ADRENERGIC RECEPTOR GENE EXPRESSION DURING PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS

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CERTIFICATE

This is to certify that the thesis entitled **"ADRENERGIC RECEPTOR GENE EXPRESSION DURING PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS"** is a bonafide record of the research work carried out by **Ms ANI DAS. V.** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

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ABBREVIATIONS USED IN THE TEXT

5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydroxy tryptamine
5-HTP	5-hydroxy tryptophan
8-OH-DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bHLH	basic Helix-loop-helix protein
B_{max}	Maximal binding
BS	Brain stem
C/EBP β	CCAAT /Enhancer binding protein β
cAMP	cyclic Adenosine mono phosphate
CC	Cerebral cortex
CNS	Central nervous system
CSF	Cerbro spinal fluid
DA	Dopamine
DEPC	Di ethyl pyro carbonate
DNA	Deoxy ribonucleic acid
DTT	Dithiothreitol
ECD	Electro chemical detector
EGF	Epidermal growth factor
EPI	Epinephrine
GABA	Gamma aminobutyric acid
GK	Glucokinase
GOD	Glucose oxidase
GTP	Guanosine triphosphate
HBSS	Hanks Balanced Salt Solution
HGF	Hepatocyte growth factor
HNF	Hepatocyte nuclear factor
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
HYPO	Hypothalamus

i.p	Intraperitoneally
IAPP	Islet amyloid polypeptide
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
IGF	Insulin like growth factor
IP_3	Inositol triphosphate
K_d	Dissociation constant
MHC	Major histocmpatibilty complex
MIF	Macrophage migration inhibiting factor
MODY	Maturity onset diabetes in the young
mRNA	messenger Ribonucleic acid
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NE	Norepinephrine
NIDDM	Non-insulin dependent diabetis mellitus
NMN	Normetanephrine
p	Level of significance
p-CPA	para-Chlorophenylalanine
PDGF	Platelet Derived Growth Factor
PDX-1	Pancreas duodenum homeobox gene-1
PEG	Polyethylene glycol
Pi	Inorganic phosphate
PKC	Protein kinase C
PMSF	Phenyl methyl sulfonyl fluoride
POD	Peroxidase
RIA	Radioimmuno assay
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SDS-PAGE	Sodium dodecyl sulphate-poly acrilamide gel electrophoresis
STZ	Streptozotocin
T_3	Tri iodothyronine
T_4	Thyroxine
TGF	Transforming growth factor

TNF	Tumor necrosis fctor
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
VMH	Ventro medial hypothalamus

CONTENTS

1. INTRODUCTION

Diabetes mellitus results as a consequence of the body's inability to respond normally to high blood glucose levels. The onset of diabetes is due to several pathological changes, which are a reflection of either the inability of the pancreatic β-cells to secrete sufficient insulin to combat the hyperglycemia or a state of insulin resistance in target tissues. However, the significance of changes in β-cell mass and decreased β-cell proliferation or growth in progression of diabetes has not been given importance till recent years. β-cells, like all other cells of our body are under the regulatory checks and balances enforced by changes in cell cycle progression. However, very little is known regarding the key components of the cell cycle machinery regulating cell cycle control of β-cells. Knowledge of key elements involved in cell cycle regulation of β-cells will improve our understanding of the replication capacity and developmental biology of β-cells. This information is essential for us to design new approaches that can be used to correct β-cell deficiency in diabetes (Rane & Reddy, 2000).

The study of the growth potential of pancreatic β-cells has elicited considerable interest during recent years because of its implications for the better understanding of the pathogenesis of diabetes and potential treatment of this disease. Reduction in the β-cell mass is a critical clinical event in the development of insulin dependent diabetes mellitus which will lead to an insulin insufficiency. The acute onset of disease is preceded by a period of progressive destruction of pancreatic islets without replacement, until the remaining mass is sufficient to respond to hyperglycemia (Kloppel *et al.*, 1985). It would therefore be of interest to examine the regulation of islet cell growth and the factors that prevent or promote replacement of lost islet cells. Glucose homeostasis is tightly controlled by insulin (Ashcroft & Ashcroft, 1992). There is a minute to minute output from the β-cell to meet the changing demands. There is also a long term adaptation of insulin production by changes in total β-cell mass (Kahn, 1998; Taylor, 1999).

The pancreas is a an organ containing two distinct populations of cells, the exocrine cells that secrete enzymes into the digestive tract and the endocrine cells that secrete hormones into the blood stream. The endocrine cells are grouped into the islets of Langerhans, which are compact spheroidal clusters embedded in the exocrine tissue.

There are four principal cell types in the endocrine tissue - α-cells, which produce glucagon, β-cells produce insulin, *delta*-cells produce somatostatin and the *pp* cells whcih produce pancreatic polypeptides. The endocrine cells are believed to originate from the pancreatic duct, which is the source of endocrine stem cells. The exocrine and the endocrine tissues are capable of regeneration after injury, but the degree of regeneration is variable. The exocrine part respond to regeneration sooner than the endocrine part (Brockenbrough *et al.*, 1988).

A variety of factors are implicated in pancreatic regeneration of which insulin and the glucose are the principal regulators. Growth factors like growth hormone, prolactin, IL-β, interferons etc. have a regulatory control over the pancreatic regeneration (Vinik, 1992). Neurotransmitters are one of the important classes of the cell cycle regulators. They are involved in the insulin secretion also. Physiological studies have shown that the insulin secretion from pancreatic β-cells is regulated by the central nervous system through sympathetic and parasympathetic nerves. Catecholamines are found to be principal inhibitors of insulin secretion (Coore & Randle, 1964).

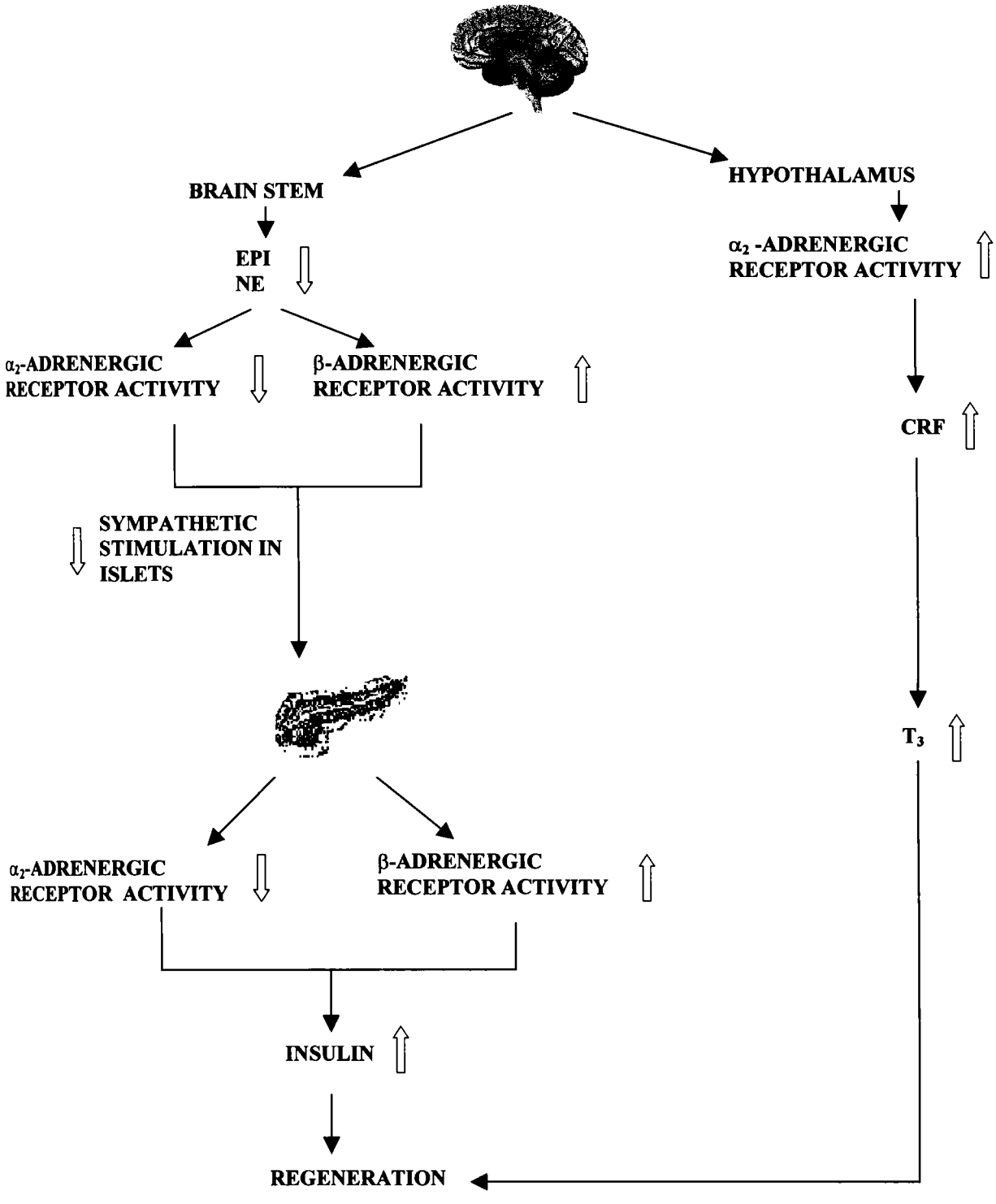
Epinephrine (EPI) and norepinephrine (NE) can regulate insulin secretion in a concentration dependent manner. At a lower concentration they can stimulate insulin secretion while at a higher concentration they can inhibit insulin secretion (Coore & Randle, 1964). It is already found that EPI and NE are increasing during diabetes (Tassava *et al.*, 1992; Fushimi *et al.*, 1984). They are also increasing with age and chances of getting diabetes is more with aging.

As EPI controls insulin secretion, it can regulate the pancreatic cell proliferation also. EPI and NE can mediate their function through same class of receptors called adrenergic receptors. There are mainly two classes of adrenergic receptors, α- and β-adrenergic receptors. α has two subtypes - α_1 and α_2; and β has - β_1, β_2 and β_3 subtypes. At a higher concentration EPI can bind to the α_2 adrenergic receptor and can inhibit both the insulin secretion and islet DNA synthesis. At a lower concentration EPI binds and activates β-adrenergic receptors which in turn stimulate insulin secretion and pancreatic regeneration (Sjoholm, 1991).

Capacity of pancreatic β-cells to proliferate, like other cell types, reflects the ability of cells to progress normally through the cell cycle. Defects or anomalies in proteins governing the regulated progression through the cell cycle may impair the capacity of β-cells to proliferate under conditions of increased functional demand on the β-cell mass, as is the case during hyperglycemia in diabetes. Modulation of cell cycle pathways in β-cells can provide alternative approaches to repopulate the β-cells in diabetic patients which will foster development of diabetes therapy.

In the present study, the changes in the brain EPI, adrenergic receptors and the receptor gene expression were investigated during pancreatic regeneration and insulin secretion. The changes in the pancreatic islet EPI and adrenergic receptors were also studied in the pancreatectomised rats. The regulatory function of EPI in association with EGF and glucose were investigated in rat islet cultures. *In vitro* studies were carried out using antagonists for adrenergic receptor subtypes to see their involvement in the islet DNA synthesis. The mechanism by which the peripheral EPI regulate insulin secretion was also investigated by studying the nuclear binding proteins in the pancreatic islets during pancreatic regeneration and diabetes.

ADRENERGIC REGULATION OF PANCREATIC REGENERATION AND INSULIN SECRETION



1.1 OBJECTIVES OF THE PRESENT STUDY

1. To study the DNA synthesis pattern in regenerating pancreas.

2. To study the epinephrine and norepinephrine content in the brain regions as well as in the pancreatic islets during regeneration.

3. To study the kinetic parameters of adrenergic receptor subtypes in brain regions - cerebral cortex, brain stem & hypothalamus and pancreatic islets of sham and pancreatectomised rats.

4. To study the circulating epinephrine and norepinephrine levels in the serum of sham and pancreatectomised young rats

5. To study the changes in the circulating insulin and T_3 levels of sham and pancreatectomised rats.

6. To study the expression of brain α_{2A}- and β-adrenergic receptor subtypes during regeneration by RT-PCR techniques.

7. To establish the role of adrenergic receptors in insulin secretion from pancreatic islets in 1 hr and 24 hr *in vitro* cultures.

8. To establish the role and mechanism of peripheral epinephrine in regulating insulin release by binding to specific nuclear protein.

9. To study the role of adrenergic receptor subtypes on islet DNA synthesis by using specific antagonists in *in vitro* in the cultures of pancreatic islets in combination with epidermal growth factor and transforming growth factor.

2. LITERATURE REVIEW

Diabetes, a disease documented in ancient Greek and Hindu writings, is among the top-ten causes of deaths in the world (Rane & Reddy, 2000; Rotter *et al.*, 1990; Ashcroft & Ashcroft, 1992). It is a disease that can arrive during the budding years (juvenile diabetes) or later (maturity or late-onset diabetes) in life. In either case, the life threatening complications associated with the disease remain the same. Despite being one of the oldest documented diseases, complete cure for the disease is still elusive which is primarily due to the lack of a complete understanding of the disease.

Diabetes results due to the inability of the body to effectively regulate the sugar balance leading to severe complications such as hyperglycemia (high blood glucose), obesity, neuropathy, nephropathy, retinopathy, limb disorders, bone disorders such as osteoporosis, coma and sometimes untimely death. The β-cells of the pancreas produce a protein, insulin, which monitors glucose level in the body. Normally, the extra-cellular concentration of glucose is restricted within a very narrow range, irrespective of variations in glucose availability and utilization. Homeostatic control of normal glucose level is achieved by co-ordinate secretion of insulin and glucagon. The basal rate of glucose utilization is approximately 10 grams per hour and to prevent hypoglycemia due to this utilization of glucose, the liver, the only source of endogenous glucose production, synthesizes glucose at a rate of 10 grams per hour. Approximately 75% of the hepatic glucose production is regulated by levels of glucagon, a product of pancreatic islet α-cells. Dysfunction of the α- and β-cells results in a disordered glucose homeostasis. If the β-cells do not respond to increased levels of glucose, hyperglycemia ensues where glucose levels exceed 10mM, a diagnostic feature of diabetes mellitus. Conversely, β-cell over-activity, observed in the case of insulinomas or β-cell tumors, leads to hypoglycemia with a possibility of brain cell injury and death (Rane & Reddy, 2000). In diabetic individuals, the regulation of glucose levels by insulin is defective, either due to defective insulin production (Type I diabetes) or due to insulin resistance (Type II diabetes).

2.1 Type 1 Diabetes or IDDM

Although, diabetes mellitus is defined simply on the basis of the ensuing hyperglycemia, it is a highly heterogeneous disease. The two forms of diabetes, IDDM

and NIDDM were distinguished in the late 1960s. This was followed by a realization that IDDM, presumably, had an autoimmune origin (Bach, 1994; Tisch & McDevitt, 1996). IDDM is a multifactorial disease with a polygenic inheritance. The genotype of the major histocompatibility complex (MHC) is the strongest genetic determinant. Several aspects of the etiology of IDDM, including the origin and pathogenesis, importance of genetic predisposition, interactions of environmental factors and characterization of the anti-β-cell immune response have been reviewed extensively (Ashcroft & Ashcroft, 1992; Bach, 1994; Tisch & McDevitt, 1996). Much of the current understanding of IDDM is based on studies using animal models, which serve as excellent tools for genetic and immunological manipulations that are impossible to carry out in human beings.

2.1.1 *Experimentally induced models*

Several experimental models have been described which provide clues to the etiology of IDDM. Streptozotocin (STZ) induced IDDM has been reported, wherein, the β-cell destruction is achieved by administration of high doses of selective β-cell toxic agent STZ (Like & Rossini, 1976; Kolb, 1987; Paik *et al.*, 1980). Repeated doses of STZ at sub-diabetogenic doses result in insulitis followed by diabetes which is immunologically mediated. Also, insulitis and diabetes can be induced in normal non-autoimmune adult rats by a combination of thymectomy and sublethal irradiation or in athymic rats by transfer of normal spleen cells (Fowell & Mason, 1993; Stumbles & Penhale, 1993; McKeever *et al.*, 1990). Transgenic mice with genetic manipulations have also provided good animal models for the study of IDDM. Selective β-cell specific expression of various transgenes can be induced, by coupling the transgenes to the insulin gene promoter. Insulitis, the primary characteristic of immunologically mediated diabetes can be induced upon transfer of the SV40 large T antigen in β-cells (Adams *et al.*, 1987). Similar results have been obtained upon transfer of the interferon alpha gene (IFNα), tumor necrosis factor (TNF) alpha and interleukin-10 genes (Stewart *et al.*, 1993; Higuchi *et al.*, 1992) (Picarella *et al.*, 1993; Wogensen *et al.*, 1993). Mice expressing the major histocompatibility complex (MHC) class I or class II genes and non-MHC molecules such as calmodulin can induce IDDM (Allison *et al.*, 1988; Markmann *et al.*, 1988; Gotz *et al.*, 1990; Epstein *et al.*, 1989). IDDM has always been recognized as a hereditary disease and familial transmission of the disease in humans, along with the data from animal models,

indicate that IDDM is both polygenic and multifactorial. This has lead to the identification of IDDM susceptibility loci in humans and the NOD mouse model. The studies provide evidence implicating both MHC-linked as well as non-MHC linked genes in the pathogenesis of IDDM (Ashcroft & Ashcroft, 1992; Bach, 1994; Tisch & McDevitt, 1996).

2.2 Type II Diabetes or NIDDM

Analogous to IDDM, pathogenesis of NIDDM is an equally complex manifestation of defects in several distinct metabolic functions of insulin and accounts for >90% of patients with diabetes (Ashcroft & Ashcroft, 1992; Taylor, 1999; Kahn, 1998). The main characteristics of NIDDM pathology being (a) peripheral insulin resistance in tissues such as skeletal muscle and adipocytes, leading to inefficient glucose uptake by these organs in response to insulin, (b) impaired insulin action to inhibit glucose production by the liver in the face of hyperglycemia and (c) aberrant insulin secretion leading to a decreased insulin output (Kahn *et al.*, 1996). NIDDM is a polygenic disease with a complex inheritance pattern. Moreover, like cancer, the incidence and degree of severity of NIDDM can be exacerbated by the presence of risk factors such as improper diet, lack of physical activity and age. Genetic factors determine the risk of developing NIDDM and susceptibility to insulin resistance and defects in insulin secretion appear to be genetically determined. The evidence of a genetic predisposition in the evolution of a diabetic phenotype is demonstrated by rare mutations in genes encoding glucokinase and transcription factors such as the hepatic nuclear factors (HNFs)-1alpha, -1beta and -4alpha, or IPF1, causing maturity onset diabetes in the young (MODY) (Yamagata *et al.*, 1996; Yamagata *et al.*, 1996; Stoffers *et al.*, 1997; Horikawa *et al.*, 1997).

2.3 FACTORS AFFECTING INSULIN REGULATION FROM PANCREATIC β-CELLS

2.3.1 *Glucose*

D-Glucose is the major physiological stimulus for insulin secretion. The mechanism of glucose induced insulin release is not completely understood. Phosphorylation of glucose to glucose-6-phosphate serves as the rate limiting step in

glucose oxidation (Schuit, 1996). Glucokinase acts as a glucose sensor during this process. Glucokinase is also linked to the phosphate potential, [ATP]/([ADP] [Pi]) (Sweet *et al.*, 1996). An increased ATP/ADP ratio is believed to close K^+-ATP channel at the plasma membrane, resulting in decreased K^+ efflux and subsequent depolarisation of the β-cell (Dunne, 1991). Depolarisation, activates voltage-dependent Ca^{2+} channels, causing an influx of extracellular Ca^{2+} (Liu *et al.*, 1996). Although intracellular Ca^{2+} activates protein kinases such as Ca^{2+} and calmodulin dependent protein kinase (Breen & Aschroft, 1997), it remains unclear how increases in intracellular Ca^{2+} leads to insulin release. Intracellular Ca^{2+} stores appears to regulate a novel plasma membrane current [Ca^{2+} release activated non-selective cation current I_{CRAN}], whose activity may control glucose activated secretion. Lesions in these pathways leads to the pathogenesis of diabetes mellitus (Dukes *et al.*, 1997). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C (PKC) within the β-cell (Harris *et al.*, 1996). It is suggested that PKC may be tonically active and effective in the maintenance of the phosphorylation state of the voltage-gated L-type Ca^{2+} channel, enabling an appropriate function of this channel in the insulin secretory process (Arkhammar *et al.*, 1994).

2.3.2 *Fatty acids*

Short chain fatty acids and their derivatives are highly active stimulators of insulin release in sheep (Horino *et al.*, 1968). A novel ester of succinic acid 1,2,3-tri-(methyl-succinyl) glycerol ester displayed stimulation of insulin release and biosynthetic activity in pancreatic islets of Goto-Kakizaki rats (Laghmich *et al.*, 1997). A monomethyl ester of succinic acid along with D-glucose is required to maintain the β-cell response to D-glucose (Fernandez *et al.*, 1996).

2.3.3 *Amino acids*

Amino acids also act as potent stimulators of insulin release. L-Tryptophan which is the precursor of 5-HT can act as a stimulator of insulin release (Bird *et al.*, 1980). L-Arginine also causes insulin release from pancreatic β-cells. Several *in vitro* studies have suggested the production of nitric oxides from islet nitric oxide system may have a negative regulation of the L-arginine induced secretion of insulin and glucagon in mice.

2.3.4 *Substrates derived from nutrients*

Substrates like pyruvate (Lisa *et al.*, 1994), citrate, ATP (Tahani, 1979), NADH and NADPH (Iain *et al.*, 1994) may involve indirect reflux stimulation triggered by food intake or local islet stimulation through the production of metabolites. Adenosine diphosphate acts as an intracellular regulator of insulin secretion. Heterotrimeric GTP-binding protein $G_{\alpha i}$ is involved in regulating glucose induced insulin release (Konrad *et al.*, 1995). GTP analogues are also important regulators of insulin secretion (Lucia *et al.*, 1987). Glucose induced insulin secretion in accompanied by an increase in the islet content of cAMP (Rabinovitch *et al.*, 1976).

2.3.5 *Glucagon*

Glucagon is the hormone secreted by pancreatic α-cells. It has been shown that glucagon has a striking stimulation of insulin release in the absence of glucose (Sevi & Lillia, 1966). The presence of specific glucagon receptors on isolated rat pancreatic β-cells as well as a subpopulation of α- and δ-cells shows the relevance of glucagon on regulation of insulin secretion. Intra-islet glucagon appears to be a paracrine regulator of cAMP *in vitro* (Schuit, 1996). Glucagon stimulates insulin release by elevating cAMP. cAMP through activation of protein kinase A, increases Ca^{2-} influx through voltage dependent L-type Ca^{2-} channels, thereby elevating $[Ca^{2-}]i$ and accelerating exocytosis (Carina *et al.*, 1993). Protein phosphorylation by Ca^{2-}/Calmodulin and cAMP dependent protein kinase play a positive role in insulin granule movement which results in potentiation of insulin release from the pancreatic β-cell (Hisatomi *et al.*, 1996).

2.3.6 *Somatostatin*

This hormone is secreted by the pancreatic δ-cells of the islets of Langerhans. Somatostatin inhibits insulin release (Ahren *et al.*, 1981). Its action is dependent on the activation of G-proteins but not associated with the inhibition of the voltage dependent Ca^{2-} currents or adenylate cyclase activity (Renstrom *et al.*, 1996).

2.3.7 *Pancreastatin*

Pancreastatin is known to be produced in islet β-cells and to inhibit insulin secretion. Pancreastatin is a modulator of the early changes in insulin secretion after

increase of glucose concentration within the physiological range (Ahren *et al.*, 1996). Pancreastatin is reported to increase Ca^{2+} in insulin secreting RINm5F cells independent of extracellular calcium (Sanchez *et al.*, 1992).

2.3.8 *Amylin*

Amylin is a 37-amino acid peptide hormone co-secreted with insulin from pancreatic β-cells. Amylin appears to control plasma glucose via several mechanisms that reduce the rate of glucose appearance in the plasma. Amylin limits nutrient inflow into the gut to blood and by its ability to suppress glucagon secretion, it is predicted to modulate the flux of glucose from liver to blood. Amylin is absolutely or relatively deficient in type I - diabetes and in insulin requiring type II - diabetes (Young, 1997). Islet amyloid polypeptide (IAPP) or amylin inhibits insulin secretion via an autocrine effect within pancreatic islets. Amylin fibril formation in the pancreas may cause islet cell dysfunction and cell death in type II - diabetes mellitus (Alfredo *et al.*, 1994).

2.3.9 *Adrenomedullin*

Adrenomedullin is a novel hypotensive adrenal polypeptide isolated from a human pheochromocytoma and is structurally related to calcitonin gene related peptide and islet amyloid polypeptide. It has been suggested that besides being an adrenal hypotensive peptide, adrenomedullin may be a gut hormone with potential insulinotropic function (Mulder *et al.*, 1996).

2.3.10 *Galanin*

Galanin is a 29 amino acid neuropeptide localised in the intrinsic nervous system of the entire gastrointestinal tract and the pancreas of man and several animal species. (Scheurink *et al.*, 1992). Among other functions galanin inhibits insulin release (Ahren *et al.*, 1991), probably via activation of G-proteins (Renstrom *et al.*, 1996) by the mediation of activated galanin receptors. However, galanin receptors are not as effective as α_2-adrenergic receptors in activating G-proteins.

2.3.11 *Macrophage migration inhibitory factor (MIF)*

MIF, originally identified as cytokines, secreted by T lymphocytes. It was found recently to be both a pituitary hormone and a mediator released by immune cells in

response to glucocorticoid stimulation. Recently it has been demonstrated that insulin secreting β-cells of the islets of Langerhans express MIF and its production is regulated by glucose in a time and concentration dependent manner. MIF and insulin were both present within the secretory granules of the pancreatic β-cells and once released, MIF appears to regulate insulin release in an autocrine fashion. MIF is therefore a glucose dependent islet cell product that regulates insulin secretion in a positive manner and may play an important role in carbohydrate metabolism (Waeber *et al.*, 1997).

2.3.12 *Other agents*

Coenzyme Q_{10} improved insulin release (Conget *et al.*, 1996) and it may also have a blood glucose lowering effect. Inositol hexa bisphosphate stimulates non-Ca^{-} mediated and purine-Ca^{2-} mediated exocytosis of insulin by activation of protein kinase C. (Efanov *et al.*, 1997). Small GTPases of the rab 3A family expressed in insulin secreting cells are also involved in the control of insulin release in rat and hamster (Regazzi *et al.*, 1996).

2.4 ROLE OF NEUROTRANSMITTERS IN INSULIN REGULATION

2.4.1 *Acetylcholine*

Acetylcholine is one of the principal neurotransmitters of the parasympathetic system. Acetylcholine, through vagal muscarinic and non-vagal muscarinic pathways (Greenberg & Pokol, 1994) increases insulin secretion (Tassava *et al.*, 1992). They function through muscarinic receptors present on pancreatic islet cells (Ostenson *et al.*, 1993).

2.4.2 *Dopamine*

High concentrations of dopamine in pancreatic islets can decrease glucose stimulated insulin secretion (Tabeuchi *et al.*, 1990). L-DOPA, the precursor of dopamine had similar effect to that of dopamine (Lindstrom & Sehlin, 1983). Dopamine D_3 receptors are implicated in the control of blood glucose levels (Alster & Hillegaart, 1996). Dopamine D_1 receptors have also been reported to be present on pancreatic β-cells (Tabeuchi *et al.*, 1990). These clearly indicate the role of dopamine in the regulation of pancreatic function.

2.4.3 *γ-Aminobutyric acid*

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in central nervous system. GABA is reported to present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson *et al.*, 1991). Glutamate decarboxylase, the primary enzyme that is involved in the synthesis of GABA, has been identified as an early target antigen of the T-lymphocyte mediated destruction of pancreatic β-cells causing insulin-dependent diabetes mellitus (Baekkeskov *et al.*, 1990). GABA through its receptors have been demonstrated to attenuate the glucagon and somatostatin secretion from pancreatic α-cells and δ-cells respectively (Gaskins *et al.*, 1995). GABA which is present in the cytoplasm and in synaptic-like microvesicles (Reetz *et al.*, 1991) is co-released with insulin from β-cells in response to glucose. The released GABA inhibits islet α-and δ-cell hormonal secretion in a paracrine manner. During diabetes the destruction of β-cells will lead to decrease in GABA release resulting in the enhancement of glucagon secretion from α-cells leading to hyperglycaemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central $GABA_A$ receptors increases plasma glucose concentration (Lang, 1995). Thus, any impairment in the GABAergic mechanism in central nervous system and/or in the pancreatic islets is important in the pathogenesis of diabetes.

2.4.4 *Serotonin*

Serotonin (5-HT) content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991); (Lackovic *et al.*, 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sumiyoshi *et al.*, 1997; Sandrini *et al.*, 1997). Ohtani *et al.*, (1997) have reported a significant decrease in extracellular concentrations of NE, 5-HT and their metabolites in the ventro medial hypothalamus (VHM). The ratio of 5-HIAA/5-HT was increased. A similar observation was reported by (Ding *et al.*, 1992) with a decrease in 5-HT in cortex (19%) and 5-HT turnover (5-HIAA/5-HT) that increased by 48%. Chu *et al.*, (1986) has reported lower 5-HT levels in both hypothalamus and brain stem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-HIAA and

accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993). There was a significant increase in 5-HIAA observed at 2-6 hours after insulin administration (Kwok & Juorio, 1987).

2.4.5 *Epinephrine and Norepinephrine*

These are secreted by the adrenal medulla. Norepinephrine (NE) is a principal neurotransmitter of sympathetic nervous system. These hormones inhibit insulin secretion, both *in vivo* and *in vitro* (Renstrom *et al.*, 1996; Porte, 1967). Epinephrine exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro *et al.*, 1996). NE and EPI, the flight and fright hormones, are released in all stress conditions and are the main regulators of glucose turnover in strenuous exercise (Simartirkis *et al.*, 1990). In severe insulin-induced hypoglycaemia, a 15 to 40 - fold increase of epinephrine plays a pivotal role in increasing glucose production independently of glucagon (Gauthier *et al.*, 1980). It is already known that, when used in high doses *in vivo* or *in vitro*, epinephrine reduces the insulin response to stimulators (Malaisse, 1972). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte *et al.*, 1966). They also inhibit insulin -stimulated glycogenesis through inactivation of glycogen synthase and activation of phosphorylase with consequent accumulation of glucose-6-phosphate. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phospho-fructokinase. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis. Adrenaline is, however, known to play a secondary role in the physiology of glucose counter-regulation. Indeed, it has been shown to play a critical role in one pathophysiological state, the altered glucose counter-regulation in patients with established insulin-dependent diabetes mellitus (Cryer, 1993). The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore and Randle (Coore & Randle, 1964), who incubated pancreatic tissue from the rabbit. As judged by Malaisse *et*

al., (1967)., the inhibitory effect of EPI on glucose-induced insulin secretion is mediated through the activation of α-adrenoreceptors

2.5 BRAIN NEUROTRANSMITTER CHANGES DURING DIABETES

A significant increase in the catecholamine contents and activity of metabolising enzymes has been reported in experimental diabetes (Gupta *et al.*, 1992). Norepinephrine has been reported to increase in several brain regions during diabetes (Tassava *et al.*, 1992; Fushimi *et al.*, 1984; Chu *et al.*, 1986; Oreland & Shasken, 1983; Wesselmann *et al.*, 1988; Chen & Yang, 1991), but a significant decrease in NE has been reported in hypothalamus (Ohtani *et al.*, 1997), pons and medulla (Ramakrishna & Namasivayam, 1995). EPI levels were significantly increased in the striatum, hippocampus and hypothalamus of diabetic rats and these changes were reversed to normal by insulin treatment (Ramakrishna & Namasivayam, 1995). Streptozotocin- induced diabetes and acute insulin deficiency were demonstrated to result in increased content of EPI in the supra chiasmatic nucleus. In addition to this, a decreased turnover of dopamine in the ventromedial nucleus in diabetes was found to be reversed by insulin treatment (Oliver *et al.*, 1989). These data indicate that experimental diabetes and acute insulin deficiency result in the rapid onset of detectable alterations in EPI and DA activity in specific hypothalamic nuclei. This can lead to the development of secondary neuroendocrine abnormalities known to occur in the diabetic condition. The DA content was increased in whole brain, (Lackovic *et al.*, 1990; Chen & Yang, 1991) corpus striatum (Chu *et al.*, 1986), cerebral cortex and hypothalamus of diabetic rats (Ohtani *et al.*, 1997; Tassava *et al.*, 1992). Serotonin (5-HT) content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991; Lackovic *et al.*, 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sumiyoshi *et al.*, 1997; Sandrini *et al.*, 1997). Brain tryptophan was also reduced during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993).

2.6 ADRENERGIC RECEPTORS

Adrenergic receptors belong to the large family of G-protein coupled receptors. These receptors form the interface between the sympathetic nervous system as well as many endocrine and parenchymal tissues (Hein & Kobilka, 1995). The most striking feature of adrenergic receptors is that they contain seven stretches of 20-28 hydrophobic amino acids that likely to represent membrane-spanning regions. Adrenergic receptors are mainly classified into α and β-adrenergic receptors. α-adrenergic receptors are then subdivided into α_1 and α_2 (Lefkowitz & Caron, 1988). α_1 has three subclasses- α_{1A}, α_{1B}, α_{1C}. (Price *et al.*, 1994) and α_2 has $\alpha_{2A/D}$, α_{2B} and α_{2C} (Hamamdzic *et al.*, 1995). β-adrenergic receptors are subclassified into β_1, β_2 and β_3 (Dohlman *et al.*, 1991). The regulation of adrenergic receptors is associated with G-proteins. Distinct, heterotrimeric G proteins, termed Gs and Gi, mediate the actions of the stimulatory and the inhibitory pathways, respectively (Gilman, 1984). EPI and NE bind to these receptors in a concentration dependant manner. At low concentration EPI and NE can bind and activate β-adrenergic receptors, which in turn stimulate the insulin secretion from pancreatic islets by activating adenylate cyclase through stimulatory G (Gs)-proteins. At high concentration they can bind to α_{2A} receptors and inhibit insulin secretion through inhibitory G_i proteins (Lacey *et al.*, 1993). α_1 receptors have also been observed to activate phospholipase A_2 and stimulate calcium influx through plasma membrane calcium channels.

2.6.1 α-adrenergic receptors

Based on the pharmacological and functional criteria, α-adrenergic receptors is further subdivided into two subclasses termed α_1 and α_2 adrenergic receptors (Langer, 1974; Hoffman & Lefkowitz, 1980).

2.6.1.1 *α_1 adrenergic receptors*

The concept of α_1-adrenoceptor subtypes was first suggested in the mid 1980s on the basis of the affinities of certain α_1-adrenoceptor agonists such as oxymetazoline, and the antagonists, WB4101 and phentolamine. α_1 adrenergic receptors have been implicated

in a variety of functions including smooth muscle contraction, regulation of hepatic glycogen metabolism, and also, in mitogenesis in certain tissues (Lomasney *et al.*, 1986). α_1-adrenergic receptors appear to be coupled to processes which lead to phosphatidylinositol 4,5-biphosphate hydrolysis (Berridge *et al.*, 1982). These receptors are found to increase the renal gluconeogenesis (Saggerson *et al.*, 1980). α_1 adrenergic receptors also play an important role in control of blood pressure via induction of vascular smooth muscle contraction (Minneman & Esbenshade, 1994). It is generally accepted that activation of α_1-adrenergic receptors stimulates phospholipase C, leading to increased hydrolysis of phosphatidylinositol 4,5-biphosphate to inositol 1,4,5-triphosphate and 1,2-diacylglycerol. Both these components play important roles as intracellular second messengers that increase intracellular Ca^{2+} concentrations and activate various isoforms of protein kinase respectively. These coupling mechanisms are typically mediated by perussis toxin-insensitive G-proteins, likely in the Gq/11 family. (Perez *et al.*, 1993). (Schwinn *et al.*, 1995). Additonally, stimulation of α_1-receptors activates phospholipase D and phospholipase A_2 via pertussis toxin-insensitive/ sensitive G-proteins (Minneman & Esbenshade, 1994). Although this predominant view of α_1 receptors signaling provides substantial insight into α_1-receptor-mediated responses in various cells, there are clear indications that these mechanisms may not explain all aspects of α_1-receptor signalling. Recent evidence demonstrates that α_1-receptor stimulated mitogenic responses in myocytes may due to activation of tyrosine protein kinases (TPKs) and MAP kinases (Thorburn *et al.*, 1994), suggesting that α_1-adrenergic receptors may share common signal pathways with tyrosine kinase receptors in the stimulation of mitogenesis.

[^{3}H]Prazosin, a hypertensive drug, which blocks these receptors, is widely used to study the characteristics of α_1 adrenergic receptors. Morrow *et al* (Morrow & Creese, 1986) suggest that [^{3}H]Prazosin labels subtypes of α_1-adrenergic receptor binding sites that are discriminated by the antagonists, phentolamine and WB4101, and the agonist pheylephrine. Apparently this drug has high affinity for post-synaptic α-receptors and relatively low affinity for pre-synaptic α-receptors (autoreceptors) (Cambridge *et al.*, 1977). Three α_1 adrenoreceptor subtypes have recently been identified by molecular cloning techniques: the α_{1A} (Stewart *et al.*, 1994), the α_{1B} (Voight *et al.*, 1990) and α_{1D} (Lomasney *et al.*, 1991) - adrenoreceptor subtypes. At the RNA level all three subtypes

appear to be present in the heart (Rokosh *et al.*, 1996). At the protein level, both the α_{1A} and α_{1D} adrenoreceptor subtypes have been reported to present in cardiac tissue, using selective receptor antagonists (Lazou *et al.*, 1994).

2.6.1.2. *α_2 adrenergic receptors*

α_2-adrenergic receptors mediate many of the physiological actions of the endogenous catecholamines, adrenaline and noradrenaline, and are targets of several therapeutic agents. α_2-adrenergic receptors are coupled by pertussis-toxin sensitive G proteins to various effectors, including adenylate cyclase and ion channels. The α_2-adrenergic receptors respond to endogenous NE and EPI to elicit a variety of physiological responses, including inhibition of neurotransmitter release, suppression of insulin release from pancreatic β-cells, activation of platelet aggregation, and contraction of arteriolar smooth muscle. The α_{2A}-adrenergic receptors subtype appears to mediate reduction in blood pressure following α_{2A} agonist administration (MacMillan *et al.*, 1998). Three human α_2-adrenoceptor subtype genes have been cloned and designated as $\alpha_{2\text{-}C10}$, $\alpha_{2\text{-}C4}$, and $\alpha_{2\text{-}C2}$, according to their location on human chromosomes 10, 4 and 2. They correspond to the previously identified pharmacological receptor subtypes α_{2A}, α_{2C} and α_{2B}. The receptor proteins share only about 50% identity in their amino acid sequence, but some structurally and functionally important domains are very well conserved. The most obvious functionally important differences between the receptor subtypes are based on their different tissue distributions; e.g. the α_{2A} subtype appears to be an important modulator of noradrenergic neurotransmission in the brain. The three receptors bind most α_2-adrenergic drugs with similar affinities, but some compounds (e.g. oxymetazoline) are capable of discriminating between the subtypes. All α_2-adrenoceptors couple to the pertussis-toxin sensitive inhibitory G proteins Gi and G(o), but recent evidence indicates that also other G proteins may interact with α_2-adrenoceptors, including Gs and Gq/11. Inhibition of adenylate cyclase activity, which results in decreased formation of cAMP, is an important consequence of α_2- adrenergic receptor activation (Aantaa *et al.*, 1995).

EPI and NE inhibit insulin secretion by α_2-adrenergic receptor activation. α_2-adrenergic receptor activation leads to inhibition of insulin release by a mechanism distal

to those regulating β-cell cyclic AMP production and [Ca^{2+}] (Ullrich & Wollheim, 1985). α_2-adrenergic receptor agonists are potent inhibitors of insulin release in the isolated islet preparation from rats (Morgan & Montague, 1985), as well as in mice *in vivo* (Skoglund *et al.*, 1986) and in man (Porte & Williams, 1966). The mechanism of action of α_2-adrenergic receptor agonists in mediation of the hyperglycaemic response is of peripheral origin and involves pancreatic β-cell post synaptic α_2-adrenergic receptors, possibly through the inhibition of insulin release (Angel & Langer, 1988). α_2-adrenergic receptors are known to have a critical role in regulating neurotransmitter release from sympathetic nerves and from adrenergic neurons in the central nervous system (Miller, 1998; Langer, 1997). Hein *et al.* (1999) have now studied neurotransmitter release in mice in which the genes encoding the three α_2-adrenergic receptor subtypes were disrupted. They found that both the α_{2A} and α_{2C}-subtypes are required for normal presynaptic control of the transmitter release from sympathetic nerves in the heart and from central noradrenergic neurons. α_{2A}-adrenergic receptors inhibit transmitter release at high stimulation frequencies, whereas the α_{2C}-subtype modulates neurotransmission at lower levels of nerve activity (Hein *et al.*, 1999).

EPI and other adrenoceptor agonists are perviously shown to induce a hyperglycaemic response following *in vivo* administration. Clonidine was used as a potent agonist for inducing hyperglycaemia by activating α_2-adrenoceptors (DiTullio *et al.*, 1984). DPI (3,4-dihydroxyphenylimino)-2-imidazolidine, a peripherally active adrenoceptor agonist, and UK 14.304, a highly selective α_2-adrenoceptor agonist, also could induce hyperglycaemia similar to clonidine (Angel & Langer, 1988). In as much as α_2 agonists have been available exclusively selective probes for α_2 adrenoceptors for several years, previous autoradiographic studies have used [^{3}H]p-Aminoclonidine and [^{3}H]Clonidine for the visualization of α_2-adrenoceptors (Unnerstall *et al.*, 1984). However, the recent development of the potent and selective radiolabelled α_2-antagonists, [^{3}H]Rauwolscine and [^{3}H]Idazoxan, has allowed to localise their respective binding sites in the rat brain (Boyagian *et al.*, 1987). The pharmacological characterisation of α-adrenoceptors has been facilitated by the introduction of [^{3}H]Yohimbine. Its use has shown that the absolute affinities and rank order of potency of a number of antagonists for

the α_2-adreneoceptor binding sites on human platelets differ from those on rat cerebral cortex membranes (Cheung *et al.*, 1982).

