DISCUSSION
5. DISCUSSION

The blood glucose level of diabetic rats was significantly elevated by administration of streptozotocin which specifically destroys the pancreatic β-cells (Junod et al., 1969). Destruction of β-cells lead to a decrease in insulin secretory efficiency of these cells. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan significantly reduced the blood glucose level. There was no significant change in the body weight of the experimental rats.

In our experiments we have used L-tryptophan instead of 5-HTP, since 5-HTP would lead to 5-HT formation at sites that do not form 5-HT physiologically. The enzyme aromatic L-amino acid decarboxylase that converts 5-HTP to 5-HT is present in many other cells other than serotonergic neurons (Fuller, 1981). Administration of L-tryptophan will specifically increase the 5-HT content in serotonergic neurons. The disadvantage in use of L-tryptophan is that, only a small percentage of the administered L-tryptophan will be converted to 5-HT. To overcome this a high dose treatment is required.

5.1. CENTRAL NERVOUS SYSTEM CONTROL OF INSULIN REGULATION

5.1.1. Brain 5-HT content is reduced during diabetes

In our experiments we have observed a significant reduction of 5-HT content in cerebral cortex (CC), brain stem (BS) and hypothalamus (Hypo) of diabetic rats. These finding agree with the previous reports of decreased 5-HT in brain regions during diabetes (Jackson & Paulose, 1999; Sandrini et al., 1997; Sumiyoshi et al., 1997; Thorre et al. 1997; Shimizu, 1991; Chu et al., 1986; Kulikov et al. 1986). In cerebral cortex the decrease in 5-HT content is due to a reduction in the conversion of 5-HTP to 5-HT. This is because of a significant decrease in 5-HTP content. Another contributing factor for the decreased 5-HT is the significant increase in the breakdown of 5-HT to 5-HIAA that is catalysed by monoamine oxidase which is known to regulate insulin secretion (Pizzinat et al., 1999).

In case of brain stem the decrease in 5-HT content is brought about by a significant
increase in the rate of synthesis of 5-HT and its breakdown to 5-HIAA. There is also no significant increase of 5-HP during diabetes. This leads to a decreased accumulation of 5-HT in the serotonergic neurons. There was no significant change in the hypothalamic 5-HT content of diabetic rats. But there was a significant reduction in the availability of the precursor 5-HP in diabetic rats. A significant reduction in the conversion of 5-HP to 5-HT is also observed during diabetes without any change in the breakdown of 5-HT. Thus it appears that in hypothalamus the breakdown of 5-HT to 5-HIAA is reduced to compensate for the decreased availability of 5-HP. The observed reduction in 5-HT synthesis will be associated with reduced transmitter release (Carndall et al., 1981).

Insulin treatment was able to significantly increase the 5-HT content in CC, BS and Hypo. This increase in the brain 5-HT content is due to the increase in tryptophan uptake through the BBB with other neutral amino acids. The carrier system for transport of tryptophan across the BBB is shared by several large neutral amino acids including tryptophan (Carndall et al., 1981). Insulin tends to release the tryptophan bound to albumin hence increasing the concentration of free tryptophan in plasma (Trulson & Mackenzie, 1978; Curzon & Mursden, 1975). Treatment of diabetic rats with tryptophan and insulin + tryptophan also significantly increased the brain 5-HT content. Jamnicky et al., (1993) have reported that administration of tryptophan in combination with insulin to diabetic rats have reversed the levels of brain tryptophan, 5-HT, 5-HIAA and serum concentrations of valine, leucine and isoleucine towards control. Oral administration of 5-HP to diabetic patients have also increased brain 5-HT content (Rossi-Fanelli, 1998).

The NE content in BS and Hypo of diabetic rats were significantly increased. There was no significant change in the NE content in CC of diabetic rats. This result is concordant with the previously published reports (Tasaka et al., 1992; Chen & Yang, 1991, Lackovic et al., 1990). Insulin, tryptophan and insulin + tryptophan treatment reversed the increased NE content in BS and Hypo to control.

It has been well documented that long term hyperglycaemia in diabetic animals can lead to chronic hypofunction of central 5-HT neurons leading to decreased brain tryptophan, 5-HT and 5-HIAA (Sandrini et al., 1997; Kwok & Juorio, 1987). The
decrease in brain 5-HT is due to the decreased availability of tryptophan in brain. The amount of tryptophan correlates with the 5-HT content in the brain (Fernstrom & Fernstrom, 1995; Fernstrom, 1991, Curzon & Mursden, 1975; Friedman et al., 1972; Ecclestron et al., 1965).

An increase in the level of insulin can result in decreased plasma concentrations of large neutral amino acids which compete with tryptophan for uptake into the brain (Cruzon & Fernando, 1977). STZ selectively destroys pancreatic β-cells and causes hypoinsulinemia leading to hyperglycaemia (Hohenegger & Rudas, 1971, Arison et al. 1967). This decrease in the circulating insulin can increase the competition of other amino acids with tryptophan for uptake into brain thereby decreasing the level of tryptophan and 5-HT in the brain of diabetic rats. Consumption of a tryptophan rich diet can also increase the brain tryptophan. This will lead to an increase in circulating tryptophan, which will reduce the competition of tryptophan with other amino acid for uptake into brain. Hutson et al. (1985) have reported a similar increase in 5-HT and 5-HIAA in the cerebrospinal fluid (CSF) as seen in brain after i.p. administration of tryptophan.

