The Mechanism of HCO_3^--Induced Insulin Secretion in Pancreatic β-cells and the Involvement in Synaptic Plasticity

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Declaration

This is to declare that the work presented in this thesis is my own and has not been submitted to this or any other institution for any degree, diploma or other qualification.

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Abstract

Cystic fibrosis (CF), which is caused by the deficiency of cystic fibrosis transmembrne conductance regulator (CFTR), is the most common autosomal recessive systemic disease with an incidence of 1: 2500 in Caucasians. Cystic fibrosis-related diabetes (CFRD), as one of the complications of CF patients, is regarded as one of the leading co-morbidity in CF patients. The mechanism of CFRD is attributed to the reduced number of islets due to pancreatic fibrosis caused by the loss of CFTR in pancreatic duct. However, the above mechanism failed to explain the dynamics of insulin secretion induced by glucose tolerance test (GTT) in some CF patients and therefore, we were forced to re-consider the mechanism for the pathogenesis of CFRD. Interestingly, the following facts imply that perhaps there is another mechanism for the onset of CFRD: decreased insulin secretion and decreased plasma HCO_3^- concentration was observed in the metabolic acidosis disease, plasma HCO_3^- level increased accompanied by the elevation of plasma insulin after food intake and CFTR accounted for HCO_3^- transport in many epithelial cells. These facts promoted us to hypothesize that the loss of HCO_3^--induced insulin secretion resulting from the deficiency of CFTR is an alternative mechanism for the onset of CFRD. Our results showed that HCO_3^- could induce insulin secretion of isolated islets from rats. Ca^{2+} imaging revealed that HCO_3^- dose-dependently induced an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in RIN-5F cells, an insulin-secreting cell line. Removal of extracellular Ca^{2+} or addition of nifedipine, the blocker of L-type Ca^{2+} channel, decreased the effect of HCO_3^- significantly, indicating the activation of L-type Ca^{2+} channel during HCO_3^- stimulation. The inhibitory effect of $BaCl_2$ implied the involvement of K^+ channel. The results that HCO_3^--induced increase in $[Ca^{2+}]i$ was reduced by PKA inhibitor and sAC blocker demonstrated that the pathway of sAC-cAMP-PKA-ATP-sentitive K^+ channel (K_{ATP} channel) was responsible for the effect of HCO_3^-. The reduction of extracellular Cl^- or the inhibitor of anion exchanger (AE) inhibited the $[Ca^{2+}]i$ increase induced by HCO_3^- significantly but the omission of external Na^+ failed. The facts that CFTR blocker decreased the effect of HCO_3^- markedly and the expression of CFTR in RIN-5F cells revealed

by western blotting suggested the CFTR-mediated HCO_3^- transport. These results suggested that HCO_3^- could induce insulin secretion in a CFTR-dependent manner, which provided a new insight into the understanding of pathogenesis of CFRD and paved the way for the therapy of CFRD.

Apart from CFRD, low cognitive skill index (CSI) was also found in CF patients and was attributed the lacking of vitamin E. Since it is known that insulin plays a role in the learning and memory, decreased plasma insulin level in CF patients is an alternative mechanism for impaired cognitive function. Although numerous studies have found that insulin can improve learning and memory, the mechanism of it is not well understood. In this study, we investigated the effect of insulin on the expression of hippocampal early-phase long-term potentiation (E-LTP) in the immature rats. Hippocampal brain slices were acutely prepared from 10-12 days and 2 months old rats and field excitatory postsynaptic potentials (fEPSCs) were recorded from CA1 region by a multi-electrode in vitro recording system. In the control group, the hippocampal slices of neonatal rats showed no increase in the magnitude of fEPSC after conventional high frequency stimulation (HFS). After pretreatment of the slices with 0.08ng/ml insulin for over one hour, there was no significant change in the magnitude of E-LTP. However，when the insulin concentration increased to 0.8ng/ml, a significant increase in the magnitude of E-LTP was observed. On the contrary, any doses of insulin failed to affect the magnitude of E-LTP of mature rats. These results suggested that insulin could dose-dependently facilitate the production of E-LTP in the hippocampus of infant rats. Application of AG-1024, an inhibitor of insulin receptor, largely abolished the insulin-dependent E-LTP in immature rats rather than adult rats, indicating the involvement of insulin signaling pathway in the insulin effect. On the other hand, increasing the concentration of glucose from 11 mM to 22 or 33 mM did not facilitate the E-LTP and application of indinavir, a blocker of insulin-sensitive glucose transporter-4, did not inhibit the effect of insulin. Therefore, it is unlikely that the facilitory action of insulin on E-LTP is via an indirect effect on glucose homeostasis or utilization. Pretreatment with the MAPK pathway inhibitor PD98059 blocked insulin-mediated E-LTP facilitation. Furthermore, the tetanic stimulation induced a

significant increase in the level of phosphorylated p42MAPK in the insulin-treated hippocampus than that in the control group. In conclusion, our results suggested that insulin could facilitate the production of hippocampal E-LTP in infant rats, which may play an important role in modulating the expression of LTP in the developing brain and perhaps is an underlying mechanism for the improving effect of insulin on learning and memory. Since insulin plays an important role in the developing brain, perhaps the deficiency of insulin effect resulted from CF patients induces the impairment of cognitive function.

摘要

囊性纖維變性是最常見的常染色體隱性遺傳疾病，由一種陰離子通道—囊性纖維變性跨膜電導調節器（CFTR）的缺陷所引起。在白色人種當中，其發病率達到 1:2500。囊性纖維變性糖尿病（CFRD）是其主要的并發癥之一。目前認為，囊性纖維變性患者由於胰腺導管上皮細胞缺乏 CFTR 通道，造成胰液的水分分泌減少，引起胰腺導管的阻塞，最終導致胰島數量減少，是囊性纖維變性糖尿病主要的發病機理。然而，這一理論并不能夠完整解釋某些 CFRD 患者胰島素分泌的特徵。有研究發現了以下事實：代謝性酸中毒患者的胰島素分泌降低，而該類患者的血漿中的 HCO_3^-含量降低；進食以後，血漿中的胰島素和 HCO_3^-含量均有所增加；在許多上皮組織中，離子通道 CFTR 參與了 HCO_3^-的轉運。綜合以上三點，我們推測，血漿當中的 HCO_3^-有可能促進胰島素的分泌，這一過程需要 CFTR 的參與。而 CFTR 的缺陷導致這一機制的缺失，最終導致囊性纖維變性糖尿病的發生。爲了驗證這一假設，我們研究了對胰島素分泌的影響，並且探索了其內在機制。

離體大鼠胰島灌流實驗表明，HCO_3^-可以引起胰島素的分泌。鈣成像技術發現，HCO_3^-能夠促進胰島 β 細胞系-RIN-5F 內的 Ca^{2+}濃度增加，而且這一效應呈現出劑量依賴性。去除細胞外液的 Ca^{2+}或者加入 L 型鈣通道阻斷劑均明顯降低這一效應。鉀通道阻斷劑的應用完全阻斷了 HCO_3^-的作用，而且 PKA 的抑製劑和 sAC 阻斷劑均可以減弱 HCO_3^-所引起的細胞內 Ca^{2+}增加。以上結果表明有可能是 sAC-cAMP-PKA-K_{ATP}通道的依次激活是 HCO_3^-的作用機制。

去除細胞外液中的 Cl^-或者 AE 阻斷劑的應用均明顯降低了 HCO_3^-的作用，但是去除細胞外的 Na^+則相反。CFTR 抑製劑也表現出明顯的阻斷作用，我們還發現 RIN-5F 細胞明顯表達 CFTR 蛋白。以上結果表明 HCO_3^-可以促進胰島素的分泌，而這一結果需要 CFTR 的參與。我們的發現可能提供了一種新穎的 CFRD 發病機制，為 CFRD 的治療提供了新方案。

有研究表明，某些 CF 患者表現出降低的認知技能指數，這一缺陷并不認為與

CFTR 的直接缺陷相關，而是與維他命 E 的缺乏有關。但是大量研究已經表明，胰島素與學習和記憶功能有著密切的聯繫，而且 CF 患者血漿當中的胰島素濃度明顯降低。因此，CF 患者的低胰島素分泌是造成學習功能下降的可能原因。儘管大量的研究已經證明胰島素可以提高學習和記憶功能，但是其內在機制并不是很清楚。在我們的研究中，我們觀察了胰島素對海馬 CA1 區域 E-LTP 的影響。

在 11-12 天大鼠，海馬腦片并不能夠誘導出 E-LTP，但是經 0.8ng/ml 的胰島素作用 1 小時后，E-LTP 被誘導出來。0.08ng/ml 的胰島素卻無此作用，表明了該作用是劑量依賴性的。在成年組大鼠，以上兩種劑量的胰島素均不能夠改變 E-LTP 的幅度。

胰島素受體的阻斷劑可以明顯阻斷胰島素的作用而不能夠影響正常成年大鼠的 E-LTP，證明了胰島素信號通路的參與。阻斷葡萄糖轉運體-4 的作用并不能夠阻斷胰島素的作用，而且增加細胞外的葡萄糖濃度也不能夠模仿胰島素的這一作用，表明胰島素的這一作用并不是通過改變腦組織的糖代謝來實現的。MAPK 通路阻斷劑的應用阻斷了胰島素的作用，而且在 E-LTP 誘導后，胰島素組海馬腦片的 p42MAPK 表達高於對照組。我們的結果表明胰島素可以易化幼年大鼠 E-LTP 的產生，而這一機制的缺陷有可能是 CF 患者學習功能低下的誘因。

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is the most common autosomal recessive systemic disease, with an incidence of 1 in 2500 live births in Caucasian population. CF results from defective production of a protein called cystic fibrosis transmembrane conductance regulator (CFTR) that functions as a cAMP-regulated Cl^- channel and plays a significant role in the transport of Cl^- and HCO_3^- in different types of epithelial cells (Morales et al., 1999; Garcia et al., 2009).

The gene that encodes CFTR protein is located on the long arm of human chromosome 7 at the position of q31.2 (Proesmans et al., 2008). More than 1500 mutations about CFTR have been identified, but the number of true disease-causing mutations may be much lower. The most common mutation of CFTR is Delta-F508 mutation, which is caused by deletion of 3 base-pair (CTT) in exon 10 of the CFTR gene and accounts for 70% of all CFTR mutations, while 10–20 less common mutations account for a further 10–15% of all mutant alleles (Garcia et al., 2009; Radpour et al., 2008).

CFTR is a member of the ATP-binding cassette (ABC) transporter super family that includes over thirty proteins and functions as a cAMP-mediated Cl^- channel with a conductance of 7-10pS. CFTR is a glycoprotein including 1480 amino acids and contains five domains: two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs) and a unique regulatory domain (RD). Each MSD is composed of six transmembrane segments which are connected by intra- and extracellular loops. Two MSDs gather to form a low conductance anion-selective pore. Each NBD includes conserved amino acid sequences that can interact with ATP. The two NBDs form a dimer by the head-to-tail connection. There are two ATP-binding sites located at the dimer interface. Site 1 is formed by the Walker A and B motifs of NBD1 and the LSGGQ motif of NBD2 and site 2 is formed by the Walker A and B motifs of NBD2 and the LSGGQ motif of NBD1. Anion transport through the

CFTR channel is controlled by the interaction of ATP with sites 1 and 2, which induces NBD dimerization and subsequent conformational changes in the MSDs. RD contains many charged amino acids and is distinguished by its multiple consensus phosphorylation sites, which is regulated by cAMP-dependent protein kinase (PKA). Phosphorylation of the RD by the PKA is essential for channel opening (Fig 1.1) (Jiang et al., 1998; Childers et al., 2007; Hwang et al., 2009).

CFTR is mainly expressed in epithelial cells which are widely distributed in the body. In epithelia cells, CFTR plays a fundamental role in salt and water movement across epithelial cells by mediating the transport of Cl^- and HCO_3^-. The expression of CFTR in non-epithelial tissues is also observed, including cardiac myocytes (Nagel et al., 1992), smooth muscular cells (Robert et al., 2005), endothelial cells (Tousson et al., 1998), erythrocytes (Sprague et al., 1998) and all types of cells in pancreatic islet (Boom et al., 2007).

Owing to the wide expression of CFTR protein, patients with CF express a variable spectrum of clinical manifestations including chronic respiratory symptoms, pancreatic insufficiency and malnutrition, bile duct obstruction, male and female infertility, high level of Cl^- in sweat and intestinal obstruction (Chan et al., 2006; Rowntree et al., 2003).

The median life expectancy of CF patients has improved in the past several decades. In the early 1940s the life expectancy was less than 1 year and a child with CF had a 20% chance of surviving over 1 year (Anderson et al., 1967). 30 years later the life expectancy increased to 16 years and in 2008 a median survival of 37.4 years was reported (Orenstein et al., 2002; Stalvey et al., 2009). In the early years, the leading co-morbidity in CF patients was pulmonary manifestations which expressed chronic inflammation and infection of the airways and then showed episodic exacerbations and tissue destruction resulting from the inflammatory response (Chmiel et al., 2002). As the age of CF patients increases, the new complications associated with aging are emerging. Recently, cystic fibrosis-related diabetes (CFRD) is regarded as one main complication of in CF patients that causes death of subjects (Costa et al., 2005).

1.2 Cystic fibrosis-related diabetes mellitus (CFRD)

1.2.1 Epidemiology of CFRD

Diabetes in CF patients was described for the first time in 1955 (Shwachman et al., 1955). 7 years later 10 patients with diabetes mellitus (DM) were reported from 1300 CF subjects and CFRD was established as a complication of CF patients from then on (Rosan et al., 1962). With the increase in life expectancy of CF patients, the prevalence of CFRD increased as well. The prevalence of CFRD was 3-10% in 1969 and then increased to 14-30% thirty years later (Milner et al., 1969; Handwerger et al., 1969; Yung et al., 1999; Charlton et al., 2001). The latest report about the prevalence of CFRD was about 20.6% in 2007 (Stalvey et al., 2009). The progressive increase in prevalence of CFRD is attributed to the dependence of CFRD onset on age. The prevalence of CFRD is 9%, 26% and 50% at the age of 5 to 9 years, 10 to 20 years and by the age of 30 years respectively (Moran et al., 1998; Lanng et al., 1993a).

CFRD is characterized by chronic hyperglycaemia resulting from the decreased insulin secretion and has some characteristics of both Type 1 and Type 2 diabetes mellitus (DM) but is also distinct from them (Moran et al., 2002). The glucose metabolism in CF patients is also affected by many factors including malnutrition, chronic infection, elevated energy expenditure, glucagon deficiency, abnormal gastrointestinal transition and liver dysfunction (Zirbes et al., 2009).

1.2.2 Pathogenesis of CFRD

1.2.2.1 β-cells function in CFRD patients

Lanng et al (1993b) investigated the insulin secretion of β-cells response to oral glucose test in 30 CF patients including CFRD patients, CF patients with impaired glucose tolerance (IGT) and CF patients with normal glucose tolerance (NGT). This study showed that there were no differences in fasting plasma insulin concentration between CF subjects and healthy controls, suggesting that the basal insulin secretion was not impaired in CFRD patients. However, after oral glucose administration,

the insulin secretion in CFRD patients decreased compared to healthy subjects. Interestingly, although CF with NGT did not express any manifestations of DM, the insulin secretion after glucose challenge decreased compared to healthy controls. Cucinotta's study observed that CF patients with and without DM expressed decreased insulin secretion response to intravenous glucose administration compared with healthy controls, which is consistent with the above study (Cucinotta et al., et al., 1994a). However, this study also found that the basal insulin secretion in CF patients was significantly lower than that of controls, which is opposite to the above. In general, the impairment of insulin secretion response to glucose in CF patients is confirmed but whether the basal insulin secretion is altered is uncertain.

Another characteristic of insulin secretion in CFRD patients is the delayed response to glucose. Moran et al (1991) observed that after intravenous administration of glucose, the time to peak insulin secretion was 20-60 minutes in healthy controls, 30-120 minutes in CF patients with NGT, 60-120 min in the CF patients with IGT and 150 min for CFRD patients. Lanng's study indicated that peak insulin secretion occurred at about 120 minutes in the CFRD patients, 60 minutes in healthy controls (Lanng et al., 1993b). Both studies demonstrated that the insulin secretion response to glucose decreased progressively with increasing IGT.

The deficiency of insulin secretion in CFRD patients is also highlighted by the loss of first phase insulin secretion. After oral glucose administration, CFRD patients showed a significantly decrease in first phase insulin secretion in comparison to CF patients with NGT. However, there were no differences in the second phase insulin secretion between the two groups (Mohan et al., 2009; DeSchepper et al., 1992; Rakotoambinina et al., 1994).

1.2.2.2 Pancreatic insufficiency

The secretion of HCO_3^- from pancreatic ductal cells is a CFTR-mediated active process which leads to the much higher HCO_3^- concentration in pancreatic juice than that in plasma and tissue fluid.

The high osmolarity in pancreatic juice resulting from high level of HCO_3^- promotes the passive secretion of water into the pancreatic duct from tissue fluid via the tight junction between ductal cells (Whitcomb et al., 2004). The deficiency of CFTR in pancreatic duct causes of HCO_3^- secretion failure and subsequent decreased water secretion (Freedman et al., 2001). Because the secretion of pancreatic enzymes is not affected by the loss of CFTR, the concentrated pancreatic juice as a result of decreased water secretion is believed to cause the obstruction of pancreatic duct and subsequent dysfunction of β-cells in islets.

In an autopsy study on CF patients, the pancreas of CFRD patients developed fat replacement of exocrine pancreas, reduced number of islets, fibrosis of islets, amyloid deposits in islets, reduced number of insulin-secreting cells in islets, islet cells atrophy and the absence of nesidioblastosis. In contrast, the pancreas from CF patients without DM expressed obvious fibrocystic alteration of the pancreas, islets without morphological changes and distinguished nesidioblastosis, suggesting that the reduced number of islets was responsible for the development of diabetes (Iannucci et al., 1984). Decreased number of β-cells in islets in CFRD patients was also reported by other separate studies (Abdul-Karim et al., 1986; Lohr et al., 1989). Interestingly, although both CFRD patients and CF patients without DM showed morphological changes in the pancreas, the islets in CF patients without DM were intact. The possible reason for this phenomenon is that the time of the developing pancreatitis is not long enough to impair islets. In fact, the pancreas CF patients without DM showed nesidioblastosis, indicating the compensation for the dysfunction of β-cells, which was consistent with the fact that even CF patients with NGT had decreased insulin secretion.

1.2.2.3 Islet amyloidosis

Islet amyloid polypeptide (IAPP) is a 37 amino acids-containing peptide and is co-localized and co-released with insulin. In human, IAPP forms fibrils which are insoluble in water by refolding to a β-sheet conformation and oligomerises. Because IAPP was found in 90% of Type 2 diabetic subjects

by the autopsy study, the deposition of amyloid in pancreatic islets is considered one of the most common pathological characteristics of Type 2 DM. Synthetic IAPP-formed fibrils shows toxic action on islets, including insertion into the lipid bilayer of cell membrane, changes in activity of ion channel on cell membrane, inducing apoptosis (Clark et al., 2004).

An autopsy study of 41 cases of CF patients showed that islet amyloid was observed in 69% subjects of CF with DM by light microscopy, 17% of subjects of CF with borderline DM and none of CF with DM, suggesting that islet amyloidosis resulting from islet amyloid polypeptide is a distinguished feature of CFRD patients (Couce et al., 1996). However, whether amyloid in islets contributes to the dysfunction of β-cells in CFRD patients is not investigated.

1.2.2.4 Insulin resistance

Andersen's study also showed an increase in ratio of insulin vs glucose in CF patients compared to healthy controls, suggesting insulin resistance in CF patients (Andersen et al., 1988). However, Mohan's study indicated the increased insulin sensitivity in CF patients (Mohan et al., 1985). These conflicting results are attributed to the changes of insulin sensitivity with the development of CFRD.

In Moran's study, CF patients were divided into three groups: CF patients with no exocrine insufficiency (NEXO), CF patients with overt diabetes (EXO-IT) and CF patients with impaired insulin secretion but with normoglycemia (EXO). Subjects in NEXO group expressed normal insulin sensitivity in peripheral and hepatic tissues. EXO individuals showed increased insulin sensitivity in peripheral tissues but decreased hepatic insulin sensitivity paradoxically, demonstrating the hepatic insulin resistance. EXO-IT subjects expressed decreased peripheral and hepatic insulin sensitivity (Moran et al., 1994). This study showed that the CFRD patients had insulin resistance at the late stage of diabetes, which may be a mechanism underlying the development of CFRD. Interestingly, CF patients with impaired insulin secretion and normoglycemia showed increased insulin sensitivity rather than insulin resistance. The possible reason is that the increased insulin sensitivity at the early stage of

CFRD is a compensation for the decreased insulin secretion in CF patients. Conflicting results were obtained from a separate study. After oral glucose test, Cucinotta's study showed that there were no significant differences in insulin sensitivity among CF patients with NGT, CF patients with IGT, CFRD patients and healthy controls. Even after a 4-year follow-up of 10 patients, although insulin responses to oral glucose decreased obviously, insulin sensitivity did not change significantly, suggesting that there was no alteration of insulin sensitivity during the development of CFRD (Cucinotta et al., 1994b).

1.2.2.5 Increased susceptibility of islet to injury

Stalvey et al (2006) investigated the effect of streptozotocin, a drug which induces injure of the pancreas and is usually used to induce Type 1 DM animal model, on islet and glucose metabolism in CFTR-/- mice and normal controls. Before intraperitoneal injection of streptozotocin, there were no differences in blood glucose levels under the baseline and glucose challenge. After administration of streptozotocin, $CFTR^{-/-}$ mice expressed much higher blood glucose level than that in controls. Since there was no difference in the number of islets left in the pancreas between the two groups, it seemed that CFTR exacerbated the dysfunction of injured islet.

1.2.2.6 Autoimmunity of islets

Using ELISA technique, Nousia-Arvanitakis et al (2000) found that islet autoantibodies were detected in 40% of 30 CF patients, which was much higher than that of healthy controls. Most of the CF patients with islet antibodies developed impaired first insulin secretion after glucose challenge, suggesting that autoimmunity might be a possible mechanism for the onset of CFRD. However, another study did not detect islet autoantibodies in CFRD patients and CF patients without DM (Minicucci et al., 2005). Thus, the role of autoimmunity in CFRD is inconclusive.

1.2.2.7 Apoptosis of β-cells in islet induced by endoplasmic reticulum (ER) stress

Endoplasmic reticulum (ER) stress is a phenomenon that occurs when excessive malfolding protein accumulates in the ER during biosynthesis. ER stress is normally a protective response and an adaptive pathway which is in favor of the survival of cells. However, long-lasting accumulation of proteins misfolding in the ER is toxic to the cells and caused the apoptosis of cells finally. Although there are not any substantial evidences about the role of ER stress in the onset of CFRD, the ER stress in the islet β-cells of CFRD patients is speculated from the following studies.

The high susceptibility of islet β-cells to ER stress has been demonstrated. It was shown that the mice with mutation in the insulin 2 gene developed hyperglycemia and this mutation resulted in the accumulation of mutated pro-insulin in the ER of β-cells, demonstrating the activated ER stress in islet β-cells and the relationship between ER stress and DM (Wang et al., 1999). It is hypothesized that the DeltaF508 mutation, which is the most common mutation of CF patients, caused the misfolding of CFTR protein, subsequent production of ER stress and final apoptosis of islet β-cells as a result of excessive malfolded CFTR in ER. In a study on transfected human epithelial cells expressing the DeltaF508-CFTR protein, the activation of UPR transducer ATF6 and ER stress sensor Grp78, both of which are ER stress markers was detected, indicating the DeltaF508-CFTR protein-induced ER stress (Kerbiriou et al., 2007).

1.3 Cognitive functions of CF patients

In a study on the cognitive function of CF patients, CF subjects were divided into 2 groups: screening (S) and control (C) in accordance to if patients were treated by Wisconsin CF Neonatal Screening Project (WCNSP), which means the diagnosis of CF in time and better nutrition for CF patients. Each group was divided into two subgroups according to their vitamin level (<300E and > 300E). This study showed that patients in C/<300E subgroup expressed significantly lower cognitive skills index (CSI) compared with other subgroups (Koscik et al., 2005). Because vitamin E can

improve learning performance (Comin et al., 2010), the impaired cognitive function in CF patients was attributed to the decreased level of vitamin E. However, in S group, there was no difference in cognitive function between <300E and >300E subgroups. The possibility is the better nutrition in S group than that in control group as a result of WCFSP. Therefore, although impaired cognitive function is observed in some CF patients, it could be due to malnutrition resulted from pancreatic insufficiency rather than the direct effect of CFTR loss.

1.4 Insulin secretion induced by glucose

1.4.1 Introduction of insulin

Insulin is well known for its main function to regulate energy and glucose metabolism in the body. Insulin is synthesized from proinsulin produced by β-cells of islets in the pancreas. After synthesis in the ER, proinsulin is transferred to the Golgi apparatus and then is cleaved by the action of proteolytic enzymes to yield insulin and a connecting peptide fragment referred to as C-peptide. Insulin is a globular protein which is composed of two peptide chains: A and B. A and B chains are connected together by two disulfide bonds and there is an additional disulfide bond within the A chain (Figure1.2). There are many physiological factors that promote the secretion of insulin in the body and glucose is the most important and common factor among them.

1.4.2 Biphasic insulin secretion induced by glucose

After stimulation of a sudden and long lasting increase in the glucose concentration which is called "square wave" stimulation, the increased insulin secretion expressed a biphasic model which has been reported for the first time by Curry in 1968 and subsequently observed by many other studies (Iversen et al., 1971; Gerich et al., 1974). The insulin secretion increases quickly to the first peak response in 10 minutes after glucose challenge which is called the first phase followed by a slow down and then increases again at a slow rate or keeps at a sustained plateau depending on species. The

biphasic model of insulin secretion was found using isolated and perfused rat pancreas in vitro (Figure1.3) (Curry et al., 1968), and then in perfused rat islets (Lacy et al., 1972). In normal human subjects, increase in plasma insulin concentration after a challenge of quick and sustained rise in plasma glucose level also expressed a similar biphasic pattern (Vila et al., 2010; Chepurny et al., 2010; Boston et al., 2009).

1.4.3 The physiological significance of biphasic insulin secretion

It has been thought that the very short first phase insulin secretion has no physiological advantages in normal subjects because of the two following reasons. First, the first phase insulin secretion response takes place only after promptly increasing extracellular glucose concentration from a substimulatory to a sustained stimulatory level. In contrast, after increasing the extracellular glucose concentration slowly, the first phase insulin secretion response disappears and the glucose-induced insulin secretion increases progressively (Curry et al., 1968). Second, the fasting plasma glucose level in normal subjects is about 5mM and even after a rich meal the plasma glucose concentration rises to only about 8mM. A very quick elevation in glucose concentration from 3 to 16.7mM that often happens in vitro never occurs physiologically in normal subject. This point has been argued by many studies. Although the first phase of insulin secretion lasts for a very short time, the quick insulin secretion in response to elevation in glucose after meal can decrease the plasma glucose concentration promptly to prevent the impairment of tissues induced by high glucose concentration. It has been shown that patients with IGT or in the early stage of type 2 diabetes were characterized by the absence of first phase insulin secretion without any decrease of the second phase insulin secretion after intravenous glucose load (Rave et al., 2010; Salinari et al., 2009; Emerson et al., 2009), indicating the physiological significance of the first phase insulin secretion. In fact, the about 8mM plasma glucose concentration after a meal is the result of the first phase insulin secretion. Actually, the absence of first phase insulin secretion would result in much higher plasma glucose concentration after a meal

(Yamanouchi et al., 2001). In agreement with this point, Mitrakou's study showed that there was an inverse correlation between the plasma glucose concentration during the second hour and the plasma insulin level 30 minutes after oral glucose test, indicating that the first phase insulin secretion significantly affected subsequent plasma glucose level to keep physiological plasma glucose levels (Mitrakou et al., 1992). In the investigation of relationship between the first phase insulin release and initial plasma glucose increase after glucose challenge, Bruce et al (1987) found that the first phase insulin secretion could inhibit the elevation in plasma glucose concentration induced by glucose challenge. On the other hand, the same study also showed that the greater the insulin release in the first phase, the more lasting the restraining effect on glucose homeostasis. Similarly, in the study of the effect of the first phase insulin secretion on hepatic glucose production, Luzi et al (1987) devided normal human subjects receiving hyperglycemic clamp studies into three groups: group 1, 150 min hyperglycemic clamp; group 2, hyperglycemic clamp with somatostatin and glucagon plus the insulin infusion with the secretion model similar to the second phase of insulin secretion but not to the first phase secretion; group 3, hyperglycemic clamp with somatostatin and glucagon plus the insulin infusion with the secretion model similar to biphasic insulin secretion. The insulin secretion in group 1 expressed normal biphasic secretion and the basal hepatic glucose production was inhibited by about 90% 20 minutes after hyperglycemic clamp and remained suppressed during hyperglycemic clamp. The similar results about basal hepatic glucose production were also found in group 3. Interestingly, in group 2, the basal hepatic glucose production was suppressed by only 50% 60 minutes after hyperglycemic clamp. In group 2, the first phase insulin secretion was suppressed by somatostatin and only the second phase insulin secretion left because of the insulin infusion (Luzi et al., 1989). Therefore, the basal hepatic glucose production could only be inhibited effectively at the presence of the first phase insulin secretion. However, the above studies only partly explain the physiological importance of the first phase insulin secretion and the full understanding of physiological advantages requires further study.

