THE FUNCTION AND MECHANISM OF ASCORBIC ACID IN THE RELEASE OF INSULIN FROM SCORBUTIC GUINEA PIG PANCREATIC ISLETS

By

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ABSTRACT

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The islets from normal guinea pigs had a rapid initial insulin secretion while the islets from scorbutic guinea pigs had delayed and lower insulin secretion in response to 20 mM glucose in the perifusion media. Scorbutic islets had increased insulin secretion in response to 20 mM glucose only in the presence of 5 mM L-ascorbic acid 2-phosphate. When the islets from control and scorbutic guinea pigs were perifused with elevated potassium (45mM) in the perifusion media, insulin secretion from scorbutic islets was as rapid as that from control islets and to the same extent. When the islets were perifused with 20 mM glyceraldehyde in the media, insulin secretion from control islets showed a rapid and increased response. Islets from scorbutic guinea pigs, in contrast, had a delayed and decreased insulin secretion. Together these observations suggest that pancreatic islets from scorbutic guinea pigs have impaired insulin secretion and that ascorbic acid can reverse this defect. The abnormal insulin secretion in pancreatic islets from scorbutic guinea pigs resides somewhere between glyceraldehyde metabolism and ATP generation, presumably by mitochondrial oxidative phosphorylation.

To

my son, Bertram Matthew Wu

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
DHA	Dehydroascorbic acid
AAH•	Ascorbic acid free radical
PDI	Protein disulfide isomerase
GSH	Glutathione
GSSG	Glutathione disulfide
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
GLP	Glucagon like peptide
GIP	Gastrointestinal inhibitory peptide
MODY	Maturity-onset diabetes of the young
IP_3	Inositol-1,4,5-triphosphate
DAG	Diacylglycerol
BCIP	5-bromo-4-chloro-3-indolyl phosphate

CHAPTER I

LITERATURE REVIEW

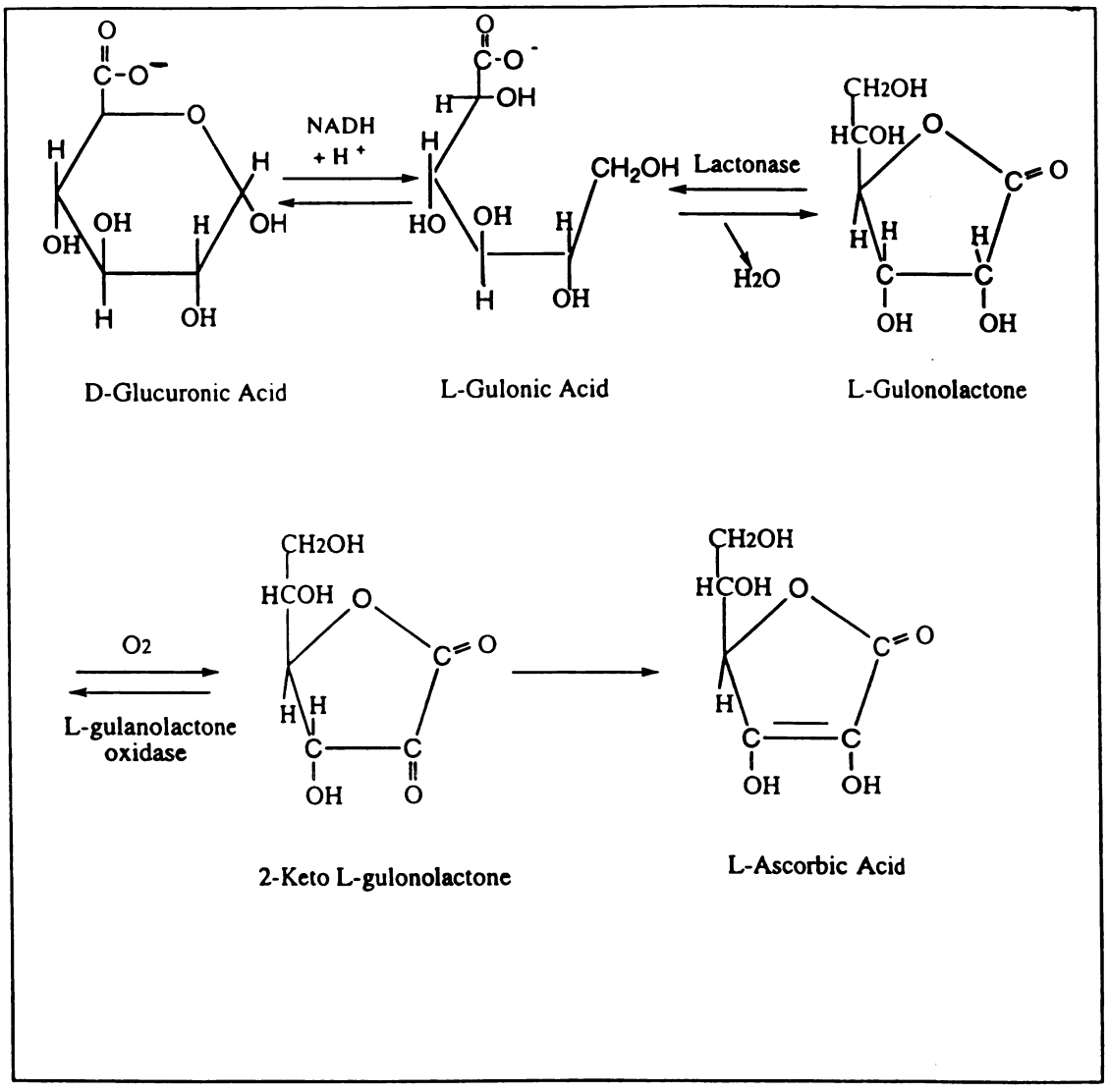
The Biological Function of Ascorbic Acid

L-ascorbic acid , $C_6H_6O_8$, can be synthesized in most mammals from glucuronic acid (Fig. 1) (1). Several species such as humans, other primates and guinea pig can not synthesize ascorbic acid (2), because they lack the enzyme L-gulonolactone oxidase, which is required for the formation of 2-keto-L-gulonolactone from 1-gulonolactone (3). The critical function of ascorbate in biological systems may involve its ability to donate electrons, while itself undergoing reversible oxidation to dehydroascorbic acid (DHA) (4). In the last decade, the biochemical importance of ascorbic acid in mammals has begun to be characterized more extensively (5). Investigators found that ascorbic acid is involved in the protein prolyl hydroxylation reaction, enzyme reducing reactions and antioxidant reactions (6).

1. Ascorbic acid and hydroxylation reactions:

Ascorbic acid accelerates hydroxylation reactions in a number of biosynthetic pathways (7). The very important role of ascorbate in the hydroxylation reaction is to function as a cofactor for prolyl and lysyl hydroxylases in the biosynthesis of collagen (8). Proline hydroxylation, an essential step in the biosynthesis of collagens, is catalyzed by prolyl-4-hydroxylase, requiring a peptide substrate, α-ketoglutarate, oxygen and ferrous ion, and releasing a hydroxylated peptide, succinate and CO_2 (9). It has been reported that ascorbate is important for optimal activation of prolyl

Figure 1. Synthesis of Ascorbic Acid from D-Glucuronic Acid.


NADH
+ H+
Lactonase
H2O
D-Glucuronic Acid
L-Gulonic Acid
L-Gulonolactone
O2
L-gulanolactone
oxidase
2-Keto L-gulonolactone
L-Ascorbic Acid

hydroxylase, hydroxylation of peptidyl proline, and enhanced secretion of procollagen that contains hydroxyproline (10). Deprivation of dietary ascorbic acid decreased the formation of collagen (11). Early studies in humans and guinea pigs showed that scurvy is characterized by poor healing of wounds and can be treated by administration of ascorbate (12). Recent molecular cloning studies (7) revealed that the β-subunit of human prolyl 4-hydroxylase is the product of the same gene as protein disulfide isomerase (PDI). Wells, et al (13) demonstrated that protein disulfide isomerase can catalyze glutathione dependent dehydroascorbic acid reduction. They speculate that the β-subunit of the hydroxylase catalyzed the reduction of dehydroascorbate (DHA) to ascorbic acid by glutathione (GSH). The regenerated ascorbic acid then functions as a cofactor in the hydroxylation reaction by a mechanism presumably as an alternate oxygen acceptor or to restore iron to the ferrous state. However, the recent studies of effects of ascorbate on collagen metabolism are fraught with contradictory results; and some evidence does not support the conclusion that the main function of ascorbate is in the hydroxylation of prolyl and lysyl residues (14). Barnes et al (15) found no difference in urinary hydroxyproline excretion in scorbutic as compared with normal guinea pigs. They concluded that even though collagen synthesis was defective in scurvy, no evidence could be found for the presence of an underhydroxylated collagen. The specific role of ascorbate in collagen synthesis has long been discussed, but is in need of further

studies.

2. Ascorbic acid and Glutathione: reducing property of ascorbic acid.

Glutathione is synthesized in many types of cells from glutamate, cysteine and glycine (16). These amino acids may be formed in cellular metabolism, so glutathione is not required in the diet (17). Cells synthesize glutathione by a two-step pathway catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase (18):

L-Glutamate + L-cysteine + ATP ⇌ L-γ-glutamyl-L-cysteine + ADP + Pi

L-γ-Glutamyl-L-cysteine + glycine + ATP ⇌ glutathione + ADP + Pi

Glutathione (GSH) provides cells with their reducing environment (19). The functions of glutathione include maintenance of the thiols of proteins and the reduced forms of other compounds such as ascorbic acid, which can augment cellular reducing environment (20). The reaction between GSH and dehydroascorbate (DHA) has been demonstrated, *in vitro* (21):

$$2GSH + DHA \leftrightharpoons GSSG + AA$$

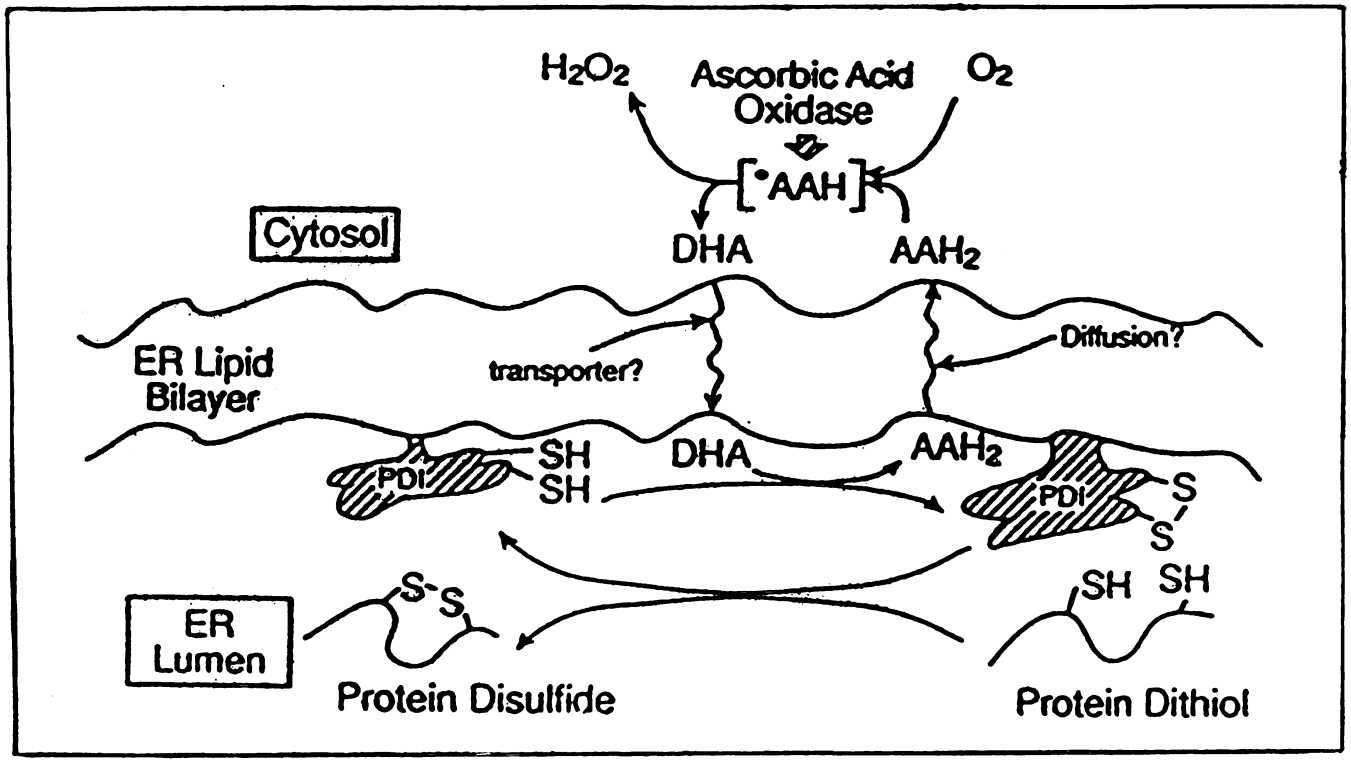
Silva (22) showed in 1927 that the oxidation product of the antiscorbutic factor, which is now known as ascorbic acid, had antiscorbutic activity when administered to vitamin C deficient guinea pigs. Szent Gyorgyi (23) indicated a possible role for GSH to restore ascorbate from DHA. Each of the two antioxidants appears to closely influence the synthesis of the other, i.e., administration of ascorbic acid can rescue glutathione-deficient newborn rats and guinea pigs and administration of glutathione