2.6.2 β-adrenergic receptors

Adrenergic receptor is a member of the large family of G protein-coupled receptors and is subjected to a complex regulation by hormones and other signalling molecules. The catecholamines, EPI and NE evoke specific responses in a variety of tissues. The β-adrenergic system has in many ways served as the premier model in which to investigate the processes by which external stimuli regulate cellular behavior. Stimulation of β-adrenergic receptor normally results in signalling by the heterotrimeric G_s protein, leading to the activation of adenylate cyclase, production of cAMP, and activation of cAMP-dependent PKA. Gu *et al.*, (2000) reported that cell death of thymocytes can be induced after stimulation of β-adrenergic receptor, or by addition of exogenous cAMP.

The concept of two subtypes of β-adrenergic receptors termed β_1 and β_2 developed by Lands *et al.* in 1967 was initially interpreted in terms of absolute organ specificity such that the heart contained exclusively β_1 and the bronchial system contained β_2 adrenergic receptors (Lands *et al.*, 1967; Lands *et al.*, 1967). Later in 1983 Tan and Curtis-Prior (Tan & Curtis-Prior, 1983) proposed the presence of another subtype of β-adrenergic receptor in the rat adipocytes which was then termed as β_3- adrenergic receptors. Isoprenaline, CGP 12177, BRL 37344 and NE are used as potent agonists of β-adrenergic receptors. The antagonists of β-adrenergic receptors widely used are propranolol, atenolol, betaxolol and practalol (Arch & Kaumann, 1993).

2.7 ADRENERGIC RECEPTORS IN DIABETES

Previous studies have shown that in diabetic condition α_{2A}-receptors are more activated which brought out the insulin inhibition and in turn hyperglycaemia (Lacey *et al.*, 1993). Rat islet cell membrane is equipped with α_{2A} (Filipponi *et al.*, 1986) which are linked to adenylate cyclase and inhibits insulin secretion. Studies conducted in C57BL/KsJ mice revealed that all of the α_1- and α_2-adrenergic receptor population were

elevated in the regional brain samples of diabetic compared with controls. However, β-adrenergic receptor populations were depressed in diabetes compared with age-matched controls (Garris, 1990). Studies from our lab, have shown that α_1-adrenoceptors expressed altered affinity in hypothalamus and brain stem of STZ-diabetic rats (Pius, 1996).

2.8 PANCREATIC β-CELL MASS AND DIABETES

Pancreatic β-cell growth is a cumulative effect of the following three phenomena during β- cell development (i) differentiation of β-cells from precursors, a process referred to as neogenesis (ii) changes in the size of individual β-cells and (iii) replication capacity of existing β-cells (Swenne, 1992). The differentiation or neogenesis of β-cells has been extrapolated upon detection of insulin-positive β-cells in pancreatic ducts. There is strong evidence to the existence of neogenesis as a plausible mechanism for changes in β-cell mass based on studies in rat models (Eriksson & Swenne, 1982; Swenne & Erikkson, 1982). In contrast, changes in size of individual β-cells is not very well documented, even though, glucose, which is the prime stimulator of β-cell replication, increases β-cell size and apparently leads to increased insulin synthesis (Borg & Andersson, 1981).

The number of functionally intact β-cells in the islet organ is of decisive importance for the development, course and outcome of diabetes mellitus. Generally speaking, the total β-cell mass reflects the balance between the renewal and loss of these cells. While factors resulting in damage and degeneration of the β-cells have attracted much attention there has been relatively little interest in the kinetics and regulation of β-cell proliferation. In early fetal life both the endocrine and exocrine pancreatic cells probably arise from a common "protodifferentiated" cell type. The renewal of β-cells in diabetes has been studied in several animal models. Both in experimental diabetes, such as that induced by alloxan, and in hereditary diabetes, exemplified by the diabetic mutant mouse (gene symbol db), there is initial stimulation of the mitotic rate during development of hyperglycaemia followed by a notable decrease in the β-cell renewal. In the diabetic mouse the decreased proliferation of β-cells is paralleled by the appearance of fulminant diabetes. These data suggest a limited capacity for β-cell proliferation, which may differ

between species. According to this hypothesis, there is an increased risk of developing diabetes once the predetermined potential for β-cell division has been exhausted (Hellerstrom *et al.*, 1976).

Hyperglycaemia, even when mild, can attenuate the secretory response of pancreatic β- and α-cells to increments in glucose and can impair insulin-mediated glucose transport, thus impeding its own correction and initiating a cycle of progressive self-exacerbation and metabolic deterioration. Both reduced islet function and insulin action may be the consequence of a generalized down-regulation and/or occupation of glucose transporters by hyperglycaemia so that the islets respond less to further increments in glycaemia. The postulated hyperglycaemic cycle can be initiated by any environmental perturbation that increases insulin demand in previously normoglycaemic patients in whom insulin secretion has already reached a maximum level of compensation for peripheral insulin resistance (Unger & Grundy, 1985). Sreenan *et al.*, (1999), observed in the NOD mouse, β-cell destruction begins soon after the onset of insulitis. Diabetes may be present when residual β-cell mass represents 30% of control levels. Defects in any one of the three pronged regulatory mechanisms, viz. insulin synthesis, insulin secretion and changes in β-cell mass, result in a relative or complete insulin deficiency leading to diabetes (Ashcroft & Ashcroft, 1992; Taylor, 1999; Kahn, 1998).

In Type 1 diabetes, β-cell mass is depleted due to autoimmune destruction and the remaining β-cells are insufficient in mounting a growth response to counter the increasing hyperglycaemia. Although, the pathogenesis of Type II diabetes is multifactorial and less well defined, there is increasing evidence that defective β-cell replication and growth may constitute an additive predisposition to the development of the disease. It is likely that in the face of defective β-cell growth, followed by insulin deficiency may develop diabetes (Kloppel *et al.*, 1985). The low growth rate and the reduced proliferation potential in Type II diabetes could be due to defects in growth regulatory proteins. However, studies aimed at identifying the key growth modulatory genes in impairment of β-cell growth and its low proliferation potential have yielded very few clues regarding mechanisms responsible for the deficient β-cell mass in these patients (Mares & Welsh, 1993; Welsh *et al.*, 1993).

2.9 PANCREATIC REGENERATION

It is generally agreed that the pancreatic β-cell plays a key role in the aetiology of diabetes mellitus. Insufficient production of biologically active insulin is a common denominator in almost all forms of diabetes and the degree of insulin deficiency determines both the severity of disease and the choice of therapy. This has stimulated world wide research efforts to elucidate the function and the natural history of the β-cell, leading to spectacular advancements in the basic understanding of the cell. Most research has been concerned with the regulation and molecular biology of insulin biosynthesis and release, and that other aspects of islet histophysiology have remained relatively neglected, despite their potential importance for a full understanding of the role of β-cell in diabetes.

The concept of the β-cell was born in 1907 (Lane, 1907; Bensley, 1911). At that time nothing was known to the hormone-producing capability of the β-cell, but there was nevertheless morphological evidence saying that this cell type would some how be involved in the development of diabetes (Schaefer, 1895; Laguesse, 1893). Today the β-cell is defined not only by its histological or cytochemical staining characteristics, but also by its ability to express a complicated set of genes which provide the cell with a unique mechanism to synthesize and store insulin and to release the hormone in exact concert with the peripheral demand. The β-cell may also express on its plasma membrane certain antigens which are specific for the cell type and which may lead to autoimmune reactions (MacLaren *et al.*, 1975) (Lernmark *et al.*, 1978; Dyrberg *et al.*, 1982; DeWinkel *et al.*, 1982). Each of these properties serves to distinguish the β-cell from other cells of the body and may be specifically involved in the pathogenesis of diabetes.

The number of functionally intact β-cells in the islet organ is of decisive importance for the development, course and outcome of diabetes mellitus. Generally speaking, the total β-cell mass reflects the balance between the renewal and loss of these cells. In early foetal life both the endocrine and exocrine pancreatic cells probably arise from a common "protodifferentiated" cell type. The islets subsequently grow according to a regular pattern characterised by a symmetrical distribution of the islet volume in relation to islet diameter. The growth of the islets reflects the replication of both α- and β-cells in individual islets. Whether new islet cells are derived also from duct epithelium or by

transformation of differentiated acinar cells remains a matter of controversy (Hellerstrom *et al.*, 1976). There is now evidence to suggest that β-cells arises from a pool of undifferentiated precursor cells in the fetal and newborn pancreas. These cells may contribute to islet growth and, if inappropriately stimulated, also to early islet hyperplasia. In the postnatal state, β-cell function is finely tuned by a complex set of incoming signals, one of which is the nutrient supply provided by the blood. Recent studies indicate that a disproportionately high fraction of pancreatic blood is diverted to the islets and that the islet blood flow is increased by glucose.

An acute stimulus to insulin release may be accompanied by a process, which enhances the distribution of the hormone to the target cells. Adaptive growth responses to an increased insulin demand occur in a number of hereditary diabetic syndromes in animals, but in some of these there is an inherited restriction on the capacity for β-cell proliferation leading to further deterioration of the glucose tolerance. Some evidence suggests that a similar mechanism may operate also in human non-insulin-dependent diabetes.

In eukaryotes, progression of the cell cycle is associated with periodic transcription activation/repression of growth-regulatory genes. Although the functional connections between transcription and cell-cycle regulators is far from being understood, recent progress has been made in connecting cell-cycle progression to dedicated components of the RNA polymerase II transcription apparatus complex (Lania *et al.*, 1999). Efrat, (1998) developed a number of highly differentiated β-cell lines in transgenic mice. These cells produce insulin amounts comparable to normal pancreatic islets and release it in response to physiological insulin secretagogues.

2.10 DIFFERENTIATION OF THE PANCREATIC β-CELL

Knowledge of the embryonic origin of the β-cell will provide important information on β-cell function later in life. It is unfortunate that methods are not available to identify directly an apparently non-differentiated precursor cell, committed to become a β-cell. In the rat foetus the growth of the β-cell mass between gestation days 20 and 22 is considerable, with a total increase of more than 100% in 48hrs. However, growth that can be accounted for the formation of new β-cells from pre-existing β-cells is only 20% and

the remaining 80% must be accounted for the mechanisms other than β-cell division (Erikkson & Swenne, 1982; Swenne & Erikkson, 1982). Recent evidence suggests that, in the early postnatal period also, differentiation of precursor cells to β-cells might contribute to islet cell growth. It was reported that when STZ was injected into 1-2 day old rats, the ensuing hyperglycemia was only transient and was completely reversed by the 14th postnatal day (Portha *et al.*, 1974; Weir *et al.*, 1981). These changes were accompanied by marked initial destruction and loss of β-cells followed by active repair (Bonner-Weir *et al.*, 1981, Cantenys *et al.*, 1981, Dutrillaux *et al.*, 1982). The latter process was characterised by the appearance of numerous insulin positive cells throughout the exocrine parenchyma and in the duct epithelium. Budding of the islets from ducts was a prominent feature. These observations suggest a rapid formation of β-cells, primarily through multiplication and differentiation of precursor cells, which may be located both in the acinar part and the ducts. It is still unclear whether precursor cells contribute to islet growth in the adult animals. While *in vitro* studies with foetal and neonatal pancreas strongly suggest that new islet tissue is derived from ductal epithelium, it is not established whether the primary cell is a committed endocrine cell or duct-like cell capable of trans-differentiation (Rosenberg, 1995).

Sarvetnick & Gu, in 1992 have shown that the pancreatic duct cells of IFN-gamma mouse are actively multiplying and that many duct cells differentiate to become endocrine cells. This islet regenerating process closely parallels the islet development during normal organogenesis in the foetus and offers a model for studying the cell lineage relationships of islet cells. They found that duct cells retain the ability to proliferate and to differentiate into islet cells. Under normal conditions, duct cells do not continue to multiply or to differentiate.suggesting that in the transgenic mice, the progenitor cells of embryonic multipotential duct cells transform into adult cells, but in the presence of appropriate signals or stimuli can resume their multipotential property.

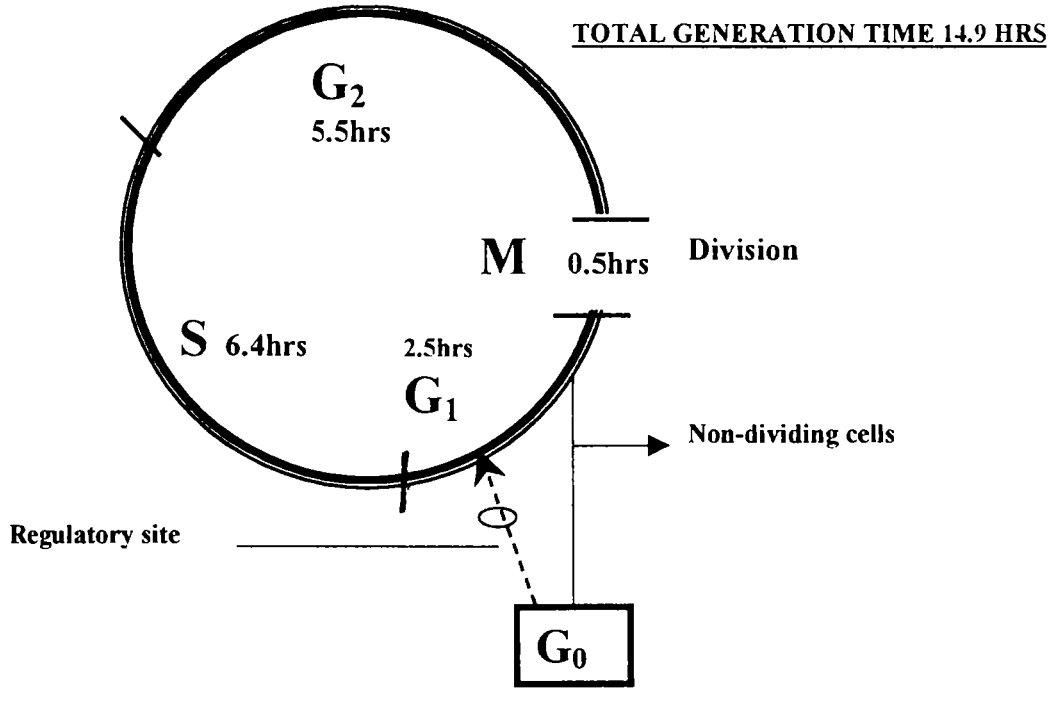
The ability of the adult pancreas to generate new insulin β-cells has been controversial because of difficulties in identifying the precursor population. Fernandes *et al.* (1997) recently determined that β-cells were generated during development from precursors that expressed the homeodomain-containing transcription factor, pancreas

duodenum homeobox gene-1 (PDX-1) and the existence of PDX-1 in the β-cell precursors and indicate that their differentiation is induced by islet injury (Fernandes *et al.*, 1997).

2.11 MECHANISM OF PANCREATIC β-CELL GROWTH

Pancreatic β-cells, like all other cells of our body are under the regulatory checks and balances enforced by changes in cell cycle progression. However, very little is known regarding the key components of the cell cycle machinery regulating cell cycle control of β-cells. Knowledge of key elements involved in cell cycle regulation of β-cells will go a long way in improving our understanding of the replication capacity and developmental biology of β-cells (Rane & Reddy, 2000). Like other somatic cells, β-cell passes through a cell cycle, which can be subdivided into several distinct phases. By knowing the normal β-cell cycle is of considerable importance for a full understanding of the mechanism β-cell proliferation.

THE CELL CYCLE OF THE PANCREATIC β-CELL



By using isolated foetal rat islets (Hellerstrom *et al.*, 1979), in which the progress of the β-cells through the cycle had been synchronised *in vitro* with the aid of hydroxyurea, Swenne recently made an extensive study of the lengths of the various

phases (Swenne, 1982; Swenne, 1982). The β-cell cycle could be subdivided into a G_1 phase of 2.5hrs, an S phase of 6.4hrs, a G_2 phase of 5.5hrs and a mitosis time of 0.5hrs, with a total generation time of 14.9hrs.

The pancreas is an organ containing two distinct populations of cells, the exocrine cells that secrete enzymes into the digestive tract, and the endocrine cells that secrete hormones into the blood stream. It arises from the endoderm as a dorsal and a ventral bud which fuse together to form the single organ. Mammals, birds, reptiles and amphibians have a pancreas with similar histology and mode of development, while in some fish, the islet cells are segregated as Brockmann bodies. Invertebrates do not have a pancreas, but comparable endocrine cells may be found in the gut or the brain. In the developing pancreatic buds, the endocrine cells start to differentiate before the exocrine cells, and co-expression of different hormones by the same cell is often observed at early stages. Although pancreatic endocrine cells produce many gene products also characteristic of neurons, evidence from *in vitro* cultures and from quailchick grafts shows that they are of endogenous and not of neural crest origin. Observational studies suggest strongly that both endocrine and exocrine cells arise from the same endodermal rudiment. Development of the pancreas in embryonic life requires a trophic stimulus from the associated mesenchyme. In postnatal life, all cell types in the pancreas continue to grow. Destruction of acinar tissue by duct ligation or ethionine treatment is followed by rapid regeneration. Surgical removal of parts of the pancreas is followed by moderate but incomplete regeneration of both acini and islets. Poisoning with alloxan or streptozotocin can lead to permanent depletion of β-cells (Slack, 1995).

2.12 FACTORS REGULATING PANCREATIC β-CELL GROWTH

The factors regulating islet cell proliferation and differentiation may permit proto-undifferentiated cells and islets to be grown in culture and to induce endocrine cell differentiation *in vitro*. Furthermore, islet cell growth factors could be used to provide 'trophic support' to islet transplants maintaining graft viability. There may also be greater scope for gene therapy when the growth factors have been isolated, purified, sequenced and cloned (Vinik *et al.*, 1993). The adult β-cell is normally virtually quiescent, but its replicatory activity can be enhanced *in vitro* by certain nutrients and growth factors. The long-term alterations in β-cell mass constitute an important means to accommodate an

increased demand for insulin (Sjoholm, 1996). Recent studies have revealed that islet cells differentiate from the epithelial cells of primitive pancreatic ducts during embryogenesis, and can regenerate in response to the loss of islet cells even in adult pancreas. The ability of islet cells to regenerate raises the possibility that impaired and decreased islets of diabetic patients can be restored.

Among the various factors, glucose is a prime regulator of β-cell replication and is known to stimulate replication in both fetal and adult rodent islets (Hellerstrom & Swenne; 1985; Swenne, 1982; Swenne, 1983). Insulin and IGF-1 stimulate islet β-cell replication in neonatal rodent pancreatic cells in culture providing evidence that insulin itself can regulate the replication capacity of β-cells in an autocrine fashion (Rabinovitch *et al.*, 1982). This study prompted the examination of several other growth factors for their role in regulating β-cell replication (Hill *et al.*, 1998). Thus, growth hormone (GH), prolactin and the related placental lactogen, IGF-1, IGF-2 and platelet-derived growth factor (PDGF) have been recognized as stimulators of β-cell replication (Nielsen, 1982; Brelje *et al.*, 1989; Brelje & Sorenson, 1991; Swenne *et al.*, 1987; Swenne *et al.*, 1988). Growth hormone has been reported to stimulate the *in vitro* replication of foetal, neonatal and adult rat β-cells. The stimulation of replication activity resulted in an increased insulin content and secretion where the effects of GH were mimicked by prolactin and its related peptide, placental lactogen (Cantenys *et al.*, 1981; Yamaoka & Itakura, 1999). GH elicits many of its actions by inducing local production of IGFs in target cells. Studies aimed at investigating a similar paracrine pathway operative in islet cells have yielded confusing results. GH, but not glucose, stimulated the release of IGF-1 from foetal and adult rat islets leading to mitogenesis (Swenne *et al.*, 1987; Swenne *et al.*, 1988; Swenne & Hill, 1989). The presence of high-affinity IGF-1 receptors on β-cells and the finding that exogenous IGF-1 stimulates β-cell replication (Schravendijk *et al.*, 1987), supported a concept that GH mitogenic activities might be mediated by a paracrine regulation involving IGF-1. Other factors which lead to a stimulation of β-cell replication include, amino acids (Swenne *et al.*, 1980), lithium (Sjoholm *et al.*, 1992), the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sjoholm, 1991), nicotinamide (Sandler & Andersson, 1986), amniotic fluid (Dunger *et al.*, 1990) and serum (Hellerstrom & Swenne, 1985).

2.12.1 *Glucose*

Glucose is one of the stimulators of β-cell replication activity. There is evidence of compensatory growth of β-cells in adult rats after short-term glucose infusion (Bonner-Weir *et al.*, 1989). Factors believed to increase the rate of replication *in vivo* include a high caloric intake, hypoglycemic sulfonylureas, various hormones and hyperglycemia. Recent studies *in vitro* have so far confirmed the mitogenic action of a high extracellular glucose concentration (Hellerstrom *et al.*, 1976). Chronic exposure to glucose in excess of basal levels can induce insulin secretion in the neonatal β-cells by increasing the cAMP levels. In late foetal life, glucose is also a strong stimulus to replication, and metabolism of glucose is a prerequisite for this process. Glucose stimulates proliferation by recruiting β-cells from a resting state into an active division state. Severe hyperglycemia, at least when induced in rats, seems to retard rather than stimulate β-cell growth (Hellerstrom & Swenne, 1991).

2.12.2 *Insulin*

Recent observations indicate that insulin can stimulate pancreatic islet β-cell growth *in vivo*. McEvoy and Herge reported that administration of insulin to diabetic rats implanted with foetal pancreas resulted in a three-fold increase in β-cell mass in some of the pancreatic recipients (McEvoy & Hegre, 1978). Rabinovitch *et al* have demonstrated that insulin can stimulate islet β-cell replication directly, possibly through a receptor for multiplication stimulating activity or another insulin like growth factor (Rabinovitch *et al.*, 1982). Insulin favored regeneration of β-cell, by activating the neogenesis of the β-cells from precursor cells (Movassat *et al.*, 1997). It is reported that mannoheptulose, an agent believed to inhibit insulin release, inhibits β-cell replication *in vitro* (King *et al.*, 1978). It has been reported that foetal rat pancreas explanted *in vitro* in the presence of added insulin had greater β-cell volume and a greater insulin content than those grown without insulin (McEvoy, 1981).

2.12.3 *Role of growth factors*

There are several reports on effects of growth factors in the normal β-cell growth. The growth hormone, prolactin and placental lactogen were found to stimulate proliferation of normal rat β-cells (Nielsen, 1986). Among the large number of protein hormones GH and lactogenic peptides, prolactin (PRL) and placental lactogen (PL) have an important role in β-cell proliferation. GH has been reported to stimulate the *in vitro* replication of foetal (Dudek *et al.*, 1984), neonatal (Brelje *et al.*, 1989) and adult rat β-cells (Swenne & Hill, 1989). In most of the studies there was also a stimulatory effect of GH on the insulin content or secretion, and the majority of effects were mimicked by PRL and PL. GH appears to elicit its biological activities by inducing local production of insulin-like growth factors (IGF) in target cells (Milner & Hill, 1984). It is reported that in both fetal and adult islets growth factors, but not glucose, stimulated release of IGF-I partially counteracted the mitogenisity of GH (Swenne *et al.*, 1987; Swenne & Hill, 1989). Culture of islets with Platelet Derived Growth Factor (PDGF) and IGF-I caused an increase in the islet content of polyamines resembling the effect of GH. These two growth factors elicited a stimulation of DNA synthesis in islets (Sjoholm *et al.*, 1990). Epidermal growth factor is known to stimulate DNA replication in many systems like hepatocytes, pituitary cells etc. Chatterjee *et al.*, (1986) have shown that EGF stimulates proinsulin biosynthesis as well as [^{3}H]thymidine uptake into pancreatic islets. They suggested that EGF behaves like glucose in stimulating both insulin biosynthesis and β-cell replication.

2.12.4 *Amino acids and polyamines*

The amino acid enrichment in the organ culture appears to favour the growth of pancreatic rudiments suggesting that metabolites other than glucose might influence the development of pancreatic β-cells. The mechanism by which amino acids provoke an increased response of growth is unknown. DeGasparo *et al.*, (1978) have shown that enrichment of amino acids in the culture medium is a factor which induce the growth of β-cells in organ culture. Amino acids are also able to stimulate β-cell replication, and it appears in the early foetal life as they are more important than glucose in this respect (DeGasparo *et al.*, 1978). Amino acids, and human amniotic fluid, were recently also identified as potent stimulators of cell proliferation in adult mouse islets (Dunger *et al.*,

1990). It is shown that glucose regulates polyamine content *in vitro*. Polyamines like, putrescine and spermidine are necessary for the maintenance of normal insulin and protein biosynthesis, whereas spermine may exert a role in some other cellular processes such as DNA replication, RNA transcription and glucose stimulated insulin release (Welsh & Sjoholm, 1988).

2.12.5 *Regulatory proteins*

A pancreatic gene celled Reg., encoding a 165-amino acid protein was isolated from regenerating rat islets after partial pancreatectomy. The *reg* gene is expressed in experimentally induced regenerating or hyperplastic islets. Unno *et al.* reported that ectopic expression of the reg gene occurs in some human colonic and rectal tumors, suggesting that enhanced reg expression may be related to the proliferative state of tumor cells. At present, any direct relationship between reg protein and β-cell replication remains to be established. However, since the reg protein is a secretory protein and reg can be expressed at an early stage of pancreatic cell differentiation, the reg protein may act on the stem cells of β-cells in an autocrine or paracrine manner. In normal mature exocrine cells, the reg gene is expressed and the gene product may be necessary to maintain adequate exocrine pancreatic function (Unno *et al.*, 1992). The reg protein is synthesised and secreted from regenerating β-cells, and that the expression of reg was closely associated with β-cell regeneration. Recently, reg protein was shown to stimulate pancreatic β-cell growth, further strengthening the notion that reg is involved in pancreatic islet growth and regeneration (Watanabe *et al.*, 1994).

2.12.6 *Inhibitors of pancreatic beta cell proliferation*

Inhibitors of pancreatic β-cell proliferation include transforming growth factor β (TGF-β), the cytokine interleukin 1β (IL1β), pancreastatin and the diazepam binding inhibitor, all of which inhibit fetal rodent β-cell proliferation. TGF-β is a multifunctional cytokine which has implicated in various biological processes including an inhibition of the epithelial, endothelial and hemopoetic cell proliferation. Recent studies have demonstrated an involvement of TGF-β in the pathogenesis of acute and chronic pancreatitis (Gress *et al.*, 1994). TGF-β inhibits glucose stimulated β-cell replication

(Sjoholm & Hellerstrom, 1991). Poypeptides like cytokines has also role in β-cell proliferation because it helps in the secretion of macrophages (Jiang & Woda, 1991) which is known to precede the onset of clinically manifest diabetes mellitus. Interleukin-1β can exert cytotoxic and cytostatic actions on β-cells in culture. Interleukin-1β can function as an inhibitor of rat insulinoma cell proliferation (Sandler *et al.*, 1989) and of adult islet cell replication in rats and mice (Southern *et al.*, 1990; Sandler *et al.*, 1991). However, the role of IL1-β in fetal islet cell proliferation is slightly complex with the first 24 hrs of stimulation leading to a suppression of β-cell proliferation followed by a potent mitogenic stimulus after 3 days of cytokine exposure. Sjoholm *et al.*, (1991) identified pancreastatin and diazepam-binding inhibitor (acyl-CoA binding protein) as inhibitors of β-cell replication. Both pancreastatin and diazepam-binding inhibitor are produced by islet cells (Chen *et al.*, 1988; Tatemoto *et al.*, 1986; Efendic *et al.*, 1987) and inhibit insulin secretion and may function as inhibitors of β-cell replication *in vivo*.

2.13 MODELS OF PANCREATIC β-CELL PROLIFERATION IN DIABETES

Since the β-cell is the only source of insulin production, mechanisms responsible for regeneration of β-cells lost or severely reduced in diabetes have been a focus of several studies. These studies have led to the generation and characterisation of many animal models, which have yielded important clues regarding the regenerative capacity of β-cells. Pancreatic β-cell toxins, alloxan or streptozotocin, have been used to selectively destroy β-cells and produce an IDDM-like state. Pancreatic β-cells, which survive the massive destruction in response to these reagents are capable of replicating, suggesting a replication capacity in a fraction of β-cells (Korcakova, 1971; Steiner *et al.*, 1970). The degree of β-cell regeneration, however, is insufficient to cure diabetes (McEvoy & Hegre, 1977). Administration of streptozotocin to neonatal rats leads to hyperglycemia due to destruction of β-cells. This is followed by evidence of increased mitotic activity in the surviving β-cells and β-cell neogenesis from undifferentiated precursor cells, resulting in reversion of the hyperglycemia to a normoglycemic state (Portha *et al.*, 1974, Bonner-Weir, 1981; Dutrillaux *et al.*, 1982).

Pancreatectomy, or removal of pancreas, is a very useful approach to demonstrate the regenerative potential of β-cells. 60% partial pancreatectomy does not result in

glucose intolerance or permanent diabetes. This maintenance of glucose homeostasis is due to a regeneration among the remaining pancreatic β-cells (Leahy *et al.*, 1988, Lohr *et al.*, 1989). However, when 85-90% partial pancreatectomy is performed, mild hyperglycemia ensues which is followed by increased β-cell replication and a 40% increased β-cell mass (Bonner-Weir *et al.*, 1983). Interestingly, 95% pancreatectomy results in severe hyperglycemia with non-existent or very minor signs of β-cell replication (Clark *et al.*, 1982). Based on the pancreatectomy models, it is evident that β-cells have a certain regenerative capacity. The relative contribution of replication, neogenesis or increased β-cell size to the increased β-cell mass is not very clear at this time. Also, it is likely that the degree of hyperglycemia may dictate the extent of the β-cell replication capacity, with severe hyperglycemia or diabetes negatively affecting the compensatory replication of β-cells. The ability of the pancreas to regenerate and the effects of trophic hormones on regeneration of the pancreas after partial pancreatectomy are not completely understood. The endogenous cholecystokinin released by FOY-305 stimulates regeneration after partial pancreatectomy. FOY-305 may be a useful agent in the treatment of pancreatic insufficiency after extensive subtotal pancreatectomy or chronic pancreatitis (Parekh *et al.*, 1991).

2.14 NEUROTRANSMITTERS AS GROWTH SIGNALS

Neurotransmitters and their receptors linked to second messengers mediate growth responses in neuronal and non-neuronal cells. Stimulation of proliferation is most often associated with activation of G-proteins negatively coupled to adenylate cyclase (Gi), or positively coupled to phospholipase C (Gq) or to pertussis toxin–sensitive pathways (Go, Gi). (Lauder.J.H, 1993).

2.14.1 *Norepinephrine*

NE is reported to amplify the mitogenic signals of both EGF and HGF by acting through the α_1 adrenergic receptor. It induces the production of EGF and HGF at distal sites and also enhances the response to HGF at target tissues (Broten, *et al* 1999). Norepinephrine rises rapidly in the plasma within one hour after PH (Knopp *et al.*, 1999). NE also enhances the mito-inhibitory effects of TGF-β1 on cultured hepatocytes isolated from the early stages of regeneration (Michalopoulose & DeFrancis, 1997). Prazosin, a

specific antagonist of α1 adrenergic receptor, as well as sympathetic denervation greatly decrease DNA synthesis at 24 hrs after PH (Cruise *et al.*, 1989). Addition of NE to hepatocytes stimulates Ca^{++} mobilisation or PI turnover and either or both of these processes was proposed to be involved in the mitogenicity of NE (Exton *et al.*, 1981; Exton *et al.* 1988; Nagano *et al.* 1999). Rat hepatomas lacked the α_{1A} and α_{1B} mRNA and receptor binding, while in the human hepato-cellular carcinoma cell line, HepG2, their expression is high but they lack receptor binding (Kost *et al.*, 1992). Hepatic neoplasm are characterised by an increase in α_2-and β-adrenergic receptors and a concomitent decline in α_1 receptors (Sanae *et al.*, 1989).

2.14.2 *Adrenergic receptors and regeneration*

Recent studies have shown that proliferation and insulin secretion of foetal rat β-cells could be significantly suppressed by α-adrenergic stimulation (Sjoholm, 1991). When α-adrenergic agonists were given together with Sp-cAMP[S] or to pertussis toxin-pretreated islets, the suppressed β-cell proliferation and insulin secretion were partially prevented, suggesting that α-adrenergic stimulation represses β-cell growth and hormone release in part by interfering with GTP binding proteins that connect cell surface receptors to adenylate cyclase (Sjoholm, 1991).

2.15 EFFECT OF AGING ON THE REGENERATIVE CAPACITY OF THE PANCREATIC β-CELLS

Since an increased insulin resistance could be expected to lead to a compensatory β-cell hyperplasia, it may be speculated that human type II diabetes becomes manifest only in those individuals who are unable to respond to an increased insulin demand with a higher rate of β-cell proliferation. The proliferating islet cells were synchronized with hydroxyurea and their progression through the cell cycle studied by pulse labeling with [^{3}H]Thymidine and it was possible to calculate the rate of formation of new β-cells from the cell cycle data. The growth rate of islet cells in *in vitro* cultures was increased with increase in the glucose concentrations and decreased with increasing age. The fraction of cells that can enter cell cycle composed about of 10% in the fetal islet cells but was less

than 3% in the adult islets. The small pool of proliferating cells in adult islets could explain why β-cell multiplication, although present in the aging rat, is insufficient to increase the insulin output to levels at which normal glucose tolerance is maintained. This forms an interesting parallel to the development of type II diabetes in man, in which an inherited low capacity for β-cell regeneration may predispose to the disease (Swenne, 1983).

Proliferation of islet cells may compensate for both increased peripheral insulin resistance and islet cell destruction but the capacity for regeneration may be genetically determined. For the latter reason, glucose-stimulated islet cell replication was estimated in both inbred C57BL/6J (BL/6) and C57BL/KsJ (BL/Ks) mice. Islets isolated from both strains were exposed to high concentrations of glucose *in vitro* or *in vivo* for a prolonged time period. In both strains high glucose concentration culture was found to increase replicatory activity of the islets but decreased with age (Swenne & Andersson, 1984).

2.16 MOLECU R BIOLOGY OF PANCREATIC β-CELL

The endocrine pancreas is an organ of enormous importance, since its dysfunction causes diabetes, one of the most common human diseases in the world. Regulation of pancreatic endocrine cell determination and differentiation requires a unique set of transcription factors, including basic helix-loop-helix and homeodomain-containing proteins. The physiological role of individual transcription factor has been characterised by gene disruption in the mouse. The results indicate that these genes are not only involved in tissue-specific activation of downstream target genes for islet-specific hormones, but also critical for the proper islet morphogenesis. Future elucidation of the genetic relationship of these genes will lead to a better understanding of the molecular mechanisms controlling endocrine pancreas formation and will contribute to the development of new therapeutic approaches to diabetes (Huang & Tsai, 2000).

The proliferative response of pancreatic islets to a prolonged glucose stimulation may be genetically determined. This may play a significant role in the development of different diabetic syndromes both in laboratory animals and man (Swenne & Andersson, 1984). At the genetic level, the regulatory regions in islet-specific genes are being

characterised. Transcription factors that interact with these regions have been cloned and these will be instructive in elucidating how islet-specific genes are regulated during development and regeneration (Steiner & James, 1992). Islet duodenal homeobox 1 (IDX-1/PF-1/STF-1/PDX-1), a homeodomain protein that transactivates the insulin promoter, has been shown by targeted gene ablation to be required for pancreatic development. The PDX-1 mRNA levels were not significantly different for common pancreatic ducts of pancreatectomised rats. PDX-1 protein expression was found to increase during active regeneration. Thus, in pancreatic regeneration PDX-1 is upregulated in newly divided ductal cells as well as in islets. The timing of enhanced expression of PDX-1 implies that it is not important in the initiation of regeneration but may be involved in the differentiation of ductal cells to β-cells (Sharma *et al.*, 1999).

2.17 PERSPECTIVE

The discovery of insulin more than 75 years ago fueled enthusiastic optimism regarding insulin therapy of diabetes. However, complications of diabetes still produce devastating consequences and it is believed that better control of glucose levels will reduce the rate and severity of these complications. Although, insulin therapy is now better due to the availability of insulin pumps and automated glucose monitoring, only a small portion of patients with IDDM obtain sufficient glycaemic control. Pancreatic and islet transplantation approaches have been experimented upon with the goal of providing exogenous sources for insulin.

In animal models of IDDM, pancreatic and islet transplantation has been successful in establishing euglycaemia. Two feasible routes for such a therapy are (1) β-cell transplantation and (2) a mechanical β-cell. The first successful islet transplants were performed in rodents in the 1970s, but unfortunately, very few human diabetic patients have received any benefit from β-cells replacement therapy. Islet allografts as a mode of therapy offer a theoretically convenient approach since islets can be delivered to the liver via the portal vein with a relatively simple procedure. However, the initial success rate of this procedure has been very low. The two major problems facing islet transplantation being (1) finding a satisfactory source of insulin producing cells and (2) how can the

transplanted cells be protected from destruction by the immune system through the processes of autoimmunity and transplant rejection (Rane & Reddy, 2000).

The source for insulin producing tissue has been from either pancreatic tissue from cadaver donors or half of the pancreas from living donors. Both of these approaches have potential drawbacks, the most striking of which is the lack of supply of available pancreatic tissue and also the possibility of inducing a diabetic state in living pancreatic tissue donors, due to a reduction in their β-cell mass. Use of fetal or neonatal pancreatic tissue, which provides an attractive source because of its increased growth potential have been explored with limited success. Efforts to expand β-cells and create insulin-producing cells with genetic engineering offer an attractive option for therapy. Although β-cells have capacity for some growth, it has not been possible to efficiently expand β-cells in the laboratory. Efforts are underway to create β-cell lines that might be useful for transplantation. By adding additional genes that influence glucose metabolism, it may be possible to manipulate these cells so they secrete insulin when exposed to glucose. These genes can be transferred into β-cells by genetic approaches. The transplanted islets can be protected from transplant rejection or autoimmune attack by similar genetic modifications, which may help the β-cell to escape recognition by the immune system (Rane & Reddy, 2000).

Knowledge about genes necessary to replicate and increase the division potential of these β-cells will definitely aid the process of genetic manipulation of β-cells and a breakthrough allowing expansion of human β-cells would solve the supply problem for transplantable insulin-producing cells. Certain nutrients, pharmacological agents and growth factors can stimulate pancreatic β-cell proliferation; however, mitogenic signal transduction pathways in β-cells have not been particularly well characterised (Rhodes, 2000).

The expansion of the β-cell mass by recruitment of β-cells to proliferate may constitute a means by which the organism can compensate for the loss or dysfunction of β-cells occurring in diabetes. Thus, if β-cells could be induced to replicate at a higher rate, which may prove beneficial in maintaining normoglycaemia, since the β-cell mass is a

major determinant of the total amount of insulin that can be secreted by the pancreas (Sjoholm, 1996).

3 MATERIALS AND METHODS

3.1 BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from Sigma Chemical Co., USA. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India. Following are the chemicals used for this study.

3.1.1 Chemicals used for the study

3.1.1.1 *Biochemicals: (Sigma Chemical Co., USA.)*

(±)Norepinephrine, (±)epinephrine, normetanephrine, 5-hydroxytyramine, 5-hydroxytryptophan, 5-hydroxy indole acetic acid, homovanillic acid, sodium octyl sulfonic acid, ethylene glycol bis (β-aminoethyl ether)-EGTA, ethylene diamine tetra acetic acid-EDTA, HEPES - [n' (2-hydroxy ethyl)] piperazine-n'-[2-ethanesulfonic acid], ascorbic acid, streptozotocin, pargyline, Tris buffer, fetal calf serum (heat inactivated), D-glucose, calcium chloride, Ascorbic acid, Catechol, benzamidine, collagenase type XI and bovine serum albumin fraction V, Transforming Growth Factor, Epidermal Growth Factor, propranolol, prazocin, yohimbine, atenolol, Phentolamine, RPMI-1640 medium.

3.1.1.2 *Radiochemicals*

Levo-[N-methyl-^{3}H]Epinephrine (Sp. activity 68.6 Ci/mmol) and L-[4-^{3}H] propranolol (21 Ci/mmol) were purchased from NEN life sciences products Inc., Boston, U.S.A, [o-methyl-^{3}H]yohimbine (Sp. activity 88Ci/mmol) and [furanyl-5-^{3}H]prazosin (Sp. Activity 27 Ci/mmol) were obtained from Amersham Life science, U.K.

Radioimmunoassay kits for insulin and tri-iodothyronine and [^{3}H]Thymidine were purchased from Bhabha Atomic Research Centre (BARC), Mumbai, India.

3.1.1.3 *Molecular Biology Chemicals*

Restriction enzymes, random hexamers, human placental Rnase Inhibitor and DNA molecular weight markers were purchased from Bangalore Genei, India. Titan™ one tube RT-PCR system was purchased from Roche Diagnostics, Germany. PCR primers used in this study were synthesised by Genemed Synthesis Inc., San Francisco, U.S.A. Tri-reagent RNA isloation kit was purchased from Sigma Chemical Co., U.S.A. DIG Chemiluminescent Detection kit for nucleic acids, was obtained Roche Diagnostics, Germany.

3.2 ANIMALS

Wistar adult rats of 200-300g and weanling rats of 80-100g body weight were purchased from Central Institute of Fisheries Technology, Cochin and used for all experiments. They were housed in separate cages under 12 hour light and 12 hour dark periods and were maintained on standard food pellets and water *ad libitum*.

