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Regulation of Pancreatic α and β Cell Function by the Bile Acid Receptor TGR5

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Regulation of Pancreatic α and β Cell Function by the Bile Acid Receptor TGR5

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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AC………………………..... Adenylyl cyclase

AEBSF………………………. 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride

ASBT……………….…......... Apical sodium dependent bile acid transporter

ATP…………………….…… Adenosine triphosphate

BA………………………….... Bile acids

BAPTA………………………. 1,2-bis(o-aminophenoxy)ethane-N,N,N^1,N^1-tetracetic acid

bp…………………………….. Base pair

BSA…………………………... Bovine serum albumin

CA………………………….... Cholic acid

cAMP………………………... Cyclic adenosine monophosphate

CAR…………………………. Constitutive androstane receptor

CDCA………………………... Chenodeoxycholic acid

CYP7A1……………………... Cytochrome P450 7A1

DCA…………………………. Deoxycholic acid

DMEM………………………... Dulbecco's modified eagle medium

DNA…………………………... Deoxyribonucleic acid

DPP-IV………………………. Dipeptidyl peptidase- 4

ELISA……………………… Enzyme linked immunosorbent assay

Epac…………………………..	cAMP-dependent exchange factor
FBS…………………………..	Fetal bovine serum
FGF19…………………………	Fibroblast growth factor 19
FPR…………………………..	Formyl-peptide receptors
FXR…………………………..	Farnesoid X receptor
GAPDH………………………..	Glyceraldehyde 3 phosphate dehydrogenase
GLP-1…………………………	Glucagon like peptide-1
GLUT………………………….	Glucose transporter 2
GPCR…………………………..	G protein coupled receptors
GSIS…………………………...	Glucose stimulated insulin secretion
HBSS…………………………..	Hank's balanced salt solution
HNF-4α…………………………	Hepatocyte nuclear factor 4 alpha
IBABP…………………………..	Ileal bile acid binding protein
IBMX…………………………..	3-isobutyl-1-methylxanthine
IP_3……………………………	Inositol triphosphate
LCA………………………….....	Lithocholic acid
LRH-1…………………………...	Liver receptor homolog-1
MRP………………………….…	Multidrug resistance-associated protein
NTCP…………………………....	Na^+-taurocholate cotransporting polypeptide
OA…………………………….…	Oleanolic acid
OATP…………………………..	Organic anion transporting polypeptide
OST……………………………..	Organic solute transporter

PC……………………………… Prohormone convertase

PCR………………………….... Polymerase chain reaction

PI………………………………. Phosphatidylinositol

PKA…………………………… Protein kinase A

PKI…………………………….. Protein kinase A inhibitor

PLC-ε……………………….….. Phospholipase C- epsilon

PXR…………………………… Pregnane X receptor

RNA…………………………… Ribonucleic acid

RPMI…………………………... Roswell park memorial institute media

S-EMCA……………………… 6α-Ethyl-23(S)-methylcholic Acid

S1PR………………………….. Sphingosine-1 phosphate receptor

SDS-PAGE………………… Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHP…………………………… Short heterodimer partner

StarD1……………………….... Steroidogenic acute regulatory domain containing protein 1

STZ…………………………… Streptozotocin

T2D…………………………… Type 2 Diabetes Mellitus

TBST…………………………. Tris buffered saline with tween-20

TGR5…………………………. Takeda G-protein coupled receptor clone 5

VDR…………………………… Vitamin D receptor

WT……………………………... Wild type

SYMBOLS

α…………………………….. Alpha

β…………………………….. Beta

δ…………………………….. Delta

UNITS

%...................................... Percent

^{0}C…………………………. Degree celsius

Da…………………………… Dalton

g………………………….. Gram

h……………………………. Hour(s)

l……………………………. Liter

M…………………………… Molar (moles/l)

min………………………….. Minutes

sec………………………….. Second(s)

vol…………………………… Volume

METRIC PREFIXES

m…………………………… Milli

μ…………………………….. Micro

n…………………………….. Nano

p……………………………. Pico

ABSTRACT

REGULATION OF PANCREATIC α AND β CELL FUNCTION BY THE BILE ACID RECEPTOR TGR5

Divya P. Kumar

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

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The discovery that bile acids act as endogenous ligands of the membrane receptor TGR5 and the nuclear receptor FXR increased their significance as regulators of cholesterol, glucose and energy metabolism. Activation of TGR5, expressed on enteroendocrine L cells, by bile acids caused secretion of GLP-1, which stimulates insulin secretion from pancreatic β cells. Expression of TGR5 on pancreatic islet cells and the direct effect of bile acids on the endocrine functions of pancreas, however, are not fully understood. The aim of this study was to identify the expression of TGR5 in pancreatic islet cells and determine the effect of bile acids on insulin secretion. Expression of TGR5 was identified by quantitative PCR and western blot in islets from human and mouse, and in α (αTC1-6) and β (MIN6) cells. Release of insulin, glucagon and GLP-1 were measured by ELISA. The signaling pathways coupled to TGR5

activation were identified by direct measurements such as stimulation of G proteins, adenylyl cyclase activity, PI hydrolysis and intracellular Ca^{2+} in response to bile acids; and confirmed by the use of selective inhibitors that block specific steps in the signaling pathway. Our studies identified the expression of TGR5 receptors in β cells and demonstrated that activation of these receptors by both pharmacological ligands (oleanolic acid (OA) and INT-777) and physiological ligands (lithocholic acid, LCA) induced insulin secretion. TGR5 receptors are also expressed in α cells and, activation of TGR5 by OA, INT-777 and LCA at 5 mM glucose induced release of glucagon, which is processed from proglucagon by the selective expression of prohormone convertase 2 (PC2). However, under hyperglycemia, activation of TGR5 in α cells augmented the glucose-induced increase in GLP-1 secretion, which in turn, stimulated insulin secretion. Secretion of GLP-1 from α cells reflected TGR5-mediated increase in PC1 promoter activity and PC1 expression, which selectively converts proglucagon to GLP-1. The signaling pathway activated by TGR5 to mediate insulin and GLP-1 secretion involved G_s/cAMP/Epac/PLC-ε/Ca^{2+}. These results provide insight into the mechanisms involved in the regulation of pancreatic α and β cell function by bile acids and may lead to new therapeutic avenues for the treatment of diabetes.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Bile acids

Bile acids, the active constituent of bile, are synthesized from cholesterol in the hepatocytes and secreted into the bile canaliculi to be stored in the gallbladder [1]. In response to a meal, the gallbladder contracts and the bile flows into the small intestine. Being amphipathic molecules, bile acids aid in the emulsification of lipids and facilitate the absorption of lipid nutrients and lipid soluble vitamins [2]. Nearly 95% of the bile acids that are secreted into the small intestine are transported back to the liver from the distal part of the ileum and colon via a process known as enterohepatic circulation (**Figure 1**). The bile acid transport is achieved by both passive diffusion as well as active transport [3]. Consequently, a significant amount of bile acids spill over into the systemic circulation, which is generally less than 5 µM under fasting conditions and increases up to 15 µM postprandially [4]. Remaining 5% of the bile acids that escape absorption from the intestine is lost in the feces, a major route for the elimination of excess cholesterol from the body [1,5]. In addition to their established roles in cholesterol homeostasis and lipid digestion, bile acids also act as signaling molecules. With the identification of the bile acid receptors, both the endocrine and paracrine

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functions of bile acids have become evident [6]. There has been an increasing development in understanding bile acid signaling, and bile acid receptors have become an attractive therapeutic target for the treatment of diseases such as metabolic syndrome and diabetes.

1.1. Bile acid synthesis and regulation

Bile acid synthesis is a complex multistep process. There are 2 major bile acid biosynthetic pathways– the classical or neutral pathway and the acidic pathway. Bile acid synthesis from the classical pathway (75%) is initiated by CYP7A1 (Cholesterol 7 alpha-hydroxylase or Cytochrome P450 7A1), which is expressed only in the hepatocytes, whereas, the acidic pathway (25%) initiated by mitochondrial CYP27A1 is expressed in macrophages and many other tissues [7]. It is noteworthy that all the essential enzymes for the conversion of cholesterol into bile acids reside only in the liver, which is the main site of synthesis [8]. The rate limiting steps in the classical pathway and the acidic pathway are 7α-hydroxylation of cholesterol by CYP7A1 and the transport of free cholesterol to the inner mitochondrial membrane by StarD1, respectively [7,9]. In humans, the primary bile acids produced in the liver are cholic acid (CA) and chenodeoxycholic acid (CDCA), whereas cholic acid and muricholic acid form the primary bile acid pool in mice. Primary bile acids are either glycine or taurine conjugated. Upon reaching the large intestine, they undergo deconjugation and dehydroxylation by the bacteria present in the gut forming secondary bile acids, which consist of lithocholic acid (LCA) and deoxycholic acid (DCA) in humans [10,11]. The secondary bile acids (unconjugated) are passively absorbed by the epithelial cells of the distal ileum, whereas the conjugated bile acids are actively transported via bile acid

transporters [12]. Bile acids consist of a steroid core and a side chain with carboxyl group. The number and position of hydroxyl groups on the steroid core determine the hydrophilic or hydrophobic nature of the bile acids. The primary bile acids are more hydrophilic than the secondary bile acids, and taurine conjugates are more hydrophilic than the glycine ones [13].

Bile acid synthesis is highly regulated. The basal rate of synthesis of bile acids in the liver is ~0.6 g per day in healthy humans, the amount sufficient to replace the lost bile acids in the feces. The total bile acid pool in the gastrointestinal tract is ~ 3 g and this pool recirculates ~ 4 to 12 times per day. If the reabsorption of bile acids is defective, de novo synthesis of bile acids in the liver increases up to 4 to 6 g per day. As high concentrations of bile acids are cytotoxic, bile acids regulate their own metabolism by exerting a negative feedback on their synthesis [5,7,14,15]. The bile acid feedback repression mechanism is governed by the interplay of several nuclear receptors to inhibit the transcription of CYP7A1 gene, the rate-limiting enzyme in bile acid synthesis [16]. In addition, factors like diet, nutrients, cytokines and hormones also regulate the expression of CYP7A1, the rate-limiting enzyme [17].

1.3. Bile Acid Transport

Bile acid homeostasis is maintained by a balance between bile acid synthesis, secretion and reabsorption [7,9,17]. Bile acids are absorbed by both passive and active mechanisms. Passive absorption of bile acids occurs along the entire small intestine and colon. At the normal pH of the intestine (5.5 to 6.5), majority of the unconjugated bile acids exist in protonated or neutral form, and a very small amount of glycine-conjugated or taurine-conjugated bile acids are protonated [11]. Thus, the unconjugated
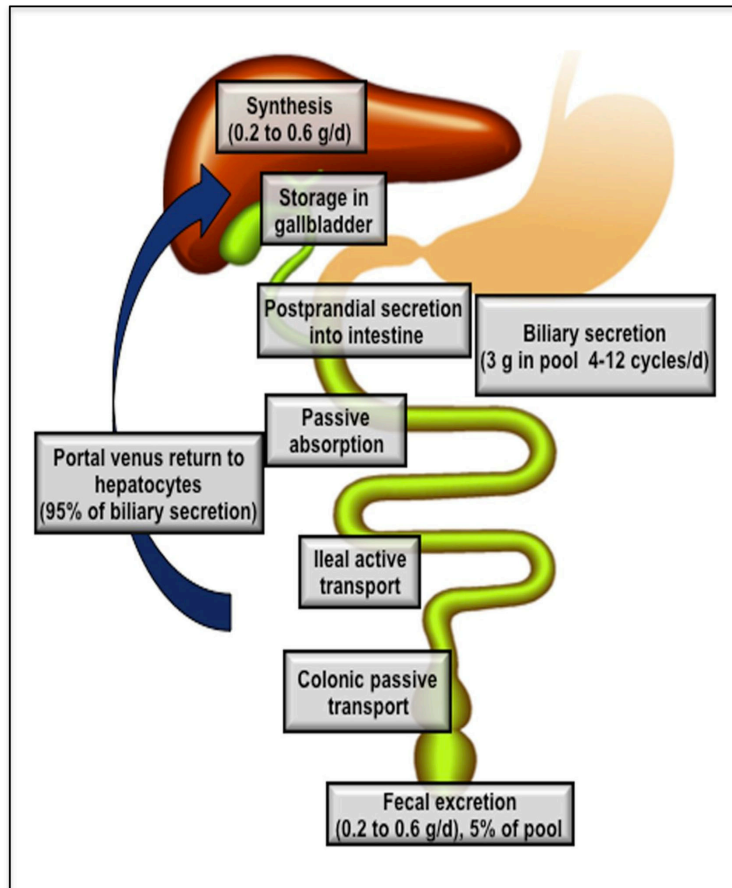
Figure 1. Enterohepatic circulation of bile acids. Bile acids synthesized from cholesterol in the liver are secreted into the bile canaliculi and stored in the gallbladder. In response to a meal, the gallbladder contracts and the bile flows into the intestine facilitating the absorption of lipid nutrients and lipid soluble vitamins. Nearly 95% of the bile acids are transported back to the liver from the distal part of the ileum and colon via a process known as enterohepatic circulation and the remaining 5% of the bile acid pool is lost in the feces. Pharmacol Rev. 2014, 66: 948-983 [9].

bile acids are absorbed by passive diffusion, however it is less intense than the active absorption [3,12]. Majority of bile acids in the ileum are conjugated and exist in anionic form. Hence the entry and exit of conjugated bile acids in the intestine and hepatocytes require active transport system, as they cannot cross the plasma membrane [5,7]. Bile acid transporters play an important role in modulating bile acid signaling by controlling the flux of bile acids [9,18].

The conjugated bile acids are actively absorbed in the terminal ileum via apical sodium-dependent bile acid transporter (ASBT) [19]. The ileal bile acid binding protein (IBABP) facilitates the intracellular transport of bile acids across the enterocyte, and the bile acids are effluxed to the portal blood via organic solute transporter α and β (OSTα/ OSTβ) [20]. Other transporters like MRP2, MRP4 and MRP3 are known to be involved in the transport of bile acids at the apical membrane and basolateral membrane of the enterocytes, respectively [21].

The circulating bile acids in the portal blood are taken up by the hepatocytes through the basolateral membrane by Na^+ dependent co-transporter system, NTCP [19,22]. It is estimated that 90% of the bile acid uptake is via Na^+-taurocholate cotransporting polypeptide (NTCP) and to a lesser extent by organic anion transporting polypeptides (OATP1 and OATP4). At the canalicular membrane of the hepatocytes, BSEP (bile salt export pump) plays a major role in the secretion of bile acids into the bile canaliculi whereas MRP2 (multidrug resistance associated protein-2) mediates secretion of bile acids along with other organic solutes [21,22]. The heterodimeric organic solute transporters (OSTα/ OSTβ), MRP3 and MRP4 mediate secretion of bile acids into the circulation at the basolateral membrane of the hepatocytes [19,20,23].

1.4. Bile acid receptors

The importance of bile acids as signaling molecules in the regulation of physiological function has gained attention with the discovery of receptors for bile acids. The most prominent and best-studied bile acid receptors are FXR and TGR5 **(Figure 2)**.

1.4.1. FXR

In 1999 three independent research groups reported that bile acids act as ligands for the farnesoid X receptor (FXR) [24-26]. The discovery of FXR was later followed by human steroid and xenobiotic receptor (SXR) and its rodent homolog pregnane X receptor (PXR), vitamin D receptor (VDR), and constitutive androstane receptor (CAR), all of which are bile acid activated nuclear receptors that are known to be functionally related [27-29].

Farnesoid **X R**eceptor (FXR) or NR1H4, a member of the nuclear receptor superfamily was identified during the search for a new heterodimer that binds to the retinoid X receptor (RXR). FXR, originally activated by farnesol was later identified as a receptor that is activated by bile acids in 1999. FXR is highly expressed in liver, intestine, kidneys and adrenal glands and is selectively activated by CDCA [24-26]. FXR was identified as a master regulator playing a pivotal role in the feedback regulation of bile acid synthesis by repressing CYP7A1, the rate-limiting enzyme of the synthetic pathway [7,9,17]. FXR activates the expression of the bile acid export transporters, multidrug resistance- associated protein 2 (MRP2) and bile salt export pump (BSEP) and simultaneously represses bile acid import to regulate bile acid transport [30,31].
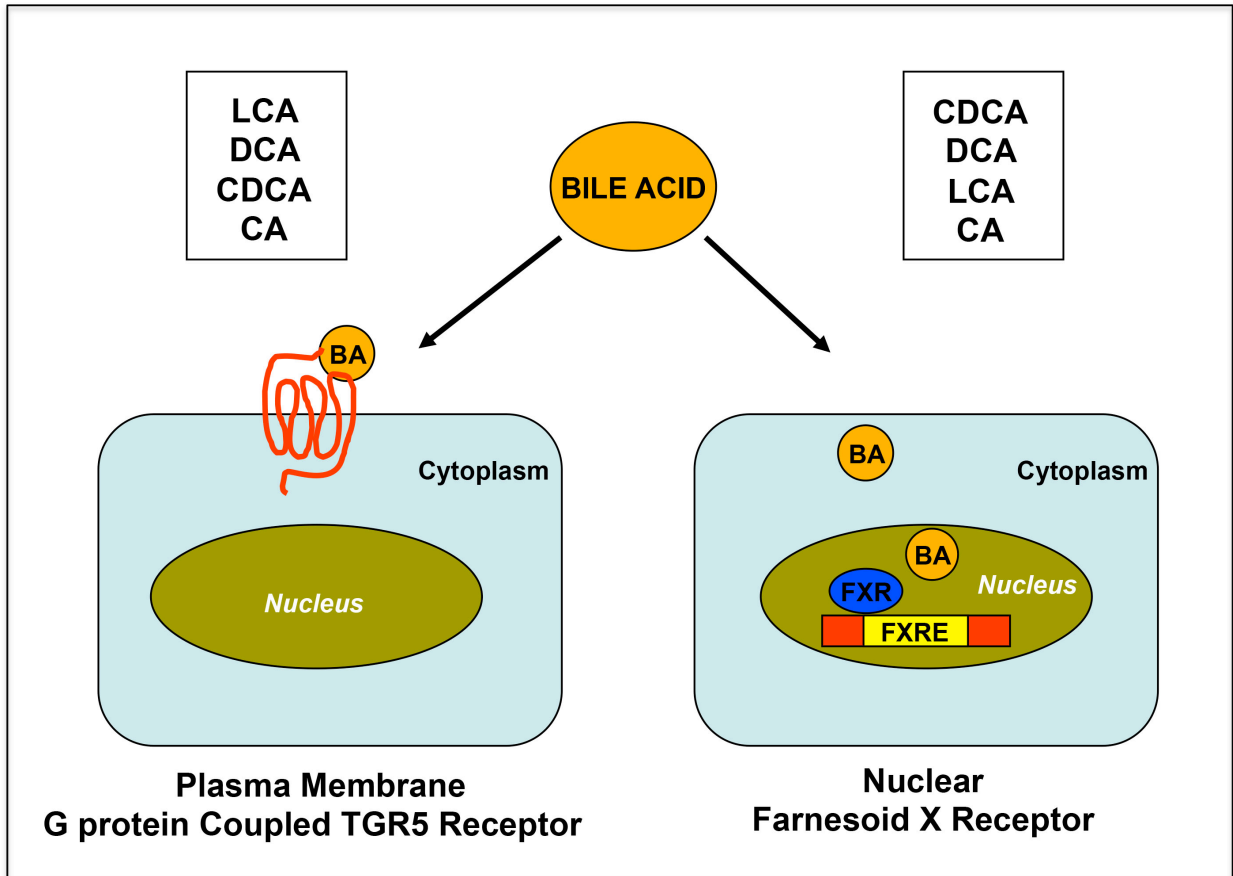
Figure 2. Schematic representation of the activation of TGR5 and FXR by bile acids. TGR5 is a plasma membrane G protein coupled receptor and FXR is a nuclear receptor that is activated by bile acids. The receptors are activated by both primary (CA and CDCA) and secondary bile acids (LCA and DCA). LCA is a potent activator of TGR5 while CDCA for FXR.

Thus, FXR functions as a sensor of metabolic signals and plays an important role in the regulation of bile acids synthesis and transport, lipid metabolism and glucose homeostasis [32-34].

1.4.2. TGR5

Takeda **G**-protein coupled **R**eceptor clone **5** (TGR5) also known as GPBAR1, M-BAR or BG37 was first discovered in 2002 by two independent research groups in Japan [35,36]. TGR5 is encoded by a single exon gene that maps to chromosome position 1c3 in mice and to chromosome 2q35 in humans. The coding region of human TGR5 gene consisting of 993 bp encodes 330 amino acids and the protein shares 82, 83, 86 and 90% homology with rat, mouse, bovine and rabbit respectively [37]. TGR5 is a member of the rhodopsin like (class A) subfamily of GPCRs and is ubiquitously expressed with varied degree of expression in different organs such as spleen, placenta, gallbladder, liver, kidney, small intestine, colon, heart, skeletal muscles, pancreas etc [38]. The bile acid receptor TGR5 is activated by different bile acids, with taurine-conjugated lithocholic acid (TLCA) being the potent agonist with an EC50 value of 0.33 μM. The other bile acids that activate TGR5 include LCA, DCA, CDCA and CA with EC50's of 0.53, 1.01, 4.43 and 7.72 μM respectively [39,40]. In addition to their cognate receptors, bile acids are also known to modulate the activity of formyl-peptide receptors (FPR), muscarinic receptors and S1PR2 (Sphingosine-1 phosphate receptor 2) [41,42]. Some of the steroids such as progesterone, androgen, estrogens, oxysterols, and pregnenolone also stimulate TGR5. However, the concentrations of these steroids required to activate TGR5 are much higher than the physiological concentration and are not considered as receptors for these steroid hormones *in vivo* [35].

1.5. Physiological significance of bile acids

In addition to their classical role in lipid digestion and absorption, bile acids are now known to play an important role in the regulation of different organ function by acting as endogenous ligands [38-40]. Bile acids via activation of its nuclear receptor, FXR or membrane receptor, TGR5 not only regulate their own synthesis and metabolism but also regulate glucose and lipid metabolism and energy homeostasis [9,16,42]. Thus bile acids activated signaling pathways serve as potential therapeutic targets for the treatment of various metabolic related diseases such as obesity, type 2 diabetes, hyperlipidemia and atherosclerosis [15,38,43,44].

1.5.1. Functions via FXR

Bile acids regulate their own synthesis by exerting a negative feedback mechanism. Activation of nuclear receptor FXR by bile acids inhibits CYP7A1, a key enzyme in the synthesis of bile acids [7,9]. In the liver, activation of FXR induces SHP (short heterodimer partner) expression. SHP exerts its inhibitory effect by interacting with LRH-1 (liver receptor homolog-1) and HNF4α (hepatocyte nuclear factor 4 alpha) and represses CYP7A1 gene expression [14]. In the intestine, bile acids activate FXR, which induces FGF19 (fibroblast growth factor 19) expression. The FGF19 released from the epithelial cells of small intestine circulates and bind to FGFR4 (fibroblast growth factor receptor 4) on the hepatocytes. The FGF19/FGFR4 signaling activates JNK (c-Jun NH$_2$-terminal kinase) pathway that inhibits CYP7A1 gene transcription [7,9]. Activation of FXR also up regulates the expression of bile acid export transporters MRP2 and BSEP and down regulates NTCP, the bile acid import transporter. FXR also positively regulates the expression of the enzymes involved in the detoxification of bile

acids such as SULT2A1 and CYP3A4 [17]. Thus, activation of FXR regulates bile acid synthesis and transport and plays an important role in hepatoprotection [17,30,45].

Bile acids via hepatic FXR activation leads to decreased triglyceride synthesis and secretion and is an important regulator of lipid metabolism [31,32]. In addition to these, the role of FXR in glucose homeostasis via the regulation of gluconeogenesis and glycogenolysis is demonstrated [46]. The activation of FXR decreases gluconeogenesis and increases glycogen synthesis, the combined effect of which reduces hyperglycemia. FXR also plays an important role in skeletal muscles and adipose tissue by increasing insulin sensitivity and is thus a therapeutic target for treating metabolic syndrome [31-34].

1.5.2. Functions via TGR5

Bile acids acting via TGR5 play an important role in the physiology of liver and gallbladder. In liver, TGR5 is expressed in the sinusoidal endothelial cells and the activation of which leads to the production of nitric oxide aiding in hepatic microcirculation, which also serves to be beneficial in the case of portal hypertension [47]. Bile acids inhibits LPS-induced cytokine expression in kupffer cells of the liver and therefore it is evident that there is an increase in the immunoreactivity by the activation of TGR5 in bile duct ligated rats (impaired bile flow and rise in cytokines) when compared to sham operated control rats [48,49]. In the cholangiocytes (epithelial cells of the bile duct), activation of TGR5 protects against lipid peroxidation and bile acid-induced injury by exerting anti apoptotic and anti-inflammatory effects [50]. Bile acids also play a role in immunomodulation and regulate the production of cytokines by the activation of TGR5 in monocytes [51]. The gallbladder volume was increased in wild

type mice when compared to TGR5 knockout mice upon the administration of LCA or INT-777 demonstrating that activation of TGR5 stimulates gallbladder filling. TGR5 also causes relaxation in the gallbladder smooth muscles [38,52].

Bile acids in the gastrointestinal tract regulate gut motility. Luminal administration of bile acids inhibits gastric motility and small intestinal transit, and the effect is mediated by activation of TGR5 present on the inhibitory motor neurons that release nitric oxide [53]. Recent studies have shown that bile acids stimulate colonic peristalsis, facilitating normal transit and digestion [54,55]. Studies by Rajagopal et al., [56] have showed that bile acid induced TGR5 activation causes gastric smooth muscle relaxation. Activation of TGR5 in primary spinal afferent and sensory neurons is known to mediate itch and analgesia, a mechanism, which contributes to pruritus and painless jaundice in patients with cholestatic liver disease [57].

The study of the role of TGR5 in the regulation of energy expenditure is gaining prime importance as obesity has become a major concern and also as it is highly associated with type II diabetes, which is a threat to the public health. Increasing physical exercise and reducing calorie intake are the first line of treatment for individuals with obesity. In this regard, bile acid induced TGR5 activation caused a significant reduction in body weight. Studies by Watanabe et al., [58] and Maruyama et al., [59] have showed that administration of cholic acid to the mice on a high fat diet caused reduction in the body weight gain and TGR5$^{-/-}$ mice when fed with high fat diet became obese when compared to wild type mice. Skeletal muscles and brown adipose tissues are the most important thermogenic sites where activation of TGR5 regulates energy metabolism [60]. The possible mechanisms involved in this process are the upregulation

of type 2 iodothyronine deiodinase (D2) expression as well as peroxisome proliferator-activated receptor-γ coactivator- 1 α (PGC-1α). It is important to note that these molecular events are purely TGR5 dependent and FXR independent [58]. The expression of D2 and PGC-1α are regulated through the cAMP-PKA (protein kinase A) dependent pathway. Both humans and mice possess cAMP responsive elements (CRE) in the promoter regions of D2 and PGC-1α [61]. The activation of D2, mediated via TGR5 leads to conversion of inactive thyroxine (T4) to active 3,5,3- tri iodothyronine (T3), which binds to the thyroid hormone receptor (THR) resulting in the transcriptional activation of key genes of energy expenditure such as uncoupling proteins (UCP-1, 2 or 3) [62,63]. Similarly increased PGC-1α induces expression of uncoupling proteins. Activation of uncoupling proteins leads to dissipation of the proton gradient and uncoupling of oxidative phosphorylation generating more heat [64]. This is consistent with the impairment of mitochondrial function in the skeletal muscle and brown adipose tissue of PGC-1α $^{-/-}$ mice [62]. The transcriptional co-activator PGC-1α binds to and increases the activity of PPARs to regulate fatty acid β-oxidation and is evident by the knockout and over-expression of PPARγ studies. PGC-1α also modulates the activity of a number of transcription factors that can increase the expression of proteins involved in fatty acid β-oxidation, the TCA cycle, and the electron transport chain [61].

Bile acids play an important role in glucose homeostasis. Studies have demonstrated that bile acids via activation of TGR5 induce GLP-1 secretion from the intestinal L cells [65]. INT-777, a specific TGR5 agonist stimulates GLP-1 secretion in mouse enteroendocrine cells, STC-1 as well as in human intestinal NCI-H716 cells [66]. GLP-1 is known to regulate postprandial glycemic response by stimulating glucose

dependent insulin secretion from the pancreatic β cells and inhibition of glucagon secretion from pancreatic α cells [67]. TGR5, in addition, to inducing insulin secretion also increases insulin sensitivity. Insulin induced by TGR5 increases glucose uptake in the skeletal muscle as well as in adipose tissue [62,63]. The possible mechanisms involve increase in GLUT-4 expression, an insulin regulated glucose transporter, leading to the uptake of glucose (postprandial glycemic control) and its oxidative phosphorylation [66]. Thus the involvement of TGR5 in glucose homeostasis was investigated *in vivo* using both gain of function and loss of function genetic approaches, and was further elucidated by the treatment with a selective TGR5 ligand and or INT-777, a specific TGR5 ligand [66].

Although the role of TGR5 in glucose homeostasis via release of GLP-1 from enteroendocrine cells is well established, the direct effect of bile acids on endocrine cells of pancreas is unclear **(Figure 3)**.

1.6. Pancreas

The Pancreas is an organ that lies in an oblique and transverse position in the upper left abdomen. In an adult, the pancreas is about 15 to 20 cm long and weighs about 75 to 100 g [68,69]. The pancreas is a dual function gland with both exocrine and endocrine functions [70].

1.6.1. Exocrine pancreas

The exocrine pancreas comprises 85-90% of the total pancreatic mass. The two major components of the exocrine pancreas are the acinar cells that are arranged into acini and the ductal system that carries the secretion into the duodenum [71].
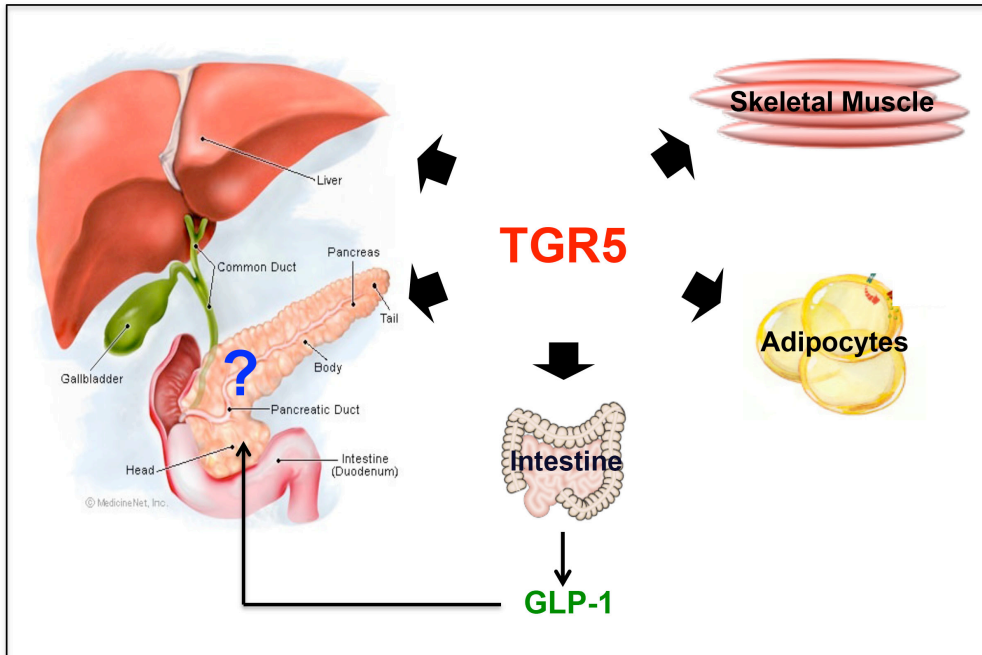
Figure 3. A diagrammatic representation of the functional significance of TGR5.
TGR5 is expressed in different tissues and is involved in the regulation of energy metabolism and glucose homeostasis. Bile acids via TGR5 modulate immune response and regulate gut motility. In the skeletal muscle and adipose tissue, TGR5 activation increases energy expenditure and insulin sensitivity. Activation of TGR5 in the intestinal L cells stimulates GLP-1 secretion, an incretin hormone that potentiates insulin secretion from pancreatic β cells. *However the direct effect of bile acids via activation of TGR5 in the regulation of pancreatic β cell function is unknown*. Adapted from Intercept Pharmaceuticals.

The exocrine pancreas functions to produce pancreatic enzymes critical to digestion and the enzymes include proteases, amylases, lipases, and nucleases with the function of breaking down specific components of the chime released from the stomach into the duodenum. In addition, bicarbonates are secreted from the epithelial cells lining these pancreatic ducts [70,71].