monoester can delay the onset of scurvy in guinea pigs (24, 25). A decrease of ascorbic acid appears to provide a metabolic signal that increases glutathione synthesis, whereas glutathione deficiency in adult mice seems to turn on ascorbic acid synthesis (26). All of these studies suggested that there may be a control mechanisms for glutathione and ascorbic acid functioning together in some of the biological metabolism (27).

3. Ascorbic acid and Diabetes:

It has been reported in the Wells lab that protein disulfide isomerase (PDI) has intrinsic dehydroascorbate reductase activity (13). A model (Fig. 2) was proposed that dehydroascorbic acid would cyclically act as a protein dithiol oxidant in a PDI catalyzed reaction. This model indicates that the oxidation of intracellular ascorbic acid by a hypothetical oxidase or peroxidase occurs at the surface of the endoplasmic reticulum in cells undergoing secretory protein synthesis. The resulting dehydroascorbic acid, moving across the endoplasmic reticulum (ER) membrane, would oxidize cysteines at the active center of PDI (28-30), which would, in turn, oxidize the nascent protein sulfhydryl groups for native disulfide conformation. Ascorbic acid, reformed from the reaction with PDI, would diffuse back into cytoplasmic space, and the reduced PDI would be reoxidized by freshly derived dehydroascorbic acid. This model was supported by the work of Krause *et al.* (31) and Venetianer and Straub (32). They reported that DHA would oxidize reduced

Figure 2. Proposed PDI catalyzed ascorbate-dehydroascorbate cycle in the disulfide protein synthesis. AAH2 = ascorbate, DHA = dehydroascorbate, PDI = protein disulfide isomerase, ER = endoplasmic reticulum


H_2O_2
Ascorbic Acid Oxidase
O_2
[•AAH]
Cytosol
DHA
AAH_2
ER Lipid Bilayer
transporter?
Diffusion?
SH
SH
PDI
DHA
AAH_2
PDI
S
S
ER Lumen
S-S
SH SH
Protein Disulfide
Protein Dithiol

inactive ribonuclease. The model was also supported by Boger (33) and Hochwald (34) who demonstrated that the serum albumin/globulin ratio is depressed in scorbutic guinea pigs and returns to normal in response to administration of ascorbic acid. In the absence of ascorbic acid, cells might synthesize defective insulin and other disulfide proteins.

Ascorbic acid also functions as an important component of cellular defense against oxygen toxicity and lipid peroxidation caused by free radical mechanisms (35). These observations suggested a possible close interrelationship between ascorbic acid and pathways known to influence diabetic processes (36). It has been reported that the plasma and tissue concentrations of ascorbic acid are decreased and dehydroascorbate increased in diabetic animals and humans (37-39). McLennan et al. (40) found low activity of prolyl hydroxylase activity in diabetic rats corresponding to the deficiency of ascorbic acid. Scorbutic guinea pigs had a depressed ability to synthesize and /or release insulin from pancreatic islets when stimulated with glucose and all of the symptoms could be treated by ascorbic acid supplement in guinea pigs (41-43). Young et al. (44) have demonstrated that ascorbate can reduce the oxidative stress in streptozotocin diabetic rats and insulin treatment can normalize ascorbate and lipid peroxidation level. Bode et al. (45) also showed that less ascorbic acid and higher levels of dehydroascorbic acid were detected in the livers of diabetic rats. They found that the enzyme activity reducing

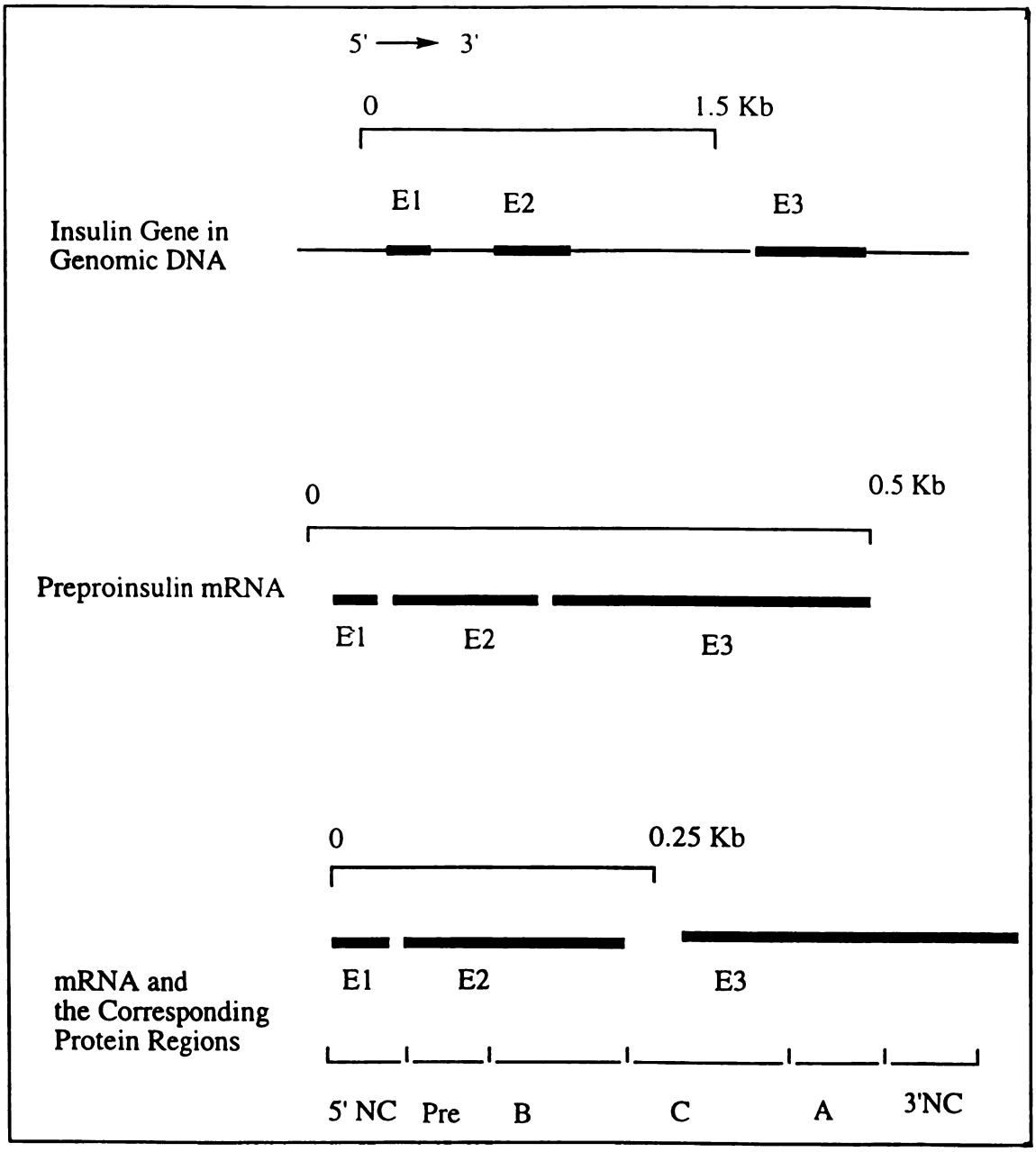
DHA in the tissue was not different in diabetic compared with the control rats, indicating that the high DHA levels was due to a defect in the regeneration of ascorbic acid from DHA in a GSH dependent manner by way of the hexose monophosphate shunt. All of these observations suggested that ascorbic acid plays an intrinsic role in the process of insulin synthesis/release through an unknown mechanism.

Mechanisms of Insulin Secretion

After the pancreatic islets had been discovered by a German medical student, Paul Langerhans in 1869 (46), Banting et al. (47) at the University of Toronto isolated insulin. Insulin was started to be used as a drug for diabetes in 1922. The human insulin gene is located at chromosome 11 and the coding region of genomic DNA was translated to a single polypeptide which is preproinsulin (Fig.3) (48). The signal portion is quickly removed leaving a proinsulin which contains insulin and the c-peptide. Using gold labeled specific antibody against proinsulin, researchers demonstrated that an equal amount of insulin and c-peptide was secreted by a fusion of the insulin containing granule with the plasma membrane when β-cells were subjected to a stimulus such as increased blood glucose (49).

Physiologically, there are two kinds of insulin secretagogues (50): nutrient

Figure 3 The Insulin Gene Structure. E = exon, Pre = preproinsulin, A = insulin A chain, B = insulin B chain, C = C-peptide, 5'NC = 5' noncoding region, 3'NC = 3' noncoding region.


5' → 3'
0
1.5 Kb
E1
E2
E3
Insulin Gene in
Genomic DNA
0
0.5 Kb
Preproinsulin mRNA
E1
E2
E3
0
0.25 Kb
E1
E2
E3
mRNA and
the Corresponding
Protein Regions
5' NC
Pre
B
C
A
3'NC

secretagogues, such as glucose, amino acids and fatty acids and nonnutrient secretagogue which are not able to initiate insulin secretion alone, but can modulate compounds. Glucose is the primary signal for insulin secretion, which is the consequence of multiple events. The stimulatory effect of glucose has been linked to its metabolism in β-cells via entry into the β-cells and generation of metabolic signals (51). Matschinsky and Ellerman showed that intracellular concentrations of glucose closely related to the extracellular glucose concentration which indicated that glucose phosphorylation by glucokinase is one of the rate limiting steps in glucose metabolism (52). Most maturity-onset diabetes of young (MODY) patients with glucokinase mutations showed altered glucose stimulated insulin secretion (53, 54). The altered threshold of glucose response is a major effect, i.e., higher glucose levels are required for insulin secretion in MODY patients than in normal individuals. Grodsky and co-workers (55) demonstrated that insulin release from an isolated perfused pancreas could be stimulated by glucose and to a lesser extent, by other metabolizable sugars such as mannose and fructose, but not by poorly metabolized sugars such as galactose, xylose, or L-arabinose. Ashcroft et al. (56) and Coore et al. (57) also demonstrated those nonmetabolizable analogs of glucose such as 2-deoxyglucose or 3-O-methyl glucose was ineffective in promoting insulin release. They also showed that inhibitors of glycolysis such as mannoheptulose or glucosamine blocked the response of β-cells to D-glucose.

