DETECTION OF HISS-DEPENDENT INSULIN ACTION:

METHOD CONSIDERATIONS

BY

Maria April Genovey Reid

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the requirements
for the Degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics
Faculty of Medicine
University of Manitoba

© August 2002

National Library of Canada

Bibliothèque nationale du Canada

Acquisitions and Bibliographic Services

Acquisitions et services bibliographiques

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-76856-2

Canadä

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

Detection of HISS-Dependent Insulin Action:

Method Considerations

BY

Maria April Genovey Reid

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

MARIA APRIL GENOVEY REID ©2002

Permission has been granted to the Library of The University of Manitoba to lend or sell copies of this thesis/practicum, to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film, and to University Microfilm Inc. to publish an abstract of this thesis/practicum.

The author reserves other publication rights, and neither this thesis/practicum nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

This thesis is dedicated to my Mom, Dad, Anthony and Devin.

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. W. Wayne Lautt for allowing me to work in his lab. His guidance and support have been incredible. His philosophy toward science is beautiful and has given me a greater appreciation for research and science.

I would like to thank my committee members Dr. Minuk, Dr. Burczynski and Dr. D'Almeida for their time and effort into making this thesis the best it could be.

I would also like to thank Dallas Legare for his patience, excellent technical training, and encouragement. I would like to thank Karen Sanders for all her help, everyday. Without her I would be both lonely and confused.

Thank you to my fellow students, Jodi Schoen, Nina Correia, and Parissa Sadri, for their friendship and advice. A very special thank you to Parissa, who guided me in my early days, taught me how to do the RIST and let me practice on her experiments. I now fully know the anxiety of letting someone else do your experiment.

To my grandparents and aunts, thank you for the time you spent with me, for all the meals, and all the little things that make family special.

To my parents John and Shelley and my brother Anthony, thank you for all you do for me. I know I would not have achieved this degree without you. You give me both confidence and shelter.

To my husband, Devin, thank you for all your support, encouragement and love. You always make my days brighter and make me believe I can do anything.

TABLE OF CONTENTS

Acknowledgements I

Table of Contents II

List of Figures VII

List of Tables VIII

List of Abbreviations IX

Abstract X

Chapter 1 – Introduction to Type 2 Diabetes and the research methods used to explore it 1

1.1 Introduction 1

1.2 Type 2 diabetes 1

1.3 Defects in insulin action 3

1.3.1 Defects in binding 3

1.3.2 Defects in signal transduction 3

1.3.3 Defects in GLUT4 translocation 4

1.3.4 Defects in glycogen synthesis 5

1.4 Defects in insulin secretion 6

1.4.1 Beta cell overwork 6

1.4.2 Glucose toxicity 7

1.4.3 Disturbed glucose handling 7

1.4.4 Lipotoxicity 7

1.4.5 Islet amyloid 8

1.4.6 Manifestations of decreased insulin secretion 9

1.5 Introduction to a new idea 10

1.6 Characterization of HISS action 11

1.7 RIST methodology 13

1.7.1 Standardized feeding 13

1.7.2 HISS-dependent vs. HISS-independent insulin action 14

1.8 Summary of the HISS hypothesis 14

1.9 Commonly used methods of assessing insulin sensitivity 15

1.10 Hypothesis tested and rationale 18

Chapter 2- Comparison of the Rapid Insulin Sensitivity Test, Insulin Tolerance Test, and Hyperinsulinemic Euglycemic Clamp, in their ability to detect HISS-dependent insulin action 19

2.1 Introduction 19

2.2 Methods 20

2.2.1 Surgical preparation 20

2.2.2 Comparison of the RIST to ITT 22

2.2.3 Comparison of the RIST to HIEC 23

2.2.4 Effect of the HIEC on insulin sensitivity using the RIST 23

2.2.5 Effect of the HIEC on insulin sensitivity using the ITT 24

2.2.6 Drugs 24

2.2.7 Data analysis 24

2.3 Results 25

2.3.1 Comparison of the RIST to the ITT 25

2.3.2 Comparison of the RIST to the HIEC 28

2.3.3 Effect of the HIEC on insulin sensitivity using the RIST 29

2.3.4 Effect of the HIEC on insulin sensitivity using the ITT 30

2.4 Discussion 34

2.4.1 Comparison of the RIST and ITT 35

2.4.2 Relationship between the RIST and HIEC 36

Chapter 3 – Introduction to pulsatile insulin release 42

3.1 Normal Physiology of pulsatile insulin release 42

3.2 Control of insulin pulsatility 43

3.2.1 Rapid pulses (5-15 minutes) 43

3.2.2 Ultradian oscillations (75-115 minutes) 45

3.3 Pulsatile insulin release and insulin sensitivity 47

3.3.1 People with type 2 diabetes 47

3.3.2 Relatives of patients with type 2 diabetes 48

3.3.3 Insulin pulsatility in relation to other conditions 49

3.4 Efficacy and effects of pulsatile versus continuous insulin infusions 50

3.5 Hypothesis tested and rationale 53

Chapter 4- The effect of insulin delivery pattern on glucose uptake and insulin sensitivity 54