1.6.2. Endocrine pancreas

The endocrine portion of the pancreas comprises small clusters of cells called islets of Langerhans or simply termed islets. The islets (comprising only 1-2% of the pancreatic mass) are found scattered throughout the pancreas. In a normal adult pancreas there are nearly 1 million islets and each islet is known to contain 1000 to 3000 cells [68,69]. The Pancreatic islets consist of five different cell types, each of which produces a different hormone. Alpha (α) cells secrete glucagon, beta (β) cells secrete insulin, delta (δ) cells secrete somatostatin, epsilon (ε) cells secrete ghrelin, and PP cells that secrete polypeptides. The islets are highly vascularized by capillary network and innervated by various nerve fibers [72]. The capillaries within the islets are fenestrated facilitating secreted hormone into the circulation [73]. The cytoarchitecture of pancreatic islets differs between species [74]. In mouse islets, more than 70% of the cells are β cells forming the core and < 20% are α cells that are towards the periphery along with δ delta cells (< 5%). In contrast, human islets are composed of ~60% of β cells and ~30% of α cells. The β cells are intermingled with α and δ cells (<5%) in the human islets [75].

The secretion of hormones from the pancreatic islet is complex and highly regulated. Interplay of neural signals, blood flow patterns, autocrine and paracrine effect

are known to control islet secretion [68,73,76]. The paracrine signaling within the islets is an important regulator of pancreatic hormone release [77]. For instance, insulin stimulates its own release but inhibits the release of glucagon from α cells. Glucagon inhibits its own release but stimulates insulin release from β cells. Somatostatin released from δ cells inhibits the release of both insulin and glucagon [78,79]. Thus there exist a co-ordination in the intraislet regulation of hormone secretion within pancreatic islets [68,76].

1.7. GPCRs and Pancreatic islets

Although glucose is the predominant trigger for insulin secretion from pancreatic β cells [80], it is also known that the effect of glucose is modulated by activation of G protein coupled receptors expressed on β cells which may either have a positive or negative effect on insulin secretion depending on the intracellular signaling pathways [81]. Generally activation of receptors coupled to $G\alpha_s$ or $G\alpha_q$ stimulate insulin secretion whereas activation receptors coupled to $G\alpha_i$ inhibit insulin secretion. **Table 1** lists some of the GPCRs expressed on the pancreatic β cells that are known to effect insulin secretion. The characterization of GPCR in pancreatic islets are largely focused on the β cells, and less is known about the role of GPCR in α cells or δ cells [82]. To date, GLP-1 based therapy represents one of the best-studied and clinically successful therapeutic strategy in targeting islet GPCR [83]. Thus GPCRs are potential therapeutic targets for the treatment of islet dysfunction or diabetes therapy due to their gaining importance in the regulation of islet function [81-83].

1.7.1. GLP-1 receptor

GLP-1 receptors belonging to class B family of GPCRs also include receptors for glucagon and GIP [84]. GLP-1 receptor (GLP-1R) was first cloned from rat pancreatic islets and is known that rat and human GLP-1R possess 95% amino acid homology and is a 64 kDa protein. GLP-1R is widely expressed in pancreatic islets as well as in other tissues including brain, heart, kidney, lung and gastrointestinal tract [85] and to date, only a single GLP-1 receptor has been identified [84,85]. Activation of GLP-1R is coupled to $G\alpha_s$ with subsequent activation of adenylyl cyclase and production of cAMP. Other signaling pathways such as MAP kinase, PI3 kinase and PKB pathways are also known to be activated by GLP-1 [86].

1.7.2. Physiological effects of GLP-1

Glucagon like peptide-1 (GLP-1) is a 30 amino acid peptide hormone secreted from the intestinal L cells located predominantly in the ileum and colon [87]. GLP-1 is a product of the proglucagon gene along with the other cleaved peptides in the enteroendocrine L cells, which includes GLP-2 and small fragment peptides like glicentin, oxytomodulin and two intervening peptides (IP-1 and IP-2). Proglucagon gene is cleaved to glucagon in α cells of the pancreas [84]. The amino acid sequence of GLP-1 is highly conserved between the species and is identical in all mammals [85]. GLP-1 is secreted in two bioactive forms, GLP-1 (7-37) and GLP-1 (7-36) amide, both of which have same half-life and identical activity [88]. Circulating levels of GLP-1 are very low at fasting state and rapidly increase following meal ingestion within 10 to 15 minutes [89]. Following its secretion into the blood, GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase- IV (DPP-IV) and the half-life of GLP-1 is less than 5 minutes.

GLP-1, an incretin hormone stimulates insulin secretion from the pancreatic β cells and the effect of GLP-1 on insulin secretion is strictly glucose dependent [90]. In addition to insulin secretion, GLP-1 is also shown to up regulate insulin gene transcription. The treatment of GLP-1 in isolated β cells increases insulin transcription through stabilization of insulin transcript and also by activation of the transcription factor PDX-1 [67]. GLP-1 is known to have trophic effects on pancreatic β cell mass as it stimulates β cell proliferation and β cell neogenesis in rat and human pancreatic duct [89]. This chronic effect of GLP-1 involving inhibition of apoptosis and stimulation of islet neogenesis has gained major interest in the treatment of diabetes [90]. The mechanisms underlying the effects of GLP-1 on islet regeneration involves upregulation of PDX-1 gene expression, PI3K activity and also transactivation of epidermal growth factor receptor [86,87]. The other key and specific effects of GLP-1 involve its inhibitory action on glucagon secretion from pancreatic α cells. This inhibitory effect of GLP-1 on glucagon secretion also contributes to an important mechanism for regulating elevated blood glucose levels in patients with type 2 diabetes [84,90].

In addition to the above-mentioned effects of GLP-1 on pancreas, it is also known to regulate gastrointestinal secretion and motility and has an inhibitory effect on gastric motility [85,87]. GLP-1 is also shown to reduce caloric intake by regulating appetite, the effects of which are mediated by the central nervous system. GLP-1 also increases insulin sensitivity in the peripheral tissues by improving insulin signaling and reducing gluconeogenesis [91,92].

1.8. Bile acids and GLP-1

Studies have demonstrated that bile acids have beneficial effects on glucose metabolism by improving postprandial glycemic control and insulin sensitivity and this is mainly associated with the activation of TGR5 by bile acids [58,63,66]. It was first reported by Katsuma et al., [65] that bile acids induce GLP-1 release in cultured mouse enteroendocrine STC-1 cells, whereas silencing of TGR5 using shRNA prevented the secretion of GLP-1 illustrating the involvement of TGR5 in bile acid induced response. GLP-1 is secreted from the enteroendocrine L cells, which are predominantly localized in the distal ileum and colon. Although the secretion of GLP-1 is stimulated by ingestion of food (carbohydrates, proteins and fats) and by neural signals, the major stimulus for the secretion of GLP-1 is the presence of nutrients, mainly fat and bile acids in the distal ileum [84,87]. This is correlated with the high levels of bile acid receptor TGR5 expression in the distal ileum and colon [35,36]. GLP-1 being an incretin hormone potentiates insulin secretion by binding to its receptors on the pancreatic β cells [88,89]. Dr. Schoonjan's group has also shown that TGR5 transgenic mice of obese and insulin resistant models are more glucose tolerant when compared to TGR5$^{-/-}$ mice, which have impaired glucose clearance [66]. The same group has also shown the release of GLP-1 from both STC-1 cells and human intestinal NCI-H716 cells by the activation of TGR5 using INT-777 (a specific TGR5 ligand). In this way, insulin secretion is indirectly regulated by TGR5 mediated GLP-1 release. *However the expression and functional significance of TGR5 receptors in the pancreatic islets is not known and hence the focus of our study* was to determine the direct effect of bile acids on pancreatic islets.

Table 1: GPCRs and their effect on insulin secretion in pancreatic β cells (modified from Ahren B, 2009)

RECEPTOR	FULL NAME	LIGAND	G PROTEIN	EFFECT ON INSULIN SECRETION
GLP-1R	Glucagon like peptide- 1 receptor	GLP-1	G_s	Stimulatory
GIPR	Glucose-dependent insulinotrophic polypeptide receptor	GIP	G_s	Stimulatory
GPR40 and GPR120	G protein-coupled receptor 40 and G protein-coupled receptor 120	Long chain fatty acids	G_q	Stimulatory
GPR41 and GPR43	G protein-coupled receptor 41 and G protein-coupled receptor 43	Short chain fatty acids	G_q	Stimulatory
GCGR	Glucagon receptor	Glucagon	G_s	Stimulatory
T1R1/T1R3	Amino acid taste receptor	Amino acids	G_q	Stimulatory
GSHR	Ghrelin receptor	Ghrelin	G_i	Inhibitory
β2	β2 adrenergic	Epinephrine	G_s	Stimulatory
α2	α2 adrenergic	Norepinephrine	G_i	Inhibitory
MT1	Melatonin receptor 1	Melatonin	G_i	Inhibitory
M3	Muscarinic receptor 3	Acetylcholine	G_q	Stimulatory
CB1	Cannabinoid receptor 1	Cannabinoids	G_i	Inhibitory
SSTR2	Somatostatin receptor 2	Somatostatin	G_i	Inhibitory
CCK1R	Cholecystokinin 1 receptor	Cholecystokinin	G_q	Stimulatory

CHAPTER 2

REGULATION OF INSULIN SECRETION IN PANCREATIC β CELLS BY

THE BILE ACID RECEPTOR TGR5

2.1 Introduction

Bile acids acting via the nuclear receptor farnesoid-X-receptor (FXR) and the membrane GPCR (TGR5) are known to play an important role in the regulation of cholesterol and triglyceride metabolism, insulin sensitivity, the intestinal endocrine response to meals and energy homeostasis [31,34,38,40,63]. Studies by Katsuma et al., have shown the role of TGR5 in mediating the release of incretins, glucagon-like peptide-1 (GLP-1) and GLP-2 from the intestinal enteroendocrine cells [65]. Both GLP-1 and GLP-2 promote insulin secretion from pancreatic β cells [88,89].

Insulin plays an important role in the regulation of glucose metabolism [93,94]. It is a peptide hormone composed of 51 amino acids with two polypeptide chains, the A- and B-chains that are linked together by disulphide bonds [68]. Insulin secretion is a highly regulated dynamic process and is modulated by nutrient status, neural, endocrine and paracrine factors [95]. Pancreatic β cells express receptors for various endogenous ligands that integrate the signals to modulate the synthesis and secretion of insulin to maintain glucose homeostasis under basal and post-prandial conditions. Insulin

promotes energy use as well as storage and thus decreases circulating blood glucose levels. The 3 major sites of insulin action are liver, skeletal muscle and adipose tissue [96]. Insulin upon binding to its cognate receptor (tyrosine kinase receptor) induces signal transduction cascade, which promotes the uptake of glucose by the glucose transporter (GLUT4) into the cells [93]. In liver, insulin promotes synthesis and storage of glycogen and reduces hepatic glucose production by inhibiting gluconeogenesis and glycogenolysis [97]. In skeletal muscle, insulin-stimulated glucose uptake accounts for energy production and in the adipose tissue, insulin increases triglyceride synthesis facilitating fat storage in the cells and also inhibits fatty acid breakdown [96].

The predominant trigger for insulin secretion in β cells is increased blood glucose level. Thus glucose-stimulated insulin secretion (GSIS) is a process coordinated by the uptake of glucose from the β cells, metabolism and Ca^{2+} triggered insulin exocytosis [98]. The β cells take up glucose by the glucose transporter 2 (GLUT-2), the metabolism of which increases the ATP:ADP ratio, causing the ATP sensitive K^+ channels to close and thereby induce membrane depolarization. This in turn opens the voltage gated Ca^{2+} channels to increase the influx of Ca^{2+} and elicit insulin release. The effect of glucose is also modulated by the activation of GPCRs, which optimize glucose-stimulated insulin secretion (**Table 1**). Pancreatic β cells also express several G-protein coupled receptors (GPCRs) and these include receptors for peptides (GLP-1, GIP, glucagon and somatostatin), amino acids (T1Rs), free fatty acids (GPR40, GPR41, GPR43, GPR84, GPR120) and acetylcholine (muscarinic m3 receptors). Activation of GPCRs coupled to G_s or G_q stimulates insulin secretion whereas activation of those coupled to G_i inhibits

insulin secretion [81,99-103]. *However the role of bile acids in the regulation of insulin secretion in the pancreatic β cells via activation of TGR5 by bile acids is unknown.*

Bile acids that are released in response to meal are found in significant amount in the systemic circulation. Under fasting conditions, circulating bile acids are less than 5 μM whereas postprandial levels rise up to 15 μM [4]. *We therefore postulated that bile acids acting via its membrane receptor, TGR5 could stimulate insulin secretion from pancreatic β cells.*

2.2. Materials and Methods

2.2.1. Materials

Oleanolic acid (OA) and lithocholic acid (LCA) were obtained from Sigma-Aldrich (St Louis, MO) and INT-777 (S-EMCA: 6α-Ethyl-23(S)-methylcholic Acid) was a generous gift from Intercept Pharmaceuticals. [^{35}S] GTPγS, myo-[^{3}H] inositol and [^{125}I]cAMP were obtained from Perkin Elmer (Boston, MA); polyclonal antibody to TGR5 was purchased from Abcam (Cambridge, MA); antibodies to Gα$_s$, Gα$_q$, Gα$_{i1}$, Gα$_{i2}$ and Gα$_{i3}$, and NF449 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); ultrasensitive mouse insulin ELISA kit was obtained from Crystal Chem. Inc. (Downers Grove, IL); ultrasensitive human insulin ELISA kit was purchased from Mercodia (Uppsala, Sweden). Collagenase P was obtained from Roche Diagnostics (Indianapolis, IN); Fura 2-AM, HEPES and RPMI were obtained from Invitrogen (Eugene, OR); U73122 and Myristoylated PKI were obtained from Calbiochem (La Jolla, CA); western blotting and chromatography materials were obtained from Bio-Rad Laboratories

(Hercules, CA). RNeasy Plus universal Mini kit and Taq PCR core kit were obtained from Qiagen (Venlo, Netherland); PCR reagents were obtained from Applied Biosystems (Foster city, CA). Dulbecco's Modified Eagle Medium (DMEM), 2-mercaptoethanol, 8-pCPT-2'-O-Me-cAMP, BAPTA-AM, Histopaque (10771 and 11191) and all other reagents were obtained from Sigma (St Louis, MO).

Wild type C57BL/6J mice were purchased from Jackson laboratories (Ban Harbor, ME) and euthanized with CO_2. The animals were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University. All procedures were conducted in accordance with the institutional Animal Care and Use Committee of the Virginia Commonwealth University.

2.2.2. Cell culture

The insulin secreting mouse pancreatic beta (β) cell line, MIN6 cells were cultured in DMEM containing L-glutamine, sodium carbonate and 2.5 mM 2-mercaptoethanol. The insulin secreting rat pancreatic β cell lines, INS-1 and 823/13 cells (a kind gift from Dr. Christopher Newgard, Duke University) were cultured in RPMI containing Hepes, L-glutamine, sodium pyruvate and 2-mercaptoethanol. All the media were supplemented with 10% fetal bovine serum and 100U/ml pencillin-streptomycin and the cells were incubated at 37°C in 5% CO_2.

2.2.3. Isolation and maintenance of mouse islets

Pancreatic islets from mice were isolated by sequential enzyme digestion of pancreas, filtration and centrifugation as described previously [104]. The mice were euthanized using CO_2. The abdominal surface was wetted with 70% ethanol and

opened with standard pair of scissors in a V shape starting from the lower abdomen and extending to the lateral portions of the diaphragm so that the organs in the peritoneal cavity are well exposed. The common bile duct was clamped off near the junction with the small intestine using John Hopkin's Bulldog clamp. About 4-5 ml of collagenase P solution (Collagenase P was dissolved in G solution- 1X HBSS, 0.35 mM sodium bicarbonate and 1% BSA) was injected into the pancreas through the common bile duct **(Figure 4A)**. The inflated pancreas was then carefully removed and incubated in 37^0C water bath for 5-9 minutes. Following digestion, the pancreatic tissue was subjected to centrifugation and filtration so as to remove the undigested tissue. Density gradient centrifugation was carried out using histopaque 1110 solution for 20 min at 1200 rpm to separate acinar tissues from the islets. The supernatant (containing the islets) was collected and washed twice with G solution by centrifuging at 1200 rpm for 4 min. Solution containing the islets was then passed through an inverted 70 μm sterile strainer or filter to remove the remaining acinar tissue and maintained in RPMI-1640 medium supplemented with 10% FBS and 100U/ml pencillin-streptomycin and incubated at 37°C in 5% CO_2. The islets are then handpicked under the microscope and counted prior to experimentation **(Figure 4B and 4C)**.

Human islets were obtained from the National Disease Research Interchange (NDRI), Philadelphia, PA.

2.2.4. RNA Isolation and RT-PCR analysis

Total RNA was isolated from cells (MIN6, INS-1 and 823/13) and mouse and human islets using RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's

instructions. 1 μg of the total RNA was reverse transcribed using high capacity cDNA reverse transcriptase kit (Applied Biosystems) with random primers in a 20 μl reaction volume. Conventional PCR was performed on 2 μl of cDNA using Taq PCR core kit (Qiagen) in a final volume of 20 μl using specific primers (Table 1). The amplified PCR products were analyzed on 2% agarose gel containing ethidium bromide using Gel Doc^{TM} EZ imager.

Real time PCR was carried out using StepOne^{TM} Real-Time PCR system (Applied Biosystems). Quantitative real-time PCR was performed for TGR5 (Mm 04212121_s1) with TaqMan probes mixed with TaqMan PCR Master-Mix for 40 cycles (95^{0}C for 15 sec, 60^{0} C for 1 min) using 2-5 ng of cDNA in a 20 μl reaction volume. Quantitative real-time PCR of the same sample was performed for β-actin (Mm 00607939_s1) expression as internal control for normalization. Each sample in the real time PCR reactions was performed in triplicates and no template control was also preformed to confirm the efficiency and lack of primer dimer formation in the reactions.

Quantification of gene expression was done using relative quantification method. In this method, the PCR signal (cycle threshold- Ct value) of the target transcript in a treatment group to that of another sample such as untreated control was compared. The difference in Ct values between TGR5 and β Actin was calculated as ΔCt values. ΔCt values of treated samples were subtracted from ΔCt values of control samples to obtain ΔΔCt values. Finally the relative fold change in the gene expression was calculated as $2^{-\Delta\Delta Ct}$.

ΔΔCt= (Ct $_{Target}$- Ct $_{Endogenous\ control}$) Treatment ***minus*** (Ct $_{Target}$- Ct $_{Endogenous\ control}$) Control

Relative gene expression (Fold Change)= $2^{-\Delta\Delta Ct}$

2.2.5. Western blot analysis

The cells were solubilized in RIPA buffer (Sigma) containing protease inhibitor cocktail (104 mM AEBSF, 80 µM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.5 mM Pepstatin A). The supernatant was collected after centrifuging the lysate at 10000g for 15 min at 4^0C and the protein concentration was determined by DC protein assay kit from Bio-Rad. Equivalent amounts of proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked in 5% non fat dry milk + tris buffered saline with 0.1% tween-20 (TBST) for 1 hour and then incubated with various primary antibodies in 5% non fat dry milk + TBST or 5% BSA + TBST for overnight at 4^0C. The blots were washed with TBST three times for 5 min and then incubated for 1 hour at room temperature with horseradish peroxidase conjugated corresponding secondary antibodies in 5% non fat dry milk + TBST. The washing steps were repeated as before and the immunoreactive proteins were visualized using SuperSignal West Pico chemiluminiscent substrate kit (Pierce, IL).

Western blot images were scanned and analyzed with ImageJ software for densitometric measurements. The average intensity obtained for each band was normalized to its respective band of β-actin. The band intensity was then presented as relative fold changes compared with the corresponding control.

2.2.6. Assay for identification of activated G proteins

MIN6 cells were homogenized and the crude membranes were solubilized following the procedure as described previously [105]. Membranes isolated from MIN6

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cells were incubated with 60 nM [35S] GTPγS containing 10 mM Hepes (pH 7.4), 0.1 mM EDTA and 10 mM MgCl2 for 30 min at 37°C in the presence or absence of OA (10 μM) and INT-777 (25 μM). The reaction was stopped by adding 10 volumes of 100 mM Tris/HCl (pH 8) containing 10 mM MgCl2, 100 mM NaCl and 20 μM GTP and was then incubated in wells precoated with specific antibodies to $G\alpha_s$, $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ for 2 h in ice. After incubation, the solution was discarded and the wells were washed with PBS containing 0.05% Tween followed by solubilization with 0.1N NaOH. The radioactivity in each well was counted by liquid scintillation and expressed in cpm/mg protein.

2.2.7. Assay for adenylyl cyclase activity

MIN6 cells were treated with 100 mM IBMX and TGR5 ligands (OA or INT-777, 25 μM), the reaction was stopped with 6% Trichloroacetic acid and the suspension was centrifuged at 2000g for 15 min at 4^0C. The supernatant was extracted with water saturated diethyl ether and lyophilized. The samples were reconstituted with 500 ul of 50 mM sodium acetate and cAMP was measured by radioimmunoassay as described previously [106]. 100 μl of aliquots were taken from each sample and acetylated with tetraethyl ammonium/acetic anhydride (2:1 v/v) for 30 min. After overnight incubation, 2 ml of 50 mM sodium acetate was added and centrifuged. The samples were decanted by discarding the supernatant and the antigen-antibody complex (pellet) was counted in the gamma counter. The data was used to construct a standard curve from which the values of the unknowns were determined. cAMP levels were expressed as percent increase above basal levels.

2.2.8. Assay for phosphoinoside (PI) hydrolysis

MIN6 cells were labeled with myo-[3H] inositol (0.5 µCi/ml) in DMEM medium for 24 h. After 24 h, cells were washed with PBS and treated with OA, INT-777, NF449 (a selective $G\alpha_s$ inhibitor), a selective Epac ligand (8-pCPT-2'-O-Me-cAMP) or U73122 (PI hydrolysis inhibitor) for 1 min. The reaction was terminated using 940 ul of Chloroform-methanol-HCl (50:100:1 v/v) and to the samples extracted, 340 ul of chloroform and 340 ul of water was added and centrifuged at 5,000 rpm for 10 min as described previously [107]. The upper aqueous phase was applied to the column prepared with Dowex AG-50W-X8 resin and water (1:1) and washed with 5 mM sodium tetraborate/60 mM ammonium formate solution. The [3H] inositol triphosphate was eluted with 0.8 M ammonium formate plus 0.1 M formic acid. Radioactivity was determined by liquid-scintillation counting and the result was expressed as percent increase above basal.

2.2.9. Measurement of intracellular calcium

MIN6 cells cultured on glass cover slips, and mouse and human islets were washed with PBS and then loaded with 5 µM fura2-AM in HBSS buffer containing 3 mM glucose for 2 h at room temperature. Clusters of cells were selected for imaging and visualized as described previously [108]. The cells were visualized through the 40X objective of Zeiss Axioskop 2 plus upright fluorescence microscope and imaged with a set up consisting of a charge coupled device camera (Imago, TILL photonics) attached to an image intensifier. The cells were alternatively excited at 340 nm and 380 nm and the increase in intracellular calcium by the TGR5 ligands (OA or LCA) was measured by determining the ratio of the fluorescence of fura-2 at 340 and 380 nm excitation.

2.2.10. Measurement of Insulin secretion

MIN6 cells were plated at a density of 5 x 10^5 cells in 24-well plates with DMEM and cultured until confluent. The cells were washed and incubated in HBSS (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, pH 7.2, 0.1% BSA) containing 3 mM glucose for 2 h at 37^0C. Cells were then treated for 30 min in HBSS containing 3 mM (basal) or 25 mM glucose (stimulated) with or without OA, INT-777 or LCA. The supernatants were collected and assayed for insulin using ELISA kit. For insulin secretion from mouse islets (20-25 islets/condition) or human islets (15-20 islets/condition), the islets were incubated at 37^0C for 2 h in HBSS with 3mM glucose. The same procedure as described above was followed.

2.2.11. Statistical Analysis

Results were calculated as means ± SEM and the experiments were performed at least three times. The experiments were performed on islets isolated from different animals. Statistical significance was analyzed using Student's t-test for paired and unpaired values. GraphPad Prism software was used for all statistical analyses and p values < 0.05 were considered significant.

2.3. Results

2.3.1. Expression of TGR5 in human and murine pancreatic islets and in pancreatic β cells

Expression of TGR5 mRNA was detected in all three pancreatic β cell lines (MIN6, INS1 and 823/13) by qRT-PCR. Expression was 11.8 fold higher in MIN6 cells compared to INS1 and 2.5 fold higher compared to 823/13 cells **(Figure 5).** RT-PCR analysis of RNA from islets of human and mice using specific primers demonstrated amplification of a PCR product of predicted size in both human islets (277 bp) and mouse islets **(Figures 6A and 6B)**. The results suggest expression of TGR5 receptors in human and mouse islets.

Further confirmation for the expression of TGR5 receptors was obtained by western blot analysis using selective antibody to TGR5. The results demonstrated the expression of TGR5 receptor of predicted size (35 kDa) in the homogenates isolated from both MIN6 and mouse islets **(Figure 6C)**.

2.3.2. Stimulation of insulin secretion by TGR5 ligands

To investigate the role of TGR5 in insulin secretion, we examined whether basal (3 mM glucose) or stimulated (25 mM glucose) insulin secretion would be modulated by the activation of TGR5 receptors. The treatment of MIN6 cells with the selective TGR5 ligand, OA stimulated insulin secretion in a dose dependent (10-50 μM) manner both at 3 mM glucose (10 μM- 1.6±0.09, 30 μM- 2.2±0.16, 50 μM- 3.7±0.37 fold increase above 3 mM glucose basal levels 32.2±1.8 ng/ml) and 25 mM glucose (10 μM- 1.2±0.03, 30 μM- 2±0.08, 50 μM- 3.2±0.07 fold increase above 25 mM glucose basal levels 64.4±1.1 ng/ml) conditions **(Figure 7)**. Consistent with the stimulatory effect of OA, the natural bile acid, lithocholic acid (LCA, 10 μM) also augmented both basal (1.5±0.09 fold increase) and stimulated (1.1±0.03 fold increase) insulin secretion **(Figure 8)**.

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Treatment of mouse pancreatic islets with OA significantly stimulated both basal and stimulated insulin secretion (1.9±0.15 fold increase above 3 mM glucose basal levels 4.5±0.4 ng/ml; 1.5± 0.07 fold increase above 25 mM glucose basal levels 10.3±0.1 ng/ml). Similar results were obtained when the islets were treated with the specific TGR5 agonist, INT-777 (1.6±0.1 fold increase above 3 mM glucose basal levels and 1.6±0.02 fold increase above 25 mM glucose basal levels) or natural bile acid LCA (1.5±0.21 fold increase above 3 mM glucose basal levels and 1.4±0.2 fold increase above 25 mM glucose basal levels). It is noteworthy that the extent of increase was similar with all three ligands used at the concentration of 25 μM each **(Figure 9)**.

Treatment of human islets with 25 μM OA, INT-777 or LCA also caused an increase in insulin secretion at both basal and stimulated conditions: OA (2.4±0.28 fold increase above 3 mM glucose basal levels 2.7±0.2 ng/ml; 1.8±0.11 fold increase above 25 mM glucose basal levels 6.05±0.1 ng/ml); INT-777 (2±0.22 fold increase above 3 mM glucose basal levels and 1.6±0.05 fold increase above 25 mM glucose basal levels); and LCA (2.3±0.37 fold increase above 3 mM glucose basal levels and 1.5±0.08 fold increase above 25 mM glucose basal levels) **(Figure 10)**.

2.3.3. Signaling mechanisms involved in TGR5-mediated insulin secretion in pancreatic β cells

After demonstrating the expression of TGR5 in β cells and secretion of insulin in response to TGR5 ligands, further studies were designed to examine the signaling pathways activated by TGR5 receptors to mediate insulin secretion. Biochemical approaches such as G protein activation, adenylyl cyclase activity and PI hydrolysis,

and intracellular Ca^{2+} were done in both MIN6 cells and islets from mouse and human. In addition, insulin secretion in response to TGR5 ligands was measured in the presence or absence of various inhibitors that block different steps in the signaling pathways.

2.3.3.1. Activation of Gα$_s$ by TGR5 selective ligands

Incubation of MIN6 cell membranes with OA **(Figure 11)** or INT-777 **(Figure 12)** caused a significant increase in the binding of [^{35}S]GTPγS selectively to Gα$_s$ (OA- 20252±389 cpm/mg protein; 309±10.86% increase and INT-777- 19267±483 cpm/mg protein; 289±12.27% increase above basal levels 4957±38 cpm/mg protein), but not to Gα$_q$, Gα$_{i1}$, Gα$_{i2}$ or Gα$_{i3}$. These results suggest that TGR5 receptors are preferentially coupled to the activation of Gα$_s$ in pancreatic β cells.

2.3.3.2. Activation of adenylyl cyclase by TGR5 selective ligands

Consistent with the activation of Gα$_s$ proteins, incubation of MIN6 cells with 25 µM, OA or INT-777 caused stimulation of adenylyl cyclase resulting in an increase in cAMP levels: OA- 56% increase in cAMP levels above basal levels (0.32 pmoles/ml); and INT-777- 75% increase in cAMP levels above basal levels (0.32 pmoles/ml) **(Figure 13)**. The results are consistent with the selective activation of Gα$_s$/cAMP pathway by OA in enteroendocrine cells and gastric smooth muscle cells [56,109].

2.3.3.3. Activation of Epac (cAMP-dependent exchange factor) by TGR5 selective ligands

To examine whether OA-induced insulin secretion involves PKA-dependent or – independent mechanism, insulin secretion in response to OA was measured in the

presence of myristoylated PKI, a selective PKA inhibitor **(Figure 14)**. The OA-induced insulin secretion was not significantly inhibited by Myr-PKI ($9\pm5\%$ inhibition at 3 mM glucose basal or $22\pm6\%$ inhibition at 25 mM glucose basal) suggesting the involvement of alternative pathway of cAMP, which is PKA-independent. The pathway could involve sequential activation of Epac (cAMP-dependent exchange factor), Epac-dependent stimulation of PLC-ε activity and release of intracellular Ca^{2+} **(Figure 15)**. Consistent with the above notion, the selective Epac ligand, 8-pCPT-2'-O-Me-cAMP (1 μM) also increased both basal (2.2 ± 0.09 fold increase above 3 mM glucose basal levels) and stimulated (2.4 ± 0.14 fold increase above 25 mM glucose basal levels) insulin secretion **(Figure 16)**.

The studies are corroborated by identification of Epac2 and PLC- ε expression. RT-PCR analysis demonstrated the expression of Epac2 and PLC-ε mRNA in MIN6 cells, and mouse and human islets **(Figure 17)**.

2.3.3.4. Activation of PI hydrolysis by TGR5 selective ligands

Previous studies in enteroendocrine L cells have shown that GLP-1 release by OA was mediated via G_s/Epac/PLC-ε/Ca^{2+} pathway [109]. Activation of a similar pathway was examined in pancreatic β cells by measurements of PI hydrolysis in response to TGR5 selective ligands, OA and INT-777 and an Epac selective ligand, 8-pCPT-2'-O-Me-cAMP. Both OA (958 ± 11.02 cpm/mg protein; $249\pm3.52\%$ increase above basal levels 274 ± 3.84 cpm/mg protein) and INT-777 (846 ± 2.52 cpm/mg protein; $209\pm4.64\%$ increase above basal levels) caused a significant and similar increase in PI hydrolysis **(Figure 18)**. The OA-induced increase in PI hydrolysis was blocked by the

selective PI hydrolysis inhibitor, U73122 (78±4.05% inhibition) or by a selective $G\alpha_s$ protein inhibitor, NF449 (75±0.98% inhibition). Similarly, INT-777 stimulated PI hydrolysis was inhibited by U73122 (55±6.96% inhibition) and NF449 (65±5.02% inhibition). These results suggest that activation of PI hydrolysis by OA and INT-777 was mediated via activation of $G\alpha_s$ proteins, probably involving activation of Epac and Epac-dependent PLC-ε. In support to this notion, the selective Epac ligand also stimulated PI hydrolysis and the extent of stimulation (849±26.77 cpm/mg protein; 209.5±5.99% increase above basal levels) was similar to INT-777. Stimulation of PI hydrolysis by Epac ligand was inhibited by U73122 (63±7.25% inhibition).

2.3.3.5. Release of Calcium by selective TGR5 ligands

To determine whether stimulation of PI hydrolysis cause increase in intracellular calcium concentration, we examined the effect of TGR5 ligands on cytosolic Ca^{2+} ($[Ca^{2+}]_I$) by calcium imaging using fura-2. Consistent with the activation of PI hydrolysis, addition of OA or LCA to MIN6 cells resulted in a rapid increase in cytosolic Ca^{2+} **(Figure 19)**. A similar increase in cytosolic Ca^{2+} in response to OA was demonstrated in islets isolated from mouse and human **(Figure 19)**. These results suggest that TGR5-mediated increase in intracellular calcium causes the release of insulin from the pancreatic human and mouse islets and β cells.