Obviously, the insulin secretion during the second phase is characterized by the gradual increase in insulin secretion as a result of glucose challenge, which results in the decrease in plasma glucose concentration after a meal. Since the second phase insulin secretion sustains for a much longer time than the first phase insulin secretion, the effect of insulin on glucose homeostasis during this phase lasts for a longer time, which is in favor of regulation of blood concentration after food intake. Therefore, the absence of second insulin secretion will lead to much higher plasma glucose level after a meal.

1.4.4 The mechanism underlying the glucose-induced insulin secretion

In Shigeto's study on MIN6 cell line, an insulin-secreting cell line, it has been demonstrated that the application of diazoxide, an opener for ATP-sensitive K^+ channel (K_{ATP} channel), blocked the first phase insulin secretion completely but did not affect the second phase insulin secretion in response to glucose challenge (Shigeto et al., 2006), suggesting the involvement of K_{ATP} -dependent pathway in the first phase insulin secretion and the involvement of K_{ATP} -independent pathway in the second phase insulin secretion.

1.4.4.1 K_{ATP}-dependent pathway

How the elevated glucose concentration promotes the insulin secretion during the first phase has been well established by many studies. In the absence of stimulatory concentrations of glucose, the rate of glycolysis in pancreatic β-cells is not very high and the relatively low ratio of ATP:ADP ensures that enough $K\text{-}_{ATP}$ channels are open in the plasma membrane and the subsequent efflux of K^+ promoted by the higher intracellular K^+ concentration through $K\text{-}_{ATP}$ channels produces resting membrane potential which is negative inside the β-cells. The voltage-dependent L-type Ca^{2+} channel are inactivated by the resting membrane potentials and the influx of Ca^{2+} is minimal, resulting in a low intracellular Ca^{2+} ($[Ca^{2+}]_i$) level and keeping the insulin secretion at a basal level.

When β-cells are treated whith much higher level of glucose, the glucose metabolism in β-cells accelerates and leads to an increase in the ratio of ATP: ADP, which induces the closure of K_{ATP} channels (Tarasov et al., 2004; Detimary et al., 1996) and in turn leads to the depolarization of membrane potential. The change in membrane potential of β-cells induces the activation the voltage-dependent L-type channel (Henquin et al., 1988) and subsequent exocytosis of insulin-containing granules as a result of the increase in $[Ca^{2+}]_i$ concentration (Lang et al., 1999; Lovis et al., 2008) (Figure1.4). Due to the involvement of K_{ATP} channels, the above processes is called K_{ATP}-dependent insulin secretion pathway.

1.4.4.1.1 VDCC in K_{ATP}-dependent pathway

The expression of several voltage-dependent Ca^{2+} channels (VDCC) on islet β-cells has been demonstrated and their roles in the insulin secretion are investigated. VDCC are classified according to their electrophysiological and pharmacological characteristics. VDCC includes high-voltage activated (HVA) and low-voltage-activated (LVA). HVA channel includes L-, N-, P-/Q-, and R-types Ca^{2+} channels, and LVA channel is designated as T-type Ca^{2+} channel (Moosmang et al., 2007).

Although the expression of R-type Ca^{2+} channel on β-cells was observed, its role in the insulin secretion remains controversial. Pereverzev' s study found that intraperitoneal addition of glucose to Ca(v)2.3-/- mice expressed increased blood glucose concentration and decreased insulin release into plasma. After glucose challenge, the insulin secretion of isolated islets from Ca(v)2.3-/- mice was not induced any more (Pereverzev et al., 2002). Vajna et al (2001) found that the addition of SNX-482，a R-type Ca^{2+} channel antagonist, slightly inhibited glucose-induced insulin secretion and reduced the non-L and non-N calcium type Ca^{2+} current in INS-1 cells. In agreement with these results, Schulla's study indicated that the application of SNX-482 decreased the voltage-dependent Ca^{2+} current by 25% (Schulla et al., 2003). The above studies suggested that R-type Ca^{2+} channel was responsible for the glucose-induced insulin secretion. Another study found that there was a higher glucose increase in

Ca(v)2.3-/- mice than that in wild type mice after glucose challenge. However, the impaired glucose tolerance was attributed to a reduction in insulin sensitivity rather than deficiency in insulin secretion (Matsuda et al., 2001). Therefore, whether R-type Ca^{2+} channel is involved in the glucose-induced insulin secretion remains to be determined by further study.

Takahashi et al found that the Ca(v)2.2-/- mice expressed a lower plasma glucose concentration and a similar pattern of insulin levels to those of wild type mice after glucose tolerance tests. Therefore, although the expression of N-type Ca^{2+} channel in the insulin-releasing cells has been observed (Takahashi et al., 2005), it seems that N-type Ca^{2+} channel doesn't play an important role in the glucose-induced insulin secretion. Satin's study showed that application of omega-conotoxin, the blocker of N-type Ca^{2+} channel, decreased peak Ca^{2+} current induced by stimulus by about 35% but couldn't block glucose-induced insulin secretion, suggesting that a N-type Ca^{2+} channel can be present without playing a role in insulin secretion (Satin et al., 1995).

Ligon et al found that the application of omega-agatoxin IVA, the blocker of P/Q type Ca^{2+} channel, inhibited the Ca^{2+} current induced by glucose, indicating that the involvement of P/Q type Ca^{2+} channel in the glucose-induced insulin secretion (Ligon et al., 1998). Another independent study found that the glucose-induced elevation of $[Ca^{2+}]_i$ in INS-1 cells, a kind of insulin-secreting cell line, was attributed to L-type Ca^{2+} channels completely, although the existence of P/Q type Ca^{2+} currents in these cells was observed (Horvath et al., 1998).

Although there are numerous studies showing the possibility that different kinds of Ca^{2+} channels are involved in the glucose-induced insulin secretion, the L-type Ca^{2+} channel is regarded as the main Ca^{2+} channel responsible for the Ca^{2+} influx during glucose stimulation according to the role of L-type Ca^{2+} channel in the insulin secretion, electrical activity and $[Ca^{2+}]_i$ oscillations (Ashcroft et al., 1994 and 1989; Mears et al., 2004; Henquin et al., 1984; Santos et al., 1991) .

1.4.4.1.2 K^+ channel in the K_{ATP}-dependent insulin secretion pathway.

The activity of L-type Ca^{2+} channel is regulated by the membrane potential which is controlled by K^+ channel. Under normal conditions, the outflow of K^+ through cation channel keeps the resting membrane potential at about -50mV (Henquin JC et al., 1982; Meissner HP et al., 1980) and in turn inactivates the L-type Ca^{2+} channel. Once stimulated by glucose, the K^+ channel is closed and then causes the depolarization of the membrane potential to about -40mV with subsequent activation of L-type Ca^{2+} channel (Henquin JC et al., 1982; Meissner HP et al., 1980). However, there are several types of K^+ channels located on the plasma membrane of β-cells, including K_{ATP} channel, a kind of voltage-dependent with large conductance (maxi-K(V) channel) and Ca^{2+}-dependent K^+ channel (Kca channel) (MacDonald et al., 2002).

It is well documented that K_{ATP} channel is a key protein during the glucose-induced insulin secretion by regulating the membrane potential of β-cells (Cook et al., 1989; Rorsman et al., 1989; Clark et al., 2010). The roles of maxi-K(V) and Kca channel are not very confirmed. Ribalet et al. found that the activity of maxi-K(V) channel was inhibited by 15mM glucose. The other insulin-secreting stimulus, mannose and 2-ketoisocaproate, which also initiate electrical activity in the islet β-cells, could decrease the activity of maxi-K(V) channel (Ribalet et al., 1988). However, whether maxi-K(V) channel is involved in the glucose-induced insulin secretion is unknown. Using RIN-5F and HIT insulin-secreting cells, Eddlestone et al found a kind of Ca^{2+}-activated K^+ channel (Kca channel) which could be blocked by glucose (Eddlestone et al et al., 1989). However, the role of Kca channel in insulin secretion was not investigated.

1.4.4.2 K_{ATP}-independent pathway

Although the role of K_{ATP}-dependent pathway in the glucose-induced insulin secretion has been manifested by many studies, numerous reports found that glucose could also elicit insulin secretion in the absence of K_{ATP} channels, demonstrating the involvement of K_{ATP}-independent pathway in the glucose-induced insulin secretion. Panten's study indicated that when the isolated pancreatic mice

islets were perfused with media containing the blockers of K_{ATP}-channels, the insulin secretion response to the increase of the glucose concentration from 10mM to 40mM expressed a transient decrease in insulin release followed by a sustained enhancement of insulin secretion (Panten et al., 1988). In Gembal's study, it is found that insulin secretion induced by high concentration of extracellular K^+ in the presence of diazoxide, the inhibitor of K_{ATP} channels closure, was dose-dependently enhanced by glucose challenge and even low level of glucose exerted an effective effect on it (Gembal et al., 1992). In agreement with the above studies, in the investigation of isolated islets, application of 16.7mM glucose combined with extracellular 50mM K^+ induces insulin secretion response which was much greater than that produced by 50mM K^+ alone and the glucose effect was not attenuated by diazoxide, suggesting that glucose still have the ability to increase insulin secretion under the conditions when K_{ATP} channels were invalid pharmacologically (Sato et al., 1992).

K_{ATP}-independent pathway is characterized by the amplifying effect of glucose on $[Ca^{2+}]_i$-induced insulin secretion and thus this pathway is also called amplifying pathway. In the study on mouse isolated islets, it is found that insulin secretion and $[Ca^{2+}]_i$ concentration increased with the step rise of glucose under the stimulation of 8mM glucose. However, $[Ca^{2+}]_i$ level reached saturation at the stimulation of 8mM glucose, the subsequent elevation of glucose concentration failed to increase $[Ca^{2+}]_i$ any more but could promote further insulin secretion (Heart et al., 2006). In another similar study, Henquin et al. divided the mouse islets into two groups: group1, challenged only with 15mM glucose; group 2: challenged by the maximally effective concentration of 500μM tolbutamide, the blocker of K^+ channels, in the presence of 1mM glucose. Although there was no difference in the magnitude of rise in $[Ca^{2+}]_i$ between the two groups, the insulin secretion response in group 1 is greater than that in group 2 during the first as well as the second phase (Henquin JC et al., 2009). The two separate studies revealed very interesting relationship between $[Ca^{2+}]_i$ and insulin secretion, i.e., increased insulin secretion without elevation in $[Ca^{2+}]_i$. From the above studies it can be seen that the inconsistence between the $[Ca^{2+}]_i$ level and insulin secretion occurred only in the presence of glucose

stimulation. Therefore, there must exist a pathway by which glucose amplifies the $[Ca^{2+}]_i$-induced insulin secretion, which is called the amplifying pathway. The mechanism of the amplifying pathway is unknown by now but perhaps is related to PKA and PKC.

In Komatsu's study, the glucose-induced insulin secretion on HIT-T15, a kind of insulin-secreting cell line, was abolished completely in the presence of nitrenpidine or severe Ca^{2+} deprivation. However, pretreatment of forskolin or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) increased the glucose-induced insulin secretion greatly in the presence of nitrenpidine or severe Ca^{2+} deprivation. Subsequent mixed pretreatment of pituitary adenylyl cyclase activating peptide (PACAP, PKC activator) and carbachol (PKA activator) showed the same results (Komatsu et al., 1996). Another study using rat islets also found that the augmentation effect of glucose on insulin secretion increased greatly when PKA and PKC were activated simultaneously (Komatsu et al., 1997). Therefore, it seems that PKA and PKC are involved in the augmentation effect of glucose on insulin secretion.

The feather of amplifying pathway plays a great role in the physiological regulation of glucose metabolism. Because there are many factors in the human body that can stimulate insulin secretion, including amino acids, fatty acids, acetylcholine, PACAP, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide 1(GLP-1) (Prentki et al., 1987; Sharp et al., 1979; Straub et al., 1996a). These factors enhance the glucose-induced insulin secretion by activating PKA and PKC to regulate the insulin secretion rapidly and accurately.

1.5 Insulin in the brain

It is well known that the main function of insulin is to regulate peripheral glucose homeostasis. Although the existence of insulin in the brain has been observed by many studies (Havrankova et al., 1978b; Baskin et al., 1988), there is no substantial evidence showing that insulin plays a physiological role in controlling glucose metabolism in the central nervous system (CNS). It is believed that insulin in the brain is derived from the transportation of plasma insulin across the blood-brain barrier (BBB)

and also via synthesis by neurons in the brain, as discussed in the following sections.

1.5.1 Insulin transport via blood-brain barrier (BBB)

Margolis' study found that the insulin level in cerebrospinal fluid (CSF) increased with elevation of peripheral plasma insulin concentration, suggesting a relationship between plasma and CSF insulin concentration (Margolis et al., 1967). Study by Banks indicated that human insulin in the mice CSF could be detected after infusion of human insulin into mice and the insulin concentration in CSF increased with the elevation of the dose of infused insulin (Banks et al., 1997a). As mice obviously can not produce human insulin, this study demonstrated conclusively that insulin can cross the BBB to enter the CSF.

The mechanism of insulin transport is a saturable process. It is demonstrated in another study by Bank et al (1997b) that the transport of human insulin radioactively labeled with iodine via BBB was inhibited by addition of human insulin or rat insulin, indicating that transport of insulin via BBB was characterized by the competitive inhibition. Although the transport of insulin via BBB is a carrier-mediated process, the transporter of insulin has not been determined up to date. The study by Miller et al (1994) suggested that insulin receptors on the brain microvessel endothelial cells are responsible for the transport of insulin. In this study, after addition of labeled insulin, the binding of labeled insulin to these receptors triggered the internalization of labeled insulin, a process which was inhibited by addition of unlabeled insulin. This study indicated that the transport of insulin via BBB was an insulin receptor-mediated process, which can explain the saturation of insulin transport via BBB.

1.5.2 Insulin synthesis by the brain

Insulin-like substance has been found in primary culture cells from rabbit fetal neuron cells using immunohistochemical technique. Apart from the cytoplasma, insulin-like substance was also found in

ER and Golgi of neuron cells. The existence of insulin-like mRNA was also observed in neuron cells by in situ hybridization and Northern blots (Schechter et al., 1994). In agreement with the above study, Devaskar et al (1994) observed the existence of insulin mRNA in the rabbit neuron cells. Therefore, the above studies indicated that neurons perhaps can produce insulin by themselves. However, insulin I and II mRNAs were not detected in fetal and adult rat brains using RT-PCR technique, suggesting that insulin could not be produced by neurons (Devaskar et al., 1993; Coker et al., 1990).

The above contrasting results may be explained by the different methods used in the studies. Immunohistochemical technique was employed in Schechter et al's study, in which there might be non-specific binding or cross-reactivity. The study by Havrankova et al (1979) indicated that the level of insulin in the CNS is many times of that in plasma in rats. On the other hand, it has been reported that the insulin level in the brain of rats was about 0.19ng/g wet wt (brain), which is much lower than that in the plasma (1.03ng/ml) (Baskin et al., 1983). It is possible that the non-specific binding or cross-reactivity is responsible for the very high insulin concentration found in the CNS (Park et al., 2001). Non-specific binding or cross reactivity was suggested previously, in which insulin was found to be distributed in the CNS uniformly (Dorn et al., 1982, 1981). Since different regions of brain have different functions and include different substances, the above results suggested that there is non-specific binding in the employment of immunostaining. Therefore, whether neurons can secrete insulin is still inconclusive.

1.6 Insulin receptor in the brain

1.6.1 The structure of insulin receptor

The insulin receptor is a transmembrane protein consisting of two extracellular α-subunits and two intracellular β-subunits (White et al., 1997). The α-subunit of peripheral insulin receptor is composed 723 amino acids and has a molecular weight of about 130kDa. The α-subunit in brain insulin receptor is different from that of peripheral tissue in the lower molecular weight, which is about

115kDa (Heidenreich et al., 1983). The insulin receptors of brain and peripheral tissue have the same structure of β-subunit which includes 620 amino acids and has the molecular weight of 95kDa (Gammeltoft et al., 1985). The α-subunit contains insulin binding site and the β-subunit includes tyrosine residues (Figure1.5). The binding of insulin to insulin receptor quickly causes the autophosphorylation of tyrosine residues and, in turn, activates the intracellular substrates, for example, insulin receptor substrate (IRS) family. Subsequently, many pathways are activated, such as phosphstidyl inositol-3 kinase (PI3K), GATPase regulators, mitogen-activated protein kinase (MAPK) pathway and others (Avruch et al., 1999).

1.6.2 The distribution of insulin receptor in the CNS

The expression of insulin receptor in the CNS has been demonstrated by many studies and it is found that different regions of brain have different levels of insulin receptor expression. The distribution of insulin receptor in CNS was observed by two independent studies. Havrankova et al (1978a) revealed that almost all the regions of brain showed the expression of insulin receptors and the olfactory bulb, cerebral cortex, hippocampus and hypothalamus contained the highest levels. Unger et al (1989) showed that olfactory bulbs, hypothalamus and median eminence, medial habenula, subthalamic nuclues, subfornical organ, CA1/2 pyramidal cell layer of the hippocampus and piriform cortex expressed the highest density of insulin receptor-like immunoreactivity. Interestingly, the study by Baskin et al (1973) indicated that the regions of brain with the highest insulin concentration are the hypothalamus and olfactory bulb, which is similar to the distribution of insulin receptor. The maldistribution of insulin receptor and insulin in the CNS implies different regulatory effects of insulin on different regions of CNS.

1.6.3 The insulin signaling pathway in the hippocampus

The binding of insulin to insulin receptor and the expression of insulin signaling were also

revealed in the hippocampus. ^{125}I-insulin could bind to the hippocampus and the binding sites were mainly found in the dentate gyrus and CA1 area of hippocampus (Doré et al., 1997). Baskin et al (1994) demonstrated the expression of insulin receptor substrate-1 (IRS-1) in hippocampus and found that IRS-1 was co-localized with insulin receptor and phosphotyrosine in the hippocampus, suggesting the structural tight integration of insulin signaling in the hippocampus. Therefore, the intact insulin signaling pathway including insulin, insulin receptor, IRS-1 and phosphotyrosine was found in hippocampus, suggesting that insulin plays an important role in the hippocampus.

1.7 The effect of insulin on learning and memory

In addition to central role of insulin in neuronal survival and food intake, accumulating evidences showing an improvement effect of insulin on learning and memory has emerged in past years (Laron et al., 2009).

1.7.1 The improvement effect of insulin on learning and memory

The improvement effect of insulin on memory has been observed in many experiments. In Seeley et al's study, rats first received an intracerebroventricular injection of insulin or heat-deactivated insulin shortly after training on a step-through passive–avoidance task. After 24 hours, the testing of the task on the rats showed that the latency to enter the dark compartment in insulin group increased significantly compared to that in heat-deactivated insulin group. The results suggested that insulin could increase memory function (Seeley et al., 2000). Since the insulin was injected after the training finished, the above study also suggested that insulin could potentiate memory consolidation. In one study, the mice receiving intraperitoneal injection of insulin expressed decreased memory compared to control group (Kopf et al., 1995). However, because the mice were given insulin peripherally, the glucose concentration in plasma might be reduced which may explain the impairment of memory. Since injection of a mixture of insulin and glucose rescued the impairment of memory, it was

suggested that it was not the insulin that damage the memory (Kopf et al., 1995). Another study also showed that insulin could increase memory function under the conditions of euglycemia (Kern, et al., 2001). In this study, healthy subjects received intravenous injection of insulin at high or low dose. To exclude the effect of insulin on glucose, glucose concentration in plasma was kept at normal level using glucose clamp technique. The subjects in high insulin group expressed a negative potential shift in the auditory evoked brain potentials, which were associated with facilitation of working memory, and the memory performance in words recalled test increased significantly compared to that in low insulin group.

In addition to the effect of insulin on healthy subjects, it has been found that insulin could rescue impaired memory function induced by damaging factors. For example, in one study, after induction of forebrain ischemia, rats were given subcutaneous injection of insulin for one week. 1-2 months after ischemia, the performance of insulin-treated rats in water maze place navigation tasks was much better than that in control groups (Voll et al., 1989). Adult rats were treated with insulin injection for 4 days prior to dorsal hippocampal lesions. In another study, one week after surgery, the rats in insulin group showed decreased deficit in avoidance acquisition and decreased amounts of freezing during the CS-US interval without any changes in the depressed struggling scores (Castro et al., 1976). Blanchard et al (1997) also showed that adult rats receiving scopolamine to injure memory function expressed poor performance in a win-shift radial arm maze, but treatment with insulin improved the impaired memory function significantly (Blanchard et al., 1997). These results indicate the facilitatory effect of insulin on functional recovery of impaired memory after brain damage.

The changes in the insulin signaling pathway in hippocampus during the memory process have also been investigated. After water maze training, the quantity of insulin receptor mRNA in CA1 area was increased and there was an elevation of insulin receptors in the cytoplasm of hippocampal synapse, demonstrating that insulin receptors maybe involved in the process of memory (Zhao et al., 1999). It was also found that the level of tyrosine phosphorylation of insulin receptor in rats increased after

training, indicating that the functional changes of insulin receptors occurred during the memory process (Zhao et al., 1999). Taken together, these results indicate the involvement of insulin-insulin receptor pathway activation in the memory formation.

1.7.2 The role of insulin in cognitive deficiency in some diseases

1.7.2.1 The role of insulin in cognitive deficiency in Alzheimer's disease

Alzheimer's disease (AD) is a progressive brain disease and the most common form of dementia. AD patients express lower CSF insulin concentration, higher plasma insulin level, and a decreased CSF/plasma insulin ratio compared with healthy subjects (Craft et al., 1988). AD patients show higher fasting plasma glucose and insulin concentration compared to healthy controls. During hyperglycemic clamp, higher steady-state glucose values and normal first- and second-phase insulin responses were recorded in AD patients (Meneilly et al., 1993). These results showed reduced insulin sensitivity index, suggesting that insulin sensitivity in AD patients is impaired. AD patients without progressive dementia showed memory facilitation and elevation in plasma insulin relative to baseline but AD patients with progressive dementia expressed significant decreases in plasma insulin and memory facilitation after 225mg/dl glucose challenge, indicating that plasma insulin concentration was positively correlated with the extent of dementia (Craft et al., 1993).

However, Frolich et al (1998) found that AD patients had stronger insulin-immunoreactivity compared with healthy subjects. In their study, insulin receptor densities of brain in AD patients were reduced in comparison to middle-aged healthy subjects, but increased compared with age-matched healthy subjects. Tyrosine kinase activity, a downstream signal transduction mechanism of insulin receptors, decreased in AD patients compared to middle-aged and age-matched healthy subjects. The increase in insulin receptors in brain is not consistent with the decreased insulin concentration in CSF. Similar results are observed in other studies. For example, rats receiving intracerebroventricular injection of streptozotocin expressed long-term and progressive deficiency in learning and memory,

indicating the impairment of working and reference memory (Lannert et al., 1998). Administration of streptozotocin could decrease the insulin-induced phosphorylation of the beta subunit of the insulin receptor, although the insulin receptor intensities increased simultaneously (Kadowaki et al., 1984). Thus, it seems that the impairment of memory is attributed to the dysfunction of insulin signaling transduction and the increased insulin receptors is perhaps a compensation for the dysfunction of insulin signaling. These studies suggested abnormal insulin concentration in CSF, impaired insulin sensitivity and/or a disturbance of insulin signal transduction in AD patients. Given the previous discussion that insulin can improve memory functions of healthy subjects, abnormality of insulin function is likely to contribute significantly in memory loss in AD patients. AD patients with intravenous insulin infusion and constant plasma glucose concentration of fasting baseline level performed much better compared to placebo controls in story recall task, suggesting that insulin alone without secondary changes in glucose metabolism could improve memory function (Craft et al., 1996).

1.7.2.2 The role of insulin in cognitive deficiency in diabetes mellitus

Although diabetes mellitus (DM) is not regarded as a neurological disease, cognitive dysfunction in DM patients was found in 1922 for the first time and was observed by many subsequent studies (Kodl et al., 2008). There are mainly two types of DM, i.e., Type 1 diabetes (also known as insulin-dependent DM) and Type 2 diabetes (also known as insulin-independent DM). Many kinds of cognitive deficits are identified in patients with Type 1 diabetes, such as slowing of information processing speed, worsening psychomotor efficiency, impaired memory and so on (Kodl et al., 2008). There are many factors contributing to the cognitive deficiency, including hyperglycaemia, hypoglycaemia and vascular disease. After injection of streptozotocin to induce Type 1 DM model, the mice expressed a notable memory retention deficiency after training of an active avoidance T-maze task and the memory deficiency could be rescued by administration of insulin (Flood et al., 1990). In another study, 11 weeks after induction of Type 1 DM by injection of streptozotocin, the rats showed

impaired spatial memory in water maze task and marked decreased long-term potentiation (LTP) in CA1 area of hippocampus (Biessels et al., 1996). However, the decreased plasma insulin concentration induced by streptozotocin could lead to increase in plasma glucose level and therefore the effect of hyperglycemia on memory function cannot be excluded. Thus, the role of insulin in the deficiency in memory function is uncertain in spite of impaired memory was found in diabetic animal models.

The most common cognitive deficits identified in patients with type 2 DM are impaired memory, decreased psychomotor speed and reduced executive function (Kodl et al., 2008). Diabetic GK rats, a Type 2 DM animal model, showed altered taste aversion, suggesting impaired memory (Marfaing-Jallat et al., 1995). GK rats are characterized by the increased basal glucose concentration and decreased insulin secretion in plasma. Therefore, the role of insulin in the impaired memory of GK rats is inconclusive. In a related study by Oomura et al (1992), the OLETF rats with the lack of cholecystokinin-A receptors were used. The OLETF rat is a kind of Type 2 DM animal model and is characterized by moderate obesity and impaired glucose tolerance. The OLETF rats showed impaired spatial memory in the Morris water-maze task. Since the absence of cholecystokinin-A receptors might contribute to the impaired memory function, the role of insulin in the impaired memory in OLETF rats is again uncertain. Zucker Diabetic Fatty (ZDF) rats, another kind of Type 2 DM animal model, expressed normal performances in Morris water maze task (Belanger et al., 2004). The LTP in CA1 area of hippocampus of ZDF rats was not altered compared to controls. ZDF rats derive from the mutation of leptin receptor gene and are characterized by the insulin resistance, hyperinsulinaemia and impaired glucose tolerance. Although ZDF rats develop has hyperglycaemia, the impairment of memory function was not found in ZDF rats, suggesting that perhaps hyperinsulinaemia rescued the impaired memory function. In these studies, the interesting fact is that impaired memory was found in GK rats with hypoinsulinaemia but not in ZDF rats with hyperinsulinaemia. There are two possible reasons for that: insulin can rescue the damaged memory functions induced by hyperglycaemia and decreased insulin results in impaired memory. Thus, there is no direct evidence proving that decreased

insulin leads to impairment of memory function in DM patients.

Taken the above together, although impaired memory function was detected in Type 1 and Type 2 DM animal models, the role of insulin in it is still not confirmed.

1.7.2.3 The role of insulin in cognitive deficiency in Parkinson's disease

Parkinson's disease (PD) is a degenerative disorder of the CNS and is characterized by muscle rigidity, tremor, a slowing of physical movement, a loss of physical movement and memory deficit. The evidences about the role of insulin in the PD are obtained from the relationship between DM and PD. It has been found that 50% to 80% of PD patients have impaired glucose tolerance (Sandyk et al., 1993). The survey from 24,831 elderly subjects in USA demonstrated that the rate of DM in PD patients was much higher than that in healthy controls (Pressley et al., 2003). Some drugs for the treatment of PD are able to affect the level of insulin. For example, levodopa induces increase in plasma insulin concentration (Craft S et al., 2004). Intracerebroventricular injection of bromocriptine, the agonist of dopamine D_2 receptor, significantly increases the insulin sensitivity of hamsters (Luo et al., 1999). The effect of these drugs on insulin may be responsible for the treatment of PD. Although the relationship between DM and PD is observed, the role of insulin in the memory loss in PD patients is unknown by now. A very early study showed that the rate of dementia increased significantly in PD patients with DM compared with that without DM (Craft et al., 2004). In PD patients, immunohistochemical studies showed that the immunoreactivity of insulin receptors in substantia nigra decreased compared with normal controls. RT-PCR revealed that the level of mRNA of beta subunit in the insulin receptor containing tyrosine kinase domain was reduced in comparison to healthy subjects (Takahashi et al., 1996). Consistent with this study, it has been shown by immunohistochemistry that neurons in the pars compacta of the substantia nigra, paranigral nucleus, parabrachial pigmental nucleus, tegmental pedunculopontine nucleus, supratrocheal nucleus, cuneiform nucleus, subcuneiform nucleus and lemniscus medialis lost insulin receptors (Moroo et al.,

1994). However, the expression of insulin receptors in hippocampus is not investigated.

The insulin concentration in CSF of PD patients was also a subject of investigation. It was observed that there were no differences in the level of insulin in CSF between healthy subjects and PD patients (Jimenez-Jimenez et al., 2000). However, this study could not exclude the role of insulin in the dementia in PD patients because PD patients with or without dementia were not identified. Another reason is that the expression of insulin receptors and the related insulin signaling pathway in the brains of PD patients were not determined. Since decreased insulin signaling pathway was found in the hippocampus from AD patients (Frolich et al., 1998), this question should be clarified by further experiments.