3.3 PARTIAL PANCREATECTOMY

Male Wistar rats, 4 - 5weeks old, were anaesthetised under aseptic conditions, the body wall was cut opened and 60-70% of the total pancreas, near to the spleen and duodenum, was removed (Pearson *et al.*, 1977). The removal of most of the pancreas was done by gentle abrasion with cotton applications, leaving the major blood vessels supplying other organs intact (Zangen *et al.*, 1997). The sham was done in an identical procedure except that the pancreatic tissue was only lightly rubbed between fingertips using cotton for a minute instead of being removed. Bodyweight and blood glucose levels were checked routinely. The rats were maintained for different time intervals and sacrificed. Diabetes was induced by a single intra-femoral dose (65 mg/kg body weight) of streptozotocin (STZ) prepared in citrate buffer, pH 4.5 (Hohenegger & Rudas, 1971, Arison *et al.*, 1967).

3.3.1 *Tissue preparation*

Rats were sacrificed by decapitation and the brain regions - cerebral cortex, brain stem and hypothalamus were dissected out quickly over ice according to the procedure of (Glowinski & Iversion, 1966). The tissues were stored at $^{-}70^{0}$ C until assay. Pancreas was also dissected out and stored.

3.3.2 *Estimation of blood glucose*

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{(GOD)} \text{Gluconic acid} + H_2O_2.$$

$$H_2O_2 + \text{Phenol} \quad \text{4-aminoantipyrene} \xrightarrow[\text{(Peroxidase)}]{} \text{Coloured complex} + H_2O$$

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm. in a spectrophotometer (Milton Roy Genesys 5 Spectronic).

3.3.3 *Estimation of circulating insulin by Radioimmunoassay*

3.3.3.1 Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [^{125}I]insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin are separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity

associated with bound fraction of sample and standards quantitates insulin concentration of samples

3.3.3.2 Assay Protocol

50µl of standards (ranging from 0 to 200 µU/ml), 50µl of insulin free serum and 50µl insulin antiserum were added together and the volume was made up to 250µl with assay buffer. They were incubated overnight at 2°C. To this mixture 50µl of [^{125}I]insulin was added and again incubated at room temperature for 3hours. This was followed by addition of 50µl of second antibody and 500µl of PEG. The tubes were vortexed and incubated for 20minutes, at the end of which they were centrifuged at 1500xg for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter. The unknown samples were assayed in the same way except that insulin free serum was avoided and the mixture contained the same volume of buffer.

A standard curve was plotted with $\%B/B_0$ on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. $\%B/B_0$ was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

Insulin concentration in the samples were determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

3.3.4 *Estimation of circulating Tri-iodothyronine by Radioimmunoassay*

T_3 levels were measured according to the procedure of BARC radioimmuno assay kit. 100µl of standards (ranging from 0 to 2.4 ng/ml), 50µl of free serum 100µl antiserum and 100µl of [^{125}I]T_3 were added together and the volume was made up to 550µl with assay buffer. The tubes were incubated at room temperature for 3hours. After incubation 1ml of PEG was added, vortexed and incubated for 20minutes, at the end of which they were centrifuged at 2000xg for 20 minutes. The supernatant was aspirated out and the

radioactivity in the precipitate was determined in a gamma counter. The unknown samples were assayed in the same way except that free serum was avoided and the mixture contained the same volume of buffer.

3.4 ISOLATION OF PANCREATIC ISLETS

Pancreatic islets were isolated from male Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell & Taylor, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers *et al.*, 1985) with the following composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 1mM KH_2PO_4, 14.3mM $KHCO_3$, 10mM HEPES with 0.2% (w/v) BSA (Fraction V), equilibrated with 5% CO_2 and pH 7.3 at room temperature. Autoclaved triple distilled water was used for making up the medium and the medium was filtered through 0.22 μm filters (Millipore).

Splenic portion of the pancreas was aseptically dissected out into a sterile petridish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2ml collagenase type XI solution (1.5 mg/ml in HBSS), pH 7.4. The collagenase digestion was carried out for 20 minutes at 37°C in an environmental shaker with vigorous shaking (300rpm/min). The tissue digest was filtered through 500 μm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS medium. Islets visible as yellowish white spheres were handpicked carefully by finely drawn pasture pipettes and aseptically transferred to HBSS. The islets prepared by this method was used for all other experiments.

3.5 *IN VIVO* DNA SYNTHESIS STUDIES IN THE PANCREAS

5μCi of [^{3}H]Thymidine was injected intra-peritoneally into partially pancreatectomised rats to study DNA synthesis at 24, 36, 72hrs, 7days and 14days of pancreatic regeneration. [^{3}H]Thymidine was injected 2hrs before sacrifice. DNA was extracted from pancreatic islets according to (Schneider, 1957). A 10% trichloroacetic acid (TCA) homogenate was made and DNA was extracted from the lipid free residue by

heating with 5% TCA at 90^0C for 15min. DNA was estimated by diphenylamine method (Burton, 1955). DNA extract was counted in a liquid scintillation counter (WALLAC 1409) after adding cocktail-T containing Triton-X 100. The amount of DNA synthesised was measured as DPM/mg DNA.

3.6 QUANTIFICATION OF BRAIN AND PANCREATIC ISLET MONOAMINES AND THEIR METABOLITES

The monoamines were assayed according to (Paulose *et al.*, 1988). The brain regions and pancreatic islets were homogenised in 0.4N perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°C (Kubota refrigerated centrifuge) and the clear supernatant was filtered through 0.45 μm HPLC grade filters and used for HPLC analysis.

Norepinephrine (NE), epinephrine (EPI), dopamine (DA) 5-hydroxy tryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptophan (5-HTP), normetanephrine (NMN) and homovanillic acid (HVA) were determined in high performance liquid chromatography (HPLC) with electrochemical detector (HPLC-ECD) (Shimadzu, Japan) fitted with CLC-ODS reverse phase columns of 5 μm particle size. The mobile phase consisted of 75 mM sodium dihydrogen orthophosphate, 1mM sodium octyl sulfonate, 50mM EDTA and 7% acetonitrile. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.45 μm filter (Millipore) and degassed. A Shimadzu (model 10 AS) pump was used to deliver the solvent at a rate of 1 ml/min. The catecholamines were identified by amperometric detection using an electrochemical detector (Model 6A, Shimadzu, Japan) with a reduction potential of + 0.8 V. The range was set at 16 and a time constant of 1.5 seconds. Twenty microlitre aliquots of the acidified supernatant were injected into the system for detection. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Shimadzu, C-R6A -Chromatopac) interfaced with the detector. Data from different brain regions of the experimental and control rats were tabulated and statistically analysed.

3.7 QUANTIFICATION OF CIRCULATING CATECHOLAMINE

1.0ml plasma was diluted in equal volume of distilled water. 50µl of 5mM sodium bisulphite was added to it followed by 250µl of 1mM Tris buffer of pH 8.6. The catecholamines were extracted from the serum using acid alumina method and assayed using HPLC (Jackson *et al.*, 1997).

3.8 PROTEIN DETERMINATION

Protein was measured by the method of (Lowry *et al.*, 1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein, which was read in a spectrophotometer at 660nm.

3.9 ADRENERGIC RECEPTOR BINDING STUDIES USING TRITIATED RADIOLIGANDS

3.9.1 *Binding studies in the Brain regions*

3.9.1.1 *[^{3}H]Epinephrine binding*

[^{3}H]Epinephrine binding assay in cerebral cortex (CC), brain stem (BS) and hypothalamus (HYPO) was done according to the modified procedure of U'Prichard *et al.*, (U'Prichard & Snyder, 1977). Brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, pH.7.7 containing 100µM Phenyl Methyl Sulfonyl Fluoride (PMSF). The homogenate was centrifuged at 900xg to remove the nuclear pellet. The supernatant was then centrifuged at 30,000xg for 60 min, the pellets were resuspended in appropriate volume of incubation buffer containing 0.1% Ascorbic acid, 1mM Catechol, 0.1mM EDTA-Na_2, 10µM Dithiothreitol (DTT), 50mM Tris-HCl and 10mM $MgCl_2$.

Binding assays were done using different concentrations i.e., 0.50-80nM of [^{3}H]EPI in the incubation buffer, pH 7.7 in a total incubation volume of 500µl containing appropriate protein concentrations (150-200µg). Non-specific binding was determined using 100µM unlabelled EPI. Competition studies were carried out with 5.0nM [^{3}H]EPI

in each tube with unlabelled ligand concentrations varying from 10^{-9} - 10^{-4}M of EPI. Tubes were incubated at 37^{0}C for 15 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold wash buffer containing 50mM Tris-HCl, 1mM Catechol, 0.1% Ascorbic acid, 10mM $MgCl_2$ (pH 7.7). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.9.1.2 *[^{3}H]Prazosin binding*

[^{3}H]Prazosin was used to study the α_1 adrenergic receptor binding status. The assay was done according to the modified procedure of Geynet *et al.* (1981). The brain regions were homogenised in 20 volumes of ice cold Tris buffer containing 4mM $MgCl_2$, 2mM EGTA, 10mM benzamidine and 5mM PMSF (pH 7.4) in a Potter-Elvejhem homogeniser. The homogenate was centrifuged at 900xg for 10 min and the supernatant again centrifuged at 30,000xg for 60min. The pellet was resuspended in 50 volumes of 50mM Tris HCl, pH 7.5 and recentrifuged at 17,000xg for another 1hour. The final pellet was resuspended in a minimum volume of incubation buffer- 50mM Tris-HCl, 10mM $MgCl_2$, 1mM EGTA, 0.8mM ascorbic acid and 3mM catechol, pH 7.7.

Membrane binding assays were performed in 0.5ml incubation volume containing appropriate protein concentrations ranging from 150-200µg and different concentrations i.e., 0.05-5.0nM of [^{3}H]Prazosin. Non-specific binding was determined using 100µM unlabelled phentolamine. Competition studies were carried out with 0.5nM [^{3}H]Prazosin in each tube with unlabelled ligand concentrations varying from 10^{-9} - 10^{-4}M of prazosin. The tubes were incubated at 25^{0}C for 30min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold buffer containing 50mM Tris-HCl and 10mM $MgCl_2$, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.9.1.3 *[^{3}H]Yohimbine binding*

The α_2 adrenergic receptor binding status was studied using [^{3}H]Yohimbine. The assay was done according to the modified procedure of Repaske *et al.* (1987). The

membrane preparation was same as done for [^{3}H]Prazosin binding. The final pellet was resuspended in a minimum volume of incubation buffer containing 25mM glycyl glycine, 10mM HEPES, 100mM NaCl, 2mM EGTA, (pH7.6).

Membrane binding assays were done in 0.5ml incubation buffer containing appropriate protein concentrations and different concentrations of [^{3}H]Yohimbine ranging from 1.0nM-12.5nM. Non-specific binding was determined using 100μM unlabelled phentolamine. Competition studies were carried out with 0.5nM [^{3}H]Yohimbine in each tube with the unlabelled yohimbine concentrations varying from 10^{-9} - 10^{-4}M. The tubes were incubated at 15^{0}C for 90 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold buffer containing 25mM glycyl glycine, pH 7.6. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.9.1.4 *[^{3}H]Propranolol binding*

[^{3}H]Propranolol is an antagonist of β-adrenergic receptors. The assay was done according to the modified procedure of Lefkowtiz *et al.* (Lefkowtiz & Williams, 1977). The membrane preparation was done by homogenising the tissue in 50mM Tris buffer containing 10mM $MgCl_2$, 25Mm Sucrose and 100μM PMSF, pH 7.4. The final pellet was resuspended in a minimum volume of incubation buffer containing 50mM Tris-HCl, 10mM $MgCl_2$, 0.8mM ascorbic acid and 3mM catechol, pH 7.4.

Membrane binding assays were done in 0.5ml incubation buffer containing appropriate protein concentrations (150-200μg) and different concentrations of [^{3}H]propranolol ranging from 0.5nM-50nM. Non-specific binding was determined using 100μM unlabelled propranolol. Competition studies were carried out using 0.5nM [^{3}H]propranolol in each tube and with unlabelled propranolol and atenolol concentrations varying from 10^{-9} - 10^{-4}M. The tubes were incubated at 37^{0}C for 30 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold buffer containing 50mM Tris and 10mM $MgCl_2$, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid

scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.9.2 *Binding studies in the Pancreatic islets*

3.9.2.1 *[^{3}H]Epinephrine binding*

Pancreatic islets were isolated and homogenised in a polytron homogeniser with 20 volumes of ice cold 50mM Tris-HCl buffer, pH.7.7 containing 100μM Phenyl Methyl Sulfonyl Fluoride (PMSF). The homogenate was centrifuged at 900xg to separate the nuclear fraction. The supernatant was then centrifuged at 30,000xg for 60 min, the pellets were resuspended in appropriate volume of incubation buffer containing 0.1% Ascorbic acid, 1mM Catechol, 0.1mM EDTA, 10μM Dithiothreitol (DTT), 50mM Tris-HCl and 10mM $MgCl_2$. Binding assays were done using different concentrations i.e., 0.50-10nM of [^{3}H]EPI in the incubation buffer, pH 7.7 in a total incubation volume of 500μl containing 50-75μg protein. Non-specific binding was determined using 100μM unlabelled EPI. Competition studies were carried out with 1.0nM [^{3}H]EPI in each tube with unlabelled ligand concentrations varying from 10^{-9} - 10^{-4}M of EPI. Tubes were incubated at 37^{0}C for 15 min. and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold wash buffer containing 50mM Tris-HCl, 1mM Catechol, 0.1% Ascorbic acid, 10mM $MgCl_2$ (pH 7.7). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.9.2.2 *[^{3}H]Prazosin binding*

The assay was done in a similar way as in the brain regions The islets were homogenised in 20 volumes of ice cold Tris buffer containing 4mM $MgCl_2$, 2mM EGTA, 10mM benzamidine and 5mM PMSF (pH 7.4) in a Potter-Elvejhem homogeniser. The homogenate was centrifuged at 900xg for 10 min and the supernatant again centrifuged at 30,000xg for 60min. The pellet was resuspended in 50 volumes of 50mM Tris HCl, pH 7.5 and recentrifuged at 17,000xg for another 1hour. The final pellet was resuspended in a minimum volume of incubation buffer- 50mM Tris-HCl, 10mM $MgCl_2$, 1mM EGTA, 0.8mM ascorbic acid and 3mM catechol, pH 7.7. Membrane binding assays were

performed in 0.5ml incubations containing appropriate protein concentrations using different concentrations i.e., 0.025-1.0nM of [^{3}H]Prazosin in the incubation buffer. Non-pecific binding was determined using 100μM unlabelled phentolamine. Competition studies were carried out with 0.05nM [^{3}H]Prazosin in each tube with unlabelled ligand concentrations varying from 10^{-9} - 10^{-4}M of prazosin. The tubes were incubated at 25^{0}C for 30min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold buffer containing 50mM Tris-HCl and 10mM $MgCl_2$, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.9.2.3 *[^{3}H]Yohimbine binding*

The membrane preparation was same as done for [^{3}H]Prazosin binding. The final pellet was resuspended in a minimum volume of incubation buffer containing 25mM glycyl glycine, 10mM HEPES, 100mM NaCl, 2mM EGTA, (pH7.6). Membrane binding assays were done in 0.5ml incubation buffer containing appropriate protein concentrations and different concentrations of [^{3}H]Yohimbine ranging from 1.0nM-50.0nM. Specific binding was determined using 100μM unlabelled phentolamine. Competition studies were carried out with 2.5nM [^{3}H]Yohimbine in each tube with unlabelled yohimbine concentrations varying from 10^{-9} - 10^{-4}M. The tubes were incubated at 15^{0}C for 90min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold buffer containing 25mM glycyl glycine, pH 7.6. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.9.2.4 *[^{3}H]Propranolol binding*

Membrane binding assays were done in 0.5ml incubation buffer containing appropriate protein concentrations (50-75μg) using different concentrations of [^{3}H]propranolol ranging from 0.1nM-10nM. Specific binding was determined using 100μM unlabelled propranolol. Competition studies were carried out with 0.5nM [^{3}H]propranolol in each tube with unlabelled propranolol and atenolol concentrations varying from 10^{-9} - 10^{-4}M. The tubes were incubated at 37^{0}C for 30min and filtered

rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 10.0 ml of ice cold buffer containing 50mM Tris and 10mM $MgCl_2$, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

3.10 ANALYSIS OF THE RECEPTOR BINDING DATA

3.10.1 *Linear regression analysis for Scatchard plots*

The data was analysed according to (Scatchard, 1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

3.10.2 *Nonlinear regression analysis for displacement curve*

The displacement data were analysed by nonlinear regression using GraphPad Prism software, GraphPad Inc., USA. The concentration of the competing drug that competes for half the specific binding was defined as EC_{50}, which is same as IC_{50} (Unnerstall, 1990). The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors (Cheng & Prusoff, 1973).

3.11 *IN VITRO* DNA SYNTHESIS STUDIES IN THE PANCREAS

Pancreatic islets were prepared by the collagenase digestion method as mentioned earlier. The isolated islets were then suspended in RPMI 1640 medium containing 10% FCS, and incubated for 16hrs at 37^0C and 5% CO_2 to remove the fibroblasts. After fibroblast removal the cells were recultured for three days to remove all other non-endocrine tissue. After the incubation the medium will be rich in β-cells. After the end of

culture period , groups of 100 islets were transferred to 1ml fresh medium containing 5% FCS, antibiotics, different concentrations of glucose (4 and 20mM), EPI (10^{-8} and 10^{-4}) and test agents like different adrenergic antagonists, EGF and TGF were added and cultured free floating for an additional 24 hrs in the presence of 1μCi of [^{3}H]thymidine (Sjoholm, 1991). The cells were harvested and the DNA isolated by TCA method. The radioactivity incorporated was determined by counting in a scintillation counter.

3.12 [^{3}H]EPI UPTAKE STUDIES BY PANCREATIC ISLETS IN VITRO

The islets isolated and were resuspended in HEPES buffered HBSS with 4mM glucose and pre-incubated for 1hour at 37^{0}C (Howell & Taylor, 1968). The islet suspension was centrifuged at 4^{0}C at 500xg to remove inherent insulin. The pre-incubated islets were then washed thrice with cold 10mM Tris HCl buffer, pH 7.4 and finally resuspended in HBSS without glucose. 200μl of islet suspension was transferred to tubes containing 10^{-8} M, 10^{-6} M and 10^{-4} M concentrations of [^{3}H]EPI and two glucose concentrations i.e., (i) 4mM glucose and (ii) 20mM glucose. The final incubation volume was made up to 0.5ml. The tubes were incubated for different time intervals- 15min, 30min., 60min and 180min respectively, at 37^{0}C in a shaking water bath.

At the end of incubation period the tubes were centrifuged at 1,500xg for 10min at 4^{0}C. The supernatant was aspirated out and pellet washed superficially with 0.2ml of HBSS twice to remove free [^{3}H]EPI. Finally the pellet was digested using 1M KOH and counted in the scintillation counter using cocktail T to measure the uptake of EPI by the whole cell.

The cells then broken by homogenisation in a glass homogeniser . Tubes were then centrifuged at 800xg for 10min at 4^{0}C to separate the nuclear fraction (Boam, 1996). The supernatant was transferred to fresh tubes and centrifuged at 30,000xg for 20min at 4^{0}C to separate the mitochondrial + plasma membrane fraction. The supernatant that consisted of cytosolic fraction was transferred to fresh tubes. The pellets then digested by incubating at 37^{0}C overnight with 100μl of 1M KOH. The bound radioactivity in nuclear fraction, mitochondrial + plasma membrane fraction and cytosolic fraction were counted in a liquid scintillation counter with cocktail-T. The DPM obtained were analysed to

determine the amount of [^{3}H]EPI bound to each subcellular fraction in the presence of different concentrations of glucose.

3.13 *IN VITRO* INSULIN SECRETION IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF EPI

3.13.1 *24 hrs incubation study*

After removing the fibroblasts, the islets were cultured for 24hrs in RPMI-1640 medium with and without epinephrine and its antagonists to study the effect of EPI and its receptor subtypes on insulin induction and release (MacDonald *et al.*, 1990). The cells were then harvested and washed with fresh KRB and then incubated for another 1hr in the presence of EPI and antagonists with different (4mM and 20mM) glucose concentrations. At the end of incubation period, the medium was collected and insulin content was measured by RIA method using kit from BARC, Mumbai.

3.13.2 *One hour incubation study*

The isolated islets were pre-incubated for 1 hr in KRB at 1hour at 37^{0}C. Islets were harvested and resuspended in fresh KRB with varying concentrations of EPI and with 4 and 20mM concentrations of glucose. After incubation, the supernatant was transferred to fresh tubes for insulin assay.

3.14 PANCREATIC ISLET NUCLEAR EPI BINDING PROTEIN STUDIES

The nuclear fraction was isolated (Boam, 1996) and resuspended in HBSS buffer of pH 7.4 and used for Scatchard and displacement assays. Binding assays were done using different concentrations [^{3}H]EPI i.e., 10nM-200nM in HBSS, pH 7.4 in a total incubation volume of 500µl. Nonspecific binding was determined using 100µM unlabelled EPI. Tubes were incubated at 37^{0}C for 30 min. Incubations were arrested by centrifugation at 10,000xg for 10 min at 4^{0}C. The pellet was digested by incubating at 37^{0}C overnight with 100µl of 1M KOH. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

3.15 DETERMINATION OF MOLECULAR WEIGHT OF PANCREATIC ISLET NUCLEAR EPI BINDING PROTEIN BY LIGAND BLOTTING TECHNIQUE

Tissue solubilisation

The pancreatic islets were isolated by collagenase digestion method as described earlier. The islets were homogenised in HBSS. The tubes were centrifuged at 800xg for 10 min at 4^0C to separate the nuclear fraction. The pellet was solubilised in 200µl solubilisation buffer by aspirating through a 12 gauge needle and syringe. The solubilising solution consisted of Tris HCl, pH 7.4, 1.6% w/v Triton X-100 and 1mM PMSF. Tubes were incubated over ice for 20 min and then centrifuged at 30,000xg for 30 min at 4^0C (Soutar & Wade, 1997).

3.16 *SDS-PAGE*

Proteins were resolved by discontinuous 10% SDS-PAGE according to (Laemmili, 1970). 200µg protein was mixed with gel loading buffer, heated for 2min in a boiling water bath and applied to each well. Standard molecular weight markers ranging from 205-kDa to 29-kDa (Sigma Chemical Co. USA) were used to determine the molecular weight of the EPI binding protein.

3.17 PROTEIN BLOTTING TO PVDF MEMBRANE AND MOLECULAR WEIGHT DETERMINATION

The gel was transferred after electrophoresis into the renaturation buffer (50mM Tris, 20% Glycerol, pH 7.4) for 20 min and then changed to transfer buffer (25mM Tris-HCl, 192mM Glycine, pH 8.3) for 1.5 hrs to remove SDS and allow the gel to swell. Two lanes on either side, with molecular weight marker and sample, were cut out and stained with coomassie blue. The protein in the remaining gel was transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting at 200mA current for 16 hrs in transfer buffer without methanol (Soutar & Wade, 1997). The membrane was incubated for 1 hr in 10ml blocking solution (HBSS, pH 7.4 containing 1% BSA) at 37^0C. The membrane was then transferred to 10ml fresh blocking solution containing 0.15µCi/ml [^{3}H]EPI and incubated for 1 hr at 37^0C with mild agitation and then washed three times in fresh HBSS to remove unbound [^{3}H]EPI. The membrane was dried and cut into 3mm slices and

counted in a liquid scintillation counter with cocktail-T (Hames, 1981). The molecular weight of the slice with maximum DPM was determined by comparing with the molecular weight of the stained band. Unlabelled EPI was used to confirm specificity of the binding.

3.18 AMINO ACID ANALYSIS OF PANCREATIC ISLET NUCLEAR EPI BINDING PROTEIN

The protein was resolved in a 10% discontinuous SDS-PAGE as described above.

After gel electrophoresis the gel was treated as follows:

1. Fixed in 50% methanol/10% acetic acid for 15 minutes
2. Staining in 10% acetic acid/0.025% coomassie blue for 30 minutes.
3. Destaining was done in 10% acetic acid for 2 x 15 minutes.

The unstained portion of the gel was also kept in 10% acetic acid but without coomassie blue stain to get uniform swelling of stained and unstained portion of the gel. The band of interest was cut out from the unstained portion of the gel by keeping it along with the stained portion. The cut out portion of the gel was homogenised with 1ml of 50mM Tris HCl. pH 7.4 containing 0.1% SDS. The homogenate was stored at 4^0C overnight and centrifuged at 10,000xg for 15 min. The supernatant was used for amino acid analysis.

3.18.1 *Amino acid analysis*

Amino acid analysis of the EPI binding protein was done by hydrolysing the protein in 6N HCl for 24 hrs at 110^OC under vacuum. The amino acid composition was analysed using HPLC LC-10A (Shimadzu).

3.19 EXPRESSION STUDIES OF ADRENERGIC RECEPTOR IN DIFFERENT BRAIN REGIONS

3.19.1 *Isolation of RNA*

RNA was isolated using Tri Reagent kit (Sigma Chemical Co., USA) with the following protocol.

25-50mg tissue was homogenised in 0.5ml Tri Reagent.

⇩

The homogenate was centrifuged at 12,000xg for 10minutes at 4^0C.

⇩

The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5minutes.

⇩

100µl of chloroform was added to it, shaken vigorously for 15minutes and allowed to stand at room temperature for 15minutes.

⇩

The tube was centrifuged at 12,000xg for 15minutes at 4^0C. Three distinct phases appeared after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and the colourless upper aqueous phase contained RNA.

⇩

The upper aqueous phase was transferred to a fresh tube and 200µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10minutes.

⇩

The tubes were centrifuged at 12,000xg for 10min at 4^0C. RNA precipitate formed a pellet on the sides and bottom of the tube.

⇩

The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5minutes at 4^0C

⇩

The pellet was briefly air dried and dissolved in minimum volume of DEPC-treated water and incubated at 60^0C for 10-15 minutes.

10µl of RNA was made up to 1ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as 1 Absorbance $_{260}$ = 42µg.

3.19.2 *RT-PCR (Reverse Transcription Polymerase Chain Reaction)*

RT-PCR was carried out using Titan™ one tube RT-PCR system (Roche Diagnostics, Germany). cDNA synthesis was performed with AMV (Avian Myeloblastosis Virus) reverse transcriptase enzyme. The PCR step was carried out with a high fidelity enzyme blend consisting of Taq DNA Polymerase and Pwo DNA Polymerase. Enzyme was stored in storage buffer (20mM Tris HCl, 100mM KCl, 0.1mM EDTA, 1mM Dithiothrietol (DTT), 0.5% Tween-20 (v/v), 0.5% Nonidet P40 (v/v), 50% Glycerol (v/v): pH 7.5 (25^{0}C).

3.19.3 *RT PCR Primers*

The following primers were used for α_{2A}-and β-adrenergic receptor mRNA expression studies.

5'-GAATTGTGAGCGATAAC-3' Forward Primer 5'-CCATTTTAGCTTCCTTAG-3' Reverse Primer	380bp	Rat α_{2A} primer
5'-TGCTGTGACTTCTTCACGAACC-3' Forward Primer 5'-GCTGATATGCGCTCTGCTCC-3' Reverse primer	550bp	Rat β primer

3.19.4 *RT-PCR of α_{2A} and β-adrenergic receptors*

RT-PCR was carried out according to the procedure of Titan™ one tube RT-PCR system from Roche Diagnostics with modifications. The reaction was carried out in a total volume of 20µl reaction mixture in 0.2ml tubes. RT-PCR was performed on an Eppendorf Personal Thermocycler. Three separate master mixes were made (Eason & Ligget, 1993). Mix# 1: consisted of 5ng RNA template, 25ng Random hexamers and RNase inhibitor (5 units). The tube was incubated at 42^{0}C for 10 min. After incubation mix # 2 was added

which consisted of reaction buffer containing 1.5mM $MgCl_2$ (5x stock containing 7.5mM $MgCl_2$ and DMSO), DTT and 5mM dNTPs (10mM stock containing mixture of dATP, dCTP, dGTP and dTTP). The tube was again incubated at 42^0C for 2 min. The reaction was started after adding mix# 3 containing 0.3μM α_{2A} adrenergic receptor forward primer (21.0μM stock), 0.3μM 5-HT_{2A} reverse primer (20.39 μM stock), enzyme mix and DEPC treated water to make up the volume to 20μl.

Mix # 1

Component	Volume	Final concentration in the RT-PCR tube	Incubate at 42^0C for 10 min.
RNA template	1.0μl	50ng	
Random hexamers	0.5μl	50ng	
RNase inhibitor	1.0μl	10U	
D H_2O	2.5μl	-	
Total	5.0μl		

Mix # 2

5 x buffer	4.0μl	1.5mM $MgCl_2$	Incubate at 42^0C for 2 min.
DTT	1.0μl	5mM	
DNTPs	0.4μl	0.2mM	
Total	5.40μl	-	

Mix # 3

Forward primer	1.0μl	0.3μM	RT-PCR according to thermocycling profile
Reverse primer	1.0μl	0.3μM	
Enzyme mix	0.4μl	AMV and Expand™ high fidelity enzyme blend	
D H_2O	7.2μl	-	
Total	9.6μl	-	
Grand Total	**20μl**	-	

3.19.5 *Thermocycling profile for RT-PCR*

Following is the thermocycling profile used for α_{2A} receptor RT-PCR

I. 42^0C -- 1 hr
II. 50^0C -- 20 min } RT step (according to the kit)

III. 94^0C -- 3 min --- Denaturation

IV. 94^0C -- 40 sec --- Denaturation
V. 52^0C -- 1.0 min --- Annealing } 29cycles
VI. 68^0C -- 1.0 min --- Extention

VII. 68^0C -- 10 min --- Final extention

Following is the thermocycling profile used for β-adrenergic receptor RT-PCR

I. 42^0C -- 1 hour
II. 50^0C -- 20 min } RT step (according to the kit)
III. 94^0C -- 3 min --- Denaturation

IV. 94^0C -- 40 sec --- Denaturation
V. 60^0C -- 1.0 min --- Annealing } 29 cycles
VI. 68^0C -- 1.0 min --- Extention

VII. 68^0C -- 10 min --- Final extention

3.19.6 *Analysis of RT-PCR product*

After completion of RT-PCR reaction 10µl of Bromophenol blue gel-loading buffer was added to 40µl reaction mixture and the total volume was applied to a 2% agarose gel containing ethidium bromide. The gel was run at constant 60V with 0.5x Tris borate EDTA buffer. The image of the bands was captured using an Imagemaster gel documentation system (Pharmacia Biotech) and densitometrically analysed using Imagemaster ID software to quantitate the α_{2A} receptor mRNA expression in control and pancreatectomised rats. A standard graph was obtained for each primer in brain regions using different concentrations (ranging from 1ng - 10ng) of control RNA. The concentration of our product of interest was found out from the standard graph, which

plotted using the pixel intensity on the Y-axis and concentration of RNA on the X-axis. The product size of α_{2A} receptor is 380 bp and size of β-adrenergic receptor is 550bp.

3.20 STATISTICS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03).

4. RESULTS

4.1 BODY WEIGHT AND BLOOD GLUCOSE LEVEL

There was no significant change in the body weights and blood glucose levels of sham operated and pancreatectomised rats (Table - 1).

4.2 DNA SYNTHESIS IN REGENERATING PANCREAS

Tritiated thymidine incorporation into replicating DNA was used as a biochemical index for quantifying the pancreatic regeneration. DNA synthesis was negligible in the pancreatic islets of sham operated rats. There was a significant ($p<0.01$) increase in the [^{3}H]Thymidine incorporation at 36 hrs and 48 hrs after partial pancreatectomy. The DNA synthesis was peaked at 72 hrs after pancreatectomy ($p<0.001$). The elevated levels of DNA synthesis reversed back to near basal level after 7 and 14 days after partial pancreatectomy (Fig. 1).

4.3 CIRCULATING INSULIN LEVEL

The insulin levels in the serum of pancreatectomised rats showed a significant increase at 48 hrs ($p<0.05$) and peaked at 72 hrs after partial pancreatectomy ($p<0.01$). The increased insulin levels then decreased to near normal by 7 and 14 days (Fig. 2).

4.4 CIRCULATING T_3 LEVEL

The circulating levels of T_3 was increased significantly ($p<0.05$) at 48 hrs after partial pancreatectomy and it further increased and peaked significantly ($p<0.01$) at 72 hrs after partial pancreatectomy. Then the T_3 level was decreased at 7days (Fig. 3)

4.5 BRAIN EPINEPHRINE AND NOREPINEPHRINE CONTENT OF RATS

In the cerebral cortex, the EPI and NE contents were significantly decreased at 72 hrs after partial pancreatecomy ($p<0.001$ and $p<0.01$ respectively). This decrease was

partially reversed to the normal level by the 7th day of partial pancreatectomy. In the brain stem and hypothalamus also the EPI and NE contents were decreased significantly ($p<0.001$) during active DNA synthesis (Table - 2).

4.6 EPINEPHRINE AND NOREPINEPHRINE CONTENT IN THE PANCREATIC ISLETS OF RATS

The HPLC analysis of isolated pancreatic islets of experimental rats showed a significant reduction ($P<0.001$) in the EPI and NE content during active regeneration. The decreased levels of these catecholamines were reversed back to near normal at 7days after partial pancreatectomy (Table - 3)

4.7 CIRCULATING EPI AND NE LEVELS OF EXPERIMENTAL RATS

The EPI and NE levels in the plasma were significantly reduced ($p<0.001$) in the pancreatectomised rats at 72 hrs after pancreatectomy (Table - 4).

4.8 CHANGES IN THE BRAIN ADRENERGIC RECEPTORS DURING PANCREATIC REGENERATION

4.8.1 *Cerebral cortex*

Scatchard analysis in cerebral cortex of rats showed two affinity sites for [^{3}H]EPI. The high affinity receptors were increased significantly ($p<0.01$) which is indicated by an increase in the B_{max}, with a significant increase in the K_d ($p<0.01$). The low affinity receptors were significantly decreased ($p<0.01$) with an increase in the affinity (Table - 5 & Fig. 4).

In displacement analysis different concentrations of unlabelled EPI were used against [^{3}H]EPI. Displacement analysis showed disappearance of low affinity site at 72 hrs after partial pancreatectomy, thus fitting the equation to a one-site model instead of the two-sited model, that is observed in the sham-operated and 7 day pancreatectomised groups. The Hill slope value in 72 hrs pacreatectomised group was above unity confirming the one-site model (Table - 6 & Fig. 5).

4.8.2 *Brain stem*

In the brain stem, the Scatchard analysis showed no significant change in the B_{max} during active DNA synthesis, but the K_d was increased significantly ($p<0.05$). The increase remained unchanged even after 7days of partial pancreatectomy (Table - 7 & Fig. 6).

The competitive curve fitted for two-sited model with Hill slope value away from unity in all experimental groups of rats. In 72 hrs pancreatectomy, the $K_{i(H)}$ was decreased with an increase in the $K_{i(L)}$ indicating that function has been taken up by the low affinity receptors. There was no significant change in log (EC_{50}) (Table - 8 & Fig. 7).

4.8.3 *Hypothalamus*

In the hypothalamus, the total adrenergic receptor number decreased significantly ($p<0.001$) in the 72 hrs pancreatectomised rats which is indicated by a decrease in the B_{max}. The K_d value also decreased significantly ($p<0.001$) in this group. These changes persisted in the 7day pancreatectomised group also (Table - 9 & Fig. 8).

The competitive curve for displacement analysis fitted to a two-sited model similar to brain stem. The $K_{i(H)}$ showed an increase without any change in the $K_{i(L)}$. Hill slope values were away from unity in all experimental groups of rats (Table - 10 & Fig. 9).

4.9 α_1 adrenergic receptors

4.9.1 *Cerebral cortex*

The α_1-adrenergic receptor status was assayed using a specific ligand, [^{3}H]Prazosin. From the Scatchard analysis, it was found that the B_{max} decreased significantly ($p<0.01$) during active pancreatic DNA synthesis with a significant decrease ($p<0.001$) in the K_d (Table - 11 & Fig. 10).

Displacement analysis showed a disappearance of low affinity site at 72 hrs after partial pancreatectomy. The competitive curve fitted to one-site model in the case of 72 hrs pancreatectomy and to a two-sited model in the case of sham and 7day

pancreatectomised group. Hill slope value was above unity in 72 hrs pancreatectomised rats (Table - 12 & Fig. 11).

4.9.2 *Brain stem*

In the brain stem the number of α_1-adrenergic receptors was reduced significantly ($p<0.05$) accompanied by a significant increase ($p<0.01$) in the affinity (Table - 13 & Fig. 12).

The competitive curve fitted to a one-sited model in sham-operated and 7day groups, where as it fitted to two-sited model in the case of 72 hrs pancreatectomised rats. An additional low affinity site also appeared at the time of active regeneration. $Ki_{(H)}$ value decreased during 72 hrs compared to the sham-operated rats (Table - 14 & Fig. 13).

4.9.3 *Hypothalamus*

Scatchard analysis in the hypothalamus showed similar changes as in the brain stem. The B_{max} and K_d were decreased significantly ($p<0.05$) during 72 hrs after pancreatectomy which were normalised after 7days of partial pancreatectomy (Table - 15 & Fig. 14).

In the displacement analysis, the 72 hrs pancreatectomised rats showed appearance of an additional low affinity site and the competitive curve fitted to a two-sited model. $Ki_{(H)}$ value was decreased during 72 hrs after partrial pancreatectomy (Table - 16 & Fig. 15).

4.10 α_2 adrenergic receptors

α_2-adrenergic receptors were assayed using [^{3}H]Yohimbine, a specific antagonist for α_2-adrenergic receptors.

4.10.1 *Cerebral cortex*

Scatchard analysis of [^{3}H]Yohimbine in the cerebral cortex of 72 hrs pancreatectomised rats showed a significant decrease ($p<0.05$) in the number of receptors and a significant increase ($p<0.05$) in the affinity (Table - 17 & Fig. 16). In the 7days pancreatectomised rats, the binding parameters remained as changed as in the case of 72 hrs group.

Displacement analysis of yohimbine against [^{3}H]Yohimbine, showed that all the groups fitted to a two-sited model. But at 72 hrs, the $Ki_{(H)}$ value decreased and $Ki_{(L)}$ increased when compared to the sham-operated rats (Table - 18 & Fig. 17).

4.10.2 *Brain stem*

The brain stem of 72 hrs pancreatectomised rats showed a significant reduction ($p<0.05$) in the B_{max} and K_d indicating that the α_2-adreneregic receptors are down-regulated during active regeneration. Within 7days after partial pancreatectomy, the reduced B_{max} and K_d were reversed near to normal (Table - 19 & Fig. 18).

The competitive curve fitted to a one-sited model in sham-operated and pancreatectomised rats. Hill slope values were above unity confirming the one-site model. There were no changes in the log (EC_{50}) values, but the Ki value was decreased in the pancreatectomised groups (Table - 20 & Fig. 19).

4.10.3 *Hypothalamus*

Scatchard analysis in the hypothalamus of 72 hrs pancreatectomised rats showed no significant change in the B_{max}, but the K_d was significantly decreased ($p<0.01$) (Table - 21 & Fig. 20).

The competition binding curve for yohimbine in the hypothalamus fitted to a one-sited model in all groups. Hill slope values were above unity. The Ki value in the

pancreatectomised rats showed a decrease when compared to sham-operated rats (Table - 22 & Fig. 21).

4.10.4 RT-PCR studies of α_{2a} adrenergic receptor in the brain regions

Quantitation of α_{2A}-adrenergic receptor mRNA by RT-PCR in the cerebral cortex (Table - 23 & Fig. 22) and brain stem (Table - 24 & Fig. 23) of the pancreatectomised rats showed a decrease in the expression at 72 hrs after partial pancreatectomy. This change was normalised at the 7^{th} day of partial pancreatectomy. These results correlate with our receptor data, which showed a decrease in the B_{max} of the α_{2A}-adrenergic receptors during pancreatic regeneration. In the hypothalamus, there was no significant change in the expression (Table - 25 &Fig. 24). The receptor number as per the receptor analysis did not show any siginificant change in the B_{max} but the affinity status changed during active DNA synthesis in the pancreatic islets.

4.11 β-adrenergic receptors

β-adrenergic receptor kinetics was studied using specific antagonist, [^{3}H]Propranolol.

4.11.1 *Cerebral cortex*

The Scatchard analysis of β-adrenergic recceptors using [^{3}H]Propranolol showed two affinity sites for propranolol binding. There was a significant increase ($p<0.05$) in the number of both low affinity and high affinity receptors during active regeneration. The K_d value was also significantly increased ($p<0.001$). The increased parameters were reversed to near normal during 7days after partial pancreatectomy (Table - 26 & Fig. 25).

Displacement analysis with propranolol in cerebral cortex of 72 hrs pancreatectomised rats showed that the competition curve fitted to a one-sited model rather than to a two-sited model, which was observed in sham-operated and 7day pancreatectomised rats. There was a shift in the low affinity site to a high affinity site during active cell proliferation. The Hill slope value in the 72 hrs pancreatectomy was above unity confirming the one-sited model (Table - 27 & Fig. 26).

4.11.2 *Brain stem*

[^{3}H]Propranolol binding in brain stem showed the presence of two affinity states. The low affinity receptors were increased significantly ($p<0.05$) without any change in the affinity during regeneration whereas the high affinity receptors remained unchanged. The increased parameters were reversed back to control in 7day pancreatectomised rats (Table - 28 & Fig. 27).

In displacement analysis, the competitive curve fitted to a one-site model in all the groups. Ki value was decreased in pancreatectomised rats when compared to sham-operated goups. The Hill slope values were near unity confirming the one-sited model (Table - 29 & Fig. 28).

4.11.3 *Hypothalamus*

The hypothalamus also showed the presence of two affinity sites. The high affinity receptor number increased significantly ($p<0.001$) accompanied by a significant reduction ($p<0.01$) in the receptor affinity. The low affinity receptors were also increased significantly ($p<0.05$) in the 72 hrs pancreatectomised group with out any change in the affinity of the rceptor (Table - 30 & Fig. 29). These results showed that the β-adrenergic receptors were up regulated during pancreatic regeneration.