2.3.3.6. Signaling pathways involved in TGR5-mediated insulin secretion in pancreatic β cells

To examine the mechanisms involved in OA-induced insulin secretion, MIN6 cells were preincubated with inhibitors of $G\alpha_s$ (NF449, 10 μM), PI hydrolysis (U73122, 10 μM) or with Ca^{2+} chelator BAPTA-AM (10 μM), and then the insulin secretion in response to 50 μM OA was measured. OA caused an increase in insulin secretion in both basal (3.7±0.14 fold increase above 3 mM glucose basal levels 29.3±0.3 ng/ml) and stimulated (3.6±0.27 fold increase above 25 mM glucose basal levels 49.4±0.1 ng/ml) condition; increase in insulin secretion was abolished by incubation of cells with NF449, U73122 or BAPTA-AM **(Figure 20)**.

In summary, pancreatic human and mouse islets, and pancreatic β cells express TGR5 receptors coupled to G_s and stimulation of adenylyl cyclase leading to generation of cAMP. Activation of TGR5 by bile acids causes insulin secretion via sequential activation $G\alpha_s$/cAMP, cAMP-dependent exchange factor Epac and Epac-dependent PLC-ε. Stimulation of PLC-ε hydrolyzes PIP_2 leading to the generation of IP_3 and IP_3-dependent Ca^{2+} release. This rise in the intracellular calcium triggers the release of insulin **(Figure 21)**.

2.4. *Discussion*

The regulated synthesis and secretion of insulin is one of the center-pieces of the body's strategy to maintain metabolic homeostasis. It is therefore not surprising that pancreatic islet β cell function is modulated by neural, endocrine and paracrine factors and cell–cell interactions [68,73,78]. A wide variety of ligands thus bind their specific receptors on islet cells, which integrates these signals to modulate its synthetic and

secretory functions. Bile acids have recently been shown as one of the ligands that affect islet function. Specifically, via binding to FXR, bile acids have been shown to affect insulin secretion. This study demonstrates that islet β cells also express TGR5 receptors, which upon activation enhance glucose-mediated insulin secretion. This discovery further supports a role for bile acids, the endogenous ligands for TGR5, in the regulation of metabolism.

Bile acids may impact islet function in both direct and indirect ways. At the level of the intestine, activation of TGR5 by bile acids enhance GLP-1 and GLP-2 release [65,109,110]. GLP-1 delays gastric emptying and thus the absorption of glucose, thereby flattening the post-prandial glycemic response [85,86]. GLP-1 also directly functions as a trophic factor for islet cells and promotes insulin secretion following a glucose challenge [67]. The current study demonstrates that bile acids promote glucose-mediated insulin secretion via TGR5 expressed on β cells and also confirm prior observations that FXR activation can enhance glucose-mediated insulin secretion [111].

At a cellular level, TGR5 ligands lithocholic acid, OA and INT-777 all increased intracellular Ca^{2+} and calcium chelation blunted the insulin secretory response indicating that the final exocytosis of insulin via TGR5 involved classical Ca^{2+}-dependent pathways used by other stimulators of insulin secretion. We further showed that bile acid induced TGR5 receptors in the pancreatic β cells is $G\alpha_s$ coupled and activation of these receptors leads to stimulation of adenylyl cyclase activity generating cAMP which in turn leads to the activation of cAMP-dependent Epac/PLC-ε pathway stimulating

insulin secretion. This mechanism was found to be similar to that used by the TGR5 expressed in the enteroendocrine cells which stimulated GLP1 release [109].

Normally, circulating glucose is the key driver of insulin secretion; islet cells sense ambient glucose levels along with fatty acids and various amino acids and activate proinsulin synthesis and insulin secretory mechanisms in response to the metabolic coupling factors [98-103]. The observed enhancement of insulin secretion by TGR5 ligands under low and high glucose conditions suggests that the known insulin-sensitizing effects of bile acids may be partly due to improved insulin secretory responses to a glucose load. Interestingly, the simultaneous use of a FXR agonist (INT-747) and a TGR5 agonist (INT-777) did not lead to an additive response although both INT-747 and INT-777 stimulated glucose mediated insulin secretion to a similar degree when used alone (data not shown). This suggests a degree of antagonism between the intracellular pathways activated by FXR and TGR5 respectively that converge on insulin secretion. This however remains to be experimentally elucidated.

The concentration of bile acids in circulation fluctuate in fasting and fed state and it is known that bile acid levels and form are affected in disease conditions [6,39]. The precise effect of bile acids *in vivo* is likely to be not only a function of its concentration but also the nature of the circulating bile acids. For example, it is well known that tauro-ursodeoxycholic acid is only a weak ligand for TGR5 and FXR whereas chenodeoxycholic acid and lithocholic acid are more potent ligands. In chronic liver disease, particularly with cholestasis, fasting levels of bile acids are elevated and there is a loss of secondary bile acids [112,113]. There is also a decrease in intestinal

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exposure to bile acids, which is expected to dampen the incretin response to meals. The contribution of these findings to the well-known susceptibility of individuals with cirrhosis to develop type 2 diabetes now need to be considered in the context of the increasing recognition of the effects of bile acids on both insulin secretion and also on insulin sensitivity.

From a public health point of view, type 2 diabetes is a leading threat to the health of human race. Fasting hyperinsulinemia and eventual β cell exhaustion are hallmarks of the insulin resistance syndrome and development of type 2 diabetes [114]. It is generally believed that a combination of increased exposure to inflammatory cytokines, fatty acids and other products of lipolysis along with increased blood sugar drive increased insulin synthesis and secretion in such cases [115]. The potential role of bile acids in modulating the insulin secretory response to these factors and as a disease modifier in the genesis of type 2 diabetes awaits elucidation.

In summary, the current study adds to the growing body of evidence supporting a key role of bile acids as a regulator of nutritional and metabolic homeostasis. It activates the release of GLP-1, a known stimulator of insulin secretion, in response to a meal in the intestine by activation of TGR5 receptors [65,66,109,110]. We now demonstrate that TGR5 receptors are present in pancreatic islet β cells and that activation of TGR5 increases insulin secretion under both low and high ambient glucose levels.
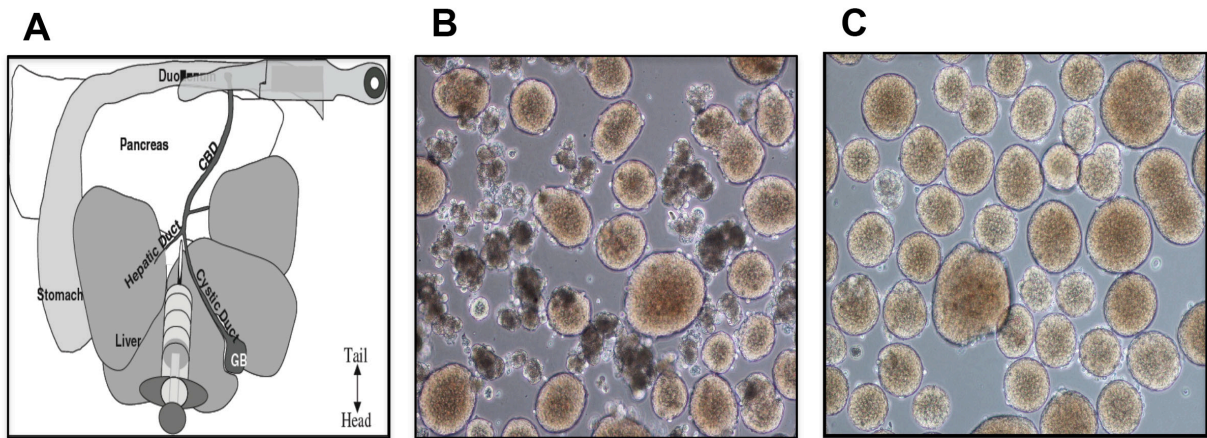
Figure 4. Isolation and culture of mouse islets. (**A**) The mouse was euthanized using CO_2 and the abdomen was completely opened from the anus to the diaphragm so that the mouse upper intraperitoneal cavity was well exposed. The common bile duct was clamped off at the duodenum and collagenase P was injected through the common bile duct. The inflated pancreas was then removed and subjected to density gradient centrifugation and series of washing. (**B**) The islets initially obtained were found along with some acinar tissues which were then removed and (**C**) the purified islets were cultured in RPMI-1640 and incubated at 37°C in 5% CO_2.
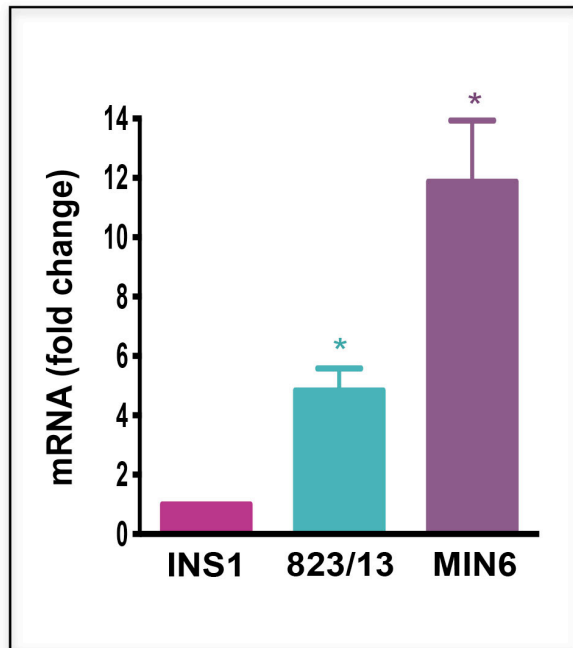
Figure 5. Expression of TGR5 in pancreatic β cell lines. Total RNA isolated from the cultured cells (INS1, 823/13 or MIN6) was reverse transcribed and TGR5 mRNA levels were measured by qRT-PCR. The results are expressed as fold change or fold difference in TGR5 expression levels in reference to INS1 after normalizing with the endogenous control, β-actin. TGR5 expression was higher in MIN6 when compared to 823/13 or INS1 cells. Values are expressed as mean ± SEM of 4 experiments. [*]p<0.05 vs. INS1.
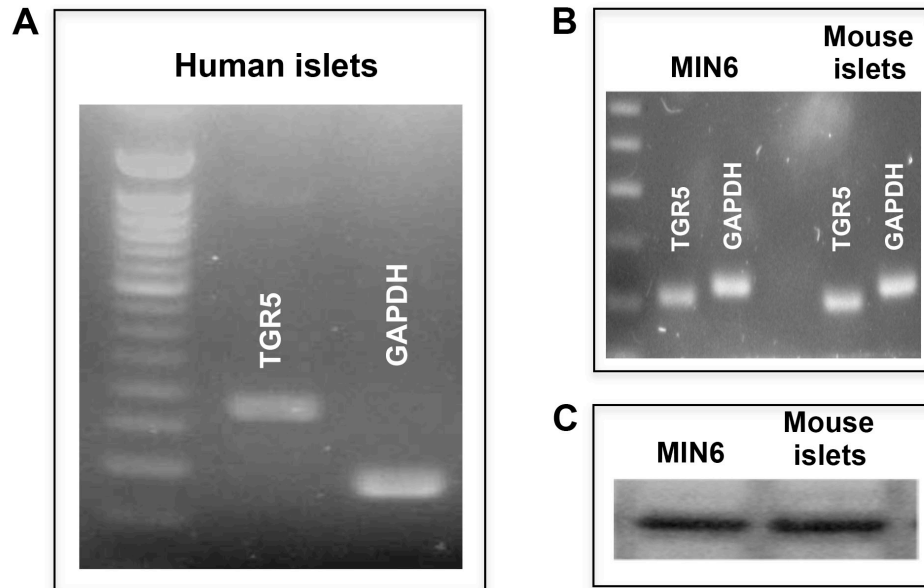
Figure 6. Expression of TGR5 in human and mouse pancreatic islets. A and B: mRNA expression- Total RNA isolated from human and mouse pancreatic islets and MIN6 cells was reverse transcribed and expression of TGR5 mRNA was examined by RT-PCR using specific primers. The results show representative PCR products (human TGR5- 277 bp or mouse TGR5- 104 bp) analyzed on 2% agarose gel containing ethidium bromide using Gel Doc[TM] EZ imager. **C: Protein expression-** Cell lysates containing equal amounts of total proteins from MIN6 cells and mouse islets were separated on SDS-PAGE and the expression of TGR5 (35 kDa) was analyzed using TGR5 selective antibody.
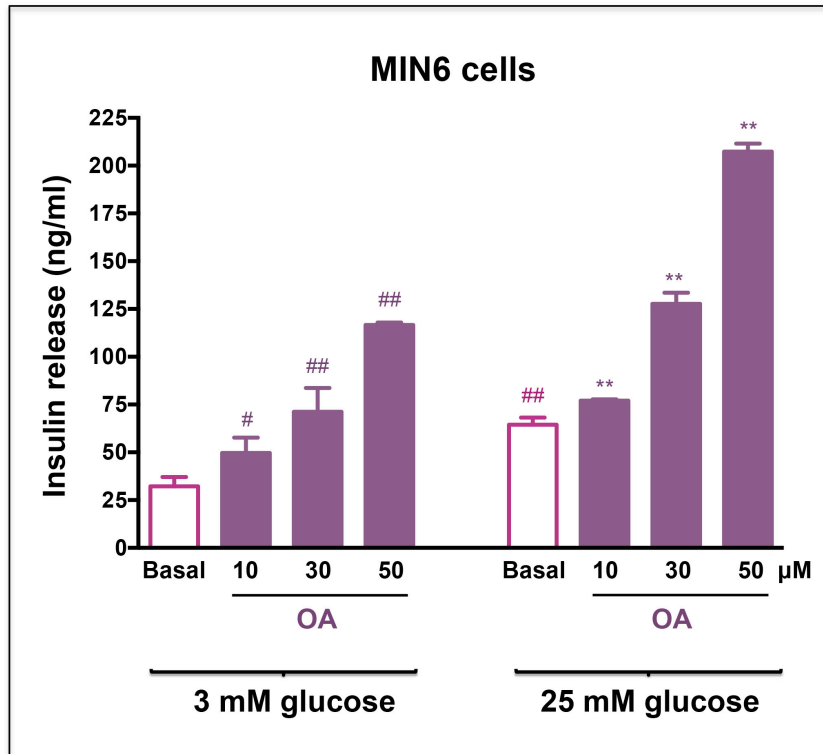
Figure 7. Stimulation of insulin secretion by oleanolic acid (OA) in MIN6 cells. MIN6 cells were plated at a density of 5×10^5 cells in 24-well plates and cultured with DMEM until confluent. The cells were washed and incubated in HBSS buffer containing 3 mM glucose for 2 h at 37^0C. The cells were then treated with different concentrations of OA (10, 30 or 50 µM) in the presence of 3 mM glucose or 25 mM glucose for 30 min. The supernatants were collected and insulin secretion was measured by ELISA. OA caused an increase in the insulin secretion in a dose dependent manner both at basal and stimulated glucose conditions. Values are expressed as mean ± SEM of 4 experiments. ##$p<0.001$ or #$p<0.05$ vs. 3 mM glucose basal; **$p<0.001$ or *$p<0.05$ vs. 25 mM glucose basal.
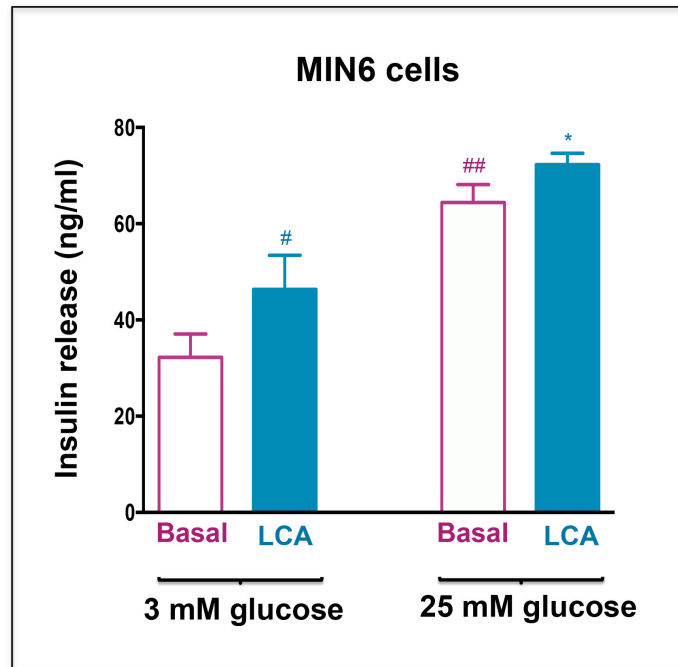
Figure 8. Stimulation of insulin secretion by lithocholic acid (LCA) in MIN6 cells.
MIN6 cells were plated at a density of 5×10^5 cells in 24-well plates and cultured with DMEM until confluent. The cells were washed and incubated in HBSS buffer containing 3 mM glucose for 2 h at 37^0C. The cells were then treated with physiological concentration of LCA (10 µM) in the presence of 3 mM glucose or 25 mM glucose for 30 min. The supernatants were collected and insulin secretion was measured by ELISA. The natural bile acid, LCA increased insulin secretion both at basal and stimulated glucose conditions. Values are expressed as mean ± SEM of 4 experiments. [##]p<0.001 or [#]p<0.05 vs. 3 mM glucose basal; [**]p<0.001 or [*]p<0.05 vs. 25 mM glucose basal.
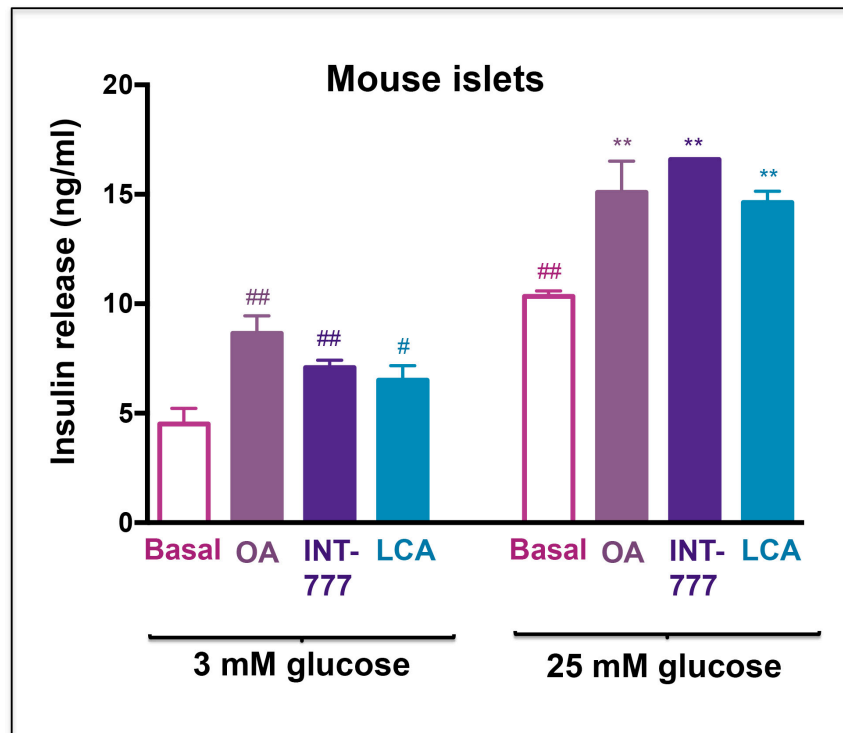
Figure 9. Stimulation of insulin secretion by TGR5 ligands in mouse pancreatic islets. Mouse islets (20-25 islets/condition) were washed and incubated in HBSS buffer containing 3 mM glucose for 2 h at 37^0C. The islets were then treated with OA (25 µM) or INT-777 (25 µM) or LCA (25 µM) in the presence of 3 mM glucose or 25 mM glucose for 30 min. The supernatants were collected and insulin secretion was measured by ELISA. Oleanolic acid, a selective TGR5 ligand; INT-777, a specific TGR5 ligand and lithocholic acid, a natural bile acid increased insulin secretion in mouse islets. Values are expressed as mean ± SEM of 3 experiments. [##]$p<0.001$ or [#]$p<0.05$ vs. 3 mM glucose basal; [**]$p<0.001$ or [*]$p<0.05$ vs. 25 mM glucose basal.
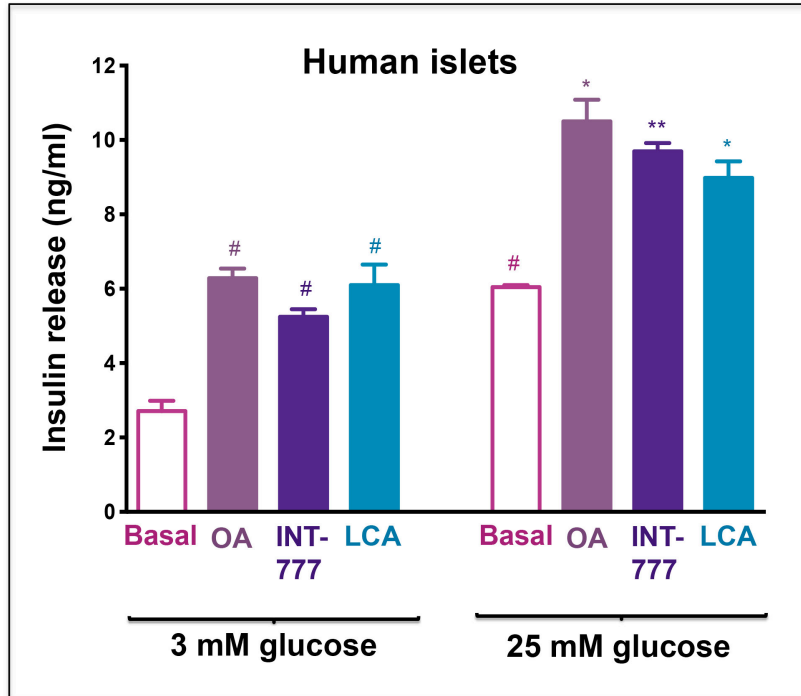
Figure 10. Stimulation of insulin secretion by TGR5 ligands in human pancreatic islets. Human islets (15-20 islets/condition) were washed and incubated in HBSS buffer containing 3 mM glucose for 2 h at 37^0C. The islets were then treated with OA (25 µM) or INT-777 (25 µM) or LCA (25 µM) in the presence of 3 mM glucose or 25 mM glucose for 30 min. The supernatants were collected and insulin secretion was measured by ELISA. Oleanolic acid, INT-777 and lithocholic acid caused an increase in insulin secretion in human islets both at basal and stimulated glucose conditions. Values are expressed as mean ± SEM of 3 experiments. [##]p<0.001 or [#]p<0.05 vs. 3 mM glucose basal; [**]p<0.001 or [*]p<0.05 vs. 25 mM glucose basal.
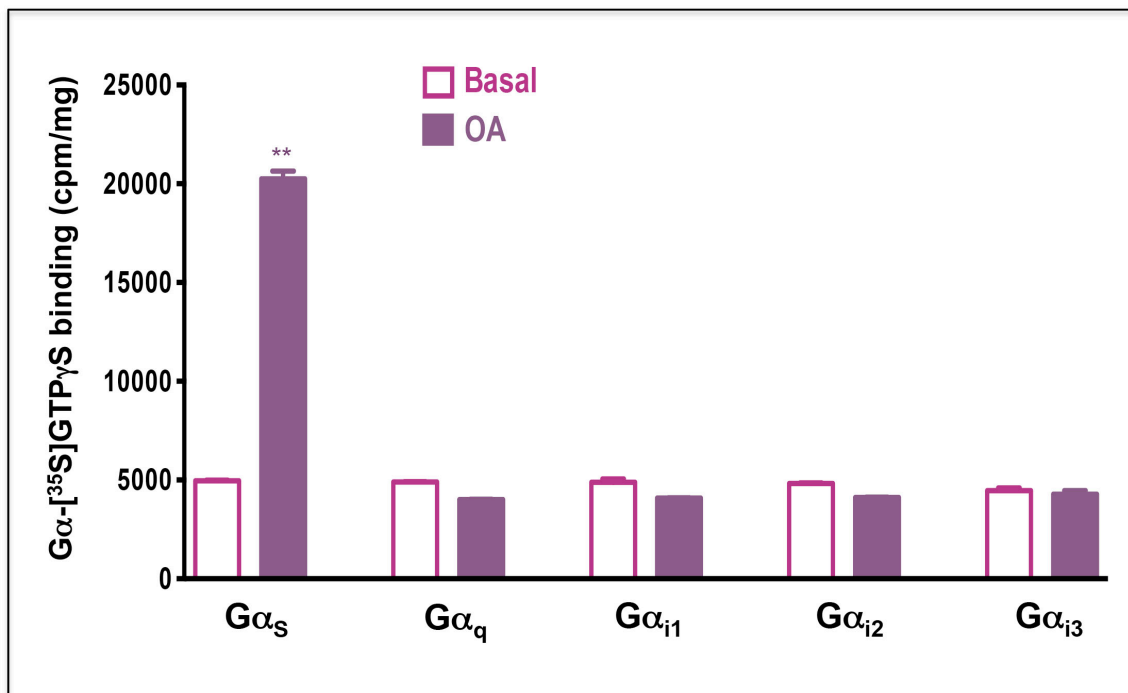
Figure 11. Selective activation of Gα$_s$ by OA in MIN6 cells. Membranes from MIN6 cells were treated with OA (10 µM) in the presence of [^{35}S]GTPγS for 30 min at 37^{0}C. Aliquots were added to the wells precoated with specific antibodies to Gα$_s$, Gα$_q$, Gα$_{i1}$, Gα$_{i2}$ or Gα$_{i3}$ and incubated for 2 h. The amount of bound radioactivity was measured by liquid scintillation and the results are expressed as counts per minute per milligram of protein. Incubation of MIN6 cell membranes with OA caused a significant increase in the binding of [^{35}S]GTPγS selectively to Gα$_s$ but not Gα$_q$, Gα$_{i1}$, Gα$_{i2}$ or Gα$_{i3}$. Values are expressed as mean ± SEM of 4 experiments. [**]$p<0.001$ vs. basal.
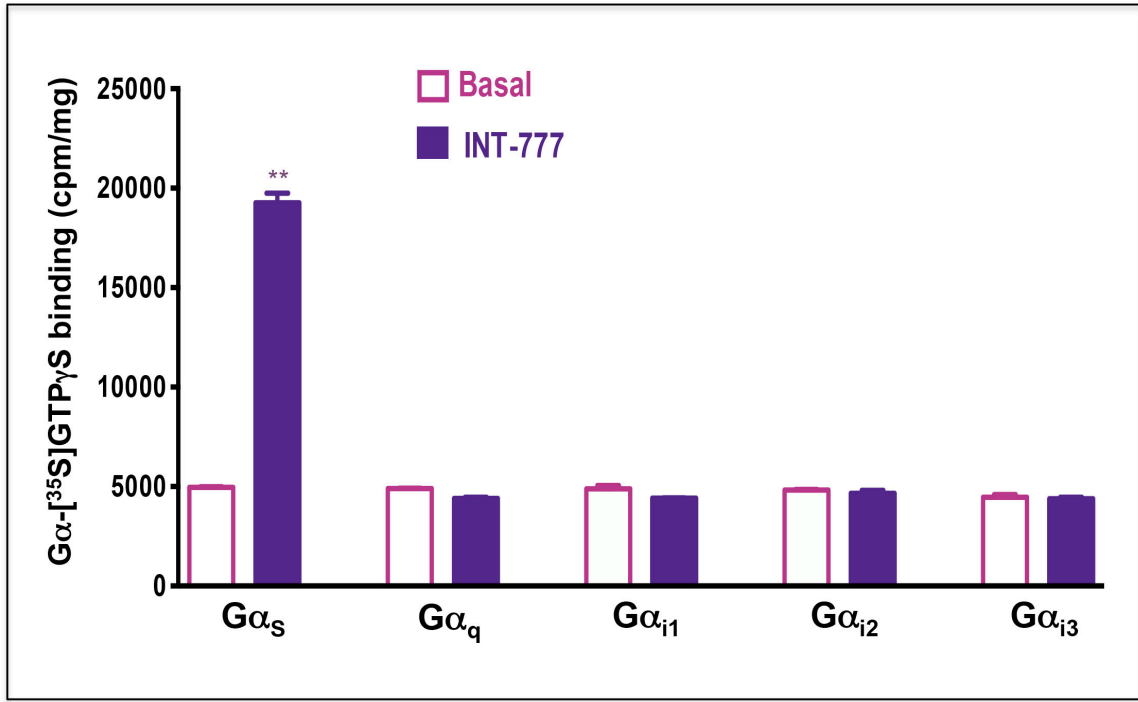
Figure 12. Selective activation of Gα$_s$ by INT-777 in MIN6 cells. Membranes from MIN6 cells were treated with INT-777 (25 µM) in the presence of [^{35}S]GTPγS for 30 min at 37^{0}C. Aliquots were added to the wells precoated with specific antibodies to Gα$_s$, Gα$_q$, Gα$_{i1}$, Gα$_{i2}$ or Gα$_{i3}$ and incubated for 2 h. The amount of bound radioactivity was measured by liquid scintillation and the results are expressed as counts per minute per milligram of protein. Selective activation of Gα$_s$ was demonstrated in response to INT-777 in MIN6 cells. Values are expressed as mean ± SEM of 4 experiments. [**]p<0.001 vs. basal.
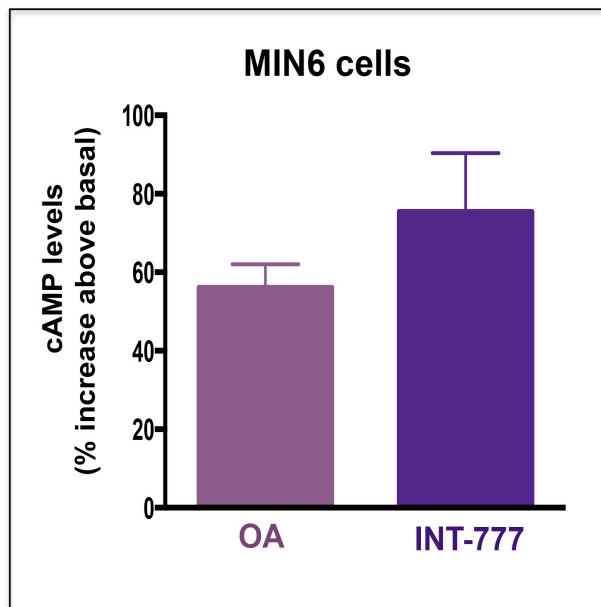
Figure 13. Activation of adenylyl cyclase by TGR5 ligands in MIN6 cells. Adenylyl cyclase activity was measured as cAMP formation in MIN6 cells. The cells were treated with TGR5 ligands (OA or INT-777, 25 µM) for 5 min in the presence of 100 µM isobutylmethylxanthine (IBMX) and cAMP formation was measured by radioimmunoassay. Results are computed from a standard curve using Prism[@] and expressed as percent increase in cAMP levels above basal levels (0.32 pmoles/ml). The TGR5 ligands, OA and INT-777 caused a significant (p<0.01) increase in cAMP formation. Values are expressed as mean ± SEM of 3 experiments.
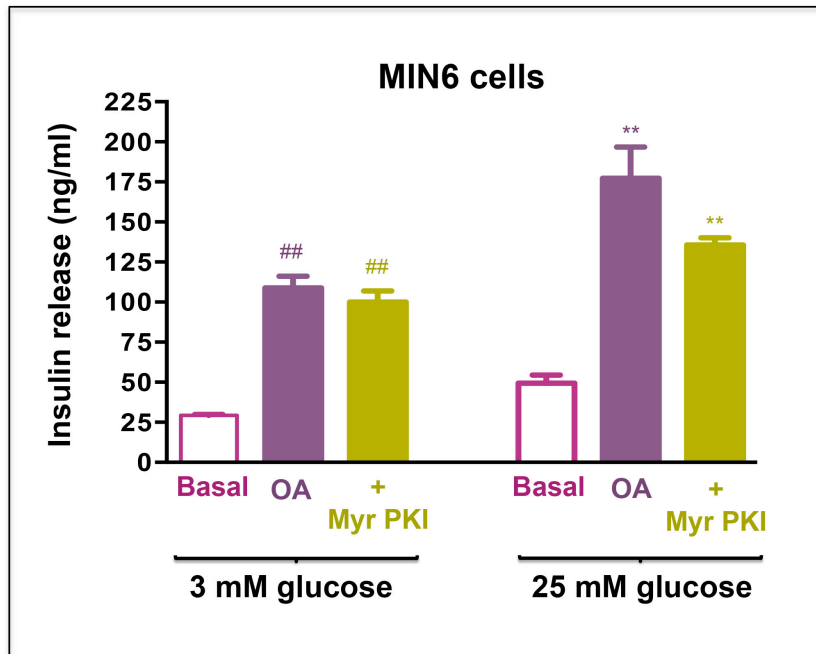
Figure 14. Effect of PKA inhibitor on OA-induced insulin secretion. MIN6 cells were treated with OA (50 μM) in the presence or absence of PKA inhibitor (myristoylated PKI, 1 μM). After 30 min, the supernatant was collected and insulin secretion was measured by ELISA. The TGR5 ligand, OA stimulated insulin secretion and the selective PKA inhibitor, Myr PKI had no significant inhibition on OA-induced insulin secretion. Values are expressed as mean ± SEM of 3 experiments. [##]p<0.001 vs. 3 mM glucose basal; **p<0.001 vs. 25 mM glucose basal.
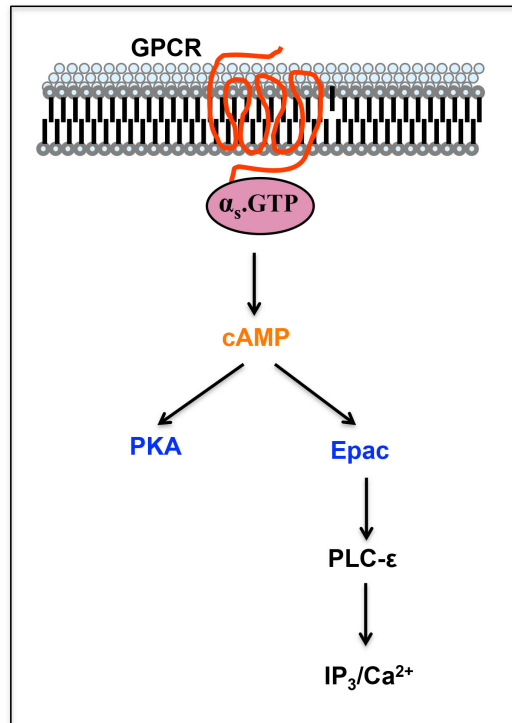
Figure 15. Schematic representation of cAMP-mediated signaling pathway. Activation of a Gα_s coupled receptors generates cAMP. The effect of cAMP is mediated by two different mechanisms: the classical cAMP-dependent protein kinase (PKA) pathway and non-canonical cAMP-dependent PKA-independent pathway involving activation of exchange factor Epac and stimulation of phosphoinositide-specific phospholipase C-ε (PLC-ε) leading to generation of IP_3 and IP_3-dependent Ca^{2+} release. This rise in the intracellular calcium triggers the release of insulin in pancreatic β cells.
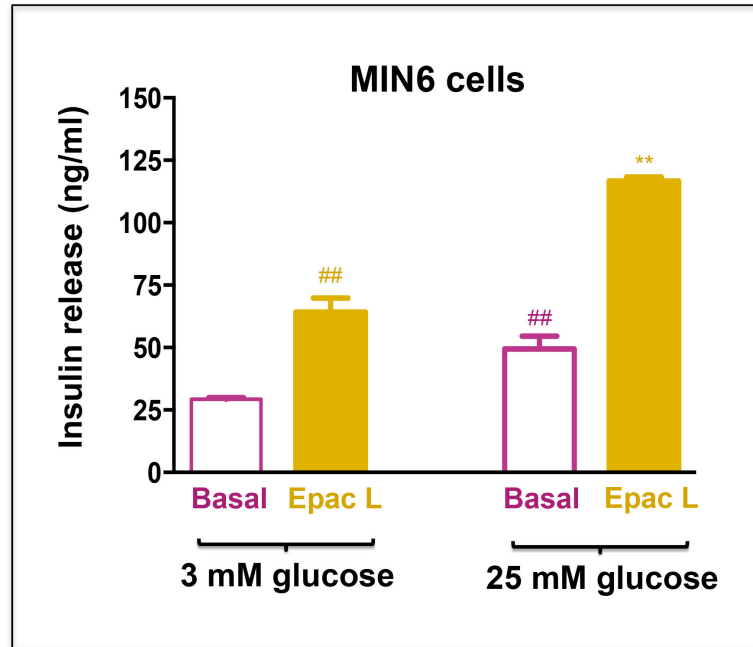
Figure 16. Stimulation of insulin secretion by the selective Epac ligand. MIN6 cells were treated with the Epac ligand, 8-pCPT-2'-O-Me-cAMP (10 μM). After 30 min, the supernatant was collected and insulin secretion was measured by ELISA. The Epac ligand stimulated insulin secretion at both basal and stimulated glucose conditions. Values are expressed as mean ± SEM of 3 experiments. [##]p<0.001 vs. 3 mM glucose basal; **p<0.001 vs. 25 mM glucose basal.
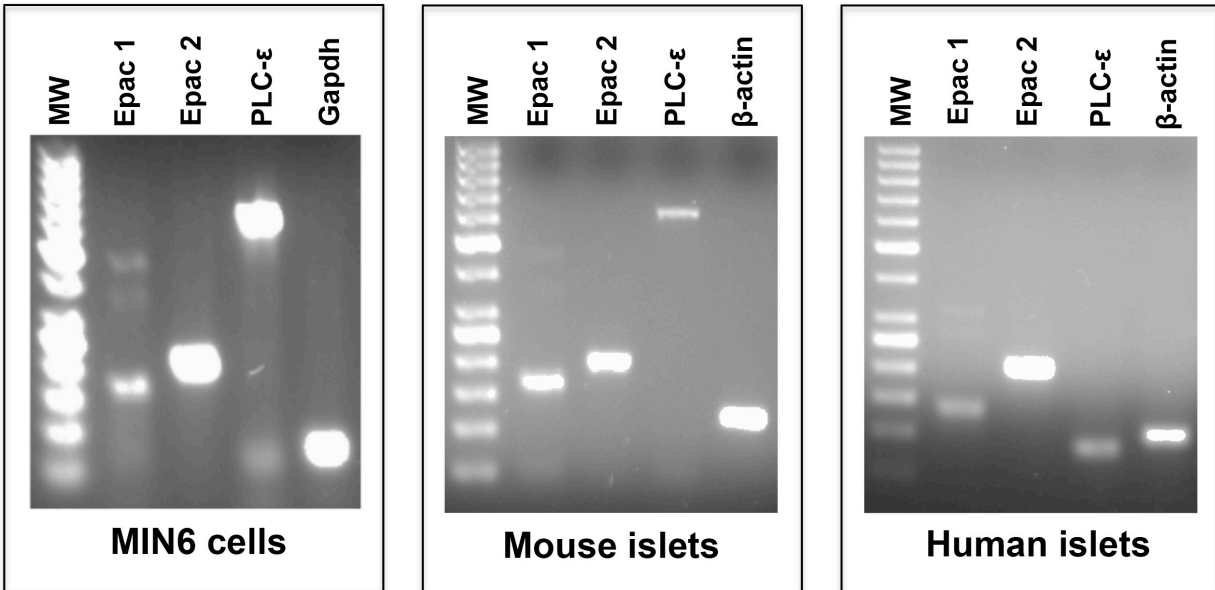
**Figure 17. Expression of Epac1, Epac2 or PLC-ε in MIN6 cells, mouse and human islets.** Total RNA was isolated from cultured MIN6 cells, mouse and human islets and reverse transcribed using 1 µg of total RNA. The cDNA was amplified using specific primers and examined for the expression of Epac1 (mouse- 159 bp, human- 130 bp), Epac2 (mouse- 195 bp, human- 203 bp) or PLC-ε (mouse- 606 bp, human- 82 bp) by RT-PCR. The results show representative PCR products analyzed on 2% agarose gel containing ethidium bromide using Gel Doc™ EZ imager. Gapdh or β-actin was used as a loading control.
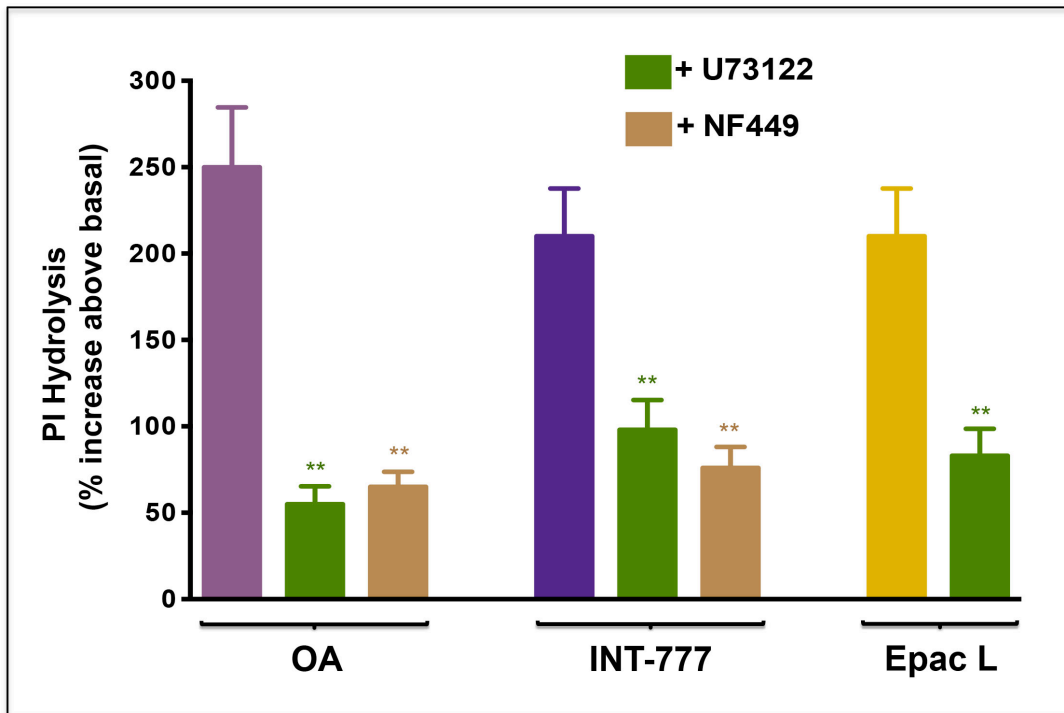
Figure 18. Activation of PI hydrolysis by TGR5 or Epac ligand in MIN6 cells.