1.7.2.4 The role of insulin in cognitive deficiency in Huntington's disease

Huntington's disease (HD), which is also called chorea, is a fatal disease with neurological and psychiatric features and is characterized by involuntary movements and cognitive deficiency. There are no direct evidences about the role of insulin in the dementia of HD patients. However, there is obvious relationship between DM and HD. In one study, 50% of HD patients developed much higher plasma glucose and lower insulin concentration after oral glucose tolerance tests, suggesting impaired glucose tolerance (IGT, Podolsky et al., 1977). Farrer et al. (1985) observed that 10.5% of a batch of 620 HD patients had DM, which was much higher that in controls. It was also found that 32% of HD patients had IGT, which was significantly higher than 3.2% in controls (Schobotz et al., 1976). Similar results were also found in animal experiments. R6/2 mice, a HD animal model, developed high fasting plasma glucose concentration, decreased insulin mRNA and reduced insulin content in β-cells compared with normal controls. Interestingly, this model developed chorea, weight loss and polyuria without eating or drinking deficits simultaneously, suggesting the relationship between DM and HD (Hurlbert et al., 1999; Andreassen et al., 2002). Although there is positive relation between DM and HD, the insulin concentration in CSF, the expression of insulin receptors and insulin signaling pathway in the brain of

HD patients are unknown. Therefore, the role of insulin in cognitive function in HD patients is still uncertain.

1.8 Long-term potentiation in hippocampus

1.8.1 Introduction of hippocampus

Hipppocampus formation is a main component of the limbic system and composed of the hippocampus, dentate gyrus (DG), subiculum and entorhinal cortex (EC). Hippocampus is composed of DG and cornu ammonis (CA) which is divided into CA1, CA2 and CA3 areas. The hippocampus receives the projection from EC which is also called perforant path. Perforant path ends in granule cells of DG and is the first pathway in hippocampus. From granule cells of DG to pyramidal cells in CA3 area there is the second path in hippocampus which is called mossy fibers. Pyramidal cells in CA3 area send their axons to CA1 area and build the third path in hippocampus which is also called Schaffer collateral pathway (Figure1.6).

Hippocampus is the most important part for many types of learning and memory and has the ability to change its neuronal connectivity, a phenomenon referred as "synaptic plasticity". Synaptic plasticity is a very common physiological phenomenon whereby synaptic efficacy was changed by the environments. Synaptic plasticity in hippocampus has been regarded as contributing cellular mechanism for information storage and memory formation. The most well known types of long-term synaptic plasticity include long-term potentiation (LTP) and long-term depression (LTD), both of which are widely considered as the cellular mechanisms underlying the learning and memory.

1.8.2 Long-term potentiation (LTP)

Long-term potentiation (LTP) is an increase in signal transmission in synapses by stimulating presynaptic and postsynaptic neurons simultaneously. LTP was first described by Bliss and Lomo in 1973 for the first time. This study found that a series of high-frequency stimulation to the perforant

pathway in hippocampus of rabbit induced a long-lasting increase in efficiency of signal transmission in the granule cells of DG (Bliss et al., 1973). By now, many kinds of LTP and the expressions of LTP in different regions of brain have been proved. LTP is divided into two temporal phases: an early-phase LTP (E-LTP) and a late-phase LTP (L-LTP). The E-LTP can be induced by a set of high frequency stimulation (HFS) and lasts for about 1 hour. The L-LTP is can be induced by multiple trains of HFSs and lasts for at least 3 hours. The L-LTP also includes two phases: the early phase in 1 hour and the late phase lasting for at least 2 hours (Zhang et al., 2009). Although the investigation about LTP lasted for over thirty years, the complete mechanisms of the production of LTP, especially that of the L-LTP, are still not entirely clear.

1.8.3 The dependence of LTP on Ca^{2+}

Intracellular injection of Ca^{2+} chelator blocked the production of LTP and increase in postsynaptic Ca^{2+} caused LTP-like changes, indicating the involvement of Ca^{2+} influx during the production of LTP (Lynch et al., 1983, 1988). There are three pathways contributing to the intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase during the LTP production: N-methyl-D-aspartate (NMDA) receptors, voltage-dependent Ca^{2+} channels (VDCC) and Ca^{2+}–induced Ca^{2+} release (CICR).

NMDA receptor is a kind of voltage-dependent Ca^{2+} and monovalent cations channel. In the absence of significant membrane depolarization, NMDA receptor is blocked by voltage-dependent binding of Mg^{2+}. Inhibition of NMDA receptors blocked the production of LTP and on the contrary, over-expression of NMDA receptors causes the enhancement of LTP (Collingridge et al., 1983; Tang et al., 1999). Ca^{2+} imaging studies with HFS, which as mentioned previously is often used to induce LTP, caused a NMDA receptor-dependent increase in $[Ca^{2+}]_i$ (Alford et al., 1993). The activation of NMDA receptors is described as follows: HFS induces the release of glutamate from the presynaptic neurons and subsequently depolarizes the membrane potential of postsynaptic neurons. The mixture of depolarization and the binding of glutamate to NMDA receptors cause the activation of NMDA

receptor and subsequent Ca^{2+} influx (Kauer et al., 1988).

In CA1 area of rat hippocampal slices, a 25-Hz HFS-induced LTP was blocked by the NMDA antagonist significantly but not by L-type VDCC inhibitor. However, a 200-Hz HFS-induced LTP was inhibited by NMDA antagonist and L-type VDCC inhibitor by 50% respectively. The above results suggested that L-type VDCC was also involved in the Ca^{2+} influx during the induction of LTP. NMDA receptor-dependent LTP can be blocked by broad-spectrum serine/threonine kinase inhibitor and L-type VDCC-dependent LTP was inhibited by tyrosine kinase inhibitor. These results showed that induction with different parameters produced different types of LTP that have different mechanisms which do not interfere with each other (Cavuş et al., 1996).

Application of thapsigargin, which exhausts $[Ca^{2+}]_i$ stores by blocking Ca^{2+} pump, blocked the induction but not the expression of LTP， suggesting the involvement of $[Ca^{2+}]_i$ stores in the elevation of $[Ca^{2+}]_i$ during the HFS stimulation (Harvey et al., 1992).

1.8.4 Ca^{2+}-activated protein kinase

As a consequence of increase in $[Ca^{2+}]_i$, several protein kinases are activated and play great roles in the production and expression of LTP. The most studied kinase is alpha-calcium-calmodulin-dependent protein kinase II (CaMK II). The expression of CaMK II in postsynaptic neurons is higher than that in presynaptic neurons, which is physiologically significant for the activation by elevation of postsynaptic $[Ca^{2+}]_i$. The fact that the inhibition of CaMK II blocked the production of LTP demonstrated the involvement of CaMK II (Malenka et al., 1989). The mice with the mutation of CaMK II expressed impaired spatial memory and deficiency in the production but showed normal postsynaptic mechanisms (Silva et al., 1992a, 1992b). Direct injection of the active form of CaMK II into pyramidal cells in CA1 area in hippocampus caused a slowly increase in the magnitude of excitatory postsynaptic currents (EPSCs). Interestingly, HFS failed to induce LTP after injection of active CaMK II, suggesting that the CaMK II-induced enhancement of EPSCs and

HFS-induced LTP shared the same mechanisms (Lledo et al., 1995). The activation of CaMK II will last and new CaMK II will be produced in the expression of LTP (Ouyang et al., 1997).

There are some evidences indicating that calcium/calmodulin-dependent protein kinase Ⅳ (CaMK Ⅳ) is responsible for the production of LTP. After HFS, CaMK IV was activated and, the phosphorylation of cAMP response element-binding protein (CREB), which is the substrate of CaMK Ⅳ, increased with the elevation of c-Fos protein which is regulated by CREB (Kasahara et al., 2001). The transgenic mice with the mutation of CaMK Ⅳ in the postnatal forebrain expressed impaired L-LTP with normal basic synaptic function and E-LTP. The mice also showed impaired consolidation/retention phase of memory and normal acquisition phase of memory (Kang et al., 2001).

1.8.5 The role of AMPA receptor in the production of LTP

The alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is a tetramer and is composed of four kinds of subunits: GluR1, GluR2, GluR3 and GluR4. AMPA receptor is also a kind of Ca^{2+} channel and the permeability can be reduced by GluR2. Many evidences indicated that AMPA receptors on the postsynaptic neurons are essential for the production of LTP. AMPA receptor is a recycling protein between the plasma membrane and cytoplasma (Malinow et al., 2002). It has been found by many studies that some excitatory synapses only contain NMDA receptor with the absence of AMPA receptor on the postsynaptic plasma membrane at resting membrane potentials which are referred as "morphological silent synapses" (Isaac et al., 1995; Liao et al., 1995). However, activation of NMDA receptors induces a rapid recruitment of AMPA receptors back to the plasma membrane in several minutes, indicating that the recycling of AMPA receptors is regulated by NMDA receptors (Liao t al., 2001). It is also observed that the "morphological silent synapses" could be translated into "functional synapses" by acquiring AMPA receptors and this translation is blocked by NMDA receptor inhibitor (Isaac et al., 1995; Liao et al., 1995). Thus, the role of AMPA receptors in the production of LTP is concluded as follows: some excitatory synapses showed the decreased AMPA receptors on the

postsynaptic plasma membrane under the conditions at resting membrane potentials. After HFS, the activation of NMDA receptors promotes the insertion of AMPA receptors on the postsynaptic plasma membrane. The glutamate released from presynaptic neurons binds to AMPA receptors and promotes the activation of AMPA receptors and then resulted in the increase in the magnitude of EPSC. In agreement with the above theory, the sensitivity of pyramidal cells in CA1 area of hippocampus to ionophoretically-applied quisqualate receptor ligands gradually increases during the production of LTP (Davies et al., 1989).

The AMPA receptor on pyramidal cells in CA1 area of hippocampus is mainly composed of GluR1 and GluR2 subunits. It is found that LTP induces the phosphorylation of AMPA receptor on Ser^{831} of GluR1. Interestingly, the phosphorylation of AMPA receptor was accompanied by the autophosphorylation of CaMK Ⅱ and was blocked by the inhibition of CaMK Ⅱ inhibitor, suggesting that the phosphorylation of AMPA receptor was induced by CaMK Ⅱ (Barria et al., 1997). The phosphorylation event can increase the single-channel conductance of AMPA receptor (Derkach et al., 1999). Similarly, the mice with the mutation of GluR2 subunit failed to produce LTP after HFS (Zamanillo et al., 1999).

Therefore, the hypothesis for induction of LTP is a sequence of events originating from the release of glutamate from presynaptic neurons induced by HFS. The released glutamate binds to the AMPA receptor on the plasma of postsynaptic neurons and depolarizes the membrane potential. The mixture of glutamate and strong depolarization induced by HFS activates NMDA receptor on the membrane of postsynaptic neurons and promote the influx of Ca^{2+} and, in turn, promotes the phosphorylation of CaMK Ⅱ. Activated NMDA receptor causes the insertion of AMPA receptor into the membrane of postsynaptic neurons and the conductance of AMPA receptor is enhanced by phosphorylation of GluR1 of AMPA receptor under the action of activated CaMK Ⅱ. The activated NMDA receptor increases the influx Ca^{2+} of and induces LTP (Collingridge et al., 1992).

1.9 The effect of insulin on hippocampus

1.9.1 Glucose metabolism

The main physiological action of insulin in the body is to regulate glucose homeostasis. Therefore, it is worth to consider the role of insulin in the glucose metabolism of the hippocampus. It is well known that glucose is the main nutrient and energy source of neurons. It has been observed that central glucose administration increased cognitive function in rodents and humans (McNay et al., 2010). Because neuron is regarded as an insulin-insensitive organ compared with peripheral tissues, this study cannot definitely prove that it is enhanced glucose metabolism that accounts for the effect of insulin. Most peripheral tissues, including skeletal muscles, cardiac muscles and fat, express a subtype of glucose transporter-4 (GlUT-4), which is responsible for the transport of glucose across the plasma membrane (Wolff et al., 2003). GLUT-4 is a membrane-spanning protein and recycles continuously between cytoplasm and plasma membrane with a higher speed of internalization than exocytosis (Klip et al., 2009). Once the binding of insulin to insulin receptors happens, the activation of insulin signaling promotes the excytosis of GLUT-4 and increases the number of GLUT-4 on the surface of membrane to enhance the uptake of glucose. Thus, GLUT-4 is insulin-sensitive and is the key protein in the regulatory protein in the effect of insulin on peripheral tissues (Huang et al., 2007). However, the main glucose transporters expressed in CNS are glucose transporter-1 (GLUT-1) and glucose transporter-3 (GLUT-3), both of which are insulin-insensitive. GLUT-1 is mainly distributed in astrocytes and the endothelial cells of cerebral microvessels while GLUT-3 is principally expressed in neurons (Vannucci et al., 1998; Simpson et al., 1999; Duelli et al., 2001). Since the two main neuronal GLUTs are unresponsive to insulin, it is generally thought that insulin is not involved in the glucose metabolism in CNS. Recently, however, the expression of GLUT-4 in CNS of rats and the regulatory effect of insulin on CNS glucose homeostasis were revealed by several studies. RT-PCR and in situ hybridization techniques demonstrated the expression of GLUT-4 in the pyramidal cells in hippocampal CA1 and CA3 areas, the granule cells of DG, subiculum, neocortical areas, piriform and

entorhinal cortices, red nucleus and cerebellar Purkinje cells in hindbrain (Messari et al., 2002). A study on SH-SY5Y, a human neuroblastoma cell line, showed that application of insulin increased the translocation of GLUT-4 to the plasma membrane and the uptake of glucose as well (Benomar et al., 2006). Intracerebroventricular administration of insulin increases the number of GLUT-4 on hippocampal plasma membranes and glucose uptake by hippocampus simultaneously, which was blocked by the inhibitor of phosphoinositide 3-kinases (PI3K). The phosphorylation of Akt induced by insulin was also observed in the same study, suggesting the involvement of PI3K pathway in the insulin-induced glucose uptake (Grillo et al., 2009). Intrahippocampal insulin improved spatial memory in a PI3K-dependent pathway, which was blocked by the inhibition of endogenous intrahippocampal insulin signaling. Furthermore, animals developing Type 2 diabetes by high-fat diet expressed impaired basal cognitive function and decreased cognitive response to hippocampal insulin administration (McNay et al., 2010). Taken the above studies together, it is reasonable to speculate that insulin-induced improvement in learning and memory may at least be contributed by its action in promoting the uptake of glucose in hippocampus.

1.9.2 MAPK pathway

Mitogen-activated protein kinase (MAPK) is an integral component of cellular signaling which responds to extracellular stimuli and regulates cellular activities. Many factors can induce the activation of MAPK pathway and the binding of insulin to insulin receptor is one of them (Gogg et al., 2009; White et al., 1998). In a study of learning and memory function of rats involving contextual fear conditioning task, Atkins et al (1998) showed that learning training induced the activation of p42MAPK significantly which was blocked by the inhibition of NMDA receptor. Application of the inhibitor of MAPK kinase, the upstream regulator of MAPK, abolished fear conditioning. English et al (1996) also showed that HFS caused a NMDA receptor-dependent activation of p42MAPK in CA1 area of hippocampus. The activation of p42MAPK could also be induced by activated NMDA receptor

and PKC. It is also found that inhibition of MAPK pathway blocked the production of LTP in hippocampus induced by theta-burst stimulation (Selcher et al., 2003). Taken together, the activation of p42MAPK pathway is induced in the process of learning and memory and appears to be an essential step.

Therefore, we speculated that the activation of MAPK pathway induced by insulin is one mechanism of the improvement effect of insulin on learning and memory. However, there are not any direct evidences supporting this speculation.

1.9.3 The effect of insulin on synaptic plasticity

The question of whether insulin can affect synaptic plasticity has been addressed by various studies. Thus, Lee et al. (2009) found that insulin rescued the impaired LTP induced by amyloid β-protein (Aβ) but failed to alter the normal LTP in the CA1 area of rat hippocmpal slices. In a similar study, addition of insulin restored the impaired LTP of hippocampal slices from streptozotocin-treated rats (Izumi et al., 2003). In agreement with this, Plitzko et al (2001) showed that insulin promotes the conversion of silent synapses to functional synapses. In the study on LTD, which is regarded as another cellular process for learning and memory function, it was found that insulin caused LTD of hippocampus by promoting the internalization of AMPA receptors (Man et al., 2000).

In a *Xenopus* oocyte expression system expressing NMDA receptor, application of insulin potentiated NMDA receptor-mediated currents (Liao et al., 1999; Liu et al., 1992). This potentiation induced by insulin was inhibited by tyrosine protein kinase inhibitor, suggesting the involvement of tyrosine phosphorylation (Liao et al., 1999; Liu et al., 1992). In agreement with the above studies, Skeberdis et al. (2001) found that insulin increased the open probability of NMDA receptor. The recruitment of NMDA receptor to the membrane surface was induced by addition of insulin and the number of NMDA receptors on the plasma membrane increased as a result of speeded NMDA receptor recuriment. Using rat hippocampal slices, it was found that exposure to insulin enhanced the

phosphorylation of NR2A and NR2B subunits of NMDA receptors (Christie et al., 1999). Because NMDA receptor is responsible for the production of LTP, the regulatory effect of insulin on NMDA receptor may contribute to the effect of insulin on the hippocampus.

1.10 Hypotheses

CFRD is characterized by chronic hyperglycaemia resulting from the decreased insulin secretion, which is distinct from both Type 1 diabetes and Type 2 diabetes (Moran et al., 2002). Due to the deficiency in glucose homeostasis, CFRD patients have a much higher mortality rate than that of CF patients without DM (Finkelstein et al., 1988). Therefore, understanding the pathogenesis of CFRD helps to decrease the mortality rate and increase the life expectancy of CFRD patients. It is well accepted that the possible reason for impaired insulin secretion in CF patients originates from the deficiency of CFTR in pancreatic duct cells. The deficiency of CFTR results in the decrease of HCO_3^- secretion and subsequently reduced pancreatic juice secretion, which causes the obstruction of pancreatic duct due to the concentrated pancreatic enzymes (Gaskin et al., 1982; Brennan et al., 2004). These activated proteases and lipases induce autolysis of the pancreas and, in turn, cause fibrosis and fat infiltration of islets (Soejimaet al., 1986; Iannucci et al., 1984). These anatomical abnormalities lead to reduced number of islets and decreased insulin secretion after exposure to oral glucose (Hinds et al., 1991; Hamdi I, et al., 1993). However, there are some problems with the above explanation. It is found in Rakotoambinina's study that the first phase insulin secretion response to intravenous glucose in CF patients with IGT was significantly lower than that in CF patients with NGT. However, there was no difference in the second phase insulin secretion during hyperglycemic clamp between the two groups (Rakotoambinina et al., 1994). Therefore, perhaps there may be another mechanism contributing to the onset of CFRD. However, what is the mechanism? Perhaps we can be illumined by metabolic acidosis, a "HCO_3^- deficiency" disease.

Metabolic acidosis is characterized by low blood pH as a result of decreased HCO_3^- concentration

in plasma (Kraut et al., 2010; Bigner et al., 1996). The consequences of metabolic acidosis can be serious, including death and coma. The symptoms of metabolic acidosis may include some mental disorders, headache, chest pain, palpitations, hypoxia, decreased visual acuity, abdominal pain, amuscle weakness and bone pains. Interestingly, the decreased insulin secretion was also observed in cows with metabolic acidosis (Bigner et al., 1996). Since acidification can lead to the increase in insulin secretion (Rebolledo et al., 1983, 1978; Ohta et al., 1991), it seems that the decreased HCO_3^- level is responsible for the impaired insulin secretion of islets β-cells.

On the other hand, it is well known that food intake leads to an increase in plasma HCO_3^- concentration, which is called "postprandial alkaline tide" (Vaziri et al., 1980). Likewise, food intake can also induce insulin secretion from β-cells in pancreas. The time-dependent similarity between insulin secretion and HCO_3^- level also suggests that perhaps there is relationship between the plasma HCO_3^- and insulin secretion. Combined with the fact that CFTR plays a great role in the transport of HCO_3^- across the plasma membrane (Hug et al., 2003), we are promoted to hypothesize that the mutation of CFTR may impair the HCO_3^- effect on insulin secretion, which is perhaps an alternative mechanism for the onset of CFRD. In our study, we investigated the effect of HCO_3^- on insulin secretion, the underlying mechanism and the role of CFTR. Our study helps to understand the pathogenesis of CFRD and is useful for the therapy of CFRD patients.

Although insulin is well known for its action on peripheral glucose homeostasis, the role of insulin in CNS was investigated by many studies (Laron et al., 2009). In addition to its central role in neuron survival and food intake, accumulating evidences about the improving effect of insulin on learning and memory have emerged. Several studies found that insulin had the ability to across the BBB to enter CSF and bind to the insulin receptor on hippocampus (Margolis et al., 1967; Banks et al., 1997a; Havrankova et al., 1978a; Doré et al., 1997). Numerous studies showed that insulin administration improved the memory of human and rats (Voll et al., 1989; De et al., 1976; Flood et al., 1990; Kern et al., 2001; Seeley et al., 2000) and the impaired memory was found in DM patients or

animal models (Flood et al., 1990; Oomura et al., 2002; Marfaing-Jallat et al., 1995; Kodl et al., 2008). These studies showed that insulin played an important role in the memory process.

Interestingly, low CSI was also found in CF patients. Thus, the fact that insulin plays a role in the process of memory suggests that decreased insulin secretion in CF patients is an alternative mechanism for impaired cognitive function although the impaired memory was attributed the lack of vitamin E. However, the mechanism underlying the improving effect of insulin on memory is unknown. Since previous studies reported that Type 1 DM animal model expressed impaired LTP in hippocampus, we hypothesize that the memory-improving effect of insulin can be attributed to the effect of insulin on LTP. In this study, we investigated the effect of insulin on LTP of hippocampus and the underlying mechanisms, which help us to understand the onset of impaired cognitive function in CF patients with decreased insulin secretion and are useful for the prevention of mental impairment.

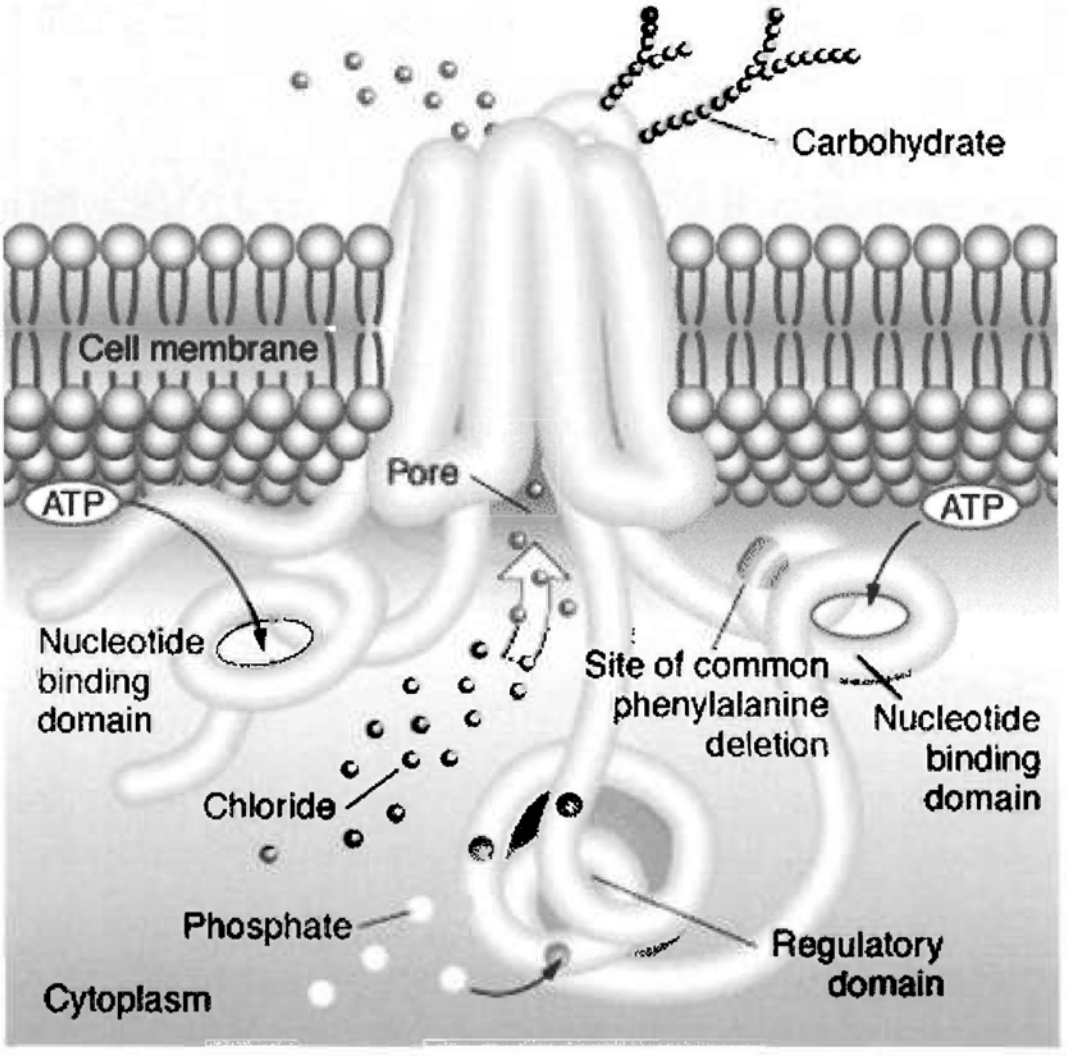

Figure 1.1 The structure of CFTR protein. CFTR is composed of five domains: two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs) and a unique regulatory domain (RD). Each MSD is composed of six transmembrane segments which are connected by intra- and extracellular loops. The two MSDs gather to form a low conductance anion-selective pore. Each NBD includes conserved amino acid sequences that can interact with ATP. The two NBDs form a dimer by the head-to-tail connection. RD contains many charged amino acids and is distinguished by it multiple consensus phosphorylation sites, which is regulated by PKA.

(http://prometheus.mse.uiuc.edu/glossary/cf/)

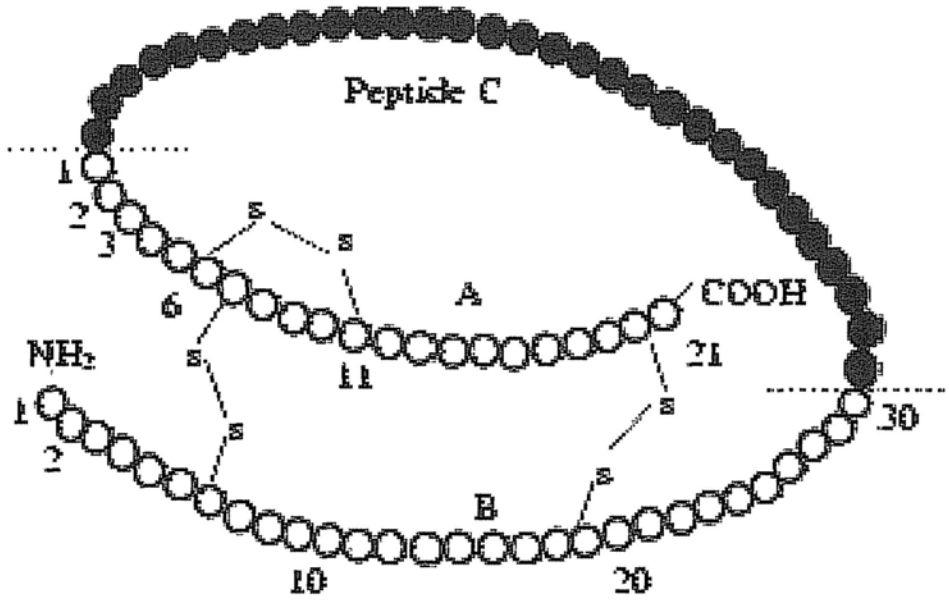

Figure 1. 2 The structure of insulin. Insulin is synthesized in the form of proinsulin, a single polypeptide chain. After catalyst by proteases, proinsulin is transformed into C peptide, A and B chains. A and B chains are connected together by two disulfide bonds and there is an additional disulfide bond within the A chain.