The stimulatory effect of glucose involves changes in the ATP:ADP ratio and inhibition of the ATP-sensitive K^+channels leading to activation of voltage-gated Ca $^{2+}$ channels (51). Holz and Habener (58) and Newgard and McGarry (51) proposed a model that glucose is initially taken up by β-cells through a glucose transporter, GlutII. The glycolysis of glucose in β-cells generates a signal, i.e., increase the ratio of intracellular ATP relative to ADP. The ATP binds to the ATP-sensitive potassium channels inducing closure of the channels. Closure of the ATP-sensitive potassium channels results in membrane depolarization which stimulates the opening of the voltage-sensitive Ca^{2+}channels. The influx of Ca $^{2+}$ triggers the insulin secretion through calcium dependent exocytosis.

The increase of ATP can come from the cytosolic glycolysis and mitochondrial oxidative phosphorylation (59). However, in glucose-stimulated insulin secreting islets, the major amount of ATP produced through the catabolism of the hexose is generated by mitochondrial oxidative reaction rather than glycolysis (60). The glycerol phosphate shuttle, activated by increased glucose metabolism, is important in transferring the reducing equivalents coupled with aerobic glycolysis to the mitochondria (61), during which the FAD-linked mitochondrial glycerophosphate dehydrogenase is activated by Ca $^{2+}$. The activity of this enzyme is much higher in islet than it is in liver homogenates (62). Malaisse (63) has reported that the enzyme activity is higher in the presence of D-glucose than in the absence of D-glucose,

indicating that the enzyme is involved in transfering the reducing equivalent generated from glucose metabolism to the mitochondria in β-cells. Recently, lower activity of mitochondrial glycerol phosphate dehydrogenase has been found in the pancreas of the strepzotocin diabetic rat than the control rat (64). The generation of ATP through the oxidative reaction in mitochondria is an important mechanism in the process of glucose induced insulin release (65).

The stimulatory effect of glucose on insulin secretion is also related to the level of reduced pyridine nucleotides (NADH and NADPH) (51). Malaisse et al. (66) and Sener et al. (67) have reported that pyruvate and lactate have mild potentiating effects in the presence of stimulatory glucose concentrations, but fail to stimulate insulin release from islets in the absence of glucose. They found that the inability of pyruvate or lactate to stimulate insulin release appears to be unrelated to the islets' capacity to oxidize these fuels, but appears to be related to their inability to generate NADH.

In the process of glucose stimulated insulin secretion, calcium, which is regulated by secondary messengers, plays a crucial role. Glucose and other fuel secretagogue can elevate intracellular calcium relying mainly on calcium influx (68). The release of calcium from intracellular storage is regulated by nonglucose secretagogue (69). Several intracellular second messengers induced by nonglucose secretagogue modify the activation of calcium channels such as inositol-1,4,5-

trisphosphate (IP_3) and cAMP (70). When pancreatic β-cells are stimulated by nonglucose secretagogue, phospholipase C is activated by a G protein connected with the appropriate receptor (71). The hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate increases the IP_3 level in islets (72). When IP_3 binds to its intracellular receptor, calcium contained in the IP_3-sensitive pools (ER) is released to the cytosol (70).

cAMP is elevated by glucose and hormones such as glucagon, glucagon-like peptide (GLP) and gastric inhibitory polypeptide (GIP). High concentrations of glucose and other fuel secretagogues cause a modest increase in islet cAMP content (73, 74). Significant rises in islet cAMP content were seen within 1-2 minutes after glucose stimulation (75) and were the consequences of the elevation of intracellular Ca^{2+} (76). An increase in cAMP concentration in pancreatic β-cells can regulate insulin gene transcription (77), increase intracellular calcium through activating L-type Ca^{2+} channel (70) and initiate rapid phosphorylation of proteins (70). cAMP-dependent protein kinase A plays a crucial role in this process (70,76,77).

Based on the above reviews that ascorbic acid metabolism is intrinsically associated with insulin synthesis or release, my study, described herein, was aimed at exploring what role ascorbic acid plays during the process of insulin synthesis and secretion, and what the functional location of ascorbic acid is in the pathway of energy dependent insulin secretion.

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CHAPTER II

PURIFICATION AND DETERMINATION OF GUINEA PIG INSULIN

Introduction

The determination of the amino acid sequence of insulin by Sanger and Yuppy showed that different species have different insulin sequences and structures (1). The first work on insulin from sheep and pig showed that variation occurred in only a limited number of amino acids confined to residues 8,9 and 10 of the A chain (2). Further sequences from horse, rabbit, human and chicken insulin confirmed the limited naturally occurring variation and suggested that a large part of the molecule was invariant. When it had been found that antibodies to bovine insulin could be produced in the guinea pig, the sequence of guinea pig insulin proved to possess greater differences from other species (3). Guinea pig insulin differs from pig insulin by eighteen residues (4). The immunological response showed great variation (5). The insulin of the guinea pig has been extracted and partially purified (6-8). This crude guinea pig insulin gave evidence of unusual structural properties, since it was not neutralizable by a large excess of anti-bovine or anti-cod insulin serum produced in the guinea pig (9). It can not be neutralized by anti-bovine or anti-porcine insulin serum produced in the horse, sheep, or rabbit. This was consistent with the strong immunological response shown by the guinea pig to bovine insulin (10). Thus, it

was suggested that guinea pig insulin would not aggregate to hexamers since residues which lie in the dimer-dimer interface of porcine insulin are larger or more hydrophilic than in guinea pig insulin (11). In addition, the lack of the zinc-coordinating B-10 histidine residue would not favor stabilization of hexamers of guinea pig insulin. On the other hand guinea pig insulin would dimerize since it retains all of the residues of the hydrophobic core of the porcine insulin dimer.

Yalow and Berson were the first to develop a chemical assay for insulin. And later on they developed a radioimmunoassay which is the basis for a common insulin measurement (12). But the radioimmunoassay has disadvantages, i.e., poor reproducibility at low insulin concentration. In 1988 Kekow (13) and colleagues described an enzyme linked immunosorbent assay (ELISA) for insulin. Briefly, the plate was coated by the rabbit antiguinea pig antibody and anti-insulin antibody which is usually raised from the guinea pig. Then to the plate were added samples or standards which was followed by the addition of rat insulin conjugated by peroxidase. The absorbance was read after the incubation with the addition of substrate for peroxidase which is o-phenylenediamine dihydrochloride (OPD) to the plate. Since guinea pig insulin can not be recognized by the antibodies against the insulin from other species, we choose another method, i.e., the indirect competitive ELISA developed by Bank (14) in 1988. In order to perform this assay, we purified guinea pig insulin from guinea pig pancreas (15,16) and conjugated it to chicken

ovalbumin to enhance the binding to 96 well plates (17).

Materials and Methods

Bovine serum albumin was purchased from United States Biochemical. Chicken egg albumin, guanidine hydrochlofide, anti-rabbit IgG (alkaline phosphatase conjugated), 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, bisbenzamide, calf deoxyribonucleic acid, bovine insulin and DEAE-Sephadex were purchased from Sigma. Sephadex G-50 was purchased from Pharmacia LKB. Bio-Gel P-30, acrylamide, N,N′-methylenebisacrylamide, ammonium persufate, SDS, and Coomassie brilliant blue R-250 were purchased from Bio-Rad. Guinea pig pancreas was purchased from Rockland (Gilbertsville, PA). Difluorodinitrobenzene was a product of Pierce. Standard guinea pig insulin and anti-guinea pig serum were kindly provided by Dr. Cecil C. Yip, University of Toronto and the Banting and Best Institute.

Insulin Purification:

Buffers and solutions were made as following: Homogenization solution: 75% ice-cold ethanol and 1.5% HCl, 2M ammonium acetate, pH 5.5, Sephadex G-50 equilibration buffer: 1M acetic acid, Bio-Gel P-30 equilibration buffer: 3M acetic acid and HPLC elution buffer: 0.2M Tris-HCl, pH 8.0 and 0.8M NaCl.

Guinea pig pancreas was homogenized with homogenization solution (4ml/g). The homogenate was extracted twice with shaking at 37 °C for 2 hours and

centrifuged at 4,000 x g for 20 min. The supernatant was neutralized with concentrated NH_4OH to pH 7.0 and allowed to precipitate overnight at 4 °C. The preparation was centrifuged as above and the supernatant was acidified with 2N HCl to pH 6.0. Ammonium acetate buffer (2 M) was added at one tenth of volume and the pH was adjusted to 6.0. Crude insulin was precipitated by the addition of 1.5 volumes of cold absolute ethanol and 2.5 volumes of cold diethyl ether. The precipitation formed overnight in the cold room was recovered by filtration on filter paper, air-dried and resuspended in 1M acetic acid. The fraction was loaded on the Sephadex-G-50 column which was equilibrated with 1M acetic acid, and the sample was eluted with the same solution. Insulin was detected at A280 as shown in Figure1. The fractions corresponding to insulin were pooled and further purified on a column of Biogel P-30 which was equilibrated with 3M acetic acid and insulin was eluted with the same solution. The fractions corresponding to insulin are shown in Figure 2. The pooled fractions were further purified with reversed anion exchange HPLC column and were lyophilized.

Dot blot Assay:

The lyophilized guinea pig insulin sample was resuspended with 0.006 N HCl and was spotted on a strip of nitrocellulose filter paper which was wet and briefly rinsed for 5 minutes with TBST buffer containing 10mM Tris-HCl (pH 8.0), 150mM NaCl and 0.05% Tween-20. The strip was blocked with TBST containing 1% bovine

Figure 1. The elution profile of Sephadex G-50 column. Insulin was eluted with 1M acetic acid and the fractions were monitored at 280nm.The eluted insulin fractions between numbers 72 and 110 were pooled and further purified by the Biogel P-30.