MIN6 cells were labeled with myo-[^{3}H] inositol for 24 h and then treated with OA (10 µM), INT-777 (25 µM) or Epac ligand (8-pCPT-2'-O-Me-cAMP, 10 µM) with or without the selective Gα_s inhibitor (NF449, 10 µM) or PI hydrolysis inhibitor (U73122, 10 µM). PI hydrolysis was measured by ion exchange chromatography and the results are expressed as percent increase above basal levels. OA or INT-777 stimulated PI hydrolysis and the stimulation was blocked with the pretreatment of cells with the inhibitors of Gα_s (NF449) and phospholipase C (U73122). Stimulation of PI hydrolysis by Epac ligand was inhibited by U73122. Values are expressed as mean ± SEM of 3 experiments. **p<0.001 significant inhibition in PI hydrolysis compared to OA, INT-777 or Epac ligand.
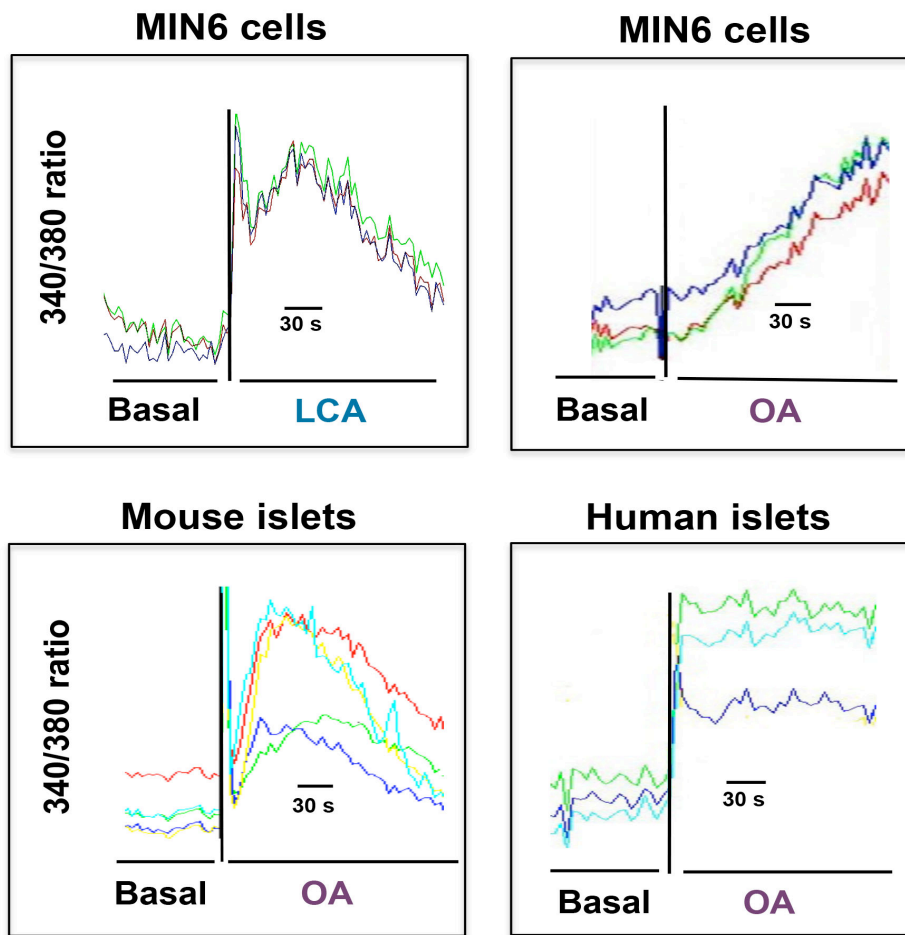
Figure 19. Release of intracellular calcium by TGR5 ligands. MIN6 cells, mouse islets and human islets were loaded with 5 µM fura-2AM in HBSS buffer for 2 h at room temperature. Clusters of cells were selected for imaging and visualization. The cells were alternatively excited at 340 nm and 380 nm and the change in cytosolic calcium in response to OA or LCA were measured by determining the ratio of the fluorescence of fura-2 at 340 and 380 nm excitation. Addition of OA or LCA to MIN6 cells resulted in a rapid increase in cytosolic Ca^{2+}. A similar increase in cytosolic Ca^{2+} in response to OA was observed in both mouse and human islets. Representative traces with each ligand are shown.
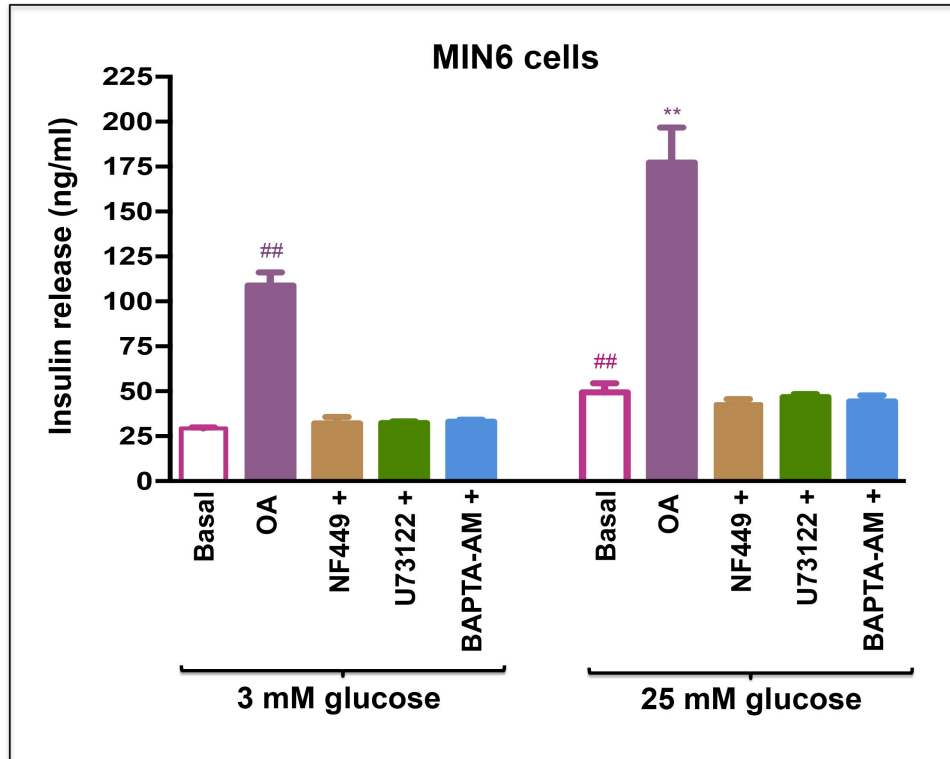
Figure 20. Effect of Gα$_s$ and PI hydrolysis inhibitor and calcium chelator on OA-induced insulin secretion. MIN6 cells were treated with OA (50 μM) in the presence or absence of selective Gα$_s$ inhibitor (NF449, 10 μM), PI hydrolysis inhibitor (U73122, 10 μM) or calcium chelator (BAPTA-AM, 10 μM). After 30 min, the supernatant was collected and insulin secretion was measured by ELISA. Basal and glucose-stimulated insulin secretion was significantly augmented by OA. The effect of OA was blocked by NF449, U73122 or BAPTA-AM. Values are expressed as mean ± SEM of 3 experiments. ##p<0.001 vs. 3 mM glucose basal; **p<0.001 vs. 25 mM glucose basal.
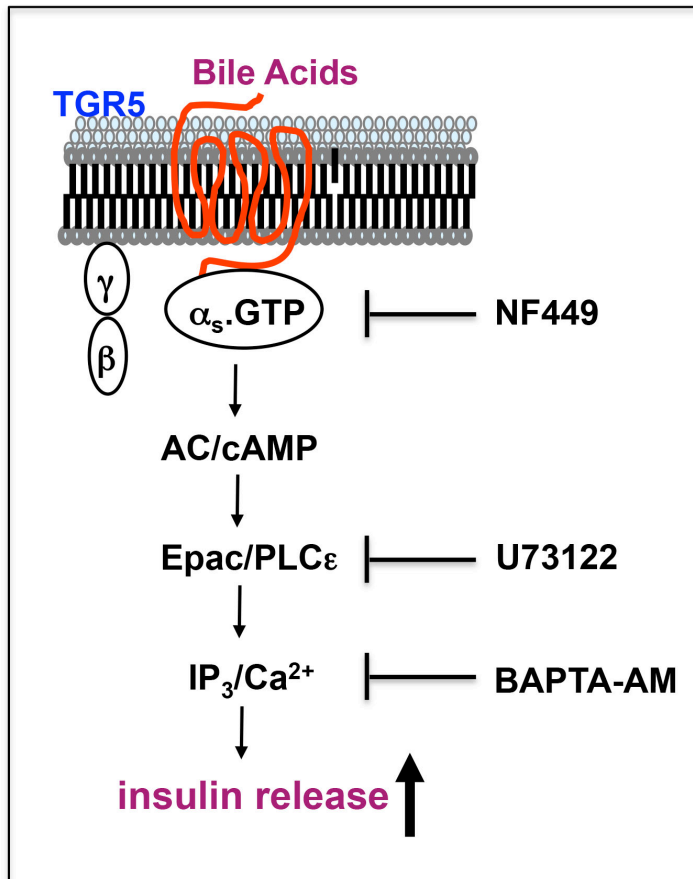
Figure 21. Schematic representation of the signaling pathway coupled to TGR5 receptors to mediate insulin secretion from pancreatic β cells. TGR5 receptors expressed in the pancreatic β cells are $G\alpha_s$ coupled and activation of these receptors by bile acids leads to the stimulation of PI hydrolysis via a cAMP-dependent mechanism involving Epac/PLC-ε/Ca^{2+} pathway and stimulation of insulin secretion. Inhibitors of Gs activation with NF449, PI hydrolysis with U73112 or chelation of intracellular Ca^{2+} with BAPTA-AM blocked the effect of TGR5 activation on insulin release.

CHAPTER 3

REGULATION OF GLUCAGON SECRETION IN PANCREATIC α CELLS BY

THE BILE ACID RECEPTOR TGR5

3.1. Introduction

We have previously shown that activation of TGR5, a novel G-protein coupled receptor by bile acids induce insulin secretion in pancreatic β cells [116]. Pancreatic islets, in addition to β cells also contain glucagon secreting α cells and somatostatin secreting δ cells along with other cell types [68,74]. The regulation of blood glucose homeostasis is maintained by the opposing actions of the dual hormonal system, which constitute insulin and glucagon [117]. Post prandial hyperglycemia (fed state) stimulates insulin secretion from pancreatic β cells, whereas hypoglycemia (fasted state) stimulates glucagon secretion from the pancreatic α cells [118].

Glucagon increases blood glucose levels, an effect that is opposite to that of insulin and is therefore known as insulin antagonist [119]. Glucagon is a peptide hormone consisting of 29 amino acids and is generated from the cleavage of proglucagon by the enzyme prohormone convertase 2 (PC2) in the pancreatic α cells [68,85]. Glucagon secretion is not only regulated by glucose concentration but is

complex and is also regulated by nutrients such as amino acids and fatty acids and by neural, hormonal and cellular interactions [68,120]. Under physiological state, in response to hypoglycemia or low glucose conditions, glucagon is secreted into the blood stream from the pancreatic α cells [118]. The secreted glucagon acts predominantly on the liver. Glucagon upon binding to its receptor (G protein coupled receptor) trigger downstream signaling pathway mostly via $G\alpha_s$ [119]. Glucagon signaling regulates the expression or activity of the enzymes involved in glucose metabolism [121]. The liver is a major site of glucagon's physiological actions and it is exposed to 2-3 times higher concentration of glucagon than other organs. In the liver glucagon promotes gluconeogenesis and glycogenolysis, and simultaneously inhibit glycolysis and glycogenesis to restore glucose homeostasis [118]. In the adipose tissue, glucagon facilitates lipolysis [117]. In addition, glucagon also stimulates insulin secretion from the pancreatic β cells and indirectly impact hepatic glucose output [118]. In summary, glucagon plays a pivotal role in maintaining fuel homeostasis along with insulin.

Hypoglycemic conditions trigger glucagon release from α cells by 3 different mechanisms: i) direct stimulatory effect of α islet cell by hypoglycemia; ii) suppression inhibitory effect of islet β cell; and iii) autonomic stimulation of α islet cell. Under hypoglycemic conditions, glucose is transported via GLUT1 and facilitates moderate activity of K_{ATP} channels in the pancreatic α cells. This event is followed by sequential activation of T type Ca^{2+} channels and voltage dependent Na^+ channels which further potentiates the opening of L type Ca^{2+} channels. The resultant firing of large amplitude

action potential leads to influx of Ca^{2+} and exocytosis of glucagon [120]. Whereas under hyperglycemic conditions, closure of K_{ATP} channels decreases α cell action potential and subsequent activation of voltage gated channels leading to inhibition of glucagon secretion [119]. This is opposite to the pattern observed in β cells in response to glucose and studies have shown that pancreatic β cells differ from α cells in being electrically active only at high glucose conditions [120].

Pancreatic α cells also express several G protein coupled receptors such as somatostatin receptors, GLP-1 receptors, fatty acid receptors, adrenergic receptors etc but unlike the overwhelming information available on GPCRs expressed on β cells, alpha cell GPCRs are not well studied [81,82]. In the present study upon examining the expression of TGR5 in αTC1-6 cells, *we postulated that activation of TGR5 at low glucose conditions stimulates glucagon secretion from* pancreatic α cells*.*

3.2. Materials and Methods

3.2.1. Materials

Oleanolic acid (OA) and lithocholic acid (LCA) were obtained from Sigma-Aldrich (St Louis, MO) and INT-777 (S-EMCA: 6α-Ethyl-23(S)-methylcholic Acid) was a generous gift from Intercept Pharmaceuticals. Antibody to TGR5 was purchased from Abcam (Cambridge, MA); Glucagon ELISA kit was obtained from R & D systems (Minneapolis, MN). Collagenase P was obtained from Roche Diagnostics (Indianapolis, IN); HEPES and RPMI were obtained from Invitrogen (Eugene, OR); western blotting and chromatography materials were obtained from Bio-Rad Laboratories (Hercules,

CA). RNeasy Plus universal Mini kit was obtained from Qiagen (Venlo, Netherland); PCR reagents were obtained from Applied Biosystems (Foster city, CA); Dulbecco's Modified Eagle Medium (DMEM), 2-mercaptoethanol, Histopaque (10771 and 11191) and all other reagents were obtained from Sigma (St Louis, MO).

Wild type C57BL/6J mice were purchased from Jackson laboratories (Ban Harbor, ME) and euthanized with CO_2. The animals were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University. All procedures were conducted in accordance with the institutional Animal Care and Use Committee of the Virginia Commonwealth University.

3.2.2. Cell culture

The insulin secreting pancreatic beta (β) cell line, MIN6 cells were cultured in DMEM containing L-glutamine, sodium carbonate, 2.5 mM 2-mercaptoethanol; glucagon secreting pancreatic alpha (α) cell line, αTC1-6 cells (obtained from ATCC) were cultured in DMEM containing HEPES, non-essential amino acids (NEAA), bovine serum albumin (BSA), sodium carbonate and somatostatin secreting pancreatic delta (δ) cell line, TGP52 cells (obtained from ATCC) were cultured in DMEM: F12 medium. All the media were supplemented with 10% fetal bovine serum and 100U/ml pencillin-streptomycin and the cells were incubated at 37°C in 5% CO_2.

3.2.3. Isolation and maintenance of mouse islets

Pancreatic islets from mice were isolated by sequential enzymatic digestion of pancreas, filtration and centrifugation as described previously [Chapter 2]. The isolated

mouse islets were cultured in RPMI-1640 medium supplemented with 10% FBS and 100U/ml pencillin-streptomycin and incubated at 37°C in 5% CO_2.

3.2.4. RNA Isolation and RT-PCR analysis

Total RNA was isolated from cells (αTC1-6, MIN6 and TGP52) and mouse islets using RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. The purified RNA was reverse transcribed to single stranded cDNA and conventional PCR was carried out as described previously [Chapter 2]. The amplified PCR products were analyzed on 2% agarose gel containing ethidium bromide using Gel Doc^{TM} EZ imager.

3.2.5. Western blot analysis

Equivalent amounts of protein were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked in 5% nonfat dry milk for 1 h followed by immunobloting with anti-TGR5 antibody and anti-rabbit IgG horseradish peroxidase secondary antibody as described previously [chapter 2]. Blots were visualized using advanced ECL western blotting detection reagents.

3.2.6. Measurement of Glucagon secretion

αTC1-6 cells were plated at a density of 5×10^5 cells in 24-well plates with DMEM medium and cultured until confluent. The cells were washed and incubated in HBSS (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, pH 7.2, 0.1% BSA) containing 16.7 mM glucose for 2 h

at 37^{0}C. Cells were then treated for 1 hour in HBSS containing 3 mM glucose with or without OA, INT-777 or LCA. The supernatants were collected and assayed for glucagon using ELISA kit. For glucagon secretion from mouse islets (25-30 islets/condition), the islets were incubated at 37^{0}C for 2 h in HBSS with 16.7 mM glucose. The same procedure as described above was followed.

3.2.7. Statistical Analysis

Results were calculated as means ± SEM and the experiments were performed at least three times. The experiments were performed on islets isolated from different animals. Statistical significance was analyzed using Student's t-test for paired and unpaired values. GraphPad Prism software was used for all statistical analyses and p values < 0.05 were considered significant.

3.3. Results

3.3.1. Expression of TGR5 in pancreatic α, β and δ cell lines and mouse islets

To study the role of TGR5 in the regulation of glucagon secretion from pancreatic alpha (α) cells, we examined the expression of TGR5 receptors by RT-PCR and western blot. RT-PCR analysis using RNA isolated from αTC1-6 cells, a PCR product of the expected size (104 bp) was obtained with TGR5 specific primers (**Figure 22A**).

We have previously shown that TGR5 is expressed in pancreatic β cell line, MIN6 cells and mouse and human islets [116]. Since islets represent a mixture of cells consisting predominantly β, α and δ cells, we also examined whether TGR5 is expressed in the pancreatic α cells (αTC1-6) and δ cells (TGP52). Western blot analysis

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using specific antibody to TGR5 demonstrated the presence of TGR5 protein (35 kDa) in the lysates derived from αTC1-6, MIN6, TGP52 cells and mouse islets (**Figure 22B**).

3.3.2. Stimulation of glucagon secretion by TGR5 ligands in αTC1-6 cells

To address the functional significance of TGR5 receptors in α cells, the effect of TGR5 ligands (OA, INT-777 or LCA) on glucagon secretion was examined. Basal levels of glucagon released under low (3 mM) conditions were 4665±5 pg/ml. Treatment of αTC1-6 cells for 1 hour at low glucose conditions with OA, INT-777 or LCA significantly stimulated glucagon secretion above basal levels (1.6±0.01 fold increase with INT-777, 1.7±0.02 fold increase with OA and 1.6±0.11 fold increase with LCA) (**Figure 23**).

The stimulatory effect of INT-777 on glucagon secretion is concentration-dependent (10 μM- 1.2±0.04, 25 μM- 1.45±0.07, 50 μM- 1.51±0.07 fold increase above 3 mM glucose basal levels, 4283±13 pg/ml). Similarly, the effect of LCA was also concentration-dependent (10 μM- 1.09±0.03, 25 μM- 1.4±0.02, 50 μM- 1.44±0.07 fold increase above 3 mM glucose basal levels) (**Figures 24A and 24B**).

3.3.3. Stimulation of glucagon secretion by TGR5 ligands in mouse islets

We also examined the effect of TGR5 ligands on glucagon secretion from mouse pancreatic islets. Basal levels of glucagon release from islets under low (3 mM) conditions were 37.8± 1.2 pg/islet. Treatment of islets with 25 μM INT-777 or OA significantly increased glucagon secretion (INT-777, 1.7±0.03 fold increase and OA, 1.5±0.08 fold increase above basal levels). Similarly, treatment of islets with 25 μM natural bile acid, LCA significantly increased glucagon secretion (1.4±0.01 fold increase

above basal level) (**Figure 25**). The extent of increase in glucose secretion with all three TGR5 ligands was similar.

3.4. Discussion

Secretion of glucagon, the counter regulatory hormone of insulin, is stimulated in response to hypoglycemia and plays a vital role in restoring normoglycemia [117]. Previously it has been shown that low glucose concentrations, amino acids (especially alanine or arginine), cholecystokinin, epinephrine or acetylcholine stimulate glucagon secretion from pancreatic α cells [119,120]. In the present study, it is demonstrated that pancreatic α cells also express TGR5 receptors, which upon activation with pharmacological ligands (OA or INT-777) and natural ligand (LCA) stimulate glucagon secretion providing evidence for an additional role of bile acids in glucose homeostasis.

Bile acids may also regulate glucagon secretion via release of GLP-1 from enteroendocrine cells. GLP-1 in addition to stimulating insulin secretion from the pancreatic β cells also suppresses glucagon secretion from α cells [67,88]. The current study demonstrates that activation of TGR5 by bile acids also promotes glucagon secretion under low conditions. We have demonstrated the glucagon secretion in response to TGR5 activation in both mouse pancreatic islets and in αTC1-6 cells, a mouse pancreatic α cell line. Hypoglycemic conditions trigger glucagon release from α cells both by direct and indirect mechanisms involving paracrine regulation via β and δ cells secretion. αTC1-6 cells are homogenous cell population that do not express insulin or somatostatin and is therefore considered a good model to examine glucagon

secretion or alpha cell gene expression [122]. The extent of stimulation of glucagon secretion appears to be similar in both αTC1-6 cells and mouse islets. These results suggest that, at least under low glucose conditions, secretion is mostly under direct effect of hypoglycemia.

Previous studies have shown that αTC1-6 cells secrete glucagon in response to nutrients and activation of G_s-coupled receptors [119]. We found that activation of TGR5 by INT-777 or LCA stimulated glucagon secretion in a dose dependent manner under low (3 mM) glucose conditions in αTC1-6 cells. Our data is similar to the previous studies, which describes the stimulatory effect of amino acids or free fatty acids to promote glucagon secretion under low glucose conditions [123,124]. Similarly we have shown that the stimulatory effect of low glucose is further amplified by the TGR5 ligands to release glucagon in mouse islets.