(http://www.pharmacorama.com/en/Sections/Insulin_1.php)

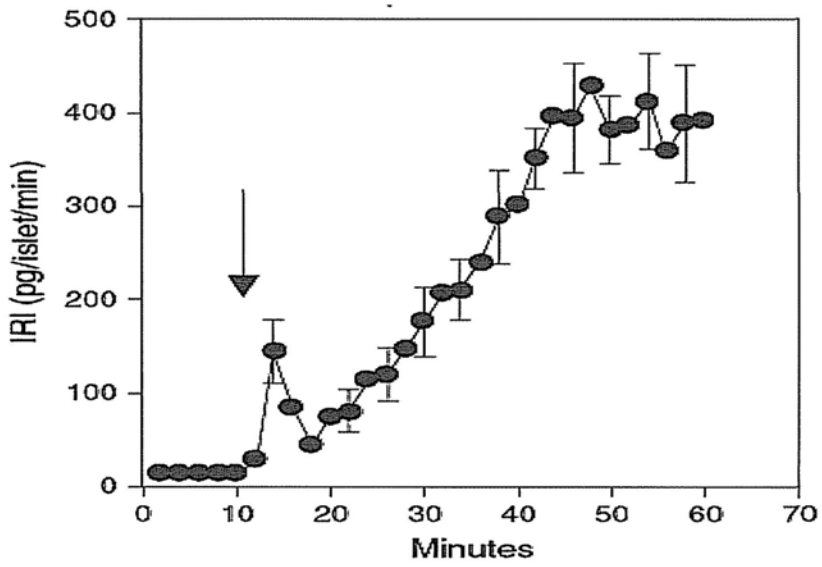

Figure 1. 3 Biphasic insulin secretion induced by glucose. After stimulation of a sudden and long lasting increase in the glucose concentration, the increased insulin secretion expressed a biphasic model which contains a quick first peak response in 10 minutes followed by a slow down and then increase to a high level again in rat islets. (Straub et al., 2002b)

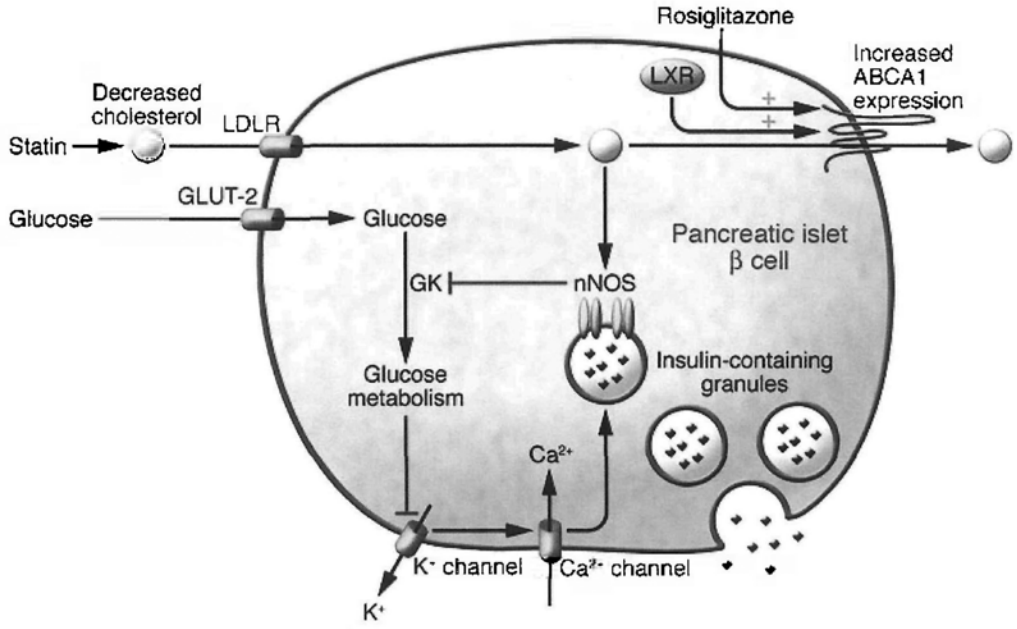

Figure 1. 4 The K_{ATP}-dependent pathway in the glucose-induced insulin secretion. When β-cells are treated by much higher level of glucose, the metabolism in β-cells accelerates and leads to an increase in the ratio of ATP: ADP, which induces the closure of K_{ATP} channels and in turn leads to the depolarization of membrane potential. The change in membrane potential induces the activation the voltage-dependent L-type channel and the subsequent exocytosis of insulin-containing granules as a result of the increase in $[Ca^{2+}]_i$.

(http://www.jci.org/articles/view/33296/files/JCI0833296.f1/medium)

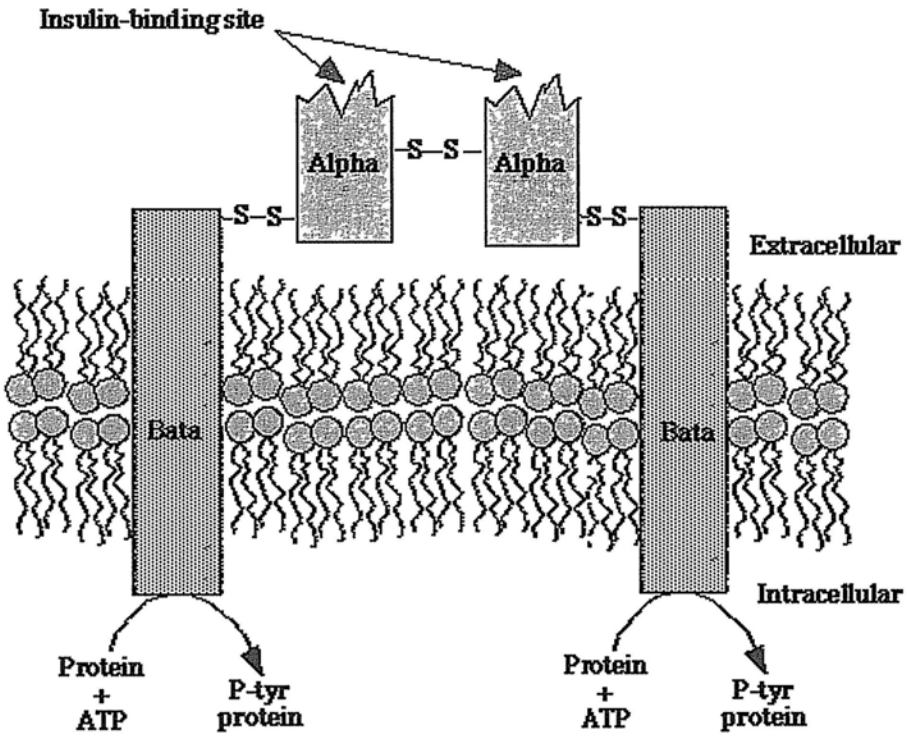

Figure 1.5 The structure of insulin receptor. The insulin receptor is composed of two extracellular α-subunits and two intracellular β-subunits. Each α-subunit has one insulin binding site. The binding of insulin to binding site activates tyrosine kinase and then promotes the phosphorylation of tyrosine residuals on β-subunits.

(http://www.pharmacology2000.com/Diabetes/InRec.gif)

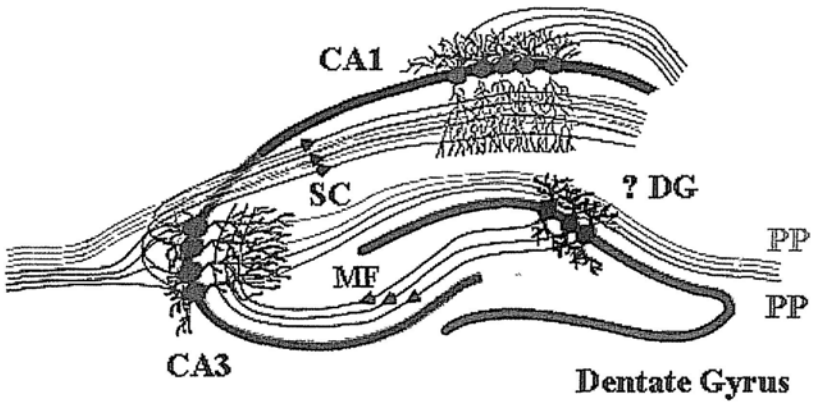

Figure 1.6 The main pathways in hippocampus. Perforant path (PP) arises from EC and terminates in granule cells in DG. Mossy fibers (MF) arise from granule cells in DG and terminate in pyramidal cells in CA3 area. Schaffer collateral (SC) arises from pyramidal cells in CA3 area and terminates in pyramidal cells in CA1 area. (Wang et al, 2005).

CHAPTER 2

GENERAL METHODS

2.1 Manipulation of cell line

2.1.1 Maintenance of cells

RIN-5F, a insulin-secreting cell line used in my experiments, was purchased from ATCC (USA). The transplantable rat islet cell tumor is induced by radiation and then is transplanted to nude mouse. Subsequently, the RIN-m cell line was separated and established from the nude mouse and is cloned into RIN-5F cell line finally. The RIN-5F cell is a kind of insulin-secreting cell line derived from the RIN-m rat islet line. Apart from insulin, the RIN-5F produces L-dopa-decarboxylase (a marker for cells with amine precursor uptake and decarboxylation) (Bhathena et al., 1984; Oie et al., 1983).

RIN-5F were cultured in the flask filled with appropriate Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium (GibcoBRL, USA) supplemented with 50 unit/ml penicillin-streptomycin and 10% v/v fetal bovine serum (FBS) (GibcoBRL, USA). The flask was placed in a humidified incubator at the temperature of 37°C with 5% CO_2 to keep the pH of culture medium at about 7.4. The culture medium was changed in 2-3 days when the color of the culture medium turned yellow. When cells reached about 80%-90% confluence, the cells would be passaged. After removing the culture medium from the flask gently, the cells were washed using 5ml Hanks' Balanced Salt Solution (HBSS) (GibcoBRL, USA) to remove remaining culture medium and then the cells were trypsinized with 1.5 ml trypsin-EDTA for several minutes. After most cells were separated from the bottom of flask, the digestion was neutralized by 3ml RPMI 1640 culture medium. After the cell suspension was transferred to a 15-ml centrifuge tube, the tube was centrifuged at 1000 rpm in a CR412 desktop centrifuge (Jouan, USA) for 5 minutes at room temperature. The supernatant was removed to wash out the residual trypsin. The cell pellet was re-suspended with fresh culture medium and transferred to new flasks. The ratio of the subcultivation was about 1:2 to 1:4.

2.1.2 Preparation of cell stock

RIN-5F cells at about 80%-90% confluence on a 60mm culture dish were washed with HBSS solution and trypsinized. After neutralization and centrifugation, the cell pellet was re-suspended using lml fresh RPMI 1640 culture medium supplemented with 50 unit/ml penicillin-streptomycin, 10% v/v FBS and 5% DMSO. The suspension of these cells were transferred to a cryotubes™ vial (Nuncbrand Demark) and then the vial was put in a freezing pot (Stratagene, CA). The pot was placed into a -80°C freezer for one night. In the freezer, the temperature of the cell stock decreased at a constant rate of about 1°C/min. After the cell stock was frozen, cell stocks were transferred into liquid nitrogen.

2.1.3 Recovery of cells from liquid nitrogen stock

The cell stock was taken out from the liquid nitrogen tank and was placed into 37°C water bath immediately. After the cell stock was thawed, 10ml RPMI 1640 with 10% v/v FBS was put into a 15-ml centrifuge tube. The 15-ml centrifuge tube was centrifuged at 1000 rpm in a CR412 desktop centrifuge (Jouan, USA) for 5 minutes at room temperature. After centrifugation, the supernatant was discarded to remove DMSO and the cell pellet was re-suspended using 4ml RPMI 1640 with 10% v/v FBS. The suspension was then transferred into a $25cm^2$ flask and incubated in the incubator. The culture medium was changed in the next day to remove residual DMSO and dead cells. When cells reached about 80%-90% confluence, the cells were passaged. After 2 passages, the cells can be used for experiments.

2.2 Collection of insulin secretion and measurement of insulin concentration

2.2.1 Preparation of isolated islets

Animal experimentation was conducted according to institutional guidelines. Healthy adult male Wistar Albino rats with the body weight between 200 to 300g and DeltaF-508 CFTR mice with different genotypes were used in our experiments. Animals were housed under controlled temperature

(23°C) with free access to water before surgery. After the rats were anesthesized with mixture of xylazine and ketamine (13 and 87 mg /kg body weight, respectively, im), the abdomen was opened by a midline abdominal incision to expose the pancreas. After ligation of bile duct nearby the pancreatic duct, a pancreatic duct cannula was made by inserting a syringe needle at the junction between the pancreatic duct and the duodenal wall for injection of 8-10ml or 2ml collagenase IV dissolved in D-Hank's solution with the concentration of 0.5mg/ml for rats and mice respectively. When the pancreas expanded, the pancreas were removed from rats or mice and put into a centrifuge tube with appropriate Hank's solution. Then the centrifuge tube was put in a 37°C water bath and incubated for 10 minutes. After shaking the centrifuge tube for 10 seconds to turn the pancreas into pieces, add appropriate Hank's solution with 10% v/v FBS to neutralize the collagenase and centrifuge the tube at 1000 rpm for 5 minutes. After centrifugation, the supernatant was removed and the cell pellet was washed with Hank's solution without FBS. Then the cell pellet was re-suspended in the RPMI 1640 culture medium with 50 unit/ml penicillin-streptomycin and 10% v/v FBS. The suspension was put in 90mm Petri dish and the islets were picked up under light microscope. These islets were used after one day's incubation in the incubator. Hank's solution contains the following ionic composition (g/l): NaCl, 8.0; KCl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.1; $MgCl_2 \cdot 6H_2O$, 0.1; $Na_2HPO_4 \cdot 2H_2O$, 0.06; $KH_2PO_4 \cdot 2H_2O$, 0.06; $CaCl_2$, 1.85; $NaHCO_3$, 0.35; glucose, 0.3. Calcium and magnesium were removed in D-Hank's solution. The pH of the solution was adjusted to 7.4. The osmolarity of the solution was kept between 290-310mOsm.

2.2.2 Perfusion of islets

The isolated islets from normal adult rats were divided into five groups. The isolates islets from CF mice were divided into two groups. The treatment in every group and all the solutions used in the perfusion of islets were list in Table 2.1 and 2. Pancreatic isolated islets were put in a small chamber and were perfused using a peristaltic pump under room temperature. The perfusion of isolated islets

was maintained at a flow rate of 1ml/min, which did not induce the impairment of insulin secretion. After beginning of the perfusion of islets, the first over 30 minutes were regarded as an equilibration stage to stabilize the physiological function of islets. Subsequently, the effluent fluid was collected before challenge of glucose or HCO_3^- as the baseline insulin secretion. And then the insulin secretion at time points of 0 minute, 5 minutes and 60 minutes after challenge were collected. The collecting time lasted for 1 minute, i.e, 1ml effluent fluid was collected every time. The effluent fluid was put into 1ml-centrifuge tube and kept in -80°C freezer for the measurement of insulin concentration.

2.2.3 Collection of insulin secretion secreted from RIN-5F cell line

After the RIN-5F cells were digested from flask, these cells were cultured in 24-well plate with the concentration of 1.5×10^6 every well. After two days, the culture medium was removed and the cells were washed by HCO_3^--free solution twice to remove residual culture medium. Then these cells were divided into two groups and added with different solutions. In control group, the HCO_3^--free solution was added which contains the following ionic composition (mM): NaCl, 130; KCl, 5; Hepes, 20; $CaCl_2$, 2.5; $MgCl_2$, glucose, 10mM. In HCO_3^- group, 40mM NaCl was replaced by 40mM $NaHCO_3$. The osmolarity of the solution was kept between 290- 310mOsm. During the experiments, the cells in control group and HCO_3^- group were put in incubator gassed with air and 5% CO_2 respectively at the temperature of 37°C. After incubation for one hour, the solution was transferred into 1ml-centrifuge tube and kept in -80°C freezer for subsequent assay for insulin concentration.

2.2.4 Measurement of insulin concentration

Insulin ELISA kit purchased from Mercodia (Sweden, Cat. No.:10-1149-10) was used to measure insulin concentration. The procedures were performed according to the guideline of the kit.

2.3 Manipulation of protein

2.3.1 Preparation of RIN-5F cell lysates

RIN-5F cells were cultured in six-well plate with 2ml culture medium in every well. After three days, the culture medium was removed carefully from adherent cells and then the cells was washed with 1ml HBSS to remove culture medium. After removal of HBSS, 250μl trypsin-EDTA solution for every well was added. After several minutes, 500μl culture medium was put into every well and transferred the mixture of trypsin-EDTA solution, culture medium and RIN-5F cells into a microcentrifuge tube and then tube was centrifuged at 13,000 rpm in a desktop centrifuge (Eppendorf, Germany) for 5 minutes. The supernatant was discarded to remove culture medium and trypsin. The cell pellet was re-suspended with 1ml PBS and was centrifuged again. The supernatant was discarded to remove residual culture medium and trypsin. The cell pellet was re-suspended with one time pellet-volume of radioimmunoprecipitation assay (RIPA) lysis buffer and then was incubated on ice for 30 minutes. All the RIPA lysis buffers were supplemented with *Pimix* (1:200 dilution) and 0.1M *PMSF* (1:100 dilution) to inhibit proteases. The mixture of RIN-5F and RIPA lysis was centrifuged at 13,000 rpm for 30 minutes at 4°C and the supernatant was collected and transferred to a new microcentrifuge tube for protein concentration measurement and the pellet was discarded. The measurement of protein concentration of the lysate was described in the following. To confirm the expression of expected proteins, the lysate was analyzed by SDS-PAGE, a kind of gel electrophoretic system for analyzing proteins, followed by western blotting.

2.3.2 Determination of protein concentration

Bradford protein assay system (Bio-Rad, USA; Catalog#:500-0006) was used to determine the protein concentration of cell lysates.

Firstly, we prepared a series of standard protein solutions using bovine serum albumin (BSA) diluted with distilled water to final concentrations of 0 (saline only), 2, 4 and 8mg BSA/l. Add 1μ l of each of the above protein standard solution and lysates to different wells of 96-well plate. Secondly,

added 200μl of diluted dye reagent (1:5 dilution with distilled water) and incubated at room temperature for over 5 minutes and then adjusted the spectrophotometer to a wavelength of 595 nm. Finally, waited for 30 seconds and read each of the standards and samples at 595nm wavelength. The readings of the serially diluted protein standard solution were plotted with linear regression to get the reference and the concentrations of these samples were calculated according to the reading results of the samples relative to the protein standards.

2.3.3 SDS-PAGE

In general, proteins in SDS-sample buffer were separated on 8% or 10% polyacrylamide gels. For preparation of separating gel mix, the pre-mixed separating gel mix (5 ml) was mixed with 25 μl of 10% w/v APS (Ammonium persulfate) and 2.5 μl of TEMED, and incubated to polymerize at room temperature. After the separating gel mixture was polymerized, the stacking gel was prepared. To get the stacking gel, 2ml stacker solution was mixed with 20μl of 10% w/v APS and 2μl of TEMED. Then the stacking gel mixture was put on the top of the polymerized separating gel carefully and a 14-well comb was inserted into the stacking gel gently. After the stacking gel mixture was polymerized, the comb was removed from the stacking gel mixture. Then the lysate sample and protein marker were put into the small wells in the stacking gel produced by the insertion of comb. The tank was filled with SDS-PAGE running buffer completely. After assembling the gel to a CBS SG-125 (CBS Scientific, USA) apparatus, the protein in the lysates samples were separated by a constant current of 25mA per gel.

2.3.4 Western blotting

The transferring of proteins from polyacrylamide gels to Hybond-ECL nitrocellulose membranes was performed using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, USA) (Amersham, USA).

After a stack of six layers of filter paper (Whatman, UK) was steeped completely using protein transfer buffer, the six-piece filter paper stack wase placed into the bottom part of the apparatus. A nitrocellulose membrane of the same size with the polyacrylamide gel was soaked with distilled water and then was steeped by transfer buffer and, in turn, was placed on the top of the six-piece filter paper stack. Then the polyacrylamide gel was placed on the nitrocellulose membrane gently. Another stack of six layers of filter paper was soaked completely and then was put on the top of polyacrylamide gel carefully. The mixture of the above was assembles and the current of $0.8mA/cm^2$ was applied for transfer for one and a half hours. After the transfer finished, put the nitrocellulose membrane in 4% milk in Tris Buffered Saline-Tween 20 (TBST) buffer for incubation for one hour for the purpose of blocking. Then the nitrocellulose membrane was then put in 2% milk TBST buffer containing the primary antibody with the appropriate dilution. After incubation in cold room with shaking overnight with the room temperature of 4°C, the nitrocellulose membrane was washed using TBST for four times with 5 minutes each time. The nitrocellulose membrane was then put in 2% milk in TBST including the secondary antibody combined with horse reddish peroxidase (Amersham Biosciences, USA) at the appropriate concentration at room temperature for 2 hours. After incubation finished, the nitrocellulose membrane was washed using TBST for six times with 5 minutes each time. After washing, the nitrocellulose membrane was incubated with 1ml ECL Western Blot Detection Reagent (Amersham Biosciences, USA) for 1 minute and then was exposed to light-sensitive films (Fuji, Japan). The exact time of exposure was determined by the intensity of the signals on the films. After exposure, the development of the films was performed using a filmprocessing machine (Eastman Kodak, USA).

2.4 Electrophysiological Experiments

2.4.1 Preparation of hippocampal slices

Acute hippocampal brain slices were used in electrophysiological experiments. After anesthesia

deeply with isoflurane (Minrad, Inc) (the death should be prevented), the rats was decapitated immediately and then put the head in the ice-cold artificial cerebrospinal fluid (ACSF) with the bubble of 95% O_2 and 5%CO_2 to decrease the metabolism of brain. ACSF contains the following ingredients (mM): NaCl, 120, KCl, 2, $MgSO_4 \cdot 7H_2O$, 1.2, KH_2PO_4, 1.2, $NaHCO_3$, 26, $CaCl_2$, 2.5, glucose, 11. In the cold ACSF, the brain was taken out from the skull as soon as possible. After immergence for 1 minute in the iced ACSF, the brain was removed to an ice-cold platform and then was cut along the midline to exposure the hippocampus. The two halves of the brain were fixed on a platform in a box filled with ice-cold ACSF bubbled with 95% O_2 and 5%CO_2. Then the hippocampus was sliced at the thickness of 300μm using a motorized vibrating microtome (Integraslice, Campden, UK). In general, only the middle part of hippocampus was used in our experiments and the other parts were discarded. The prepared slices were transferred to a glass box full of oxygenated ACSF under room temperature and were trimmed using scalpel to get the slices containing the main structure of hippocampus, especially the CA1 area. At last, the trimmed slices were gently transferred to a beaker filled with ACSF bubbled by of 95% O_2 and 5%CO_2 under the surrounding temperature of 37℃. The incubation time in the beaker was longer than 2 hours to remove the effect of stress on slices induced by the operation. The whole process which was from the decapitation to the accomplishment of prepared slices should be finished within 20 minutes to avoid hypoxia impairement.

2.4.2 Multi-electrode recording setup

The field excitatory postsynaptic potentials (fEPSCs) of hippocampal slices were recorded from CA1 region by a relatively new planar multi-electrode recording system which is called MED 64 system (Alpha Med sciences Co., Ltd, Tokyo, Japan). The MED64 system is a 64 planar microelectrodes array system and is composed of four parts: the integrated amplifier (SU-MED640), splitter (Med B02), connector, probe and the related software (performer 2.0, Conductor and MED 64 Recorder for evoked response) for the management of the MED64 system. The integrated amplifier

includes internal stimulator which can stimulate and record the signals from all the electrodes of the 64 array transmitted from the probe. The splitter is an optional component of the system which is connected to the integrated amplifier and divided the 8×8 array into 4 independent grids with each of them including 4×4 microelectrodes. Therefore, it is possible that the signals from 4 slices in 4 different probes are monitored and recorded simultaneously. The connector is an intermediator between the MED probe and the amplifier for the transmission and provides a stable platform for the MED probe. The Med probe is used for the transmission of stimulation to the hippocampal slice and the recording of fEPSPs of hippocampus, and then transmits them to the connector. There are several types of probes for the experiments and P210A is used in our experiments. P210A has 20μm of array size and 100μm of interpolar distance which is suitable for the hippocampal slices of rats.

2.4.3 Perfusion of slices

To prepare the perfusion of the hippocampal slices, the ACSF was warmed in 37℃ water bath and bubbled with 95% O_2 and 5%CO_2 15 minutes before the slices were transferred to the Med probe from incubating beaker. The probe was filled with warmed and oxygenated ACSF. After the slices were taken out of the incubating beaker gently, the slice was put in the probe quickly. Under the monitoring of microscope (MIC-D, Olympus Ltd., Japan), the slice was manually moved to the correct position to make sure that the CA1 area of hippocampus was in the centre of the microelectrode arrays. The image of the slice in the probe was taken for reference during the stimulation. Subsequently, the probe was placed in the connector and the perfusion of ACSF with the temperature of 37℃ and the mixture of 95% O_2 and 5%CO_2 was continued at the rate of 1.5ml/minute.

2.4.4 Induction of early-phase long-term potentiation

The early-phase long-term potentiation (E-LTP) was recorded in our experiments. Firstly, the appropriate stimulation electrode and recording electrode were determined according to the shape of

the fEPSPs and the position of electrodes in the probe. Secondly, the stimulus intensity was 30-40% of that causing maximum response. After a stable baseline for over 30 minutes, a conventional high frequency stimulation (HFS) containing a train with the 100Hz stimulation was applied and then he fEPSPs was recorded for over 1 hour. E-LTP was quantified as the percentage the average magnitude of the fEPSPs during 50-60 minutes after HFS over that of baseline within the 30 minutes before HFS.

2.5 Measurement of intracellular Ca^{2+}

RIN-5F cells grown on coverslips were used for measurement of intracellular Ca^{2+}. The coverslips were sterilized using 70% alcohol for 1 day and then were put into 35mm Petri dish. After the RIN-5F cells were digested from flask, these cells were cultured on coverslips after washing off residual trypsin and culture medium. After one day's growth, the coverslips were washed three times using PBS and then the mixture of 1ml HCO_3^--free solution, 3μM Fura-2-acetoxy-methylester (Fura-2AM) and 1.6μM pluronic F127 was put in the Petri dish. After incubation for over 45 minutes, the coversilp was taken from Petri dish and put in a specially designed miniature chamber. The measurement of intracellular Ca^{2+} concentration was recorded in an inverted Olympus 1X70 microscope equipped with a CCD camera. Fura-2AM crosses cell membranes and the acetoxymethyl groups are cut by intracellular esterases in the interior of the cells. The maximum excitation wavelengths for fura-2-Ca^{2+} and fura-2-complex are 380nm and 340nm. The ratio of the emissions at those wavelengths was directly proportional to the amount of intracellular Ca^{2+} concentration and the images were captured by using MetaFluro from Universal Imaging Corp. (Washington, DC). The HCO_3^--free solutions used in the experiments are listed in Table 2.3. All the solutions were gassed with air because of the omission of HCO_3^-.

2.6 Statistical analysis

One-Way ANOVA was used for the comparison of the measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

and the magnitude of LTP. Repeated measurement ANOVA and paired *t*-test are used for the insulin secretion induced by HCO_3^-. Tests for equal variances and normality should be performed before One-Way ANOVA and Repeated measurement ANOVA. Non parametric test was used when heterogeneity of variance and/or asymmetrical distribution were found. $P<0.05$ is considered a statistical significance.

Composition (mM)	NaCl	Glucose	$NaHCO_3$
A solution (low glucose concentration without HCO_3^-)	130	3.3	
B solution (high glucose concentration without HCO_3^-)	130	20	
C solution (high glucose concentration with 60mM HCO_3^-)	70	20	60
D solution (low glucose concentration without HCO_3^-)	130	3.3	
E solution (low glucose concentration with 60mM HCO_3^-)	70	3.3	60
F solution (low glucose concentration with 25mM HCO_3^-)	105	3.3	25
G solution (low glucose concentration with 20mM HCO_3^-)	110	3.3	20
H solution (low glucose concentration with 40mM HCO_3^-)	90	3.3	40

Table 2.1 The composition of different solutions used for the perfusion of isolated rats. All the solutions also contain the following composition: KCl, 5mM; Hepes, 20mM; $CaCl_2$, 2.5mM; $MgCl_2$, 1mM; 0.1%BSA. The pH of the solution was adjusted to 7.4. The osmolarity of the solution was kept between 290- 310 mOsm.

	Treatment	Equilibtation	Challenge
Glucose challenge for healthy rats	3.3mM glucose to 20mM glucose without HCO_3^-	A solution	B solution
	3.3mM glucose without HCO_3^- to 20mM glucose with 60mM HCO_3^-	A solution	C solution
HCO_3^- challenge for healthy rats	0mM HCO_3^- to 25mM HCO_3^-	D solution	F solution
	20mM HCO_3^- to 40mM HCO_3^-	G solution	H solution
	40mM HCO_3^- to 60mM HCO_3^-	H solution	E solution
HCO_3^- challenge for CF mice	20mM HCO_3^- to 40mM HCO_3^-	G solution	H solution

Table 2.2 The treatment on isolated islets in groups of perfusion experiments.

Composition(mM)	normal	Na+-free	Cl^--free	Low Cl	Ca^{2+}-free
NaCl	130				130
KCl	5	5			5
HEPES	20	20	20	20	20
Glucose	10	10	10	10	10
$CaCl_2$	2.5	2.5		2.5	
$MgCl_2$	1	1			1
NMDG-Cl		130			
Na-gluconate			130	130	
K-gluconate			5	5	
1/2Ca-gluconate			5		
$MgSO_4$			1	1	

Table 2.3 The ionic composition of different solutions used in the experiments. The osmalarity in these solutions were regulated to 290-310 mOsm using D-mannitol.

CHAPTER 3

THE EFFECT OF HCO_3^- ON INSULIN SECRETION

3.1 Summary

Cystic fibrosis (CF), which is caused by the deficiency of cystic fibrosis transmembrne conductance regulator (CFTR), is the most common autosomal recessive systemic disease with an incidence of 1: 2500 in Caucasians. Cystic fibrosis-related diabetes (CFRD), as one of the complications of CF patients, is regarded as the leading co-morbidity in CF patients. The mechanism of CFRD is attributed to the reduced number of islets as a result of pancreatic fibrosis caused by the loss of CFTR in pancreatic duct. However, the above mechanism failed to explain the dynamics of insulin secretion induced by glucose tolerance test (GTT) in some CF patients and therefore, we were forced to re-consider the mechanism for the pathogenesis of CFRD. Interestingly, the following facts imply that perhaps there is another mechanism for the onset of CFRD: decreased insulin secretion was observed in metabolic acidosis with decreased plasma HCO_3^- concentration, plasma HCO_3^- level increased accompanied by the elevation of plasma insulin after food intake and CFTR accounted for HCO_3^- transport in many epithelial cells. These facts promoted us to hypothesize that the loss of HCO_3^--induced insulin secretion resulting from the deficiency of CFTR is an alternative mechanism for the onset of CFRD. Our results showed that HCO_3^- potentiated glucose-induced insulin secretion of isolated islets from healthy rats. In the absence of glucose challenge, HCO_3^- dose-dependently caused insulin secretion. Using CF animal model, we showed that insulin secretion of islets isolated from DeltaF508-CFTR mutation and wild type mice increased by about 46.7±13.1% and 100.7±1.2% respectively, however there was no significant difference between the two groups. In conclusion, our results showed that HCO_3^- could induce insulin secretion irrespective of glucose challenge, which may be helpful for the treatment of patients with HCO_3^- deficiency. The role of CFTR in the HCO_3^- effect on insulin secretion should be investigated by further study.