5.1.2. Plasma 5-HT and EPI concentrations is increased during diabetes

During diabetes there was a significant increase in plasma 5-HT concentration. This agrees with our earlier report on the increase in platelet 5-HT content during diabetes (Jackson et al., 1997). There is also a significant increase in the plasma 5-HTP concentration in diabetic rats. The increase in plasma 5-HTP can be related to an increase in plasma tryptophan. In the absence of insulin, plasma tryptophan remains bound to albumin which is not taken up readily through the BBB since only free tryptophan is taken up through the BBB. This leads to an accumulation of tryptophan in the plasma of diabetic rats. Plasma EPI concentration was also significantly increased in diabetic rats. The increased NE and EPI concentrations in plasma is due to increased sympathetic stimulation during diabetes centrally and peripherally (Jackson et al. 1997; Chaouloff et al., 1990a; McCall & Hornis, 1988, Hoyer, 1988c). Insulin, tryptophan and insulin + tryptophan treatment effectively reversed the 5-HT and EPI levels to control.
5.1.3. **Brain 5-HT$_{2A}$ receptor activity and expression is increased during diabetes**

One of the major findings of this study is that there is an increase in affinity of 5-HT$_{2A}$ receptors in cerebral cortex and hypothalamus without any change in its number and there is an appearance of a low affinity site during STZ-induced diabetes. In the case of brain stem 5-HT$_{2A}$ receptors there is an up-regulation of 5-HT$_{2A}$ receptors accompanied by a decrease in its affinity. These alterations of 5-HT$_{2A}$ receptors in the brain regions is a compensatory mechanism for the decreased 5-HT content reported during diabetes in the brain regions (Jackson & Paulose, 1999; Sandrini et al., 1997).

In our experiments, treatment of diabetic rats with insulin effectively reversed the altered 5-HT$_{2A}$ receptors to control. The increase in circulating insulin favours the increased uptake of tryptophan into the brain that in turn increases the brain 5-HT content thereby bringing a decrease in the 5-HT$_{2A}$ receptors. It is reported that the up-regulation of 5-HT$_{2A}$ receptors during diabetes is a secondary effect of hypoinsulinemia (Sumiyoshi et al., 1997). This up-regulation of the receptor can have a possible role in the regulation of insulin secretion. The increased affinity and increase in number of 5-HT$_{2A}$ receptors in CC, Hypo and BS respectively can increase the sympathetic nerve discharge thereby increasing the circulating NE and EPI levels. This increased NE and EPI might then bind to $\alpha_2$ adrenergic receptors and inhibit insulin secretion from pancreatic islets with simultaneous increase in glucagon level. It is already reported that the 5-HT$_2$ agonist 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI) was able to produce a tremendous increase in sympathetic nerve discharge, thus increasing EPI concentration. 5-HT$_2$ antagonists, ketanserin and LY53857 were able to reverse the increase in sympathetic nerve discharge produced by DOI (Jackson et al., 1997; Chaouloff et al. 1990a; McCall & Hornis, 1988; Hoyer, 1988c). McDonald, (1996) have reported increased expression of 5-HT$_2$ receptor mRNA in islets maintained for 1 day at 20mM glucose than in islets maintained at 1mM glucose. Trulson & Mackenzie, (1978) have reported that after 4 weeks of administration of streptozotocin, the brain tryptophan content was decreased by 27%. Insulin administration was able to bring back the brain tryptophan and 5-HIAA levels to normal. Tryptophan uptake across the BBB is increased in the presence insulin. Insulin enhances the uptake of branched chain amino acids into the muscles thereby decreasing their plasma concentration. Since these amino acids
compete with tryptophan for transport into brain, there is a resultant increase in brain tryptophan (Curzon & Mursden, 1975).

During diabetes there is a significant reduction of brain tryptophan, 5-HT and 5-HIAA content (Sandrini et al., 1997; Kwok & Juorio, 1987). The decreased brain 5-HT content leads to an up-regulation of 5-HT\textsubscript{2\text{A}} receptors in BS and an increased affinity of these receptors in cerebral cortex (Jackson & Paulose, 1999; Sandrini et al., 1997; Sumiyoshi et al., 1997). This leads to increased sympathetic stimulation and thereby decreases insulin secretion from pancreatic islets mediated by EPI release from adrenal glands (Chaouloff et al., 1990d). An up-regulation of 5-HT\textsubscript{2\text{A}} receptors also increases the risk of diabetes induced depression (Mann et al., 1986; Stanley, 1983). The increase in brain 5-HT reverses the altered 5-HT\textsubscript{2\text{A}} receptor binding parameters in cerebral cortex and brain stem and reduces sympathetic nerve stimulation thus reducing the inhibitory effect of EPI on insulin secretion.