The insulin producing β cells and glucagon producing α cells are featured with unique set of ion channels and the two cell types are well coordinated within the pancreatic islet to fine tune the secretion of hormone and thus regulate glucose homeostasis [118,120]. Glucagon secretion is inhibited by hyperglycemia, in contrast to increase in insulin secretion. The threshold for suppression of glucagon release is lower than β cells suggesting that α cells are more sensitive to glucose than β cells [119]. In contrast to opposing effect of glucose on glucagon and insulin secretion, amino acids stimulate release of both glucagon and insulin [120]. Free fatty acids also affect glucagon secretion. Saturated fatty acids are more effective in stimulating glucagon secretion than unsaturated fatty acids [125]. The mechanisms involved in TGR5-

mediated glucagon secretion from pancreatic α cells remains to be elucidated. It is possible that TGR5 utilizes the same Gs/cAMP/Epac/PLC-ε/Ca^{2+} pathway to mediate glucagon secretion as this pathway was shown to be involved in insulin secretion from islet β cells and GLP-1 secretion from enteroendocrine cells in response to activation of TGR5 [109]. The physiological significance of TGR5 in α cells is also unclear. Levels of systemic bile acids in hypoglycemic (fast state) conditions are 3 times lower than the post prandial state and increased glucose levels under post prandial state suppress glucagon release from α cells. Previous studies have shown that other neurohumoral agents (e.g., acetylcholine, sphingosine-1-phosphate) also interact with TGR5 receptors in different cell types. The significance of the possibility of TGR5 activation by ligands other than bile acids under hypoglycemic conditions remains to be determined.

In conclusion, the current study provide evidence to the regulation of pancreatic α cell function by bile acids via activation of TGR5 in addition to the regulation of insulin secretion from β cells [116]. With a better understanding of the intra islet regulation of insulin secretion, new therapeutic strategies for the treatment of diabetes can be targeted not only on the approaches that suppress liver glucose production but also on enhancing intraislet glucagon action. Unlike antidiabetic drugs like sulphonylurea derivatives, which pose the risk of hypoglycemia [87,91], the use of TGR5 ligands may serve a better therapeutic approach in the management of T2D and awaits further elucidation.
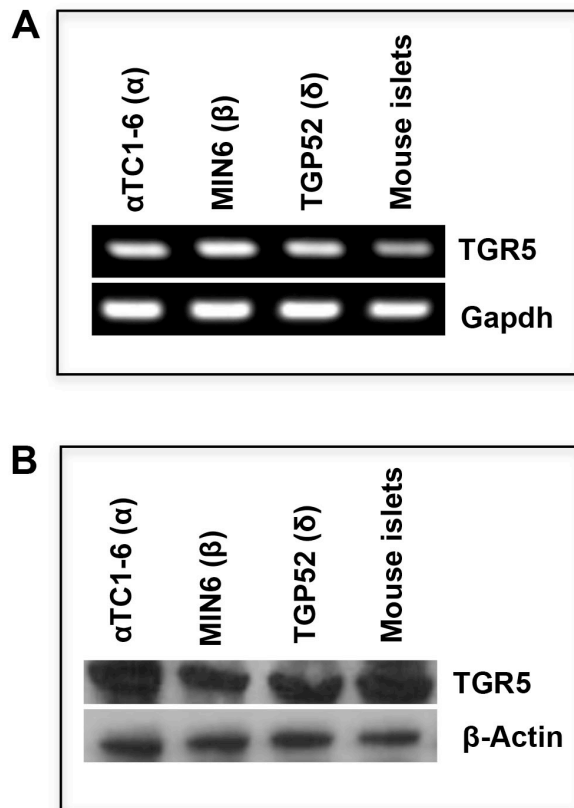
Figure 22. Expression of TGR5 in pancreatic α, β and δ cell lines and mouse islets. A: mRNA expression- Total RNA isolated from αTC1-6 (α cell line), MIN6 (β cell line), TGP52 (δ cell line) cells and mouse islets was reverse transcribed and expression of TGR5 mRNA was examined by RT-PCR using specific primers. The results show representative PCR products (TGR5- 104 bp, Gapdh- 122 bp) analyzed on 2% agarose gel containing ethidium bromide using Gel Doc^{TM} EZ imager. **B: Protein expression-** Cell lysates containing equal amounts of total proteins from αTC1-6, MIN6, and TGP52 cells and mouse islets were separated on SDS-PAGE and the expression of TGR5 was analyzed using TGR5 selective antibody. A band corresponding to 35 kDa was detected by chemilumiscence. The membrane was reprobed for β-actin (42 kDa), an endogenous loading control.
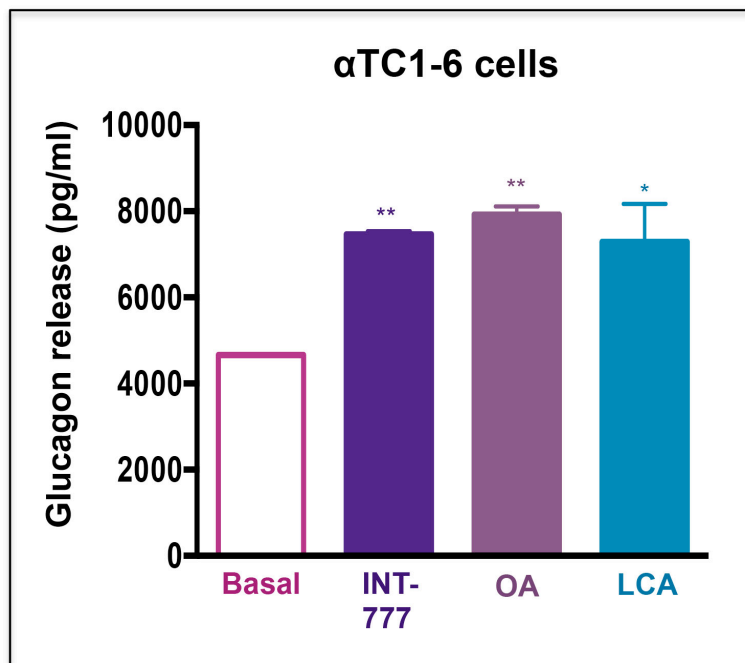
Figure 23. Stimulation of glucagon secretion by TGR5 ligands in αTC1-6 cells. αTC1-6 cells were plated at a density of 5 x 10^5 cells in 24-well plates and cultured with DMEM until confluent. The cells were washed and incubated in HBSS buffer containing 16.7 mM glucose for 2 h at 37^0C. Cells were then treated for 1 hour with TGR5 ligands (OA, INT-777 or LCA- 25 µM) in HBSS containing 3 mM glucose. The supernatants were collected and glucagon secretion was measured by ELISA. The TGR5 ligands increased glucagon secretion in αTC1-6 cells. Values are expressed as mean ± SEM of 4 experiments. [**]$p < 0.001$ or [*]$p < 0.05$ vs. 3 mM glucose basal.
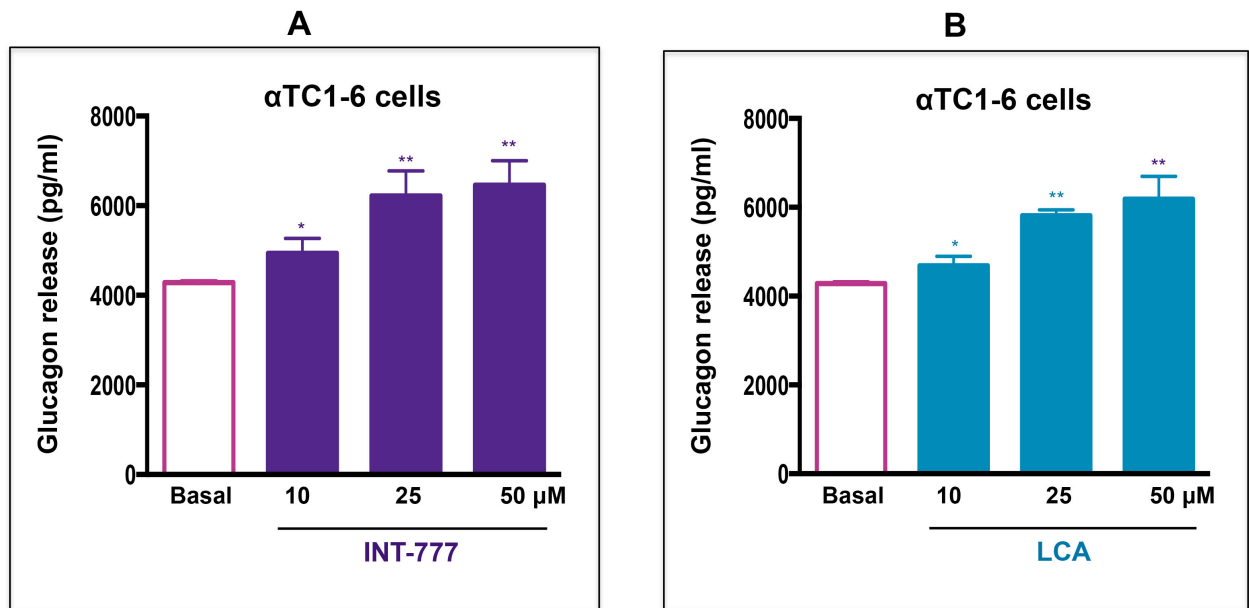
Figure 24. Stimulation of glucagon secretion by INT-777 or LCA in αTC1-6 cells. αTC1-6 cells were plated at a density of 5 x 10^5 cells in 24-well plates and cultured with DMEM until confluent. The cells were washed and incubated in HBSS buffer containing 16.7 mM glucose for 2 h at 37^0C. Cells were then treated for 1 hour with different concentrations of INT-777 (10, 25 or 50 μM) or LCA (10, 25 or 50 μM) in HBSS containing 3 mM glucose. The supernatants were collected and glucagon secretion was measured by ELISA. INT-777 **(A)** or LCA **(B)** caused an increase in the glucagon secretion in a dose-dependent manner. Values are expressed as mean ± SEM of 4 experiments. $^{**}p<0.001$ or $^*p<0.05$ vs. 3 mM glucose basal.
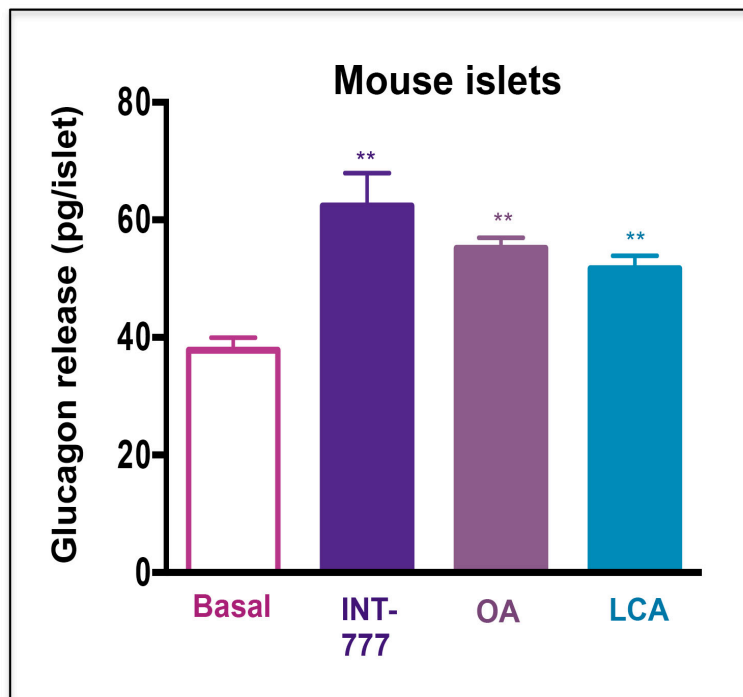
Figure 25. Stimulation of glucagon secretion by TGR5 ligands in mouse pancreatic islets. Mouse islets were washed and incubated in HBSS buffer containing 16.7 mM glucose for 2 h at 37^0C. The islets (25-30 islets/condition) were then treated with INT-777 (25 µM) or OA (25 µM) or LCA (25 µM) in the presence of 3 mM glucose for 1 hour. The supernatants were collected and glucagon secretion was measured by ELISA. INT-777 (specific TGR5 ligand), oleanolic acid (selective TGR5 ligand) and lithocholic acid (natural bile acid) significantly increased glucagon secretion in mouse islets. Values are expressed as mean ± SEM of 3 experiments. [**]p<0.001 vs. 3 mM glucose basal.

CHAPTER 4

REGULATION OF HYPERGLYCEMIA-INDUCED PC1 EXPRESSION AND

GLP-1 SECRETION IN PANCREATIC α CELLS BY TGR5

4.1. Introduction

Bile acids via activation of TGR5 regulate glucose homeostasis by improving postprandial glycemic levels and insulin sensitivity [38,40,63]. Bile acids that are released in response to a meal stimulate GLP-1 secretion from the intestinal enteroendocrine cells via activation of TGR5 receptors [65,109,110]. GLP-1 is an incretin hormone that stimulates insulin secretion from the pancreatic β cells in a glucose dependent manner [67,88]. In addition, GLP-1 exerts a variety of physiological effects and plays an important role in integrating nutrient and energy metabolism [86,87].

GLP-1 is a 30 amino acid peptide hormone that is processed from proglucagon by the enzyme prohormone convertase 1 (PC1) in the enteroendocrine L cells [85,89]. GLP-1 secretion is highly regulated by nutrients such as fats, carbohydrates and proteins as well as via neural signals, endocrine and paracrine factors [83-85]. The fasting circulatory levels of GLP-1 are 5-15 pM and increases up to 20-30 pM after ingestion of meal [88]. Following its secretion into the blood, GLP-1 is rapidly degraded

by the enzyme dipeptidyl peptidase- IV (DPP-IV), and the half life of GLP-1 is less than 5 minutes [89,91].

GLP-1 exerts its action via binding to its receptor which is a seven transmembrane G protein coupled receptor expressed on pancreatic islets as well as in other tissues including brain, gastrointestinal tract, lung and kidneys [85]. In pancreatic islet, GLP-1 stimulates insulin secretion from the β cells in a glucose dependent manner [87]. GLP-1 also increases insulin synthesis and promotes β cell proliferation and inhibits glucagon secretion from α cells [67,86]. In addition, GLP-1 inhibits gastric emptying and food intake and improves insulin sensitivity [85,86].

We have recently shown that bile acids activate TGR5 in pancreatic β cells to stimulate insulin secretion in [116]. Insulin secretion is a highly dynamic process and in addition to nutrients and neural inputs it is also regulated by hormonal factors such as glucagon secreted from the pancreatic α cells and glucagon-like peptide 1 (GLP-1) by the intestinal L cells [68,91,95]. Both glucagon and GLP-1 are processed from the same precursor, proglucagon in a cell type specific manner. In the pancreatic α cells, proglucagon undergoes alternative splicing (post translational modification) by the enzyme prohormone convertase 2 (PC2) to yield glucagon whereas proglucagon is processed by PC1 to GLP-1 in the enteroendocrine L cells [126,127]. This tissue specific processing is due to differential expression of the enzymes PC1 and PC2 [126-128].

We have also shown that TGR5 receptors are expressed on pancreatic α cells and activation of these receptors by bile acids at low glucose conditions stimulates

glucagon secretion from αTC1-6 cells and mouse islets (Chapter 2). Previous studies have shown that under hyperglycemic conditions, there is activation of PC1 in the pancreatic α cells leading to GLP-1 production and this was evident in different mouse models of diabetes such as STZ-induced diabetic mice, prediabetic NOD mice, db/db mice and ob/ob mice [129,130]. These studies suggested that hyperglycemia causes a switch in the processing of proglucagon by up regulating PC1 expression leading to GLP-1 secretion from the pancreatic α cells [131-133]. Recent studies have shown that circulating bile acid concentration are higher in obese patients with type 2 diabetes when compared to lean healthy individuals and increased systemic bile acid concentrations are associated with the pathophysiology of obesity and type 2 diabetes [134,135]. *We therefore postulated that under hyperglycemia, activation of TGR5 by bile acids in the pancreatic α cells could further upregulate PC1 expression with subsequent GLP-1 production and thereby also regulate insulin secretion.*

4.2. Materials and Methods

4.2.1. Materials

Oleanolic acid (OA) and lithocholic acid (LCA) were obtained from Sigma-Aldrich (St Louis, MO) and INT-777 (S-EMCA: 6α-Ethyl-23(S)-methylcholic Acid) was a generous gift from Intercept Pharmaceuticals. PC1 antibody and PC1 plasmid was a generous gift from Dr. Lakshmi Devi (Icahn School of Medicine, Mount Sinai, NY) and Dr. Theodore Friedman (Charles R. Drew University of Medicine and Science, Los Angeles, CA) respectively. NF449 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); antibodies to PC2, p-CREB and CREB were obtained from Cell

Signaling Technology (Danvers, MA); ultrasensitive human insulin ELISA kit was purchased from Mercodia (Uppsala, Sweden); High sensitivity GLP-1 ELISA kit was obtained from Millipore (Billerica, MA). Collagenase P was obtained from Roche Diagnostics (Indianapolis, IN); HEPES, lipofectamine 2000 and RPMI were obtained from Invitrogen (Eugene, OR); U73122, and Myristolyated PKI were obtained from Calbiochem (La Jolla, CA); western blotting and chromatography materials were obtained from Bio-Rad Laboratories (Hercules, CA). RNeasy Plus universal Mini kit and Taq PCR core kit were obtained from Qiagen (Venlo, Netherland); PCR reagents were obtained from Applied Biosystems (Foster city, CA); ultrasensitive human insulin ELISA kit was purchased from Mercodia (Uppsala, Sweden); BrightGlo lucifearase assay kit and pGL2 basic vector were obtained from Promega (Madison, WI). Dulbecco's Modified Eagle Medium (DMEM), 2-mercaptoethanol, 8-pCPT-2'-O-Me-cAMP, BAPTA-AM, Exendin (9-39), Histopaque (10771 and 11191) and all other reagents were obtained from Sigma (St Louis, MO).

Wild type C57BL/6J mice, db/db mice (homozygous for leptin receptor) and control mice (C57BLKS/J) were purchased from Jackson laboratories (Ban Harbor, ME) and euthanized with CO_2. The animals were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University. All procedures were conducted in accordance with the institutional Animal Care and Use Committee of the Virginia Commonwealth University.

4.2.2. Cell culture

αTC1-6, MIN6 and TGP52 cells were cultured in their respective media as described previously [Chapter 3]. The enteroendocrine cells, STC-1 (obtained from ATCC) were cultured in DMEM containing L-glutamine, sodium pyruvate **(Figure 26)**. All the media were supplemented with 10% fetal bovine serum and 100U/ml pencillin-streptomycin and the cells were incubated at 37°C in 5% CO_2.

4.2.3. Isolation and maintenance of mouse islets

Pancreatic islets from mice were isolated by sequential enzymatic digestion of pancreas, filtration and centrifugation as described previously [Chapter 2]. The isolated mouse islets were cultured in RPMI-1640 medium supplemented with 10% FBS and 100U/ml pencillin-streptomycin and incubated at 37°C in 5% CO_2.

4.2.4. RNA Isolation and RT-PCR analysis

Total RNA was isolated from cells (αTC1-6, MIN6 and TGP52) and mouse islets using RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. The purified RNA was reverse transcribed to single stranded cDNA and conventional PCR was carried out as described previously [Chapter 2]. The amplified PCR products were analyzed on 2% agarose gel containing ethidium bromide using Gel Doc^{TM} EZ imager. Real time PCR was carried out using StepOne^{TM} Real-Time PCR system (Applied Biosystems) and quantification of gene expression was done using relative quantification method as described previously [Chapter 2].

4.2.5. Western blot analysis

Equivalent amounts of protein were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked in 5% nonfat dry milk for 1 h followed by immunobloting with respective primary antibody and anti-rabbit IgG horseradish peroxidase secondary antibody as described previously [chapter 2]. Blots were visualized using advanced ECL western blotting detection reagents.

4.2.6. Assay for phosphoinoside (PI) hydrolysis

αTC1- 6 cells cultured in 25 mM glucose for 6-7 days were labeled with myo-[3H] inositol (0.5 μCi/ml) in DMEM medium for 24 h. After 24 h, cells were washed with PBS and treated with INT-777, NF449 (a selective $G\alpha_s$ inhibitor), 8-pCPT-2'-O-Me-cAMP (a selective Epac ligand), U73122 (PI hydrolysis inhibitor) or ESI-05 (Epac2 inhibitor) for 1 min. The reaction was terminated, samples were extracted and ion-exchange chromatography was carried out as described previously [chapter 2].

4.2.7. Transfection of PC1 plasmid into αTC1- 6 cells

αTC1- 6 cells were plated at a density of 5×10^5 cells in 96-well plates in DMEM and cultured until confluent. One day before the transfection, the media was changed to antibiotic free medium. The cells were then transiently transfected with 0.2 μg PC1 plasmid (human PC1 luciferase promoter construct, -971 to -1 bp relative to the translation initiation codon inserted in the pGL2-basic vector) using lipofectamine 2000 for 24 hours. Cells were also transfected with plasmid containing red fluorescence (pSIREN-DNR-DsRED) and the transfection efficiency was monitored by the expression

of the red fluorescent protein using FITC filters. Control cells were transfected with vector (pGL2-basic vector) alone. All transfections were done in quadruplets.

4.2.8. PC1 promoter activity assay

αTC1- 6 cells cultured in 96-well plate was transfected using lipofectamine 2000 as previously described. After 24 hours of transfection, cells were treated with 5 mM glucose or 25 mM glucose with or without INT-777 in the presence of specific inhibitors for 24 hours. Luciferase activity was measured in a luminometer using BrightGlo assay kit (Promega).

4.2.9. Measurement of GLP-1 secretion

αTC1-6 cells were plated at a density of 2 x 10^5 cells in 24-well plates with DMEM containing 5 mM glucose or 25 mM glucose and cultured for 6-7 days. The cells were then washed and incubated in DMEM containing 5 mM or 25 mM glucose with or without OA, INT-777 or LCA for 24 hours. The supernatants were collected and assayed for GLP-1 using ELISA kit. For GLP-1 secretion from control mouse islets, the islets were cultured in RPMI-1640 medium containing 5 mM glucose or 25 mM glucose for 6-7 days and then treated with or without OA, INT-777 or LCA for 24 hours. The islets isolated from db/db mice were cultured in RPMI-1640 medium with or without OA, INT-777 or LCA for 24 hours. The supernatant were collected and assayed for GLP-1 secretion. 25-30 mouse islets were used in each experimental condition. DPP-4 inhibitor was added to prevent GLP-1 degradation at a concentration of 10 μl/ml of the medium.

4.2.10. Measurement of Insulin secretion

Human islets were cultured under low (5 mM) or high (25 mM) glucose conditions for 7 days. The islets were washed and incubated in HBSS (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, pH 7.2, 0.1% BSA) containing 3 mM glucose for 2 h at 37^0C. Human islets (30-35 islets/condition) were then treated for 1 hour in HBSS containing 3 mM (basal) or 25 mM glucose (stimulated) with INT-777 or exendin (9-39) or both. The supernatants were collected and assayed for insulin using ELISA kit.

4.2.11. Statistical Analysis

Results were calculated as means ± SEM and the experiments were performed at least three times. The experiments were performed on islets isolated from different animals. Statistical significance was analyzed using Student's t-test for paired and unpaired values. GraphPad Prism software was used for all statistical analyses and p values < 0.05 were considered significant.

Table 2. RT-PCR primer sequences. Primers used for the amplification of different genes. TGR5- Taketa G-Protein coupled receptor 5, PC1- prohormone convertase 1, PC2- prohormone convertase 2, Epac- exchange proteins activated by cAMP, PLC-ε- phospholipase C-epsilon, Gapdh- glyceraldehyde 3 phosphate dehydrogenase.

Primer set	Forward 5'-3'	Reverse 3'-5'	Size (bp)
Mouse TGR5	GAGCGTCGCCCACCACTAGG	CGCTGATCACCCAGCCCCATG	104
Mouse Gapdh	AGAAACCTGCCAAGTATGATG	AGAAACCTGCCAAGTATGATG	122
Human TGR5	CTGGCCCTGGCAAGCCTCAT	CTGCCATGTAGCGCTCCCCGT	277
Human Gapdh	AGGTGAAGGTCGGAGTCAACGG	TTCCCGTTCTCAGCCTTGACGGT	190
Mouse PC1	TCTGGTTGTCTGGACCTCTGAGT	CAAGCCTGCCCCATTCTTT	73
Mouse PC2	CACCTCCAAGCGGAACCA	GCCAAAGAGGTGATTAAATTCA	85
Mouse Epac1	GAGACTGGAAGAACACGGCA	TCCGGTCTCATAGCCTCCAA	159
Mouse Epac2	GTCTCACGCCAAAGGAGGAA	TCCCGAAGAACAATGGAGGC	195
Mouse PLC-ε	TGGGAATGCCAACCCGCCAC	CCCCTGCGGCACAGACGTTT	606
Human PC1	CACACATGGGGAGAGAACCC	GAGAAGAGGTCCCGTGCAAA	121
Human Epac1	CTGGGACTTGGGGTCCATTC	GGAACCGGTAGAATTGGGCA	130
Human Epac2	TGATTGCTGCCCTCAAGGAG	TGTGTAGGTGTGGTCCATGC	203
Human PLC-ε	CGGAAACAGTACGTCAGCCT	CACCAAACAACTCCACAGCG	82
Human β-actin	AAGATCATTGCTCCTCCTGAGC	TCCTGCTTGCTGATCCACATC	101

Table 3. Primary and secondary antibodies. Primary and secondary antibodies and their catalog number, product size, company name and dilution ratio.

Antibody	Catalog #	Product size	Company name	Dilution	Species
TGR5	Ab72608	35-37 kDa	Abcam	1:1000	Rabbit
PC2	Sc-30166	68 and 75 kDa	Santa Cruz	1:200	Rabbit
CREB	9197	43 kDa	Cell Signaling	1:1000	Rabbit
p-CREB	9198	44 kDa	Cell Signaling	1:1000	Rabbit
β-actin	A3854	42 kDa	Sigma	1:25,000	Mouse
Anti-mouse	7076		Cell Signaling	1:3000	
Anti-rabbit	7074		Cell Signaling	1:3000	

4.3. Results

4.3.1. Expression of TGR5 under hyperglycemia

To investigate the role of bile acids via TGR5 under hyperglycemic conditions, we firstly examined whether the expression of TGR5 is modulated by glucose concentrations. Expression of TGR5 mRNA was measured by qRT-PCR in MIN6 cells, αTC1-6 cells or control mouse islets cultured under low (5 mM) or high (25 mM) glucose conditions for 7 days. Expression of TGR5 was also measured in islets isolated from control and diabetic (db/db) mice. Expression of TGR5 was similar in islets from control and diabetic (db/db) mouse islets **(Figure 27A)**. Similarly there was no change in TGR5 expression in control mouse islets **(Figure 27B)**, MIN6 cells **(Figure 28A)** or αTC1-6 cells **(Figure 28B)** cultured under low (5 mM) concentrations compared to islets or cells cultured under high (25 mM) glucose concentrations. Thus diabetes *in vivo* or hyperglycemia *in vitro* had no effect on the expression of TGR5 expression.

4.3.2. Differential expression of PC1 and PC2 in pancreatic α, β and δ cell lines and mouse islets

The effect of GLP-1 on pancreatic islet β cell function could be modulated by neural, endocrine or paracrine mechanisms [67]. Recent studies have shown that under hyperglycemia or type 2 diabetes, there is up regulation of PC1 expression in α cells resulting in GLP-1 secretion [129-133]. Thus under hyperglycemic conditions, pancreatic alpha cells are also known to be a source of GLP-1 raising the possibility that α cells have positive effect on insulin secretion from β cells via GLP-1 release in a paracrine fashion **(Figure 29)**. This is mainly because the precursor for glucagon and

GLP-1 is the same i.e. Proglucagon. The processing of proglucagon by alternative splicing generates glucagon or GLP-1 in a cell type-specific manner due to differential expression of the enzymes PC1 and PC2 **(Figure 30)**. Therefore we examined the basal expression of PC1 and PC2 in pancreatic α cell line, αTC1-6 cells by RT-PCR and western blot. RT-PCR analysis of RNA from αTC1-6 cells using specific primers demonstrated amplification of a PCR product of predicted size for both PC1 (73 bp) and PC2 (85 bp). However, expression of PC2 was much higher when compared to PC1 **(Figure 31A)**. We also examined the differential expression of PC1 and PC2 in MIN6 and TGP52 cells and mouse islets.

Further confirmation on the differential expression of PC1 and PC2 was obtained by western blot analysis using selective antibody to PC1 and PC2. The results demonstrated the expression of PC1 (70 kDa) and PC2 (68 kDa- mature form, 75 kDa-precursor form) of predicted size in the homogenates isolated from αTC1-6, MIN6 and TGP52 cells and mouse islets **(Figure 31B)**. In α cells, expression of PC2 was much higher than PC1. In enteroendocrine cells (STC-1), in contrast, expression of PC1 was much higher than PC2.

4.3.3. Hyperglycemia-induced PC1 expression is augmented by INT-777

As mentioned previously, under hyperglycemia or type 2 diabetes, there is increased expression of PC1 [129-133]. Recent studies have also shown that there is an elevation in the post prandial bile acid concentration in obese patients with type 2 diabetes compared to normal healthy individuals [134]. To investigate the role of TGR5 receptors under hyperglycemia, the effect of INT-777 on α cell PC1 expression was

measured at low (5 mM) or high (25 mM) glucose conditions for 7 days. Expression of PC1 was 2.9 fold higher in αTC1-6 cells cultured under high (25 mM) glucose conditions compared to cells cultured under low glucose. The effect of glucose was further augmented when the cells were treated with INT-777 (4.1 fold increase above 5 mM glucose basal) **(Figure 33)**. Notably there was no change in the expression of PC2 in αTC1-6 cells cultured under high glucose conditions compared to low glucose **(Figure 32)**.

Expression of PC1 was also increased in mouse islets cultured under high glucose compared to islet cultured under low glucose (3.6 fold increase above 5 mM glucose basal). The effect of glucose was further augmented when the islets were treated with 25 µM INT-777 for 48 h under high glucose (5.2 fold increase above 5 mM glucose basal) **(Figure 34)**. Expression of PC1 was significantly higher in islets from db/db mouse compared to islets from control mice (3 fold increase above control islets). Treatment of islets with INT-777 further augmented the increase in PC1 expression (4 fold increase above control islets) **(Figure 35)**.

Expression of PC1 was also increased in human islets cultured under high glucose compared to islet cultured under low glucose (1.8 fold increase above 5 mM glucose basal). The effect of glucose was further augmented when the islets were treated with 25 µM INT-777 for 48 h under high glucose (2.3 fold increase above 5 mM glucose basal) **(Figure 36)**. These results suggest that activation of TGR5 under hyperglycemic conditions promotes PC1 expression in islet α cells.

4.3.4. Signaling mechanisms involved in TGR5-mediated PC1 expression in pancreatic α cells

The notion that PC1 expression was increased by TGR5 activation was further examined in αTC1-6 cells transfected with human PC1 luciferase promoter construct. Treatment of αTC1-6 cells with 25 mM glucose caused a significant increase in the promoter activity (1.8±0.15 fold increase above 5 mM glucose basal) that was further augmented by INT-777 (3±0.24 fold increase above 5 mM glucose basal) **(Figure 37B).** Neither glucose nor INT-777 had an effect on the promoter activity of control vector (pGL2 basic vector) **(Figure 37A)**.

To examine the mechanisms involved in INT-777-induced PC1 expression, αTC1-6 cells were preincubated with INT-777 in the presence or absence of inhibitors of $G\alpha_s$ (NF449, 10 μM) or PKA (myristoylated PKI, 1 μM) and then PC1 promoter activity in response to 25 μM INT-777 was measured. INT-777 caused an increase in PC1 promoter activity at high (25 mM) glucose conditions; the increase was significantly inhibited by NF449 (75.6±3.87% inhibition) or myristoylated PKI (100% inhibition) **(Figure 38)**. These results suggest that TGR5 receptors in pancreatic α cells are G_s coupled and TGR5-mediated PC1 promoter activity involves PKA-dependent pathway.

PC1 promoter activity was shown to be regulated by the transcription factor CREB [131]. We next examined the effect of INT-777 on the activation of transcription factor CREB. Activation was measured as increase in phosphorylation of CREB in response to INT-777. Consistent with the activation of PC1 promoter activity, treatment of αTC1-6 cells with INT-777 caused activation of CREB **(Figure 39)**. Similarly,

treatment of islets from db/db mice with INT-777 caused activation of CREB compared to treatment of islets from control mice **(Figure 40)**.

In summary, the results suggest that activation of TGR5 leads to stimulation of PC1 promoter activity via G_s/PKA/CREB pathway. The activated CREB bound to CBP then binds to the cAMP response element (CRE) region on the PC1 gene and increases its expression **(Figure 41)**.

4.3.5. Hyperglycemia-induced GLP-1 secretion is augmented by TGR5 ligands

Previous studies have shown that PC1 gene expression and synthesis of GLP-1 are co-regulated, and increased PC1 expression in rat islet α cells under hyperglycemia leads to an increase in GLP-1 secretion [129-133]. Since we found that activation of TGR5 by INT-777 augmented PC1 expression in αTC1-6 cells and control mouse islets cultured under hyperglycemic conditions and also in diabetic mouse islets, we next examined the effect of TGR5 ligands on GLP-1 secretion in αTC1-6 cells as well as mouse islets cultured under low (5 mM) and high glucose (25 mM) conditions.