3.2 Introduction

Cystic fibrosis (CF) is the most common autosomal recessive systemic disease with an incidence of 1: 2500 in Caucasians (Brennan et al., 2004). CF is derived from the dysfunction of cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated Cl^- channel (Morales et al., 1999). As CFTR is widely distributed in the body, the deficiency in CFTR leads to many clinical complications in CF patients, including chronic respiratory symptoms, pancreatic insufficiency, bile duct obstruction, male and female infertility, high Cl^- concentration in the sweat and intestinal obstruction (Chan et al., 2006; Rowntree et al., 2003).

In 1955, CF-related diabetes (CFRD) was described as one of the complications of CF patients for the first time (Shwachman et al., 1955). From then on, the number of CF patients developing diabetes mellitus (DM) increased when the life expectancy of CF patients increased from 1 year to 37 years. Recently, the incidence of CFRD is even increased to about 43% of CF patients and CFRD has been regarded as the leading co-morbidity in CF patients (Rana et al., 2010; Moran et al., 1998; Alves et al., 2007; Costa et al., 2005).

CFRD is characterized by chronic hyperglycaemia resulting from the decreased insulin secretion and is distinct from both Type 1 diabetes and Type 2 diabetes (Moran et al., 2002). Owing to the deficiency in glucose homeostasis, CFRD patients have a much higher mortality rate than that of CF patients without DM (Finkelstein et al., 1988). Therefore, understanding the pathogenesis of CFRD may help to decrease the mortality rate and increase the life expectancy of CFRD patients.

It is well accepted that the possible reason for impaired insulin secretion in CF patients originates from the deficiency of CFTR in pancreatic duct cells. The deficiency of CFTR results in the decrease of pancreatic juice secretion due to the reduced HCO_3^- level and subsequent obstruction of pancreatic duct (Gaskin et al., 1982; Brennan et al., 2004). The accumulation of proteases and lipases in the pancreatic duct cause decreased insulin secretion as a result of reduced number of islets (Hinds et al., 1991; Hamdi et al., 1993; Soejima et al., 1986; Iannucci et al., 1984). However, in Rakotoambinina's

study, it is found that only the first phase insulin secretion in CF patients was impaired and the second phase insulin secretion was not (Rakotoambinina et al., 1994), which is not consistent with the above explanation. Therefore, perhaps there is another mechanism contributing to the onset of CFRD and metabolic acidosis can provide some clues.

Clinically, metabolic acidosis is characterized by low blood pH and decreased HCO_3^- concentration in plasma with the decreased insulin secretion (Bigner et al., 1996; Kraut et al., 2010; Bigner et al., 1996). Obviously, low blood pH is not the mechanism of the decreased insulin secretion as acidification is considered a stimulating factor of insulin secretion (Rebolledo et al., 1983, 1978; Ohta et al., 1991). Therefore, it seems that the decreased HCO_3^- level is responsible for the impaired insulin secretion of islets β-cells. Combined with the fact that CFTR plays a great role in the transport of HCO_3^- across the plasma membrane (Hug et al., 2003), we hypothesize that HCO_3^- has the ability to stimulate insulin secretion of β-cells in which CFTR perhaps is involved.

3.3 Materials

3.3.1 Animals

Male healthy adult Wistar Albino rats weighing between 200 to 300g were supplied by the laboratory animal service center (LASEC) of the faculty of medicine, the Chinese University of Hong Kong (CUHK). Animals were maintained in a room with fixed temperature of 23°C, the controlled humidity and a 12-h cycle of light/dark. Animals were housed in polycarbonate cages and had free access to food and water. A lot of bedding of pine shavings was put on the bottom of the cages. The bedding is changed once in three days.

DeltaF508-CFTR mutation mice, a CF animal model in which CFTR protein contains the mutation of Delta-F508, were used in my experiments. This CF animal model is caused by deletion of a 3 base-pair (CTT) deletion in exon 10 of the CFTR gene, leading to the loss of codon 508 (phenylalanine) of CFTR protein (Okay TS et al., 2005). DeltaF508-CFTR mutation mice were

maintained by LASEC under the condition described above. (Animal license No: (07-240) in DH/ORHI/8/2/1 pt. 10))

3.3.2 Biochemicals and reagents

Collagenase Ⅳ, NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $Na_2HPO_4 \cdot 2H_2O$, $KH_2PO_4 \cdot 2H_2O$, $CaCl_2$, $NaHCO_3$, glucose, Na-gluconate, Ca-gluconate, K-gluconate and NMDG were purchased from Sigma Aldrich (USA). Ethanol was obtained from MERK (Germany).

3.4 Results

3.4.1 Insulin secretion induced by HCO_3^- in the presence of glucose challenge

We first investigated the effect of HCO_3^- on insulin secretion of isolated rat islets upon glucose challenge using perfusion technique. The glucose-induced insulin secretion is characterized by a first phase in which the peak response occurs from 3 to 7 minutes and a subsequent slow increase to the second peak response (Curry et al., 1968; Lacy et al., 1972; Vila et al., 2010).

In our study, there was a significant increase in insulin secretion from 0.52 ± 0.02ng/ml to 0.79 ± 0.09ng/ml 5 minutes after elevating the glucose concentration from 3.3mM to 20mM in the absence of HCO_3^- (Figure 3.1A，n=4, $P<0.05$). At the same time point after elevation of glucose level combined with addition of 60mM HCO_3^-, the insulin secretion increased significantly from 0.47 ± 0.06ng/ml to 1.04 ± 0.15ng/ml (Figure 3.1A, n=4, $P<0.01$). Although glucose challenge could promote insulin secretion irrespective of the presence of HCO_3^-, the degree of the increase in insulin secretion in HCO_3^-–free group was only 49.8% over the baseline, which is much lower than that of HCO_3^--treated group (118.7%)(Figure 3.1B, $P<0.01$), indicating that HCO_3^- could enhance glucose-induced insulin secretion of the first phase.

However, as observed in Figure 3.1A, it seemed that the second phase response to glucose challenge disappeared in my study. Sixty minutes after glucose load during when the second phase

occurs, there was an obvious but not significant increase in insulin secretion over the baseline in HCO_3^--free group (40.8%, P>0.05, Figure 3.1A). Similarly, in the HCO_3^--treated group, a slight and non-significant increase in insulin secretion during the second phase was observed (8.8%, P>0.05, Figure 3.1A).

The above results showed that HCO_3^- could increase the glucose-induced insulin secretion in the first phase but not in the second phase after a "square wave" stimulation of higher glucose concentration. However, the above experiments were performed in the presence of glucose load and we could not determine the effect of HCO_3^- alone on insulin secretion. Therefore, the role of HCO_3^- in the insulin secretion in the absence of glucose challenge was investigated in the following experiments.

3.4.2 Insulin secretion induced by HCO_3^- in the absence of glucose challenge

In these experiments, the islets were treated by HCO_3^- challenge in the absence of glucose. Five minutes after shift of HCO_3^- concentration from 0, 20, and 40mM to 25, 40 and 60mM respectively, the insulin secretion increased from 0.74±0.08ng/ml, 0.32±0.02ng/ml and 0.50±0.02ng/ml to 1.79 ±0.32ng/ml (n=3), 0.59±0.07ng/ml (n=3) and 0.58±0.07ng/ml (n=5), increased by about 139%, 80.5% and 15.6% respectively (Figure 3. 2A, B, C and D, P<0.05), indicating that HCO_3^- alone could induce insulin secretion in a concentration-dependent manner. Interestingly, the degree of the increase in insulin secretion also decreased with the raise of HCO_3^- level (Figure 3. 2D), suggesting that the maximum effect of HCO_3^- on insulin secretion happened in the range from 0 to 25mM.

Sixty minutes after increase in HCO_3^- level from 0, 20, and 40mM to 25, 40 and 60mM respectively, the insulin secretion were 0.73±0.21ng/ml, 0.36±0.06ng/ml and 0.51±0.03ng/ml respectively, there were no statistical significances observed in comparison to the baseline (Figure 3. 2A, B and C, P>0.05), demonstrating that HCO_3^- could not enhance insulin secretion 60 minutes after challenge.

3.4.3 The role of CFTR in the HCO_3^-–induced insulin secretion

In many epithelial tissues, the transport of HCO_3^- across plasma membrane is CFTR-mediated and thus we hypothesized that CFTR was involved in the insulin secretion induced by HCO_3^-. To test this hypothesis, DeltaF-508 CFTR mice, a kind of CF animal model, was used. In Figure 3. 3A, the isolated islets from wild type mice showed an increase in insulin secretion from 0.478±0.021ng/ml to 0.959±0.036ng/ml 5 minutes after elevation of HCO_3^- level from 20 to 40mM (n=2). In the group of DeltaF-508 CFTR mice, the same treatment increased the insulin secretion from 0.477±0.012ng/ml to 0.701±0.072ng/ml (n=4). However, there was no statistical difference in the increase in insulin secretion between wild type and DeltaF-508 CFTR mice (Figure 3. 3B, 100.7±1.2% vs 46.7± 13.1%).

Sixty minutes after HCO_3^- challenge, the insulin secretion in wild type and DeltaF-508 CFTR mice were 0.504±0.031ng/ml/min and 0.465±0.006ng/ml/min respectively, with no statistical changes compared to that of baseline.

3.5 Discussion

3.5.1 The physiological significance of HCO_3^--induced insulin secretion

HCO_3^-, which has a concentration of about 20mM in the plasma, is a very important anion in the body and has many physiological functions, such as regulating the extracellular and intracellular pH and osmolarity, involvement in the processes of spermatogenesis and sperm capacitation, promoting digestion of food in the duodenum and so on (Xu et al., 2007; Chen et al., 2009). The level of HCO_3^- in plasma is affected by many factors, including breath, sports and food intake. It is well known that food intake leads to an increase in plasma HCO_3^- concentration from 20 to 25 mM, which is called "postprandial alkaline tide" (Vaziri et al., 1980; Ozaki J et al., 2000). Interestingly, food intake can also induce insulin secretion from β-cells in the pancreas. The time-dependent similarity between insulin secretion and HCO_3^- level promotes us to consider if there is relationship between them. Since HCO_3^-

in the plasma can reach most of the tissues in the body, of course, including pancreatic β cells, an interesting question is raised whether increased HCO_3^- level plays a significant role in the regulation of insulin secretion after food intake?

In our studies, addition of HCO_3^- enhanced insulin secretion of isolated islets from normal rats in the presence or absence of glucose load, demonstrating that HCO_3^- may play an important role in the regulation of insulin secretion after food intake. This idea is supported by the clinical features of metabolic acidosis.

Metabolic acidosis is characterized by the decreased pH resulted from reduced HCO_3^- concentration in plasma (Kraut et al., 2010). It is found that glucose tolerance test caused a higher plasma glucose level and lower insulin secretion in metabolic acidosis cows than those in normal controls, suggesting that the insulin secretion was impaired during metabolic acidosis. The impaired insulin secretion was partly restored by oral administration of $NaHCO_3$ before glucose tolerance test (Bigner et al., 1996), which is consistent with the fact that HCO_3^- has the ability to stimulate insulin secretion.

3.5.2 The effect of HCO_3^- in different phases on insulin secretion

Interestingly, our study also showed that HCO_3^- promoted insulin secretion 5 minutes rather than 60 minutes after HCO_3^- challenge, what does such difference between the two time points mean? The time points of 5 and 60 minutes belong to different phases of insulin secretion induced by glucose. After stimulation of a sudden and long lasting increase in the glucose concentration which is also called "square wave" stimulation, the increased insulin secretion expressed a biphasic model which has been proved by many subsequent studies (Iversen et al., 1971; Gerich et al., 1974). The insulin secretion increased quickly to the first peak response in 10 minutes which is called first phase followed by a slow down and then increase again at a slow rate or keep a sustained plateau depending on the species used. The biphasic model of insulin secretion was found using isolated and perfused rat

pancreas in vitro (Curry et al., 1968) and then in perfused rat islets (Lacy et al., 1972) and normal human subjects (Vila et al., 2010; Chepurny et al., 2010; Boston et al., 2009). Thus, the different effects of HCO_3^- at different time points showed that HCO_3^- could promote insulin secretion during the first phase.

The results about the second phase insulin secretion observed in our study were unexpected. As observed in Figure 3. 1A, the insulin secretion 60 minutes after glucose challenge failed to exhibited statistical increase over the baseline. The possible reason for this phenomenon is different experimental conditions compared the other laboratories. However, the two groups were performed under the same experiments conditions and therefore, the fact that HCO_3^- did not exert any effect on insulin secretion during the second phase was suggested.

3.5.3 The physiological significance of first phase insulin secretion induced by HCO_3^-

The first phase insulin secretion lasts only for several minutes, and thus the following question is raised: does the first phase insulin secretion play an important role in the regulation of glucose metabolism in the body?

Although the first phase of insulin secretion lasts for a very short time, the quick insulin secretion in response to elevation in glucose after meal can decrease the plasma glucose concentration promptly to prevent the impairment of tissues induced by high glucose concentration. This idea is suggested by the fact that some patients with IGT or type 2 diabetes were characterized by the absence of the first phase insulin secretion without any decrease of the second phase insulin secretion after intravenous glucose load (Rave et al., 2010; Salinari et al., 2009; Emerson et al., 2009). The inhibitory effect of the first phase insulin secretion on the elevation in plasma glucose concentration induced by glucose challenge and the inverse correlation between the plasma glucose concentration during the second hour and the plasma insulin level 30 minutes after oral glucose test provide substantial evidences for the above idea (Mitrakou et al., 1992; Bruce et al., 1987).

However, it is well documented that the first phase insulin secretion is mainly caused by glucose, and therefore, the question is raised, can the deficiency in HCO_3^--induced insulin secretion cause defective insulin secretion after a meal? Namely, does HCO_3^-–dependent insulin secretion play a physiological role in the glucose metabolism?

In cows with metabolic acidosis, decreased insulin secretion was observed only in less than 60 minutes after oral glucose test, especially at the 30 minutes, suggesting that the deficiency of first phase insulin secretion could be due to decreased HCO_3^- level (Bigner et al., 1996). Thus, the increase in HCO_3^- concentration after food intake may play a physiological role in the regulation of glucose homeostasis.

3.5.4 The role of HCO_3^--induced insulin secretion in CFRD

There is evidence showing that CFTR play a significant role in the transport of HCO_3^- across plasma membrane (Mizumori et al., 2009; Wheat et al., 2000; Hug et al., 2003) and therefore, we hypothesized that CFTR was involved in the effect of HCO_3^- by mediating its transport. In our study, the increase in HCO_3^-–induced insulin secretion was about 100.7% in wild type mice, which is twofold of that in CFTR mutant mice (46.7%). Unfortunately, the statistical significance between the two groups was not found. There are two possibilities about the statistical result. One is that CFTR is not involved in the HCO_3^- effect. The other is that the number in the group of wild type mice is too small (n=2), which perhaps affects the statistical result. Therefore, this study failed to determine the role of CFTR in the effect of HCO_3^-.

Figure:

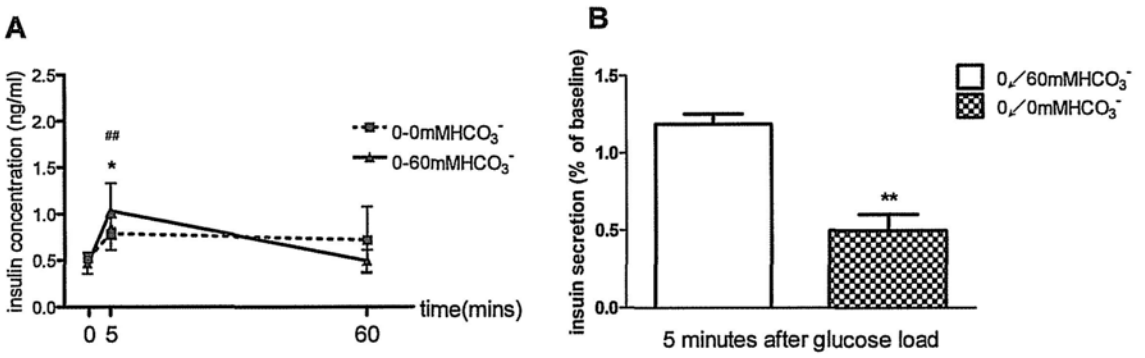

Figure 3. 1 The effect of HCO_3^- on insulin secretion in the presence of glucose challenge. A: There was an increase in insulin secretion compared with the baseline in the presence or absence of HCO_3^- application 5 minutes but not 60 minutes after glucose challenge. * *P<0.05* in HCO_3^--free group, ## *P<0.01* in HCO_3^- challenge group **B:** 5 minutes after glucose challenge, insulin secretion increased by about 118.7±6.5% (n=4) in the HCO_3^- challenge group, which is significantly higher than that in the HCO_3^--free group (49.8±10.4%, n=4). * *P<0.05*, Data were expressed as mean±S.E.M

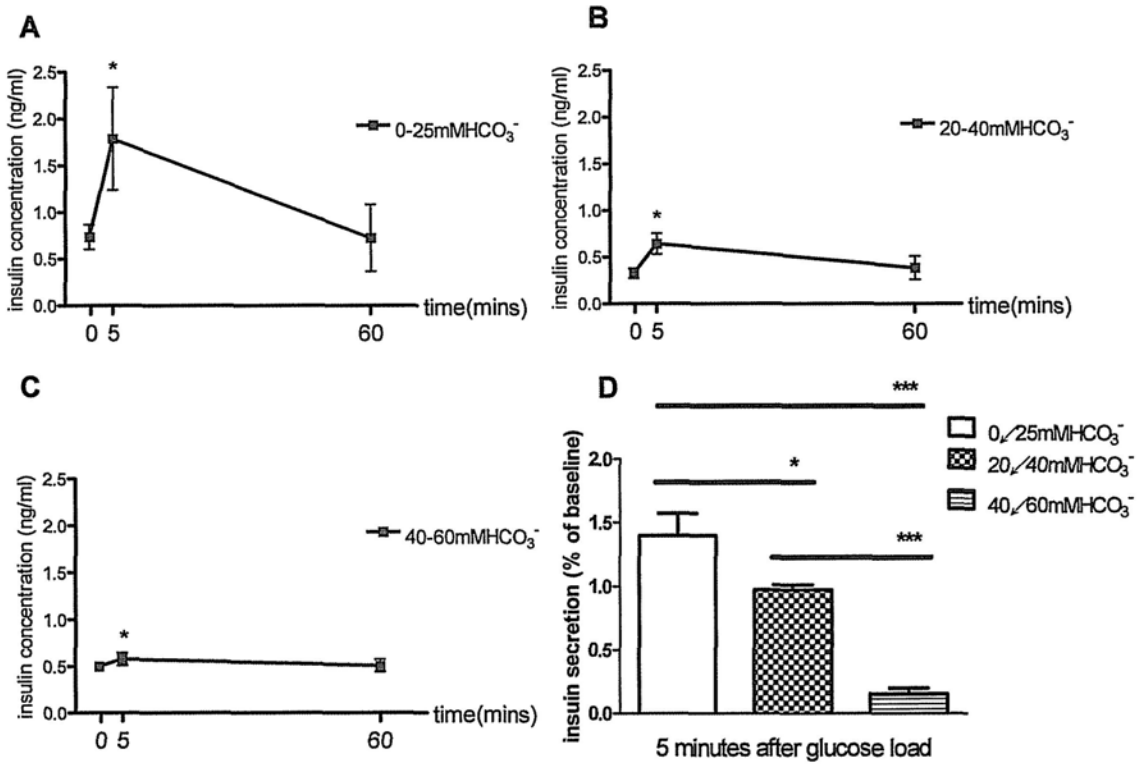

Figure 3. 2 The effect of HCO_3^- on insulin secretion in the absence of glucose challenge. A, B and C: The insulin secretion increased by about 139.7±17.4%, 97.0±4.0% and 15.5±3.9% respectively compared with the baseline secretion 5 minutes after HCO_3^- elevation but no change in insulin secretion were observed at 60 minutes. **D:** The degree of the increase in insulin secretion decreased with the raise of HCO_3^- concentration. Data were expressed as mean±S.E.M, * *P<0.05*, *** *P<0.001*

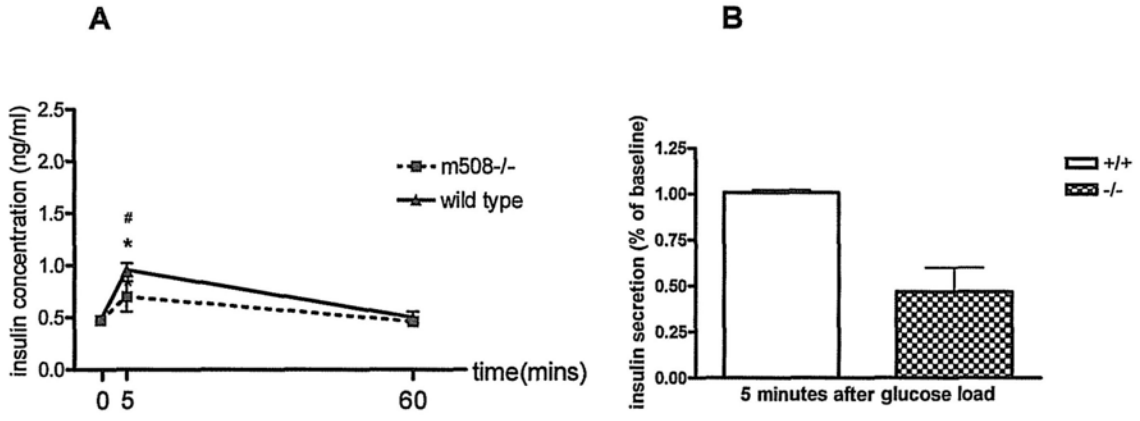

Figure 3. 3 The effect of HCO_3^- on insulin secretion of isolated islets from different genotypes of DeltaF-508 CFTR mice. A: There was an increase in insulin secretion 5 minutes rather than 60 minutes after elevation of HCO_3^- from 20 to 40mM compared with the baseline in the absence of glucose challenge. * *P<0.05* in DeltaF-508 CFTR mice group, # *P<0.05* in wild type group. **B:** 5 minutes after elevation of HCO_3^- from 20 to 40mM, the insulin secretion increased by about 100.7± 1.2% and 46.7±13.1% in wild type and DeltaF-508 CFTR mice respectively. Data were expressed as mean±S.E.M

CHAPTER 4

THE EFFECT OF HCO_3^- ON INTRAECELLULAR Ca^{2+} OF β-CELLS AND THE UNDERLYING MECHANISMS

4.1 Summary

As a new secretagogue for insulin secretion, the mechanism of HCO_3^--induced insulin secretion is unknown. There are evidences showing that HCO_3^- can activate intracellular soluble adenylyl cyclase (sAC) to increase cAMP level. Numerous studies suggested that cAMP could increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) through three different pathways, including closure of ATP-sensitive K^+ channel (K_{ATP} channel), activation of voltage-dependent Ca^{2+} channel (VDCC) and induction of Ca^{2+} release from intracellular stores. Thus, we hypothesized that HCO_3^- promoted insulin secretion via sAC-cAMP pathway. Since insulin exocytosis is the last step of insulin secretion and is triggered by $[Ca^{2+}]_i$, we investigated the effect of HCO_3^- on $[Ca^{2+}]_i$ and the underlying mechanisms using RIN-5F, an insulin-secreting cell line, to test our hypothesis. Addition of $NaHCO_3$ dose-dependently induced the increase in $[Ca^{2+}]_i$ but NaCl, Na-gluconate and Na_2HPO_4 failed to affect $[Ca^{2+}]_i$, suggesting that it was HCO_3^- responsible for the increase in $[Ca^{2+}]_i$. Removal of extracellular Ca^{2+} or addition of nifedipine, the blocker of L-type Ca^{2+} channel decreased the effect of HCO_3^- significantly, indicating the involvement of L-type Ca^{2+} channel. The inhibitory effect of $BaCl_2$ on the increase in $[Ca^{2+}]_i$ suggested the involvement of K^+ channel. The HCO_3^--induced increase in $[Ca^{2+}]_i$ was reduced by PKA inhibitor and sAC blocker, indicating that the pathway of sAC-cAMP-PKA was responsible for the effect of HCO_3^-. The reduction of extracellular Cl^- or the inhibitor of anion exchanger (AE) significantly blocked the $[Ca^{2+}]_i$ increase induced by HCO_3^- but the omission of external Na^+ failed. The facts that CFTR blocker decreased the effect of HCO_3^- markedly and the expression of CFTR in RIN-5F cells demonstrated by western blotting suggested the involvement of CFTR in mediating HCO_3^- effect. In conclusion, our results suggested the possibility that HCO_3^- enter β-cells through a

CFTR- and AE-dependent pathway, and then, induced the closure of K_{ATP} channel and subsequent activation of L-type Ca^{2+} for the influx of extracellular Ca^{2+}, in which intracellular sAC-cAMP may be involved. The involvement of CFTR in the HCO_3^--induced increase in $[Ca^{2+}]_i$ suggests that the lack of HCO_3^- effect due to deficiency in CFTR may be related to the onset of CFRD.

4. 2 Introduction

There are many physiological factors in the body which can enhance insulin secretion, including glucose, fatty acids, amino acids, glucagon, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), acetylcholine and HCO_3^- which is a new secretagogue (Haber et al., 2006). Although the mechanisms underlying insulin secretion induced by these factors may be different, the exocytosis of insulin is the last step. Insulin exocytosis is a process that is triggered by $[Ca^{2+}]_i$ (Hou et al., 2009). The facts that most of the above stimulating factors promote insulin secretion by increasing $[Ca^{2+}]_i$ via different pathways indicate that perhaps HCO_3^- can affect $[Ca^{2+}]_i$. However, the question is raised on how HCO_3^- increase $[Ca^{2+}]_i$ to regulate insulin secretion.

HCO_3^- can increase intracellular soluble adenylyl cyclase (sAC) activity in a pH-independent manner in vivo and in vitro (Litvin et al., 2003; Chen et al., 2000; Tresguerres et al., 2010). sAC is a protein with the molecular weight of about 48kD and is distinguished from the G protein-responsive transmembrane adenylyl cyclase (Litvin et al., 2003; Chen et al., 2000; Tresguerres et al., 2010). The activation of sAC catalyzes the conversion of ATP to cAMP and subsequently results in an increase in intracellular levels of cAMP. There are numerous studies showing that cAMP could increase $[Ca^{2+}]_i$ through three different pathways, including closure of ATP-sensitive K^+ channel (K_{ATP} channel), activation of voltage-dependent Ca^{2+} channel (VDCC) and induction of Ca^{2+} release from intracellular stores .

K_{ATP} channel is gated by intracellular ATP and plays a fundamental role in insulin secretion induced by some secretagogues. During the glucose-induced insulin secretion, the metabolism of

glucose in pancreatic β-cells increases the ratio of ATP:ADP and then closes K_{ATP} channel to depolarize the membrane. The depolarization leads to the activation of L-type Ca^{2+} channel with subsequent Ca^{2+} influx. The binding of GLP-1 to GLP-1 receptor could also inactivate K_{ATP} channel to induce the elevation of $[Ca^{2+}]_i$ and subsequent insulin secretion (Holst et al., 2007). Apart from ATP, increase in intracellular cAMP is an inhibitory regulator for the K_{ATP} channel on the membrane of β-cells (Holz et al., 1993; Kang et al., 2008).

Influx of Ca^{2+} through VDCC accounts for the insulin exocytosis in the process of glucose-induced insulin secretion. The Ca^{2+} current of VDCC could be potentiated by cAMP analogue as well as GLP-1 through PKA-dependent pathway (Kanno et al., 1998; Suga et al., 1997; Furman et al., 2010). It was observed that forskolin and IBMX induced the phosphorylation of the cardiac-type alpha 1 subunit of VDCC as well the insulin secretion in a kind of insulin-secreting cell line (Leiser et al., 1996).

Ca^{2+} release from intracellular stores is regarded as one mechanism for the increase in $[Ca^{2+}]_i$ after glucose stimulation (Varadi et al., 2004; Roe et al., 1993). GLP-1 was shown to enhance Ca^{2+} release from endoplasmic reticulum (ER) in a cAMP-PKA pathway by activation of ryanodine channel and/or through phosphorylation of the IP3 receptor on the ER (Kang et al., 2005; Holz et al., 1999; Islam et al., 1998; Dyachok et al., 2004; Tsuboi et al., 2003).

Since HCO_3^- can increase the production of intracellular cAMP which is a promoter of insulin secretion, we hypothesize that HCO_3^- induces the increase in $[Ca^{2+}]_i$ in a cAMP-dependent manner in which K_{ATP} channel, VDCC and CICR are perhaps involved.

4.3 Materials

4.3.1 Animals and cell line

Insulin-secreting cell line, RIN-5F, is used in my experiments, was purchased from ATCC (USA). The transplantable rat islet cell tumor is induced by radiation and then is transplanted to nude mouse.

Subsequently, the RIN-m cell line was established from the nude mouse and is cloned into RIN-5F cell line. The RIN-5F cell is a kind of insulin-secreting cell line derived from the RIN-m rat islet line.