In the displacement analysis, the competitive curve fitted to a two-sited model in all groups with an increase in the log (EC_{50}) of 72 hrs pancreatectomised rats. Ki(H) value of pancreatectomised rats was increased when compared to sham. The Hill slope values were away from unity, which confirmed the two-sited model (Table - 31 & Fig. 30).

4.11.4 RT-PCR studies of β-adrenergic receptor in the brain regions

RT-PCR analysis of of β-adrenergic receptor mRNA in the cerebral cortex (Table - 32 & Fig. 31), brain stem (Table - 33 & Fig. 32) and hypothalamus (Table - 34 & Fig. 33) of the pancreatectomised rats showed an increase in the expression at 72 hrs after partial pancreatectomy. By the 7th day of partial pancreatectomy the changes in the expression of

β-adrenergic receptors were reversed to normal in the brain stem, but in the cerebral cortex and hypothalamus it did not show any reversal. These results correlate with our receptor data, which showed an increase in the B_{max} of the β-adrenergic receptors in all the brain regions during pancreatic regeneration. These results showed that the β-adrenergic receptor expression is increased at the time of active islet cell proliferation.

4.12 CHANGES IN THE PANCREATIC ADRENERGIC RECEPTORs DURING PANCREATIC REGENERATION

Total adrenergic receptors were studied using [^{3}H]Epinephrine. The Scatchard analysis showed a significant decrease ($p<0.001$) in the B_{max} accompanied by a significant decrease ($p<0.01$) in the K_d (Table - 35 & Fig. 34).

The displacement analysis showed appearance of an additional high affinity site in 72 hrs pancreatectomised rats. The competition curve in the 72 hrs pancreatectomy fitted to a two-sited model whereas in sham and 7day pancreatectomised groups it fitted to a one-sited model. Hill slope values also confirmed these results (Table - 36 & Fig. 35).

4.12.1 *α_1-adrenergic receptors*

The α_1-adrenergic receptor status was assayed using [^{3}H]Prazosin. From the Scatchard analysis, it was found that the B_{max} decreased significantly ($p<0.05$) during active pancreatic DNA synthesis without any significant change in the K_d (Table - 37 & Fig. 36).

Displacement analysis showed a one-site model in all groups. Hill slope value was above unity confirming the one-sited model (Table - 38 & Fig. 37).

4.12.2 *α_2-adrenergic receptors*

Scatchard analysis of [^{3}H]Yohimbine binding in the pancreatic islets showed a significant decrease in the B_{max} ($p<0.001$) and K_d ($p<0.05$) during active pancreatic DNA synthesis (Table - 39 & Fig. 38).

In displacement analysis the competitive curve fitted to a one-site model in all groups. Ki value was decreased in 72 hrs pancreatectomised rats when compared to sham. Hill slope value was above unity confirming the one-sited model (Table - 40 & Fig. 39).

4.12.3 *β-adrenergic receptors*

Scatchard analysis of [^{3}H]Propranolol binding against propranolol in the pancreatic islets showed a significant increase ($p<0.001$) in the B_{max} during active pancreatic DNA synthesis without any change in the K_d (Table 41 & Fig.40).

Displacement analysis showed that the competitive curve fitted to a one-sited model in sham-operated rats whereas it fitted to a two-sited model in the pancreatectomised rats. Hill slope value was away from unity in the pancreatectomised rats confirming the two-sited model, but it was above unity in the case of sham thus confirming the one-sited model (Table 42 & Fig. 41).

4.13 *IN VITRO* STUDIES

4.13.1 *Effect of EPI and glucose on insulin secretion in vitro*

The isolated islets were incubated for 1 hr with various concentrations of epinephrine and different adrenergic receptor antagonists. The pancreatic islets were incubated with 10^{-8}, 10^{-6} and 10^{-4} M EPI at different glucose concentrations(0, 4 and 20mM). The insulin content was increased significantly ($p<0.01$) with the increase in the glucose concentration in the controls. Lower concentration of EPI could increase the insulin concentration significantly ($p<0.05$) at all glucose concentrations. But at 10^{-4} M concentration it was found to significantly decrease ($p<0.01$) the insulin concentration with increase in glucose concentration (Fig. 42).

4.13.2 *Effect of different adrenergic receptor antagonists on insulin secretion in vitro*

Prazosin, an α_1-adrenergic antagonist, at 20mM glucose and 10^{-8}M EPI concentration decreased the insulin secretion significantly ($p<0.05$), whereas at low

glucose and high EPI concentration prazosin could increase the insulin secretion (Fig. 43a). When blocked with yohimbine, a specific α_2-antagonist, the insulin secretion was increased significantly ($p<0.01$ & $p<0.05$ respectively) at 4mM and 20mM glucose concentrations. The increase was significant only at higher concentration of EPI (Fig. 43b). β-adrenergic receptor antagonist could block insulin secretion significantly ($p<0.01$) at all concentrations of glucose and EPI (Fig. 43c).

4.13.3 *Effect of EPI and glucose on insulin secretion in 24 hrs islet cultures*

Glucose coould induce the same changes on insulin secretion as in the case of 1 hr cultures. 20mM glucose stimulated the insulin secretion significantly ($p<0.001$). 10^{-4}M EPI at both glucose concentrations decreased the insulin secretion significantly ($p<0.001$) whereas 10^{-8} M EPI, significantly stimulated the insulin secretion in the presence of 20mM glucose ($p<0.001$) (Fig. 44).

4.13.4 *Effect of adrenergic receptor antagonists on insulin secretion in 24 hrs islet cultures*

In 24hrs culture also, Prazosin significantly ($p<0.05$) blocked the insulin secretion at lower concentration of EPI and higher concentration of glucose (Fig. 45a). Yohimbine significantly increased ($p<0.001$) the insulin secretion at all concentrations of EPI and glucose (Fig. 45b). The insulin secretion was decreased significantly ($p<0.001$) in the presence of propranolol (Fig. 45c).

4.14 [^{3}H]EPI UPTAKE AND BINDING TO WHOLE CELL AND SUBCELLULAR FRACTIONS OF PANCREATIC ISLETS AT DIFFERENT TIME INTERVALS

4.14.1 *[^{3}H]EPI uptake into whole cell*

Our results showed a significant increase ($p<0.01$) in [^{3}H]EPI uptake by pancreatic islets in the presence of 4mM and 20mM glucose with the increase in the time (Fig. 46a). 4mM and 20mM glucose in the incubation medium can be considered equivalent to normal and diabetic states respectively. These results show that there is a rapid uptake of

EPI into the pancreatic islets regardless of the glucose concentration and the uptake depends only on the time of incubation.

4.14.2 *[^{3}H]EPI in different fractions of pancreatic islets*

We observed that [^{3}H]EPI is taken up by the whole cell and then it is binding to the nuclear fraction. Binding of EPI to the nuclear fraction increased with the increase in the incubation time at 4mM and 20mM glucose concentrations. The increase was significant ($p<0.05$) at 3hrs when compared with other time intervals (Fig. 46b). Membrane fraction also showed [^{3}H]EPI binding which is significantly increased ($p<0.01$) with the increase in time when incubated with 4mM and 20mM glucose (Fig. 46c). Cytosolic fraction showed the presence of [^{3}H]EPI but there was no significant binding (Fig 46d). This explains that EPI is binding to the membrane receptors and it can enter into the cell where it binds to some nuclear proteins.

4.15 SCATCHARD ANALYSIS OF EPI-BINDING NOVEL NUCLEAR PROTEIN IN THE PANCREATIC ISLETS OF PANCREATECTOMISED RATS

[^{3}H]EPI binding parameters were analysed in the islet nuclear fraction of pancreatectomised rats to study the status of the EPI-binding protein during regeneration. The Scatchard analysis showed that the B_{max} of nuclear protein increased significantly ($p<0.01$) during active pancreatic regeneration without any change in the affinity (Table - 43 & Fig. 47).

4.16 SCATCHARD ANALYSIS OF EPI-BINDING NUCLEAR PROTEIN IN THE PANCREATIC ISLETS OF DIABETIC RATS

Scatchard analysis of EPI-binding nuclear protein was done using [^{3}H]EPI in the diabetic rats in order to study the role of this protein in insulin secretion. Scatchard analysis of [^{3}H]EPI in the nuclear fraction showed a significant decrease in the B_{max} and K_d ($p<0.01$; $p<0.05$ respectively) (Table - 44 & Fig. 48). This shows that during insulin deficiency, the nuclear EPI-binding receptors are down regulated.

4.17 MOLECULAR WEIGHT DETERMINATION OF NUCLEAR EPI-BINDING PROTEIN

PVDF membrane slice No. 25 showed maximum DPM after incubation with [^{3}H]EPI (Fig. 49). The band corresponding to this slice was compared with known markers stained with coomassie blue and the molecular weight was calculated from the standard curve. The molecular weight of the EPI- binding protein was determined as 70-kDa (Fig. 50).

4.18 AMINO ACID COMPOSITION OF 70-kDa EPI-BINDING PROTEIN

The 70-kDa EPI binding protein was mainly composed of Histidine (69.92%) followed by glycine (5.63%) and aspartic acid (2.03%) (Table 45, Fig. 51).

4.19 70-kDa EPI-BINDING PROTEIN SHOWED MAXIMUM HOMOLOGY TO THE MOUSE ZINC FINGER PROTEIN

The Swiss-Prot online protein composition homology search showed maximum homology to mouse zinc finger proteins, ZFP-35 and ZFP-90, which may function as transcription activators. This protein is a DNA binding protein and has a wide similarity to all other zinc finger proteins in the mouse and human. This protein might also be involved in the control of gene activity during cell division.

4.20 *IN VITRO* DNA SYNTHESIS STUDIES IN PANCREATIC ISLETS

4.20.1 *Effect of glucose on DNA synthesis in the pancreatic islets*

Glucose, at 4mM and 20mM concentrations significantly ($p<0.05$ & $p<0.001$ respectively) stimulated the DNA synthesis of pancreatic islets. Glucose in combination with EGF also significantly increased ($p<0.001$) the DNA synthesis in the cultured islets (Fig. 52).

4.20.2 *Effect of EGF and TGF on glucose-induced DNA synthesis in the pancreatic islets in vitro*

EGF, the principal growth factor, stimulated the DNA synthesis in the pancreatic islets significantly ($p<0.001$). When it is used along with 20mM glucose it could enhance the DNA synthesis very significantly than when used alone or with 4mM glucose. TGF inhibited DNA synthesis in islets significantly ($p<0.001$). TGF when used in combination with EGF could inhibit EGF induced DNA synthesis significantly ($p<0.01$) in the pancreatic islets (Fig. 53).

4.20.3 *Effect of epinephrine on DNA synthesis in pancreatic islets in vitro*

EPI at 10^{-8}M concentration enhanced the stimulatory effect of EGF, but the higher concentration of EPI inhibited the DNA synthesis. EPI in the presence of TGF and in combination with EGF and TGF also inhibited the islet cell proliferation *in vitro* (Fig. 54). These results show that the EPI can act both as stimulatory and inhibitory to DNA synthesis in the pancreatic islets.

4.20.4 *Dose dependent effect of epinephrine on DNA synthesis in pancreatic islets in vitro*

The dose-dependant study showed that the lower concentrations of EPI, i.e., 10^{-8}, 10^{-7} and 10^{-6} M, could increase the rate of DNA synthesis at 4mM and 20mM glucose concentrations. The higher concentrations of EPI inhibited the glucose-induced DNA synthesis in the pancreatic islets (Fig. 55). These results revealed that the pancreatic islet cell proliferation is decreased with the increase in the EPI concentration irrespective of the glucose concentration.

4.20.5 *Effect of different adrenergic receptor antagonists on islet DNA synthesis in vitro*

At 10^{-4} M EPI concentration, prazosin significantly ($p<0.05$) stimulated the *in vitro* DNA synthesis in the islets whereas at 10^{-8} M EPI concentration, prazosin inhibited the DNA synthesis significantly ($p<0.001$). This suggests that the α_1-adrenoreceptors act both as stimulatory and inhibitory according to the EPI concentration (Fig. 56a). The α_2-adrenergic receptor antagonist, yohimbine could increase the DNA synthesis significantly

($p<0.001$) in the islets at all concentrations of glucose and EPI suggesting that α_2-adrenergic receptors are inhibitory to the islet cell proliferation (Fig. 56b). Propranolol, a selective blocker of the β-adrenergic receptors, inhibited the in vitro DNA synthesis in the islets significantly ($p<0.001$). This proves that the β-adrenergic receptors are stimulating the pancreatic growth (Fig. 56c).

Table - 1

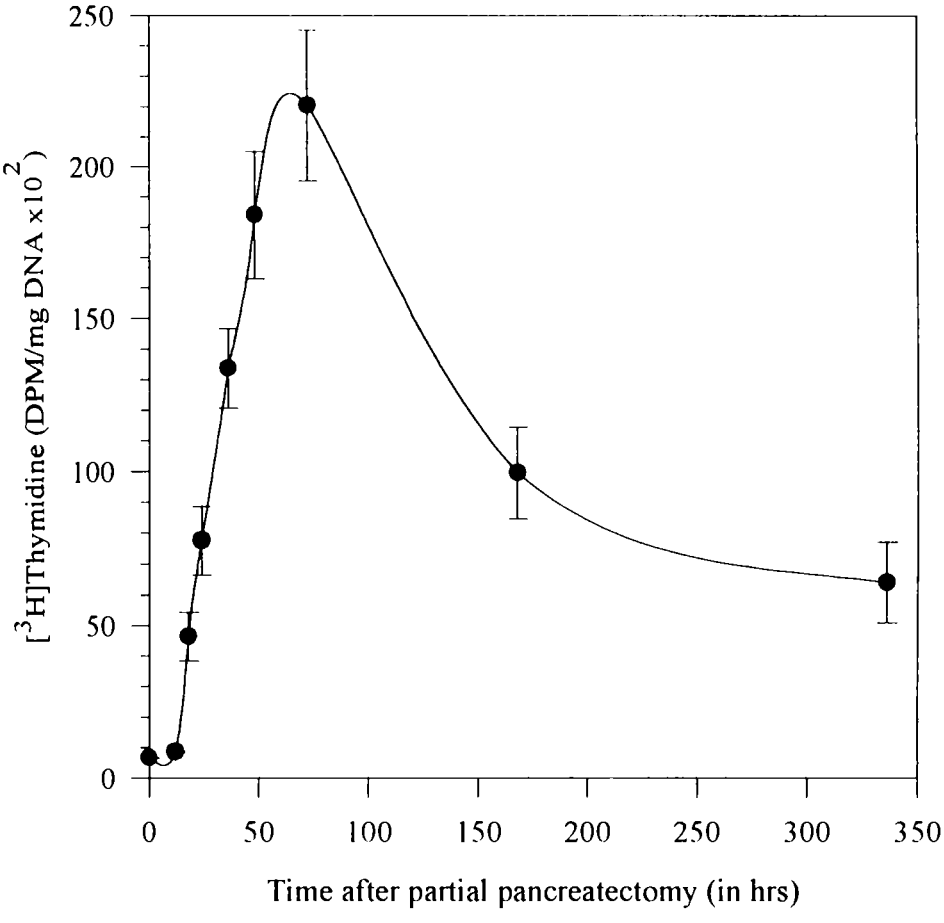
Blood glucose level and body weight of experimental groups

Animal status	Blood glucose level (mg/dl)	Body weight (g)
Sham	123.17 ± 21.33	80 ± 4
72 hrs pancreatectomy	100.477 ± 6.1	60 ± 5
7days pancreatectomy	122.02 ± 24.78	60 ± 5

Values are mean ±S.E.M of 4 - 6 separate experiments

Figure -1

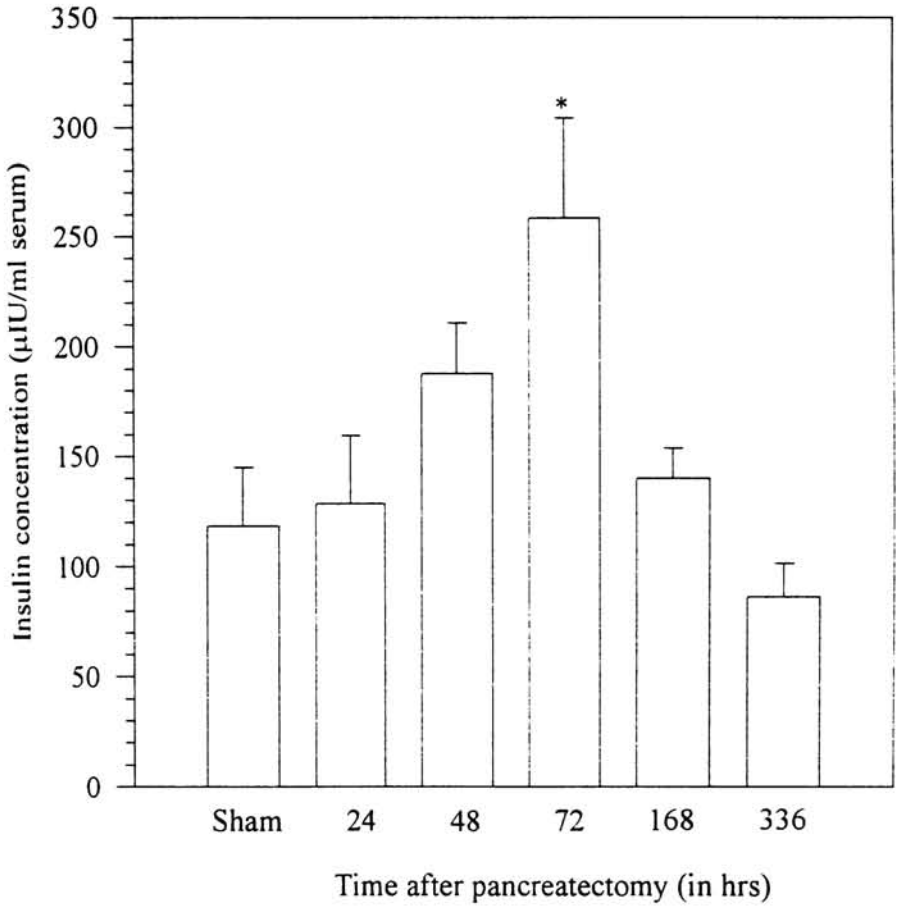
DNA synthesis in the regenerating pancreas of young rats



Values are Mean ± S.E.M. 4-6 separate determinations

Figure - 2

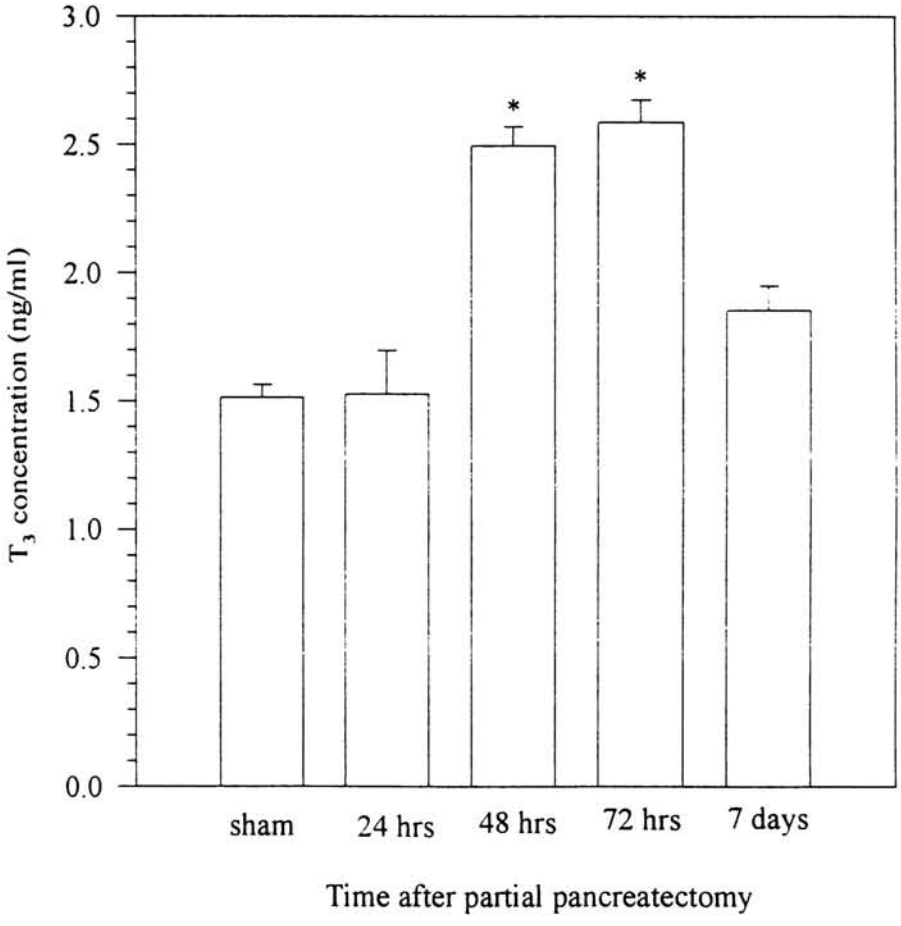
Circulating insulin levels of the sham and pancreatectomised young rats



Values are Mean ± S.E.M. of 4-6 separate determinations
*$p<0.05$ when compared to sham

Figure - 3

Circulating T_3 levels of sham and pancreatectomised rats



Values are mean ± S.E.M of 4-6 separate determinations
*$p<0.05$ when compared to sham

Table - 2

Epinephrine and Norepinephrine content (nmoles/g wet wt. of tissue) of brain regions of sham and pancreatectomised young rats

Animal status	Brain regions					
	Cerebral cortex		Brain stem		Hypothalamus	
	NE	EPI	NE	EPI	NE	EPI
Sham	2.03 ± 0.31	0.53 ± 0.11	28.86 ± 0.36	34.99 ± 11.58	30.91 ± 0.69	23.03 ± 1.07
72hr pancreatectomy	0.29 ± 0.13**	0.21 ± 0.09***	10.05 ± 0.06***	ND	3.29 ± 0.58***	1.31 ± 0.18***
7day pancreatectomy	1.74 ± 0.26†††	0.73 ± 0.11*†††	29.47 ± 0.49†††	46.67 ± 9.02	6.09 ± 0.52***†	44.59 ± 5.65***†††

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to sham
**p<0.01 when compared to sham
*p<0.05 when compared to sham
†††p<0.001 when compared to 72hr pancreatectomy
†p<0.05 when compared to 72hr pancreatectomy
NE - Norepinephrine; EPI - Epinephrine

Table -3

Epinephrine and norepinephrine content (nmoles/g wet wt, of tissue) in the pancreatic islets of sham and pancreatectomised young rats

Animal status	NE	EPI
Sham	45.37 ± 3.51	210.26 ± 10.21
72hrs pancreatectomy	17.68 ± 3.32***†††	104.14 ± 7.35***†
7days pancreatectomy	47.95 ± 4.28	144.11 ± 8.98***

Values are mean ± S.E.M of 4-6 separate experiments

*** $p<0.001$ when compared to sham

† $p<0.05$ when compared to 7days

††† $p<0.001$ when compared to 7days

Table -4

Epinephrine and norepinephrine content (nmoles/g wet wt, of tissue) of plasma of sham and pancreatectomised young rats

Animal status	NE	EPI
Sham	2.21 ± 0.44	3.26 ± 0.26
72hrs pancreatectomy	0.88 ± 0.09***	0.83 ± 0.12***†††
7days pancreatectomy	1.01 ± 0.06***	2.75 ± 0.28

Values are mean ± S.E.M of 4-6 separate experiments

*** $p<0.001$ when compared to sham

††† $p<0.001$ when compared to 7days

Figure - 4

Scatchard analysis of [^{3}H]Epinephrine binding against epinephrine in the cerebral cortex of sham and pancreatectomised young rats

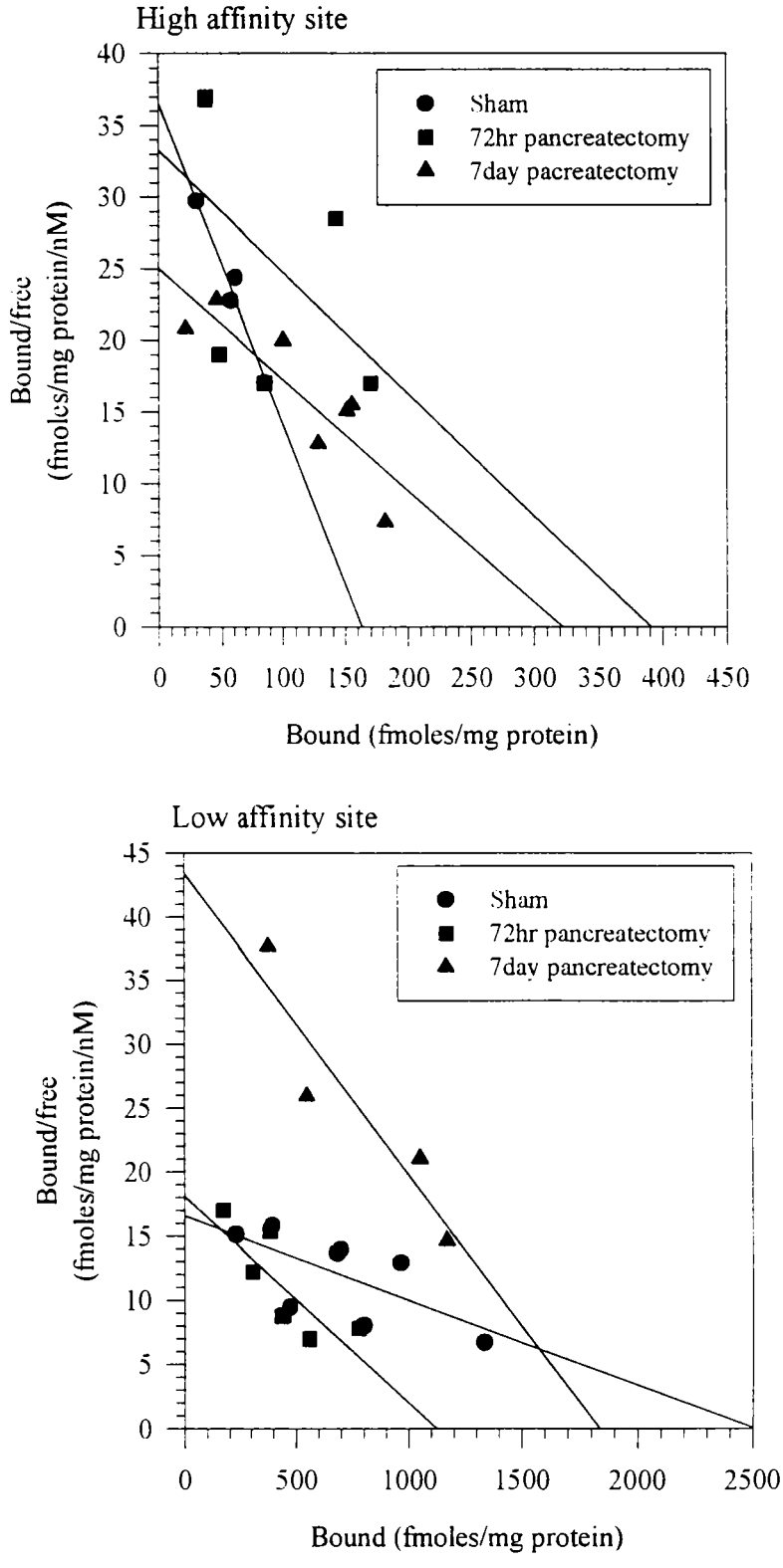


Table-5

Scatchard analysis of [^{3}H]Epinephrine binding against epinephrine in the cerebral cortex of sham and pancreatectomised young rats

Animal status	[^{3}H]Epinephrine binding			
	High affinity		Low affinity	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
Sham	91.17 ± 11.12	5.96 ± 0.68	3116.67 ± 120.00	160.00 ± 5.24
72hr pancreatectomy	209.33 ± 11.35**	10.66 ± 0.81**	1240.00 ± 41.79**	71.07 ± 2.23*
7day pancreatectomy	170.00 ± 4.00*	9.38 ± 0.56*	1800.00 ± 103.75**	41.38 ± 3.33**

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared to sham
*p<0.05 when compared to sham
B_{max} - Maximal binding; K_d - Dissociation constant

Table - 6

Binding parameters of [^{3}H]Epinephrine against epinephrine in the cerebral cortex of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.30	-4.08	2.75x10-9	2.42x10-9	-0.61
72hrs pancreatectomy	One-site	-7.64	-	1.26x10-8	-	-1.73
7days pancreatectomy	Two-site	-8.35	-5.24	4.46x10-5	3.15x10-6	-0.42

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 5

Displacement of [^{3}H]Epinephrine with epinephrine in the cerebral cortex of sham and pancreatectomised young rats

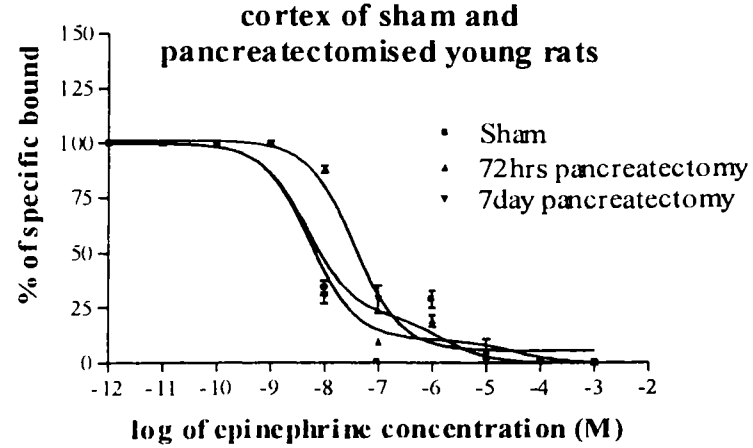


Figure - 6

Scatchard analysis of [^{3}H]Epinephrine binding against epinephrine in the brain stem of sham and pancreatectomised young rats

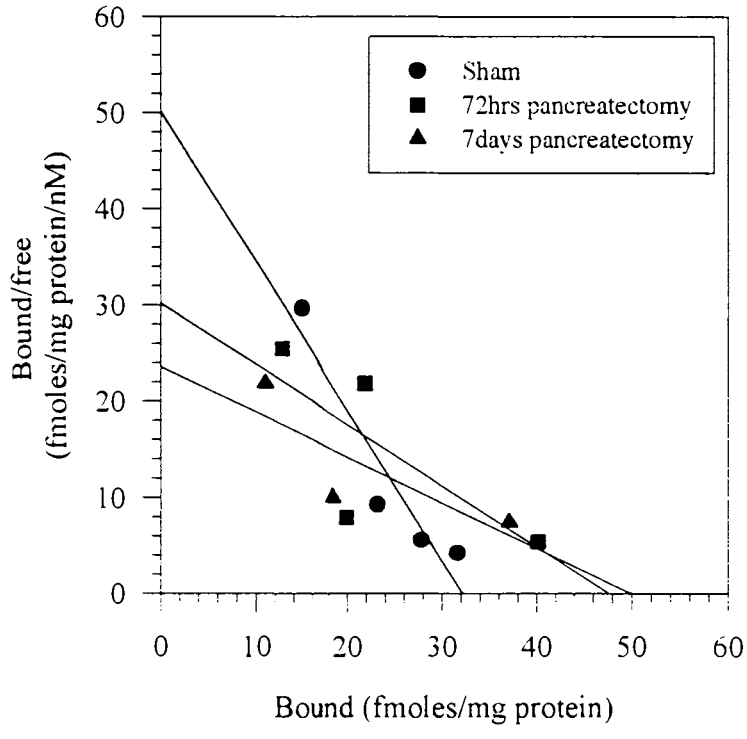


Table - 7

Scatchard analysis of [^{3}H]Epinephrine binding against epinephrine in the brain stem of sham and pancreatectomised young rats

Animal status	[^{3}H]Epinephrine binding	
	B_{max}(fmol/mg protein)	K_d (nM)
Sham	58.5 ± 6.63	0.61 ± 0.04
72hrs pancreatectomy	39.00 ± 9.00	1.96 ± 0.08*
7days pancreatectomy	68.00 ± 4.50	2.58 ± 0.17**

Values are Mean ± S.E.M. of 4-6 separate experiments

**p<0.01 when compared to sham, *p<0.05 when compared to sham.

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 8

Binding parameters of [^{3}H]Epinephrine against epinephrine in the brain stem of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.18	-4.77	$2.52x10^{-9}$	$6.43x10^{-6}$	-0.32
72hrs pancreatectomy	Two-site	-8.84	-4.55	$5.05x10^{-10}$	$1.06x10^{-5}$	-0.35
7days pancreatectomy	Two-site	-8.72	-5.02	$7.20x10^{-10}$	$3.63x10^{-6}$	-0.34

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Figure - 7

Displacement of [^{3}H]Epinephrine with epinephrine in the brain stem of sham and pancreatectomised young rats

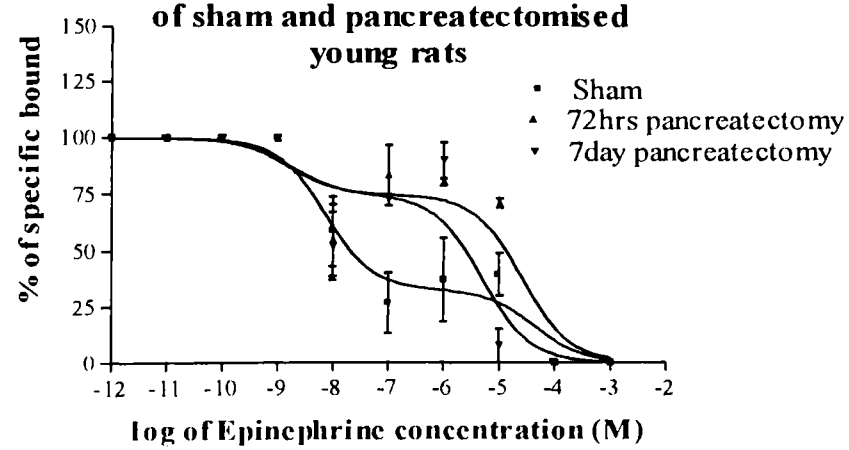


Figure - 8

Scatchard analysis of [^{3}H]Epinephrine binding against epinephrine in the hypothalamus of sham and pancreatectomised young rats

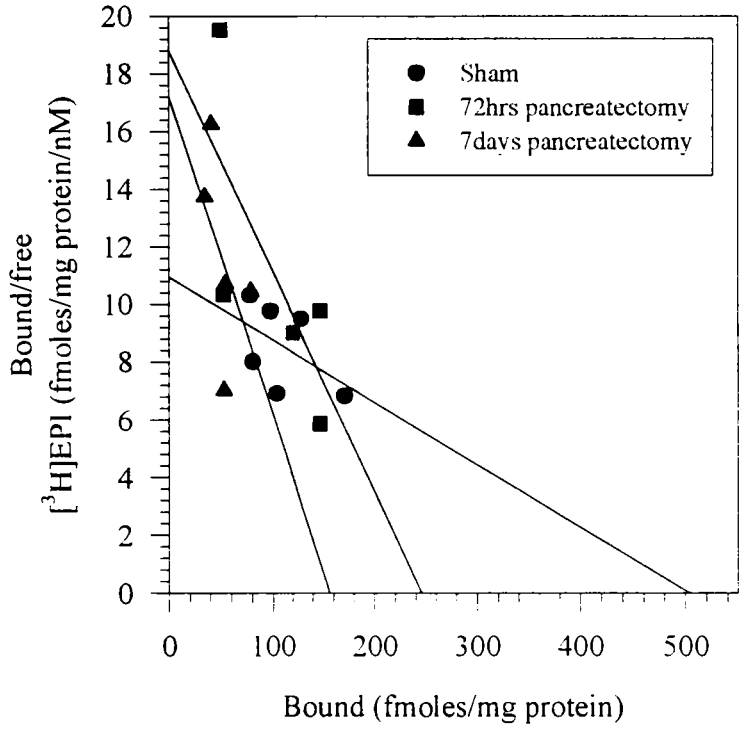


Table - 9

Scatchard analysis of [^{3}H]Epinephrine binding against epinephrine in the hypothalamus of sham and pancreatectomised young rats

Animal status	[^{3}H]Epinephrine binding	
	B_{max}(fmol/mg protein)	K_d (nM)
Sham	486.67 ± 56.25	55.04 ± 14.76
72hrs pancreatectomy	183.33 ± 15.90***	9.46 ± 1.54***
7days pancreatectomy	106.67 ± 10.92***	5.80 ± 1.70***

Values are Mean ± S.E.M. of 4-6 separate experiments

***$p<0.001$ when compared to sham

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 10

Binding parameters of [^{3}H]Epinephrine against epinephrine in the hypothalamus of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.59	-4.29	$2.46x10^{-9}$	$4.86x10^{-5}$	-0.22
72hrs pancreatectomy	Two-site	-8.25	-4.45	$5.34x10^{-9}$	$3.43x10^{-5}$	-0.29
7days pancreatectomy	Two-site	-7.57	-5.71	$2.55x10^{-8}$	$1.85x10^{-6}$	-0.65

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Figure - 9

Displacement of [^{3}H]Epinephrine with epinephrine in the hypothalamus of sham and pancreatectomised young rats

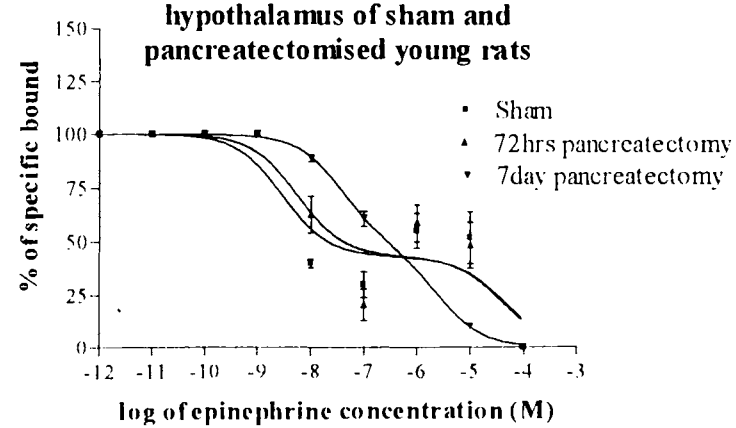


Figure - 10

Scatchard analysis of [^{3}H]Prazosin binding against phentolamine in the cerebral cortex of sham and pancreatectomised young rats

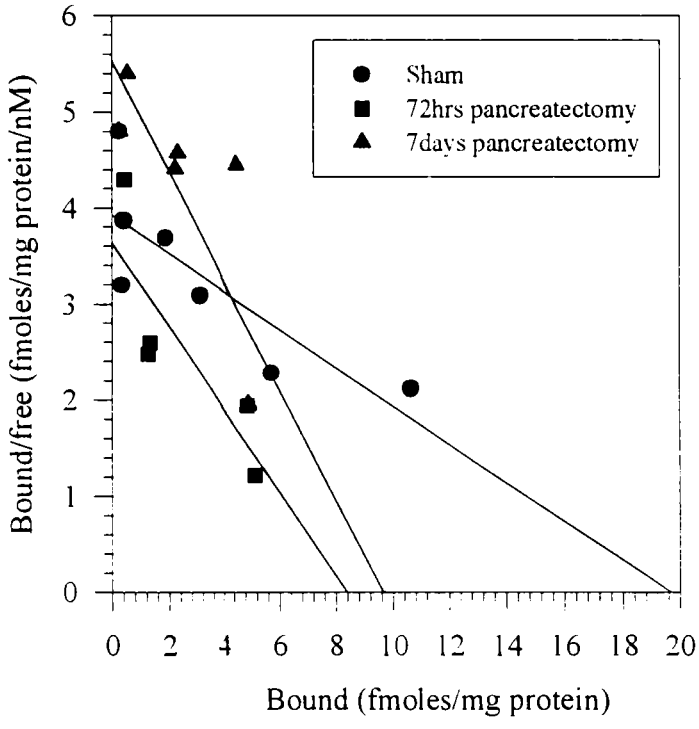


Table-11

Scatchard analysis of [^{3}H]Prazosin binding against phentolamine in the cerebral cortex of sham and pancreatectomised young rats

Animal status	[^{3}H]Prazosin binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	18.50 ± 5.50	5.36 ± 1.30
72hrs pancreatectomy	9.00 ± 3.22**	2.10 ±0.64***
7days pancreatectomy	9.33 ± 3.30*	1.56 ± 0.88***

Values are Mean ± S.E.M. of 4-6 separate experiments

***p<0.001 when compared to sham, **p<0.01 when compared to sham

*p<0.05 when compared to sham

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 12

Binding parameters of [^{3}H]Prazosin against prazosin in the cerebral cortex of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.52	-4.86	$2.95x10^{-9}$	$1.36x10^{-5}$	-0.30
72hrs pancreatectomy	One-site	-8.46	-	$3.43x10^{-9}$	-	-5.40
7days pancreatectomy	One-site	-7.92	-3.62	$1.17x10^{-8}$	$2.33x10^{-4}$	-0.58

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 11

Displacement of [^{3}H]Prazosin with prazosin in the cerebral cortex of sham and pancreatectomised young rats

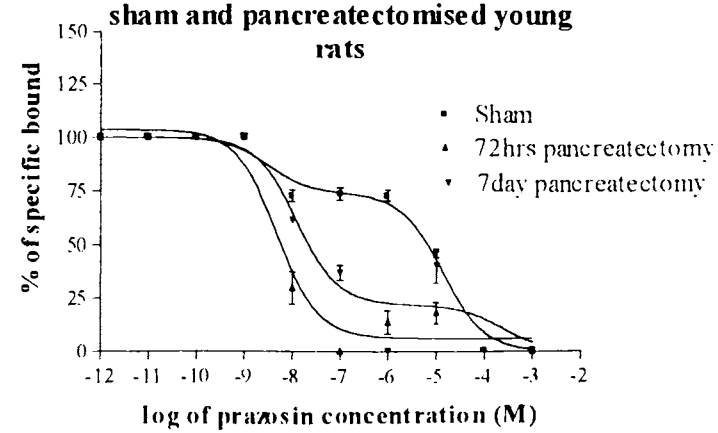


Figure - 12

Scatchard analysis of [^{3}H]Prazosin binding against phentolamine in the brain stem of sham and pancreatectomised young rats