GLP-1 secretion from αTC1-6 cells cultured under 25 mM glucose was significantly higher compared to cells cultured under 5 mM glucose (2.0±0.12 fold increase above 5 mM glucose basal). The effect of glucose on GLP-1 secretion was further augmented upon treatment of cells with INT-777 (1.8±0.3 fold increase above 25 mM glucose basal). Similar augmentation in GLP-1 secretion was also obtained upon treatment of cells with LCA (1.7±0.13 fold increase above 25 mM glucose basal) **(Figure 42)**.

We also examined the effect of TGR5 ligands on GLP-1 secretion from mouse pancreatic islets cultured in low (5 mM) or high (25 mM) glucose condition for 7 days. GLP-1 secretion from islets cultured under 25 mM glucose was significantly higher compared to islets cultured under 5 mM glucose (2.6±0.17 fold increase above 5 mM glucose basal). The effect of glucose on GLP-1 secretion was further augmented upon treatment of islets with INT-777 (1.5±0.12 fold increase above 25 mM glucose basal). Similar augmentation in GLP-1 secretion was also obtained upon treatment of islets with LCA (1.4±0.11 fold increase above 25 mM glucose basal) **(Figure 43)**. Similarly treatment of diabetic (db/db) islets with 25 µM INT-777 significantly augmented GLP-1 secretion (1.5±0.11 fold increase above db/db basal) compared to control mouse islets **(Figure 44)**.

Similar pattern of GLP-1 secretion was obtained with human islets. GLP-1 secretion from islets cultured under 25 mM glucose was significantly higher compared to islets cultured under 5 mM glucose (1.7±0.05 fold increase above 5 mM glucose basal). The effect of glucose on GLP-1 secretion was further augmented upon treatment of islets with INT-777 (1.3±0.12 fold increase above 25 mM glucose basal) **(Figure 45)**.

4.3.6. Signaling mechanisms involved in TGR5-mediated GLP-1 secretion in pancreatic α cells

We next investigated the mechanisms by which INT-777 augments GLP-1 secretion in pancreatic α cells under hyperglycemic (25 mM) conditions. To examine whether INT-777 induced GLP-1 secretion involves PKA-dependent or PKA-independent mechanism, GLP-1 secretion in response to INT-777 was measured in the

presence of myristoylated PKI, a selective PKA inhibitor. The INT-777 induced GLP-1 secretion was unaffected by myristoylated PKI (2.2±0.05 fold increase above 25 mM glucose basal) **(Figure 46)** suggesting that GLP-1 release from αTC1-6 cells was not mediated by PKA. Treatment of αTC1-6 cells with a cAMP analog, 8-pCPT-2'-O-Me-cAMP (Epac ligand) that activates the exchange factor, Epac (but not PKA) caused an increase in GLP-1 secretion (1.7±0.13 fold increase above 25 mM glucose basal) **(Figure 47)**.

We have previously shown that bile acids via activation of TGR5 stimulates insulin secretion from pancreatic β cells and is mediated via G_s/Epac/PLC-ε/Ca^{2+} pathway. This pathway was similar to the one involved in TGR5-mediated GLP-1 release in the enteroendocrine L cells [109]. RT-PCR studies also demonstrated the expression of Epac2 and PLC-ε in αTC1-6 cells **(Figure 48)**.

We next examined the effect of INT-777 and Epac selective ligand 8-pCPT-2'-O-Me-cAMP on PI hydrolysis in α cells. Both INT-777 and Epac ligand caused significant increase in PI hydrolysis **(Figure 49).** INT-777-induced increase in PI hydrolysis (1201±38 cpm/mg protein; 185±3% increase above basal levels) was blocked by selective inhibitors of $G\alpha_s$ protein (NF449, 76±5% inhibition), PI hydrolysis (U73122, 77±6% inhibition) or Epac2 (ESI-05, 80±4% inhibition). These results suggest that stimulation of PI hydrolysis by INT-777 was mediated via activation of $G\alpha_s$ proteins, and cAMP-dependent Epac/PLC-ε activity. In support to this notion, the selective Epac ligand also stimulated PI hydrolysis (1154±20 cpm/mg protein; 173±4% increase above basal levels). Stimulation of PI hydrolysis by Epac ligand was inhibited by U73122

(66.6±3.5% inhibition) or ESI-05 (65.0±2.35% inhibition) but not by NF449 (1033±10 cpm/mg protein; 143±35% increase above basal levels).

To further confirm the signaling pathway involved in TGR5-mediated GLP-1 secretion in pancreatic α cells, we measured the effect of INT-777 on GLP-1 release in the presence or absence of NF449, U73122, BAPTA-AM or ESI-05. INT-777-induced GLP-1 secretion (2.4±0.07 fold increase above 25 mM glucose basal) was abolished by incubation of cells with NF449 (Gα_s inhibitor), U73122 (PI hydrolysis inhibitor), BAPTA-AM (calcium chelator) or ESI-05 (Epac 2 inhibitor) **(Figure 50)**.

In summary, hyperglycemia induces PC1 expression in islet α cells leading to GLP-1 secretion. The effect of glucose on PC1 promoter activity, PC1 expression and GLP-1 secretion was augmented by activation of TGR5 receptors in response to bile acids. The pathway coupled to TGR5 involved sequential activation of G$_s$, cAMP-dependent Epac and Epac-mediated PLC-ε activity and Ca^{2+} release **(Figure 51)**

4.3.7. Regulation of pancreatic β cell function by GLP-1 released from α cells in response to bile acids

We have previously shown that bile acids promote glucose-induced insulin release via TGR5 in pancreatic β cells [116]. TGR5 receptors are expressed on pancreatic β cells as well as α cells and our present data suggests that activation of TGR5 by INT-777 under hyperglycemic conditions augments PC1 expression in pancreatic α cells leading to GLP-1 secretion. This raises the possibility that α cells have positive effect on the insulin secretion from β cells via GLP-1 release in a paracrine fashion. This notion was examined by measurement of insulin secretion from

human islets cultured under hyperglycemic conditions for 7 days. In these islets, the effect of INT-777 on insulin secretion was measured in the presence or absence of 0.5 μM exendin (9-39), a GLP-1 receptor antagonist. INT-777 augmented glucose-induced insulin secretion in islets cultured under both 5 mM (1.9±0.02 fold increase) and 25 mM glucose (1.5±0.06 fold increase) **(Figure 52).** The effect of INT-777 was inhibited by the GLP-1 receptor antagonist, exendin (9-39) in islets cultured only under 25 mM glucose. (52±10% inhibition) **(Figure 52)**. The results are consistent with the augmentation of glucose-induced PC1 expression by INT-777.

These results suggest that insulin release in response to bile acids under hyperglycemic conditions results from combined activation of TGR5 receptors and GLP-1 receptors on β cells. Activation of the latter reflects the paracrine effect of GLP-1 released from α cells upon bile acid-induced stimulation of TGR5 receptors. In *in vivo* the paracrine effect of GLP-1 could act in concert with the endocrine effect involving GLP-1 release from enteroendocrine cells in response to bile acids **(Figure 53)**.

4.4. Discussion

The link between plasma levels of bile acids and regulation of key metabolic components suggests the possibility that modulation of bile acids levels and their effects could be a therapeutic approach. TGR5 is one of the two main receptors of bile acids and plays an important role in the regulation of energy metabolism and glucose homeostasis [63,66]. In the previous chapters, we have demonstrated that TGR5 is expressed in both islet α and β cells and activation of these receptors causes release of glucagon from α cells and insulin from β cells. Activation of TGR5 expressed on the enteroendocrine L cells, on the other hand, causes release of GLP-1. Both GLP-1 and glucagon are processed from the same precursor, proglucagon by the enzymes PC1 and PC2, respectively. Both qPCR and western blot analysis revealed that PC2 is preferentially expressed in α cells, whereas PC1 is preferentially expressed in enteroendocrine L cells. In the present chapter, we have demonstrated that PC1 expression can be induced in α cells by high glucose and the effect of glucose was further augmented by activation of TGR5 by bile acids. The conclusive evidence for the involvement of TGR5 in the upregulation of PC1 expression and GLP-1 secretion was provided using several complimentary approaches: 1) both qPCR and western bolt analysis showed an increase in the expression of PC1 in αTC1-6 cells and islets; 2) luciferase assay using human PC1 promoter construct showed an increase in promoter activity; and 3) ELISA measurements of GLP-1 showed an increase in GLP-1 secretion. The mechanism observed in αTC1-6 cells was confirmed in islets from mouse and human and in islets from db/db mice. In addition, we have also identified the signaling

pathways activated by TGR5 to mediate upregulation of PC1 expression and GLP-1 release. PC1 upregulation was mediated via G_s/cAMP/PKA/CREB pathway, whereas GLP-1 secretion was mediated via Gs/cAMP/Epac/PLC-ε/Ca^{2+} pathway; the latter pathway also mediates insulin release from islet β cells and GLP-1 release from the enteroendocrine L cells.

The major stimulus for the secretion of GLP-1 from enteroendocrine L cells is the presence of nutrients and is secreted very rapidly (within 10-15 minutes) from the intestinal L cells in response to meal ingestion [89,91]. Recent studies using TGR5$^{-/-}$ mice have demonstrated that activation of TGR5 was involved in GLP-1 secretion from enteroendocrine L cells in response to bile acids [66]. Studies by Kohli et al., provided clinical significance of the mechanism by demonstrating that following Rouxen Y gastric bypass (RYGB) surgery there is increased circulating bile acid concentrations leading to GLP-1 secretion [136]. Thus the enteroendocrine L cells are not only sensitive to nutrients but also to bile acids to stimulate GLP-1 secretion. The pivotal role of GLP-1 is the glucose dependent insulinotrophic effect by binding to its specific receptors present on the cell membrane of the pancreatic β cells [85]. GLP-1 is also known to stimulate β cell proliferation and also enhance the differentiation of new β cells from progenitor cells present in the pancreatic duct epithelium [67]. In addition to the positive effect on insulin secretion, GLP-1 is also known to inhibit glucagon secretion, a counteractive hormone from α cells [86] ensuing an optimal effect on glucose homeostasis. However, GLP-1 is rapidly degraded by an endoprotease, dipeptidyl peptidase-4 (DPP-4) and making plasma GLP-1 levels short lived (1-2 minutes) [89]. This rapid metabolism of GLP-1 has

paved new therapeutic avenues in developing DPP4 inhibitors or GLP-1 receptor agonists for the treatment of diabetes [87,91].

Previous studies have demonstrated that there is increased bile acid pool and composition in animal models of type 1 diabetes (T1D) [136,137]. Therefore the present study was aimed to determine the role of the bile acids via activation of TGR5 on the expression and activity of PC1 in pancreatic α cells under normal and hyperglycemic conditions with the hypothesis that the locally produced GLP-1 may have a paracrine effect on the β cells to regulate insulin secretion. Our results demonstrated that priming of α cells with high glucose is necessary for the activation of TGR5 to up regulate PC1 expression and stimulate GLP-1 secretion. Thus, TGR5 activation in control islets cultured under high (25 mM) glucose conditions or islets from diabetic (db/db) mice further augmented the effect of glucose on PC1 expression and GLP-1 secretion. We have also demonstrated that release of GLP-1 from α cells, in turn, regulates insulin release from β cells. This paracrine pathway provides an attractive alternative mechanism to regulate glucose levels. The increase in insulin secretion in response to TGR5 activation under hyperglycemic conditions was not due to upregulation of TGR5 expression. Our results also showed that hyperglycemia had no effect on TGR5 expression in both α and β cells. Expression of TGR5 was also similar in islets from control and db/db mice. Our studies are done *in vitro* using cell lines, mouse islets and human islets; therefore the *in vivo* relevance of this paracrine mechanism needs to be demonstrated.

Our data showed an increase in PC1 expression under high glucose conditions in consistent with previous studies by other groups [131-133]. Increase in GLP-1 secretion from α cells was demonstrated *in vitro* in αTC1-6 cells cultured under high glucose conditions, and *in vivo* in both type 1 and type 2 diabetic models such as NOD mice, STZ treated mice and ob/ob, db/db mice [129-131]. Cell therapy involving transplantation of encapsulated PC1 expressing α cells improved glucose handling in db/db mice [138-140]. It is important to note that high glucose up regulated PC1 but not PC2 expression. Our results are in consistent with studies by McGirr et al., [131]

Taken together, the studies provide evidence on the role of bile acids via TGR5 in enabling pancreatic α cells to produce GLP-1 under high glucose conditions that can act locally in a paracrine mechanism to improve β cell function. Given the half life of plasma GLP-1, a paracrine mechanism rather than the endocrine action seems more effectual and physiological. In support to this notion, it is also reported that there exist direct intracellular contacts between α and β cells in the human islets [75,141]. Acetylcholine secreted by human pancreatic α cells sensitizes β cells to respond to the increase in glucose concentrations by acting in a paracrine manner [142]. In addition to factors such as high glucose, inflammatory cytokines (IL-6) [143], β cell destruction, bile acids could also aid in the reprogramming of pancreatic α cells causing a switch in the processing of proglucagon by up regulating PC1 expression and increasing GLP-1 production, an adaptation under pathological conditions such as obesity or diabetes. Thus the findings in the present study opens new avenues in considering bile acids or bile acid mimetics as a therapeutic approach for treating type 2 diabetes.
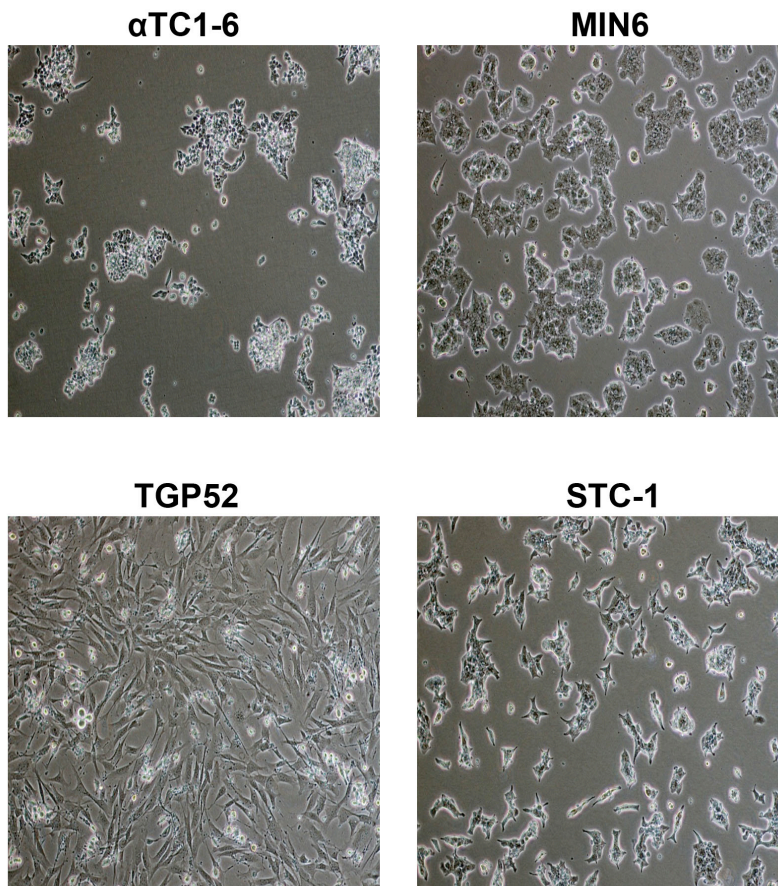
Figure 26. Cell lines used in the study. Pancreatic α (αTC1-6), β (MIN6) and δ (TGP52) and enteroendocrine cell line (STC-1) were grown in their respective culture media and the cells were incubated at 37°C in 5% CO_2.
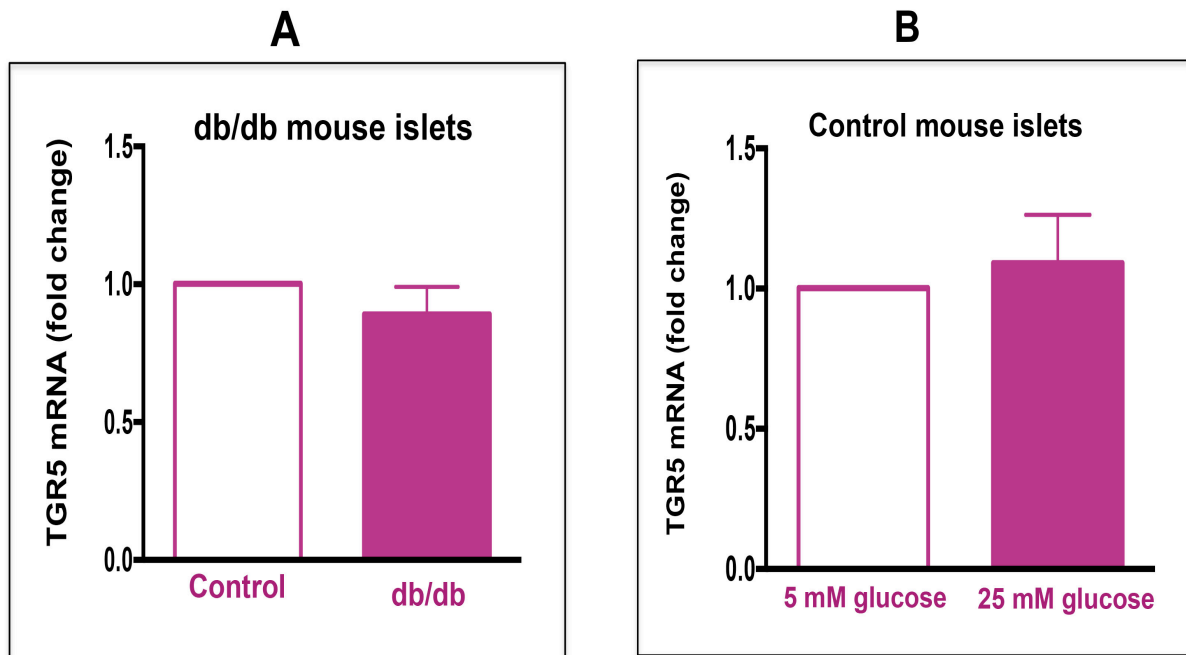
Figure 27. Effect of glucose on TGR5 expression in mouse islets. Total RNA isolated from the islets of control or diabetic (db/db) mice **(A)** and islets from control mice cultured for 7 day under low (5mM) or high (25 mM) glucose conditions **(B)** was reverse transcribed and TGR5 mRNA levels was measured by qRT-PCR. The results are expressed as fold change in TGR5 expression in reference to control (A) or 5 mM glucose basal (B) after normalizing with the endogenous control, β-actin. TGR5 expression was found to be similar between control and db/db mouse islets and between islets cultured under 5 mM versus 25 mM glucose concentration. Values are expressed as mean ± SEM of 3 experiments.
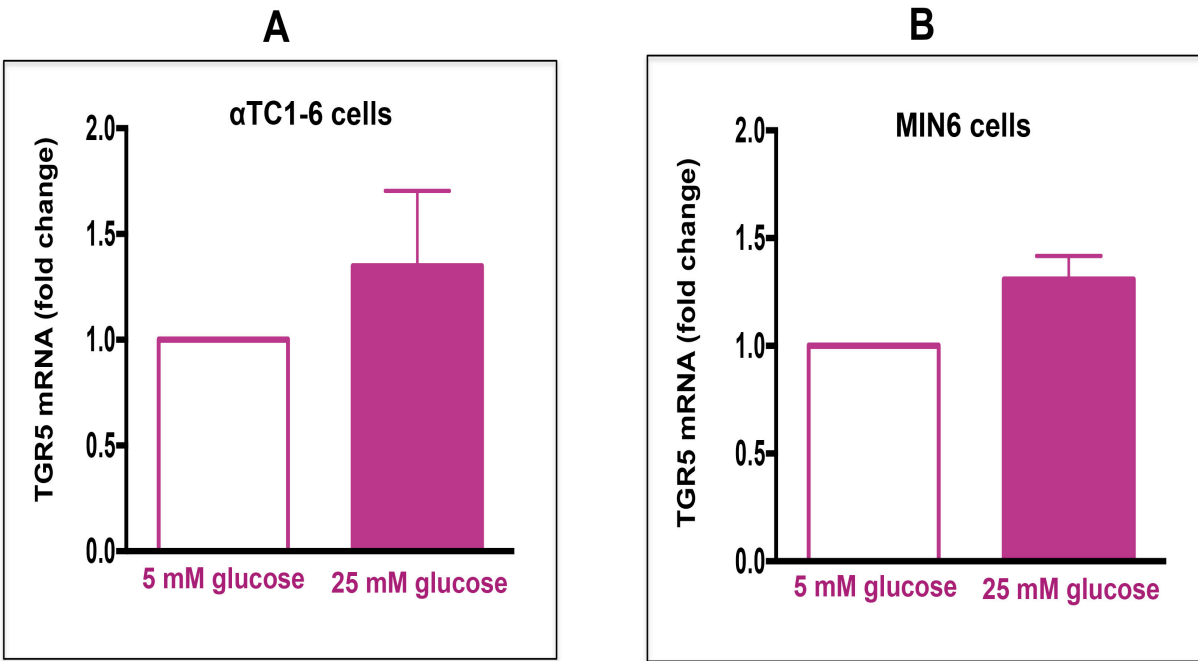
Figure 28. Effect of glucose on TGR5 expression in αTC1-6 and MIN6 cells. Total RNA isolated from αTC1-6 (A) and MIN6 (B) cells cultured for 7 days under low (5mM) or high (25 mM) glucose conditions was reverse transcribed and TGR5 mRNA levels was measured by qRT-PCR. The results are expressed as fold change in TGR5 expression in reference to 5 mM glucose basal after normalizing with the endogenous control, β-actin. High glucose had no effect on TGR5 expression in both αTC1-6 and MIN6 cells. Values are expressed as mean ± SEM of 3 experiments.

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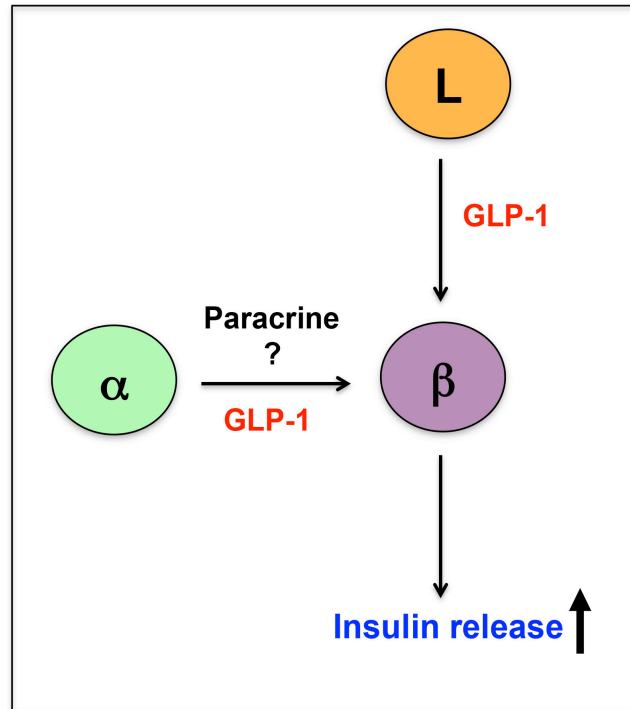
Figure 29. Regulation of insulin secretion from pancreatic β cells via GLP-1 (Endocrine and paracrine mechanisms). GLP-1, the gut peptide is secreted from the intestinal L cells in response to bile acids. The released GLP-1 reaches the systemic circulation and exerts its effect upon binding to its receptors on the pancreatic β cells and stimulates glucose dependent insulin secretion in an endocrine fashion. Studies have shown that under hyperglycemic conditions, pancreatic α cells produce GLP-1 in response to high glucose concentrations, which in turn acts as a paracrine stimulate β cells to release insulin. However the role of bile acids on the paracrine mechanism is unknown.
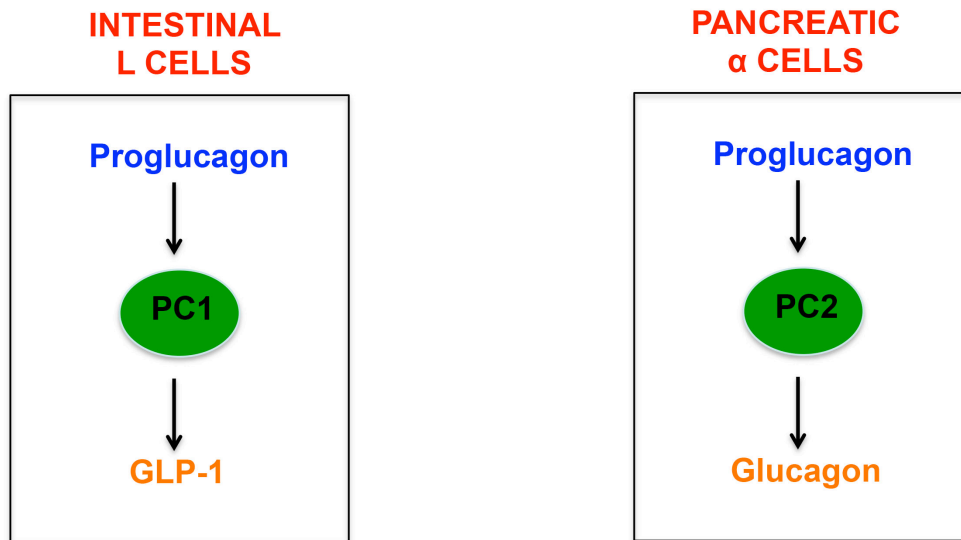
Figure 30. Processing of proglucagon. Proglucagon is the precursor for both GLP-1 and glucagon and is processed in a cell type specific manner. Proglucagon is processed to GLP-1 by prohormone convertase 1 (PC1) in the enteroendocrine L cells while in α cells of the pancreatic islets, proglucagon is processed to glucagon by prohormone convertase 2 (PC2). This tissue specific processing of proglucagon is due to differential expression of enzymes PC1 and PC2.
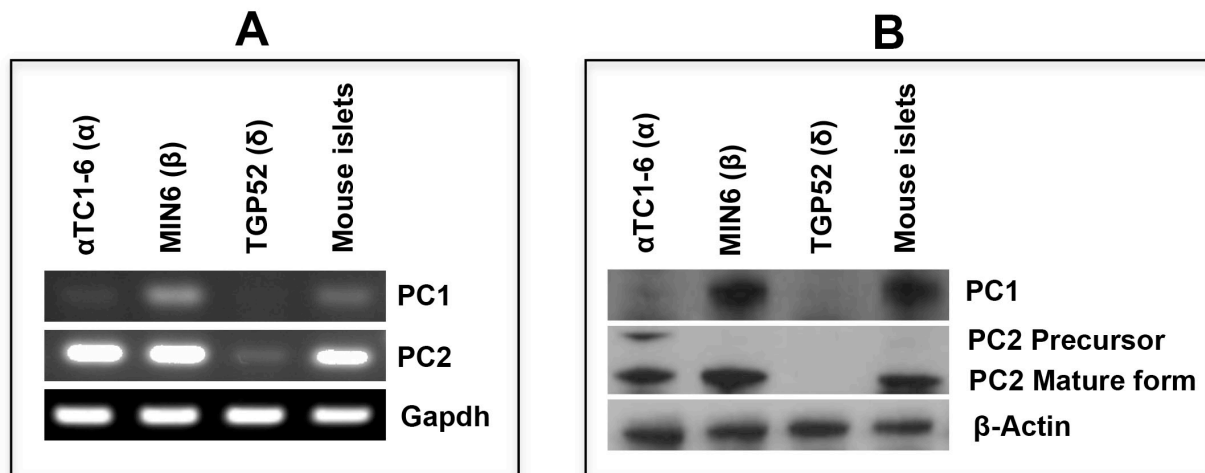
Figure 31. Differential expression of PC1 and PC2 in pancreatic α, β and δ cell lines and mouse islets. A: mRNA expression- Total RNA isolated from αTC1-6 (α), MIN6 (β), TGP52 (δ) cells and mouse islets was reverse transcribed and expression of PC1 and PC2 mRNA was examined by RT-PCR using specific primers. The results show representative PCR products (PC1- 73 bp, PC2- 85 bp or Gapdh- 122 bp) analyzed on 2% agarose gel containing ethidium bromide using Gel Doc™ EZ imager. **B: Protein expression-** Cell lysates containing equal amount of total proteins from αTC1-6, MIN6, TGP52 cells and mouse islets were separated on SDS-PAGE and the expression of PC1 and PC2 was analyzed using selective antibody. PC1 with the molecular weight of 70 kDa and PC2 with a molecular weight of 75 kDa (precursor) and 68 kDa (mature form) were detected by chemilumiscence. The membrane was reprobed for β-actin (42 kDa), an endogenous loading control.
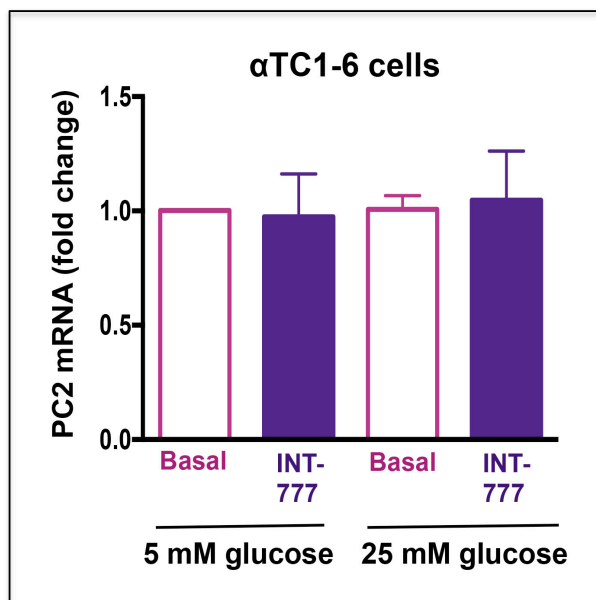
Figure 32. Effect of glucose and INT-777 on PC2 expression in αTC1-6 cells. Total RNA isolated from αTC1-6 cells cultured for 7 days under low (5mM) or high glucose (25 mM) glucose conditions in the presence or absence of INT-777 (48 hours) was reverse transcribed and PC2 mRNA levels was measured by qRT-PCR. The results are expressed as fold change in PC2 expression levels in reference to 5 mM or 25 mM glucose basal after normalizing with the endogenous control, β-actin. PC2 expression was found to be unchanged under conditions of hyperglycemia (25 mM glucose) and INT-777 also had no effect on the expression of PC2. Values are expressed as mean ± SEM of 3 experiments.
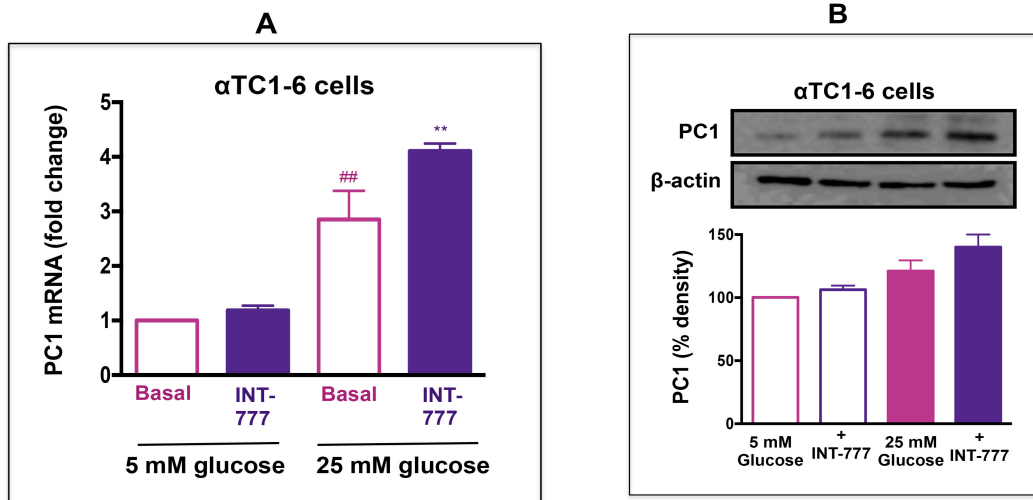
Figure 33. Increased PC1 expression in response to glucose and INT-777 in αTC1-6 cells. A: qRT-PCR- Total RNA isolated from αTC1-6 cells cultured for 7 days under low (5mM) or high (25 mM) glucose conditions in the presence or absence of INT-777 (48 hours) was reverse transcribed and PC1 mRNA levels was measured by qRT-PCR. The results are expressed as fold change in PC1 expression levels after normalizing with the endogenous control, β-actin. PC1 expression was increased under high (25 mM) glucose conditions and the increase was further augmented upon treatment with INT-777. Values are expressed as mean ± SEM of 3 experiments. [##]$p<0.001$ vs. 5 mM glucose basal; **$p<0.001$ vs. 25 mM glucose basal. **B: Protein expression-** Cell lysates containing equal amounts of total proteins from αTC1-6 cells cultured for 7 days under low (5 mM) or high (25 mM) glucose conditions in the presence or absence of INT-777 (48 hours) were separated on SDS-PAGE and the expression of PC1 (70 kDa) was analyzed using selective antibody. Protein bands were visualized with enhanced chemiluminescence, images were quantified and densitometric values were calculated after normalization to β-actin density.