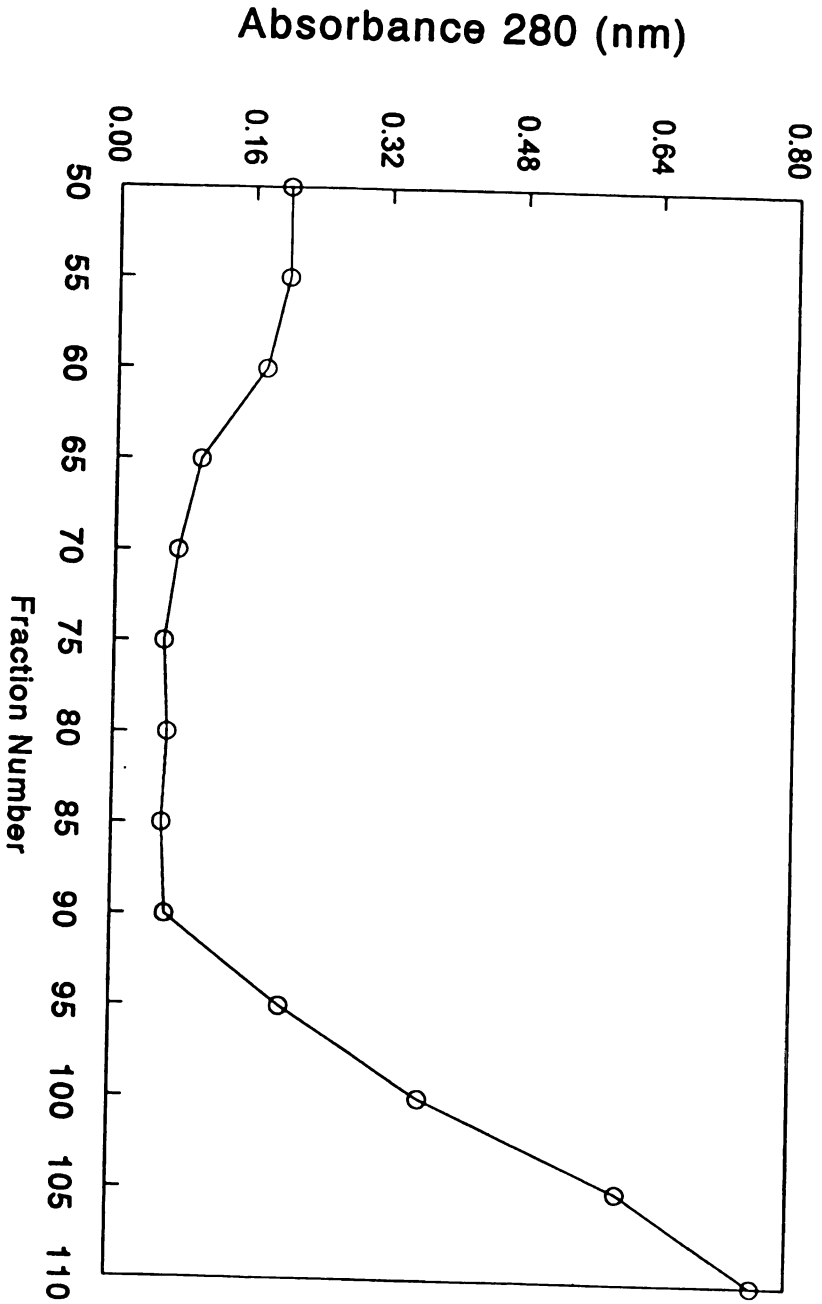
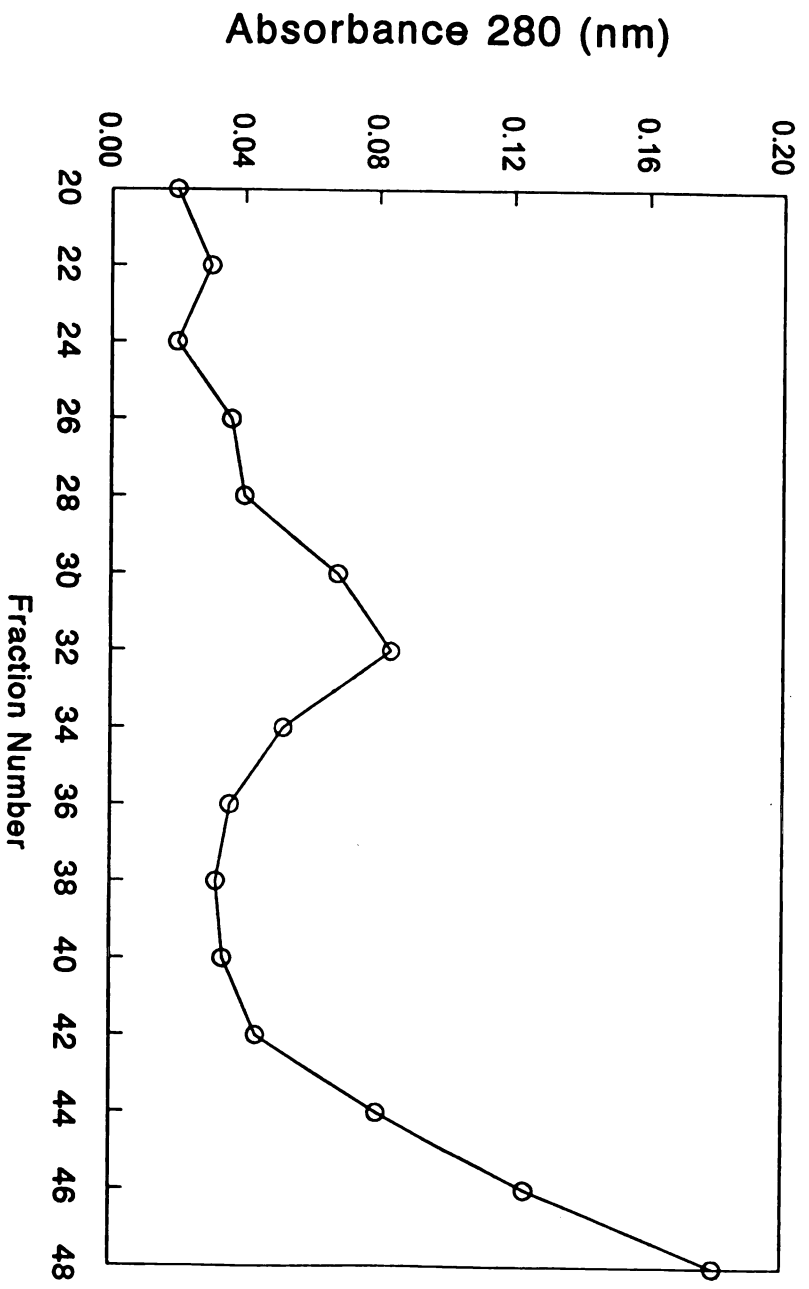

Absorbance 280 (nm)
0.00
0.16
0.32
0.48
0.64
0.80
50
55
60
65
70
75
80
85
90
95
100
105
110
Fraction Number

Figure 2.The elution profile of the Biogel P-30 column. Insulin was eluted with 3M acetic acid and the fractions were monitored at A280nm.The eluted insulin fractions between numbers 25 and 35 were pooled and further purified by HPLC.


Absorbance 280 (nm)
0.00
0.04
0.08
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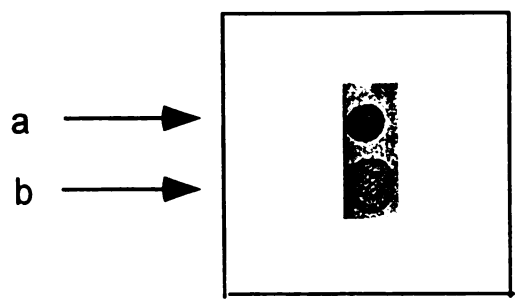
0.16
0.20
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
Fraction Number

serum albumin for 30 min at room temperature. The nitrocellulose filter paper was incubated with primary antibody which is rabbit anti guinea pig insulin (1:6000 dilution) at room temperature for 60 minutes. The strip of filter paper was washed again with TBST for 5 minutes for three times and was incubated with the secondary antibody which is goat anti rabbit IgG conjugated with alkaline phosphatase, 1: 15,000 dilution. The strip of paper was washed again as indicated before. Substrate reaction occurred in the presence of 0.5ml Solution A and 0.5ml Solution B in 99ml AP buffer indicated as following. AP Buffer: 100mM NaCl, 100mM Tris-HCl and 5mM $MgCl_2 \cdot 6H_2O$. Solution A: 0.75mg BCIP and 0.5ml DMF. Solution B: 15mg NBT, 0.35ml DMF and 0.15ml H_2O. The reaction was incubated at room temperature for 30 min under the dark. The result of dot blot analysis is shown in Figure 3.

SDS PAGE: The electrophoresis analysis of guinea pig insulin followed the method of Herbert Ley (18).

The stock solutions were made as following: Resolving gel solution (15%) was made with 18.02g urea, 0.69g sodium phosphate monobasic, 24.5 ml acrylamide:bis-acrylamide stock (30%:0.8%) and 0.5 ml of 10 % SDS (pH 7.2). Stacking gel solution (7.5%) consisted of 3.60g urea, 0.14g sodium phosphate monobasic , 2.44ml acrylamide:bis stock (30%:0.8%) and 0.1ml 10% SDS. The running buffer consisted of 0.1M sodium phosphate, pH 7.2 and 0.1% SDS. The sample buffer (10ml) contained 4.2g urea, 0.01g sodium phosphate monobasic,

Figure 3. The dot blot assay for purified guinea pig insulin. a: guinea pig insulin from Yip. b: purified guinea pig insulin. The primary antibody is rabbit anti-guinea pig insulin serum (1:6,000) and the secondary antibody is goat anti-rabbit IgG conjugated with alkline phosphate (1:15,000).


a
b

1.0ml of 10% SDS, 0.1ml of 2-mercaptoethanol and 1.0ml of 0.1% Bromphenol Blue, adjusted to pH 7.2. The staining solution consisted of 0.1% Coomassie Brillant Blue, 25% isopropanol and 10% acetic acid. The destaining solution contained 50ml glacial acetic acid and 75ml methanol made up to 500ml with distilled water.

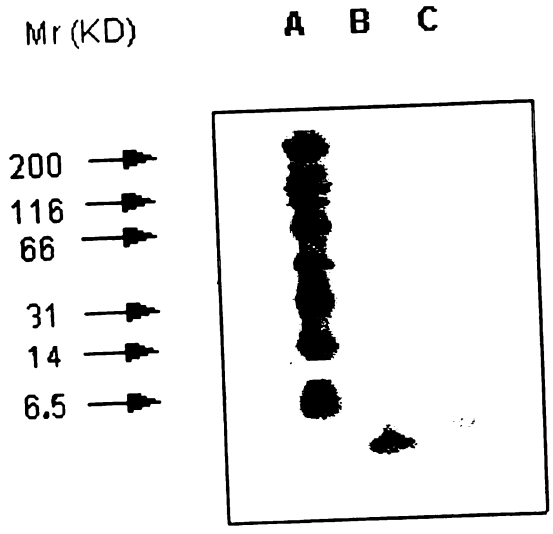
The resolving gel was made by mixing 5ml of resolving gel solution, 9μl 20% ammonium persulfate and 4μl TEMED, and was pipeted immediately into the slab gel set-up, overlayed with water and allowed to polymerize. The stacking gel solution was made by mixing 3ml of stacking gel solution, 10μl 20% ammonium persulfate and 2μl TEMED, and was immediately pipetted onto the top of the resolving gel. The standard molecular weight marker and the samples were loaded onto the gel after they were treated by boiling 8 parts of the sample buffer with 1 part of standard molecular weight marker or the sample for 3 minutes. The electrophoresis was performed at constant low potential of 60 volts at room temperature until the dye reached the bottom of the gel. The gel was removed and stained for 30 minutes in the staining solution. Following the staining, the gel was put into the destaining solution until the clear protein bands appeared. The result of purified guinea pig insulin on electrophoresis is shown on Figure 4.

Conjugation of guinea pig insulin to chicken ovalbumin:

The following stock solutions and buffers were made: 7M guanine hydrochloride, 0.1 M potassium phosphate, pH 7.2, 3% of 1,5-difloro-2,4-

Figure 4. The Electrophoresis of purified guinea pig insulin.

A. BIO-RAD low molecular weight marker. B: bovine insulin from sigma. C: purified guinea pig insulin. The electrophoresis was performed under low electrical potential (60V) at room temperature. The gel was stained with 0.1% Commassie Brillant Blue.


Mr (KD)
A
B
C
200
116
66
31
14
6.5

dinitrobenzene (DFDNB) dissolved in pure methanol, cold ether, saturated sodium borate (pH 10) and 7 M Urea.

Insulin was dissolved in guanidine hydrochloride-potassium phosphate solution at the concentration of 5mg/0.5ml. The solution was mixed with DFDNB (30mg/ml) and was left at room temperature for 15 minutes before it was placed on ice. The preparation was mixed vigorously with 4 times volume of cold ether. The upper layer was removed and the lower layer was mixed vigorously with a triple volume of ether and the upper layer was removed before adding 10 times the volume of cold ether. After the final precipitation formed on ice for one minute, the ether was decanted and the tubes drained. Chicken ovalbumin was dissolved in saturated sodium borate (10mg/ml) and added to the insulin precipitation. Guanidine hydrochloride (7 M) was added in a dropwise manner to facilitate rapid solution in the mixture. The dissolved sample was loaded onto Sephadex G-50 which was eluted with 7M urea at room temperature. The fractions corresponding to the conjugated insulin (dot blot analysis) were pooled (Figure 5) and subjected to SDS PAGE electrophoresis (19) after they were concentrated with centriprep 30 (Figure 6).