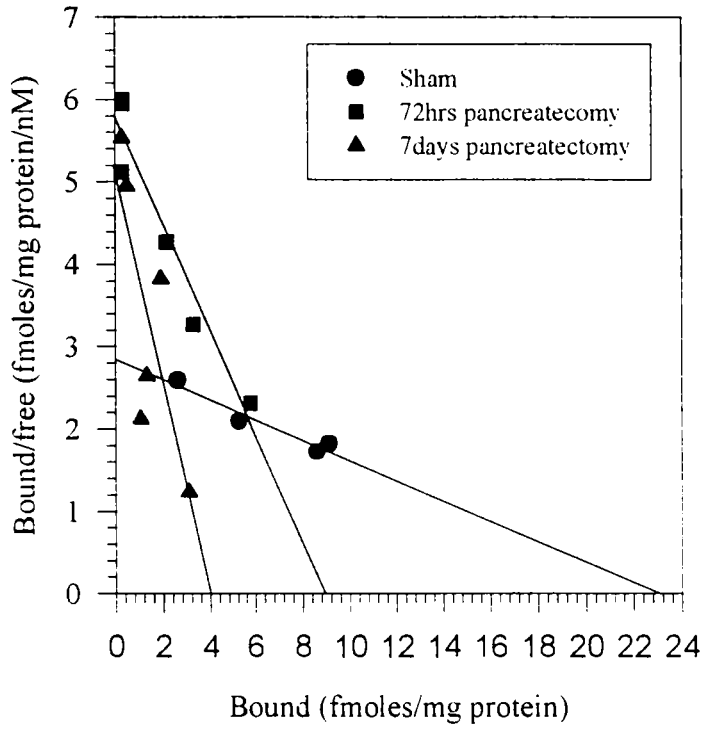


Table-13

Scatchard analysis of [^{3}H]Prazosin binding against phentolamine in the brain stem of sham and pancreatectomised young rats

Animal status	[^{3}H]Prazosin binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	23.33 ± 4.67	6.84 ± 0.62
72hrs pancreatectomy	7.08 ± 4.24*	1.58 ± 0.82**
7days pancreatectomy	3.45 ± 0.26*	0.82 ± 0.08***

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to sham
**p<0.01 when compared to sham
*p<0.05 when compared to sham
B_{max} - Maximal binding; K_d - Dissociation constant

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One-site	-6.38	-	$4.11x10^{-7}$	-	-0.85
72hrs pancreatectomy	Two-site	-8.65	-5.80	$2.22x10^{-9}$	$1.57x10^{-6}$	-0.45
7days pancreatectomy	One-site	-6.93	-	$1.15x10^{-7}$	-	-0.99

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 13

Displacement of [^{3}H]Prazosin agianst prazosin in the brain stem of sham and pancreatectomised young rats

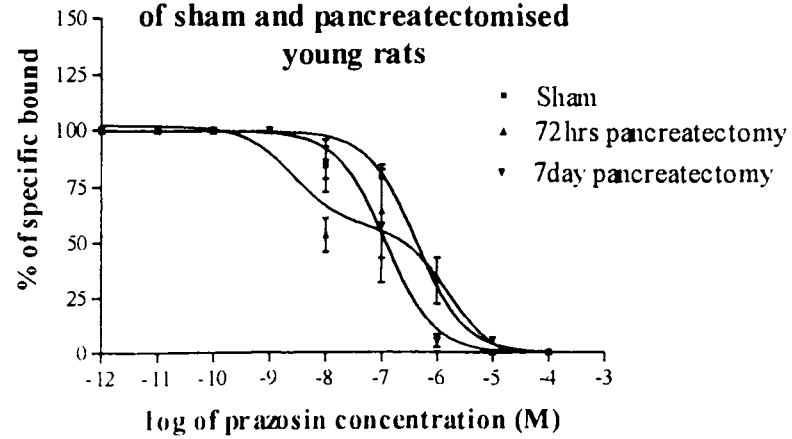


Figure - 14

Scatchard analysis of [^{3}H]Prazosin binding against phentolamine in the hypothalamus of sham and pancreatectomised young rats

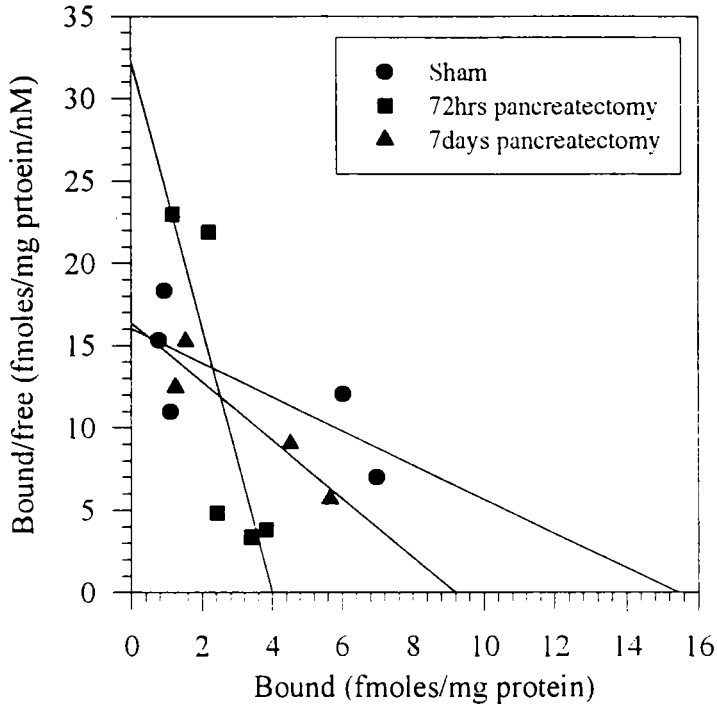


Table-15

Scatchard analysis of [^{3}H]Prazosin binding against phentolamine in the hypothalamus of sham and pancreatectomised young rats

Animal status	[^{3}H]Prazosin binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	23.33 ± 4.67	6.84 ± 0.62
72hrs pancreatectomy	7.08 ± 4.24*	1.58 ± 0.82**
7days pancreatectomy	3.45 ± 0.26*	0.82 ± 0.08***

Values are Mean ± S.E.M. of 4-6 separate experiments

***p<0.001 when compared to sham

**p<0.01 when compared to sham

*p<0.05 when compared to sham

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 16

Binding parameters of [^{3}H]Prazosin against prazosin in the hypothalamus of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One-site	-6.88	-	$1.10x10^{-7}$	-	-1.56
72hrs pancreatectomy	Two-site	-7.60	-7.54	$2.08x10^{-8}$	$2.38x10^{-8}$	-0.16
7days pancreatectomy	One-site	-7.60	-	$2.06x10^{-8}$	-	-1.10

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 15

Displacement of [^{3}H]prazosin with prazosin in the hypothalamus of sham and pancreatectomised young rats

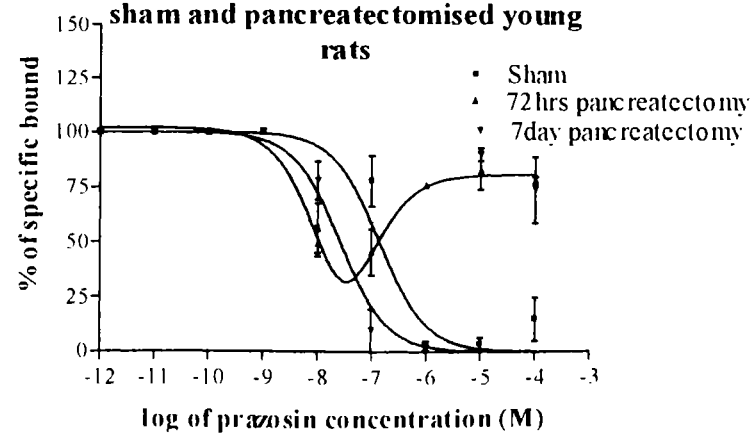


Figure - 16

Scatchard analysis of [^{3}H]Yohimbine binding against phentolamine in the cerebral cortex of sham and pancreatectomised rats

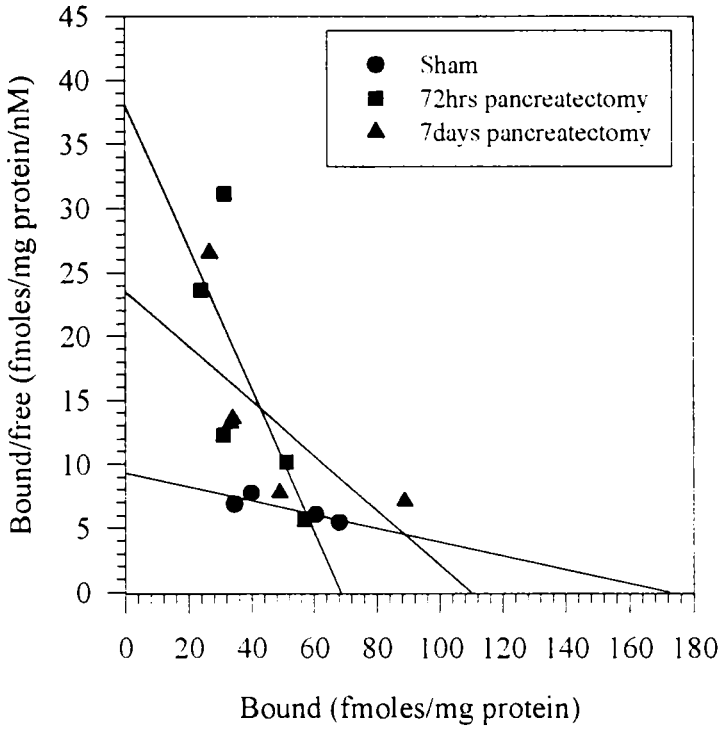


Table-17

Scatchard analysis of [^{3}H]Yohimbine binding against yohimbine in the cerebral cortex of sham and pancreatectomised young rats

Animal status	[^{3}H]Yohimbine binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	135.33 ± 18.49	8.32 ± 1.02
72hrs pancreatectomy	69.50 ± 11.70 *	3.33 ± 0.61*
7day pancreatectomy	77.33 ± 13.53*	2.12 ± 0.15

Values are Mean ± S.E.M. of 4-6 separate experiments
*$p<0.05$ when compared to sham
B_{max} - Maximal binding; K_d - Dissociation constant

Table -18

Binding parameters of [^{3}H]Yohimbine against yohimbine in the cerebral cortex of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-6.70	-7.87	$1.04x10^{-8}$	$1.50x10^{-4}$	-0.33
72hrs pancreatectomy	Two-site	-8.91	-4.32	$1.12x10^{-9}$	$3.72x10^{-5}$	-0.30
7days pancreatectomy	Two-site	-8.59	-5.84	$2.00x10^{-9}$	$1.70x10^{-6}$	-0.38

Valueas are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 17

Displacement of [^{3}H]Yohimbine with yohimbine in the cerebral cortex of sham and pancreatectomised young rats

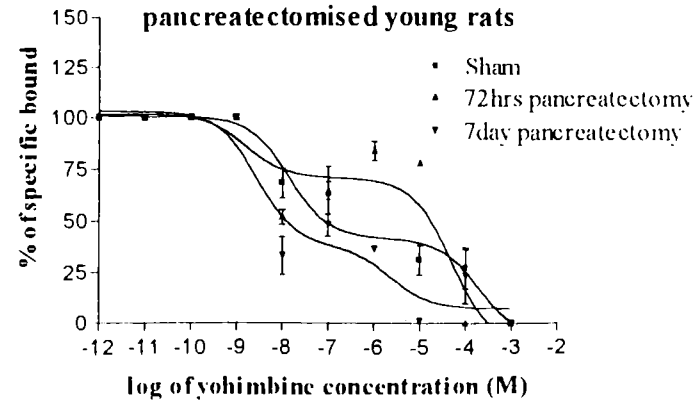


Figure - 18

Scatchard analysis of [^{3}H]Yohimbine binding against phentolamine in the brain stem of sham and pancreatectomised young rats

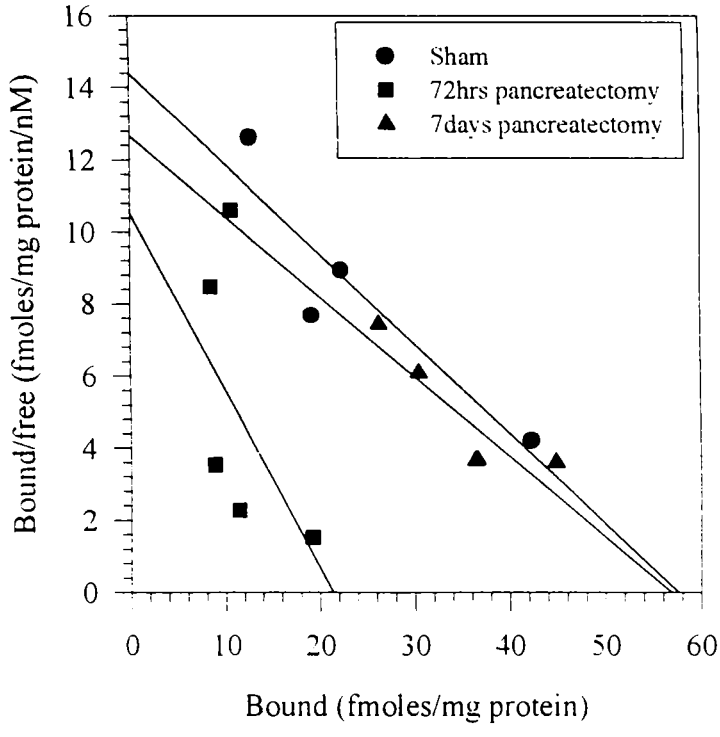


Table - 19

Scatchard analysis of [^{3}H]Yohimbine binding against yohimbine in the brain stem of sham and pancreatectomised young rats

Animal status	[^{3}H]Yohimbine binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	67.00 ± 9.00	4.28 ± 0.25
72hrs pancreatectomy	24.33 ± 28.00*	2.59 ± 0.81*
7days pancreatectomy	71.00 ± 13.53	7.63 ± 2.90

Values are Mean ± S.E.M. of 4-6 separate experiments
*$p<0.05$ when compared to sham
B_{max} - Maximal binding; K_d - Dissociation constant

Table - 20

Binding parameters of [^{3}H]Yohimbine against yohimbine in the brain stem of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One-site	-7.17	-	$4.25x10^{-8}$	-	-4.72
72hrs pancreatectomy	One-site	-7.43	-	$2.35x10^{-8}$	-	-2.67
7days pancreatectomy	One-site	-8.23	-	$3.68x10^{-9}$	-	-4.47

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 19

Displacement of [^{3}H]yohimbine with yohimbine in the brain stem of sham and pancreatectomised young rats

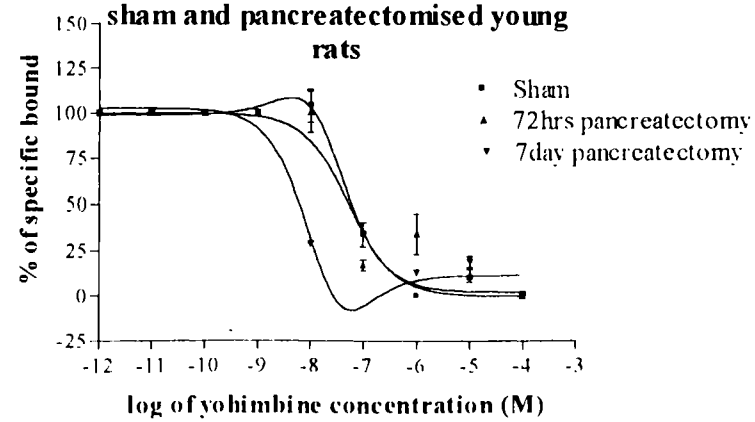


Figure - 20

Sctachard analysis of [^{3}H]Yohimbine binding against phentolamine in the hypothalamus of sham and pancreatectomised young rats

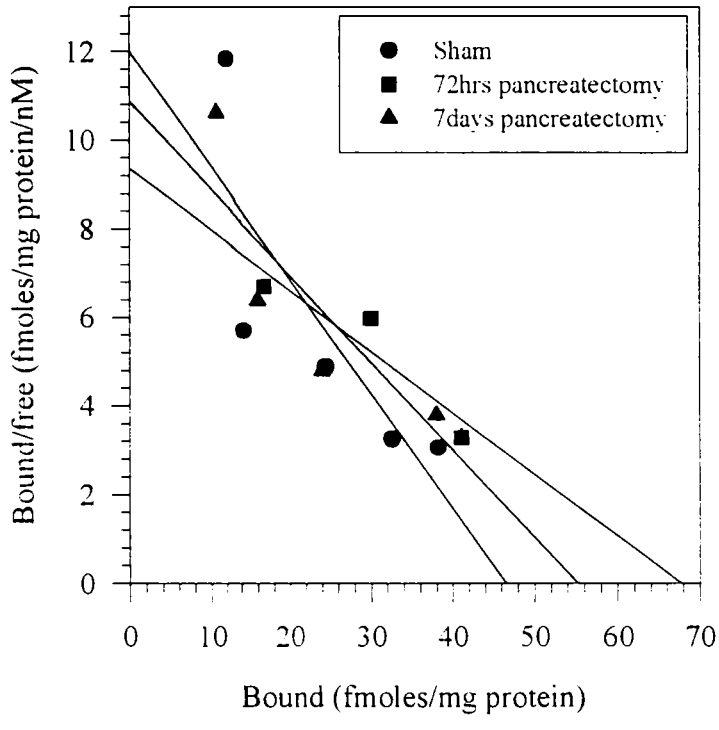


Table-21

Scatchard analysis of [^{3}H]Yohimbine binding against yohimbine in the hypothalamus of sham and pancreatectomised young rats

Animal status	[^{3}H]Yohimbine binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	46.00 ± 1.53	4.11 ± 0.20
72hrs pancreatectomy	41.00 ± 6.08	1.65 ± 0.18**
7days pancreatectomy	50.00 ± 8.08	3.54 ± 0.59

Values are Mean ± S.E.M. of 4-6 separate experiments

**p<0.01 when compared to sham

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 22

Binding parameters of [^{3}H]Yohimbine against yohimbine in the hypothalamus of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One-site	-7.93	-	$7.27x10^{-9}$	-	-1.42
72hrs pancreatectomy	One-site	-8.46	-	$2.15x10^{-9}$	-	-5.90
7days pancreatectomy	One-site	-8.44	-	$2.26x10^{-9}$	-	-4.47

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 21

Displacement of [^{3}H]yohimbine with yohimbine in the hypothalamus of sham and pancreatectomised rats

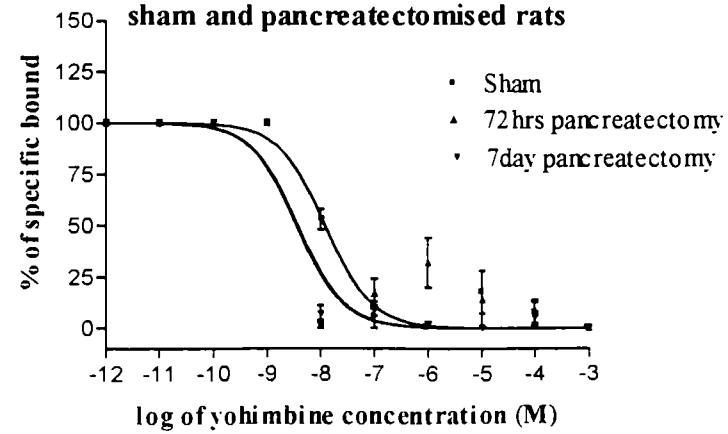


Figure - 22

RT-PCR amplification product of α_{2A}-adrenergic receptor mRNA from the cerebral cortex of experimental rats

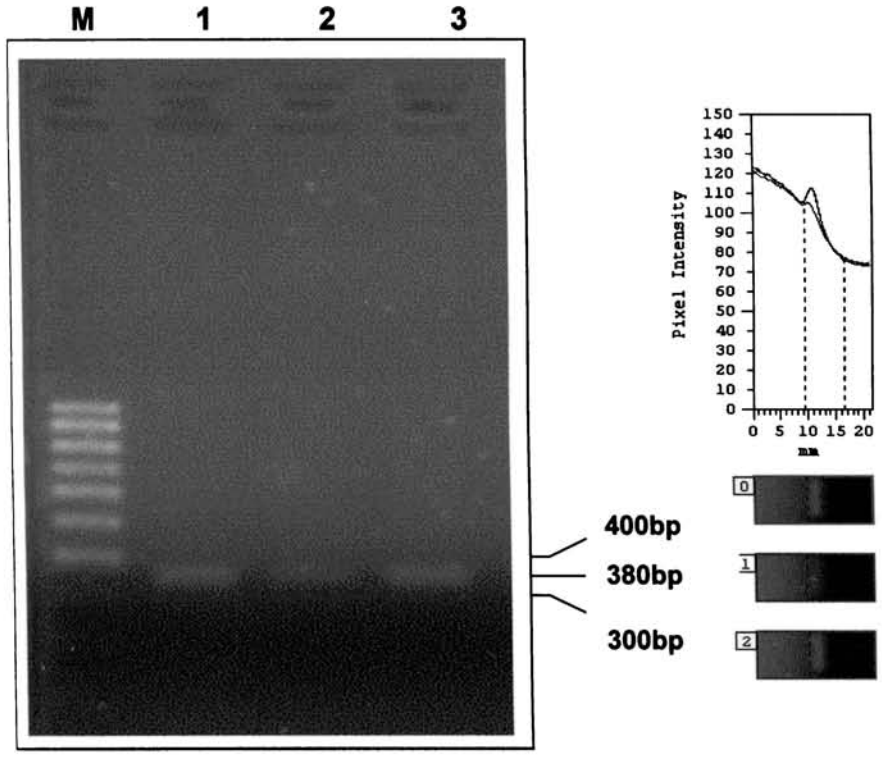


Table - 23

Concentration of RT-PCR amplification product of α_{2A}-adrenergic receptor mRNA in the cerebral cortex of experimental rats

LANE No.	LANE IDENTIFICTION	CONCENTRATION (ng)
M	DNA molecular weight Marker (100bp - 1000bp ladder)	-
1	Sham	2.8 ± 0.09
2	72 hrs pancreatectomy	$2.0 \pm 0.15^{***}$
3	7 days pancreatectomy	$2.9 \pm 0.03^{\dagger\dagger\dagger}$

Values are mean ± S.E.M. of separate determinations

*** $p<0.001$ when compared to sham

††† $p<0.001$ when compared to sham

Figure - 23

RT-PCR amplification product of α_{2A}-adrenergic receptor mRNA from the brain stem of experimental rats

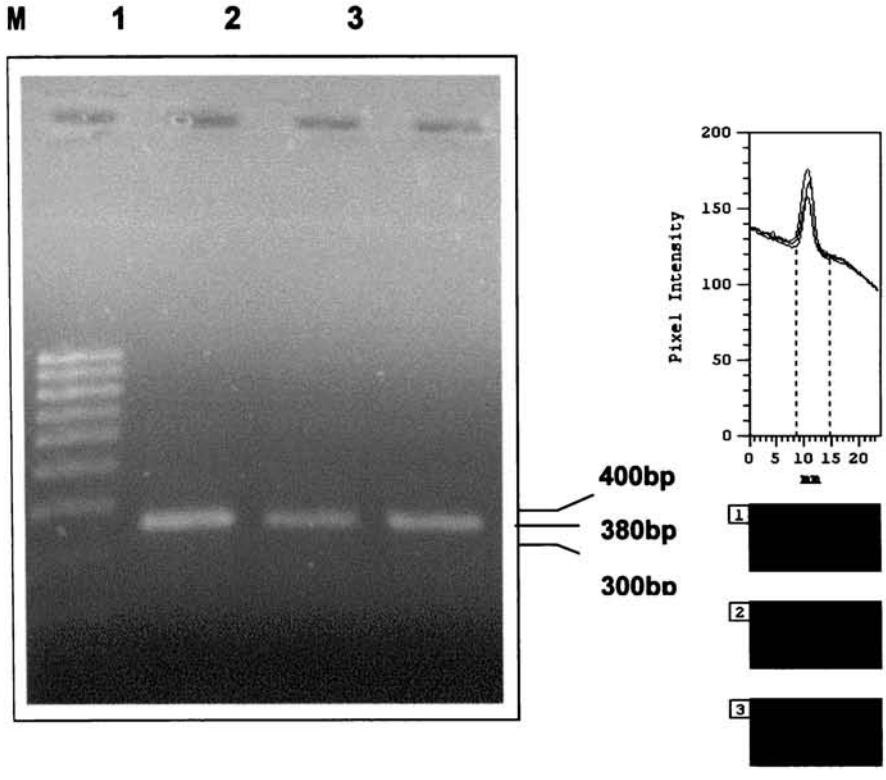


Table - 24

Concentration of RT-PCR amplification product of α_{2A}-adrenergic receptor mRNA in the brain stem of experimental rats

LANE No.	LANE IDENTIFICTION[A]	CONCENTRATION (ng)
M	DNA molecular weight Marker (100bp - 1000bp ladder)	-
1	Sham	11.4 ± 0.12
2	72 hrs pancreatectomy	$9.3 \pm 0.09^{***}$
3	7 days pancreatectomy	$10.5 \pm 0.15^{\dagger\dagger}$

Values are mean ± S.E.M. of separate determinations

*** $p<0.001$ when compared to sham

†† $p<0.01$ when compared to sham

Figure - 24

RT-PCR amplification product of α_{2A}-adrenergic receptor mRNA from the hypothalamus of experimental rats

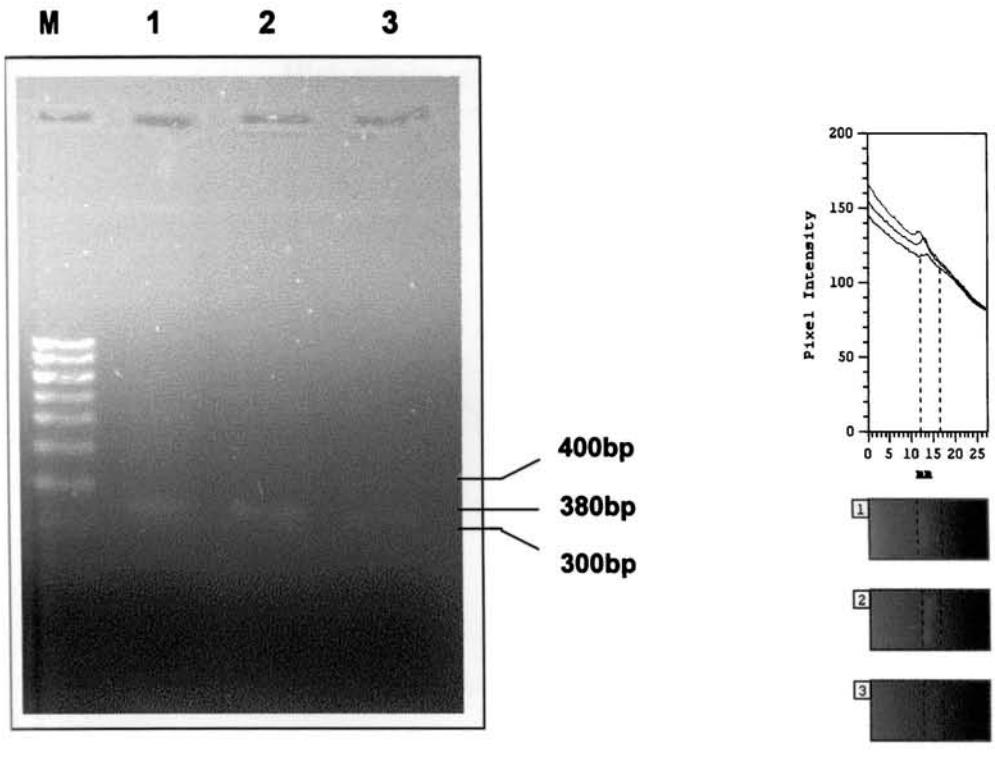


Table - 25

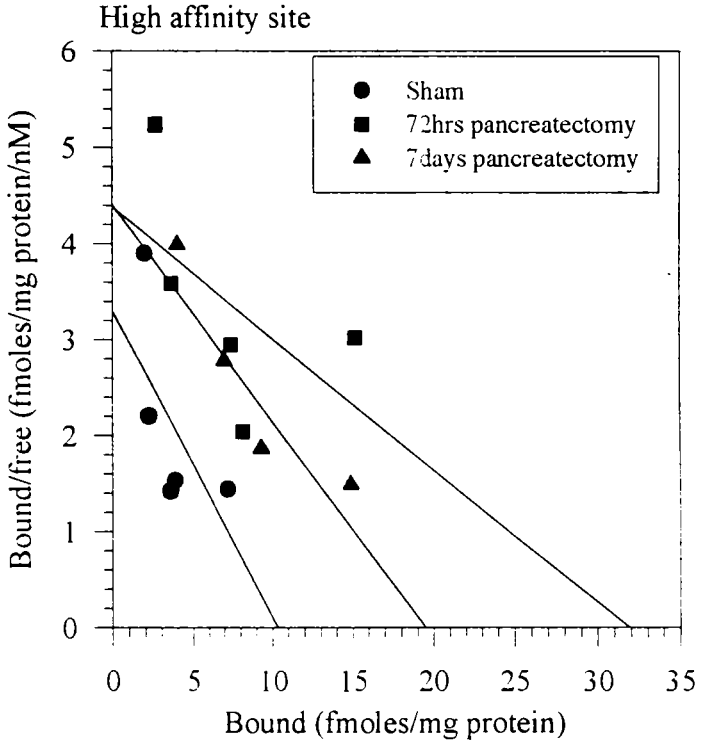
Concentration of RT-PCR amplification product of α_{2A}-adrenergic receptor mRNA in the hypothalamus of experimental rats

LANE No.	LANE IDENTIFICTION	CONCENTRATION (ng)
M	DNA molecular weight Marker (100bp - 1000bp ladder)	-
1	Sham	5.9 ± 0.89
2	72 hrs pancreatectomy	5.3 ± 0.15
3	7 days pancreatectomy	4.0 ± 0.13†

Values are mean ± S.E.M. of separate determinations

Figure - 25

Scatchard analysis of [^{3}H]Propranolol binding against propranolol in the cerebral cortex of sham and pancreatectomised young rats



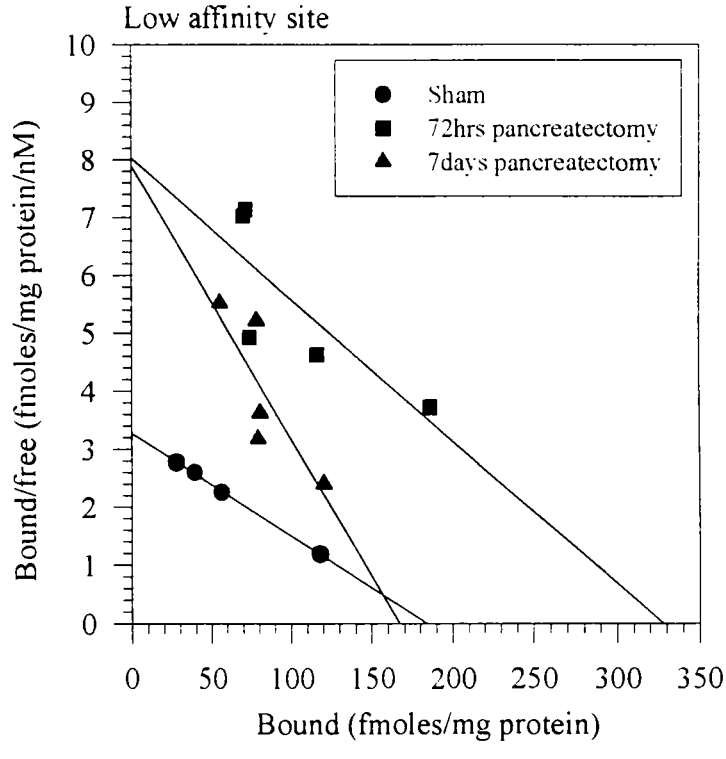


Table-26

Scatchard analysis of [^{3}H]Propranolol binding against propranolol in the cerebral cortex of sham and pancreatectomised young rats

Animal status	[^{3}H]Propranolol binding			
	High affinity		Low affinity	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
Sham	7.67 ± 1.86	0.81 ± 0.07	147.67 ± 26.28	13.58 ± 0.95
72hrs pancreatectomy	26.67 ± 6.22*	4.29 ± 0.44***	314.33 ± 57.20*	113.44 ± 16.21***
7days pancreatectomy	10.50 ± 2.01	2.26 ± 0.33*††	145.00 ± 25.50	40.26 ± 5.36

Values are Mean ± S.E.M. of 4-6 separate experiments

$^{***}p<0.001$ when compared to sham

$^{*}p<0.05$ when compared to sham

$^{††}p<0.01$ when compared to 72hr pancreatectomy

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 27

Binding parameters of [^{3}H]Propranolol against propranolol in the cerebral cortex of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.54	-2.42	$1.28x10^{-9}$	$1.70x10^{-3}$	-0.28
72hrs pancreatectomy	One-site	-8.37	-	$1.92x10^{-9}$	-	-2.79
7days pancreatectomy	Two-site	-8.68	-6.13	$9.38x10^{-10}$	$3.33x10^{-7}$	-0.36

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 26

Displacement of [^{3}H]Propranolol with propranolol in the cerebral cortex of sham nd pancreatectomised young rats

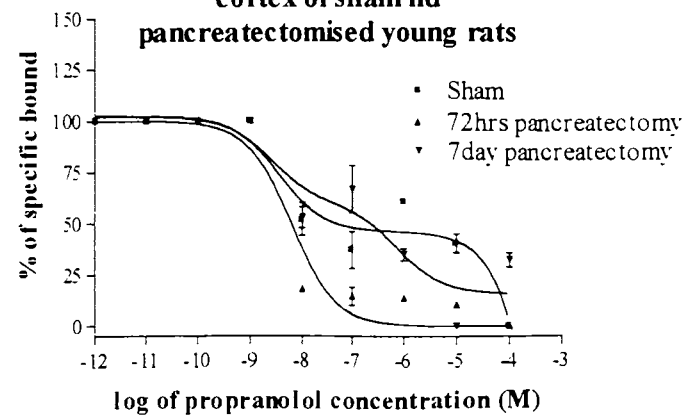


Figure - 27

Scatchard analysis of [^{3}H]Propranolol binding against propranolol in the brain stem of sham and pancreatectomised young rats

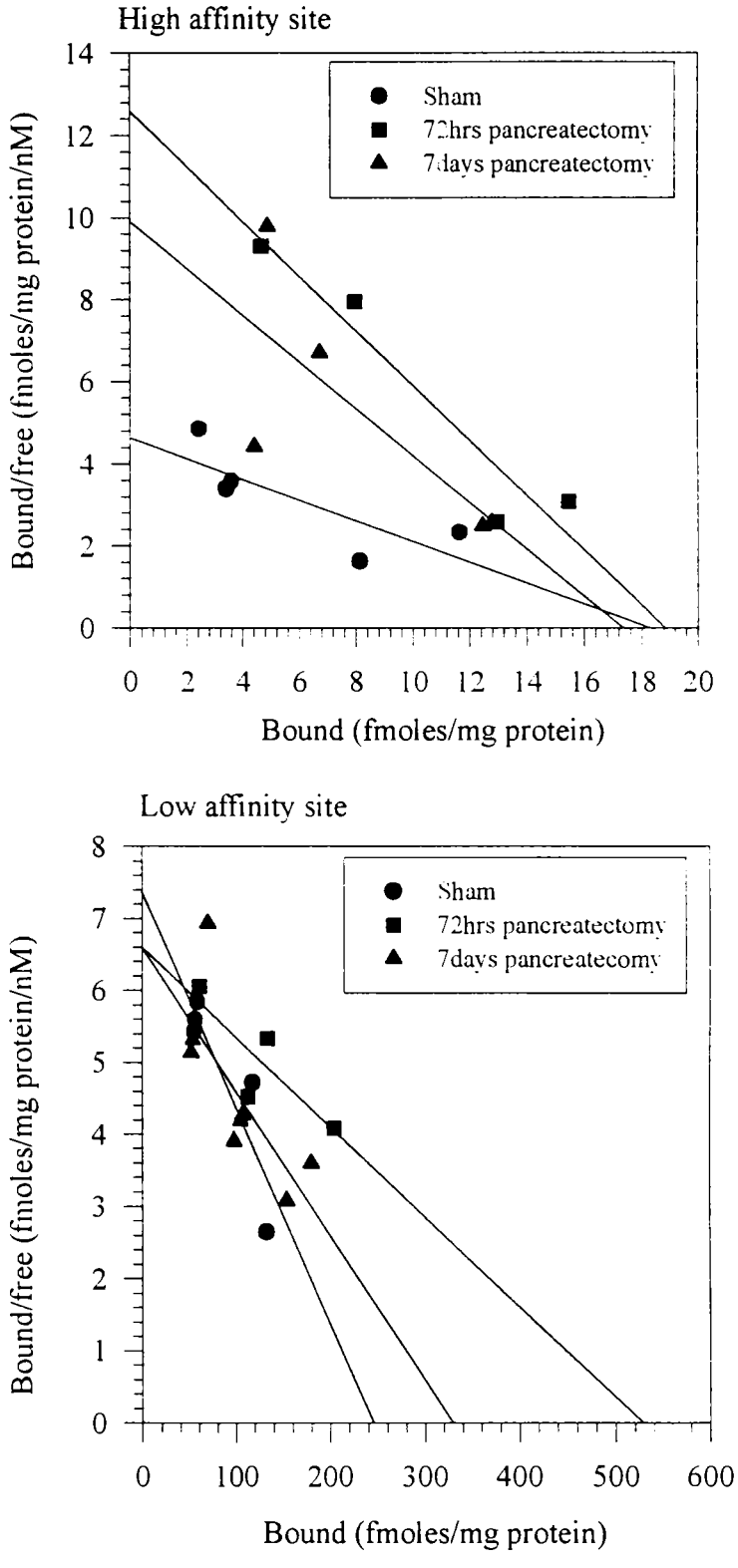


Table-28

Scatchard analysis of [^{3}H]propranolol binding against propranolol in the brain stem of sham and pancreatectomised young rats

Animal status	[^{3}H]propranolol binding			
	High affinity		Low affinity	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
Sham	22.50 ± 5.95	3.40 ± 0.86	280.00 ± 60.00	44.70 ± 6.20
72hr pancreatectomy	31.00 ± 9.71	2.49 ± 0.60	546.00 ± 53.00*	54.17 ± 12.74
7day pancreatectomy	17.50 ± 2.50	2.16 ± 0.55	306.67 ± 12.02	43.97 ± 3.90

Values are Mean ± S.E.M. of 4-6 separate experiments
*$p<0.05$ when compared to sham
B_{max} - Maximal binding; K_d - Dissociation constant

Table - 29

Binding parameters of [^{3}H]Propranolol against propranolol in the brain stem of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One -site	-5.84	-	$1.12x10^{-6}$	-	-1.51
72hrs pancreatectomy	One-site	-6.38	-	$3.23x10^{-7}$	-	-1.10
7days pancreatectomy	Two -site	-8.21	-4.62	$4.72x10^{-9}$	$1.86x10^{-9}$	-0.38

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Figure - 28

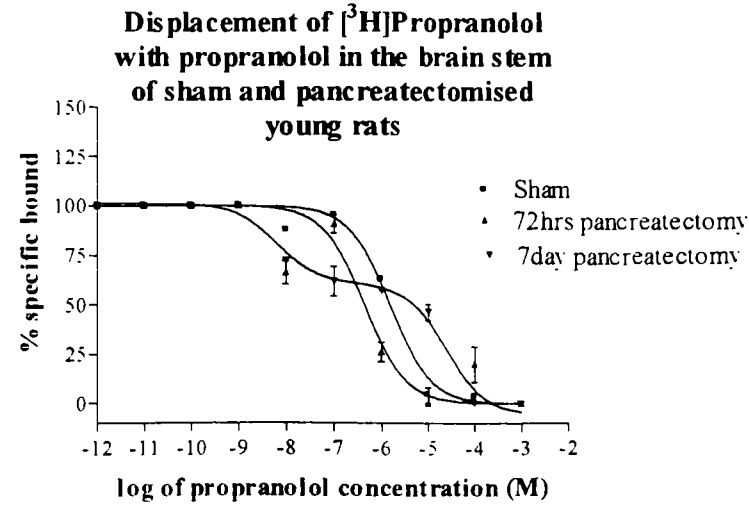
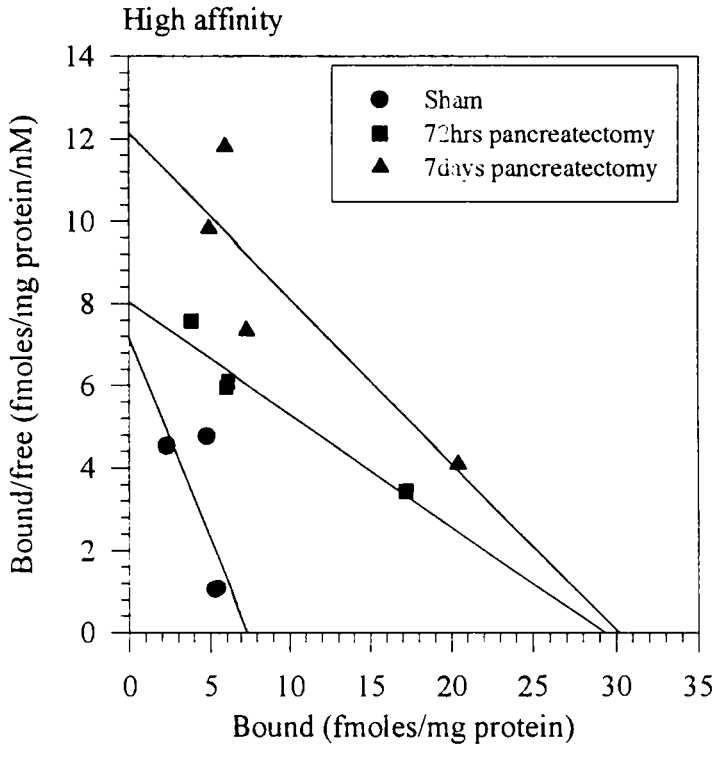