Figure 34. Increase in PC1 expression in response to glucose and INT-777 in control mouse islets. Total RNA isolated from control mouse islets cultured for 7 days under low (5 mM) or high (25 mM) glucose conditions in the presence or absence of INT-777 (48 hours) was reverse transcribed and PC1 mRNA levels was measured by qRT-PCR. The results are expressed as fold change in PC1 expression levels in reference to 5 mM glucose basal after normalizing with the endogenous control, β-actin. PC1 expression was increased under high glucose condition and the increase was further augmented upon treatment with INT-777. Values are expressed as mean ± SEM of 3 experiments. [##]p<0.001 vs. 5 mM glucose basal; **p<0.001 vs. 25 mM glucose basal.
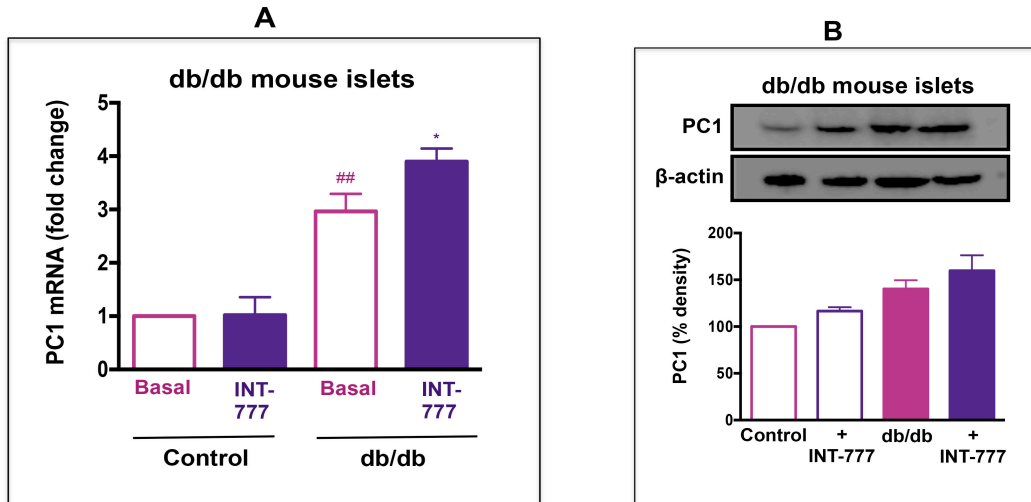
Figure 35. Increase in PC1 expression in response to INT-777 in diabetic mouse islets. A: qRT-PCR- Total RNA isolated from control and diabetic (db/db) mouse islets cultured for 2 days in the presence or absence of INT-777 was reverse transcribed and PC1 mRNA levels was measured by qRT-PCR. The results are expressed as fold change in PC1 expression levels in reference to control basal after normalizing with the endogenous control, β-actin. PC1 expression was increased in db/db mouse islets and the increase was further augmented upon treatment with INT-777. Values are expressed as mean ± SEM of 3 experiments. [##]$p<0.001$ vs. 5 mM glucose basal; *$p<0.05$ vs. 25 mM glucose basal. **B: Protein expression-** Cell lysates containing equal amounts of total proteins from control and diabetic (db/db) mouse islets cultured for 48 hours in the presence or absence of INT-777 were separated on SDS-PAGE and the expression of PC1 (70 kDa) was analyzed using selective antibody. Protein bands were visualized with enhanced chemiluminescence, images were quantified and densitometric values were calculated after normalization to β-actin density.
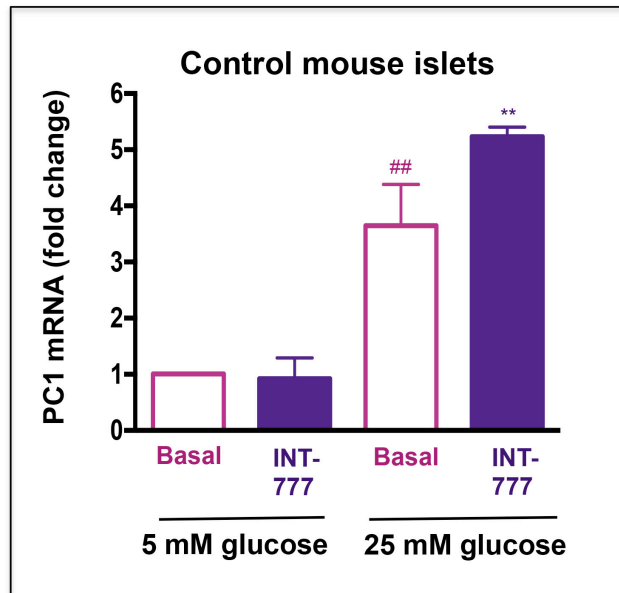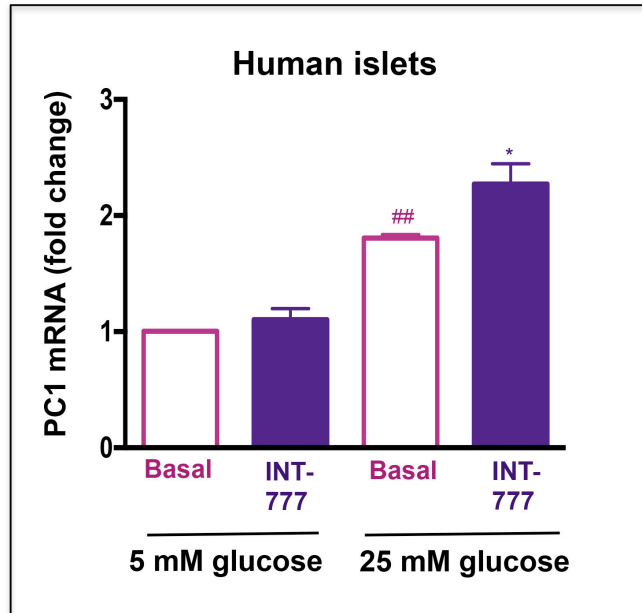
Figure 36: Increased PC1 expression in response to glucose and INT-777 in human islets. Total RNA isolated from human islets cultured for 7 days under low (5 mM) or high (25 mM) glucose conditions in the presence or absence of INT-777 (48 hours) was reverse transcribed and PC1 mRNA levels was measured by qRT-PCR. The results are expressed as fold change in PC1 expression levels in reference to 5 mM glucose basal after normalizing with the endogenous control, β-actin. PC1 expression was increased under high glucose condition and the increase was further augmented upon treatment with INT-777. Values are expressed as mean ± SEM of 3 experiments. ##p<0.001 vs. 5 mM glucose basal; *p<0.05 vs. 25 mM glucose basal.
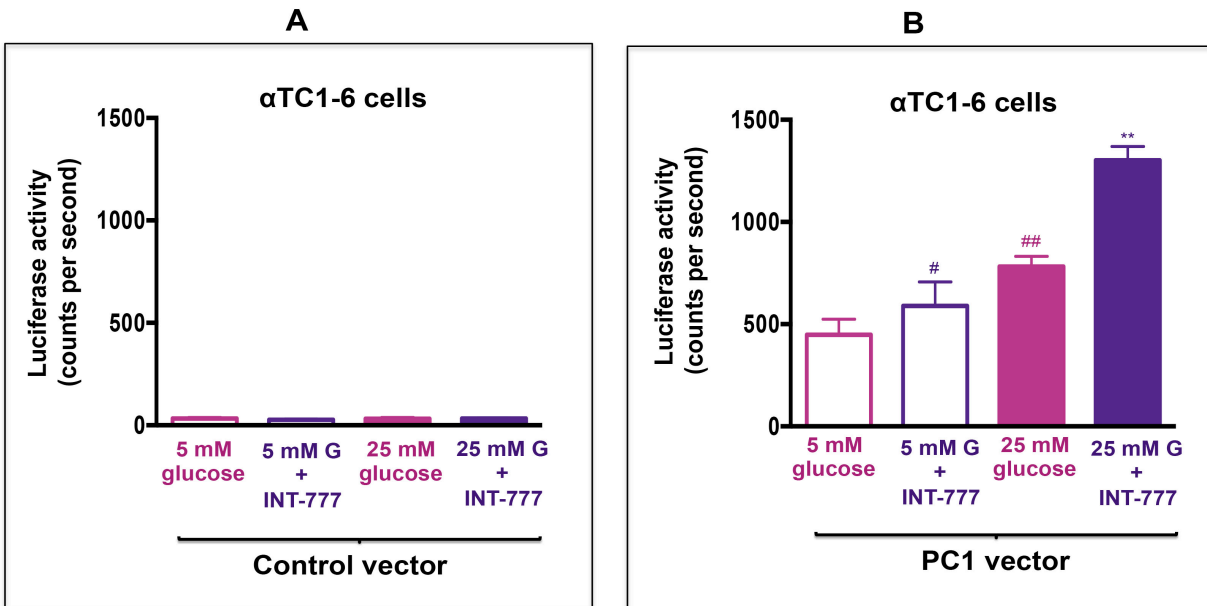
Figure 37. Increase in PC1 promoter activity in response to glucose and INT-777 in αTC1-6 cells. The PC1 promoter-luciferase reporter construct containing CRE motifs- DNA from 971 to 1 bp (PC1 vector) or pGL2 basic vector (control vector) were transiently transfected into αTC1-6 cells. After 24 hours of transfection, cells were treated with 5 mM or 25 mM glucose with or without INT-777 for 24 hours. Cells were lysed and luciferase activity was measured in the cell supernatants using BrightGlo assay kit in a luminometer. High (25 mM) glucose-induced PC1 promoter activity and the increase were further augmented by INT-777. The results are expressed as mean ± SEM of 5 experiments. [##]p<0.001 or [#]p<0.05 vs. 5 mM glucose basal; **p<0.001 vs. 25 mM glucose basal.
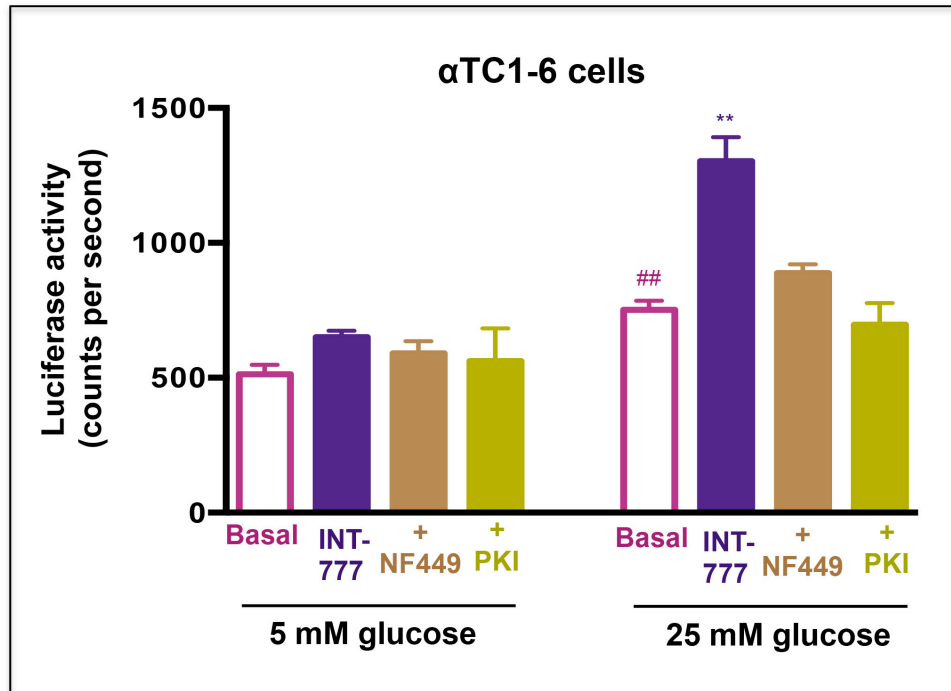
Figure 38. Effect of Gα$_s$ and PKA inhibitors on INT-777-induced PC1 promoter activity. αTC1-6 cells were transiently transfected with PC1 promoter-luciferase reporter construct using lipofectamine. Cells were treated with 5 mM glucose or 25 mM glucose with or without INT-777 for 24 hours in the presence or absence of selective Gα$_s$ inhibitor (NF449, 10 µM) or PKA inhibitor (myristoylated PKI, 1 µM). Cells were lysed and luciferase activity was measured in the cell supernatants using BrightGlo assay kit in a luminometer. High (25 mM) glucose-induced PC1 promoter activity was augmented by INT-777 and the effect of INT-777 was inhibited by NF449 or myristoylated PKI (PKI). The results are expressed as mean ± SEM of 4 experiments. [##]$p<0.001$ vs. 5 mM glucose basal; **$p<0.001$ vs. 25 mM glucose basal.
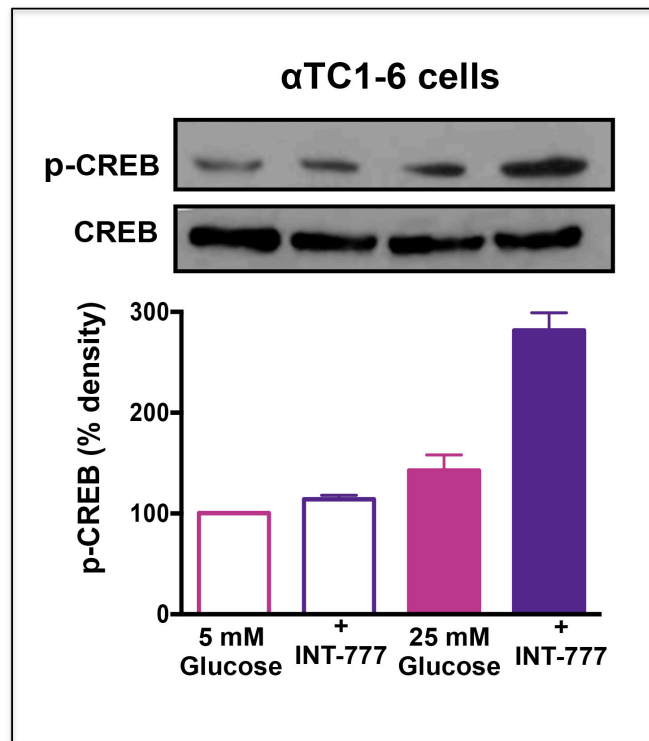
Figure 39. Activation of CREB in response to glucose and INT-777 in αTC1-6 cells. αTC1-6 cells were cultured under low (5mM) or high (25 mM) glucose conditions for 7 days. The cells were then washed and treated with INT-777 for 1 hour. Cell lysates containing total proteins were separated on SDS-PAGE and the expression of CREB and p-CREB (43 kDa) was analyzed using selective antibody. Protein bands were visualized with enhanced chemiluminescence, images were quantified and densitometric values were calculated after normalization to CREB density.
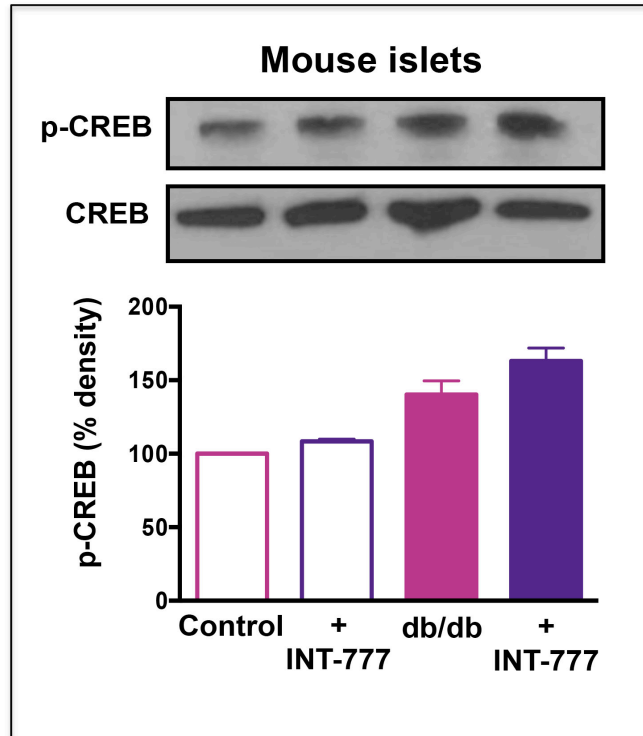
**Figure 40. Activation of CREB in response to INT-777 in db/db mouse islets.**

Cell lysates containing equal amounts of total proteins from control and diabetic (db/db) mouse islets treated with INT-777 for 1 hour were separated on SDS-PAGE and the expression of CREB and p-CREB (43 kDa) was analyzed using selective antibody. Protein bands were visualized with enhanced chemiluminescence, images were quantified and densitometric values were calculated after normalization to CREB density.
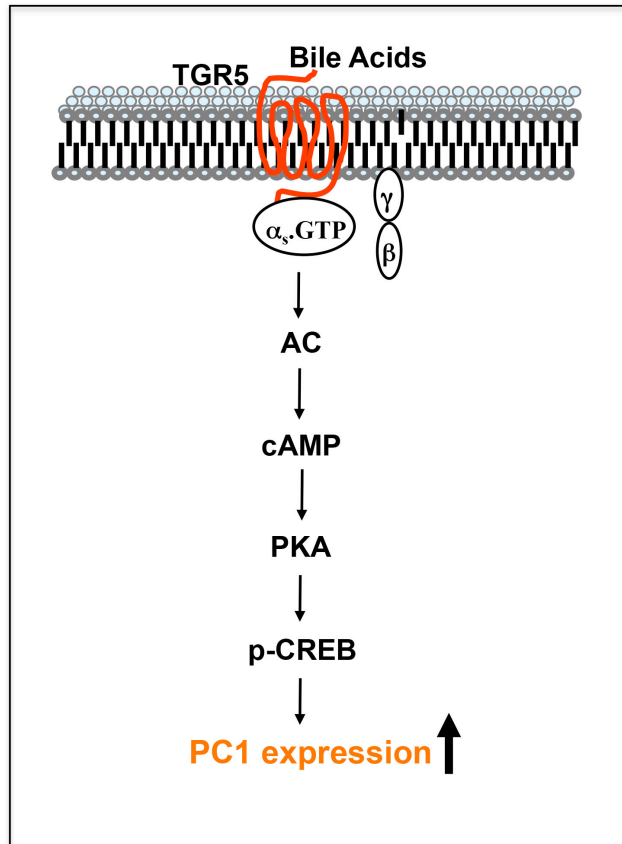
Figure 41. Schematic representation of the signaling pathway coupled to TGR5 receptors to mediate PC1 expression in pancreatic α cells. TGR5 receptors expressed in the pancreatic α cells are $G\alpha_s$ coupled, and activation of these receptors by bile acids leads to the generation of cAMP and activation of PKA. Activation of PKA leads to activation of CREB and the activated CREB in the presence of CBP binds to the cAMP response element (CRE) region on the PC1 gene and increases its expression.
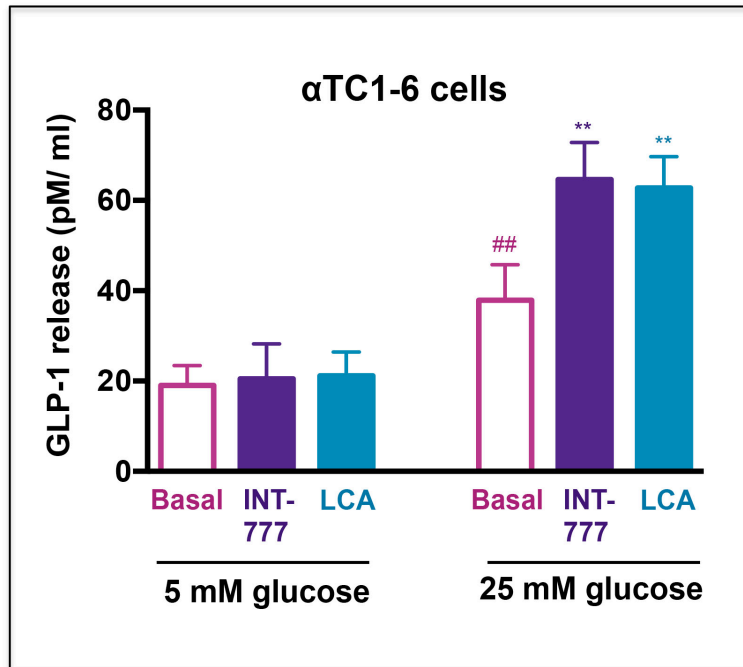
Figure 42. Augmentation of hyperglycemia-induced GLP-1 secretion by TGR5 ligands in αTC1-6 cells. αTC1-6 cells were plated at a density of 2 x 10^5 cells in 24-well plates with DMEM containing 5 mM glucose or 25 mM glucose and cultured for 6 days. The cells were then washed and incubated in DMEM containing 5 mM or 25 mM glucose with or without INT-777 or LCA for 24 hours. The supernatants were collected and GLP-1 secretion was measured by ELISA. High (25 mM) glucose induced GLP-1 secretion and the increase was further augmented by INT-777 or LCA. Values are expressed as mean ± SEM of 4 experiments. [##]p<0.001 vs. 5 mM glucose basal; [**]p<0.001 vs. 25 mM glucose basal.
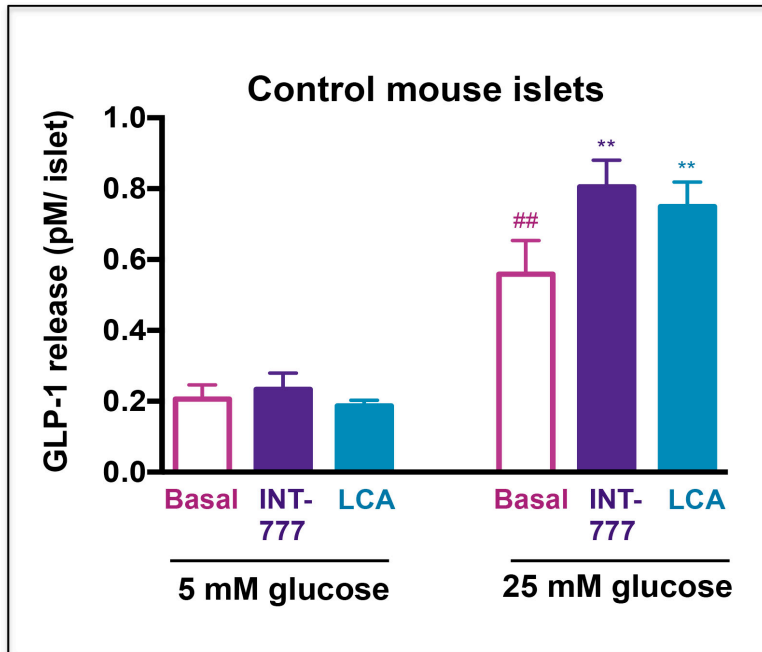
Figure 43. Augmentation of hyperglycemia-induced GLP-1 secretion by TGR5 ligands in control mouse islets. The islets isolated from the control mice were cultured in RPMI-1640 medium containing 5 mM glucose or 25 mM glucose for 6 days. The islets (25-30 islets/condition) were then washed and incubated in RPMI-1640 containing 5 mM or 25 mM glucose with or without INT-777 or LCA for 24 hours. The supernatants were collected and GLP-1 secretion was measured by ELISA. High (25 mM) glucose induced GLP-1 secretion and the increase was further augmented in response to activation of TGR5 by INT-777 or LCA. Values are expressed as mean ± SEM of 4 experiments. [##]$p<0.001$ vs. 5 mM glucose basal; [**]$p<0.001$ vs. 25 mM glucose basal.
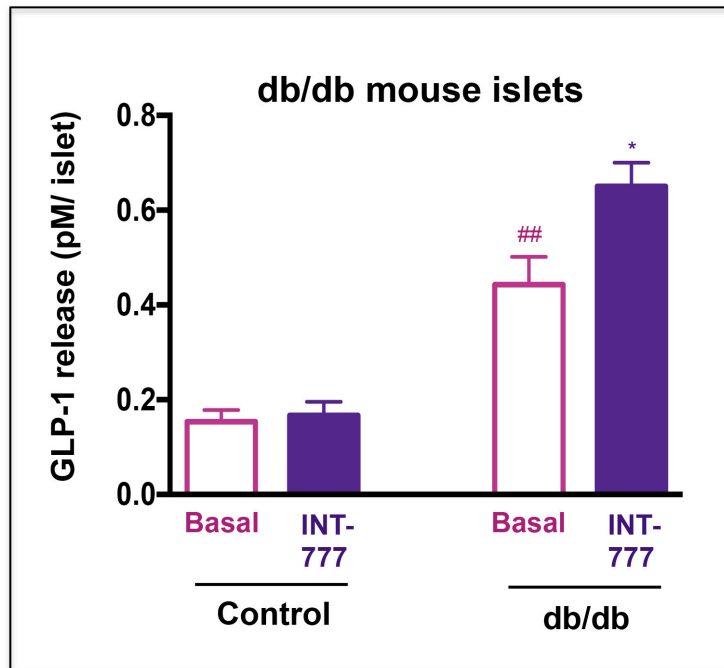
Figure 44. Augmentation of GLP-1 secretion by INT-777 in diabetic (db/db) mouse islets. The islets isolated from the control or diabetic (db/db) mice were incubated in RPMI-1640 with or without INT-777 for 24 hours. The supernatants were collected and GLP-1 secretion was measured by ELISA. GLP-1 secretion was higher in diabetic islets when compared to control mouse islets. Activation of TGR5 by INT-777 augmented GLP-1 secretion from db/db mouse islets, but not from control mouse islets. Values are expressed as mean ± SEM of 4 experiments. $^{\#\#}p<0.001$ vs. control basal; $^{*}p<0.05$ vs. db/db basal.
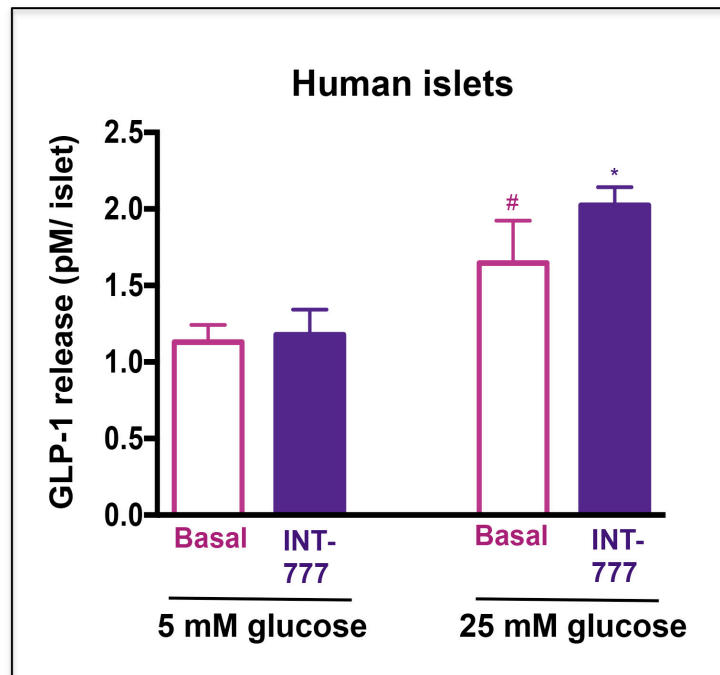
Figure 45: Stimulation of hyperglycemia-induced GLP-1 secretion by TGR5 ligands in human islets. The human islets were cultured in CMRL-1066 medium containing 5 mM glucose or 25 mM glucose for 6 days. The islets (30-35 islets/condition) were then washed and incubated in CMRL-1066 containing 5 mM or 25 mM glucose with or without INT-777 for 24 hours. The supernatants were collected and GLP-1 secretion was measured by ELISA. High (25 mM) glucose induced GLP-1 secretion and the increase was further augmented in response to activation of TGR5 by INT-777. Values are expressed as mean ± SEM of 3 experiments. [#]$p<0.05$ vs. 5 mM glucose basal; [*]$p<0.05$ vs. 25 mM glucose basal.
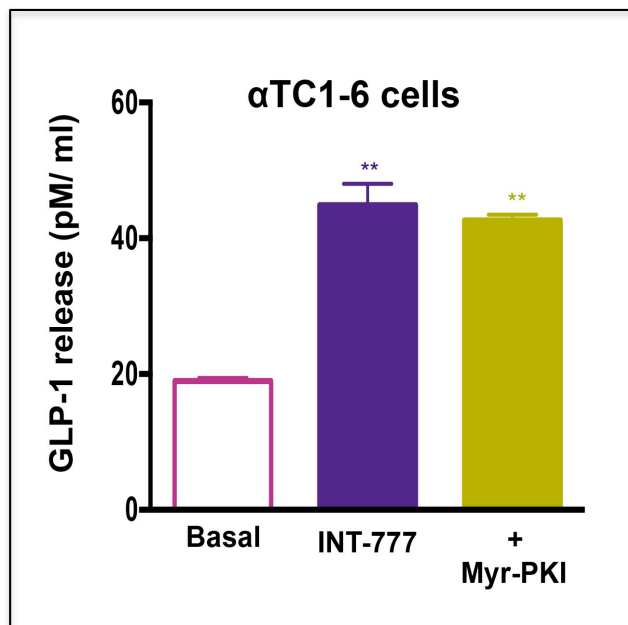
Figure 46. Effect of PKA inhibitor on INT-777-induced GLP-1 secretion. αTC1-6 cells cultured for 7 days under high (25 mM) glucose were treated with INT-777 (25 μM) in the presence or absence of PKA inhibitor (myristoylated PKI, 1 μM). After 1 hour, the supernatant was collected and GLP-1 secretion was measured by ELISA. The TGR5 ligand, INT-777 stimulated GLP-1 secretion and the selective PKA inhibitor, myristoylated PKI (Myr PKI) had no effect on INT-777-induced GLP-1 secretion. Values are expressed as mean ± SEM of 4 experiments. **$p < 0.001$ vs. 25 mM glucose basal.
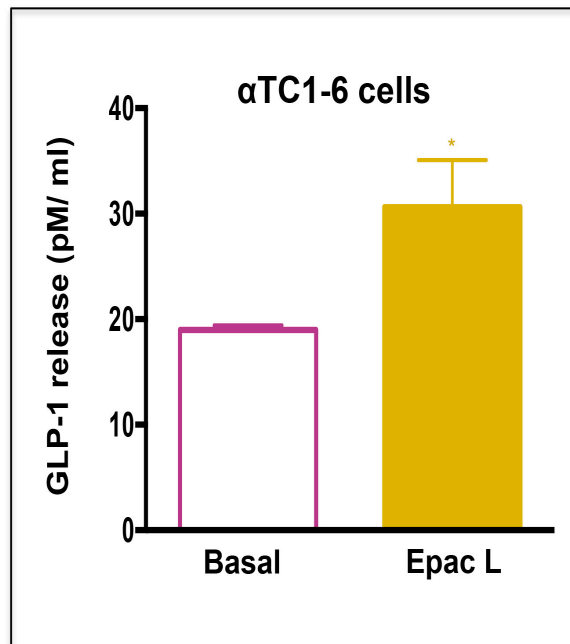
Figure 47. Stimulation of GLP-1 secretion by Epac ligand. αTC1-6 cells cultured for 7 days under high (25 mM) glucose were treated with the Epac ligand, 8-pCPT-2'-O-Me-cAMP (10 μM). After 1 hour, the supernatant was collected and GLP-1 secretion was measured by ELISA. The Epac ligand significantly stimulated GLP-1 secretion from αTC1-6 cells. Values are expressed as mean ± SEM of 4 experiments. *p<0.05 vs. 25 mM glucose basal.
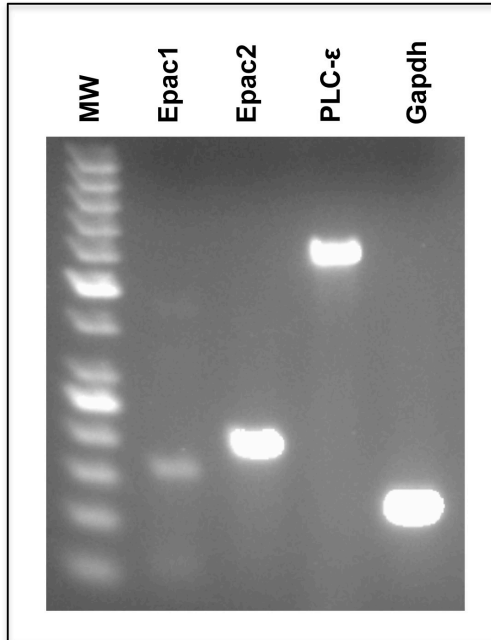
Figure 48. Expression of Epac1, Epac2 or PLC-ε in αTC1-6 cells. Total RNA was isolated from cultured αTC1-6 cells and reverse transcribed using 1 µg of total RNA. The cDNA was amplified using specific primers and examined for the expression of Epac1 (159 bp), Epac2 (195 bp) or PLC-ε (606 bp) by RT-PCR. The results show representative PCR products analyzed on 2% agarose gel containing ethidium bromide using Gel Doc[TM] EZ imager. Gapdh (122 bp) was used as a loading control.
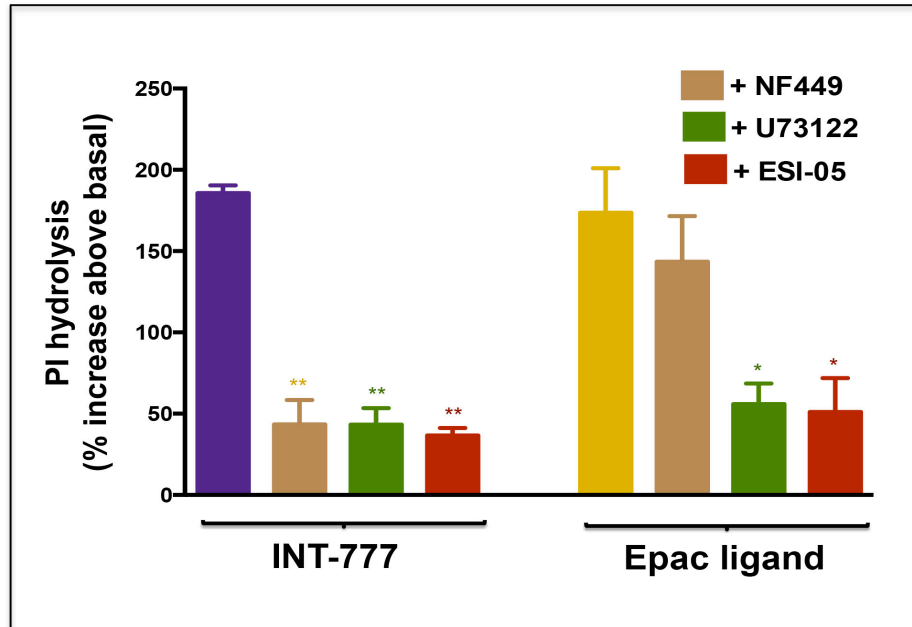
Figure 49. Activation of PI hydrolysis by TGR5 or Epac ligand in αTC1-6 cells.