DNA Determination:

Reagents and Buffers: Phosphate-saline buffer was 0.05 M sodium phosphate, pH 7.4 and 2.0 M NaCl, 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl] - 6 -(1-methyl- 4 piperzyl) - benzmidazol•3HCl (H33258), 50μg/ml, was dissolved in phosphate-

Figure 5. Dot blot assay for conjugated guinea pig insulin. a, purified guinea pig insulin conjugated with chicken ovalbumin. b, purified guinea pig insulin. The primary antibody is rabbit anti-guinea pig insulin serum (1:6,000). The secondary antibody is goat anti-rabbit IgG conjugated with alkaline phosphate (1:15,000).

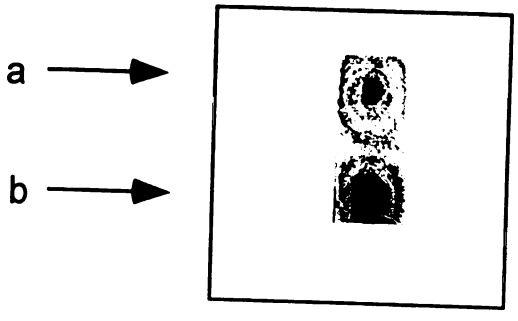
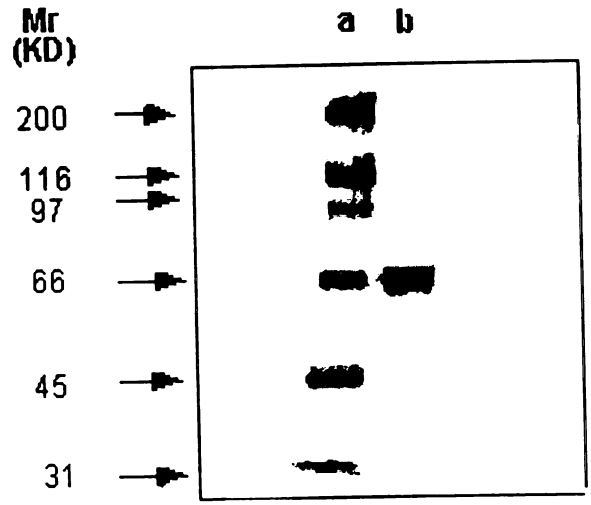

a
b

Figure 6. The SDS PAGE analysis for conjugated guinea pig insulin. a, Bio-Rad high molecular weight marker. b, guinea pig insulin conjugated with chicken ovalbumin. The electrophoresis was performed at a potential of 100V at room temperature. The gel was stained with Coomassie Brillant Blue.


Mr
(KD)
a
b
200
116
97
66
45
31

saline buffer, calf thymus DNA , 5μg/ml, was dissolved in phosphate-saline buffer.

The islets were removed from the perifusion chamber with phosphate saline buffer and were homogenized and centrifuged in micro filter tubes (spin-X) at 8,000 rpm for 20 minutes to remove the Biogel P-2 from the preparation. The amount of DNA in the filtrate was determined by adding filtrate sample to the test tubes which contained H-33258 (0.1 μg/ml). Various concentrations of thymus DNA were set up for the standard instead of samples. The final assay volume was made to 2ml with the phosphate saline buffer. The reaction was allowed to proceed for 1 hour at room temperature in the dark. The fluorescence of the sample or standard DNA was read in a Turner fluorometer with the maximum emission at 458 nm and the maximum excitation at 356 nm. The total amount of DNA for the islets was calculated mathematically based on the concentration of the standard DNA by a computer program.

Insulin Determination - Indirect Competitive ELISA:

Buffers: The PBS buffer consisted of 137mM NaCl, 1.1mM KH_2PO4, 8.1mM Na_2HPO4 and 2.7mM KCl, pH 7.3. The coating buffer contained 0.3% aprotinine, 10mM HEPES and 0.01% merthiolate(v/v) in the PBS buffer. The washing buffer was 0.05% Tween-20 in PBS. The competition buffer contained 1% bovine serum albumin (RIA grade) and 0.05% Tween-20 in PBS buffer. The conjugated guinea pig insulin was coated to the microtiter plate (solid phase) and incubated overnight

at 4°C. The plate was washed 3 times with washing buffer before blocking the non specific binding with competition buffer for 45 minutes at 37 °C. After the plate was washed 3 times with washing buffer, the insulin samples (or insulin standard) were added competed with the solid phase guinea pig insulin for the antibody which was rabbit anti-guinea pig insulin serum (1:3300 dilution). After the samples were incubated at 37°C for one hour, the plate was washed 3 times with washing buffer and the secondary antibody which is goat anti rabbit IgG labeled with alkaline phosphatase (1:9,000) was added. After the plate was incubated at 37 °C for 1.5 hour, chromogenic substrate, i.e., p-nitrophenyl phosphate (1mg/ml, 200μl) was added to the plate after washing 3 times with washing butter. After 30 minutes reaction, the plate was read immediately at 405nm on a microtiter plate reader. The data were plotted with a computer program.

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CHAPTER III

EFFECT OF ASCORBIC ACID ON INSULIN RELEASE FROM SCORBUTIC GUINEA PIG PANCREATIC ISLETS

Introduction

Scorbutic guinea pigs have impaired release of insulin from pancreatic β-cells when stimulated with glucose (1-3). The syptoms of the animals caused by the decreased insulin could be treated by the administraion of ascorbic acid. It also has been reported that scorbutic guinea pigs had increased dehydroascorbate and decreased GSH (4). Thus ascorbate and GSH are involved in insulin biosynthesis and /or release by an unknown mechanism.

It has been demonstrated in the Wells lab that protein disulfide isomerase (PDI) has dehydroascorbate reductase activity (5). They proposed a model in which ascorbic acid can be regenerated from dehydroascorbate in the presence of protein thiols and PDI at the surface of the endoplasmic reticulum when cells are undergoing secretory protein synthesis (6). In the absence of ascorbate, cells may produce proteins lacking disulfide bonds with loss of normal functions. To test this hypothesis, we designed the following experiments in which isolated pancreatic islets from normal and scorbutic guinea pigs were stimulated with glucose in the presence or absence of ascorbate 2-phosphate in the perifusion media. The L-ascorbate 2-phosphate is a derivative of ascorbic acid that stabilizes ascorbic acid (7). In the

studies here, we found that islets from scorbutic guinea pigs had abnormal response to glucose compared with those from control guinea pigs. Ascorbate 2-phosphate, after it was dephosphorylated by islet alkline phosphatase, rapidly normalized the insulin release from the scorbutic guinea pig islets in response to glucose.

Materials and Method

Guinea pigs were purchased from the Michigan Department of Public Health and Charles River Breeding Laboratories. Ascorbic acid-free diet for guinea pigs and bovine serum albumin (BSA; RIA grade) were purchase from United States Biochemical. Collagenase (type V), chicken egg albumin, anti rabbit IgG (alkaline phosphatase conjugated) and p-nitrophenyl phosphate were from Sigma. Standard guinea pig insulin and anti-guinea pig serum were kindly provided by Dr. Cecil C. Yip (University of Toronto). Bio-Gel P-2 was purchased from Bio-Rad. Nylon mesh (10 μm, pore size) was purchased from Whatman.

The scorbutic guinea pig model: Guinea pigs of the scorbutic group were fed an ascorbic acid-free diet from USB. At about three weeks, the guinea pigs presented the symptoms of scurvy starting with decreased gain of body weight, less movement, and decreased food uptake. The control animals were fed the same ascorbic acid-free diet but received 0.1% neutralized ascorbic acid in their drinking water daily.

Isolation of pancreatic islets: The guinea pig pancreatic islets were isolated by collagenase according to J. Chen and D. Romsos (8).

Buffers: A stock (10x) salt buffer contained 1.18 M NaCl, 0.05 M KCl, 0.012 M KH_2PO_4, 0.025 M $CaCl_2$ and 0.012 M MgSO4. The perifusion buffer contained 10% Salt Buffer, 0.005 M $NaHCO_3$ and 0.01 M HEPES adjusted to pH 7.4 after bubbling with 95% O_2:5% CO2 for 15 minutes (9).

The guinea pigs were sacrificed after they became scorbutic (21 to 28 days of feeding) and the pancreas was removed rapidly. Control guinea pigs were sacrificed at equivalent ages. The pancreas was inflated by injecting the solution containing collagenase V (4mg/ml) and chicken ovalbumin (16mg/5ml) in perifusion buffer. The tissue was minced and put into a glass tube which contained 3ml of digestion solution consisting of collagenase V (6mg/3ml) and chicken ovalbumin (48mg/3ml) in the perifusion buffer. The tissue was digested for 2 to 2.5 minutes with shaking gently at 37^{0}C. The digestion was stopped by adding 15ml of cold perifusion buffer. The digested acinar tissue was washed out and removed by asperation three to four times with cold 15 ml of perifusion buffer allowing the heavier islets to sediment in the washing tube for 4-5 minutes after each wash. The islets were selected under a dissection microscope with the aid of a pipetman.

Perifusion of the pancreatic islets: The perifusion apparatus consisted of a multiple-channel pump, polyethylene tubing and a perifusion chamber which was made by cutting a 2 ml syringe. The islets were sandwiched between Biogel P-30 with a nylon mesh (20μm) placed at the bottom of the chamber, which was connected

to small diameter tubing by a connector. The perifusion rate was 0.5ml per minute. The islets were washed with the perifusion buffer containing 1.7mM glucose for 30 minutes followed by the different patterns of the perifusion for up to two hours at 37°C immersed in a water bath. The fractions (2.5ml) of the perifusion were collected every 5 minutes and frozen at -70°C immediately until insulin determination. The islets and gel were collected from the chamber with the PBS buffer for DNA determination as indicated above (Chapter II). In the first experiment, the islets from the control (n = 3) and the scorbutic (n = 3) guinea pigs were perifused in the system described above with 20 mM glucose for 120 minutes after 30 minutes of basal perifusion. In the second experiment, the islets from scorbutic (n = 9) and the control guinea pigs (n = 5) were perifused with 20 mM glucose in the perifusion media for 60 minutes after 30 min of basal perifusion. At this point, 5 mM L-ascorbate 2-phosphate was added to the perifusion media for an additional 60 minutes period. In the third experiment, the islets from the control (n = 3) and the scorbutic (n = 5) guinea pigs were perifused for 120 minutes with 20 mM glucose and ascorbate 2-phosphate immediately after the 30 minutes basal perifusion.

Results

In the first experiment, the release of insulin from the control islets was immediately increased in response to 20 mM glucose, followed by the secondary responses. The scorbutic pancreatic islets had impaired immediate response to 20 mM glucose and the delayed response was much lower than that in the control (Figure 1).

In the second experiment, insulin release from the control islets was the same as that seen in the first experiment. Scorbutic pancreatic islets had impaired immediate insulin release during the first 60 minute of the perifusion with 20 mM glucose. Ascorbic acid could correct this impairment, i.e., the scorbutic islets had the same rapid response to 20 mM glucose in the presence of 5 mM ascorbate 2-phosphate in the perifusion media as the control (Figure 2). In the third experiment, the insulin release from the control was the same as that seen in the first and the second experiment. For scorbutic pancreatic islets, the release of insulin was elevated as rapidly as the control islets in response to 20 mM glucose with the presence of 5 mM L-ascorbate 2-phosphate in the perifusion media (Figure 3).

This experimental study indicated that scorbutic guinea pig pancreatic islets have defective insulin release in response to elevated glucose and ascorbic acid precursor, L-ascorbate 2-phosphate could restore the ability to respond to elevated glucose.