It is reported that diet can also influence the brain 5-HT content. Consumption of tryptophan deficient diet can also lead to reduced circulating tryptophan and brain 5-HT content (Fernstrom, 1994). DeMarte & Enesco, (1985) maintained a group of mice for 78 weeks on tryptophan restricted, protein restricted and control diet. They found that brain 5-HT levels were significantly reduced only in mice on the tryptophan-restricted diet, but not for mice on the protein restricted diet. It is not only tryptophan that is influenced by the diet but other amino acids such as tyrosine that is the precursor for dopamine and norepinephrine. The same process is applicable for the uptake of choline which is the precursor of acetylcholine (Fernstrom, 1994). From this it appears that diet can also play an important role in the induction of diabetes through the serotonergic system by reducing the brain 5-HT content. In addition to the central 5-HT\textsubscript{2\text{A}} receptors the peripheral 5-HT\textsubscript{2\text{A}} receptors may also play a major role in regulation of insulin, since the pancreatic islets contain a large amount of endogenous serotonin (DeMarte & Enesco, 1985; Bird et al., 1980).

Thus, from our study we conclude that STZ induced diabetes causes an increase in affinity of 5-HT\textsubscript{2\text{A}} receptors in cerebral cortex and hypothalamus without any change in
the number of receptors. The brain stem 5-HT$_{2A}$ receptors are up-regulated accompanied by the appearance of a low affinity site which was reversed to control by insulin treatment. The enhanced 5-HT$_{2A}$ receptor binding observed in brain regions can mediate an increased sympathetic nerve discharge leading to inhibition of insulin release from pancreas and can also mediate diabetes induced depression. Alterations in the apparent number of 5-HT$_{2A}$ receptors have also been reported in several central nervous system disorders such as schizophrenia, Parkinson's disease and Alzheimer's disease (Conn & Sanders-Bush, 1987).

We have observed an increase in the expression of 5-HT$_{2A}$ receptors of diabetic BS. The CC and Hypo showed only an increase in affinity for 5-HT during diabetes without any change in its expression. This can be due to the fact that only BS has direct nerves originating from it and extending to the pancreas (Coldman & Dampney, 1998). The CC and Hypo does not have direct inervation to the end organ. The CC co-ordinates the overall function of the brain. Hypo acts through the hypathalamo-pituitary end organ axis. In CC and Hypo, a decrease in 5-HT content during diabetes brings about a compensatory increase in the affinity of 5-HT$_{2A}$ receptors without increasing the mRNA levels. This compensation can be considered as a momentary change to overcome the decrease in 5-HT. In the case of BS the decreased 5-HT is compensated by increasing the level of 5-HT$_{2A}$ receptor mRNA which in turn will inhibit insulin secretion by direct sympathetic stimulation. This compensation is not a momentary change but it affects the transcription of the 5-HT$_{2A}$ receptor gene. It has been reported that 5-HT can regulate the expression of 5-HT$_{2A}$ receptors. The 5-HT dependent transcription activity depends upon the presence of functional 5-HT$_{2A}$ receptors (Yun-Long et al., 1995). Therefore, once there is a decrease in 5-HT in BS, the 5-HT$_{2A}$ receptors increase its affinity to bind to the available 5-HT. This in turn regulates the transcription of the 5-HT$_{2A}$ receptors in BS. The significant increase in 5-HT$_{2A}$ receptors observed during diabetes in pancreatic islets may be due to an increase in pancreatic 5-HT content.

5.1.4. Brain 5-HT$_{1A}$ receptor activity is increased during diabetes

5-HT$_{1A}$ has already been reported to have a similar role in the inhibition of insulin secretion. Scatchard analysis of high affinity 5-HT$_{1A}$ receptors in CC and Hypo showed a significant decrease in the number of receptors without any significant change in its
affinity during diabetes. But the low affinity receptors in these two regions showed a significant increase in its number during diabetes. From these results it seems that the decreased 5-HT is able to up-regulate the low affinity 5-HT\textsubscript{1A} receptor and down-regulate the high affinity 5-HT\textsubscript{1A} receptors. As mentioned in the results the high affinity and low affinity 5-HT\textsubscript{1A} receptors are actually two different proteins coded by two distinct mRNA and are not inter-convertable states of the same protein (Nenonene et al. 1994). Therefore, such a differential regulation shows that in CC and Hypo, the alterations in the 5-HT\textsubscript{1A} receptors during diabetes are mediated by the low affinity 5-HT\textsubscript{1A} receptors. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan did not reverse the altered high affinity and low affinity 5-HT\textsubscript{1A} receptors. Nenonene et al., (1994) have demonstrated the heterogeneity of \([3H]8\text{-OH-DPAT}\) binding in rat cerebral cortex and hippocampus. The first high affinity binding site, 5-HT\textsubscript{1A}\textsuperscript{HIGH} represents the classic 5-HT\textsubscript{1A} receptor based on its pharmacological profile and the effects of Gpp(NH)p. The second binding site, 5-HT\textsubscript{1A}\textsuperscript{LOW}, is also labelled by \([3H]8\text{-OH-DPAT}\) It has a micromolar affinity for 5-HT and it is not coupled to G proteins.