4.1 Introduction 54

4.2 Methods 55

4.2.1 Comparison of the efficacy of three delivery methods 55

4.2.2 Effect of a continuous insulin infusion on insulin sensitivity 56

4.2.3 Effect of three insulin pulses given 20 minutes apart on insulin sensitivity 56

4.2.4 Effect of three insulin pulses given according to glucose uptake on insulin sensitivity 57

4.2.5 Effect of a bolus insulin infusion on insulin sensitivity 57

4.2.6 Effect of various durations of continuous insulin infusion on on insulin sensitivity 57

4.2.7 Drugs 58

4.2.8 Data analysis 58

4.3 Results 58

4.3.1 Efficacy of different delivery methods 58

4.3.2 Effect of continuous insulin delivery on insulin sensitivity 62

4.3.3 Effect of three insulin pulses given 20 minutes apart on insulin sensitivity 62

4.3.4 Effect of three pulses given according to glucose uptake on insulin sensitivity 62

4.3.5 Effect of a bolus insulin infusion on insulin sensitivity 66

4.3.6 Effect of different durations of continuous insulin infusion on insulin sensitivity 67

4.4 Discussion 70

4.4.1 Efficacy of continuous versus pulsatile insulin delivery 70

4.4.2 Effect of continuous and pulsatile insulin infusions on insulin sensitivity 73

4.4.3 Effect of different durations of continuous insulin infusion on insulin sensitivity 75

Chapter 5 – Final Remarks 77

5.1 Conclusions 77

5.2 Speculations 78

5.3 Future direction 79

References 81

LIST OF FIGURES

Figure 2.1 – Arterial-venous blood sampling shunt 21

Figure 2.2 – Correlation comparison of the RIST and ITT 26

Figure 2.3 a – Glucose infusion profile for the RIST

b - Hypoglycemic response to ITT

c – HISS action profiles calculated from the RIST and ITT 27

Figure 2.4 – Glucose infusion rates for HIEC in both control and HISS blocked state 28

Figure 2.5 – Correlation comparison of the RIST and HIEC 29

Figure 2.6 – RIST indexes, for fed and fasted rats, before and after the HIEC 31

Figure 2.7 – Correlation comparison of the RIST index to the initial slope of the HIEC 32

Figure 2.8 – HISS action profile calculated from the first 30 minutes of the HIEC 32

Figure 2.9 – ITT before and after HIEC and atropine 33

Figure 4.1 – Glucose uptake in response to three pulses of insulin 59

Figure 4.2 – Glucose uptake in response to six pulses of insulin 60

Figure 4.3 – Glucose uptake in response to a continuous insulin infusion 60

Figure 4.4 – *top* HISS action profile for three pulses of insulin; *middle* HISS action profile for six pulses of insulin; *bottom* HISS action profile for continuous insulin infusion 61

Figure 4.5 – Effect of continuous insulin infusion on insulin sensitivity 63

Figure 4.6 – Effect of three pulses of insulin, given 20 minutes apart, on insulin

sensitivity 64

Figure 4.7 – Effect of three pulses of insulin, given after the previous pulse's action is gone, on insulin sensitivity 65

Figure 4.8 – Effect of a bolus of insulin on insulin sensitivity 66

Figure 4.9 – Percent inhibition in insulin sensitivity due to various durations of continuous insulin infusion 69

LIST of TABLES

Table 4.1 – Data from various durations of continuous insulin infusions 68

COMMONLY USED ABBREVIATIONS

3-morpholinosydnonimine – SIN-1

Adenosine triphosphate - ATP

Arterial-venous shunt – AV shunt

Glucose infusion rate – GIR

Glucose transporter – GLUT

Guanidine triphosphate – GTP

Hepatic Insulin Sensitizing Substance – HISS

High-density lipoprotein – HDL

HISS-dependent insulin resistance – HDIR

Hyperinsulinemic euglycemic clamp – HIEC

Insulin receptor substrate – IRS

Insulin Tolerance Test – ITT

Intraperitoneal – i.p.

Intraportal venous – i.p.v.

Intravenous - i.v.