Discussion

In the current experiment, the application of the ascorbic acid precursor, L ascorbate 2-phosphate was crucial because a low concentration of ascorbate would be rapidly oxidized to dehydroascorbic acid (10) in the perifusion media with the presence of small amount of metals at pH 7.4. Dehydroascorbic acid has been reported to inhibit insulin secretion from nouse pancreatic islets at concentration of

Figure 1. Comparison of insulin release from control (●) and scorbutic (○) islets in response to elevated D-glucose (20 mM). Pancreatic islets from control (n=3) and scorbutic (n=3) guinea pigs were perifused with KRB medium supplemented with 0.1% BSA and 1.7 mM D-glucose at a rate of 0.5 mL/min at 37°C. D-glucose was elevated as indicated by the arrow after 30 min, and the perifusion continued for another 120 min. Values are mean ± SD

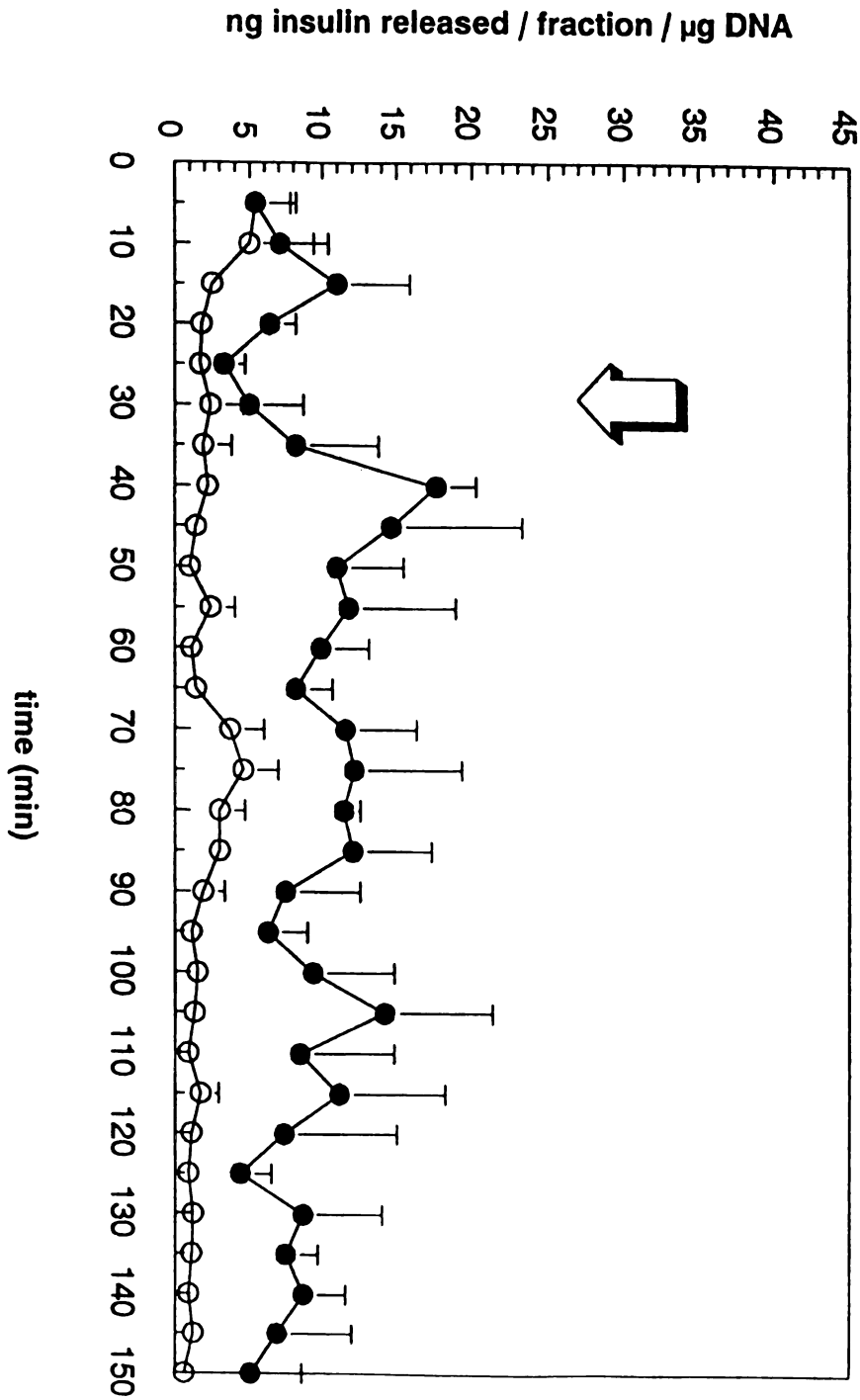
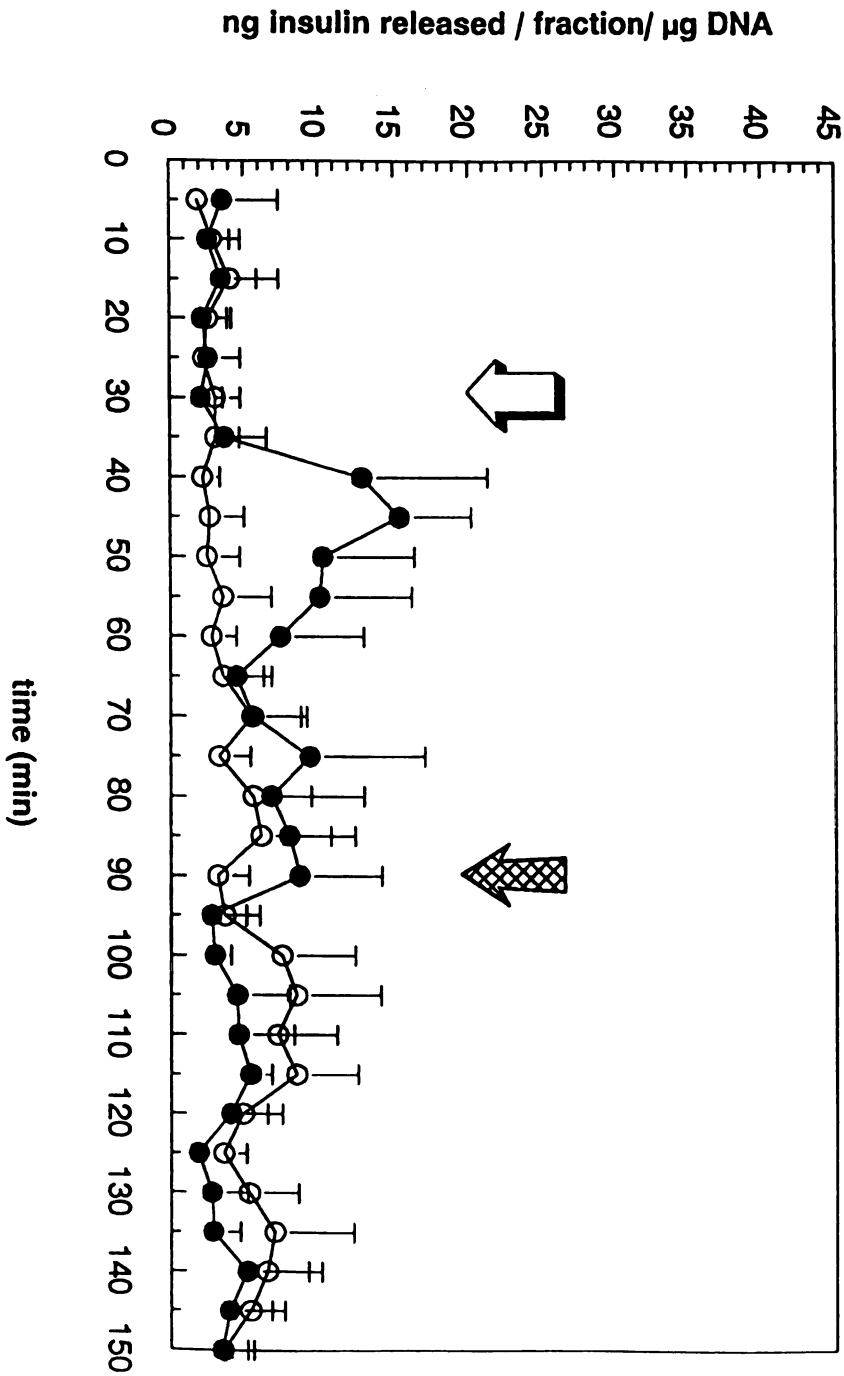

ng insulin released / fraction / µg DNA
0
5
10
15
20
25
30
35
40
45
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
time (min)

Figure 2. Comparison of insulin release from control (●) and scorbutic (○) islets in response to elevated D-glucose and delayed ascorbate 2-phosphate (5 mM). Pancreatic islets from control (n = 5) and scorbutic (n = 9) guinea pigs were perifused with KRB medium supplemented with 0.1% BSA and 1.7 mM D-glucose at a rate of 0.5 ml/min at 37 °C. D-glucose was elevated as indicated by the open arrow after 30 min and 5 mM ascorbic acid 2-phosphate was added as indicated by the cross-hatched arrow after 90 min. Values are mean ± SD.


ng insulin released / fraction/ µg DNA
0
5
10
15
20
25
30
35
40
45
0
10
20
30
40
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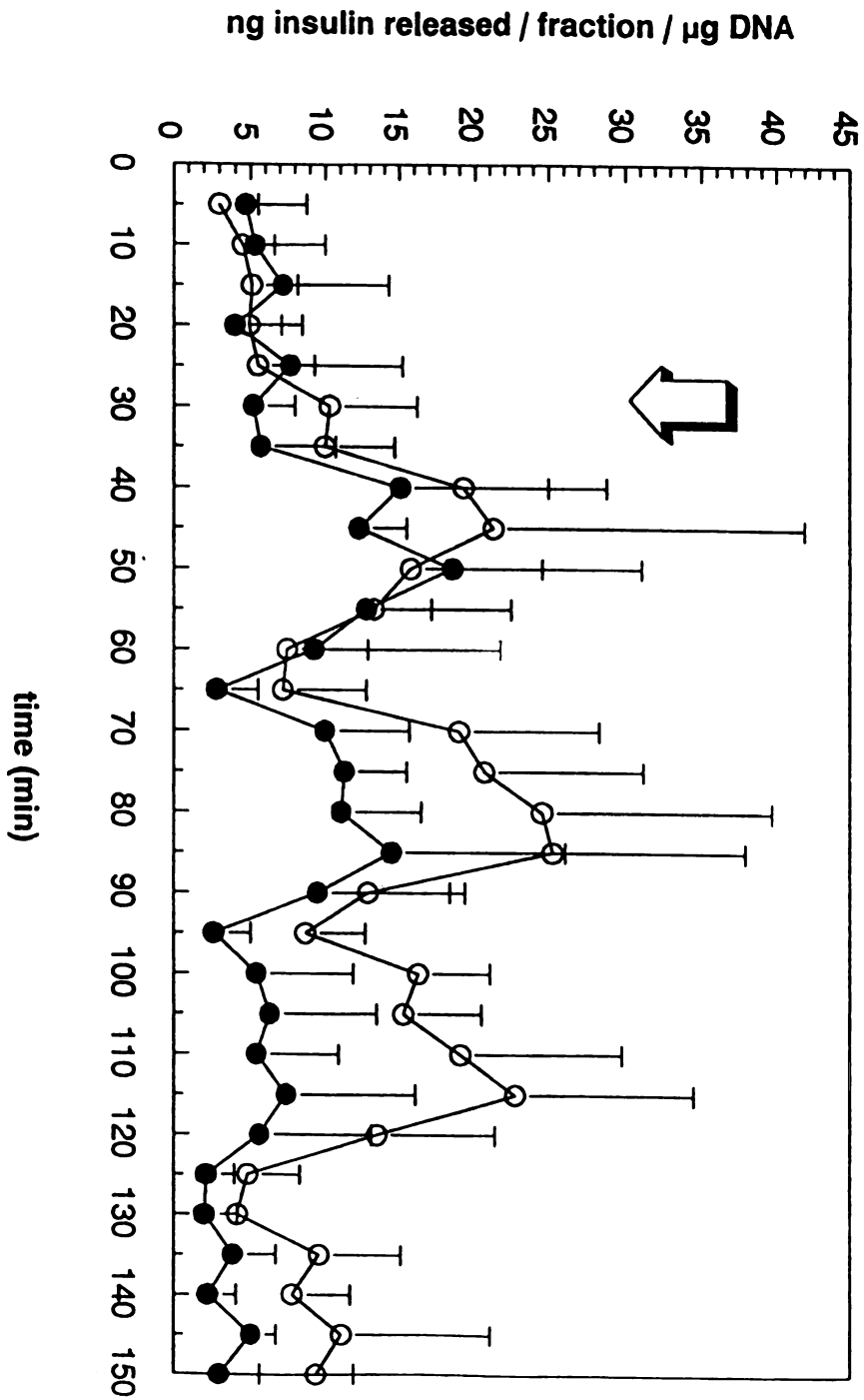
60
70
80
90
100
110
120
130
140
150
time (min)

Figure 3. Comparison of insulin release from control (●) and scorbutic (○) islets in response to the elevated D-glucose (20mM) and L-ascorbic acid 2-phosphate (5 mM). Pancreatic islets from control (n = 5) and scorbutic (n = 9) guinea pigs were perifused with KRB medium supplemented with 0.1% BSA and 1.7 mM D-glucose at a rate of 0.5 ml/min at 37 °C. D-glucose was elevated concurrently with 5 mM ascorbic acid 2-phosphate as indicated by the arrow. Values are mean±SD.


ng insulin released / fraction / µg DNA
0
5
10
15
20
25
30
35
40
45
time (min)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

2 mg% (11). In the present study, the concentration of dehydroascorbic acid was likely to be very minimal because ascorbic acid was at low level.