In case of brain stem, there is no significant change in number of low affinity 5-HT\textsubscript{1A} receptor but there is an increase in its affinity during diabetic state. The high affinity 5-HT\textsubscript{1A} receptors in BS show a significant increase in its number without any change in affinity. This is reversed by insulin, tryptophan and insulin + tryptophan treatment. From these results it appears that in CC and Hypo the low affinity 5-HT\textsubscript{1A} receptors are more involved during diabetes and in BS the high affinity 5-HT\textsubscript{1A} receptors are involved in mediating the effect of decreased 5-HT. An increase in the 5-HT\textsubscript{1A} receptors in CC and BS has been previously reported (Sandrini et al., 1997; Sumiyoshi et al., 1997). But they have not reported any difference in the high and low affinity states of these receptors.

The increase in number of the 5-HT\textsubscript{1A} receptors in response to the decreased 5-HT content in CC and BS will stimulate the sympathetic nerves and increase the vagal tone. An increased sympathetic activity will induce increased EPI output from the adrenal medulla that will inhibit insulin secretion (Bauhelal & Mir, 1993, Bauhelal & Mir, 1990a). This was proved by injecting the specific 5-HT\textsubscript{1A} receptor agonist 8-OH-DPAT to normal
rats. The rats showed a very rapid increase in blood glucose level that reached its peak within 30 min. A similar observation was reported from a number of laboratories (Laude et al., 1990; Bauhelal et al., 1990b; Chaouloff & Jeanrenard, 1988; Chaouloff & Jeanrenaud, 1987). Administration of 8-OH-DPAT also produced a sustained fall in blood pressure and heart rate. All these symptoms were abolished by idazoxan pre-treatment and by adrenalectomy (Bauhelal & Mir, 1993). 8-OH-DPAT treatment can also increase plasma levels of corticosterone that can also inhibit insulin secretion (Chaouloff et al., 1990d; Aulakh et al., 1988; Koenig et al., 1987).

The 5-HT_{1A} is coupled to guanine nucleotide regulatory proteins or G proteins and its labelling by [3H]8-OH-DPAT decreases in the presence of GTP (Hall et al., 1985). GTP analogue caused a decrease in affinity of 5-HT_{1A} receptors to their natural ligand 5-HT in CC and BS of diabetic rats. This indicates a desensitisation of the 5-HT_{1A} receptors on G-protein association. In control rats the inhibition of 1nM [3H]8-OH-DPAT binding by increasing concentrations of 5-HT shifted to the lower affinity state in the presence of Gpp[NH]p and the K_i value increased in agreement with previous results using serotonergic agonists (Hamon et al., 1988). The reduction of 1nM [3H]8-OH-DPAT high affinity confirms that this site corresponds to the known 5-HT_{1A} receptor. The other component of 1nM [3H]8-OH-DPAT binding i.e., the 5-HT_{1A,low} sites remained insensitive to 100μM Gpp[NH]p. A similar observation was reported by Emerit et al. (1991).

5.1.5. Insulin synthesis is inhibited by stimulation of sympatho-adrenal activity through 5-HT_{1A} and 5-HT_{2A} receptors

In case of 5-HT_{1A} and 5-HT_{2A} receptors in brain regions of diabetic rats we have found a compensatory increase in either the number of the receptors or an increase in its affinity to bind to the decreased brain 5-HT content. Thus, from our study we conclude that the enhanced 5-HT_{2A} and 5-HT_{1A} receptor binding observed in brain regions of diabetic rats can mediate an increased sympathetic nerve discharge (Bauhelal & Mir, 1993; Bauhelal & Mir, 1990a). Simultaneously there is an increased EPI release from the adrenal medulla adding to the sympathetic stimulation. This lead to an inhibition of insulin release from pancreas.
5.1.6. Hypothalamo-pituitary-thyroid axis also plays an important role in inhibition of insulin secretion

The hypothalamo-pituitary-thyroid status is also affected during STZ induced diabetes. We have observed a significant decrease in hypothalamic 5-HT content and circulating T₃ and T₄ levels. This agrees with the previous reports of TSH, T₃ and T₄ reduction in STZ diabetes (VanHaasteren et al., 1997; Wilber et al., 1981). We have also observed an increase in affinity of 5-HT₂A and increase in number of low affinity 5-HT₁A receptors in hypothalamus of diabetic rats. A decrease in 5-HT content in hypothalamus will lead to decreased TSH secretion. Dakshinamurti et al. (1985) have reported a reduction in hypothalamic 5-HT content in pyridoxine-deficient hypothyroid rats. This decreased 5-HT reduces the synthesis and release of TSH from the pituitary through TRH secretion (Dakshinamurti et al., 1986). Van Haasteren et al. (1997) have reported that the reduced hypothalamic TRH release during diabetes is probably not caused by decrease in TRH synthesis or transport to the median eminence, but seems to be due to impaired TRH release from the median eminence which can be related to the lack of insulin. Therefore, the decreased T₃ and T₄ in circulation may be due to a decreased release of TRH from the hypothalamus that is mediated by an altered 5-HT₂A and 5-HT₁A receptors. The postulate that 5-HT neurons stimulate TSH secretion in rats is supported by the observation that injection of 5-HT into the third ventricle caused rapid increase in serum TSH (Smythe et al., 1982). Balsa et al., (1998) have reported that 5-HT can stimulate the secretion of GH, ACTH and LH acting directly at pituitary level on the posterior pituitary. Treatment with insulin, tryptophan and insulin + tryptophan reversed the hypothalamic 5-HT content and the altered 5-HT₁A and 5-HT₂A receptors. But the T₃ and T₄ content in circulation was not reversed to control level by these treatments. This may be due to the delayed thyroid response to the treatments.