Intravenous glucose tolerance test – IVGTT

N-monomethyl-L-arginine acetate – L-NMMA

N-nitro-L-arginine methyl ester – L-NMMA

Non-insulin-dependent diabetes mellitus – NIDDM

Oral glucose tolerance test – OGTT

Phosphatidylinositol 3 kinase – PI3 kinase

Rapid Insulin Sensitivity Test – RIST

ABSTRACT

The objective of this project was to compare the rapid insulin sensitivity test (RIST), insulin tolerance test (ITT) and the hyperinsulinemic euglycemic clamp (HIEC) in their ability to detect Hepatic Insulin Sensitizing Substance (HISS) -dependent insulin action, and to determine the effect of pulsatile and continuous insulin infusions on HISS-dependent insulin action. This was accomplished by setting up conditions where HISS release was known to occur and where it was blocked, then assessing insulin sensitivity with the RIST, ITT and HIEC. By giving insulin in pulses or continuously, before and after atropine, it was possible to determine HISS action in response to continuous and pulsatile insulin infusion. Performing the RIST before and after continuous, bolus, and pulsatile insulin infusions assessed the change in insulin sensitivity due to the infusion pattern. The ITT and RIST were equally able to detect HISS release and the two tests had a significant relationship ($r^2 = 0.84$). In contrast, the HIEC and RIST were not comparable. The HIEC was only able to detect HISS release during its initial rising slope and not during the classically reported final 30 minutes. Also, use of the HIEC blocked HISS release in subsequent tests, as determined by both the RIST and ITT. HISS-dependent, HISS-independent and total insulin action were similar in quantity for the continuous and pulsatile insulin infusions. However, continuous, but not pulsatile or bolus, insulin infusions caused full HISS-dependent insulin resistance in subsequent tests. The conclusions drawn from these experiments were that the RIST and ITT are comparable, thus, the RIST has been validated against this standard. The HIEC and RIST are not comparable probably because use of the HIEC induces HISS-dependent insulin resistance. The HIEC-induced insulin resistance is very likely due to the

continuous insulin infusion used during this methodology since continuous but not pulsatile insulin infusions induced HISS-dependent insulin resistance in subsequent tests.

CHAPTER 1 – Introduction to Type 2 Diabetes and the research methods used to explore it

1.1 Introduction

The work presented in this thesis as well the work conducted in the past and present, by colleagues of the laboratory, represent work done under a new paradigm. A paradigm is defined as the philosophical and theoretical framework of a scientific school or discipline within which theories, laws and generalizations and the experiments performed in support of them are formulated (Merriam-Webster online dictionary). Our general research area is type 2 diabetes. The paradigm under which we work in based on the characterization of a putative neurohumoral factor called Hepatic Insulin Sensitizing Substance (HISS), which acts in concert with insulin to produce maximal insulin action. This thesis is divided into two areas, the first being a validation of the method that was used in the characterization of HISS, which was developed within the lab. The second arose from observations made during the validation and is an investigation of the importance of insulin pulsatility in the maintenance of HISS release.

1.2 Type 2 diabetes

Type 2 diabetes is a condition characterized by a defect in insulin action and insulin secretion. Which occurs first is much like the age-old question, which came first the chicken or the egg; thus, there are arguments for each situation. One idea is that the progression of type 2 diabetes is from a state where body tissues become less sensitive, or resistant, to insulin and the pancreas attempts to compensate by increasing the insulin output, to a state where the pancreas fails and can no longer produce adequate amounts of

insulin (DeFronzo et al 1992). Another idea is that a defect in insulin release must exist for type 2 diabetes to develop (Ostenson 2001).

Type 2 diabetes is preceded by a period of glucose intolerance, which may be asymptomatic and unrecognized for many years before diagnosis. Studies on twins have concluded that there is a genetic factor that predisposes people to develop type 2 diabetes (Barnett et al 1981). The thrifty genotype theory, described by Neel (1962), proposes that the people which develop diabetes today are the people that would have survived periods of famine because their bodies are efficient in food intake and use. This 'gene' would have survived evolution because it was beneficial, however, we presently live in a society of abundance where such a trait is troublesome. High fat diets of convenience are common, and both obesity and weight gain during adulthood are associated with diabetes (Chan et al 1994). The progression from impaired glucose tolerance to diabetes can be prevented, by 50%, with a low fat and high complex carbohydrate diet and exercise (Pan et al 1997). Even persons diagnosed with diabetes can decrease its severity and progression with exercise and diet modifications (Bourn et al 1994). Thus, while there is evidence for a genetic predisposition to the disease there is a definite lifestyle component. This is best exemplified by a study that compares two groups of Pima Indians, one that, because of westernization, was unable to grow and eat a traditional diet and another that continued in traditional ways. The amount of physical labor was significantly less and blood cholesterol levels and body mass index were higher, in the westernized compared to the traditional population. Thus, the finding that only 6.3 % of men and 10.5% of women in the traditional population had diabetes, compared to 54% of men and 37% of women in the westernized population, is not surprising (Ravussin et al 1994).

1.3 Defects in insulin action

Defect in insulin action is otherwise known as insulin resistance. The exact mechanisms of insulin resistance and why certain conditions, such as hyperglycemia or hyperlipidemia, cause insulin resistance are still unknown. Thus, all we have are observations from which researchers hope to devise mechanisms, some of which are presented here. Skeletal muscle is the most significant site of peripheral insulin resistance and there are 4 general areas that could be implicated, insulin binding, signal transduction, GLUT4 translocation and glycogen synthesis (DeFronzo et al 1992).