Figure - 29

Scatchard analysis of [^{3}H]Propranolol binding against propranolol in the hypothalamus of sham and pancreatectomised young rats



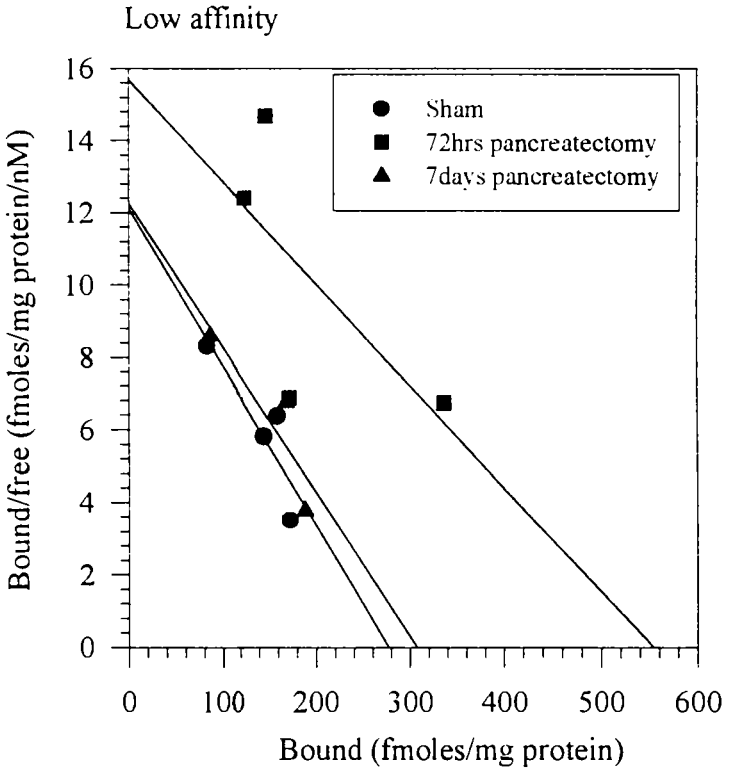


Table-30

Scatchard analysis of [^{3}H]propranolol binding against propranolol in the hypothalamus of sham and pancreatectomised young rats

Animal status	[^{3}H]propranolol binding			
	High affinity		Low affinity	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
Sham	9.16 ± 1.67	1.32 ± 0.13	370.00 ± 20.00	44.07 ± 4.22
2hr pancreatectomy	48.00 ± 5.69***	4.65 ± 0.64**	551.67 ± 16.42*	36.99 ±1.96
1day pancreatectomy	36.66 ± 4.41**	3.02 ± 0.78	456.67.± 86.67	33.37 ± 7.54

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to sham
**p<0.01 when compared to sham
*p<0.05 when compared to sham
B_{max} - Maximal binding; K_d - Dissociation constant

Table - 31

Binding parameters of [^{3}H]Propranolol against propranolol in the hypothalamus of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.84	-1.89	$8.20x10^{-10}$	4.44x	-0.19
72hrs pancreatectomy	Two -site	-8.53	-4.99	$1.69x10^{-9}$	$5.77x10^{-6}$	-0.44
7days pancreatectomy	Two-site	-8.74	-4.77	$1.02x10^{-9}$	$9.49x10^{-6}$	-0.32

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 30

Displacement of [^{3}H]Propranolol with propranolol in the hypothalamus of sham and pancreatectomised young rats

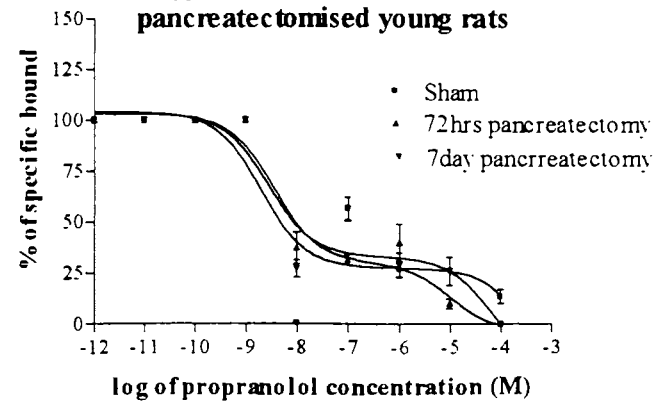


Figure - 31

RT-PCR amplification product of β-adrenergic receptor mRNA from the cerebral cortex of experimental rats

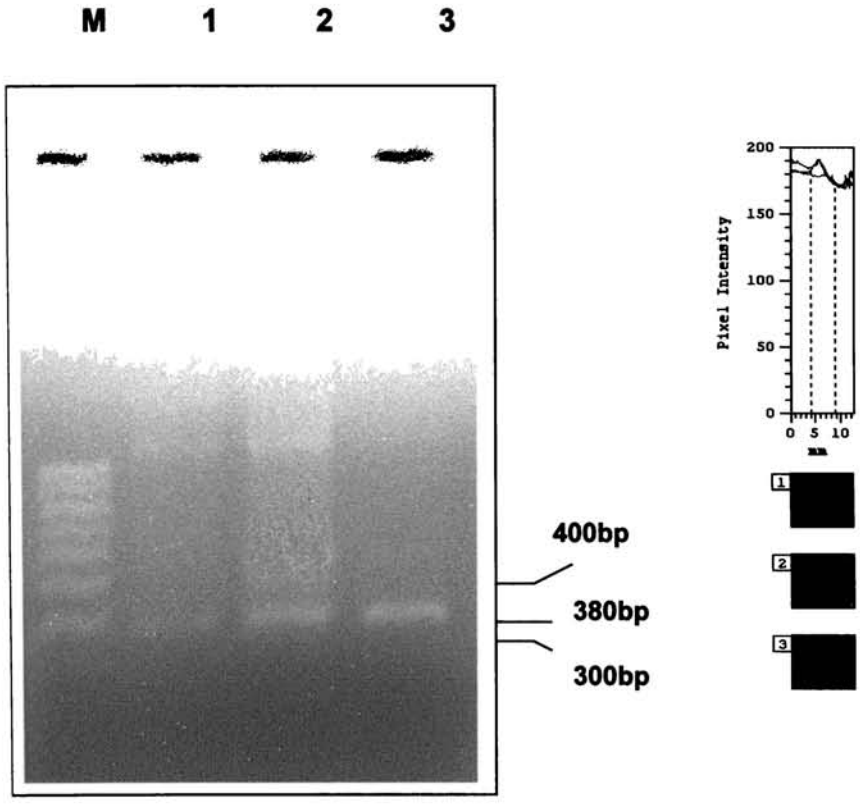


Table - 32

Concentration of RT-PCR amplification product of β-adrenergic receptor mRNA in the cerebral cortex of experimental rats

LANE No.	LANE IDENTIFICTION	CONCENTRATION (ng)
M	DNA molecular weight Marker (100bp - 1000bp ladder)	-
1	Sham	3.8 ± 0.06
2	72 hrs pancreatectomy	5.2 ± 0.06***
3	7 days pancreatectomy	5.5 ± 0.21***

Values are mean ± S.E.M. of separate determinations

*** $p<0.001$ when compared to sham

Figure - 32

RT-PCR amplification product of β-adrenergic receptor mRNA from the brain stem of experimental rats

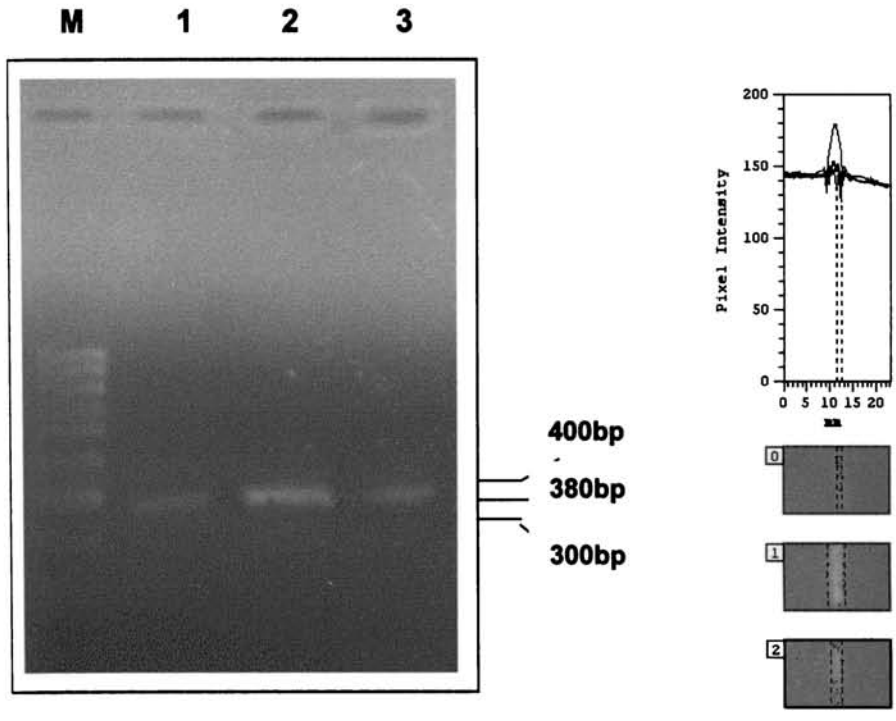


Table - 33

Concentration of RT-PCR amplification product of β-adrenergic receptor mRNA in the brain stem of experimental rats

LANE No.	LANE IDENTIFICTION[A]	CONCENTRATION (ng)
M	DNA molecular weight Marker (100bp - 1000bp ladder)	-
1	Sham	0.8 ± 0.06
2	72 hrs pancreatectomy	4.1 ± 0.06***
3	7 days pancreatectomy	0.9 ± 0.06†††

Values are mean ± S.E.M. of separate determinations

*** p<0.001 when compared to sham

††† p<0.001 when compared to control

Figure - 33

RT-PCR amplification product of β-adrenergic receptor mRNA from the hypothalamus of experimental rats

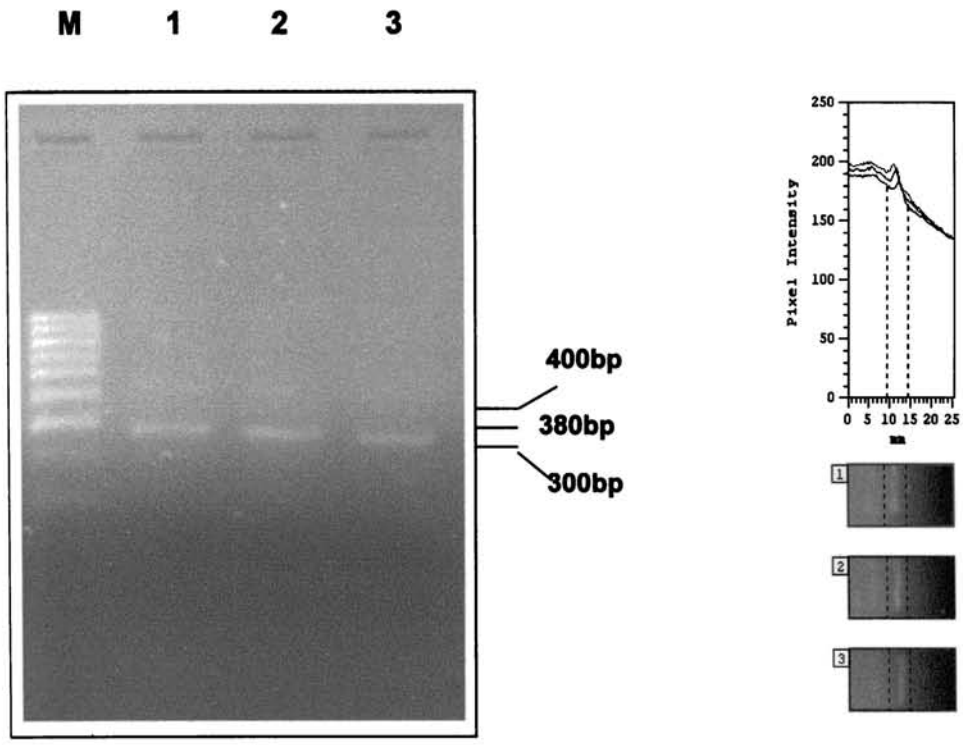


Table - 34

Concentration of RT-PCR amplification product of β-adrenergic receptor mRNA in the hypothalamus of experimental rats

LANE No.	LANE IDENTIFICTION	CONCENTRATION (ng)
M	DNA molecular weight Marker (100bp - 1000bp ladder)	-
1	Sham	7.0 ± 0.06
2	72 hrs pancreatectomy	5.7 ± 0.08***
3	7 days pancreatectomy	5.0 ± 0.06***

Values are mean ± S.E.M. of separate determinations

*** $p<0.001$ when compared to sham

Figure - 34

ırd analysis of [^{3}H]Epinephrine binding against epinephrine in the membrane fraction of pancreatic islets of sham and pancreatectomised young rats

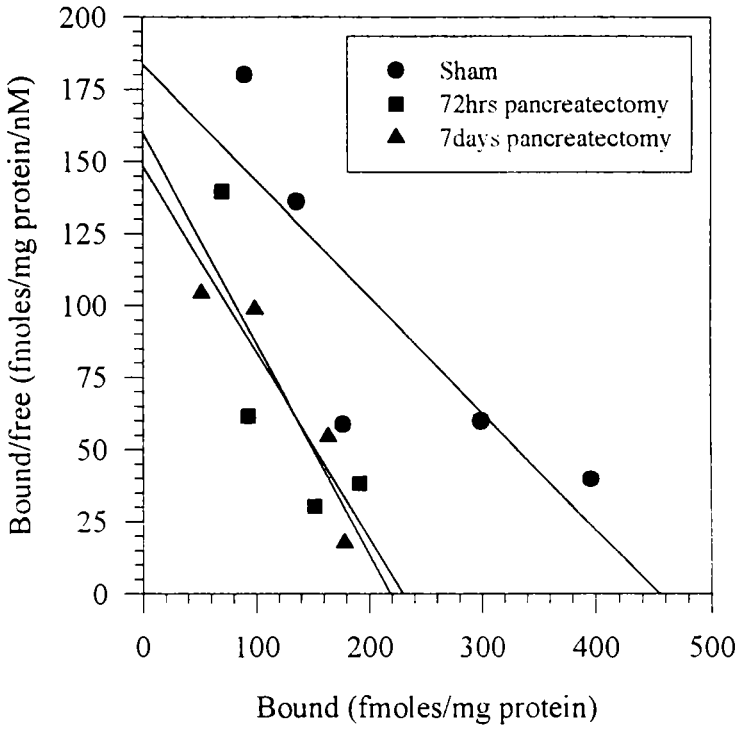


Table -35

Scatchard analysis of [^{3}H]Epinephrine binding against epinephrine in the membrane fraction of pancreatic islets of sham and pancreatectomised young rats

Animal status	B_{max}(fmol/mg protein)	K_d (nM)
Sham	440.33 ± 18.49	2.32 ± 0.20
72hrs pancreatectomy	220.50 ± 1.70**	1.38 ± 0.12*
7days pancreatectomy	205.33 ± 13.53*	1.41 ± 0.15*

Values are mean ± S.E.M of 4-6 separate experiments
**p<0.01 when compared to sham, *p<0.05 when compared to to sham
B_{max} - Maximal binding, K_d - Dissociation constant

Table - 36

Binding parameters of [^{3}H]Epinephrine against epinephrine in the membrane fraction of Pancreatic islets of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One-site	-8.33	-	$3.37x10^{-9}$	-	-1.26
72hrs pancreatectomy	Two-site	-7.46	-4.81	$2.46x10^{-8}$	$1.12x10^{-5}$	-0.25
7days pancreatectomy	One-site	-4.78	-	$1.20x10^{-5}$	-	-1.31

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Figure - 35

Displacement of [^{3}H]Epinephrine with epinephrine in the membrane fraction of sham and pancreatectomised young rats

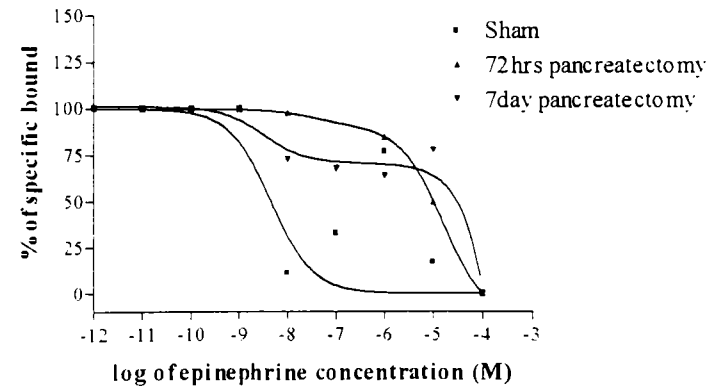


Figure - 36

Scatchard analysis of [^{3}H]prazosin binding against phentolamine in the islets of sham and pancreatectomised young rats

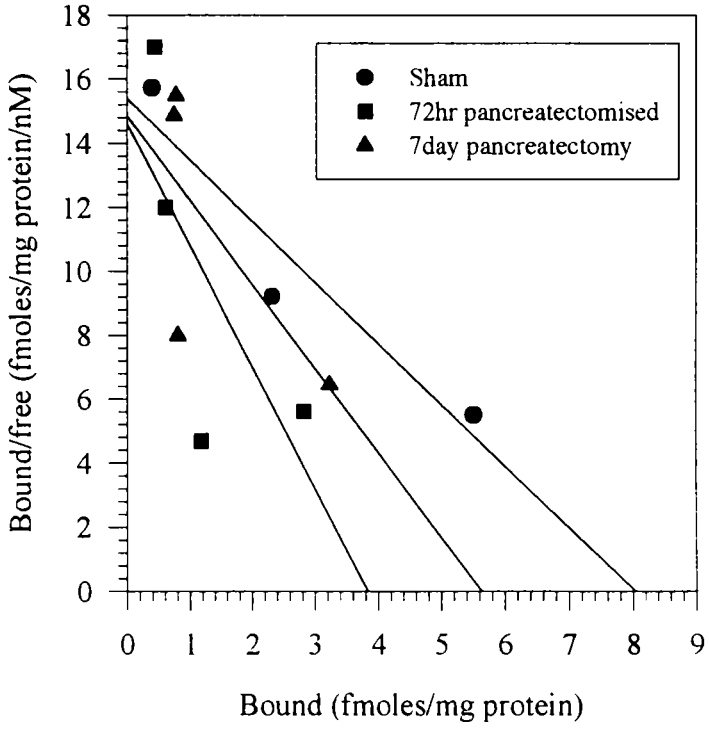


Table-37

Scatchard analysis of [^{3}H]Prazosin binding against phentolamine in the pancreatic islets of sham and pancreatectomised young rats

Animal status	[^{3}H]Prazosin binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	8.73 ± 0.73	0.58 ± 0.05
72hr pancreatectomy	3.87 ± 0.29*	0.37 ± 0.07
7day pancreatectomy	6.97 ± 1.12	0.40 ± 0.03

Values are Mean ± S.E.M. of 4-6 separate experiments

*$p<0.05$ when compared to sham

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 38

Binding parameters of [^{3}H]Prazosin against prazosin in the pancreatic islets of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One -site	-7.54	-	$2.63x10^{-8}$	-	-9.74
72hrs pancreatectomy	One-site	-7.50	-	$2.90x10^{-8}$	-	-10.06
7days pancreatectomy	One -site	-7.34	-	$4.34x10^{-8}$	-	-1.61

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Figure - 37

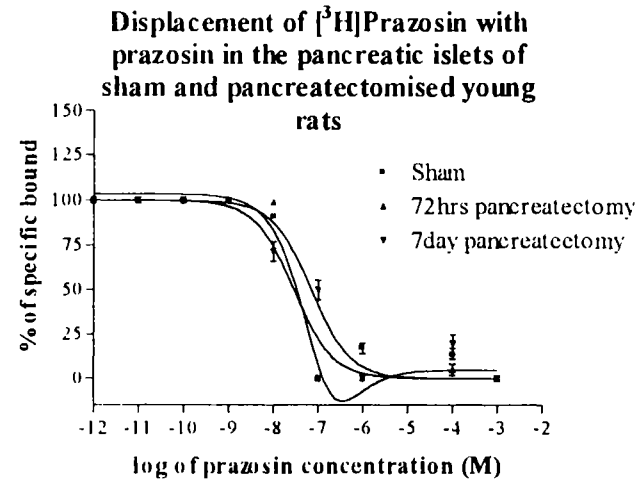


Figure - 38

Scatchard analysis of [^{3}H]Yohimbine binding against yohimbine in the pancreatic islets of sham and pancreatectomised young rats

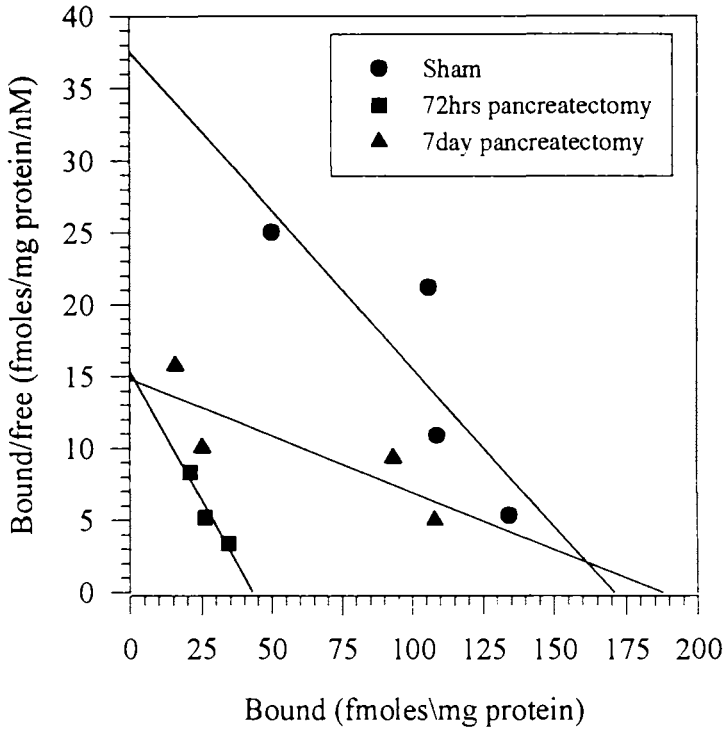


Table-39

Scatchard analysis of [^{3}H]Yohimbine binding against yohimbine in the pancreatic islets of sham and pancreatectomised young rats

Animal status	[^{3}H]Yohimbine binding	
	B_{max} (fmol/mg protein)	K_d (nM)
Sham	170.00 ± 13.53	4.59 ± 0.20
72hr pancreatectomy	40.00 ± 6.08***	2.67 ± 0.11*
7day pancreatectomy	190.00 ± 15.88†††	6.67 ± 0.98†††

Values are Mean ± S.E.M. of 4-6 separate experiments

$^{***}p<0.001$ when compared to sham

$^{*}p<0.05$ when compared to sham

$^{\dagger\dagger\dagger}p<0.001$ when compared to sham

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 40

Binding parameters of [^{3}H]Yohimbine against yohimbine in the pancreatic islets of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One -site	-5.72	-	$1.28x10^{-6}$	-	-2.09
72hrs pancreatectomy	One-site	-7.57	-	$1.82x10^{-8}$	-	-1.17
7days pancreatectomy	One -site	-8.43	-	$2.47x10^{-9}$	-	-7.40

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 39

Displacement of [^{3}H]Yohimbine with yohimbine in the pancreatic islets of sham and pancreatectomised young rats

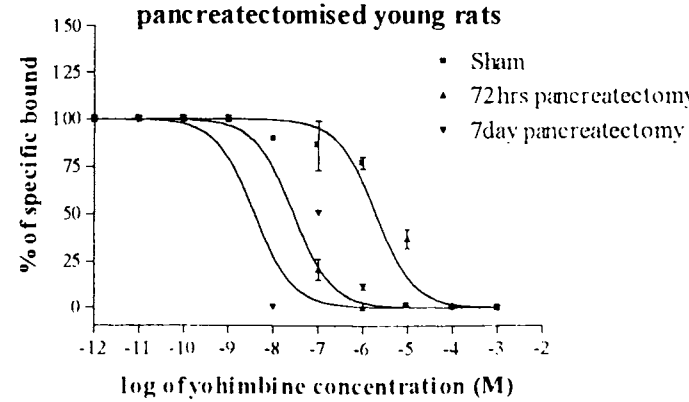
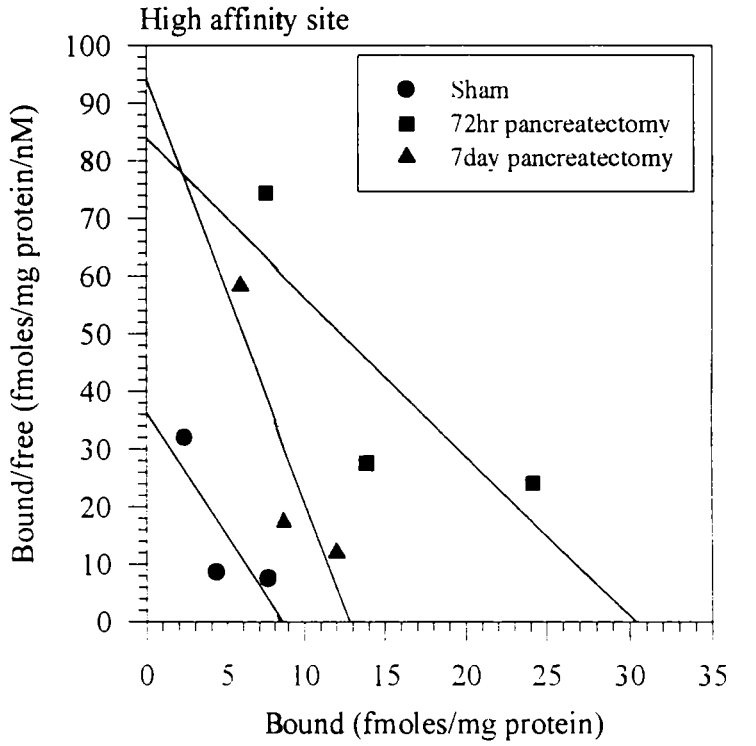


Figure - 40

Scatchard anlysis of [^{3}H]propranolol binding against propranolol in the islets of sham and pancreatectomised young rats



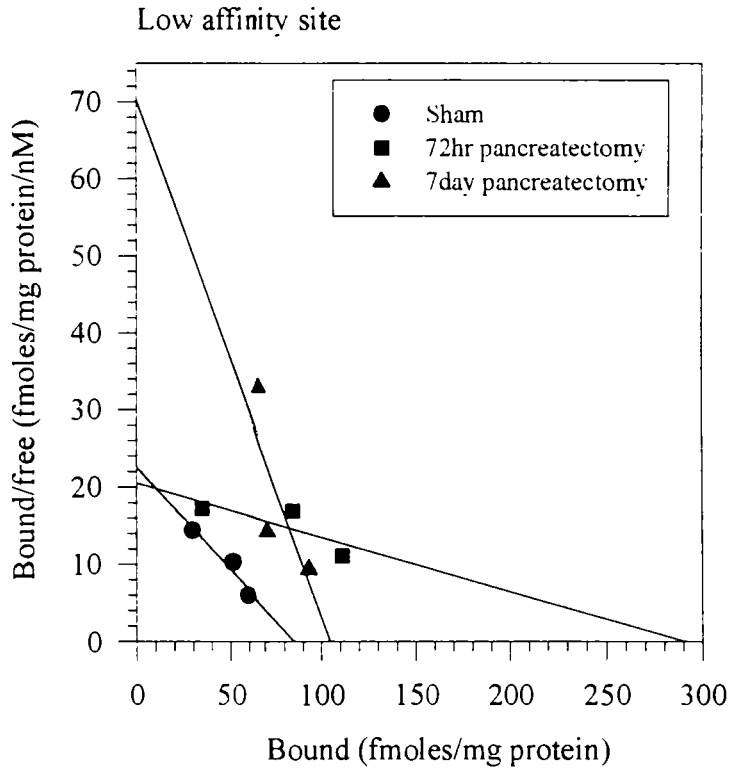


Table-41

Scatchard analysis of [^{3}H]Propranolol binding against propranolol in the pancreatic islets of sham and pancreatectomised young rats

Animal status	of [^{3}H]Propranolol binding			
	High affinity		Low affinity	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
Sham	7.50 ± 1.98	0.21 ±0.05	90.00 ± 9.00	4.09 ± 0.89
2hr pancreatectomy	30.00 ± 9.00***	0.36 ± 0.08	290.00 ± 10.58***	3.81 ±0.91
1day pancreatectomy	13.00 ± 1.46*	0.14 ± 0.02	110.00 ± 14.33	2.57 ± 0.58

Values are Mean ± S.E.M. of 4-6 separate experiments

***p<0.001 when compared to sham

*p<0.05 when compared to sham

B_{max} - Maximal binding; K_d - Dissociation constant

Table - 42

Binding parameters of [^{3}H]Propranolol against propranolol in the pancreatic islets of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC50)-1	Log (EC50)-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	One -site	-8.27	-	$1.60x10^{-9}$	-	-1.60
72hrs pancreatectomy	Two-site	-7.83	-7.81	$4.39x10^{-}$	$4.63x10^{-9}$	-0.60
7days pancreatectomy	Two-site	-8.84	-3.07	$4.26x10^{-10}$	$2.55x10^{-4}$	-0.32

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism and a one-site Vs two-site model. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50}. The equation built into the programme is defined in terms of the $\log(EC_{50})$.

Figure - 41

Displacement of [^{3}H]Propranolol with propranolol in the pancreatic islets of sham and pancreatectomised young rats

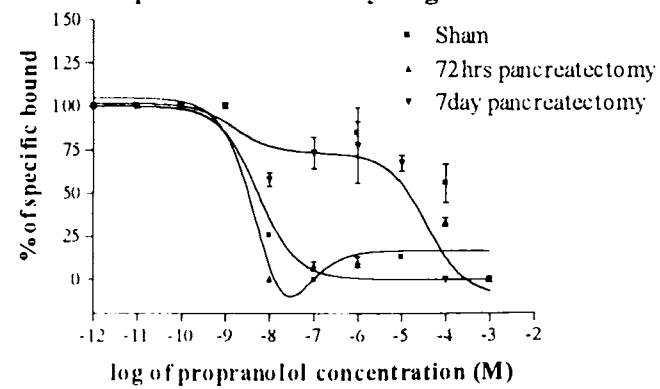
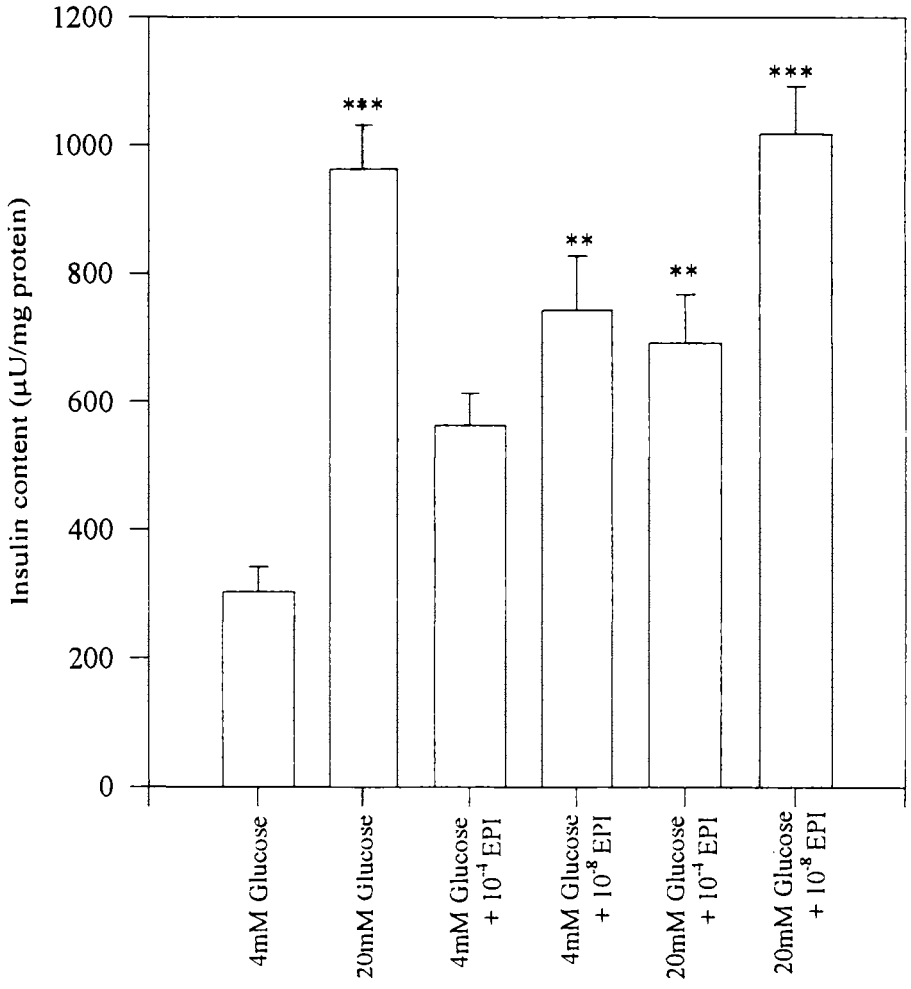


Figure - 42

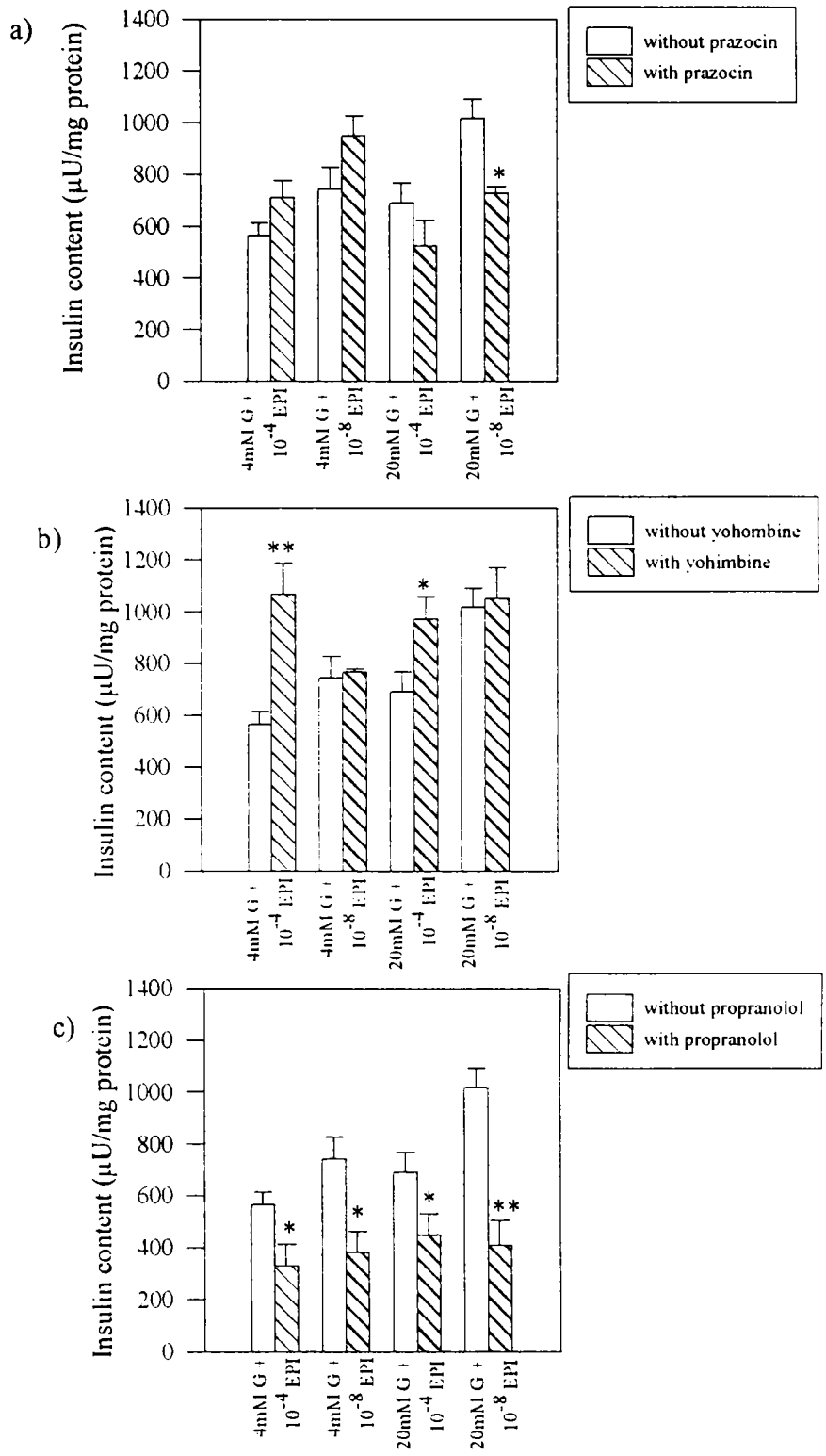
Glucose induced insulin secretion from pancreatic islets in 1hr *in vitro*



Values are mean ± S.E.M of 4-6 separate determinations
***p<0.001 when compared to 4mM glucose
**p<0.01 when compared to 4mM glucose
EPI - Epinephrine

Figure - 43

Effect of different adrenergic receptor antagonists on insulin secretion from pancreatic islets in 1hr in vitro culture



Values are Mean ± S.E.M. of 4-69 separate determinations

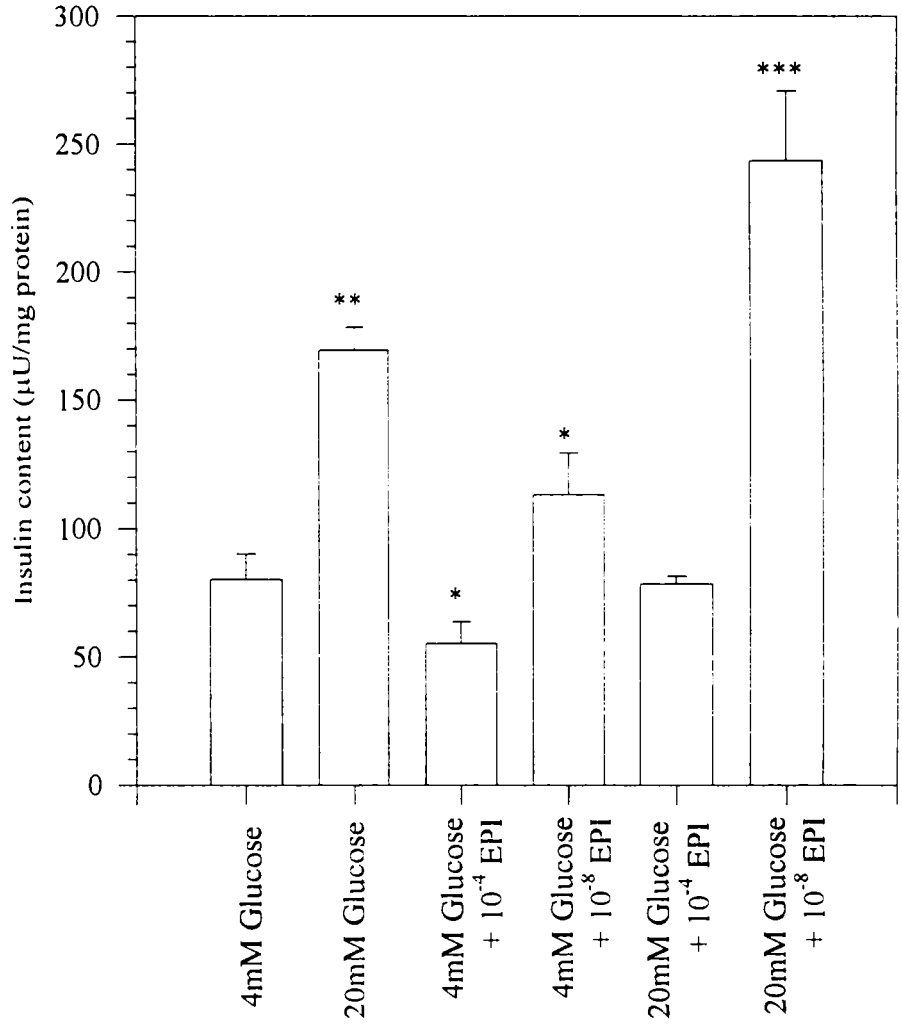
**p<0.01 when compared to without antagonists