5.2. PERIPHERAL CONTROL OF INSULIN SECRETION

5.2.1. Pancreatic islet 5-HT content is increased during diabetes

Pancreatic islet is considered as a tissue rich in 5-HT (Bird et al., 1980). Within the islets 5-HT is stored along with insulin granules (Bird et al., 1980; Jain-Etchevery &
Zieher, 1968; Falck & Hellmann, 1963). Our results show a significant increase in pancreatic 5-HT content in diabetic rats. There is also a significant increase in the level of 5-HTP which acts as precursor of 5-HT. The presence of increased pancreatic content of 5-HTP in diabetic rats will lead to increased synthesis of 5-HT which is evident from our results. In presence of high glucose 5-HTP is rapidly taken up into the islets which stimulates insulin secretion. But the enzyme 5-Hydroxy tryptophan decarboxylase readily converts it into 5-HT that inhibits insulin secretion (Sundler et al., 1990; Lindstrom & Sehlin, 1983). Tryptophan which is a precursor of 5-HTP, is reported to have a stimulatory effect on insulin release from hamster pancreas (Bird et al., 1980). The presence of monoamine oxidase enzyme that catabolises 5-HT within the β-cells and specifically nuclear membrane show an effective metabolism of 5-HT within the pancreas (Pizzinat et al., 1999; Gujrati et al., 1996; Feldman & Chapman, 1975). Treatment with insulin, tryptophan and insulin + tryptophan effectively decreased the pancreatic 5-HTP content which in turn decreased the 5-HT content. The decreased 5-HT will in turn reduce the inhibition of insulin secretion. 5-HT can also act as a marker for insulin secretion. 5-HT is taken up into insulin granules and co-released with insulin on stimulation of pancreatic β-cells by glucose (Zhou & Misler, 1996). All these evidences show the presence of 5-HT within the pancreatic islets and has a role in the regulation of insulin secretion from the B-cells. The EPI content in pancreas of diabetic rat showed a significant increase. At high concentrations, EPI binds and stimulates α2-adrenergic receptors which in turn inhibits insulin secretion.

5.2.2. Diabetes induces 5-HT uptake into pancreatic islets and inhibits insulin secretion

A number of laboratories have reported the presence of 5-HT and other monoamines within the pancreatic islets (Bird et al., 1980), but there was no evidence for the actual mechanism by which 5-HT control insulin secretion from the β-cells. We have observed a significant uptake of [3H]5-HT into the pancreatic islets in the presence of 20mM glucose, which can be considered equivalent to diabetic state in vivo. Incubation of islets with 1nM and 5nM [3H]5-HT showed a marked increase in 5-HT uptake in presence of 20mM glucose concentration compared to islets incubated with 4mM glucose. But cells incubated with 10nM [3H]5-HT did not show any significant increase in 5-HT uptake. These results show that 5-HT is taken up by the islets only in the presence of
glucose. Thus, it can be considered that glucose is the determining factor for the uptake of 5-HT into the islets. There are also reports which state that 5-HT is taken up into the insulin granules and secrete 5-HT/insulin in a pulsatile fashion on stimulation of pancreatic islet β-cells under physiologic conditions (Zhou & Misler, 1996). Our results confirm the earlier findings where in a normal islet, an increase in glucose level will lead to increased uptake of 5-HT into the islets. 5-HT is co-released with insulin on glucose stimulation maintaining a steady equilibrium. But in the case of diabetic islets the increase in glucose leads to increased uptake of 5-HT into the islets. Since the glucose induced stimulus for insulin secretion is less, there is a decreased insulin output. This leads to an increased accumulation of 5-HT within the islets. Extensive studies on transmembrane transport of 5-HT have been carried out with neurons and thrombocytes. These cells generally have similar uptake mechanisms (Sneddon, 1973; Abrams & Solomon, 1969). The following are the two hypotheses for the mechanism of 5-HT uptake into the cells:

(i) 5-HT transport is mediated by islet high affinity low capacity active transport mechanism as well as by passive diffusion (Stahl & Meltzer, 1978).

(ii) 5-HT has two active transport systems working in parallel - one with saturable high affinity and low capacity and another with non-saturable low affinity and high capacity (Shaskan & Snyder, 1970).