1.3.1 Defects in binding

Insulin binding must occur to stimulate efficient glucose uptake into a muscle cell. However, insulin receptor binding is decreased in muscle biopsies taken from diabetic subjects, compared to control (Maegawa et al 1991). *In vitro,* cells chronically exposed to insulin display decreased insulin binding at low concentrations (Livingston et al 1978). *In vivo,* animals made insulin resistant by high fat feeding display a decrease in insulin receptor quantity and glucose transport (Grundleger and Thenen 1982). Decreased receptor numbers cannot totally account for the degree of impaired glucose transport, indicating that post-receptor signaling defects significantly contribute to insulin resistance (Grundleger and Thenen 1982). Receptor and post receptor defects are involved in insulin resistance.

1.3.2 Defects in signal transduction

Insulin binds to its receptor initiating the first step of signal transduction, autophosphorylation of tyrosine kinase. In diabetic individuals autophosphorylation is decreased, potentially impairing further signal transduction (Maegawa et al 1991). The

activated tyrosine kinase phosphorylates insulin receptor substrate (IRS) proteins; muscle biopsies from diabetic subjects display decreased IRS-1 phosphorylation (Bjornholm et al 1997). Gene-therapy reversible insulin resistance occurs in IRS-1 knock out mice (Ueki et al 2000), and restoring the IRS-1 protein restores insulin sensitivity, indicating its importance to the signal transduction pathway. There are numerous targets for IRS-1 including the phosphorylation of phosphatidylinositol 3-kinase (PI3-kinase), which has decreased activity in diabetic subjects (Bjornholm et al 1997). In some instances, the decreased activation of PI3-kinase is due to an amino acid polymorphism in IRS-1, ($Gly \rightarrow Arg^{972}$); this mutation is most often observed in people with type 2 diabetes (Hribal et al 2000). PI3-kinase is involved in activating both the glycogen synthesis pathway and Atk kinase, an initiator of GLUT-4 translocation (Zierath et al 2000). Atk kinase protein levels are unchanged in obese animal models but display decreased insulin stimulated phosphorylation (Shao et al 2000). Animals made insulin resistant with high fat feeding also exhibit decreased Akt kinase activity (Tremblay et al 2001). Atk kinase activity can be restored with normalization of glycemia in diabetic animal models (Krook et al 1998). Just a few of the many proteins involved in insulin signal transduction have been mentioned here. There are more to be identified and any of these may also be defective in insulin resistance.

1.3.3 Defects in GLUT4 translocation

Translocation of GLUT4 to the cell surface is necessary for glucose entry into the cell, and defects in GLUT4 translocation machinery can contribute to insulin resistance (Garvey et al 1998). When muscle biopsies from insulin stimulated control and diabetic subjects are analyzed, the cellular localization of GLUT4 differs. This

indicates that GLUT4 does not reach its proper destination in people with type 2 diabetes (Garvey et al 1998). High fat feeding decreases the amount of GLUT4 in the cell membrane, possibly because it decreases PI3-kinase activity (Zierath et al 1997; Tremblay et al 2001). Whatever the cause, abnormal trafficking or decreased signal transduction, decreased GLUT4 transporters in the cell membrane equals decreased glucose transport. However, even if normal amounts of GLUT4 are present in the cell membrane, glucose transport can be diminished due to decreased GLUT4 activation by p38 MAP kinase (Sweeny et al 2001).

1.3.4 Defects in glycogen synthesis

Glycogen synthesis is decreased in insulin resistant individuals (Cefalu 2001), and in prolonged experimental hyperinsulinemia (Iozzo et al 2001). With good glycemic control it is possible to increase glucose disposal and glycogen synthesis in people with type 2 diabetes (Pratipanawatr et al 2002). A target for PI3-kinase is the glycogen synthase pathway, thus, one could infer that decreases in PI3-kinase activity could contribute to decreased glycogen synthesis. Some, (Shulman 2000), believe that rather than a specific defect in any of the glycogen synthesis pathway enzymes the problem is decreased glucose transport into the cell. Others, (Thorburn et al 1990), believe that the reduction in glycogen synthesis is independent of glucose uptake.

Besides skeletal muscle, the liver is an important site of insulin resistance. Type 2 diabetic subjects, in the postabsorptive phase, show 30% more glucose release from their liver compared to control (Meyer et al 1998). Increased glucose output can contribute to hyperglycemia. Contrary to these results, a study by Pigon et al (1996) showed that in lean diabetics, with moderate fasting hyperglycemia (7 mmol/l), glucose release did not

differ from control. They (Pigon et al 1996) proposed that in the early stages of diabetes, when glucose levels are still near normal, liver tissue is not yet insulin resistant and increased glucose output develops with progression of the disease; it is not a primary defect.