*p<0.05 when compared to without antagonists

EPI - Epinephrine

Figure -44

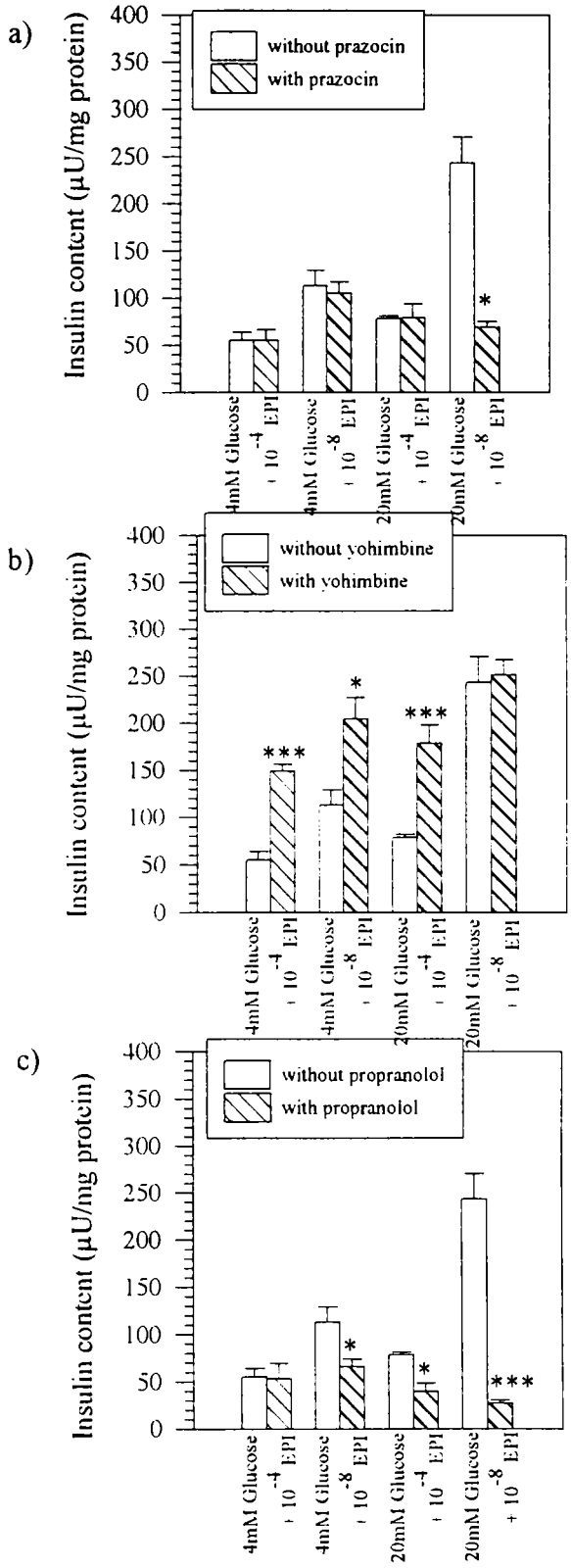
Glucose induced insulin secretion from pancreatic islets of young rats in 24hrs *in vitro* cultures



Values are mean ± S.E.M of 4-6 separate determinations
***p<0.001 when compared to 4mM glucose
**p<0.01 when compared to 4mM glucose
*p<0.05 when compared to 4mM glucose
EPI - Epinephrine

Figure - 45

Effect of different adrenergic receptor subtypes on insulin secretion from pancreatic islets in 24hr *in vitro* culture



Values are Mean ± S.E.M. of 4-6 separate determinations

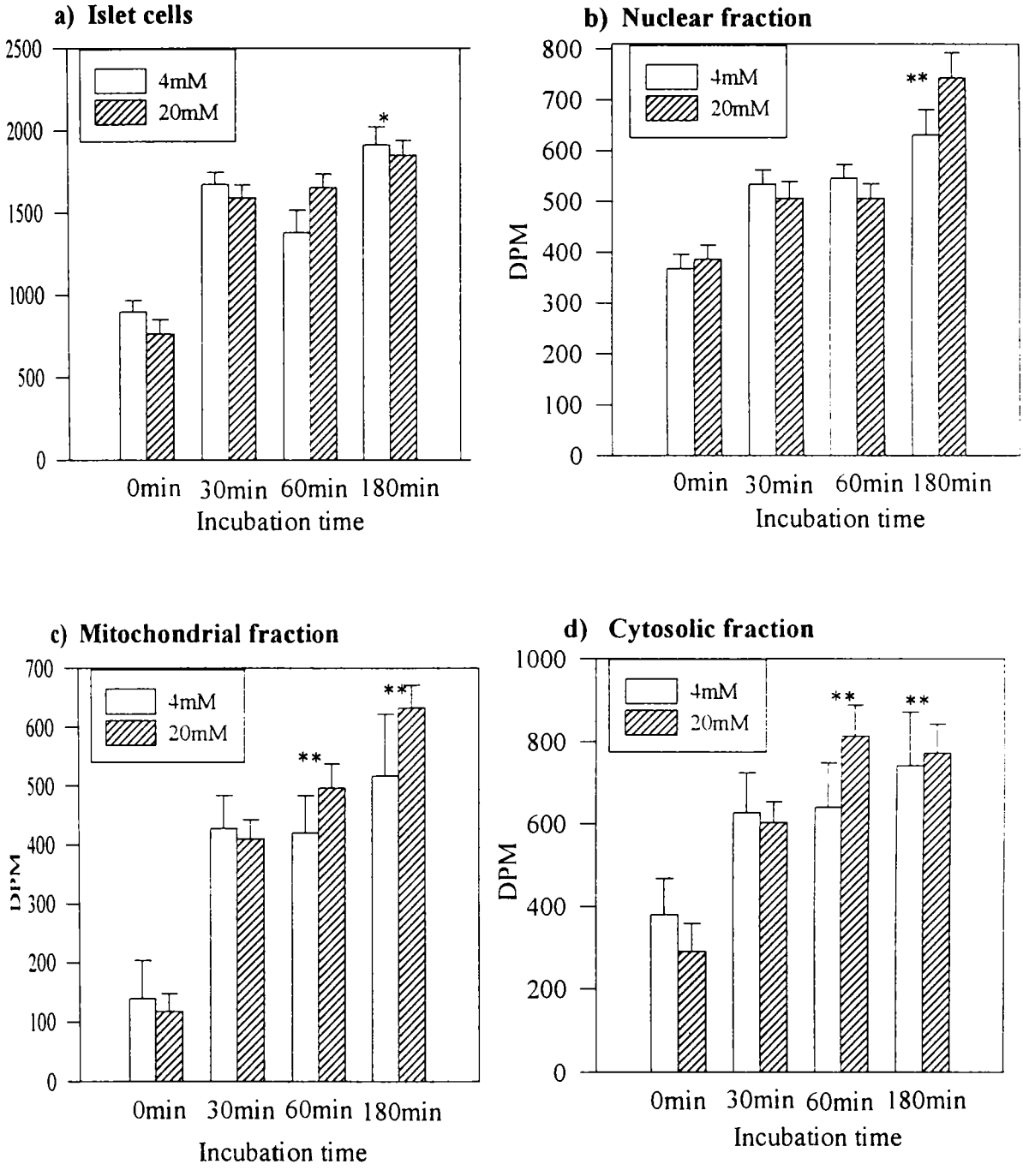
***p<0.001 when compared to without antagonists

*p<0.01 when compared to without antagonists

EPI - Epinephrine

Figure - 46

Binding of [^{3}H]EPI to different fractions of rat pancreatic islets at different time intervals



Values are mean ± S.E.M of 4-6 separate determinations
**p<0.01 when compared to 4mM glucose
*p<0.05 when compared to 4mM glucose
EPI - Epinephrine

Figure - 47

Scatchard analysis of [^{3}H]EPI binding in the nuclear fraction of the pancreatic islets of sham and pancreatectomised young rats

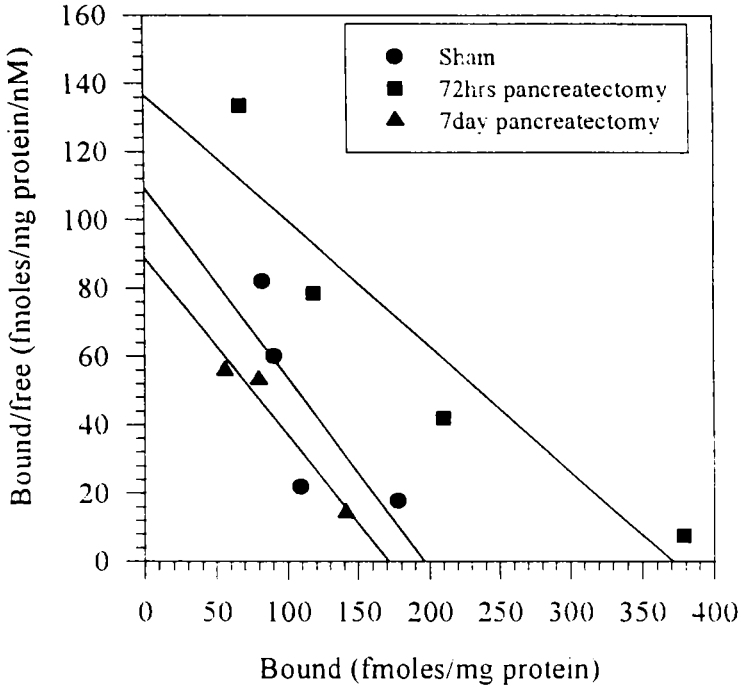


Table -43

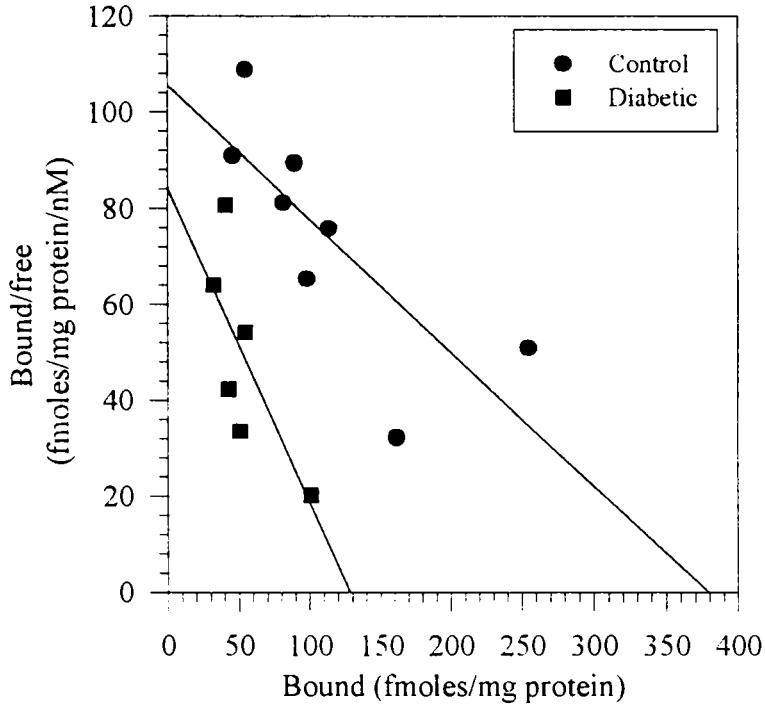
[^{3}H]Epinephrine binding parameters in the nuclear fraction of pancreatic islets of sham and pancreatectomised young rats

Animal status	B_{max}(fmol/mg protein)	K_d (nM)
Sham	190.00 ± 15.00	1.76 ± 0.13
72hrs pancreatectomy	370.33 ± 18.49*†	2.72 ± 0.12
7days pancreatectomy	170.33 ± 13.53	1.90 ± 0.15

Values are mean ± S.E.M of 4-6 separate experiments
*$p<0.05$ when compared to sham, †$p<0.01$ when compared to 7 day
B_{max}. Maximal binding, K_d. Dissociation constant

Figure - 48

Scatchard analysis of specific nuclear binding of [^{3}HEpinephrine in the pancreatic islets of STZ-diabetic rats



STZ - Streptozotocin

Table -44

[^{3}H]Epinephrine binding parameters in the nuclear fraction of pancreatic islets of STZ-diabetic rats

Animal status	B_{max}(fmol/mg protein)	K_d (nM)
Control	350.00 ± 13.00	3.33 ± 0.25
Diabetic	120 ± 9.23**	1.53 ± 0.16*

Values are mean ± S.E.M of 4-6 separate experiments

**p<0.01 when compared to sham

*p<0.05 when compared to sham

B_{max}. Maximal binding

K_d. Dissociation constant

Figure - 49

Identification of [^{3}H]EPI binding nuclear protein in pancreatic islets by ligand blotting method

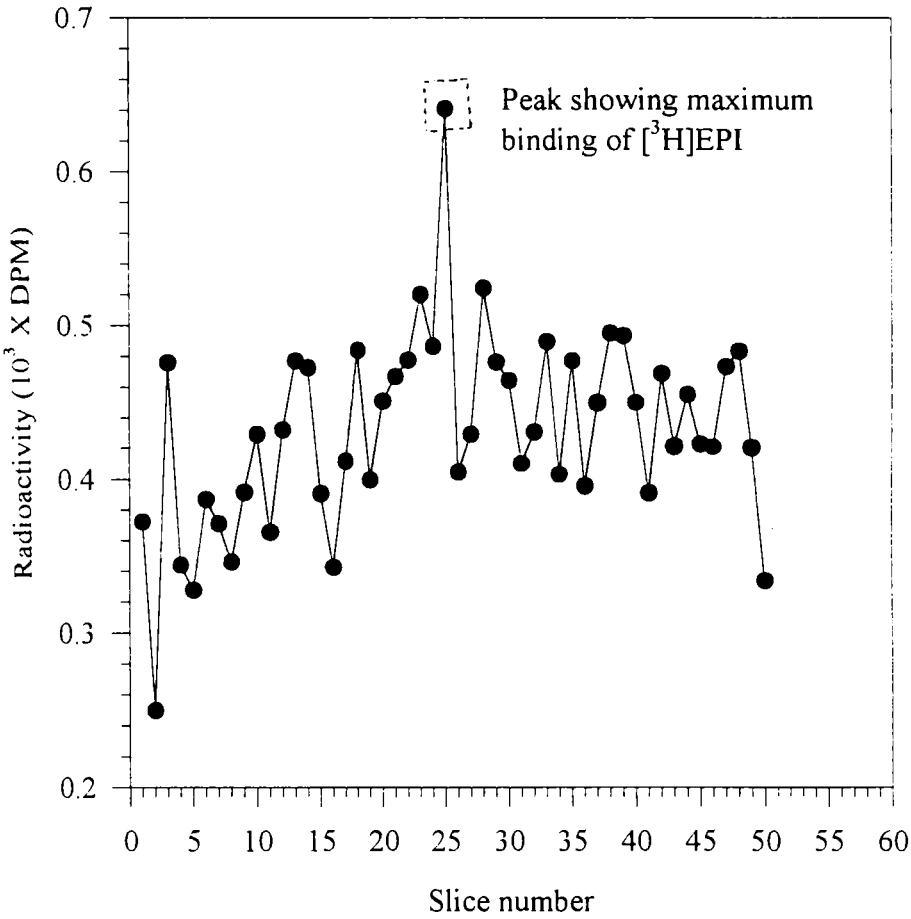
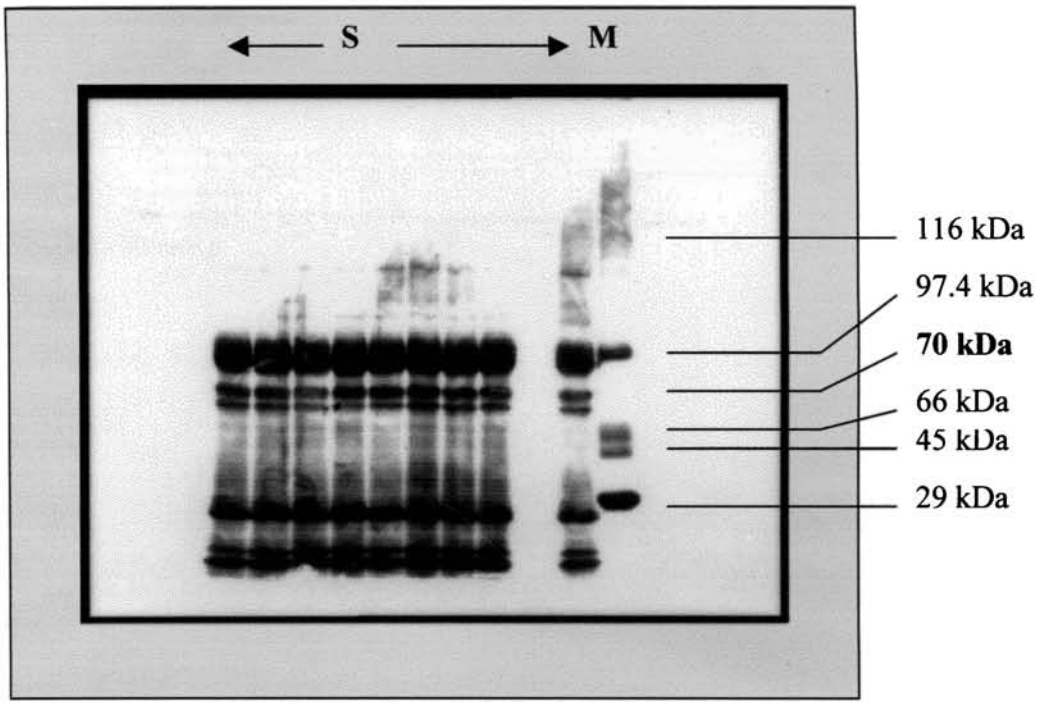


Figure - 50

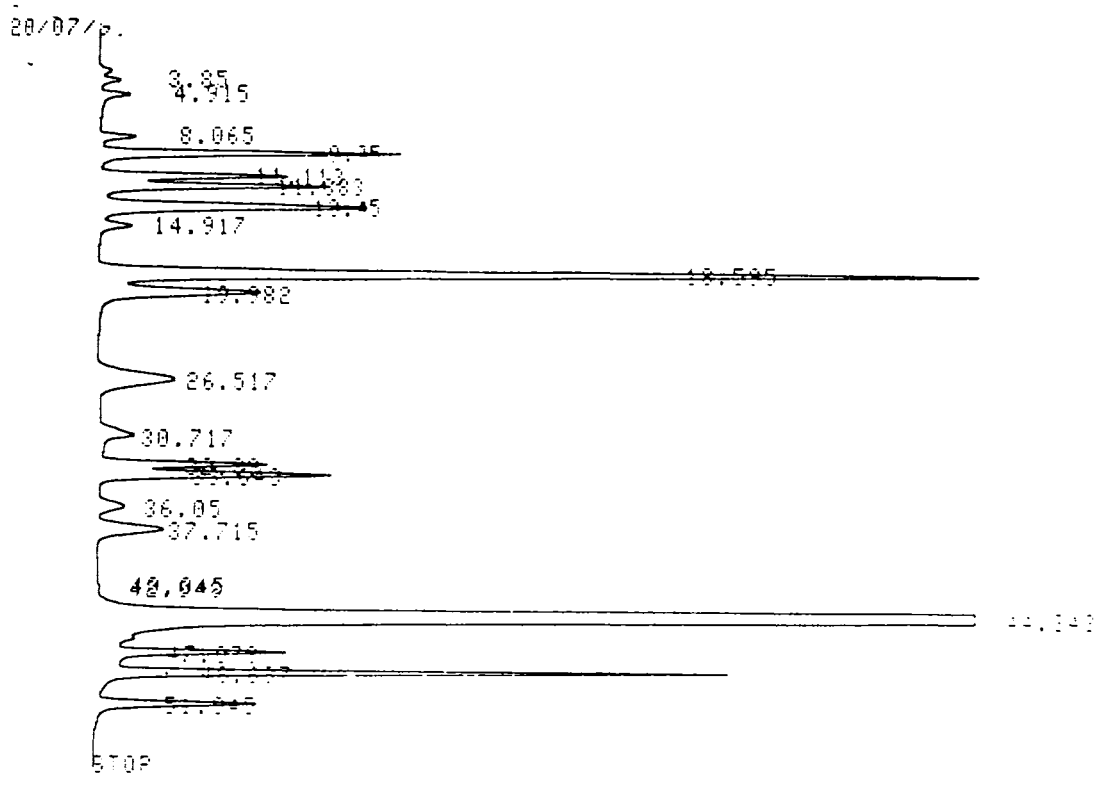
SDS-PAGE of specific EPI-binding nuclear protein in the pancreatic islets of rats



M - SDS MOLECULAR WEIGHT MARKER

S - SAMPLE

Chromatogram of aminoacid composition of 70-kDa nuclear specific binding protein from pancreatic islets



CHROMATOGRAM 3 MEMORIZED

CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 44
REPORT NO 1277 SAMPLE WT 100

PKNO	TIME	AREA	TYPE	CONC	NAME
1	3.85	463			
2	4.915	896			
3	8.065	912			
4	9.35	8538			
5	11.113	5670		0.0026	SSP
6	11.883	6943	V	0.0031	SER
7	13.45	10704	V 4	0.0063	GLU
8	14.917	1180	V 5	0.0004	PRO
9	18.595	33383	6	0.0211	GLY
10	19.982	7562	V 6	0.0048	GLY
11	26.517	5862	8	0.0028	VAL
12	30.717	2054	10	0.0012	MET
13	32.89	5183	V 11	0.0022	ILE
14	33.643	7949	V 12	0.0033	LEU
15	36.05	1320	13	0.0019	TYR
16	37.715	3543	14	0.0017	PHE
17	40.045	239			
18	42.048	121	V		
19	44.343	258536	BV 15	0.0693	HIS
20	47.078	4771	T 16	0.0006	LYS
21	48.637	16370	V 17	0.0025	AMM
22	51.045	5474	V 18	0.0005	ARG

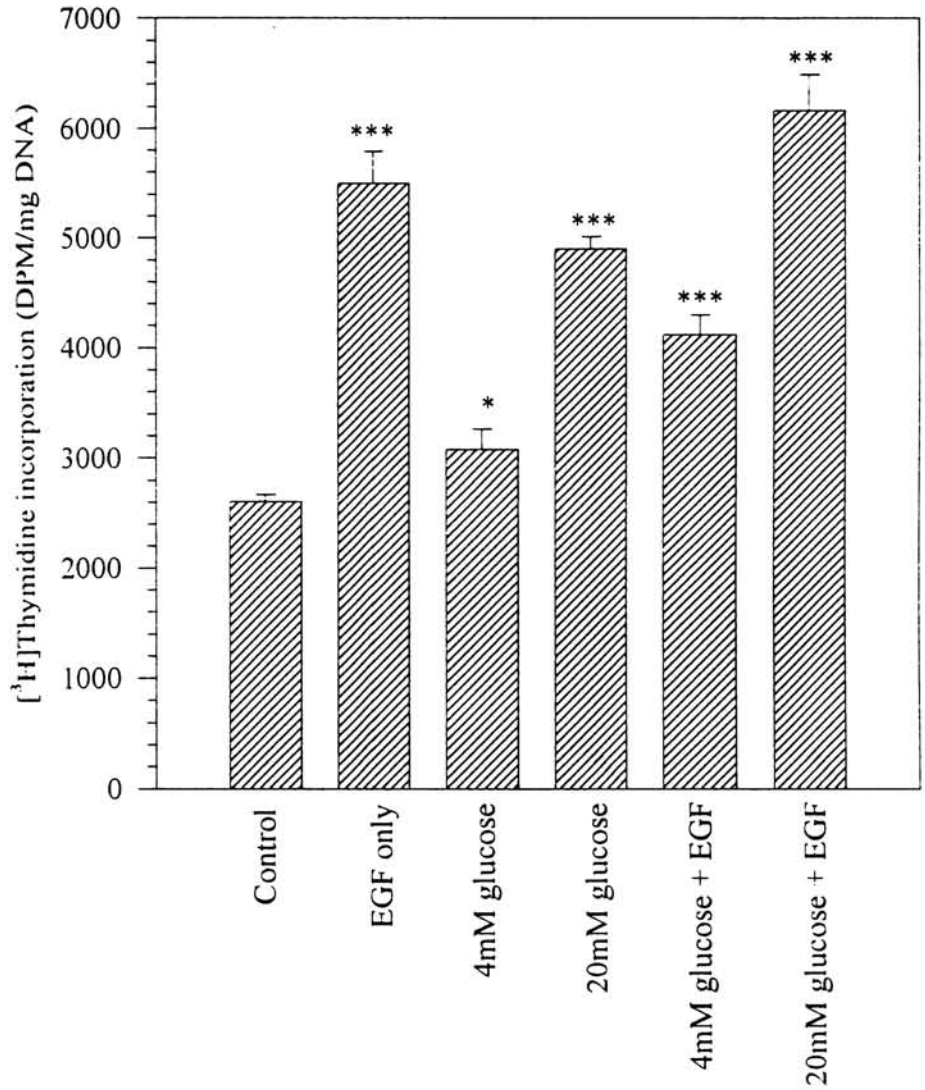
Table -45

Amino acid composition of 74kDa EPI-binding nuclear protein in the pancreatic islets

AMINOACID COMPOSITION (%)	
Aspartic acid	2.03
Histidine	**69.92**
Alanine	1.50
Arginie	1.34
Isoleucine	0.94
Lysine	1.63
Glutamic acid	3.06
Glycine	5.63
Proline	0.98
Valine	1.93
Leucine	1.91
Serine	1.17
Threonine	0.99
Tyrosine	0.68
Methionine	0.54
Phenyl alanine	0.96

Figure - 52

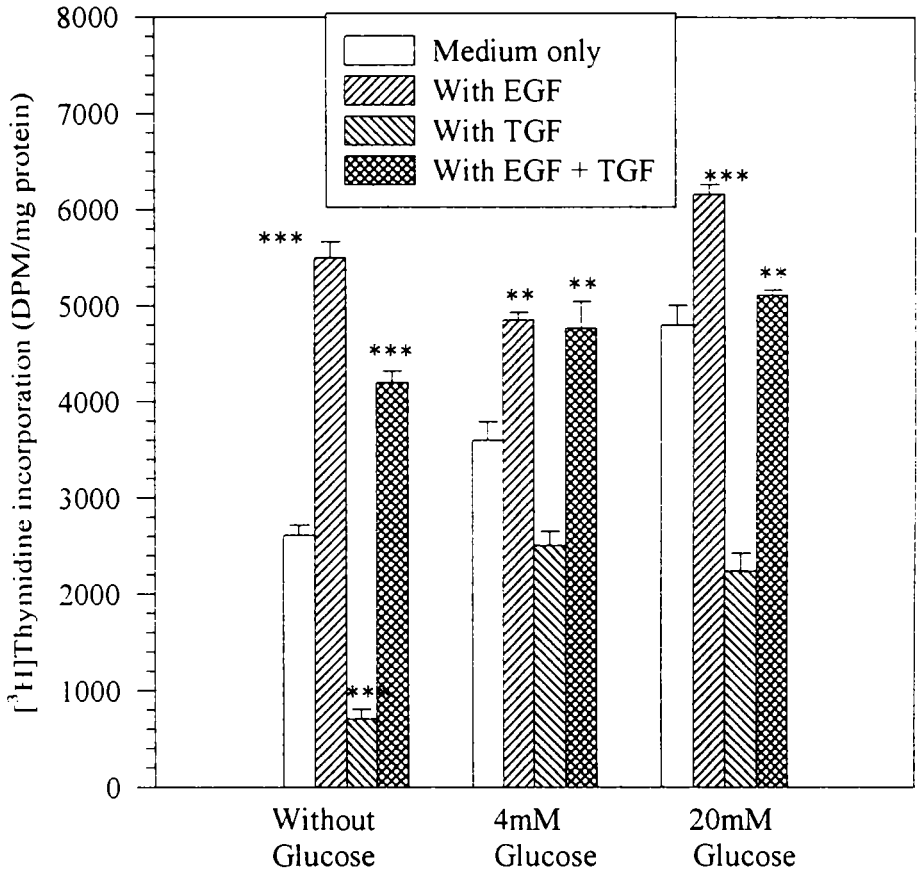
Effect of glucose on DNA synthesis in the pancreatic islets *in vitro*



Values are Mean ± S.E.M. of 4-6 separate determinations
*p<0.001 when compared to medium only
***p<0.05 when compared to medium only
EGF - Epidermal growth factor

Figure - 53

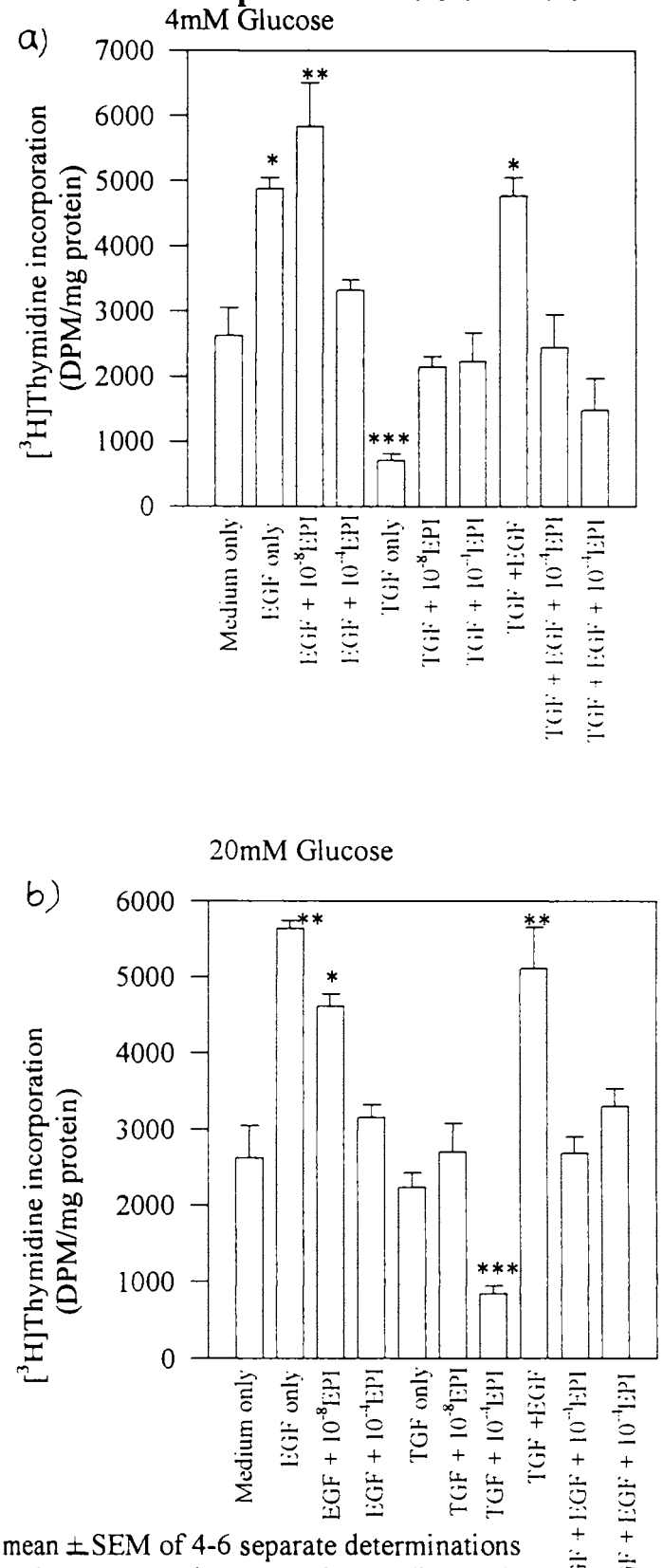
Effect of EGF and TGF on glucose-induced DNA synthesis in the pancreatic islets *in vitro*



Values are mean ± S.E.M. of 4-6 separate determinations
***p<0.001 when compared to respective medium only
**p<0.02 when compared to respective medium only
EGF - Epidermal growth factor; TGF - Transforming growth factor

Figure - 54

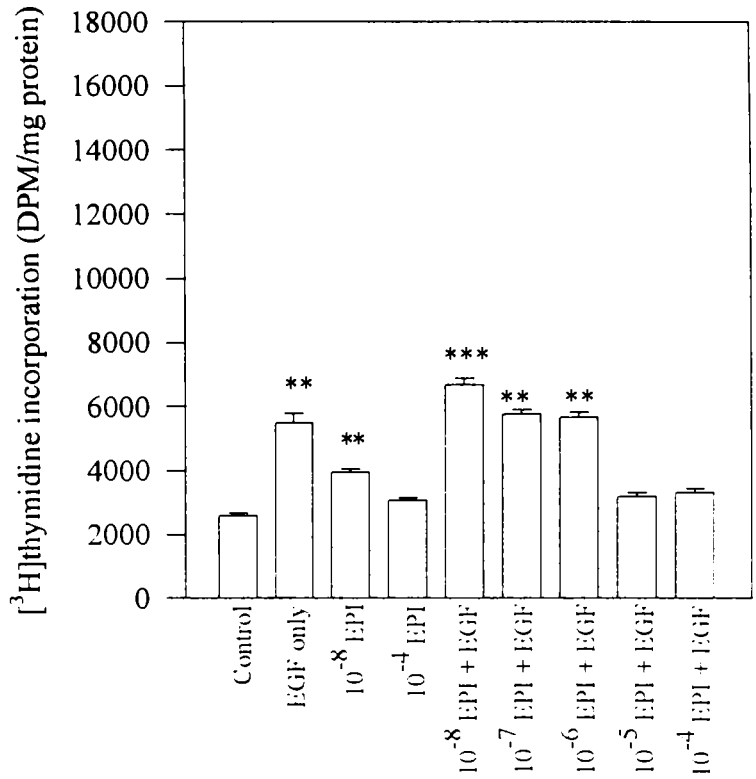
Effect of Epinephrine on DNA synthesis of pancreatic islets *in vitro*



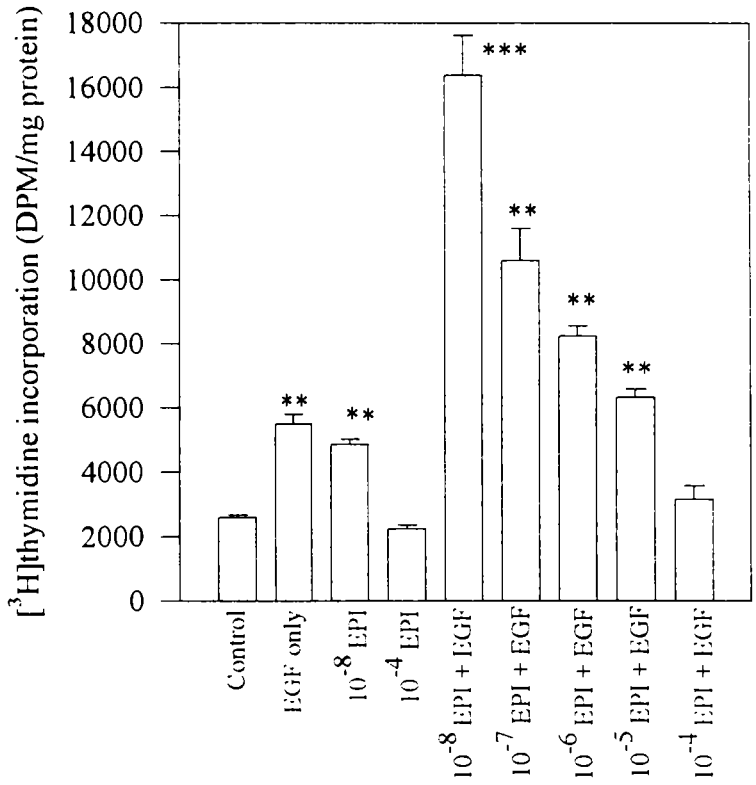
alues are mean ± SEM of 4-6 separate determinations
"'p<0.001 when compared to respective medium only,
"p<0.01 when compared to respective medium only
'<0.05 when compared to respective medium only

Dose dependent effect of epinephrine on DNA synthesis of pancreatic islets *in vitro*

a)



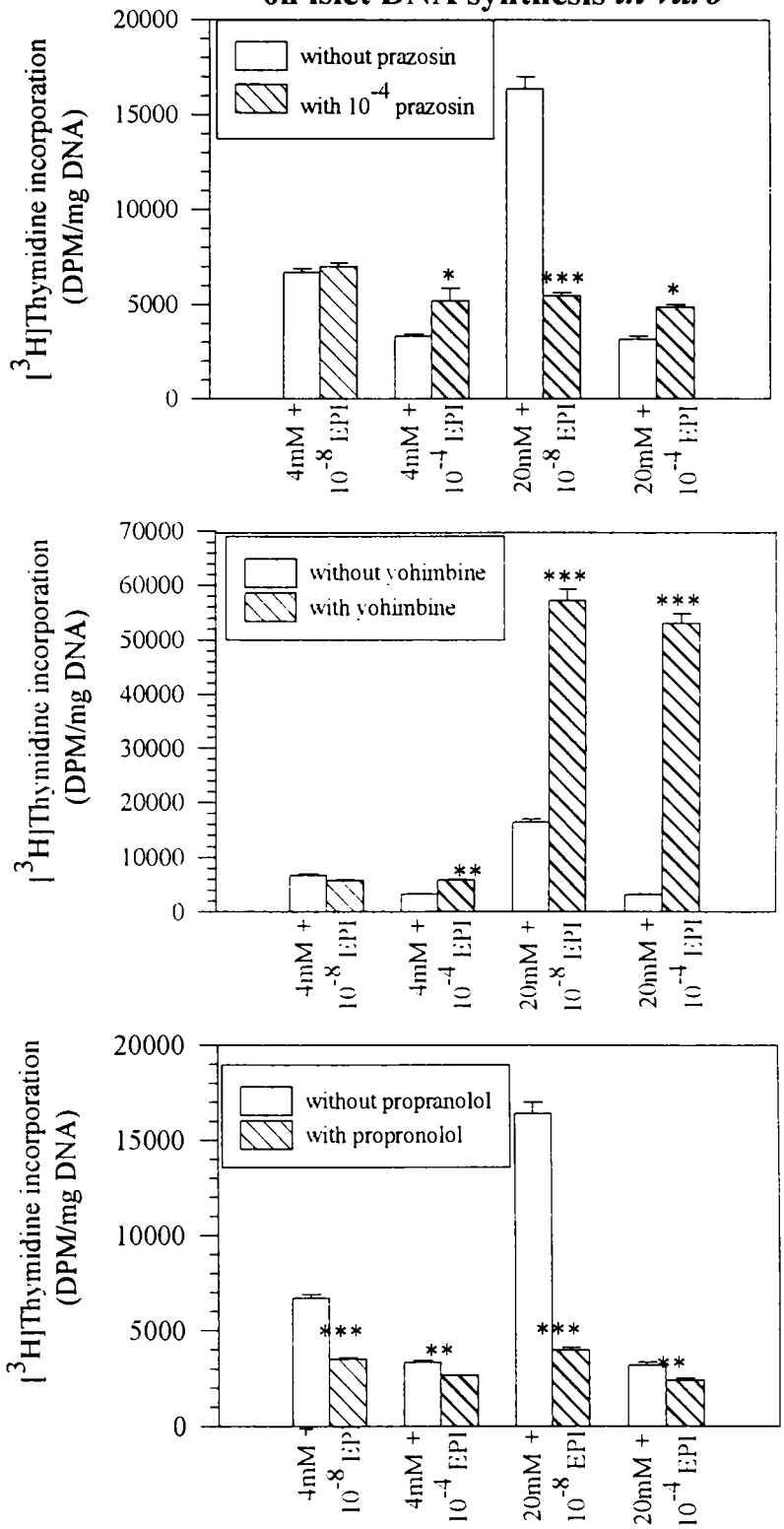
b)



Values are mean ±S.E.M. of 4-6 separate determinations
***p<0.001 when compared to respective medium only
**p<0.02 when compared to respective medium only
EPI - Epinephrine EGF - Epidermal growth factor

Figure -56

Effect of different adrenergic receptor antagonists on islet DNA synthesis *in vitro*



Values are Mean ± S.E.M. of 4-6 separate determinations

***p<0.001 when compared with respective without antagonists

**p<0.01 when compared with respective without antagonists

EPI - Epinephrine

5 DISCUSSION

Pancreatectomy is a very useful approach to demonstrate the regenerating potential of the β-cells. Removal of 60% of the total pancreas did not cause any reduction in the body weight and elevation in the blood glucose levels of the pancreatectomised rats. This maintenance of glucose homeostasis is due to a regeneration among the remaining pancreatic β-cells and their excess production of insulin (Leahy *et al.*, 1988, Lohr *et al.*, 1989).

[^{3}H]Thymidine incorporation into the islet DNA was used to monitor the entry of quiescent islet cells into the DNA synthetic phase. Sham operated rats showed negligible change in the DNA synthesis. But at 36 hrs and 48 hrs after partial pancreatectomy, DNA synthesis showed a significant increase which was peaked at 72 hrs. This elevated level of DNA synthesis was reversed to near normal after 7days. This pattern of DNA synthesis is concordant with previous reports (Brockenbrough *et al.*, 1988; Pearson *et al.*, 1977).

During active DNA synthetic period, i.e.; at 72 hrs after 60% pancreatectomy, we found that the insulin secretion is increased. Previous studies suggest that the increase in the β cell proliferation is related to the degree to which insulin biosynthesis and/or release is increased (King & Chick, 1976; Chick *et al.*, 1975). Recent observations indicate that insulin can stimulate pancreatic β-cell growth *in vivo*. McEvoy and Hegre, in 1978, reported that administration of insulin to diabetic rats implanted with foetal pancreases showed a three-fold increase in β-cell mass. Insulin caused a significant increase in the [^{3}H]Thymidine incorporation as well as the mitotic activity in β-cells of neonatal rats maintained in monolayer cultures. It is suggested that insulin can enhance islet β-cell replication directly, possibly through a receptor for multiplication stimulating activity (MSA) or another insulin-like growth factor (Rabinovitch *et al.*, 1982). Buchner *et al* reported that pancreatic resection resulted in suppression of liver DNA synthesis at 24 hrs after partial hepatectomy in untreated rats but not in animals that received peripheral injections of insulin and glucagon. (Rabinovitch *et al.*, 1982).

T_3 levels were increased during pancreatic regeneration. Studies on the role of thyroid hormones in influencing DNA synthesis have shown that T_3 can induce

proliferative responses after subcutaneous administration in the intact liver (Francavilla *et al.*, 1984). It is reported that thyrotropin through the cyclic AMP cascade, and in the presence of insulin or IGF-1, activates the proliferation of dog thyroid cells (Taton & Dumont, 1995). TSH induces DNA synthesis, in the presence of insulin. It is proposed that insulin induces the increase of cell mass that is a prerequisite for the mitogenic effect of TSH. Results from our laboratory indicate that thyroid hormones can influence DNA synthesis during liver regeneration by regulating the activity of thymidine kinase, which is a key enzyme for DNA synthesis (Tessy *et al.*, 1997). Insulin can stimulate TSH and TSH stimulation causes increased production of T_3. It is reported that in FRTL-5 cells TSH has a mitogenic effect (Takahashi *et al.*, 1990).

5.1 Epinephrine and Norepinephrine content is decreased in the brain regions, pancreatic islets and plasma during pancreatic regeneration.