4.3.2 Biochemicals and reagents

Collagenase Ⅳ, nifidipine, H_{89}, 2-HE, CFTR(inh)-172, NPPB, NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $Na_2HPO_4 \cdot 2H_2O$, $KH_2PO_4 \cdot 2H_2O$, $CaCl_2$, $NaHCO_3$, glucose, Na-gluconate, Ca-gluconate, K-gluconate and NMDG were purchased from Sigma Aldrich (USA). Ethanol was obtained from MERK (Germany). Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium, Fetal bovine serum (FBS), Hanks' Balanced Salt Solution (HBSS) and Tris base were purchase from GIBCO BRL/ Invitrogen (USA). Anti-rabbit Ig antibody conjugated with horse reddish peroxidase, Karnamycin, ECL Western Blot Detection Reagent, Hybond-ECL nitrocellulose membranes, were from Amersham Biosciences. FITC labeled goat anti-rabbit secondary antibody were purchased from Promega (USA). Acrylamide, Bis N-N'-methylene-bis-acrylamide, Coomassie blue were obtained from Bio-Rad laboratories (USA).

4.4 Results

4.4.1 The effect of HCO_3^- on $[Ca^{2+}]_i$

We first examined the effect of HCO_3^- on insulin secretion of RIN-5F cells. As observed in Figure 4.1A, the insulin concentration was about 18.6±1.3ng/ml and 14.2±7.5ng/ml in 40mM $NaHCO_3$ group (n=4) and control group (addition of 40mM NaCl, n=4) respectively. Compared with control group, insulin secretion in $NaHCO_3$ group increased by about 31.1% (Figure 4.1A, $P<0.05$), indicating that HCO_3^- could promote insulin secretion in RIN-5F cells, which is similar to that of isolated islets.

It is well known that insulin secretion from β-cells is a process of exocytosis which is triggered by $[Ca^{2+}]_i$. Therefore, we next investigated if HCO_3^- had the ability to affect $[Ca^{2+}]_i$. External addition of $NaHCO_3$ induced an increase in fura-2 fluorescence ratio which contained a transient peak followed

by a sustained plateau (Figure 4.1B), suggesting that $NaHCO_3$ had the ability to increase $[Ca^{2+}]_i$. Both the peak and plateau were induced dose-dependently by $NaHCO_3$ and with an EC50 of 14.17mM and 2.73mM respectively (Figure 4.1C and D). Interestingly, the peak lasted for a very short time and the plateau showed long-lasting sustain without decay. Obviously, the increase in $[Ca^{2+}]_i$ expressed in the form of plateau was the main mechanism for $NaHCO_3$-induced insulin secretion. Thus, we focused on the plateau induced by $NaHCO_3$ in all the subsequent experiments using Ca^{2+} imaging technique.

Addition of $NaHCO_3$ not only elevated HCO_3^- level but also increased the osmolarity, pH and Na^+ concentration of extracellular solution. Therefore, the role of HCO_3^- alone in the effect of $NaHCO_3$ on $[Ca^{2+}]_i$ was investigated in the following experiments.

As showed in Figure 4.1B, addition of 40mM $NaHCO_3$ induced a markedly increase in fura-2 fluorescence ratio (0.123 ± 0.017, n=6). However, the increase in fura-2 fluorescence ratio induced by application of 40mM NaCl and Na-gluconate were only about 0.033 ± 0.010 (Figure 4.2A, n=12) and 0.030 ± 0.011 (Figure 4.2B, n=13) respectively, which were much lower than that in $NaHCO_3$ group (Figure 4.2A and B, $P<0.001$), indicating that increased osmolarity and Na^+ level were not involved in the effect of $NaHCO_3$ on $[Ca^{2+}]_i$. Addition of 55mM Na_2HPO_4 induced an increase in fura-2 fluorescence ratio with the magnitude of about 0.021 ± 0.008, which was significantly lower than that in $NaHCO_3$ group (Figure 4.2C, $P<0.01$, n=8) and thus excluded the role of increased pH in the effect of $NaHCO_3$ on $[Ca^{2+}]_i$. In general, the above results indicated that it was HCO_3^- but not other factors to induce an increase in $[Ca^{2+}]_i$ after $NaHCO_3$ stimulation.

4.4.2 The mechanisms of HCO_3^--induced increase in $[Ca^{2+}]_i$

There are two pathways which are responsible for the increase in $[Ca^{2+}]_i$ of β-cells during insulin secretion: the influx of extracellular Ca^{2+} and the release of Ca^{2+} from ER (Dukes et al., 1993; Worley et al., 1994). In our study, removal of extracellular Ca^{2+} decreased the HCO_3^--induced increase in $[Ca^{2+}]_i$ by about 75% (Figure 4.3A, $P<0.01$, n=5), suggesting the involvement of extracellular Ca^{2+}

influx in the effect of HCO_3^-. It has been reported that there are several Ca^{2+} channels on the membrane of β-cells and L-type Ca^{2+} channel is regarded as the main one which contributes to the Ca^{2+} influx during the glucose-induced insulin secretion (Leech et al., 1994; Ligon et al., 1998; Satin et al., 1995). To investigate if the HCO_3^--induced increase in $[Ca^{2+}]_i$ was due to the activation of L-type Ca^{2+} channel, nifedipine, the inhibitor of L-type Ca^{2+} channel was used. Figure 4.3B showed that addition of nifedipine(1μM), blocked the HCO_3^--induced increase in $[Ca^{2+}]_i$ completely and significantly (n=8) in comparison to that in control group, proving that L-type Ca^{2+} channel played an important role in the effect of HCO_3^-.

L-type Ca^{2+} channel is regulated by the membrane potential which is controlled by K_{ATP} channel. K_{ATP} channel on the membrane of β-cells can be inactivated by exposure to glucose and then L-type Ca^{2+} channel is activated as a result of depolarization of membrane potential (Jahanshahi et al., 2009). To examine the role of K^+ channel in the effect of HCO_3^-, $BaCl_2$, the inhibitor of K^+ channel, was employed. As observed in Figure 4.4A, application of $BaCl_2$, abolished the HCO_3^--induced increase in $[Ca^{2+}]_i$ completely and significantly compared with that of control group ($P<0.001$), indicating that K^+ channel was involved in mediating the HCO_3^- effect.

To determine the mechanism for activation of K^+ channel by HCO_3^-, the role of cAMP in the effect of HCO_3^- was investigated. Pretreatment of PKA inhibitor, H89 (4μM), decreased the HCO_3^--induced increase in $[Ca^{2+}]_i$ by about 44% (Figure 4.4B, $P<0.05$), suggesting that cAMP played an important role in the HCO_3^- effect. Since it has been observed that HCO_3^- could activate sAC to increase intracellular cAMP level (Tresguerres et al., 2010), we investigated if sAC was involved in mediating HCO_3^- effect. Pretreatment of 2-HE (20μM), the blocker of sAC, decreased the HCO_3^--induced $[Ca^{2+}]_i$ increase by about 49% (Figure 4.4C, $P<0.01$). Consistent with this result, the expression of sAC in RIN-5F cells was demonstrated by western blot analysis (Figure 4.4D). Combined with the fact that intracellular cAMP was another inhibitory regulator for K_{ATP} channel (McQuaid et al., 2006; Kang et al., 2006), these results suggest that HCO_3^- increases $[Ca^{2+}]_i$ by

activating K_{ATP} channel in the sAC-cAMP pathway.

4.4.3 The HCO_3^- entry into β-cells?

There are several studies showing the expression of Na^+-HCO_3^--cotransporter (NBC) and Na^+-driven Cl^-/HCO_3^--exchanger (NCBE) on β-cells (Wang et al., 1994; Soyfoo et al., 2009; Bulur et al., 2009). To determine the roles of NBC and NBCE in the transport of HCO_3^-, the external Na^+ was removed to block the action of NBC and NBCE. Figure 4.5A showed that the removal of extracellular Na^+ significantly enhanced rather than reduced the HCO_3^--induced increase in $[Ca^{2+}]_i$ by 218.6% (n=6) compared with that in control group (Figure 4.5A, $P<0.01$), suggesting that both NBC and NCBE were not responsible for the transport of HCO_3^-. As observed in Figure 4.5B, removal extracellular Cl^- for over 30 minutes significantly decreased the HCO_3^--induced increase in $[Ca^{2+}]_i$ by about 68% ($P<0.05$, n=5).The similar results was also found after decreasing the external Cl^- level from 142mM to 5mM. As observed in Figure 4.5C, the reduction of Cl^- level inhibited the effect of Ca^{2+} completely and significantly ($P<0.05$, n=3), implying that perhaps Cl^-/HCO_3^--exchanger (anion exchanger, AE) which is widely distributed in epithelial cells of the body was involved. To further confirm this result, H_2DIDS, the inhibitor of AE was used. Application of H_2DIDS (100μM) decreased HCO_3^--induced increase in $[Ca^{2+}]_i$ by about 83% (Figure 4.5D, $P<0.05$, n=9), indicating the possibility that AE was involved in the transport of HCO_3^- across plasma membrane.

The fact that CFTR-mediated HCO_3^- transport was demonstrated in many tissues (Hanrahan et al., 2004) promoted us to hypothesize that CFTR was also involved in the influx of extracellular HCO_3^-. Addition of NPPB(100μM) or CFTR(inh)-172(2.5μM), two inhibitors of CFTR, blocked HCO_3^--induced increase in $[Ca^{2+}]_i$ significantly (Figure 4.6 A and B, NPPB: $P<0.001$, n=7; 172: $P<0.05$, n=3), demonstrating that CFTR is involved in the HCO_3^--induced $[Ca^{2+}]_i$ increase. To further confirm the role of CFTR, the expression CFTR in RIN5F cells was investigated by western blotting analysis. Figure 4. 6C showed the expression of CFTR protein of RIN-5F cells, which is consistent

with that has been reported (Boom A et al., 2007).

4.5 Discussion

4.5.1 HCO_3^--induced increase in $[Ca^{2+}]i$

In this chapter, we investigated the effect of HCO_3^- on $[Ca^{2+}]_i$ in RIN-5F cells. First of all, we examined if HCO_3^- could induce insulin secretion of RIN-5F cells. As expected, our results showed that $NaHCO_3$ increased insulin secretion by RIN-5F cells significantly and thus indicated that RIN-5F cells was an appropriate in vitro model for the study of mechanisms underlying the HCO_3^--induced insulin secretion.

Although the experiments of Ca^{2+} imaging showed that $NaHCO_3$ induced an increase in $[Ca^{2+}]_i$, we couldn't determine the role of HCO_3^- in this phenomenon because addition of 40mM $NaHCO_3$ could increase HCO_3^- level as well as the osmolarity, pH and Na^+ concentration, all of which could affect insulin secretion. In a study on HC9 cells, another β-cell line, decrease of external osmolarity induced a transient secretion of insulin, demonstrating the influence of osmolarity on insulin secretion (Straub et al., 2002). It is found that increase of extracellular Na^+ concentration enhanced glucose-induced insulin secretion of perfused pancreas isolated from rats (Mokuda et al., 1998). In our study, the results that addition of 40mM NaCl, Na-gluconate almost could not induce any increase in $[Ca^{2+}]_i$, indicating that it was not the increase in Na^+ level and/or osmolarity to induce the elevation of $[Ca^{2+}]_i$ after treatment of $NaHCO_3$. Manning's study observed that external alkalization failed to stimulate insulin secretion (Manning et al., 2006) and our study also found that addition of 55mM Na_2HPO_4 did not induce elevation of $[Ca^{2+}]_i$. Therefore, the role of increased pH in the effect of $NaHCO_3$ was excluded.

4.5.2 The role of L-type Ca^{2+} channel in the $[Ca^{2+}]_i$ increase induced by HCO_3^-

There are two pathways contributing to the increase in $[Ca^{2+}]_i$ of β-cells during the insulin

secretion: the influx of extracellular Ca^{2+} via Ca^{2+} channel and the release of Ca^{2+} from ER (Dukes et al., 1993; Worley et al., 1994). In our study, removal of extracellular Ca^{2+} completely reduced the HCO_3^--induced increase in $[Ca^{2+}]_i$ significantly, indicating that influx of extracellular Ca^{2+} was responsible for the elevation of $[Ca^{2+}]_i$.

VDCC includes L-, N-, P-/Q-, R-, and T-types Ca^{2+} channels (Moosmang et al., 2007). Although the expression of R-, N-, and P-/Q-type Ca^{2+} channel on β-cells was observed, their roles in the glucose-induced insulin secretion were not confirmed (Pereverzev et al., 2002; Vajna et al., 2001; Takahashi et al., 2005; Satin et al., 1995; Ligon et al., 1998; Horvath et al., 1998). In fact, L-type Ca^{2+} channel is regarded as the main Ca^{2+} channel for the influx of extracellular Ca^{2+} during glucose stimulation according to the role of L-type Ca^{2+} channel in the insulin secretion, electrical activity and $[Ca^{2+}]_i$ oscillations (Ashcroft et al., 1994 and 1989; Mears et al., 2004; Henquin et al., 1984; Santos et al., 1991) .

In this study, the application of nifedipine, the inhibitor of L-type Ca^{2+} channel blocked the HCO_3^--induced increase in $[Ca^{2+}]_i$ completely, demonstrating that L-type Ca^{2+} channel may account for the Ca^{2+} influx induced by HCO_3^-.

In addition, the Ca^{2+} current of L-type Ca^{2+} channel could be potentiated by cAMP analogue as well as GLP-1 through PKA-dependent pathway (Kanno et al., 1998; Suga et al., 1997; Furman et al., 2010). It was observed that forskolin and IBMX induced the phosphorylation of the cardiac-type alpha 1 subunit of the voltage-sensitive Ca^{2+} channel as well the insulin secretion in a kind of insulin-secreting cell line (Leiser et al., 1996). Since HCO_3^- could increase intracellular cAMP via activation of sAC, another possibility is that L-type Ca^{2+} channel could be activated during HCO_3^- stimulation. However, our study found that application of K^+ channel blocker completely blocked the effect of HCO_3^-, suggesting that L-type Ca^{2+} channel could not be activated directly without the opening of K^+ channel during HCO_3^- stimulation.

4.5.3 The role of Ca^{2+} release from ER in the HCO_3^- effect

Ca^{2+}-induced Ca^{2+} release (CICR) from endoplasmic reticulum (ER) is regarded as one mechanism for the increase in $[Ca^{2+}]_i$ after glucose stimulation (Varadi et al., 2004; Roe et al., 1993). Because the inhibition of inflow of external Ca^{2+} could also block CICR, the role of CICR in the increase in $[Ca^{2+}]_i$ after HCO_3^- stimulation can not be excluded although the role of L-type Ca^{2+} channel was confirmed.

Interestingly, removal of extracellular Ca^{2+} failed to abolish the HCO_3^-–induced increase in $[Ca^{2+}]_i$ completely, suggesting a mechanism of increase in $[Ca^{2+}]_i$ which was different from CICR and Ca^{2+} influx through VDCC. This phenomenon suggested that HCO_3^- had the ability to enhance Ca^{2+} release from intracellular stores directly. The similar result was also observed in Roe's study. It is found that glucose could still caused Ca^{2+} release from ER in the absence of extracellular Ca^{2+} (Roe et al., 1993). There are several studies suggesting that GLP-1 stimulates $[Ca^{2+}]_i$ release in a cAMP-PKA pathway by activation of ryanodine channel and/or through phosphorylation of the IP3 receptor (Kang et al., 2005; Holz et al., 1999; Islam et al., 1998; Dyachok et al., 2004; Tsuboi et al., 2003). Combined with the observed inhibition of HCO_3^- effect by PKA inhibitor, we speculate that HCO_3^- promoted Ca^{2+} release from intracellular stores via cAMP-PKA pathway. However, we do not have direct evidences to support this.

4.5.4 The role of K_{ATP} channel in the HCO_3^--induced increase in $[Ca^{2+}]_i$

L-type Ca^{2+} channel is regulated by the membrane potential which is controlled by K^+ channel and there are several types of K^+ channels located on the plasma membrane of β-cells, including K_{ATP} channel, a kind of voltage-dependent with large conductance (maxi-K(V) channel) and Ca^{2+}-dependent K^+ channel (Kca channel) (MacDonald et al., 2002). However, the roles of maxi-K(V) and Kca channel are not very confirmed and it is well accepted that K_{ATP} channel is a key protein during the glucose-induced insulin (Cook et al., 1989; Rorsman et al., 1989; Clark et al., 2010; Ribalet et al.,

1988; Eddlestone et al., 1989).

Apart from ATP, intracellular cAMP is also an inhibitory regulator of K_{ATP} channel (McQuaid et al., 2006; Kang et al., 2006; Tresguerres et al., 2010). In some studies, it has been suggested that HCO_3^- can increase intracellular sAC activity in a pH-independent manner in vivo and in vitro (Litvin et al., 2003; Chen et al., 2000). sAC is a protein with the molecular weight of about 48kD and is distinguished from the G protein-responsive transmembrane adenylyl cyclase (Litvin et al., 2003; Chen et al., 2000). The activation of sAC catalyzes the conversion of ATP to cAMP and subsequently results in an increase in intracellular level of cAMP. In our study, both cAMP antagonist and sAC inhibitor decreased the HCO_3^--induced increase in $[Ca^{2+}]_i$ significantly. In addition, the expression of sAC in RIN-5F cells was confirmed by western blotting. Therefore, it is likely that the sequence of events underlying the HCO_3^- effect involves HCO_3^--sAC-cAMP-K_{ATP} channel.

4.5.5 HCO_3^- transport across the plasma membrane of β-cells

Since the expression of two kinds of HCO_3^- transporter, Na^+-HCO_3^--cotransporter (NBC) and Na^+-driven Cl^-/HCO_3^- -exchanger (NCBE) in rat pancreatic islets has been reported (Soyfoo et al., 2009; Bulur et al., 2009; Wang et al., 1994), we hypothesized that perhaps NBC and/or NBCE mediated the transport of HCO_3^- across the plasma membrane. However, completely removal of extracellular Na^+ to block the action of NBC and NBCE didn't decrease the elevation of $[Ca^{2+}]_i$ induced by HCO_3^- at all, indicating that NBC and NBCE were not what we expected. On the contrary, removal of extracellular Na^+ increased the HCO_3^--induced increase in $[Ca^{2+}]_i$ by about 218%, which was an interesting result. Possibly, this phenomenon is related to the activity of Na^+-Ca^{2+} exchanger (NCX) on the plasma membrane of pancreatic islet β-cells. The action of NCX is to mediate the outflow of Ca^{2+} in exchanger for Na^+ after elevation of $[Ca^{2+}]_i$ (Ximenes et al., 2003; Herchuelz et al., 2007).The removal of external Na^+ induced the decrease of the activity of NCX and the much higher increase in $[Ca^{2+}]_i$ as a result of reduced Ca^{2+} outflow.

In Pace's study, it is found that application of DIDS, the inhibitor of AE, blocked the electrical activity of β-cells induced by glucose, indicating the possibility of the existence of AE on β-cells (Pace et al., 1982). We showed that inhibition of AE by application of AE inhibitor or the removal of extracellular Cl^- blocked $[Ca^{2+}]_i$ increase significantly, indicating the involvement of AE.

Although CFTR is known to be a Cl^- channel, the role of CFTR in the transport of HCO_3^- in epithelial cells has been reported (Hug et al., 2003). Thus we hypothesized that CFTR was also involved in the transport of HCO_3^- in RIN-5F cells. Consistent with what we expected, application of CFTR inhibitors blocked the increase in $[Ca^{2+}]_i$ significantly. Taken together, we have elucidated that AE transported HCO_3^- into cells with the exchange of intracellular Cl^- which enters into cells via CFTR again. Therefore, the recycle of Cl^- through CFTR and AE was responsible for the HCO_3^- transport. Similar model has been suggested in epithelial cells by other studies (Mizumori et al., 2009; Wheat et al., 2000). However, there are no direct evidences demonstrating the expression of AE in islets and we do not have evidences to support this model. Therefore, the above model should be proved by further study.

Figures:

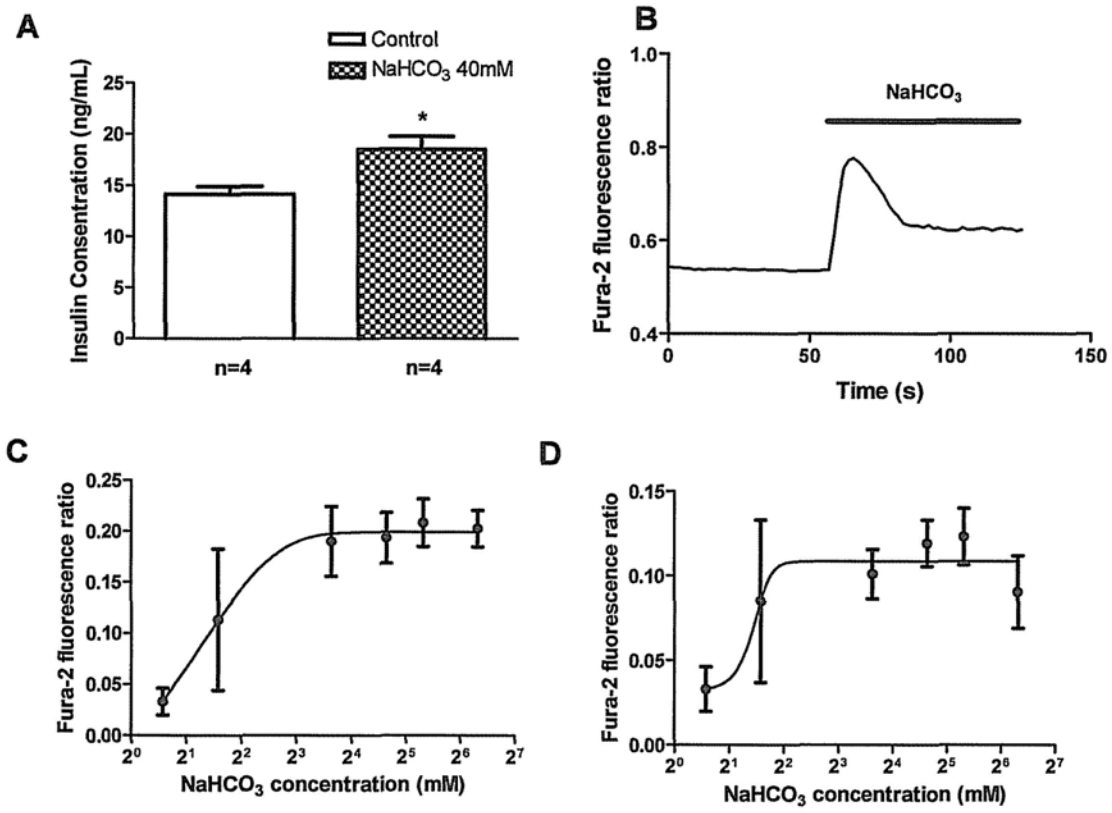

Figure 4.1 The effect of HCO_3^- on RIN-5F. A: Application of 40mM $NaHCO_3$ increased insulin secretion by about 31%. **B:** Addition of $NaHCO_3^-$ induced an increase in $[Ca^{2+}]_i$, which contained a transient peak followed by a sustained plateau. $NaHCO_3$ induced the peak **(C)** and plateau **(D)** dose-dependently. * *P<0.05*, Data were expressed as mean±S.E.M

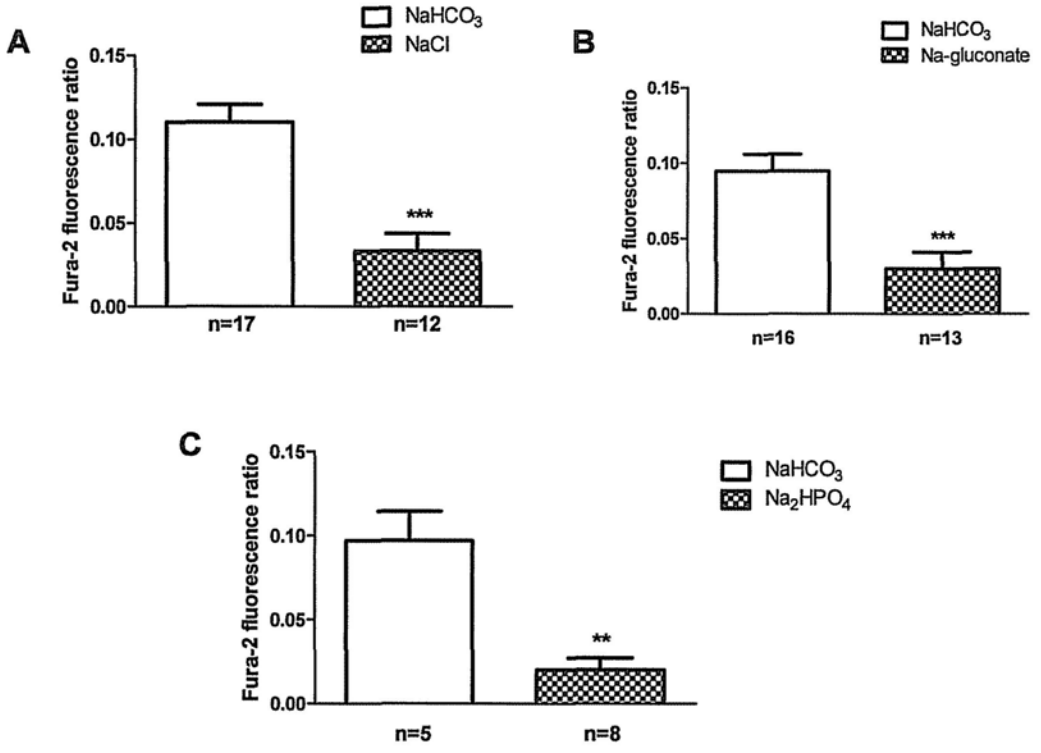

Figure 4.2 The role of HCO_3^- in the $NaHCO_3$-induced increase in $[Ca^{2+}]i$. Application of 40mM NaCl **(A)** and Na-gluconate **(B)**, 55mM Na_2HPO_4 **(C)** almost couldn't elevate $[Ca^{2+}]_i$. ** *P<0.01*, *** *P<0.001*, Data were expressed as mean±S.E.M,

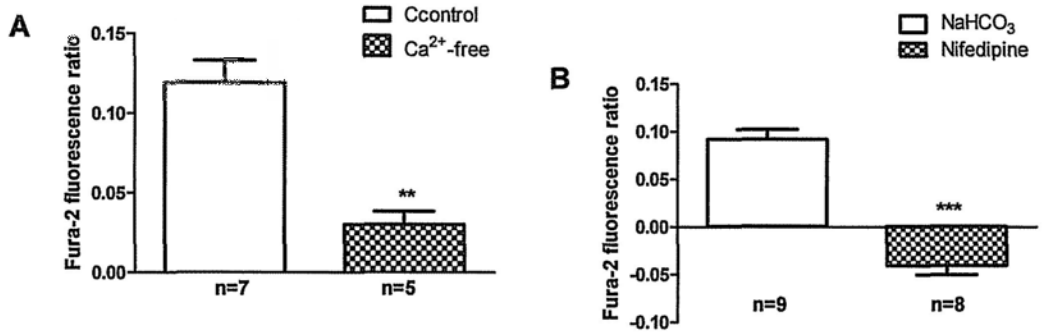

Figure 4.3 The roles of extracellular Ca^{2+} and L-type calcium channels in the effect of HCO_3^-. A: Removal of extracellular Ca^{2+} decreased the HCO_3^--induced increase in $[Ca^{2+}]_i$ by about 75%. **B:** Addition of nifedipine(1μM), the inhibitor of L-type calcium channels, blocked the HCO_3^--induced increase in $[Ca^{2+}]_i$ completely. ** *P<0.01*, *** *P<0.001*, Data were expressed as mean±S.E.M,

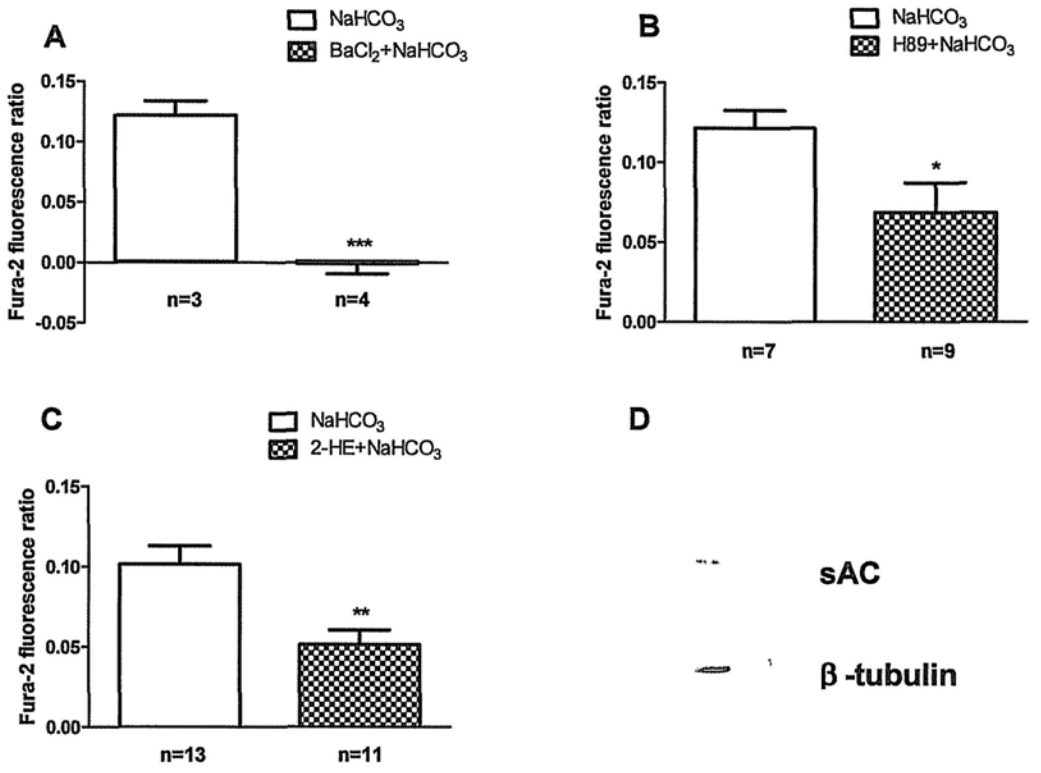

Figure 4.4 The role of K_{ATP} channel in the effect of HCO_3^-. A: Application of $BaCl_2$, the inhibitor of K^+ channel, decreased the HCO_3^--induced $[Ca^{2+}]_i$ increase completely. **B, C:** Pretreatment of H89 (4μM), a kind of cAMP antagonist, or 2-HE (20μM), the blocker of sAC, decreased the HCO_3^--induced $[Ca^{2+}]_i$ increase by about 44% and 49% respectively. **D:** Western blot showed the expression of sAC in RIN-5F cell line. * *P<0.05*, ** *P<0.01*, *** *P<0.001*, Data are expressed as mean±S.E.M,

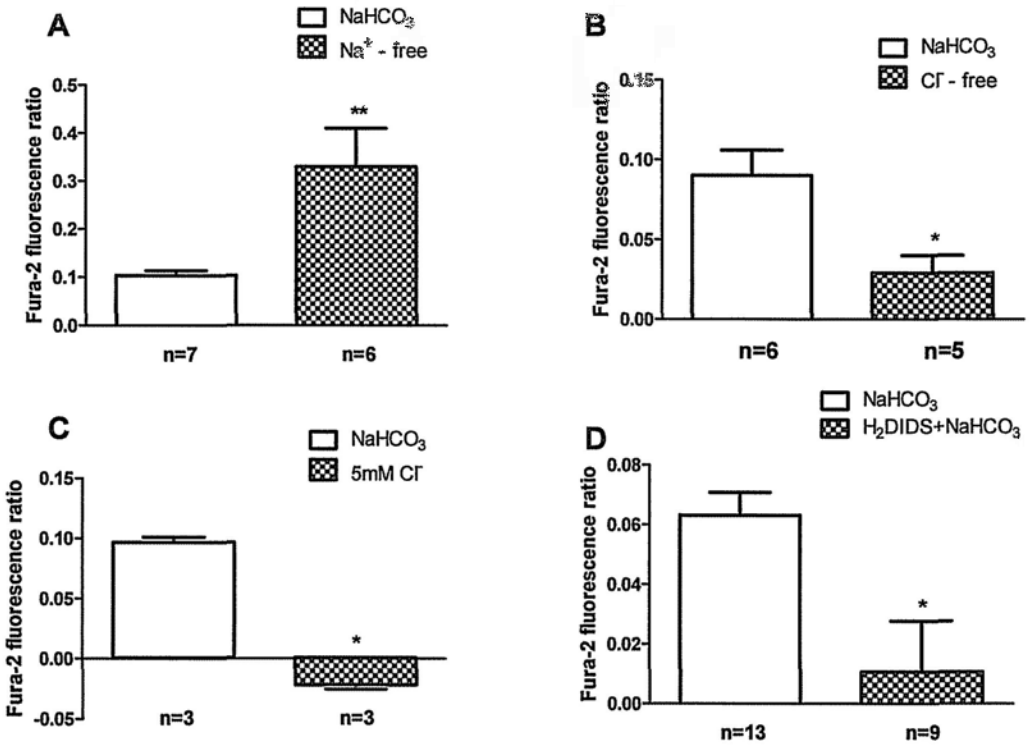

Figure 4.5 The mechanisms of HCO_3^- transport across plasma menbrane. A: Removal of extracellular Na^+ increased the HCO_3^--induced increase in $[Ca^{2+}]_i$. **B:** Removal of extracellular Cl^- decreased HCO_3^- effect by about 68%. **C:** Decrease of extracellular Cl^- level to 5mM abolished HCO_3^- effect completely. **D:** Application of H_2DIDS(100μM), the inhibitor of AE, decreased HCO_3^- effect by about 83%. * *P<0.05*, ** *P<0.01*, Data were expressed as mean±S.E.M.