The current experiments supported the other reports that scorbutic guinea pig pancreatic islets have defective release of insulin (1-3). The early suggestions that scorbutic guinea pigs have impaired insulin synthesis was not observed in our experiments. In contrast, the amount of insulin from pancreas of scorbutic guinea pig was increased compared with that from the control as detected by gel electrophoresis (12). We believed this discrepancy is because the experimental methods used in the early study, which used a hyperglycemia bioassay of guinea pig insulin in rabbits, was not accurate, since guinea pig insulin has different immunological and biological properties compared with insulin from other species (13,14).

The current experimental results and the other experimental reports in my laboratory (12) are not supportive to our original proposal, i.e., ascorbic acid redox cycling plays an important role in the disulfide formation in insulin biosynthesis (15). However, these experimental results imply an essential function for ascorbic acid in enhancing the competency of glucose in the activation of the insulin release process, but not an effect on the insulin biosynthesis (16).

The mechanism of glucose induced insulin release has been widely reviewed (17, 18, 19). Glucose metabolism in the β-cell generates a signal, an increased ATP/ADP ratio, to induce the deplorization of the cell membrane by closing the potassium

channel (20). The deplorization of the membrane opens the voltage sensitive calcium channel on the membrane. The influx of calcium trigers insulin release from β-cell through the exocytosis process (21). Our experimental results offer an explaination for the early observations (1), i.e., scorbutic guinea pig pancreatic islets had abnormal responses to D-glucose stimulated insulin release and implied a new role of ascorbic acid in the glucose stimulated insulin release from pancreatic β-cells.

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CHAPTER IV

STUDIES OF THE MECHANISM OF ASCORBIC ACID IN THE RELEASE OF INSULIN FROM GUINEA PIG PANCREATIC ISLETS

Introduction

The previous study showed that scorbutic guinea pig pancreatic islets had impaired release of insulin, and that ascorbic acid was an essential factor for glucose induced insulin release from the scorbutic pancreatic islets (1). The mechanism of glucose induced insulin release has been widely studied (2). The elevation of intracellular ATP is a signal for increasing intracellular Ca^{+2} through plasm membrane depolarization by closure of the ATP/ADP sensitive potassium channel (3). Smith et al. (4) showed that the addition of 20 mM glucose resulted in a continuous electrical activity which depolarized the plasma membrane to initiate an action potential. Martinez (4) also demonstrated that the infusion of KCl (0.5 mEq/Kg/h) intravenously to dogs increased the insulin level in the portal vein. Further studies (6) demonstrated that glyceraldehyde, which was metabolized to glyceraldehyde-3-phosphate, stimulated insulin release from cultured RINm5F cells. Pyruvate alone can not stimulate insulin secretion (7). This failure is due to a lack of elevation of reduced nicotinamide nucleotide, and no increased ATP generation through mitochondrial oxidative phosphorylation (8-10). My present study was designed to further explore the mechanism of ascorbic acid action on the glucose

induced release of insulin, i.e., to narrow the range of the regulatory site of ascorbic acid in the pathway of glucose induced insulin release in guinea pig pancreatic islets. We used the same perifusion system as indicated in Chapter II and Chapter III. The isolated pancreatic islets from normal and scorbutic guinea pigs were perifused with elevated potassium chloride (45mM) or with D-glyceraldehyde (20mM)in the perifusion media. We found that the site of ascorbic acid on insulin release lies between triosephosphate metabolism and ATP generation, i.e., the process of oxidative phosphorylation in the mitochondria.

Effect of Potassium on the Release of Insulin from the Scorbutic Pancreatic Islets:

KRB buffer (see Chapter III) containing 45 mM KCl as the perifusion medium was made by replacing 40mM NaCl with 40mM KCl in the KRB buffer. Other buffers for DNA determination and insulin determination were the same as indicated in Chapter II.

The islets were isolated from control or scorbutic guinea pig pancreatic tissues and put into a perifusion chamber as indicated in Chapter III. The islets were perifused with KRB containing 1.7 mM glucose at the rate of 0.5 ml/min for 30 min after they were equilibrated with oxygen/carbon dioxide (95:5, %). Then the islets were continuously perifused with 45 mM KCl in the same media throughout the next 120 min. The perifusion fractions (2.5ml) were collected every 5 min and stored

for insulin determination as indicated in Chapter II. After the perifusion, the contents of the chamber were transferred to a plastic tube with PBS buffer and stored at -70°C for DNA determination as indicated in Chapter II.

Effect of D-glyceraldehyde on the release of insulin from the guinea pig pancreatic islets:

To further identify the site of ascorbic acid function on glucose dependent insulin release, islets from control or scorbutic guinea pigs were perifused with D-glyceraldehyde. Reagents and buffers for perifusion, DNA determination and ELISA were the same as indicated in Chapter II. The D-glyceraldehyde was from the Aldrich Chemical Co.

Islets were isolated from control or scorbutic guinea pig pancreatic tissue and put into the perifusion chamber as indicated in Chapter II and Chapter III. Oxygen / carbon dioxide equilabrated KRB buffer containing 1.7 mM glucose was use to perifuse the islets at a rate of 0.5 ml/min for 30 min. At this time, 20 mM D-glyceraldehyde was added to the previous medium and the perifusion continued for 120 min. The perifusion fractions (2.5ml) were collected every 5 min and stored at -70°C for insulin determination. After the perifusion, the islets and the biogel were transfered to plastic tubes and stored for the DNA determination as indicated in Chapter II.

RESULTS

In the potassium study, the release of insulin from the control islets increased in response to the elevation of potassium in the perifusion media, followed by several secondary peaks of insulin release. (Figure 1). The scorbutic islets had the same response to the elevation of potassium as seen in the control islets.

These results indicated that potassium can stimulate insulin release equally well from both the pancreatic islets of control and scorbutic guinea pigs. Moreover, the results indicated that scorbutic islets have a defective site of insulin release located somewhere between glucose transport, its metabolism and ATP generation by mitochondrial oxidative phosphorylation.

In the experiment of D-glyceraldehyde perifusion, the insulin release of pancreatic islets from normal guinea pigs in response to 20 mM glyceraldehyde increased immediately with an initial response followed by secondary responses (Figure 2). In contrast, pancreatic islets from scorbutic guinea pigs failed to respond rapidly to 20 mM glyceraldehyde and a delayed response was significantly lower than that of the control islets preparations. These results indicated that scorbutic islets have an impaired function of insulin release presumably in the generation of ATP by islet mitochondria, which lies between triose phosphate metabolism and oxidative phosphorylation.

Figure 1. Comparison of the effect of 45 mM KCl on the release of insulin from control (●) and scorbutic (○) guinea pig pancreatic islets. The pancreatic islets from control (n = 10) and scorbutic (n = 8) guinea pigs were perifused with oxygen/carbon dioxide (95:5, %) equilibrated KRB medium supplemented with 0.1% BSA and 1.7 mM glucose at a rate of 0.5 ml/min at 37 °C. KCl was elevated from 5mM to 45 mM as indicated by the arrow. Values are the mean ± SD.