The high affinity mechanism is sodium dependant and inhibited by metabolic blockers, whereas the low affinity component is not sodium dependant (Stahl & Meltzer, 1978). It is also reported that metabolic inhibition and sodium deficiency reduced the initial uptake of 5-HT in the presence of low extracellular 5-HT concentration but had no or less pronounced effects at higher 5-HT concentrations. This explains the decreased 5-HT uptake into the islets at 10nM [3H] 5-HT concentration seen in our results whereas at low [3H] 5-HT concentration (1nM, 5nM) there was a significant increase in glucose dependant [3H] 5-HT uptake.

To study the possible action of 5-HT on insulin regulation, we fractionated the islets into membrane + mitochondrial, cytosolic and nuclear fractions. Only the nuclear fraction showed positive binding to [3H]5-HT in all the experimental groups compared to
the membrane and cytosolic fractions which did not show any direct binding. Further confirmation on the uptake and binding of 5-HT to the nuclear fraction was made by incubating the fractionated islets with 5nM \[^{3}H\]5-HT for different time intervals and at different glucose concentrations (4mM and 20mM). The whole cells showed a glucose dependant uptake of \[^{3}H\]5-HT as discussed above. The mitochondrial and membrane fractions did not show any significant change in binding in the presence of glucose whereas the cytosolic fractions showed a significant increase of \[^{3}H\]5-HT uptake/transport in the presence of 20mM glucose at 1hour and 3hours incubations. The nuclear fraction also showed a significant increase in glucose dependent \[^{3}H\]5-HT uptake at 3hour incubations. We conclude from these results that 5-HT is taken up into the islet in a glucose dependant manner and binds to the nuclear protein. The presence of increased amounts of \[^{3}H\]5-HT in the cytoplasm signifies the 5-HT taken up which then binds to the nuclear protein.

The 5-HT binding protein on the nuclear membrane was identified by ligand blotting as a 14-kDa protein. Amino acid composition of this protein showed the presence of high concentrations of histidine (79.08%) followed by glycine (6.18%) and glutamine (11.2%). This 14-kDa 5-HT binding protein showed a marked similarity and homology with respect to amino acid composition and molecular weight to a helix-loop-helix protein2 (bHLH2) which belongs to the basic helix-loop-helix family of transcription factors.

The catabolic breakdown of 5-HT and other monoamines are also carried out in the pancreatic islets. Catacholamines are inactivated mainly by two mechanisms, through the enzyme catechol-o-methyltransferase (CDMT) 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\(_2\)O\(_2\)). The generation of H\(_2\)O\(_2\) may affect the redox state of the \(\beta\)-cell glutathione system, the balance of which is known to influence nutrient-induced insulin release (Miller, 1981).
Monoamine oxidase exists in two isoforms, MAO-A and MAO-B, which are separate gene products, exhibiting different substrate specificity and whose activity may be differentially inhibited by specific inhibitors (Pizzinat et al., 1999; Fowler et al., 1978; Johnston, 1968). 5-HT is the preferred substrate for MAO-A whereas phenylethylamine (PEA) is preferentially deaminated by MAO-B. Tyramine, EPI, NE and dopamine are common substrate for both isoforms. In addition to cytoplasm of the cell, monoamine oxidase has been reported to be present in the nuclei of placental cells (Gujrati et al. 1996).

The uptake of 5-HT into the islets can directly inhibit insulin synthesis within the pancreatic islets. The islets incubated with 1nM and 5nM 5-HT in the presence of 20mM glucose showed a significant inhibition of insulin secretion. But the islets incubated with 10nM 5-HT showed a lesser degree of inhibition compared to 1nM and 5nM 5-HT. This is due to the decreased uptake of 5-HT due to high concentration of 5-HT in the medium. In contrast to this, in the absence of glucose, 5-HT was able to stimulate insulin secretion. This stimulation was further reduced in the presence of 4mM glucose. These results show that the 5-HT acts as a stimulant for insulin secretion in the absence of glucose but inhibits insulin in the presence of glucose.

5.2.3. 14-kDa nuclear 5-HT binding protein is regulated by 5-HT

Scatchard analysis of the 5-HT binding protein (bHLH) in islet nuclear fraction shows a significant down-regulation during diabetes that is evident from the reduced $B_{max}$. The $K_d$ of the diabetic group is also significantly reduced showing an increased affinity for 5-HT. This can be a natural mechanism of down-regulation similar to the 5-HT$_{2A}$ receptor by the increased content of 5-HT (Leysen & Pauwels, 1990; Eison et al., 1989). This is consistent with the traditional adaptive pattern of regulation of brain monoamine receptors (Yun-Long et al., 1995). The increased affinity of the nuclear 5-HT binding protein leads to increased binding of 5-HT present within the islets to this protein. This leads to a feedback down-regulation of the 5-HT binding protein and inhibits insulin gene transcription during diabetes since this protein acts as an enhancer for insulin gene transcription.
SEROTONIN (5-HT) AND INSULIN REGULATION IN NORMAL PANCREATIC β-CELL

Figure shows normal pancreatic islet cell with normal amount of 5-HT, nuclear 5-HT binding protein and insulin output.