1.4 Defects in insulin secretion

According to Kahn (2001), it is an accepted fact that for hyperglycemia to exist in type 2 diabetes there must be β-cell dysfunction. There are many potential causes of dysfunction including β-cell overwork, glucose toxicity, disturbed glucose handling, lipotoxicity, and amyloid deposition. There are also many manifestations of altered secretion including increased proinsulin levels, abnormal pulsatility, loss of first phase insulin response and decreased glucose-stimulated insulin response.

1.4.1 Beta cell overwork

Diabetic subjects have decreased glucose-stimulated insulin secretion (Ward et al 1984). Subjects made acutely insulin resistant with nicotinic acid show an increase in β-cell secretion so that fasting plasma insulin levels are doubled and euglycemia is maintained (Kahn et al 1989). When presented with a glucose stimulus the insulin response is heightened in these insulin resistant subjects (Kahn et al 1989). Cockburn et al (1997) showed that, in response to glucose, the isolated islets from 5-week old Zucker Diabetic Fatty rats secreted more insulin compared to control, but at 12 weeks they secreted less insulin than control. The beta cell is initially able to adapt to insulin resistance with hypersecretion but this adaptation eventually fails.

1.4.2 Glucose toxicity

Glucose toxicity is more than just glucose desensitization because it creates irreversible changes in β-cell function. The process from desensitization to toxicity is gradual and can probably be prevented by tight glycemic control. When hyperglycemia is corrected in diabetic patients, there is an observed improvement in insulin secretion and sensitivity (Rossetti et al 1990). High glucose levels impair insulin gene transcription, which leads to a decrease in insulin production (Robertson et al 1994), possibly accounting for the decrease in insulin secretion. Beta cells incubated with high glucose concentrations are subject to apoptosis (Efanova et al 1998), indicating that uncorrected hyperglycemia could reduce β-cell mass.

1.4.3 Disturbed glucose handling

Glucose handling can be disturbed by a decrease in GLUT2 transporters in the pancreas. GLUT2 transporters are primarily responsible for glucose transport into β-cells. Diabetic rat models show a decrease in GLUT2 mRNA that is related to a decrease in insulin secretion (Johnson et al 1990), or an increase in hyperglycemia (Thorens et al 1990). Arginine-stimulated insulin release is still possible without GLUT2 transporters, but because the animals are unable to respond to glucose they are left in a diabetic state (Johnson et al 1990).

1.4.4 Lipotoxicity

Lipotoxicity can occur in obese subjects. In dogs, fed a high fat diet, there is a decrease in insulin sensitivity that is not accompanied by an increase in glucose-stimulated insulin release (Kaiyala et al 1999). Animal models of diabetes show that free fatty acid levels rise before the occurrence of hyperglycemia and loss of glucose-

stimulated insulin secretion (Lee et al 1994). In obese Zucker diabetic fatty rats, high levels of circulating free fatty acids cause fat deposition in islets, followed by apoptosis within the islet (Shimabukuro et al 1998). Cell cultures acutely (8 hr) exposed to palmitate showed increased insulin release, but chronic (48 hr) exposure led to decreased insulin release, decreased GLUT-2 protein levels and suppressed insulin biosynthesis (Yoshikawa et al 2001). Cell cultures exposed to the monounsaturated fatty acid palmitoleic acid caused beta cell proliferation (Maedler et al 2001). Exposure to the saturated fatty acid palmitic acid did not cause proliferation but rather induced apoptosis (Maedler et al 2001).

1.4.5 Islet amyloid

Islet amyloid deposits are found in healthy individuals and those with diabetes (Johnson et al 1989). Islet volumes are decreased in diabetic subjects, compared to non-diabetic subjects, due to amyloid deposits, and amyloid deposits are associated with beta cell damage (Westermark and Wilander 1978). While islet amyloid polypeptide is a natural product of the beta cell, it is unknown what initiates the deposits. Isolated islets cultured with high levels of glucose, to stimulate hormone secretion, produce a greater proportion of amyloid precursors, however, it is unknown if they are able to form into the fibrils (Hou et al 1999). This may not be important, since Janson et al (1999) showed that beta cell death is not induced by exposure to mature amyloid fibrils, *in vitro,* instead beta cell death is induced by intermediate-sized amyloid peptide aggregates that cause membrane disruption.