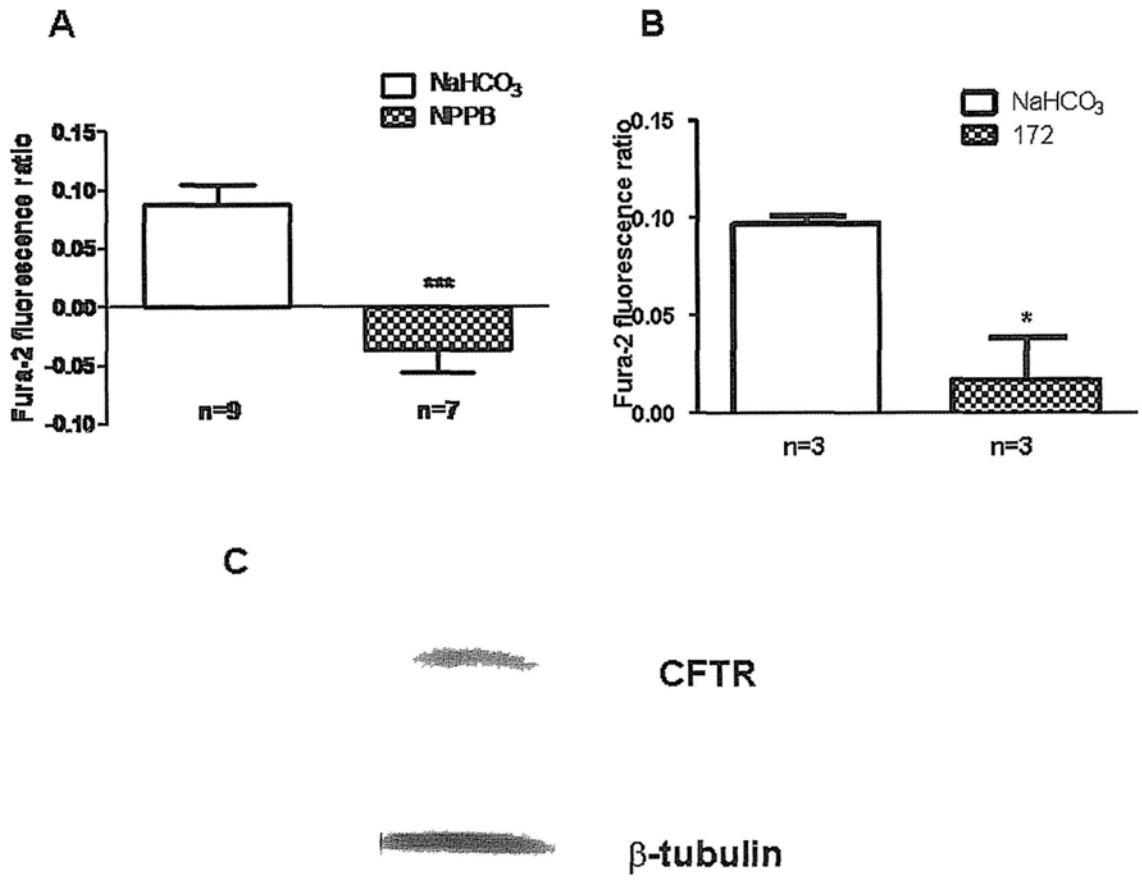

Figure 4.6 The role of CFTR in HCO_3^--induced increase in $[Ca^{2+}]_i$. A: The application of NPPB(100μM), the inhibitor of CFTR, decreased the HCO_3^--induced increase in $[Ca^{2+}]$i completely. **B:** The application of CFTR(inh)-172(2.5μM), the inhibitor of CFTR, decreased the HCO_3^--induced increase in $[Ca^{2+}]_i$ by about 83%. **C:** The expression of CFTR in RIN-5F cell line showed by western blot. * *P<0.05*, ** *P<0.01*, Data were expressed as mean±S.E.M,

CHAPTER 5

PERMISSIVE ROLE OF INSULIN IN THE EXPRESSION OF LONG-TERM POTENTIATION IN THE HIPPOCAMPUS OF IMMATURE RATS

5.1 Summary

Many studies indicate that learning and memory deteriorate in conditions in which insulin signaling is impaired including diabetes and Alzheimer's disease. However, previous studies fail to establish a clear role of insulin on long-term potentiation (LTP) in the hippocampus. In this study, we investigated the effect of insulin on the expression of LTP in the hippocampus of immature (11-12 days) and adult (2 months) rats *in vitro*. While insulin pretreatment did not affect the magnitude of hippocampal LTP in the adult group, it facilitated the expression of LTP in the young rats, which otherwise did not express LTP in response to standard high frequency stimulation (HFS). Application of AG-1024, an inhibitor of insulin receptor, largely abolished the insulin-dependent LTP in the young rats indicating the involvement of insulin receptor. On the other hand, increasing the concentrations of extracellular glucose failed to facilitate the LTP and application of the insulin-responsive glucose transporter-4 inhibitor indinavir did not impair the effect of insulin. These results suggest that the facilitatory action of insulin on LTP is not via an indirect effect on glucose homeostasis or utilization. Involvement of the MAPK pathway was revealed by pretreatment with the specific inhibitor PD98059 which blocked the insulin-mediated LTP facilitation and that HFS induced a significant increase in the level of phosphorylated p42MAPK in the insulin-treated hippocampus. The present study suggests that insulin may be an essential factor in the developing brain allowing the expression of LTP to facilitate learning and memory.

5.2 Introduction

Although insulin is well known for its action on peripheral glucose homeostasis，the roles of insulin as a neuromodulator and neurotrophic factor in the brain have received much attention in recent

years (Chiu and Cline, 2010; Van der Heide et al., 2006; Kodl & Seaquist, 2008; McNay, 2007; Laron Z et al., 2009). The interest on a central action of insulin was prompted by the early unexpected finding that the brain contains insulin and insulin receptor. Margolis et al (1967) found that the insulin concentration in CSF increased with elevation of peripheral plasma insulin level, suggesting the relationship of insulin between plasma and CSF. Banks et al (1977a) observed that human insulin in CSF was detected after infusion of human insulin into mice and the insulin concentration in CSF increased with the elevation of the dose of infused insulin. Because mouse can not produce human insulin, the above study indicated that insulin had the ability to enter into CSF across the blood-brain barrier (BBB). Additionally, insulin-like substance was detected in neurons by several studies. Insulin-like substance was observed in primary culture cells from rabbit fetal neuron cells and rats brain using immunohistochemical technique (Schechter et al., 1994; Havrankova et al., 1978b). Apart from cytoplasm, insulin-like substance was also found in endoplasmic reticulum and Golgi of neuron cells. The existence of insulin-like mRNA was suggested in neuron cells by in situ hybridization and Northern blots (Schechter et al., 1994). In agreement with the above, Devaskar's study observed the existence of insulin mRNA in the rabbit neuron cells (Devaskar et al., 1994). However, no studies identified the insulin-like substance found in neurons as insulin secreted from pancreatic β-cells. Moreover, insulin I and II mRNAs were not detected in fetal and adult rats using RT-PCR technique. Therefore, whether neurons could produce insulin is inconclusive.

The increased awareness of the possible causal link between insulin resistance and cognitive deficits in diabetes suggested that CNS is a target organ for insulin (Kodl & Seaquist, 2008). Winocur et al (2005) found that obese Zucker rat developed impaired memory function and insulin resistance as well. Diabetic GK rats, a Type 2 DM animal model, expressed impaired memory (Marfaing-Jallat et al., 1995). The OLETF rat, another Type 2 DM animal model, is characterized by moderate obesity and impaired glucose tolerance and developed impaired spatial memory in the Morris water-maze task (Oomura et al., 2002). Clinically, different kinds of cognitive deficits are identified in patients with

both Type 1 and Type 2 DM, including slowing of information processing speed, worsening psychomotor efficiency, impaired memory (Kodl et al., 2008). Furthermore, there is evidence that defective insulin signaling contributes to the pathophysiology of Alzheimer's diseases (AD) (Kodl & Seaquist, 2008; McNay, 2007). It is found that AD patients expressed a decreased CSF/plasma insulin ratio compared with healthy subjects. Interestingly, the difference was positively related to the development of AD (Craft et al., 1988).

Experimental studies from human and animals in general point to a facilitatory role of insulin in cognitive performance. In an in vivo study, the rats received an intracerebroventricular injection of insulin or heat-deactivated insulin shortly after training on a step-through passive–avoidance task. After 24 hours, the testing of the task on the rats showed that the latency to enter the dark compartment in insulin group increased significantly compared to that in heat-deactivated insulin group, indicating that insulin could increase memory function (Seeley et al., 2000; Park et al., 2000). The same result was also observed in Kern's experiments. Healthy human subjects received intravenous injection of insulin with high or low dose. To exclude the effect of insulin on glucose, glucose concentration in plasma was kept at normal level using glucose clamp technique. The subjects in high insulin group expressed a negative potential shift in the auditory evoked brain potentials, which were associated with facilitation of working memory, and the memory performance in words recalled test increased significantly compared to that in low insulin group (Kern et al., 2001). Administration of insulin intranasally in human also resulted in enhancement of memory functions (Benedict et al, 2007).

Insulin also exerts a restorative action on memory impairment in various settings, including drug-induced experimental model of diabetes, model of stress (Moosavi et al., 2007) and also in Alzheimer's disease patients (Craft et al., 1999). After induction of forebrain ischemia, the rats were given subcutaneously injection of insulin for one week. 1-2 months after ischemia, the performance of insulin-treated rats in water maze place navigation tasks was much better than that in control groups (Voll et al., 1989). Similarly, adult rats were treated with insulin injection for 4 days prior to dorsal

hippocampal lesions. One week after surgery, the rats in insulin group showed decreased deficiency in avoidance acquisition and decreased amounts of freezing during the CS-US interval without any changes in the depressed struggling scores, demonstrating the facilitatory effect of insulin on functional recovery of impaired memory after brain damage (De et al., 1976). Consistent with the above studies, the impairment of memory was also observed in DM animal model. After injection of streptozotocin to induce Type 1 DM animal model, the mice and rats expressed a marked memory deficiency after train of an active avoidance T-maze task or Morris water maze task and the memory deficiency could be rescued by administration of insulin (Flood et al., 1990; Biessels et al., 1998). The impaired memory was also enhanced by treatment of insulin in AD patients and the rats with treatment of chronic restraint stress (Moosavi et al., 2007; Craft et al., 1999).

The above findings are consistent with the observation that insulin receptors are especially abundant in the hippocampus in both rat and human. Havrankova's study indicated that almost all the regions of brain showed the expression of insulin receptors and the olfactory bulb, cerebral cortex, hippocampus and hypothalamus contained the highest levels (Havrankova et al., 1978a). Similarly, Unger's results showed that olfactory bulbs, hypothalamus and median eminence, medial habenula, subthalamic nuclues, subfornical organ, CA1/2 pyramidal cell layer of the hippocampus and piriform cortex expressed the highest density of insulin receptor-like immunoreactivity (Unger et al., 1989). The concentrated expression of insulin receptors in hippocampus was also revealed in other independent studies (Marks et al., 1990; Hopkins & Williams, 1997). Moreover, the binding of insulin to insulin receptor and the expression of insulin signaling were also found in hippocampus. Doré's study demonstrated that ^{125}I-insulin could bind to the hippocampus and the binding sites were mainly found in the dentate gyrus and CA1 area of hippocampus (Doré et al., 1997). Baskin's experiments indicated the expression of insulin receptor substrate-1 (IRS-1) and found that IRS-1 was co-localized with insulin receptor and phosphotyrosine in the hippocampus, suggesting the structural tight integration of insulin signaling in the hippocampus (Baskin et al., 1994). On the other hand, the

expression of insulin receptors in the hippocampus increased in the process of memory. After water maze training, it was found that IR mRNA in the CA1 and dentate gyrus of the hippocampus was up-regulated (Zhao et al., 1999; Dou et al., 2005).

It is widely accepted that long-term synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus is the mechanism that underlies some forms of learning and memory. To investigate the mechanisms of the role of insulin in cognitive function, the effect of insulin on LTD and LTP was studied. In Huang's study, application of insulin for 10 minutes dose-dependently caused a reduction of fEPSPs, which was called insulin-induced LTD-like phenomenon (Huang et al., 2004). However, this effect of insulin need to be confirmed due to the fact that the lowest concentration of insulin used in this study was about ten millions and three millions times of that in CSF of rats and humans respectively (Stein et al., 1983; Ratzmann et al., 1980). In addition, the streptozotocin-induced diabetic rats developed enhanced rather than impaired LTD compared with control groups, indicating that perhaps LTD was not involved in the effect of insulin on learning and memory (Gardoni et al., 2002).

Likewise, the effect of insulin on LTP remains unclear. In fact, insulin has not been reported to facilitate high frequency stimulation (HFS)-induced LTP. Also, although several studies show that in experimental diabetes, LTP is impaired and can be rescued by insulin (Biessels et al., 1996, 1998; Izumi et al., 2003), there are reports showing that LTP is preserved in this condition (Bélanger et al., 2004). Thus, the exact relationship between insulin and LTP is still obscure.

In this study, we addressed the question of whether insulin application has any effect on LTP expression in the hippocampus of normal rats. Since it is known that insulin receptor expression is developmentally regulated (Chiu and Cline, 2010; Park et al., 2009), we examined and compared the effect of insulin on LTP in both immature and adult rats. Our experiments reveal that insulin plays a critical permissive role in LTP expression in the immature brain via insulin receptors but not through its influence in glucose utilization/homeostasis. This finding suggests that insulin may be an

indispensible endogenous factor allowing the expression of LTP in the normal young animals for learning and memory, and provides an explanation for the well known observation of declined cognitive function in children with insulin-dependent, type 1 diabetes.

5.3 Materials

5.3.1 Animals

Male healthy Sprague-dawley rats with the age of 10-12 days and 2 months were supplied by the laboratory animal service center (LASEC) of the faculty of medicine, the Chinese University of Hong Kong (CUHK). Animals were maintained in a room with fixed temperature of 23°C, the controlled humidity and a 12-h cycle of light/dark. Animals were housed in polycarbonate cages and had free access to food and water. A lot of bedding of pine shavings was put on the bottom of the cages. The bedding is changed once in three days.

5.3.2 Biochemicals and reagents

DMSO, NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$, D(+)-glucose, $CaCl_2$, K_2HPO4 and insulin were purchase from sigma (USA), indinavir was obtained from TRC (Canada), PD98059 was from Cayman (USA) and AG1024 was purchased from Spectrum (USA).

5.4 Results

5.4.1 Differential effects of insulin on LTP in young and adult rats

We first examined the ability of the hippocampal slices from 2 month old (adult) and 11-12 days old (young) rats to exhibit LTP. After baseline recording for at least 30 mins, standard HFS (100 Hz, 1 s) stimuli were delivered to the Schaffer collateral pathway and fEPSPs were recorded in the CA1 dendritic layer for at least 60 mins. As shown in Figure 5. 1A, in the adult group, the fEPSP amplitude was potentiated to 165.8 $\pm$ 13.0% of baseline (12 slices, 8 rats). On the other hand, HFS failed to

induce potentiation of the fEPSP in the young rat measured at the end of 60 mins (Figure 5. 1B, 100.3 ± 6.4% of baseline, 8 slices, 4 rats).

To test the effect of insulin on LTP, some hippocampal slices from both age groups were pre-treated with insulin (0.08 ng/ml or 0.8 ng/ml) for at least 1 hr before delivery of HFS. As shown in Figure 5. 1A, in the adult group, the magnitude of LTP was not altered by insulin at both 0.08 ng/ml (158.5 ± 14.4%, 8 slices 5 rats, $P > 0.05$ compared with control) and the higher concentration of 0.8 ng/ml (149.3 ± 10.3%, 10 slices 5 rats, $P>0.05$ compared with control). Strikingly, however, in the young rats in which HFS could not induce LTP, pre-treatment with insulin induced potentiation of the fEPSP in a dose-dependent manner (Figure 5. 1B). At a concentration of 0.08 ng/ml, the fEPSP measured at the end of 60 min was 102.6 ± 3.4% of baseline ((Figure 5. 1B, 9 slices from 6 rats, $P > 0.05$ compared with control). Significant potentiation of the fEPSP, i.e. LTP, was enabled at an insulin concentration of 0.8ng/ml. The amplitude of the of the fEPSP at 60 min after HFS was 120.4 ± 4.4% of baseline (Figure 5. 1B, 10 slices from 6 rats, $P < 0.01$, compared with control). These results indicate that under the experimental conditions of the present study, insulin plays a permissive role in the expression of LTP in young rats while it is without effect on the LTP in the adult hippocampus.

5.4.2 Insulin-dependent LTP is mediated by insulin receptor

To determine the involvement of insulin receptor in mediating the effect of insulin on synaptic plasticity, we studied the effect of tyrosine kinase inhibitor, tyrphostin AG-1024, a known insulin receptor inhibitor, on the observed insulin-dependent LTP in the young rats. Incubation of the hippocampal slices with 80nM of AG-1024 did not affect the basal synaptic transmission (Figure 5. 2A). However, as shown in Figure 5. 2B, pre-treatment of the slices with AG-1024 for 30 mins before HFS and throughout the experiment largely abolished the insulin-dependent LTP. The magnitude of LTP in the presence of AG-1024 was 105.8 ± 2.5% (15 slices from 9 rats), which was significantly lower than that of the insulin alone group (Figure 5. 2B, 130.4± 5.7%; 14 slices from 7 rats, $P <$

0.001).

We also tested the effect of AG-1024 on the LTP expressed by the adult hippocampus. In the control group, the LTP magnitude was 143.8 ± 9.4% (Figure 5. 2C, 12 slices from 6 rats). Treatment with AG-1024 did not affect the LTP (148.6 ± 8.3%, 11 slices 6 rats, $P > 0.05$; Figure 5. 2C). These results indicate that AG-1024 itself does not have a direct effect on LTP and is in line with the notion that the LTP expressed in the adult hippocampus is not dependent on insulin.

5.4.3 The facilitation of LTP expression is not via regulation of glucose utilization

The effect of insulin on glucose metabolism in the brain is uncertain, and is still much of a debate (Laron, 2009). To address the possibility that the facilitatory action of insulin on LTP expression in the young animal is via its effect on glucose utilization, we performed two sets of experiments. In the first experiment, we studied the effect of indinavir, an insulin-sensitive glucose transporter-4 (GLUT-4) inhibitor, on the insulin-dependent LTP. In the insulin-treated group, the magnitude of LTP was 122.2 ± 4.9% (Figure 5. 3A, 10 slices from 5 rats). Pre-incubation of the slices with indinavir (100μM) for over 30 minutes did not affect the magnitude of LTP observed (Figure 5. 3A, 118.7 ± 9.1%, 11 slices from 6 rats, $P > 0.05$). In the second set of experiment, we tested the impact of increasing the extracellular glucose concentration on the neonatal LTP. As shown in Figure 5. 3B, elevation of glucose concentration from 11mM to 22mM or 33mM did not affect the magnitude of LTP in the young rat (104.6 ± 3.2%, 9 slices from 6 rats and 106.7 ± 2.2%, 10 slices from 6 rats respectively, $P > 0.05$). Taken together, these data strongly suggest that the facilitatory action of insulin on LTP expression is not an indirect effect via the regulation of glucose availability to the neurons.

5.4.4 Involvement of the MAPK pathway

We next explored the intracellular signaling pathway of the insulin-dependent LTP. Specifically, we tested the involvement of MAPK pathway, which has been shown to be a major signaling pathway

mediating the effect of insulin in the peripheral organs (Saltiel & Kahn, 2001). Figure 5. 4A shows that in the insulin treatment group, the average of magnitude of fEPSP obtained in 8 slices from 4 rats was 119.4 ± 3.2%. When PD98059 (20μM), the inhibitor of MAPK pathway, was applied before and continued throughout the experiment, the LTP magnitude was reduced to 106.6± 3.3% (Figure 5. 4A , 10 slices from 5 rats, $P < 0.05$).

To further confirm the involvement of MAPK pathway in the effect of insulin, we examined the expression of phosphorylated MAPK in the hippocampus of young rats after HFS stimulation by Western blot analysis. As shown in Figure 5. 4B, the ratio of phosphorylated p42 MAPK vs total p42 MAPK increased significantly in the presence of insulin compared to that in control group (Figure 5. 4B, 0.0768±0.0254 vs 0.0452±0.0187, n=3, $P<0.05$). However, the ratio of phosphorylated p44 MAPK vs total p44 MAPK did not changed compared to that in control group (Figure 5. 4B, 0.0384±0.0171 vs 0.0407±0.0173, n=3, $p>0.05$).

5.5 Discussion

5.5.1 The effect of insulin on LTP

Despite different lines of evidence supporting a role of insulin in facilitating learning and memory, the relationship between insulin and LTP, the best cellular candidate underlying memory formation, remains elusive up to date. By investigating the action of insulin on the expression of hippocampal LTP in healthy adult and young rats, we show that insulin plays a permissive role in the expression of LTP in young rats but has minimal effect in the adult rats. This conclusion is based on the fact that the hippocampus obtained from 11-12 days old rats, in contrast to that of the adult, could not express LTP in response to the conventional HFS paradigm. However, addition of 0.8ng/ml (or 137nM) of insulin allowed the expression of a decent LTP, which amounts to over 20% of potentiation in fEPSP. On the other hand, insulin application had no effect on the magnitude of the LTP in the adult hippocampus, which is in agreement with previous studies (van der Heide, 2005; Lee et al., 2009).

These findings lead to some interesting speculations. First, it has been shown by others (Harris & Teyler, 1984; Jackson et al., 1993; Lu et al., 1996) and also in the present study that hippocampal slices from rats younger than 2 weeks are much weaker in the ability to express LTP, and therefore are regarded as developmentally immature. Our study suggests that the hippocampus at 11-12 days is capable of expressing LTP in response to the standard one train HFS. The fact that they could not express LTP could be due to the removal of endogenous factors like insulin in the incubation fluid rather than due to inherent deficit. We would like to point out that, unlike many studies on insulin, the concentration of insulin that we used in the present study was low, at sub-nanomolar concentration, which is likely to be within the physiological range (Stein et al., 1983; Ratzmann et al., 1980). Second, insulin as a neuromodulator is particularly important for learning and memory in young subjects. This speculation is in line with the observation that the expression of insulin receptor is developmentally regulated, being highest in early postnatal stages and decreases with age (Chiu & Cline, 2010; Park et al., 2009), and can explain the cognitive dysfunction found in children with type 1 diabetes in which there is deficiency in insulin production (Gaudieri et al., 2008). In the adult hippocampus, it is possible that other factors, e.g. BDNF, or mechanisms serve to ensure the expression of LTP which as a result relies less on insulin.

Our results imply the involvement of insulin receptor in mediating the insulin-dependent LTP. This is based on the fact that the insulin receptor inhibitor AG-1024 and the MAPK pathway inhibitor PD98059 significantly suppressed the effect of insulin. However, due to the similarity between insulin receptor and that of insulin-like growth factor-1 (IGF-1), and that both insulin and IGF-1 can act on these two types of receptors, we cannot rule out a role of IGF-1. In fact, the tyrosine kinase inhibitor AG-1024 inhibits both receptors. However, since the expression of insulin receptor and IGF-1 receptor within the hippocampus is different, with the insulin receptor being higher in CA1 region (Bondy et al., 1992), it is likely that insulin is the more important endogenous factor in permitting the expression of LTP in the CA3-CA1 pathway *in vivo*.

5.5.2 The role of glucose metabolism in the effect of insulin

What is the mechanism that insulin facilitates the expression of LTP? It is well known that glucose is the main nutrient and energy source of neurons (McNay et al., 2010). However, there are great differences in glucose metabolism between brain and peripheral tissues. Most of peripheral tissues, including skeletal muscles, cardiac muscles and fats, express a subtype of glucose transporter-4 (GlUT-4), which is responsible for the transport of glucose across the plasma membrane (Von et al., 2003). GLUT-4 is a membrane-spanning protein and recycles continuously between cytoplasm and plasma membrane with higher speed of internalization than exocytosis (Klip et al., 2009). Once the binding of insulin to insulin receptors happens, the activation of insulin signaling promotes the exocytosis of GLUT-4 and increases the number of GLUT-4 on the surface of membrane to enhance the uptake of glucose. Thus, GLUT-4 is insulin-sensitive and is the key protein in the effect of insulin on peripheral tissues (Huang et al., 2007). However, the main glucose transporters expressed in CNS are glucose transporter-1 (GLUT-1) and glucose transporter-3 (GLUT-3), both of which are insulin-insensitive. GLUT-1 is mainly distributed in astrocytes and the endothelial cells of cerebral microvessels while GLUT-3 is principally expressed in neurons (Vannucci et al., 1998; Simpson et al., 1999; Duelli et al., 2001). Since the two main neuronal GLUTs are resistance to insulin, it is generally regarded that brain was an insulin-insensitive organ (Lund-Andersen, 1979).