SEROTONIN (5-HT) AND INSULIN REGULATION IN DIABETIC PANCREATIC β-CELL

Figure shows inhibition of insulin secretion from a diabetic pancreatic β-cell. Glucose-induced 5-HT uptake down-regulates the nuclear 5-HT binding protein. Since this protein can act as an enhancer for insulin transcription, down-regulation of this protein reduces the insulin output.
Insulin replacement and a combination of treatment with insulin, tryptophan and insulin + tryptophan to diabetic rats failed to reverse the feedback control of the 5-HT binding protein. This is evident from the significant decrease in $B_{\text{max}}$ compared to the controls. Thus, from our results we can infer that the feedback inhibition of the binding protein is triggered in the islets by hyperglycaemia induced increase in islet 5-HT. The down-regulation of the 5-HT binding protein cannot be completely corrected by insulin, tryptophan and insulin + tryptophan treatments. This is opposite to the results seen in the brain regions where we have observed a complete reversal of the 5-HT binding parameters to control, by insulin, tryptophan and insulin + tryptophan treatments.

5.2.4. Possible transcriptional regulation of insulin gene by basic Helix-Loop-Helix protein2 (bHLH 2)

Based on the amino acid homology the 14-kDa nuclear 5-HT binding protein was identified as a basic helix-loop-helix protein. The bHLH2 protein acts as a transcriptional activator of insulin promoter. They bind to E-box of the insulin promoter and enhance the transcription of the insulin gene (Vienna & Melson, 1995, Peers et al., 1994; Robinson et al., 1994). An array of A and E elements constitute symmetrical enhancers that cooperatively account for >90% of the transcriptional activity of the insulin gene promoter (Ohlsson et al., 1988). The E elements contain a core sequence CANNTG, which are recognition motifs for transcription factors in the basic helix-loop-helix family E12 and E47 which activate the insulin promoter in close synergism with A element binding homeobox transcription factors, such as IDX-1. The A elements consist of a core sequence TAAT which constitutes a binding site for homeodomain transcription factors. In addition to transcriptional activation there are various repressors of insulin gene promoter. Chronic hyperglycaemia may contribute to the pancreatic $\beta$-cell dysfunction observed in patients with type II diabetes which is mainly due to the phenomenon of glucose toxicity (Robertson et al., 1994). Studies, in vivo in animal models and in vitro studies with immortalised $\beta$-cell line have shown a reduction in insulin gene transcription mediated by glucose toxicity. Such a condition is associated with loss of transactivator protein such as IDX-1/IPF-1/STF-1 and PIPE3b1-binding protein (Zangen et al., 1997; Poitout et al., 1996; Sharma et al., 1995, Olson et al., 1995; Olson et al., 1993, Robertson...
Since insulin gene transcription is both positively and negatively controlled, the repressors also play an important role in insulin regulation. Glucose induced repression of insulin gene is mainly mediated through CCAAT/enhancer binding protein (C/EBP). C/EBPs are a family of transcription factors that regulate genes of the acute phase response, cell growth, differentiation and the expression of cell type specific genes (Mandrup & Lane, 1997; Pope et al., 1994; Vasseur-Cognet & Lane, 1993; Descombes et al. 1990; Poli et al. 1990).

The C/EBPs bind to DNA exclusively as dimers and contain a conserved C-terminal basic region, Leucine-Zipper domain, that is characterised by a DNA-contacting basic region linked to a leucine-zipper dimerisation motif (Trautwein et al., 1996). In pancreatic β-cell C/EBP specifically interacts by direct protein-protein interactions with a heptad leucine repeat sequence within activation domain 2 (AD2) of the basic helix-loop-helix transcription factor E47, thereby inhibiting the DNA binding activity and the transactivation potential of E47. This interaction leads to the inhibition of both dimerisation and DNA binding of bHLH2 protein to the E element of the insulin promoter that reduces insulin gene transcription.

In our results we have observed a decrease in 5-HT binding protein (considered as the bHLH2 protein based on amino acid homology) in islets incubated in hyperglycaemic medium. This decrease in bHLH2 protein can reduce its binding to the E element of the insulin promoter and decrease insulin gene transcription in diabetic state. The inhibition of insulin gene transcription is further aggravated by the induction of the repressor C/EBP in pancreatic β-cells by chronically elevated glucose levels (Ming et al. 1997). This increased C/EBP can bind to the AD2 domain of bHLH2 protein and prevent it from binding to the E element of the insulin promoter thus inhibiting insulin gene transcription during diabetes.

There is another report that agrees on the inhibition of insulin transcription by decreased bHLH2 protein (Dumonteil et al., 1998). The bHLH2 protein E47 is able to dimerise with another protein BETA2 (β-cell E-box transactivator2). This BETA2/E47 complex is able to regulate both insulin promoter and glucagon promoter by binding to the
E-box. Over expression of E47 (bHLH2 protein) is able to inhibit E-box mediated glucagon gene expression and stimulate E-box mediated insulin gene transcription.

**NORMAL TRANSCRIPTION OF INSULIN GENE IN PRESENCE OF bHLH2 PROTEIN**

![Diagram showing normal transcription of insulin gene in presence of bHLH2 protein.]