EPI and NE contents decreased in the brain regions as well as in the pancreatic islets of 72 hrs pancreatectomised rats. They have an antagonistic effect on insulin secretion and glucose uptake (Porte *et al.*, 1966). It is reported that EPI and NE contents in the brain regions were increased significantly in the STZ-diabetic rats (Lackovic *et al.*, 1990; Chen & Yang, 1991; Tasaka *et al.*, 1992). This shows that during pancreatic degeneration the sympathetic stimulation is increasing. The increased sympathetic activity can inhibit the insulin secretion from the pancreatic islets (Efendic *et al.*, 1978; Renstrom *et al.*, 1996; Porte, 1967). Since NE and EPI inhibit insulin secretion they can suppress DNA synthesis as insulin is required for the proliferation of pancreatic islets.

The pancreatic islets form a highly innervated organ, receiving sympathetic neural inflow via the splanchnic nerves (Miller, 1981). A significant increase in the EPI and NE contents in the pancreas was reported during STZ-diabetes. The inhibitory effect of EPI on insulin secretion induced by glucose was reported by Coore and Randle (Coore & Randle, 1964). A limited β-cell regeneration has been observed in STZ-treated neonatal rats (Weir *et al.*, 1981). NE assists the mito-inhibitory effects of TGF-β_1 on other cell types like hepatocytes (Michalopoulose & DeFrancis, 1997). Our results showed a significant decrease in the EPI and NE contents in the 72 hrs pancreatectomised rats,

which increased the insulin secretion, thereby stimulating the regenerative capacity of the pancreatic islets.

EPI and NE levels in the plasma showed a significant decrease during active regeneration of pancreatic islets. Decreased levels of EPI and NE facilitate DNA synthesis in the pancreatic islets via increasing the insulin secretion. The insulin secretion is controlled by the sympathetic stimulation. Higher EPI and NE stimulate α_2-adrenergic receptors and inhibit the insulin secretion, but at low concentrations, they activate β-adrenergic receptors thus stimulating insulin secretion from the pancreatic islets (Coore & Randle, 1964). The effect of EPI on islet hormone secretion is dependent on its plasma level. At low levels of EPI, both insulin and somatostatin secretions are stimulated and at extreme stress levels, it produced inhibition (Ahren *et al.*, 1988).

5.2 Brain adrenergic receptors are altered during pancreatic regeneration in rats.

Scatchard analysis of [^{3}H]EPI binding showed an increase in the number of high affinity adrenergic receptors in cerebral cortex and the low affinity receptors showed a decrease. The hypothalamic adrenergic receptors are decreased with an increased affinity. Brain stem did not show any change in the receptor number, but the affinity was decreased significantly. All these data showed that the total adrenergic receptors are down regulated during active cell proliferation of pancreatic islets. Decreased adrenergic activity facilitates the insulin secretion and cell proliferation. It is already reported that the insulin secretion is inhibited by the increased sympathetic activity, which is observed in the diabetic state. Chronic adrenergic hyperinnervation acts directly or indirectly on ion flux to partially inhibit insulin release (Grodsky *et al.*, 1997).

5.3 α_1-adrenergic receptors are down regulated during active islet cell proliferation

[^{3}H]Prazosin binding studies in the cerebral cortex, brain stem and hypothalamus showed that there is a significant decrease in the B_{max} with an increase in the affinity during active regenerative phase. Thus, from our results it is clear that the α_1-adrenoreceptors are down regulated at the time of regeneration of pancreatic islets. Sjoholm *et al.* (1991) reported that the α_1-agonist phenylephrine is a potent inhibitor of

islet DNA synthesis and it inhibited insulin secretion also. Down regulation of α_1-adrenergic receptors was reported in liver regeneration after partial hepatectomy (Cruise *et al.*, 1989). α_1-adrenergic agonists are known to inhibit insulin secretion (Nilsson *et al.*, 1989). It is suggested that the α_1-adrenergic stimulation may cause a reduction in the cAMP synthesis, inhibits insulin secretion and islet DNA synthesis (Sjoholm, 1991).

5.4 α_2-adrenergic receptor activity is decreased in the brain regions during islet regeneration

[^{3}H]Yohimbine binding to α_2-adrenergic receptors in the cerebral cortex and brain stem of 72 hrs pancreatectomised rats showed a significant reduction in the receptor number with an increased affinity. This decrease in the number of α_2-adrenergic receptors indicates that these receptors are down regulated during pancreatic islet cell proliferation. α_2-adrenergic receptors are one of the potent inhibitors of insulin secretion from the islets (Moratinos *et al.*, 1988; John *et al.*, 1990). α_2-adrenoceptor stimulation by the endogenous catecholamines could lead to inhibition of insulin release, masking any potentiated response that otherwise should have appeared from α_1-and β-adrenoceptor stimulation (Garcia-Barrado *et al.*, 1998). In diabetic condition, α_{2A}-adrenoreceptors are more activated which brought out the insulin inhibition and in turn hyperglycaemia (Lacey *et al.*, 1993). The displacement analysis data showed that in the cerebral cortex, at 72 hrs after partial pancreatectomy, $Ki_{(H)}$ value was found to be decreased but the $Ki_{(L)}$ was increased. The function of low affinity receptors is increased at the time of active regeneration. In the hypothalamus, there was no change in the B_{max}, but the affinity of the receptors was significantly increased suggesting that hypothalamic α_2-adrenergic receptor activity is increased during pancreatic regeneration. Although there are reports, which say the role of brain α_2-adrenergic receptors in the insulin secretion, there are not much studies about the inhibitory action of this receptor subtype on the islet DNA synthesis. Sjoholm in 1991, has suggested that the rate of DNA synthesis, insulin secretion and cAMP content in the isolated pancreatic islets were markedly inhibited by long term exposure to the α_2-adrenergic agonist, clonidine (Sjoholm, 1991). Our results showed that in the brain regions the α_2-adrenergic function is decreasing during pancreatic

regeneration which then stimulated the insulin secretion and in turn the DNA synthesis in the islets.

RT-PCR analysis of α_{2A}-adrenergic receptor mRNA showed that its expression is decreased in the cerebral cortex and brain stem of experimental rats during active islet cell proliferation. This result is in concordant with the receptor data where we observed a significant decrease in the number of receptors at 72 hrs after partial pancreatectomy. Cerebral cortex co-ordinates the overall function of the brain and the brain stem has direct nerves originating from it that extends to the pancreas, the receptor changes observed in these regions can have a direct effect on the pancreatic function (Coldman & Dampney, 1998). Decreased levels of cAMP led to an increase in α_{2A}-adrenergic receptor mRNA abundance (Sakaue & Hoffman, 1991). It is reported that the cAMP content is decreased during islet DNA synthesis (Sjoholm, 1991). In the hypothalamus, expression of α_2-adrenergic receptor mRNA remained unchanged confirming the receptor data obtained from the binding analysis in which there was no change in the receptor number. There was only the affinity change in the hypothalamus during islet regeneration. Thus, our results show that the brain α_2-adrenergic receptors are down regulated during active islet cell proliferation.

5.5 Brain β-adrenergic receptors are up regulated at the time of active DNA synthesis in the pancreatic islets

In cerebral cortex and hypothalamus, both the low and high affinity β-adrenergic receptors increased in numbers in the 72 hrs pancreatectomised rats and the affinity of the receptors decreased. The displacement analysis of these receptors showed that at 72 hrs after partial pancreatectomy there was a decrease in the affinity confirming the data obtained from the Scatchard analysis. In the brain stem, low affinity receptors were found to increase without change in the affinity. Thus, from our data it is clear that the total β-adrenergic receptors are up regulated during islet regeneration. β-adrenergic receptors have been reported to couple directly to calcium channels through a stimulatory G -protein (Brown & Birnbaumer, 1988) thereby, stimulating insulin secretion. It is already reported that β-adrenergic receptor populations were decreased in diabetes (Garris, 1990). There are also reports saying that thyroid hormones can regulate β-adrenergic receptor number.

In a hyperthyroid state the cardiac β-adrenergic activity is enhanced (Williams *et al.*, 1977). We also observed an increase in the T_3 level during active regeneration in pancreatectomised rats. In T_3-treated rats myocardial β-adrenergic receptors, as measured by [^{3}H]dihydroalprenolol binding, were significantly increased [Scarpace, 1981 #335].

RT-PCR analysis of β-adrenergic receptor mRNA in the brain regions showed that the mRNA expression is increasing confirming the receptor data. β-adrenergic receptor expression and receptor mRNA levels are down regulated by β-adrenergic agonists and up regulated by glucocorticoids (Hadcock *et al.*, 1989) (Hadcock & Malbon, 1988). Here we observed an up regulation, because in the brain region agonists EPI and NE , are found to be decreasing. Decrease in cAMP levels results in down regulation of the β_2-AR (Hosoda *et al.*, 1995) and it is already known that the cAMP content is increased during islet DNA synthesis (Sjoholm, 1991; Swenne, 1982). These data imply that variations in cellular content of β_2-adrenergic receptor mRNA account for differences in receptor number Up regulation of the receptor can result from increased production of mRNA due to an increase in the transcription of the gene for the receptor which is stimulated by thyroid hormone or by glucocorticoids (Idem, 1988).

5.6 Pancreatic adrenergic receptors are decreased during active islet cell proliferation

[^{3}H]EPI binding studies in the pancreatic islet membrane showed that the total adrenergic receptors are decreasing. The affinity of the remaining receptors was increased. The EPI and NE contents in the pancreatic islets were found to be decreasing at 72 hrs after partial pancreatectomy. This result was in concordant with the results obtained in the brain regions. Alterations of central neurotransmission and environmental factors can change the relative contribution of sympathetic outflow to the pancreas, liver, adrenal medulla and adipose tissues, leading to the modulation of glucose and fat metabolism (Nonogaki, 2000). The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several different neurotransmitters are stored within the terminals of these nerves, both the classical neurotransmitters, acetylcholine and noradrenaline, and several neuropeptides. Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion. Thus, insulin

secretion is stimulated by parasympathetic nerves and inhibited by sympathetic nerves (Ahren, 2000). Previous studies have shown that the level to which β-cell proliferation increased is related to the degree to which insulin biosynthesis and/or release is enhanced (Chick *et al.*, 1973). Since the adrenergic activity is decreasing it will eventually help in the insulin secretion thereby enhancing the DNA synthesis in the islets.

5.7 α_1-adrenergic receptors in the islets are down regulated during regeneration

α_1-adrenergic receptor binding studies in the pancreatic islets showed that the receptor number is decreased without any significant change in the affinity. It is reported that the α_1-agonist phenylephrine inhibited the islet DNA synthesis (Sjoholm, 1991). α_1-adrenergic receptors are reported to inhibit insulin secretion and they are found to be increased in the streptozotocin diabetic state (Pius. 1996). It was reported in hepatic regeneration that the α_1-adrenergic receptors are decreased without any change in their affinity (Sandnes *et al.*, 1986). Reciprocal changes in the expression of α_1 and β-adrenergic receptors have been demonstrated to occur in the primary cultures of rat hepatocytes (Kunos *et al.*, 1995).

5.8 Islet α_2-adrenergic receptors are down regulated during pancreatic regeneration

Scatchard analysis of [^{3}H]Yohimbine binding in the pancreatic islets showed a significant decrease in the α_2-adrenergic receptor number during active islet DNA synthesis. Affinity of the remaining receptors is found to decrease. We also observed a similar change in the brain regions. α_2-adrenergic receptors are known to have a critical role in regulating neurotransmitter release from the sympathetic nerves and from the adrenergic neurons in the central nervous system (Miller, 1998). Decreased α_2-adrenergic activity can evoke β-adrenergic receptor mediated stimulation of insulin release from the pancreatic islets, which will then enhance the DNA synthesis. It was shown that α_2-adrenergic receptor agonist clonidine, supresses insulin secretion from the islets *in vitro* (Nilsson *et al.*, 1989). The function of α_2-adrenergic receptors is mediated through an inhibitory G-protein which decrease the cAMP content in the islet cells (Gillman, 1987). Decreased cAMP content will inhibit the insulin secretion as well as the DNA synthesis in the pancreatic islets. It is suggested that the long term stimulation of α_2-adrenergic

receptor interferes with signalling through pertussis toxin-sensitive G-protein which by suppressing cAMP production inhibits β-cell DNA synthesis and insulin secretion.

5.9 β-adrenergic receptors in the islets are up regulated during regeneration

β-adrenergic receptors are found to be increased without any change in the affinity during active regeneration in the pancreatic islets. Thus from our data it is clear that the total β-adrenergic receptors are up regulated at 72 hrs after partial pancreatectomy. The brain β-adrenergic receptors are also increased at the time of active DNA synthesis. The β-receptor is linked to a stimulatory G-protein leading to the activation of the adenylate cyclase which in turn catalyses the conversion of ATP to cAMP. cAMP is responsible for the physiological response, the nature of which differs with the type of cell (Fraser, 1993). Increased intracellular levels of cAMP have been proposed to mediate inhibitory as well as stimulatory effects in DNA synthesis and mitosis in fibroblast cell lines (Pessin *et al.*, 1983). It is reported that during primary culture of rat hepatocytes β-adrenergic response is increased (Sandnes *et al.*, 1986). β-adrenergic receptor stimulation evokes an increase in cAMP level which then activates cAMP dependent protein kinase and insulin release.

5.10 Adrenergic receptor antagonists regulate insulin secretion in 1hr incubation and long term culture of islet cells

Insulin secretion is increased with the increase in the glucose concentration. Similar changes were observed by Bombara *et al* (Bombara *et al.*, 1995) and Castro *et al* (Castro *et al.*, 1992). cAMP system is positively responsive to increasing glucose concentration. cAMP and the insulinotropic peptides, glucose-dependently increase the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in pancreatic β-cells, which is tightly linked to the potentiation of glucose-induced insulin release (Yaekura *et al.*, 1996).

Epinephrine is known to regulate the insulin secretion from the pancreatic islets. It exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro *et al.*, 1996).]. Our data showed that the EPI regulate the insulin secretion from pancreatic islets in two opposite ways. It is already known that, when used in high doses *in vivo* or *in vitro*, epinephrine reduces the insulin response to stimulators

(Malaisse, 1972). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte *et al.*, 1966). As judged by Malaisse *et al*, the inhibitory effect of EPI upon insulin secretion induced by glucose is related to its ability to activate α-adrenoreceptors which then inhibit insulin secretion (Malaisse *et al.*, 1967).

There are different subtypes for adrenergic receptors, of which we studied the involvement of three subtypes in regulation of insulin secretion from the islets. α_1-adrenergic receptors are found to regulate the insulin secretion in two ways, depending upon the concentration of EPI used in the culture. At lower concentrations of EPI, α_1-adrenergic receptors act as stimulatory to insulin secretion. We conclude that α_1-adrenoceptor subtypes are differentially regulated by agonist treatment even if they are expressed in the same cell type. Yang *et al.*, reported that the down regulation of α_1-adrenoceptor subtypes by 100μM phenylephrine was time-dependent, and significant reductions were observed already after 2-4 hrs incubations. In contrast, incubation of α_{1D}-adrenoceptor-expressing cells with phenylephrine increased receptor number in a time and concentration-dependent manner (Yang *et al.*, 1999). We found that the insulin secretion is regulated by the α_1-adrenoceptors possibly mediated through subtypes differentially expressed according to the EPI concentration in the pancreatic islets.

α_2-adrenergic receptors are inhibitory to the insulin secretion. α_2-adrenergic receptors are coupled by Gi proteins to various effectors, including adenylate cyclase and ion channels. The α_{2A} adrenergic receptors respond to endogenous NE and EPI to elicit a variety of physiological responses, including inhibition of neurotransmitter release and suppression of insulin release from pancreatic β-cells (MacMillan *et al.*, 1998). Our studies with yohimbine showed that the insulin secretion from the pancreatic islets is significantly increased confirming that the α_2-adrenergic receptors inhibited the insulin secretion from the pancreatic islets. Thus, EPI, a potent activator of this subclass of adrenergic receptors, is a potent inhibitor of insulin secretion from the islets. α_2-adrenergic receptor activation leads to inhibition of insulin release by a mechanism distal to those regulating β-cell cyclic AMP production and $[Ca^{2+}]_I$ (Ullrich & Wollheim, 1985). The mechanism of action of α_2-adrenergic receptor agonists in mediation of the hyperglycaemic response is of peripheral origin and involves pancreatic β-cell post

synaptic α_2-adrenergic receptors, possibly through the inhibition of insulin release (Angel & Langer, 1988).

Our experiments with *in vitro* cultures of islets showed that propranolol blocking resulted in a marked decrease in the insulin secretion. This explains the stimulatory role of β-adrenergic receptors in the insulin secretion. Stimulation of β-adrenergic receptor normally results in signalling by the stimulatory G protein, leading to the activation of adenylate cyclase, production of cAMP, and activation of cAMP-dependent protein kinase (PKA) in turn stimulating the insulin secretion. EPI and NE, when present at low concentrations, stimulate the β-adrenergic receptors thereby increasing the insulin release from the pancreatic islets.

The long-term insulin secretion studies showed the same pattern of changes as in the 1 hr incubations. The presence of insulin activators or stimulators in the islet cell cultures for 24 hrs showed that they capacitate or loss the ability of the viable cells to synthesise and secrete the insulin. We have done the 24 hrs islet cell culture to study the long-term effect of different adrenergic antagonists to stimulate or block the insulin synthesis and release from the isolated islets.

Thus our results show that the EPI acts through different subclasses of adrenergic receptors bringing about the changes in the insulin secretion. α_1-adrenergic receptors can act both as stimulatory and inhibitory, while α_2-adrenergic receptors are strictly inhibitory and β-adrenergic receptors are stimulatory to the insulin release from the islets.

5.11 EPI is taken up by the islet cells and binds to different subcellular fractions of islets

[^{3}H]EPI uptake in the islet cells at different time intervals showed that EPI can go inside the islet cell and bind to the nuclear proteins in a time-dependent manner regardless of the glucose concentration. This explains that the EPI uptake by the islets is not glucose-induced. With the increase in the time of incubation, the concentration of EPI uptake is increased. If the sympathetic stimulation from CNS is increased it can cause a parallel increase in the islet EPI content. It is reported that the EPI and NE content is increased in

the pancreatic islets of young and old diabetic rats (Abraham, 1998). Most released EPI and NE are efficiently removed by neuronal and extraneuronal uptake (Eisenhofer *et al.*, 1992). A number of laboratories have reported the presence of monoamines like 5-HT within the islets (Bird *et al.*, 1980). NE and EPI transporters are present in the plasma membrane of the islets, which can help the passage of EPI in to the interior of the islets. We propose that the presence of increased EPI activate the transporter proteins to transport the EPI which then binds to some protein present in the nuclear fraction. Catecholamines are inactivated mainly by two mechanisms, through the enzyme catechol-o-methyltransferase (COMT) and monoamine oxidase (MAO). A number of reports have appeared with regard to the role of MAO in islets, since the process of deamination of biogenic and exogenic amines lead to concomitant production of hydrogen peroxide (H_2O_2). The generation of H_2O_2 may affect the redox state of the β-cell glutathione system, the balance of which is known to influence nutrient-induced insulin release (Miller, 1981). According to our data it is clear that the EPI uptake by the islets can be irrespective of the glucose concentration and bind to the membrane as well as nuclear fractions of pancreatic islets. The insulin secretion is decreased when we incubated the islets with high concentrations of EPI. This led us to a conclusion that there are some proteins in the nuclear fraction of the islets to which EPI can bind and regulate the insulin secretion.

5.12 The EPI-binding nuclear protein in the islets is identified as a 70-kDa Hisitidine-rich protein

The EPI-binding nuclear protein was identified by ligand blotting method. The protein was identified as a 70-kDa protein. The amino acid analysis of the protein revealed that the protein is rich in Histidine (69.92%). 70-kDa EPI-binding nuclear protein showed a similarity to a mouse zinc finger protein, which may function as transcription activator. This protein has also a regulatory role during cell division (Cunliffe *et al.*, 1990; Cunliffe *et al.*, 1990).

5.13 EPI-binding nuclear proteins are increased in the islets of pancreatectomised rats

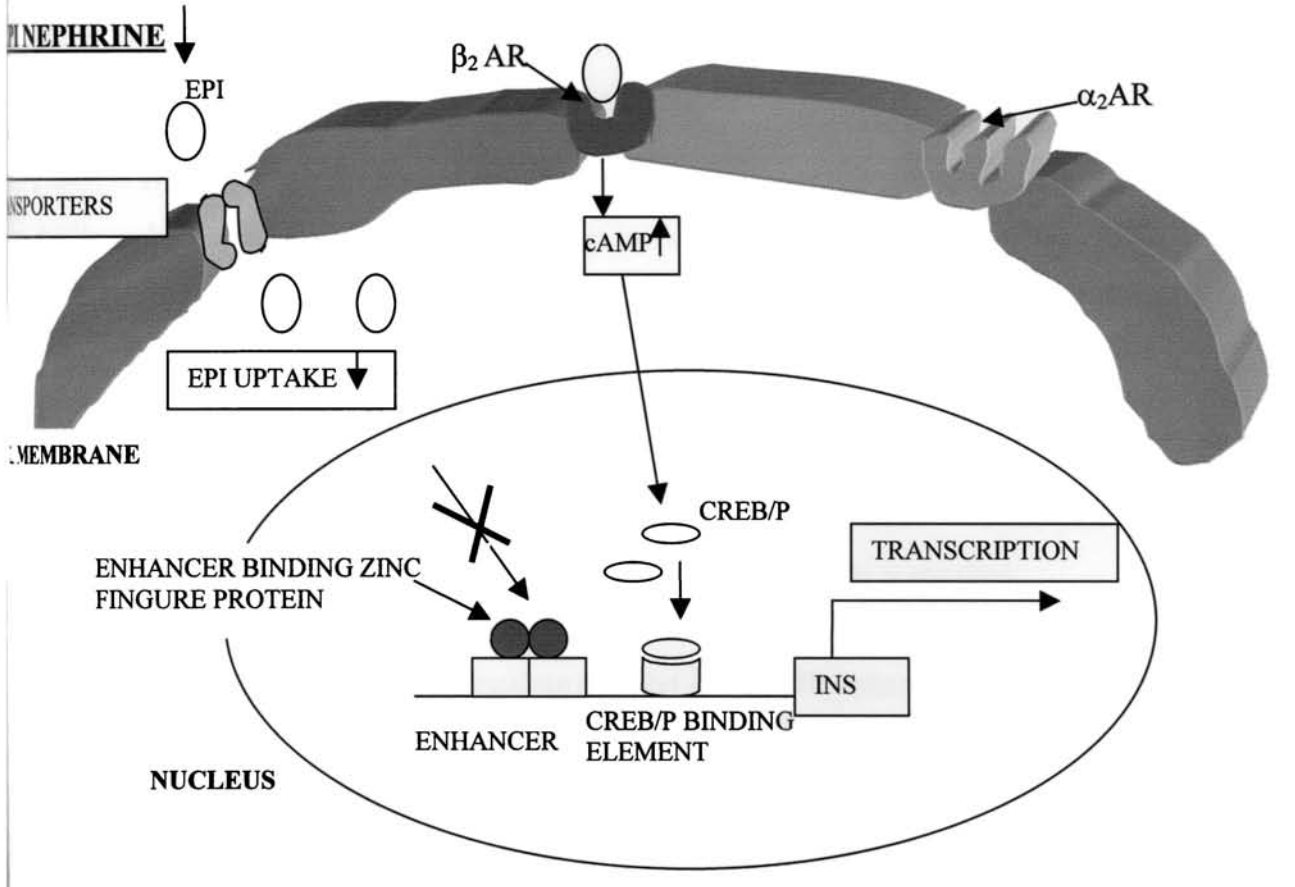
Radioreceptor analysis in the nuclear fraction of the islets using [^{3}H]EPI showed an increase in the number of EPI-binding specific nuclear protein in the pancreatic islets during active regeneration. The affinity of the proteins is also increased. The EPI content in the islets was decreased. The decreased EPI can cause increase in the EPI-binding proteins, which may increase the insulin secretion, observed during regeneration. Central sympathetic system has a parallel control over the peripheral system. Decrease in the brain EPI content causes a significant decrease in the islet EPI. The low EPI content in the islets regulate the membrane and nuclear receptors in two ways. The decrease in EPI results in the reduction of membrane receptors and elevation in the nuclear receptors.

5.14 EPI binding nuclear proteins are down regulated in the pancreatic islets of STZ-diabetic rats

This experiment was done to see the changes in the EPI-binding nuclear proteins in the islets in an insulin-deficient state in rats. We observed that in the STZ-diabetic stage, the nuclear proteins are decreased with an increased affinity. This result showed that the EPI-binding nuclear proteins are down regulated during diabetes. It is reported that the EPI and NE content in the islets are increased in the diabetic rats when compared to control. Thus, from this data it is clear that increase in EPI/NE can decrease the EPI-binding nuclear protein, which eventually decreased the insulin secretion.

5.15 Possible regulation of insulin transcription by the 70-kDa protein

There is no evidence so far explaining the involvement of a 70-kDa zinc finger protein in regulation of insulin gene. The insulin gene has some regulatory sequences which contained many binding sites for highly cell-specific and ubiquitous protein factors (Boam *et al.*, 1990). These proteins when bound to their specific DNA motifs, determine the temporal and spacial expression of the gene and its inducibility by various external stimuli, such as second messenger molecules (Imagawa *et al.*, 1987). It is reported that in the insulinoma cells a 70-kDa DNA-binding protein was identified and reported to bind to the -CACC- promoter element of Gastrin gene. This protein is known to interact with


PINEPHRINE
EPI
β_2 AR
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NSPORTERS
cAMP
EPI UPTAKE
MEMBRANE
CREB/P
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ENHANCER BINDING ZINC
FINGURE PROTEIN
INS
ENHANCER
CREB/P BINDING
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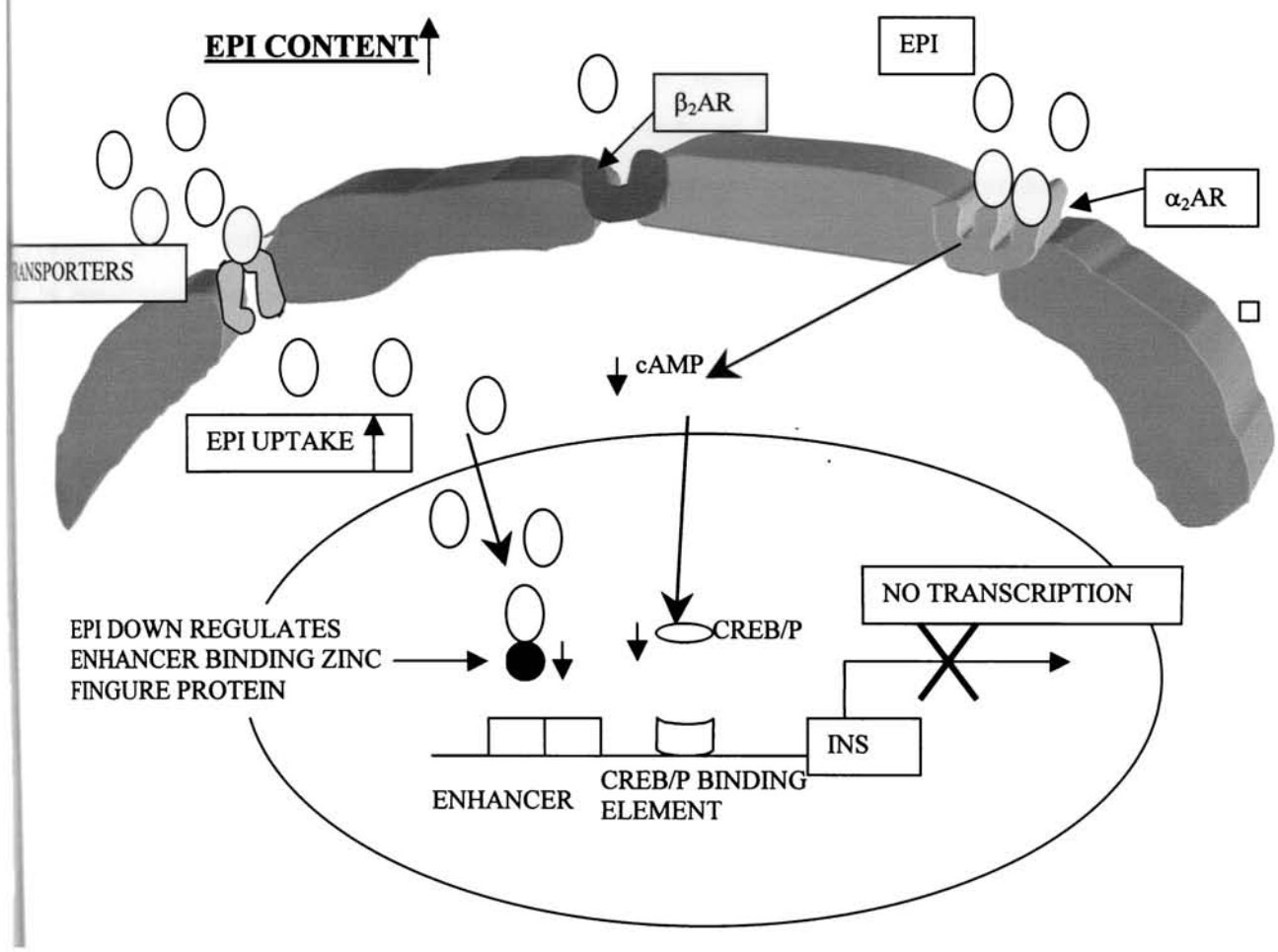
EPI CONTENT
EPI
β_2AR
α_2AR
TRANSPORTERS
cAMP
EPI UPTAKE
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EPI DOWN REGULATES
ENHANCER BINDING ZINC
FINGURE PROTEIN
CREB/P
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ENHANCER
CREB/P BINDING
ELEMENT

other islet nuclear proteins to synergically activate transcription in a cell-specific manner (Tilloson *et al.*, 1994). We assume that the 70-kDa zinc-finger protein bind directly to the insulin enhancer factor (IEF) binding region in the insulin gene or interact with other DNA-binding proteins to enhance the gene transcription. EPI at high concentrations down regulate these nuclear binding proteins and at lower concentrations it causes up regulation. Thus, it is evident from our results that EPI has a direct role not only in the insulin secretion but also in the insulin gene transcription. As it can involve in the insulin gene transcription, it might have involved in the β-cell regeneration also. To confirm the regulatory role of EPI in the insulin gene transcription further study is required.

5.16 Glucose and growth factors regulate the islet cell proliferation *in vitro*

Glucose is widely accepted as an important stimulus to the development to β-cell growth and function. Glucose infusion *in vivo* increased the insulin level in rats (Kervan & Gitard, 1974). In monolayer culture also D-glucose is the principal stimulator that acted as a β-cell mitogen (DeGasparo *et al.*, 1978). We also found in the suspension cultures glucose could increase the DNA synthesis as monitored by the [^{3}H]Thymidine incorporation. Glucose also enhanced the EGF-induced DNA synthesis in the *in vitro* cultures. One of the mechanisms by which the glucose enhances the DNA synthesis is by increasing the insulin secretion. Some reports say that glucose controls β-cell proliferation by regulating the number of cells which enter the cell cycle (Swenne, 1982). After entering into the cell cycle, then it proceeds irrespective of the glucose concentration. Increase in the glucose will increase the cAMP level which then trigger the mitogenesis in the β-cells (Sjoholm, 1997). Swenne *et al.*, also reported that nutrients like glucose and amino acids have a big role in the regulation of insulin biosynthesis and β-cell growth *in vitro* (Swenne *et al.*, 1980).

EGF is a prototype stimulator of most epithelial cells. It has mitogenic effect on many of the tissues like hepatocytes. EGF is reported to have a mitogenic role in the pancreatic islets also. Barton *et at* (Barton *et al.*, 1991) have shown that EGF stimulates the proliferation of pancreas. We also found that the EGF stimulated the DNA synthesis significantly. Chatterjee *et al.* (1986) have also shown that EGF stimulates proinsulin biosynthesis as well as [^{3}H]Thymidine incorporation into the pancreatic islet DNA. They

suggested that EGF behaves like glucose in stimulating both insulin biosynthesis and β-cell replication. TGF-β_1 is known to have inhibitory regulation in the proliferation of various cell types. It is an inhibitor of hepatocyte proliferation in cultures (Michalopoulose & DeFrancis, 1997). TGF-β_1 has been implicated as an inhibitor of cell proliferation and a potent inducer of apoptosis *in vitro* and *in vivo* after its the administration in high doses (Fan *et al.*, 1998). . Konturek *et al.* (1997) have reported that in acute pancreatitis the TGF-β_1 showed a marked increase. Our data showed that TGF inhibited the DNA synthesis in the islet cell cultures. But when used in combination with EGF, it suppressed the EGF-induced DNA synthesis

5.17 EPI controls islet cell proliferation in a concentration dependent manner

EPI at lower concentrations enhanced the EGF-induced [^{3}H]Thymidine incorporation in the islets cell culture whereas higher concentrations inhibited the DNA synthesis. As suggested earlier, the high concentrations of EPI inhibit and low concentrations stimulate the insulin secretion as well as DNA synthesis in the islets. NE offsets the mito-inhibitory effects of TGF-β_1 on other cell types like hepatocytes (Michalopoulose & DeFrancis, 1997). The dose-dependent studies showed that the inhibitory effect is increasing with the increase in the EPI concentration. Our results confirm that EPI has both the stimulatory as well as inhibitory effect on the DNA synthesis which is concentration dependent.

5.18 α-adrenergic receptors inhibit and β- adrenergic receptors stimulate the DNA synthesis in islet cells

The pancreatic islets form a highly innerved organ, receiving sympathetic neuron inflow via the splanchnic nerves (Miller, 1981). Ever since the original discovery by Coore and Randle (Coore & Randle, 1964) that the sympathetic neurotransmitter epinephrine inhibits insulin secretion from rabbit pancreas, there has been a great number of studies investigating the effects of adrenergic drugs on insulin secretion. Sjoholm in 1991, has studied the effect of adrenergic agonists and antagonists on the rate of DNA synthesis, insulin secretion and cAMP content in isolated islets. He found that α_1-agonist, phenylephrine, the α_2-agonist clonidine and β-adrenoceptor antagonist propranolol were

all potent inhibitors of islet cell DNA synthesis and insulin secretion. Our results are in concordant with these results. We observed that α_1-adrenergic receptors have two different roles depending upon the concentration of EPI present in the medium. This subclass has the same effect on insulin secretion, which we observed from our insulin secretion studies. The α_2-adrenergic receptors are found to be strictly an inhibitor of islet DNA synthesis. β-adrenergic receptors studied using propranolol, stimulated the EGF-induced DNA synthesis in the islet cells. The possible role of adrenergic receptors in regulating the islet cell proliferation is mediated by cAMP. α-adrenergic receptor activation decreases the cAMP content in the islets whereas β-adrenoreceptor activation causes an increase in the cAMP levels (Sjoholm. 1991). It is suggested from our results that EPI regulates the pancreatic islet DNA synthesis through its different adrenergic receptors. According to the concentration of EPI different subclasses of adrenergic receptors are activated and its function, either stimulatory or inhibitory, is mediated through these receptors. Also, our results suggest that the central and pancreatic adrenergic receptor function have an important role to play in the regulation of islet cell proliferation and insulin homeostasis.

6. SUMMARY

Partial pancreatectomised (60%) rats were used as the model for studying the pancreatic regeneration in rats.

Suspension cultures of pancreatic islets were used as the *in vitro* model system to study the insulin secretion and DNA synthesis.

[^{3}H]Thymidine incorporation into the pancreatic islets was used as the index to study the DNA synthesis after partial pancreatectomy and in cultured pancreatic islets.

The DNA synthesis was peaked at 72 hrs and started decreasing at 7 days after partial pancreatectomy.

The circulating insulin and T_3 levels were significantly elevated during active DNA synthesis in the pancreatic islets.

The EPI and NE contents were significantly decreased in the cerebral cortex, brain stem and hypothalamus during active pancreatic islet regeneration. EPI and NE contents in the pancreatic islets also decreased at the peak of DNA synthesis.

Circulating NE and EPI levels were decreased at the time of active regeneration in pancreatic islets.

The brain adrenergic activity is reduced. The α_1-adrenergic receptors are decreased significantly in all brain regions. The α_2-adrenergic receptors were down regulated in the cerebral cortex and brain stem during pancreatic regeneration. The hypothalamic α_2-adrenergic receptor affinity was increased at the period of peak DNA synthesis. In all brain regions, the β-adrenergic receptors were up regulated at 72 hrs after partial pancreatectomy.

RT-PCR studies confirmed the decreased α_{2A}-adrenergic receptors in the cerebral cortex and brain stem and the increased β-adrenergic receptors in the cerebral cortex, brain stem and hypothalamus.

The decreased α_2-adrenergic and increased β-adrenergic receptor function will lead to an increase in the insulin secretion and DNA synthesis in the pancreatic islets.

In the pancreatic islets, the α_2-adrenergic receptors were down regulated and β-adrenergic receptors were up regulated which lead to an increase in the insulin secretion as well as DNA synthesis.

In vitro studies of pancreatic islets with EPI showed that it regulates the insulin secretion in a concentration-dependent manner. Higher concentrations of EPI inhibited the glucose-mediated insulin secretion and lower concentrations stimulated the glucose-mediated insulin secretion from the islets.

Insulin secretion studies in the presence of different adrenergic antagonists revealed that α_1-adrenergic receptor can act both as stimulatory and inhibitory depending upon the EPI concentration. α_2-adrenergic receptors are purely inhibitory and β-adrenergic receptors are stimulatory to insulin secretion in both 1 hr and 24 hrs islet cell cultures.

In vitro incubation of pancreatic islets with [^{3}H]EPI showed that EPI uptake by the islets is time-dependent irrespective of the glucose concentration. It is also found out that EPI can bind to a specific nuclear protein.

By using ligand blotting method the protein was identified as a 70-kDa protein. The amino acid analysis showed that this protein is rich in Hisitdine. The homology search in SWISS-PROT revealed that it has a homology to a mouse zinc-finger protein, which may act as a transcriptional activator.

During regeneration the 70-kDa EPI-binding nuclear protein was up regulated and in insulin-deficiency it was down regulated. From our data it is clear that the 70-kDa EPI-binding nuclear protein is acting as an enhancer of insulin synthesis thereby promoting the islet cell regeneration.

The *in vitro* DNA synthesis studies showed that EPI at low concentrations could enhance the EGF-induced DNA synthesis and at high concentrations inhibit the EGF-induced DNA synthesis in the pancreatic islets. α_1-adrenergic receptors act both as stimulatory and inhibitory according to the EPI concentration. α_2-adrenergic receptors inhibited the DNA synthesis while the β-adrenergic receptors stimulated the DNA synthesis in the islet cell cultures.

Thus from our results it is evident that α- and β-adrenergic receptors regulate the insulin secretion and islet cell proliferation. The changes in the brain adrenergic receptors were similar to the islet receptor changes. This suggests that the brain adrenergic receptor gene expression alters the pancreatic adrenergic receptor function. The balance between α- and β-adrenergic receptor functional correlation controls the insulin secretion and pancreatic β-cell proliferation in diabetes.

7. CONCLUSION

The adrenergic system regulates the insulin secretion and islet cell proliferation. The regulation is suggested to be mediated through the central nervous system directly and/or indirectly affecting the peripheral adrenergic system at the pancreatic level. The DNA synthesis was peaked at 72 hrs and started decreasing at 7 days after partial pancreatectomy as observed by the [^{3}H]thymidine incorporation studies. There was a significant elevation in the circulating insulin and T_3 levels during active DNA synthesis in the pancreatic islets. The EPI and NE contents were significantly decreased in the cerebral cortex, brain stem and hypothalamus as well as in the pancreatic islets during active pancreatic islet regeneration. Circulating NE and EPI levels were found to decrease at the time of active regeneration of pancreatic islets. The decreased brain EPI content lead to a decreased receptor function in the brain as well as in the pancreatic islets. The α_1-and α_2-adrenergic receptors were down regulated in the brain regions and pacreatic islets during pancreatic regeneration. The β-adrenergic receptors were up regulated in the brain regions and islets at 72 hrs after partial pancreatectomy. RT-PCR studies confirmed the receptor data. The decreased α_{2A}-adrenergic and increased β-adrenergic receptor function will lead to an increase in the insulin secretion and DNA synthesis in the pancreatic islets.

In vitro studies of pancreatic islets showed that EPI regulates the insulin secretion in a concentration-dependent manner. Higher concentrations of EPI inhibited the glucose-mediated insulin secretion and lower concentrations stimulated the glucose-mediated insulin secretion from the islets. The α_1-adrenergic receptors act both stimulatory and inhibitory depending upon the EPI concentration. α_2-adrenergic receptors are inhibitory and β-adrenergic receptors are stimulatory to insulin secretion in both 1 hr and 24 hrs islet cell cultures. Peripherally the EPI uptake by the islets is time-dependent irrespective of the glucose concentration. EPI is able to bind with a novel 70-kDa nuclear protein within the islets. During regeneration, this protein was found to be up regulated and in diabetic condition it was down regulated. It is clear from our results that the 70-kDa EPI-binding nuclear protein is acting as an enhancer of insulin synthesis thereby promoting the islet cell regeneration. The *in vitro* DNA synthesis studies showed that EPI at low concentrations could enhance the EGF-induced DNA synthesis and at high concentrations

inhibit the EGF-induced DNA synthesis in the pancreatic islets. α_1-adrenergic receptors act both as stimulatory and inhibitory according to the EPI concentration. α_2-adrenergic receptors inhibited and β-adrenergic receptors stimulated the DNA synthesis in the islet cell cultures.

Thus we conclude that EPI can regulate the pancreatic islet cell proliferation by controlling the insulin synthesis and secretion. The brain adrenergic receptor gene expression and functional correlation regulate the pancreatic adrenergic receptors. The functional balance of α- and β-adrenergic receptors controls the insulin secretion and pancreatic β-cell proliferation, which will have immense clinical significance in the treatment of Diabetes mellitus.

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