αTC1-6 cells were labeled with myo-[^{3}H] inositol for 24 h and then treated with INT-777 (25 µM) or Epac ligand (8-pCPT-2'-O-Me-cAMP, 10 µM) with or without the selective Gα$_s$ inhibitor (NF449, 10 µM), PI hydrolysis inhibitor (U73122, 10 µM) or Epac2 inhibitor (ESI-05, 5 µM). PI hydrolysis was measured by ion exchange chromatography and the results are expressed as percent increase above basal levels. INT-777 or Epac ligand stimulated PI hydrolysis and the stimulation were blocked with the pretreatment of cells with U73122 and ESI-05. NF449 inhibited PI hydrolysis stimulated by INT-777 but not by Epac ligand. Values are expressed as mean ± SEM of 3 experiments. **p<0.001 significant inhibition in PI hydrolysis compared to INT-777 or Epac ligand.
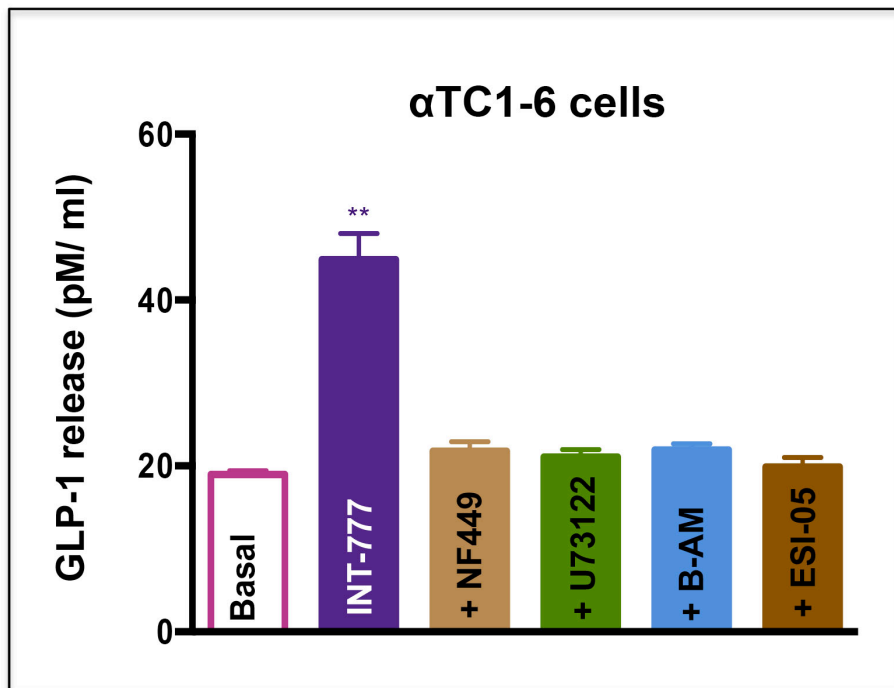
Figure 50. Effect of Gα$_s$, PI hydrolysis, Epac2 inhibitor and calcium chelator on INT-777-induced GLP-1 release. αTC1-6 cells cultured for 7 days under high (25 mM) glucose were treated with INT-777 (25 μM) in the presence or absence of selective Gα$_s$ inhibitor (NF449, 10 µM), PI hydrolysis inhibitor (U73122, 10 µM), Epac2 inhibitor (ESI-05, 5 µM) or calcium chelator (BAPTA-AM, 10 µM). After 1 hour, the supernatant was collected and GLP-1 secretion was measured by ELISA. The TGR5 ligand, INT-777 stimulated GLP-1 secretion and stimulation was abolished by the blockade of Gα$_s$ activation, PI hydrolysis, Epac2 activation or chelation of Ca^{2+}. Values are expressed as mean ± SEM of 3 experiments. **p<0.001 vs. 25 mM glucose basal.
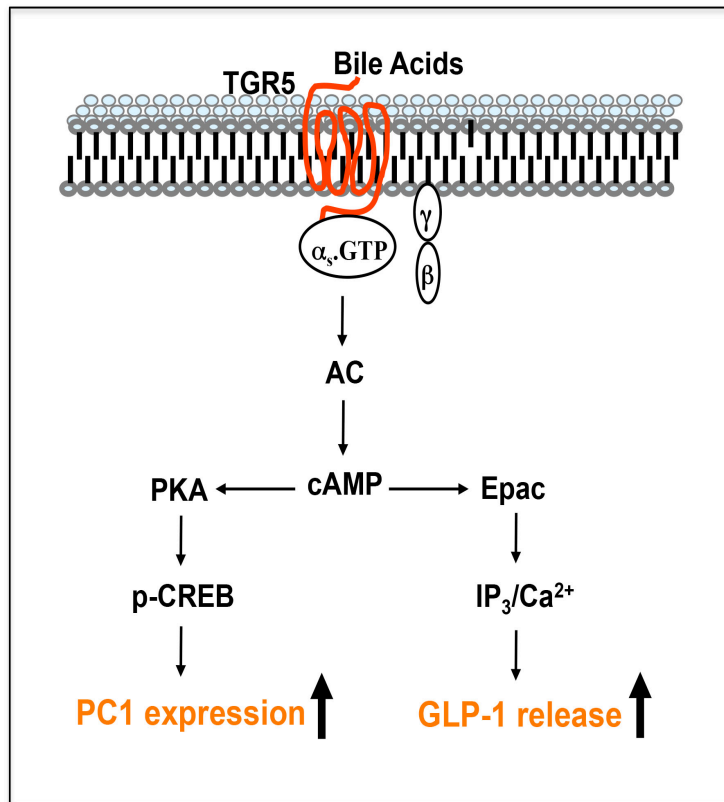
Figure 51. Schematic representation of the signaling pathway coupled to TGR5 receptors to mediate PC1 expression and GLP-1 secretion from pancreatic α cells. TGR5 receptors expressed in the pancreatic α cells are $G\alpha_s$ coupled and activation of these receptors by bile acids leads to the generation of cAMP and activation of PKA. Increase in PC1 expression is mediated via PKA-dependent mechanism involving activation of CREB while stimulation of GLP-1 release involves PKA-independent mechanism via Epac/PLC-ε/Ca^{2+} pathway.
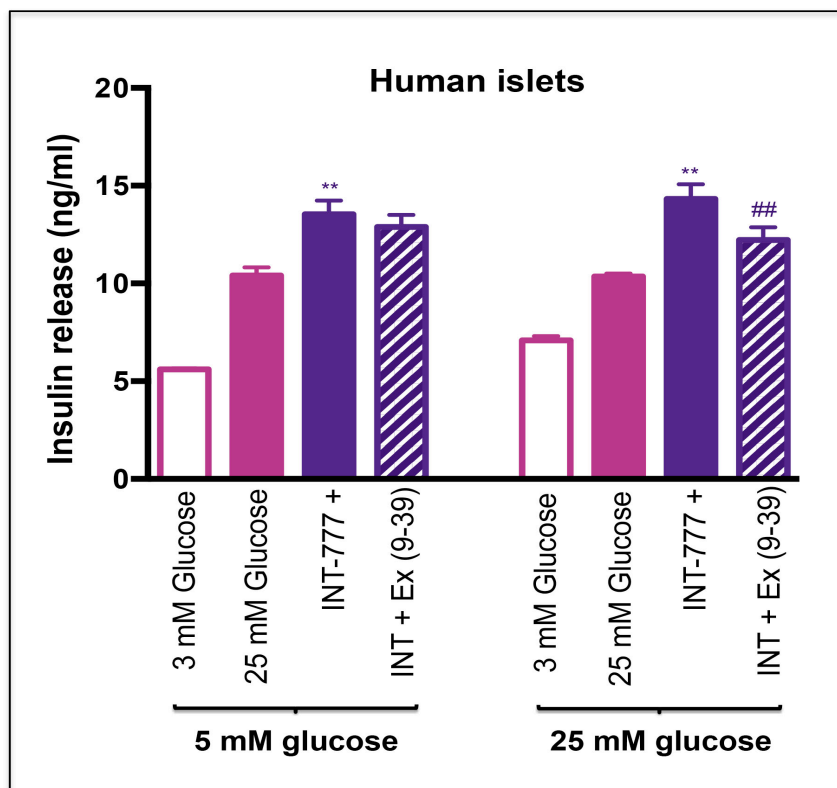
Figure 52: Effect of Exendin (9-39) on INT-777-induced insulin secretion in human islets. The human islets were cultured in CMRL-1066 medium containing 5 mM glucose or 25 mM glucose for 7 days. The islets (30-35 islets/condition) were treated with INT-777 (25 µM) in the presence or absence of exendin (9-39) for 1 hour. The supernatants were collected and insulin secretion was measured by ELISA. INT-777 augmented glucose-induced insulin secretion in islets cultured under both 5 mM and 25 mM glucose. The effect of INT-777 was inhibited by the GLP-1 receptor antagonist, exendin (9-39) in islets cultured only under 25 mM glucose. Values are expressed as mean ± SEM of 3 experiments. [**]$p<0.001$ vs. 25 mM glucose basal; [##]$p<0.001$ significant inhibition of INT-777-induced insulin secretion.
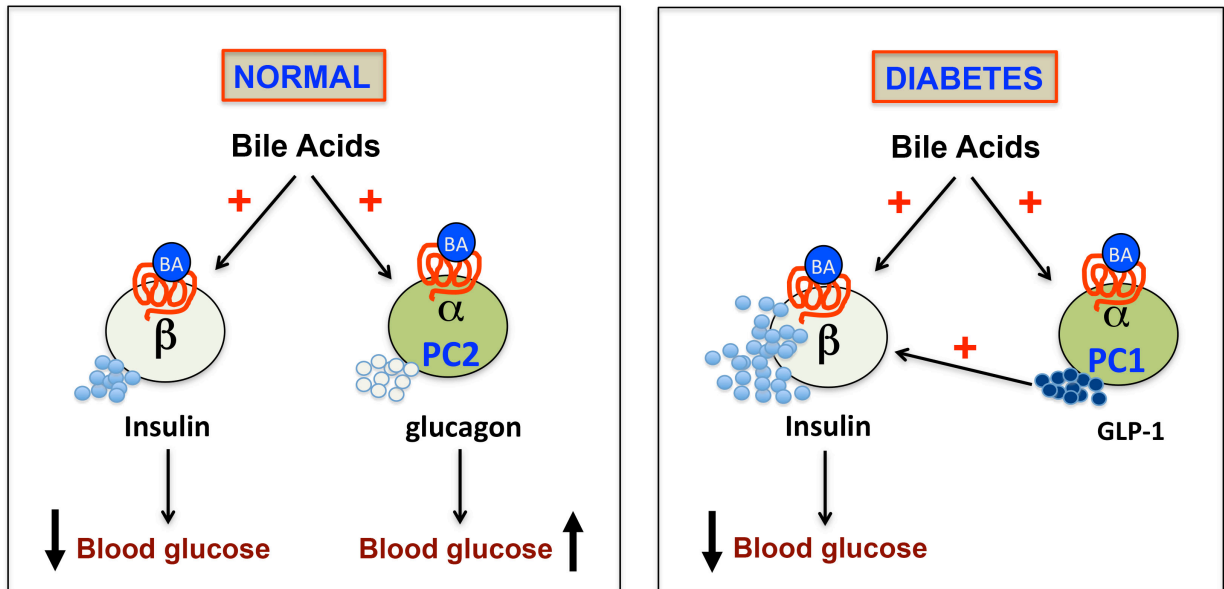
Figure 53. Regulation of pancreatic α and β cell function by the bile acid receptor TGR5. (Left panel) Under normal conditions, activation of TGR5 receptors on the pancreatic β cells by bile acids leads to release of insulin. Activation of TGR5 receptors on the pancreatic α cells by bile acids leads to release of glucagon, which is processed from proglucagon by the selective expression of prohormone convertase 2 (PC2) in these cells. Insulin and glucagon act to oppose each other action to regulate blood glucose levels. **(Right panel)** Under chronic hyperglycemia or diabetes, activation of TGR5 receptors on α cells augments glucose-induced PC1 expression and GLP-1 secretion, which in turn, acts in a paracrine manner to promote insulin secretion from β cells. Thus, in diabetes, secretions of α and β cells act in concert to regulate blood glucose levels.

CHAPTER 5

GENERAL DISCUSSION

Bile acids, the end product of cholesterol metabolism, are made in hepatocytes and stored in gallbladder [1]. In humans, cholic acid and chenodeoxycholic acid are the primary bile acids, and deoxycholic acid and lithocholic acid are the secondary bile acids. Bile acids are released in response to a meal upon gallbladder contraction. This results in an increased bile acid concentration in the gut postprandially [4]. The physiological function of bile acids as emulsifying agents to facilitate lipid digestion and absorption in the gut is well established [2]. Most of the bile acids secreted into the duodenum are reabsorbed in the distal ileum and redirected to liver via hepatic portal circulation [5]. The concentration of bile acids within the intestinal lumen and in the circulation varies in an episodic fashion. After a meal, bile acid levels increase in the portal vein and liver and also in the systemic circulation [5,40]. The concentration of bile acids reaches up to 15 μM under postprandial condition [5].

Recent studies have established the role of bile acids as signaling molecules with the identification of specific nuclear (FXR) and a plasma membrane G-protein coupled receptors (TGR5) for which bile acids are physiological ligands [24-26,35,36]. Activation of these receptors plays an important role in several physiological functions such as

hepatic bile flow, immune responses, cell proliferation, gastrointestinal motility and secretion, energy metabolism and glucose homeostasis. The expression of FXR and TGR5 is ubiquitous and are known to have distinct functional roles [30-35, 38-42].

The role of TGR5 in the regulation of glucose metabolism via release of GLP-1 from enteroendocrine L cells has gained a lot of interest as GLP-1 exerts its biological effects via the activation of GLP-1 receptors to release insulin from the pancreatic β cells [65,67]. Insulin secretion, in addition to glucose, is regulated by fatty acids, amino acids and by neurohumoral agents via activation of specific G protein coupled receptors [81,99-103]. The expression and function of TGR5 in pancreatic islets are unknown. Hence our studies were aimed to examine the direct effect of bile acids via TGR5 on the pancreatic α and β cells.

Role of TGR5 in pancreatic β cell function

We have identified the expression of TGR5 receptors on the β cells and demonstrated that activation of these receptors by bile acids stimulates insulin secretion in a glucose dependent manner. Evidence for the specific involvement of TGR5 in insulin secretion was obtained in MIN6 cells (β cell line) and islets isolated from human and mouse pancreas using various ligands: oleanolic acid (selective ligand for TGR5), INT-777 (specific ligand for TGR5) and lithocholic acid (physiological ligand that preferentially activates TGR5). LCA at the physiological concentrations (10-25 μM) induced insulin secretion underlying the physiological significance of TGR5 receptors in β cells. We further elucidated the signaling mechanism involved in TGR5-mediated insulin secretion in β cells using both biochemical and pharmacological approaches. We

provided evidence that β cell TGR5 receptors are coupled to G_s and stimulation of adenylyl cyclase leading to generation of cAMP. Insulin release in response to TGR5 activation uses non-canonical pathway that is independent of PKA. Activation of cAMP dependent Epac/PLC-ε/Ca^{2+} pathway stimulates insulin secretion. Activation of TGR5 in β cells leads to stimulation of PI hydrolysis and increase in intracellular Ca^{2+}, a biochemical measure of PLC-ε activity and IP_3-dependent Ca^{2+} release pathway. In Our studies, we also demonstrated that insulin secretion in response to TGR5 activation was blocked by inhibitors of G_s (NF449), and PI hydrolysis (U73122) and intracellular Ca^{2+} chelator (BAPTA-AM), but not by inhibitor of PKA (myristoylated PKI). We have also demonstrated the expression of Epac and PLC-ε in β cells and release of insulin in response to Epac-selective ligand, 8-pCPT-2'-O-Me-cAMP. These studies provide conclusive evidence that activation of β cell TGR5 by bile acids causes insulin release via G_s/cAMP/PLC-ε/Ca^{2+} pathway. Thus, bile acids, in addition to regulating insulin release via GLP-1 secretion from enteroendocrine L cells, also directly acts on TGR5 receptors in β cells to promote insulin release. The signaling pathway involved in insulin secretion from β cells was found to be similar to the secretion of GLP-1 from enteroendocrine L cells in response to TGR5 activation [109].

Pancreatic islets are composed of insulin producing β cells, glucagon producing α cells along with other cell types [68,74,75]. The blood glucose levels are well regulated by the opposing actions of insulin and glucagon secreted from the islets [119]. The normal glycemic levels in the body are determined by the insulin to glucagon ratio. Under postprandial conditions, the secretion of insulin from β cells reduces blood

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glucose levels and during the fasted state, the secretion of glucagon from α cells acts as a counter regulatory hormone and increases blood glucose levels [118]. Studies have shown that secretion of glucagon from α cells are modulated by not only low glucose concentrations but also by nutrients such as amino acids, fatty acids; neurotransmitters like acetylcholine, epinephrine and also hormones such as cholecystokinin, GLP-1, somatostatin etc [68,120]. In our next study we examined the effect of bile acids on the regulation of glucagon secretion from α cells.

Role of TGR5 in pancreatic α cell function

We have identified the expression of TGR5 receptors in α cells and demonstrated that activation of these receptors by bile acids stimulates glucagon secretion under low glucose conditions. Evidence for the specific involvement of TGR5 in glucagon secretion was obtained in αTC1-6 cells (α cell line) and islets isolated from mouse pancreas using pharmacological (OA and INT-777) and physiological (LCA) ligands. The physiological significance of TGR5 receptors in α cells needs to be determined given the fact that glucagon secretion occurs under hypoglycemic condition and concentration of bile acids are lower under 'fasted state'. Glucagon and GLP-1 are derived from the same precursor, proglucagon by the enzymes PC2 and PC1, respectively. Activation of TGR5 in enteroendocrine L cells causes GLP-1 secretion, whereas activation in α cells causes glucagon secretion. This is due to cell-specific expression PC1 and PC2. PC2 is preferentially expressed in α cells and PC1 is preferentially expressed in enteroendocrine L cells. Recent studies have shown that in islet α cells under hyperglycemic conditions there is a switch in the processing of

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proglucagon to GLP-1 [131-133]. Transplantation of encapsulated α cells into db/db mice improved glucose handling in these mice [139-140] suggesting that secretion from α cells facilitate insulin secretion from β cells. Recent studies have also shown that there is increase in bile acid pool in animal models of diabetes. These studies led us to examine the hypothesis that activation of TGR5 receptors in α cells under hyperglycemia up regulates PC1 expression leading to GLP-1 secretion.

Paracrine regulation of insulin secretion by GLP-1 released from α cells

Our studies suggest that bile acids could aid in the reprogramming of pancreatic α cells causing a switch in the processing of proglucagon by up regulating PC1 expression and increasing GLP-1 production, an adaptation under pathological conditions such as obesity or diabetes. Under normal conditions, PC2 expression is more abundant in α cells. Hyperglycemic conditions, however, up regulated PC1, but not PC2 expression in α cells and the effect of glucose was further augmented by TGR5 activation. Glucose-induced GLP-1 release from α cells was also augmented by the activation of TGR5 receptors. Augmentation of glucose-induced PC1 expression and GLP-1 secretion by bile acids was demonstrated in αTC1-6 cells and in islets from mouse and human. Furthermore, our studies showed that release of GLP-1 from diabetic (db/db) mice islets was augmented by TGR5 receptor activation. We have also identified distinct signaling pathways involved in the activation of PC1 and release of GLP-1 in response to TGR5 activation. Stimulation of PC1 promoter activity involved G_s/cAMP/PKA pathway and PKA-dependent activation of the transcription factor, CREB, whereas stimulation of GLP-1 release involved G_s/cAMP/Epac/PLC-ε/Ca^{2+}

pathway. GLP-1 released from α cells acts on GLP-1 receptors on β cells and facilitates insulin secretion. This paracrine pathway provides a compensatory mechanism under hyperglycemic conditions to regulate glucose homeostasis. This paracrine mechanism involving GLP-1 release from α cells will be more significant given the fact that concentrations of GLP-1 from enteroendocrine L cells are short-lived due to degradation via peptidases. In support to this notion, it is also reported that there exist direct intracellular contacts between α and β cells in the human islets [75,141].

Potential implications on human health

Type 2 diabetes mellitus (T2D) is a major cause of morbidity and mortality in many parts of the world. It is driven by insulin resistance and pancreatic β cell stress eventually leading to β cell exhaustion and death [114,115]. GLP-1, in addition to its insulinotrophic effect in type 2 diabetes, is also known to regulate β cell mass by increasing β cell proliferation and survival by exerting anti-apoptotic effect [67,86]. Therefore TGR5 signaling in T2D in humans resulting in GLP-1 secretion from pancreatic α cells is very promising with the hope of considering TGR5 ligands for the management and therapy of T2D.

The current studies may also have implications for another devastating disease namely pancreatic adenocarcinoma. This malignancy often develops in those with diabetes and insulin resistance is a well-known risk factor [144]. GLP-1 receptors are also present in pancreatic ductal cells and acinar tissues [67]. We hypothesize that prolonged GLP-1 secretion by the pancreatic islets under hyperglycemic conditions

such as that seen in diabetes may trigger pancreatic ductal hyperplasia and cancer. This possibility is supported by the data that incretin based therapies mediate increased ductal cell proliferation in patients with obesity or T2D, a risk factor for pancreatic adenocarcinoma [145]. An important future direction for our work will be to confirm or refute this possibility.

In conclusion, we have identified the expression of TGR5 in both pancreatic α and β cells. Activation of these receptors by bile acids causes release of insulin from β cells and glucagon from α cells. Under normal conditions, glucagon counter regulates the function of insulin. Under hyperglycemic conditions, however, release of GLP-1 due to upregulation of PC1 expression promotes insulin release to facilitate glucose homeostasis. Thus the role of TGR5 activated by bile acids makes it a potential target to treat type 2 diabetes and metabolic syndrome. The knowledge gathered from this study has promising therapeutic potential and opens new avenues in considering TGR5 agonists for the treatment of metabolic syndrome.

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EDUCATION

M.S.	Biotechnology	University of Mysore, Mysore, India	2005-2007
B.S.	Biochemistry	St. Philomena's College, Mysore, India	2002-2005

AWARDS and HONORS

"Research Recognition Award" from **APS Endocrinology and Metabolism Section** Experimental Biology Meeting, 2014.

Invited speaker at Gordon Research Seminar on Phosphorylation and G-protein mediated signaling networks, Maine, 2012.

"The best outgoing student award" of the batch 2002- 2005 for all round participation in curricular and extra- curricular activity, St. Philomena's College, Mysore, India.

RESEARCH EXPERIENCE

Doctoral Research: Department of Physiology and Biophysics, Virginia Commonwealth University, 2011–2014 (Research advisors- Dr. Arun J. Sanyal and Dr. Murthy S. Karnam)
> **Regulation of Pancreatic α and β Cell Function by the Bile Acid Receptor TGR5**

Research Technician: Department of Physiology and Biophysics, Virginia Commonwealth University, 2010- 2011(Research advisor- Dr. Qinglian Liu)
> **Biochemical and Structural analysis of Hsp70 chaperone system.**

Clinical Research Associate: Vikram Hospital and Heart Care, Mysore, India, 2007-2009 (Research advisor- Dr. Arun Srinivasan)
> **The phase III - study of stroke prevention in Atrial Fibrillation, called RE-LY™.**

Project Trainee: Vikram Hospital and Heart Care, Mysore, India, 2007-2009 (Research advisor- Dr. Anjali Arun)
> **Postoperative hyperbilirubinemia in cardiac surgery: Incidence, risk factors and clinical significance.**

MEMBERSHIP

American Physiological Society

TEACHING EXPERIENCE

Teaching Assistance: Undergraduate Human Physiology laboratory, Virginia Commonwealth University, 2014

PUBLICATIONS

1. **Kumar DP**, Vorvis C, Sarbeng EB, Cabra Ledesma VC, Willis JE, Liu Q. The four hydrophobic residues on the Hsp70 inter-domain linker have two distinct roles. *J Mol Biol* 411: 1099–1113, 2011.

2. Xu X, Sarbeng EB, Vorvis C, **Kumar DP**, Zhou L, Liu Q. Unique peptide substrate binding properties of 110-kDa heat-shock protein (Hsp110) determines its distinct chaperone activity. *J Biol Chem* 287: 5661–5672, 2012.

3. **Kumar DP**, Rajagopal S, Mahavadi S, Mirshahi F, Grider JR, Murthy KS, Sanyal AJ. Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic β cells. *Biochem Biophys Res Commun* 427: 600–605, 2012.

4. Rajagopal S, **Kumar DP**, Mahavadi S, Bhattacharya S, Zhou R, Corvera CU, Bunnett NW, Grider JR, Murthy KS. Activation of G protein-coupled bile acid receptor, TGR5, induces smooth muscle relaxation via both Epac- and PKA-mediated inhibition of RhoA/Rho kinase pathway. *Am J Physiol Gastrointest Liver Physiol* 304: G527–G535, 2013.

5. Mahavadi S, Bhattacharya S, **Kumar DP**, Clay C, Ross G, Akbarali HI, Grider JR, Murthy KS. Increased PDE5 activity and decreased Rho kinase and PKC activities in colonic muscle from caveolin-1-/- mice impair the peristaltic reflex and propulsion. *Am J Physiol Gastrointest Liver Physiol.* 305: G964–G974, 2013.

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8. Nalli AD, **Kumar DP**, Mahavadi S, Al-Shboul O, Alkhatani R, Kuemmerle JF, Grider JR, Murthy KS. Hypercontractility of intestinal longitudinal smooth muscle induced by cytokines is mediated by NFκB/AMP-activated kinase/MLC kinase pathway. *J Pharmacol Exp Ther* 350: 89–98, 2014.

9. Mahavadi S, Nalli AD, **Kumar DP**, Grider JR, Kuemmerle JF, Murthy KS. Cytokine-induced iNOS and ERK1/2 inhibit adenylyl cyclase type 5/6 (AC5/6) activity and stimulate phosphodiesterase 4D5 (PDE4D5) activity in intestinal longitudinal smooth muscle. *Am J Physiol Cell Physiol* 307: C402–C411, 2014.

10. Nalli AD, **Kumar DP**, Al-Shboul O, Mahavadi S, Grider JR, Murthy KS. Regulation of Gβγi-dependent PLC-β3 activity in smooth muscle: inhibitory phosphorylation of PLC-β3 by PKA and PKG and stimulatory phosphorylation of Gαi-GTPase activating protein RGS2 by PKG. *Cell Biochem Biophys* 70: 867–880, 2014.

11. Bala V, Rajagopal S, Mahavadi S, Nalli AD, **Kumar DP**, Zhou R, Sanyal AJ, Grider JR, Murthy KS. Release of GLP-1 and PYY in response to the activation of bile acid receptor TGR5 is mediated by Epac/PLCε-pathway and modulated by endogenous H_2S. *Front Physiol.* (Ahead of Print).

SCIENTIFIC MEETINGS/ABSTRACTS

1. Senthil Rajagopal, **Divya P. Kumar**, Sayak Bhattacharya, Sunila Mahavadi, Ruizhe Zhou, Carlos Corvera, Nigel Bunnett, John R. Grider and Karnam S. Murthy. Activation of G Protein-Coupled Bile Acid Receptor, TGR5 Induces Muscle Relaxation via PKA-and Epac-Mediated Inhibition of RhoA/Rho Kinase Pathway. Keystone Symposium, Canada, 2012.

2. **Divya P. Kumar**, Sunila Mahavadi, Senthilkumar Rajagopal, Faridoddin Mirshahi, Michael Fuchs, Karnam S. Murthy, and Arun J. Sanyal. Expression and Function of the Transmembrane Bile Acid Receptor TGR5 in human and murine Pancreatic β Cells. Digestive Disease Week, San Diego, 2012.

3. Sunila Mahavadi, Wimolpak Sriwai, **Divya P. Kumar**, Ruizhe Zhou, John R. Grider, Karnam S. Murthy. Down-regulation of microRNA-133a due to oxidative stress mediates up-regulation of RhoA expression and increase in Rho kinase activity and gastric muscle contraction in diabetes. Digestive Disease Week, San Diego, 2012.

4. Vanitha Bala, Senthilkumar Rajagopal, **Divya P. Kumar**, Sunila Mahavadi, Ruizhe Zhou, Zachary L. Bradley, Nigel W. Bunnett, Carlos U. Corvera, Johan Auwerx, John R. Grider, Arun J. Sanyal and Karnam S. Murthy. Hydrogen Sulfide (H_2S) Inhibits Bile Acid Receptor TGR5-Mediated GLP-1 Release from Enteroendrocrine Cells: Possible involvement of H_2S in Altered Glucose Metabolism in Diabetes. Digestive Disease Week, San Diego, 2012.

5. Senthilkumar Rajagopal, **Divya P. Kumar**, Sayak Bhattacharya, John R. Grider and Karnam S. Murthy. Characterization of Gz-Coupled Dopamine D3 Receptors in Gastric Smooth Muscle. Experimental Biology, San Deigo, 2012.

6. **Divya P. Kumar**, Sunila Mahavadi, Faridoddin Mirshahi, John R. Grider, Karnam S. Murthy, and Arun J. Sanyal. Expression and function of bile acid receptor TGR5 in glucagon secreting pancreatic α cells: hyperglycemia induced expression of PC1 and release of GLP-1 in response to activation of TGR5 by bile acids. Experimental Biology, San Deigo, 2014.

7. Sunila Mahavadi, Ancy D. Nalli, **Divya P. Kumar**, Sayak Bhattacharya, Ruizhe Zhou, John R. Grider and Karnam S. Murthy. Increased expression of caveolin-1 is associated with upregulation of the RhoA/Rho kinase pathway and smooth muscle contraction in diabetes. Experimental Biology, San Deigo, 2014.

8. **Divya P. Kumar**, Sunila Mahavadi, Faridoddin Mirshahi, John R. Grider, Karnam S. Murthy, and Arun J. Sanyal. Bile acids increase PC1 promoter activity and GLP-1 release via Gs-coupled TGR5 in pancreatic α cells of diabetic mice. Gordon Research Conference, Maine, 2014.