1.4.6 Manifestations of decreased insulin secretion

Increased proinsulin is a manifestation of β-cell dysfunction. Proinsulin levels are increased in both the fasting and glucose stimulated states of subjects that have impaired glucose tolerance or type 2 diabetes (Yoshioka et al 1988). When insulin secretion is over-stimulated for many days, an increase in circulating proinsulin is observed in normal individuals. An even greater increase is observed if β-cell dysfunction is already present (Ward et al 1987). Another manifestation is an aberration in pulsatile insulin release. Normal insulin release is pulsatile and oscillatory. Healthy individuals have insulin pulsations every 10.5 minutes while diabetic subjects have pulses every 8.8 minutes (Lang et al 1981). It has been suggested that insulin sensitivity decreases as pulse frequency increases (Zarkovic et al 1999). When presented with equivalent glycemic stimuli diabetic patients secrete less insulin, compared to control individuals (Perely and Kipnis 1967). When presented with a glucose stimulus, the normal insulin response includes a rapid first phase that peaks within five minutes and subsides, followed by a sustained second phase response (Daniel et al 1999). When diabetic subjects are presented with a glucose stimulus the first phase response is absent (Bagdade et al 1967). A significant relationship exists between glucose intolerance and decreased first phase insulin response (Bagdade et al 1967).

The information presented in the past two sections is only a small portion of the research done in an attempt to understand the mechanisms of type 2 diabetes. There is proof for defects at nearly every identifiable stage of insulin signaling or release, making it a complex disease for which no definitive mechanism has ever been found.

1.5 Introduction to a new idea

"Under physiologic conditions, the concentration of blood glucose fluctuates only in a narrow range despite alterations in periods of food intake and fasting. This stability is due to a remarkably efficient hormonal system that exerts opposite effects on the organs of glucose storage and production. Whereas several hormones can prevent the blood glucose level from falling dangerously low by stimulating glycogenolysis and gluconeogenesis, insulin is the only efficient means by which the organism can prevent exaggerated elevations in blood glucose level" (Henquin 1994). This statement sums up the paradigm under which conventional diabetes research is conducted. To paraphrase, glucose homeostasis is very important and essential, and while there are many ways the body prevents glucose levels from decreasing too much it only has one, single, solitary way to prevent it from becoming too high, and that is insulin. Considering that normal physiology often displays redundant control mechanisms it is very difficult to believe that insulin acts alone.

Our laboratory's research is unique. It defines that the mechanism of insulin resistance in many situations to be the loss of hepatic insulin sensitizing substance (HISS), a putative neurohumoral factor with insulin-like action. Conventional findings are applicable to this theory because conventional findings do not focus on a mechanism, only characterized events in the disease. For instance, insulin hypersecretion could be compensatory for loss of HISS action. Hyperglycemia will have the same cellular toxicity and hyperlipidemia could result in pancreatic damage and loss of pulsatility or first phase insulin secretion. Decreased post-receptor enzyme activity could be due to

lack of activation by HISS. As we learn more about the nature of HISS we will be better able to answer these questions.

1.6 Characterization of HISS action

Xie et al (1993) observed that when the hepatic anterior plexus in cats was denervated there was an inhibition in the whole body response to insulin, as assessed by the insulin tolerance test. Neither total hepatic denervation or bilateral vagotomy changed the level of insulin resistance; the authors concluded that glucose balance is dependent on an intact functional hepatic anterior nerve plexus. Further, it was suggested that the parasympathetic, rather than sympathetic, nerves played a permissive role in allowing full expression of insulin effects. Support for this idea was provided when an intraportal venous (i.p.v.) atropine infusion caused a similar decrease in insulin sensitivity as the denervation (Xie and Lautt 1994, 1995a). Atropine, however, is a nonselective muscarinic antagonist and, to date, 5 receptor subtypes have been identified. Pirenzepine, a selective M_1 antagonist, produced the same degree of insulin resistance as atropine, suggesting that the M_1 receptor subtype may be specifically responsible for regulating the insulin resistance (Xie and Lautt 1995b). Using arterial-venous glucose gradients, skeletal muscle was identified as the site of insulin resistance (Xie and Lautt 1996a). The resistance induced by hepatic denervation was further shown to be reversible by intraportal but not intravenous acetylcholine (Xie and Lautt 1996b). This confirmed that the liver was the target organ for reversing the resistance. Acetylcholine itself has no effect on blood glucose levels, since it is only after insulin infusion that the greater glucose uptake response is triggered. This supports the idea that the parasympathetic nerves play a permissive role in insulin sensitivity.

Fasted rats are insulin resistant compared to fed rats (Lautt et al 1998). Insulin resistance induced by fasting is gradual and based on the length of time an animal is fasted. Insulin sensitivity can be increased by placing food in an anesthetized rat's stomach (Lautt et al 2001). It was proposed that the liver releases a factor called hepatic insulin sensitizing substance, or HISS, which affects the whole body response to insulin. Sadri et al (1997) and Sadri and Lautt (1998) also observed that blocking nitric oxide (NO) synthase with N-nitro-L-arginine methyl ester (L-NAME) or N-monomethyl-L-arginine acetate (L-NMMA) caused a decrease in insulin sensitivity, identifying nitric oxide as a mediator of the HISS release pathway. Sadri and Lautt (1999) showed that intravenous (i.v.) L-NAME does not cause insulin resistance but i.p.v. L-NAME does, indicating that the liver is the controlling organ. Intraportal venous, and not i.v., administration of SIN-1, an NO donor, causes reversal of L-NMMA induced insulin resistance (Sadri and Lautt 1999).