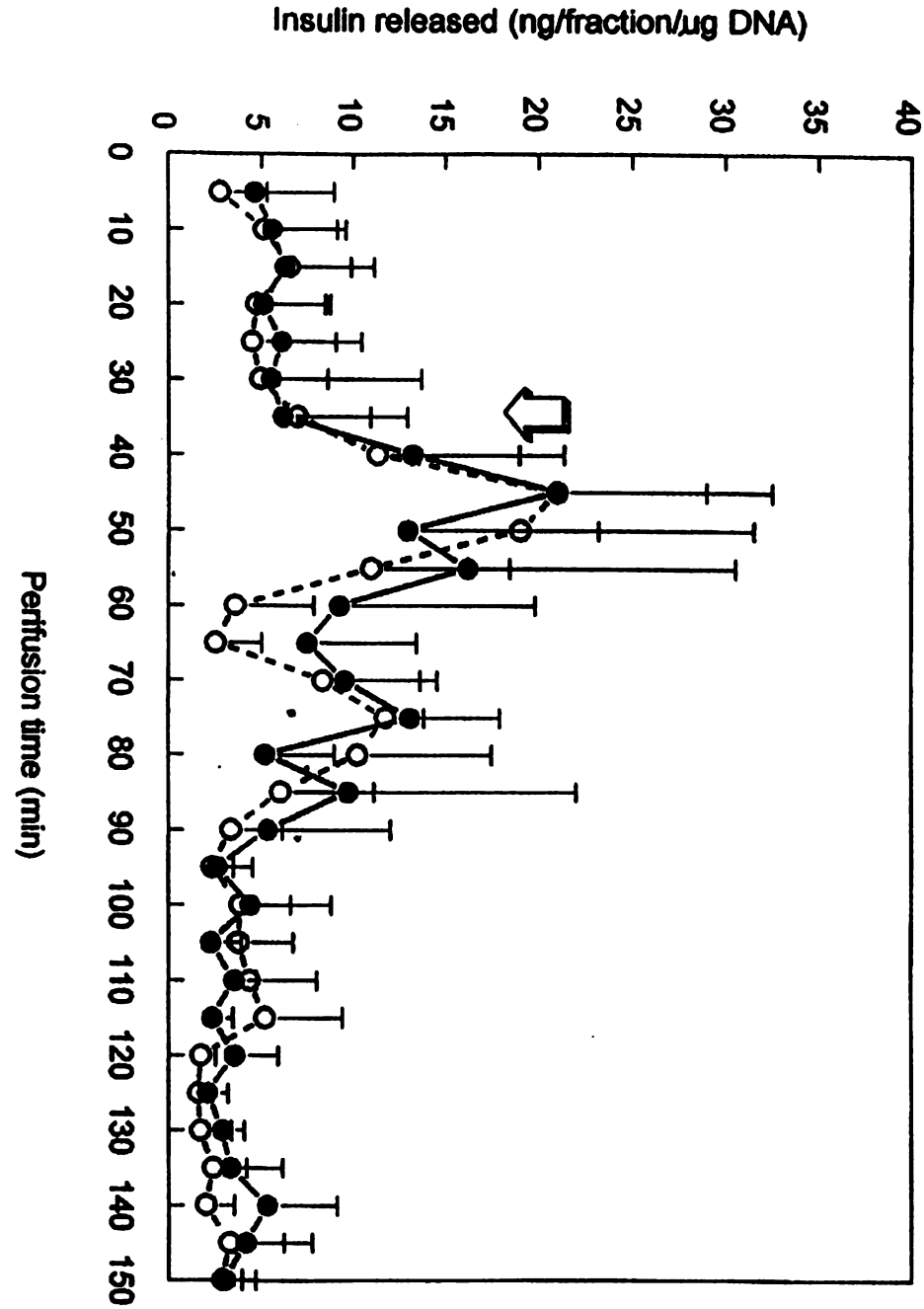
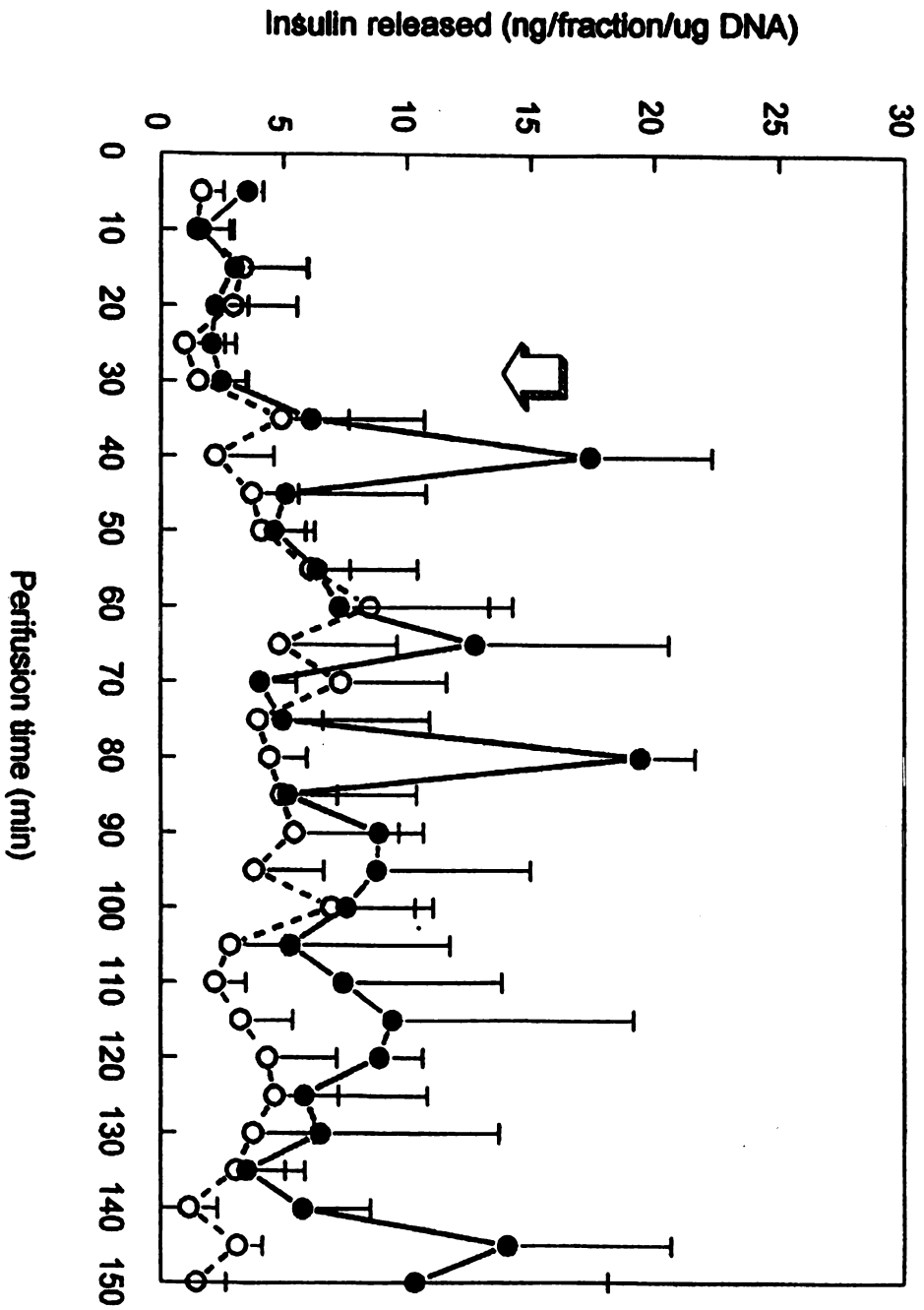

Insulin released (ng/fraction/µg DNA)
0
5
10
15
20
25
30
35
40
Perfusion time (min)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

Figure 2. Comparison of the effects of 20 mM D-glyceraldehyde on the release of insulin from control (●) and scorbutic (○) guinea pig pancreatic islet.

The pancreatic islets from control (n = 3) and scorbutic (n = 4) guinea pigs were perifused with oxygen/carbon dioxide (95:5, %) equilibrated KRB medium supplemented with 0.1% BSA and 1.7 mM glucose at a rate of 0.5 ml/min at 37 °C. D-glyceraldehyde was added at a concentration of 20mM after 30 min perifusion followed by a 120 min perifusion period as indicated by the arrow. Values are the mean ± SD.


Insulin released (ng/fraction/ug DNA)
0
5
10
15
20
25
30
Perifusion time (min)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

Discussion

The present experimental results indicated that pancreatic islets from scorbutic guinea pigs can release insulin in response to potassium, but not in response to D-glyceraldehyde. This leads to the notion that the defective site of glucose induced insulin release of scorbutic pancreatic islets resides in the ATP generation pathway located between glyceraldehyde metabolism and ATP generation through mitochondrial oxidative phosphorylation.

It is still puzzling from work done by others (5) that glyceraldehyde can stimulate insulin secretion, as confirmed by my studies, but pyruvate can not in the absence of glucose. In order to explain these results, pharmacologic agents, which can block the electron transport system, have been employed. It was found that glucose stimulated insulin release can be reduced by inhibitors of electron transport or of ATP generation, such as antimycin A, rotenone or cyanide (8). All of these facts suggested that mitochondria must play an important role in glucose or other fuel stimulated insulin secretion, especially the process of transferring reducing equivalents to oxygen coupled with ATP generation. A recent study by Dukes et al. (11) showed that incubation of β-cells with inhibitors of the citric acid cycle and inhibitors of pyruvate transport had no significant effect on glucose or D-glyceraldehyde induced closure of potassium channels. An inhibitor of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), iodoacetate, can completely

inhibit glucose-induced closure of ATP sensitive potassium channels of the β-cells and inhibit the stimulation of insulin release. The conclusion from their experiments was that ATP generation from glycolysis, i.e., substrate phosphorylation, and the citric acid cycle has no significant function in closure of potassium channels that regulate fuel-mediated insulin release.

The critical signaling event was the generation of NADH in the G3PDH reaction. The islets treated with the glucose analog, streptozotocin, which caused a depletion of NAD^+, abolished glucose-induced closure of the potassium channel. Nicotinamide treatment of these cells to restore NAD^+ resulted in recovery of glucose sensing. The further speculation is that NADH generated at the G3PDH step fuels mitochondrial ATP via the malate-aspartate and glycerolphosphate-dihydroxyacetone phosphate shuttles, funneling reducing equivalents into the electron transport system. ATP generated by this pathway can induce the closure of the potassium channels and trigger the influx of calcium. Soejima (12) demonstrated that mitochondrial gene knock out cell line had no increased insulin release after stimulation with 25 mM D-glucose, indicating that mitochondrial respiratory chain function is necessary for the glucose stimulated insulin secretion. Giroix et al. (13) have reported that mitochondrial glycerophosphate dehydrogenase, which accepts reducing equivalents in the electron transport process, coupled with glycolysis in the islet β-cells by the glycerol phosphate shuttle, is deficient in some non-insulin-

dependent diabetic patients. McDonald et al. (14) reported that the mitochondrial FAD-linked glycerol phosphate dehydrogenase in pancreatic islets greatly exceeded that in other tissues and plays a crucial role in the process of transferring reducing equivalents from cytosol to β-cell mitochondria. In the present study whereby β-cells of the scorbutic guinea pig pancreatic islets can not respond to the glyceraldehyde, the possible cause is due to a defect in the activity of this enzyme, mitochondrial glycerophosphate dehydrogenase.

An earlier report by Harrer and King showed that succinic dehydrogenase and cytochrome c oxidase were diminished in the heart and skeletal muscle of scorbutic guinea pigs (15). A deficiency of these enzymes opens other possible mechanisms in which ascorbic acid may play an essential role in energy metabolism not only related to the release of insulin from the scorbutic guinea pig pancreatic islets, but also in those tissues where a similar defect may compromize energy metabolism in scorbutic animals.

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CHAPTER V

SUMMARY AND FURTHER DIRECTION

Scorbutic guinea pigs have decreased insulin secretion from pancreatic islets when stimulated with glucose (1-3), and this impairment can be treated by ascorbic acid. Scorbutic guinea pig pancreatic islets failed to respond to elevated glucose in the perifusion media, whereas ascorbic acid precursor L-ascorbate 2-phosphate, supplemented in the perifusion media, led to full recovery of the response of the scorbutic guinea pig pancreatic islets to elevated glucose. This indicated that ascorbic acid was essential for glucose stimulated insulin release from scorbutic pancreatic islets (4). It will be of interest to study the effects of other secretgogues, such as leucine and acetylcholine, on the release of insulin from scorbutic guinea pig pancreatic islets.

The mechanism of glucose induced insulin release is related to the elevation of intracellular ATP which is a signal for increasing intracellular Ca^{2+} through the membrane depolarization by closure of the ATP/ADP sensitive potassium channel (5). When guinea pig pancreatic islets were perifused with elevated potassium in the perifusion media, scorbutic islets had the same response as the control indicating that the β-cells from the scorbutic pancreatic islets have normal cell membrane depolarization and downstream events, i.e., the opening the voltage sensitive channel and protein kinase cascades (6). The site of ascorbic acid in the release of insulin in response to elevated glucose is somewhere between the oxidation of triose phosphate,

production of NADH, and ATP generation. It will be interested to study the metabolism of triose-phosphate on insulin release from scorbutic pancreatic islet and to study the enzymes on this pathway from different tissues of scorbutic and normal guinea pigs.

D-Glyceraldehyde can stimulate insulin release, whereas pyruvate can not. However, at a concentration of 30 mM, pyruvate can stimulate insulin release, but only in the presence of elevated concentrations of glucose (7, 8). D-Glyceraldehyde (20 mM) in the perifusion media stimulated insulin secretion from the normal guinea pig pancreatic islets but not from the scorbutic guinea pig pancreatic islets, indicating that ascorbic acid is required somewhere between glyceraldehyde metabolism and ATP generation. It will be of interest to study whether ascorbic acid can induce recovery from the defect in islets from scorbutic and normal guinea pigs perifused with 20mM D-glyceraldehyde and the presence of 5mM L-ascorbate 2-phosphate in the perfusion media.

Mitochondrial glycerophosphate dehydrogenase (mGPDH) is an important enzyme candidate (9). Two other enzymes previously reported to be decreased in skeletal muscle of scorbutic guinea pigs are succinate dehydrogenase and cytochrome c oxidase (10). It will be of interest to study these enzymes in scorbutic guinea pig pancreatic islets to find out which enzyme or enzymes are responsible for decreased ATP generation in the process of glucose induced insulin release in the β-

cells and other tissues. Such studies may locate a novel site or sites for the participation of ascorbic acid in metabolism. It will be of interest to isolate the mitochondria from normal and scorbutic pancreatic islets to set up an assay system allowing ascorbic acid addition to the tissue, which can lead to a direct study of the role mitochondria plays in energy dependent insulin release. It will also be of interest to study these enzymes in β-cell lines and to study the effect of ascorbic acid on these enzymes.

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