**INHIBITION OF TRANSCRIPTION BY DIMERISATION OF bHLH2 WITH C/EBPβ INDUCED BY HYPERGLYCAEMIA**

![Diagram showing inhibition of transcription by dimerisation of bHLH2 with C/EBPβ induced by hyperglycaemia.]

**INHIBITION OF TRANSCRIPTION BY DOWN-REGULATION OF bHLH2 BY 5-HT**

Decreased E47 (bHLH2)

![Diagram showing inhibition of transcription by down-regulation of bHLH2 by 5-HT.]

In our experiment we have found a decrease in bHLH2 protein induced by hyperglycaemia. This prevents the inhibition of glucagon gene transcription leading to increased glucagon and glycogenesis thereby increasing blood glucose level. Therefore, the heterodimer BETA2/E47 can be considered as an islet specific factor whose ratio can control both insulin and glucagon gene transcription.

In addition to the above mentioned mechanisms these transcription factors can...
influence insulin gene promoter by regulating glucose and hormones, which elevate β-cell [Ca2+] and cAMP levels and possibly protein kinase C activity (Goodison et al. 1992).

5.3. CONCLUSION

We conclude from our studies that the serotonergic system can regulate insulin secretion from the pancreatic islets. The regulation is suggested to be mediated through the central nervous system directly and/or indirectly affecting the sympathetic stimulation and the peripheral control at the pancreatic level. We have observed a decrease of 5-HT content in CC, BS and Hypo. This decrease in 5-HT content led to an increase in the expression of 5-HT2A receptors in BS along with an increase in its affinity for 5-HT in CC and Hypo. The high affinity 5-HT1A receptors in BS was up-regulated and the low affinity 5-HT1A receptors of CC and Hypo showed an increase in affinity for 5-HT. This increased activity of the 5-HT1A and 5-HT2A receptors will inhibit insulin secretion by increasing the sympathetic activity during diabetes. An increased sympathetic activity will increase the circulating EPI content that directly controls insulin secretion. Peripherally 5-HT is rapidly taken up into pancreatic islets which inhibits insulin secretion. Our in vitro studies show that there is a significant decrease in the amount of glucose induced insulin secretion in the presence of 5-HT. Within the islets 5-HT is able to bind to a novel 14-kDa nuclear protein and down-regulate it during diabetes. Based on the amino acid composition homology, this 14-kDa nuclear 5-HT binding protein was identified as a basic helix-loop-helix (bHLH2) transcription factor that acts as an enhancer of insulin gene promoter. Since 5-HT can bind and down-regulate this 14-kDa bHLH2 protein, it will lead to inhibition of insulin secretion from the pancreatic β-cells. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan reversed the altered brain 5-HT1A and 5-HT2A receptors by increasing the brain 5-HT content. But these treatments were not able to up-regulate the nuclear 5-HT binding protein.

Thus we conclude that serotonin can regulate insulin secretion through increased 5-HT2A receptor gene expression, up-regulation of 5-HT1A receptors in brain regions and down-regulation of nuclear 5-HT binding bHLH2 protein in pancreatic islets. Manipulation of these receptor gene expressions at the molecular level can have a clinical significance in the control of diabetes mellitus.
5.4. SUMMARY

1. Streptozotocin induced diabetic rats were used as model to study the role of 5-HT and its receptors in insulin regulation.

2. The 5-HT content is decreased in cerebral cortex, brain stem and hypothalamus of diabetic rats. This is due to decrease in uptake of L-tryptophan through the blood-brain-barrier during diabetes.

3. RT-PCR studies confirmed an increase in expression of brain stem 5-HT2A receptor and an increase in its affinity in cerebral cortex and hypothalamus. The high affinity 5-HT1A receptors in brain stem was up-regulated and the low affinity 5-HT1A receptors of cerebral cortex and hypothalamus showed an increase in affinity for 5-HT.

4. The increased function of the brain 5-HT1A and 5-HT2A receptors will lead to inhibition of insulin secretion by increasing the sympathetic activity. An increase in sympathetic activity will decrease insulin secretion from the pancreatic islets.

5. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan reversed the altered 5-HT1A and 5-HT2A receptor binding parameters in cerebral cortex, brain stem. The 5-HT2A receptor binding parameters were reversed by these treatments in hypothalamus but 5-HT1A receptors did not reverse to the control state.

6. In vitro incubation of pancreatic islets with [3H]5-HT and increased concentration of glucose showed a glucose mediated 5-HT uptake into the islets.

7. Glucose mediated insulin secretion was inhibited in islets incubated with high glucose and 5-HT concentrations.

8. During hyperglycaemic state the increased 5-HT in the islets can bind and down-regulate a novel 14-kDa nuclear 5-HT specific binding protein. Based on amino acid
composition homology, this 14-kDa protein was identified as a basic helix-loop-helix (bHLH2) transcription factor that acts as an enhancer of insulin gene promoter.

9. Down-regulation of the bHLH2 protein during diabetes by 5-HT will inhibit insulin gene transcription, since this protein acts as an enhancer of insulin gene promoter.