Insulin resistance observed in many disease models was also identified as being caused by a decrease in HISS action. Chronic bile duct ligation was shown to produce insulin resistance that was reversible by intraportal acetylcholine administration (Lautt and Xie 1998). Thioacetamide induced cirrhotic rats displayed decreased insulin action due to a blockade of HISS release (unpublished observation). Ribeiro et al (2001a) suggested that insulin resistance in spontaneously hypertensive rats was HISS-dependent. Insulin resistance in Zucker fa/fa rats, a model of obesity, is both HISS-dependent and HISS-independent (Ribeiro et al 2001b). Rats fed a high sucrose diet display HISS-dependent insulin resistance (Ribeiro et al 2001c). Fetal ethanol exposure has also been

shown to induce insulin resistance in the adult rat via HISS blockade (Sadri, Legare, Takayama and Lautt, unpublished results).

1.7 RIST methodology

The development of the Rapid Insulin Sensitivity Test (RIST) has been key to the characterization of HISS action, and has been described and revised in the literature (Xie et al 1996; Lautt et al 1998). In all except the first experimental series the RIST has been used to quantify insulin sensitivity.

To perform the RIST insulin, 50 mU/kg, is infused intravenously into the animal over 5 minutes. Euglycemia, as dictated by the animals own basal glycemia, is maintained with a variable rate glucose infusion that is adjusted according to blood samples taken every 2 minutes and analyzed for blood glucose levels. Once the animal can maintain euglycemia on its own with no glucose infusion, the RIST is completed. Insulin sensitivity is measured as the amount of glucose infused, expressed in mg/kg, and is called the RIST index. The duration of the RIST is variable but is usually complete within 30-35 minutes in a sensitive animal and in less time in an insulin resistant animal.

1.7.1 Standardizing feeding

Control animals are generally fasted for 8 hours and then fed for 2 hours before surgery. This ensures a high response to insulin. Lautt et al (2001) reported a decrease in insulin sensitivity that is related to increased duration of fasting. By fasting the animals we ensure that they will want to eat, and doing experiments within two hours of refeeding standardizes the prandial status of the animal. Anesthesia maintains the prandial status for many hours, since five consecutive RISTs can yield the same response (Xie et al 1996), however, in the conscious animal insulin sensitivity decreases about 10% per hour

after feeding (Latour and Lautt 2002b). It is quite possible that HISS has been overlooked because most studies use fasted subjects.

1.7.2 HISS-dependent vs. HISS-independent insulin action

Generally, the RIST index after blocking HISS release physiologically by fasting, pharmacologically by blockade of muscarinic receptors with atropine or hepatic NO synthase with L-NMMA or L-NAME, or surgically by denervating the liver is very similar. This portion of insulin sensitivity is referred to as HISS-independent insulin sensitivity because HISS action had no part of the response since it was blocked. In a fed animal, where HISS release occurs, the insulin sensitivity is much higher and is made up of both the HISS-dependent and HISS-independent portions of insulin sensitivity. By subtracting the blocked response from a paired control response it is possible to determine the HISS-dependent portion of insulin sensitivity. Generally fluctuations in whole body insulin sensitivity are, in fact, fluctuations in HISS-dependent insulin sensitivity. When HISS release is blocked, a state of HISS-dependent insulin resistance (HDIR) occurs, where the whole body glucose uptake in response to insulin is decreased by approximately 55% (Lautt et al 2001).

1.8 Summary of HISS hypothesis

Hepatic Insulin Sensitizing Substance (HISS) is a putative neurohumoral hormone that is released from the liver in response to a bolus of insulin. HISS acts on skeletal muscle to aid glucose uptake (Xie and Lautt 1996a). When HISS release is blocked, a state of HISS-dependent insulin resistance occurs, and insulin sensitivity is reduced by approximately 55% (Lautt et al 2001). HISS release can be blocked physiologically by fasting (Lautt et al 2001; Sadri and Lautt 2000), pharmacologically by blockade of

hepatic muscarinic cholenergic receptors using atropine (Takayama et al 1999, 2000; Xie and Lautt 1994, 1995a) or blockade of hepatic nitric oxide synthase using L-NMMA or L-NAME (Sadri and Lautt 1998, 1999), and surgically by denervating the liver (Xie and Lautt 1994, 1996a, 1996b; Sadri and Lautt 2000; Xie et al 1993; Latour and Lautt 2002a). Two important things allowed for the characterization of HISS, the prandial status of the animal and the development of the Rapid Insulin Sensitivity Test (RIST).

