty acids, which are more commonly associated with beta-cell failure and diabetes.

VII. REFERENCES

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