However, recent studies suggest the presence of the insulin-responsive GLUT-4 in the brain, and insulin can induce GLUT-4 translocation to the plasma membrane in neurons. RT-PCR and in situ hybridization techniques demonstrated the expression of GLUT-4 in the pyramidal cells of hippocampal CA1 and CA3 areas, the granule cells of DG, subiculum, neocortical areas, piriform and entorhinal cortices, red nucleus and cerebellar Purkinje cells in hindbrain (Messari et al., 2002). Insulin treatment increased translocation of GLUT-4 to the plasma membrane in hippocampal slices of rats and human neuroblastoma cell line (Benomar et al., 2006; Piroli et al., 2007). Intracerebroventricular administration of insulin increases the number of GLUT-4 on hippocampal plasma membrane and

glucose uptake by hippocampus simultaneously, which was blocked by the inhibitor of phosphoinositide 3-kinases (PI3K). The phosphorylation of Akt induced by insulin was also observed in the same study, suggesting the involvement of PI3K pathway in the insulin-induced glucose uptake (Grillo et al., 2009). Intrahippocampal insulin improved spatial memory in a PI3K-dependent pathway, which was blocked by inhibition of endogenous intrahippocampal insulin signaling. The animals developing Type 2 diabetes by a high-fat diet expressed impaired basal cognitive function and decreased cognitive response to hippocampal insulin administration due to the insulin resistance (McNay et al., 2010). Therefore, an insulin-induced increase in glucose utilization and subsequent increase in energy supply is a possible mechanism underlying the facilitatory effect of insulin on LTP.

To test this hypothesis, the inhibitor of GLUT-4 was used. Indinavir is a kind of protease inhibitor used for the treatment of acquired immune deficiency syndrome (AIDS). However, the AIDS patients treated with indinavir expressed very high prevalence of insulin resistance (Safrin et al., 1999). Further study found that indinavir reduced glucose uptake by inhibition of GLUT-4 rather than other GLUTs, suggesting that indinavir was a selective blocker of GLUT-4 (Murata et al., 2000, 2002). In our study, indinavir which was used as an inhibitor of GLUT-4, didn't express any effects on insulin-mediated E-LTP, suggesting that insulin-sensitive GLUT-4 didn't play a role in the insulin-dependent E-LTP and then ruled out the involvement of glucose metabolism induced by insulin. To further confirm this result, we investigated the increased extracellular glucose concentration on the E-LTP of neonatal rats. Our results showed that elevation of glucose level from 11mM to 22 or 33mM didn't affect the magnitude of E-LTP of immature rats. In general, the involvement of insulin-induced glucose metabolism in the effect of insulin was not supported by our results.

5.5.3 The role of p42MAPK in the effect of insulin

Mitogen-activated protein kinase (MAPK) is an integral component of cellular signaling which responds to extracellular stimuli and regulates cellular activities.

In a study of learning and memory of rats in contextual fear conditioning task, Atkins et al.

showed that learning training induced the activation of p42MAPK significantly in a NMDA receptor-dependent manner. Application of the inhibitor of MAPK kinase, the upstream of MAPK, abolished fear conditioning (Atkins et al., 1998). English's study showed that HFS caused a NMDA receptor-mediated activation of p42MAPK in CA1 area of hippocampus. The activation of p42MAPK could also be induced by activated NMDA receptor and PKC (English et al., 1996). It is also found that inhibition of MAPK pathway blocked the production of LTP in hippocampus induced by theta-burst stimulation (Selcher et al., 2003). Thus, the activation of p42MAPK pathway is induced in the process of learning and memory and may play an essential role in learning and memory. Owing to the facts that the binding of insulin to insulin receptor leads a sequential activation of Raf, MEK and MAPK (Gogg et al., 2009; White et al., 1998), we hypothesized that insulin-activated MAPK pathway was responsible for the effect of insulin on E-LTP.

We found that in our study application of PD98059, the inhibitor of MAPK pathway, almost blocked the insulin-dependent E-LTP completely, indicating the involvement of MAPK pathway. Consistent with the above results, tetanic stimulation induced a significant increase in the expression of phosphorylated p42MAPK in insulin-treated slices in comparison to that in control group.

However, how MAPK pathway facilitates the production of E-LTP of neonatal rats? In general, the induction of LTP is a sequent events originating from the release of glutamate from presynaptic neurons induced by tetanic stimulation. The released glutamate binds to the AMPA receptors on the plasma membrane of postsynaptic neurons and depolarizes the membrane potential. The mixture of glutamate and strong depolarization induced by tetanic stimulation activates NMDA receptor on the membrane of postsynaptic neurons and promotes the influx of Ca^{2+} and, in turn, promotes the phosphorylation of CaMK Ⅱ (Malenka, et al., 1989; Silva et al., 1992a, 1992b; Lledo et al., 1995; Alford et al., 1993; Collingridge et al., 1983; Tang et al., 1999). Activated NMDA receptor causes the insertion of AMPA receptor into the membrane of postsynaptic neurons and the conductance of AMPA receptor is enhanced by phosphorylation of GluR1 of AMPA receptor under the action of activated

CaMK II (Barria et al., 1997; Derkach V et al., 1999).The activated AMPA receptor increases magnitude of EPSC (Collingridge et al., 1992). From the above description, AMPA receptor may play an important role in the induction of LTP.

However, the synapses in the hippocampus of neonatal rats are different from that of adult rats. It has been proved by many studies that excitatory synapses in infant rats only contain NMDA receptors with the absence of AMPA receptors on the postsynaptic plasma membrane at resting membrane potentials and these synapses are referred as "morphological silent synapses" (Isaac et al., 1995; Liao et al., 1995; Petralia et al., 1999; Takumi et al., 1999). During the first two weeks after birth, a gradual decreasing relative occurrence of these "silent" synapses are observed in the hippocampus of neonatal rats (Durand et al., 1996; Liao and Malinow, 1996; Hsia et al., 1998) by conversion of these synapses from NMDA to dual NMDA and AMPA receptor-containing synapses in an activity-dependent manner (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996). On the other hand, the hippocampal fEPSPs in neonatal rats after titanic stimulation express different forms compared to that of mature rats. After stimulation, the magnitude of fEPSPs increased within 30-40 minutes and then decayed to the baseline, suggesting the expression of short-term potentiation (STP) with the lack of LTP of neonatal hippocampus (Figurov et al., 1996; Muller et al., 1989). These facts promote us to conclude that the absence of AMPA receptor is responsible for the lack of LTP.

Several studies indicated that insulin and MAPK pathway have the ability to alter the distribution of AMPK receptor. It is also found that the insertion of the GluR1 subunit of AMPA receptor was stimulated by application of insulin (Passafaro et al., 2001). Treatment of 17-Beta-estradiol resulted in increased membrane levels of GluR1 in hippocampal slices in an MAPK-dependent pathway (Zadran et al., 2009).

According to these studies, we speculated that insulin-induced insertion of AMPA receptor into plasma membrane of postsynaptic neurons may be responsible for the facilitatory effect of insulin on E-LTP from developing hippocampus. Of course, we do not have any evidences to support the above

hypothesis.

5.5.4 Other possible mechanisms underlying the effect of insulin

There are other potential mechanisms by which insulin can facilitate the expression of LTP. For example, it has been found that insulin promotes cell surface expression of NMDA receptors (Skeberdis et al, 2001), which can lead to enhanced synaptic transmission. Insulin also enhances NMDA receptor-mediated current in hippocampal neurons (Liu et al., 1995). These effects could lower the threshold for the induction of LTP in the hippocampus and allows its expression in response to the HFS. Insulin may also faciliate the expression of LTP by increasing the protein expression of the dendritic scaffolding protein PSD-95 (Lee et al., 2005) and the recruitment of the insulin receptor substrate IRSp53, which has been shown to be translocated to the synapses in response to neuronal activity (Hori et al., 2005). Obviously, further work is needed to dissect the detailed mechanism.

In conclusion, our results identify an acute, restorative action of insulin on LTP expression in the young animals suggesting its importance in the synaptic plasticity, and therefore learning and memory, of the developing brain. This finding highlights a new fact of insulin signaling in addition to the growing belief that this protein is important for synapse maturation and maintenance during the development process (Chiu & Cline, 2010). Since cognitive impairment is a common feature in children suffering from the insulin-dependent type 1 diabetes, understanding the role of insulin in the developing nervous system is important not only scientifically but also has clinical implication.

Figures:

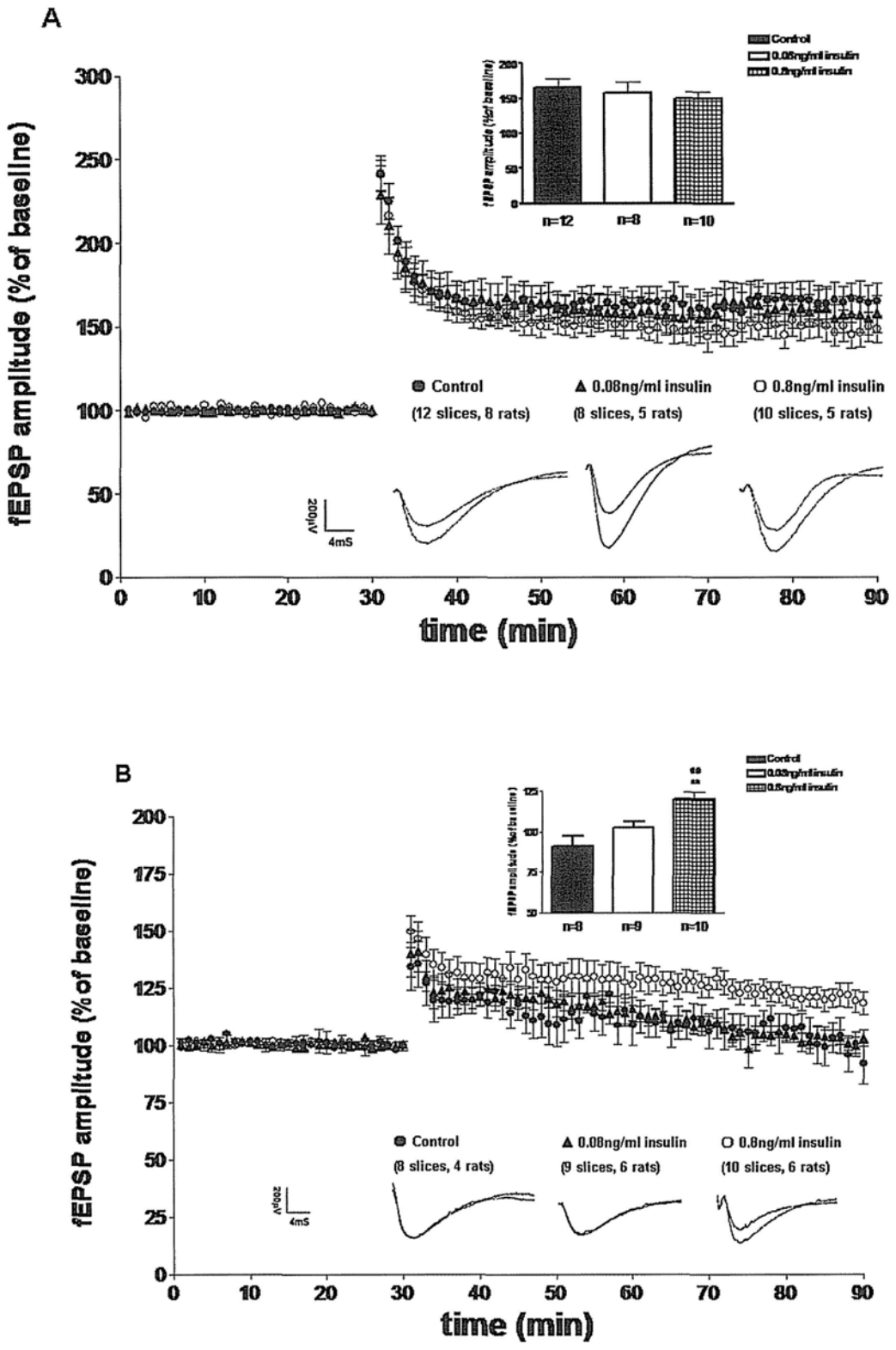

Figure 5.1: The effect of insulin on E-LTP in the CA1 area of hippocampal slices from immature and mature rats. A: fEPSPS of adult rats were recorded and titanic stimulation (1 train, 100 Hz, 1 s)

was used to induce E-LTP. No significance was found among control and insulin treatment groups. **B:** fEPSPS of infant rats were recorded. 0.8ng/ml insulin significantly increased the potentiation of fEPSPs induced by HFS compared with those of control group and 0.08ng/ml insulin group. ** *P<0.01* compared with control group, ##: *P<0.01* compared to 0.08ng/ml insulin group, Data are expressed as mean±S.E.M.

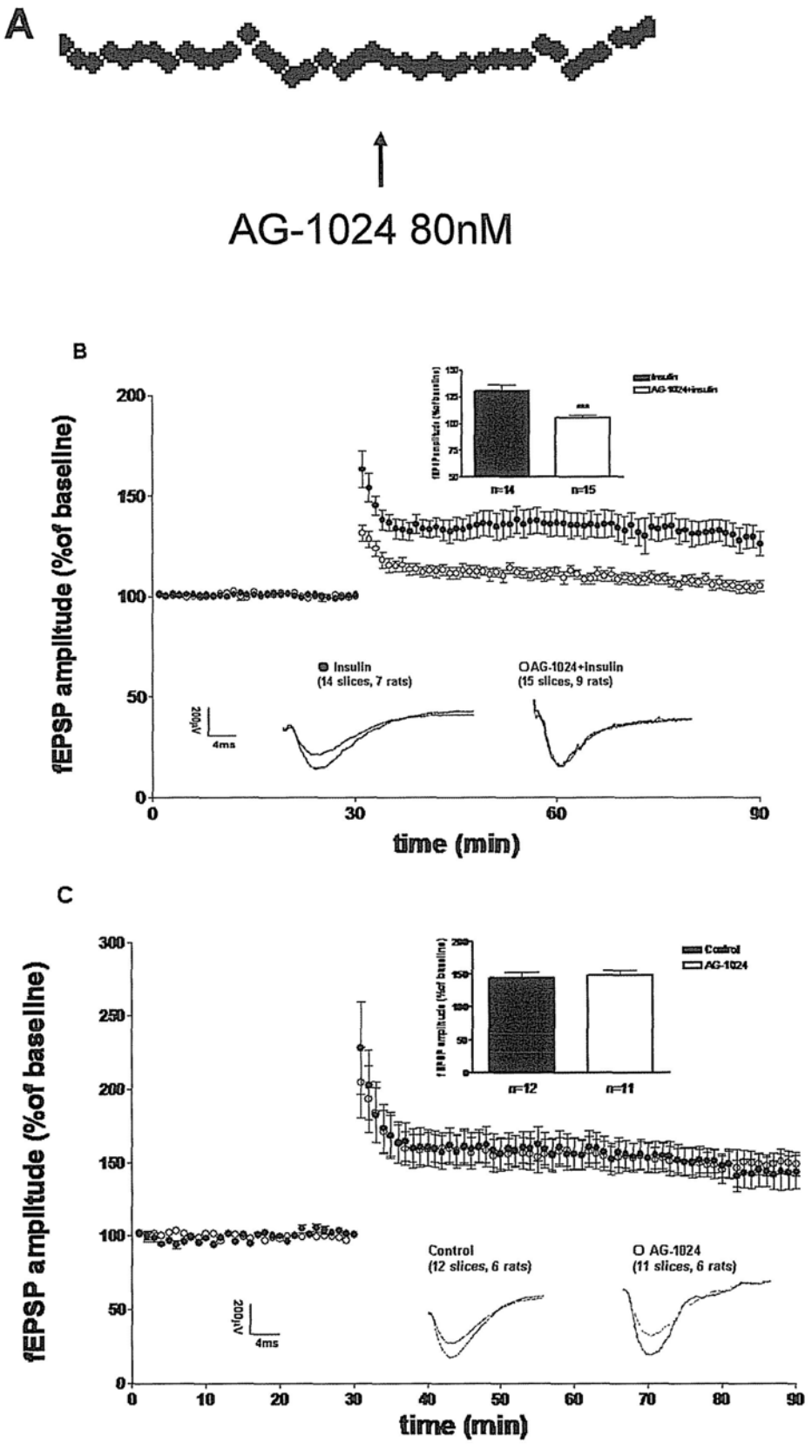

Figure 5.2: The effect of AG-1024 on E-LTP. A: Incubation of the hippocampal slices with 80nM of

AG-1024, the inhibitor of insulin receptor, did not affect the basal synaptic transmission. **B:** AG-1024 significantly decreased the magnitude of insulin-mediated E-LTP of neonatal rats. **C:** Addition of AG-1024 didn't affect the magnitude of E-LTP from mature rats. *** $P<0.001$. Data are expressed as mean±S.E.M.

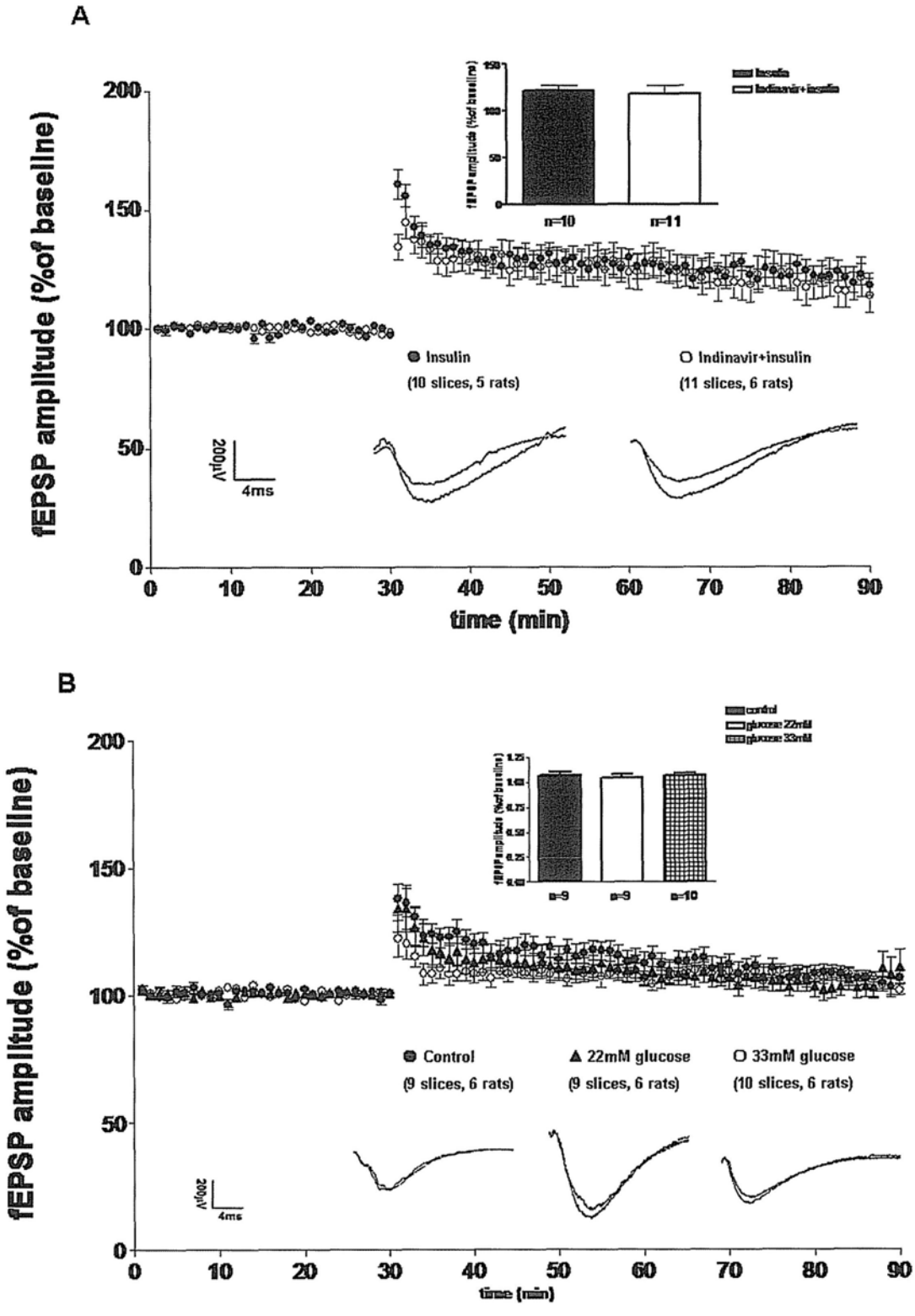

Figure 5.3: The role of glucose metabolism in the E-LTP. A: Application of indinavir (100μM), the inhibitor of insulin-sensitive GLUT-4, didn't exert any effect on the magnitude of E-LTP of infant rats in the presence of insulin treatment. **B:** Increase of glucose concentration from 11mM to 22 or 33mM didn't facilitate the production of E-LTP of immature rats. Data are expressed as mean±S.E.M.

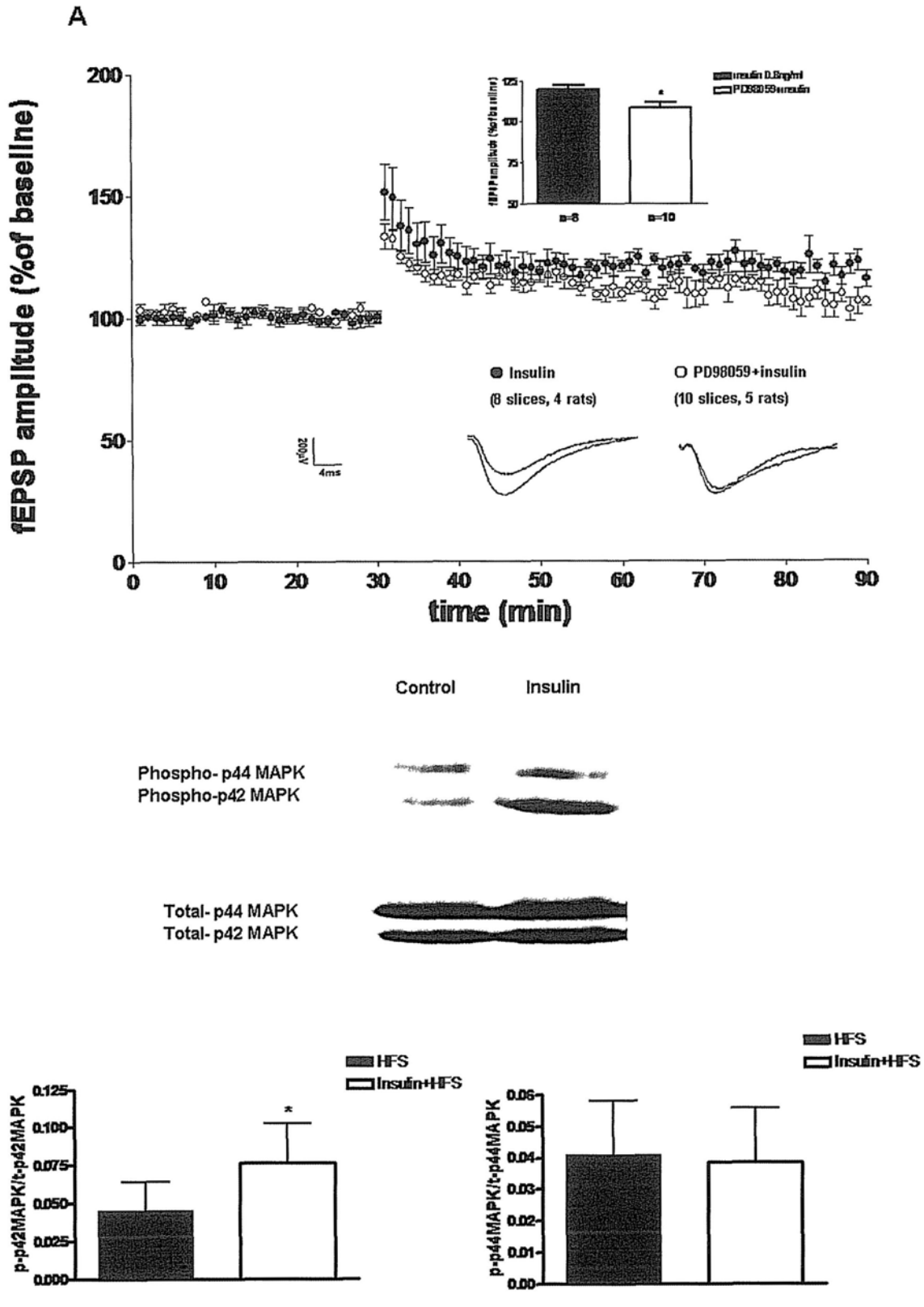

Figure 5.4: The involvement of activated p42MAPK in the insulin-dependent E-LTP. A: Pretreatment of PD98059 (20μM), the inhibitor of MAPK pathway, significantly decreased the

magnitude of insulin-mediated E-LTP of postnatal rats. **B:** Western blotting showed that titanic stimulation induced an increase in the expression of phosphorylated p42MAKPK in 10 minutes in the insulin-treated hippocampal slices in comparison to that in control group using infant rats. * $P<0.05$. Data are expressed as mean±S.E.M.

CHAPTER 6

GRNERAL DISCUSSION

6.1 The role of HCO_3^--induced insulin secretion in CFRD

CFRD is characterized by chronic hyperglycaemia caused by the decreased insulin secretion and is distinct from both Type 1 diabetes and Type 2 diabetes (Moran et al., 2002). The mechanism of dysfunction of insulin secretion in CFRD patients has been investigated by many studies. The possible reason for impaired insulin secretion in CF patients originates from pancreatic insufficiency as a result of the deficiency of CFTR in pancreatic duct cells. However, this explanation is not supported by the fact that some CF patients developed the absence of the first insulin secretion with intact the second insulin secretion after glucose challenge (Rakotoambinina et al., 1994).

Our results provide another possible explanation for the dysfunction of insulin secretion in CFRD. We observed that inhibition of CFTR blocked the HCO_3^--induced increase in $[Ca^{2+}]_i$ significantly, indicating that CFTR was involved in the effect of HCO_3^- on β-cells. CFRD is also characterized by the reduced first phase insulin secretion (Mohan e tal., 2009; DeSchepper et al., 1992), which is consistent with the enhancement effect of HCO_3^- on first phase insulin secretion. Taken together, it seems that the absence of HCO_3^--induced insulin secretion as a result of CFTR deficiency was one possible reason for the defective insulin secretion in CFRD.

Although our study proposes a novel theory for the onset of CFRD, the previous explanation can not be discarded because the reduced islets in the pancreas was observed and the decrease of second phase insulin secretion was also found in some CF patients with IGT and NGT (Soejima et al., 1986; Iannucci et al., 1984; Austin et al., 1994). In fact, the two explanations are not contradictory to each other and perhaps are responsible for different stages of CFRD or different types of CFRD.

6.2 The role of insulin in the impaired cognitive function of CF patients

Only a very few studies focus on the central nervous system (CNS) of CF patients on account of

the well accepted idea that the dysfunction of CFTR doesn't exert any effects on the function of neurons. Although lower cognitive skills index (CSI) was observed in some CF patients, this phenomenon was attributed to the decreased level of vitamin E resulting because of malnutrition (Koscik et al., 2005). However, the decreased insulin secretion provided another mechanism for the impaired cognitive function in CF patients.

Insulin is well known for its action on peripheral glucose homeostasis. The location of insulin and insulin receptor in CNS suggest that CNS is a target organ for insulin (Margolis et al., 1967; Banks et al., 1997a; Havrankova et al., 1978a; Unger et al., 1989). In addition to its central role in neuron survival and food intake, accumulating evidences about the improving effect of insulin on learning and memory has emerged (Seeley et al., 2000; Kern et al., 2001). In addition, diabetic patients and animal models expressed impaired memory (Kodl et al., 2008; Marfaing-Jallat et al., 1995; Oomura et al., 2002). Numerous studies proved that insulin could rescue the impaired cognitive function (Lee et al., 2009; Izumi et al., 2003; Voll et al., 1989; De et al., 1976). Our study demonstrated that insulin could facilitate the production of hippocampal E-LTP in infant rats. Over all, the decreased insulin secretion is a possible reason for the impaired cognitive function in CF patients.

Abbreviations

AD	Alzheimer's disease
AE	anion exchanger, Cl^-/HCO_3^--exchanger
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	blood-brain barrier
CaMK II	alpha-calcium-calmodulin-dependent protein kinase II
CaMK Ⅳ	calcium/calmodulin-dependent protein kinase
CICR	Ca^{2+}–induced Ca^{2+} release
CNS	central nervous system
CF	cystic fibrosis
CFRD	cystic fibrosis related diabetes mellitus
CFTR	cystic fibrosis transmembrane conductance regulator
CREB	cAMP response element-binding protein
CSF	cerebrospinal fluid
CSI	cognitive skills index
DM	diabetes mellitus
E-LTP	early-phase LTP
ER	endoplasmic reticulum
fEPSP	field excitatory postsynaptic potentials
GAD	glutamate decarboxylase
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GLUT	glucose transporter
HD	Huntington's disease
HFS	high frequency stimulation
HVA	high-voltage activated
IAPP	islet amyloid polypeptide
IGT	impaired glucose tolerance
IRS	insulin receptor substrate
K_{ATP} channel	ATP-sensitive K^+ channel
Kca channel	Ca^{2+}-dependent K^+ channel
L-LTP	late-phase LTP
LTD	long-term depression
LTP	long-term potentiation
LVA	low-voltage-activated
MAPK	mitogen-activated protein kinase
MF	mossy fibers
maxi-K(V) channel	voltage-dependent with large conductance K^+ channel
NBC	Na^+-HCO_3^--cotransporter
NCBE	Na^+-driven Cl^-/HCO_3^--exchanger
NCX	Na^+-Ca^{2+} exchanger
NGT	normal glucose tolerance
NMDA	N-methyl-D-aspartate
PD	Parkinson's disease

PI3K	phosphoinositide 3-kinases
PP	perforant path
sAC	soluble adenylyl cyclase
SC	Schaffer collateral
STP	short-term potentiation

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