1.9 Commonly used methods of assessing insulin sensitivity

There are numerous methods for assessing insulin sensitivity described in the literature. Each method has its place in research or clinical practice and has yielded valuable information.

Clinically, the World Health Organization (W.H.O.) recommends that the diagnostic criteria for diabetes mellitus be a fasting plasma glucose concentration of 7.0 mmol l^{-1} and above or 6.1 mmol l^{-1} and above for whole blood. They recommend that for epidemiological studies the oral glucose tolerance test (OGTT) be employed. After an overnight fast and blood sample, subjects are presented with an oral glucose challenge, which consists of 75g of anhydrous glucose. A blood sample is taken after 2 hours to determine plasma glucose levels, and a concentration above 11.1 mmol l^{-1} is indicative of diabetes mellitus (W.H.O. 1999). This test is easy to perform in large populations, however, a limitation of this test is that it requires endogenous insulin release.

The intravenous glucose tolerance test (IVGTT) consists of an intravenous glucose injection that will stimulate an insulin response. In the simple IVGTT, the rate of glucose disappearance reflects the sensitivity of tissues to endogenous insulin (Scheen et al 1994). If both insulin and glucose concentrations are monitored, a mathematical

model, called the minimal model, can be applied to the collected data to define the insulin sensitivity index, which is the dependence of fractional glucose disappearance on plasma insulin concentrations (Bergman et al 1979). The IVGTT with minimal modeling is feasible for large populations because it is easy to perform. It also allows the investigator to observe both first and second phase insulin secretion (Bergman et al 1979). By including a labeled glucose tracer it is possible to determine which tissues are responding to insulin and to monitor glucose disposal without the influence of hepatic glucose output (Avogaro et al 1996). This test is more favorable than the OGTT, even though they are based on the same principle, because it eliminates variation due to gut motility and absorption. However, the response is also different between the two tests and the OGTT provides a higher insulin release to glucose concentration, indicating that blood glucose concentration is not the only factor controlling the insulin response (Perely and Kipnis 1967). A limitation of this test is that it requires endogenous insulin release.

The hyperglycemic clamp technique consists of a constant glucose infusion that is adjusted to keep blood glycemia at a predetermined level, basal glucose plus 5 mmol (Elahi 1996). It assesses glucose stimulated insulin secretion, or beta cell sensitivity to glucose, and quantifies the amount of glucose metabolized by the body following hyperglycemia (DeFronzo et al 1979). By measuring insulin levels both first and second phase insulin responses are easily identifiable. This is important because first phase insulin secretion is lost early in the progression of diabetes (DeFronzo et al 1979). Tissue sensitivity to endogenously secreted insulin is expressed as the ratio of glucose metabolized to plasma insulin concentration (DeFronzo et al 1979) and is well correlated to tissue sensitivity determined by the euglycemic clamp (DeFronzo et al 1979; Mitrakou

et al 1992). The hyperglycemic clamp works only if insulin secretion is possible, and under the assumption that hepatic glucose output is suppressed (DeFronzo et al 1979).

The hyperinsulinemic euglycemic clamp (HIEC) technique is considered to be the 'gold standard' test for insulin sensitivity. This test provides an estimate of tissue sensitivity to insulin while euglycemia is maintained (DeFronzo et al 1979). It involves a constant insulin infusion and a variable glucose infusion to maintain euglycemia. Ideally the test period is 2-3 hours, making this test extremely long and laborious for researchers. Insulin sensitivity is defined as the rate of glucose infusion used to maintain euglycemia during the final 30 minutes of the test, which is also considered to be the amount of glucose metabolized in response to the insulin (DeFronzo et al 1979); others define it as the ratio of glucose metabolized to plasma insulin concentration during the clamp (Bonora et al 1989). When combined with radioactive glucose, this test, as well as the hyperglycemic clamp, can identify the tissues most responsive to insulin (DeFronzo et al 1979). A further discussion on this method follows in Chapter 2.

The insulin tolerance test (ITT) is a very simple test. It involves injecting a bolus of insulin and observing the change in blood glucose concentration. It is a very rapid test since it measures insulin sensitivity by the rate of decline in blood glucose (Akinmokun et al 1992) or by the ratio of change in blood glucose to basal levels (Scheen et al 1994). The greatest concern when using this test is the potential to develop hypoglycemia. This has been averted by lowering the amount of insulin given (Gelding et al 1994) or by administering glucose after 30 minutes (Bonora et al 1989), since counter-regulatory hormones would influence any data collected after this point. A further discussion of this method follows in Chapter 2.

1.10 Hypothesis tested and rationale

In the following chapter the hypothesis, that the RIST method is comparable to the ITT and the HIEC, is tested. We felt that each of these tests has been validated and extensively used in the literature. Since an early study in the HISS series used the ITT it became a good candidate against which to validate the RIST. The HIEC, considered to be the gold standard, is simply a longer version of the RIST, and is therefore, another excellent comparative test. It was essential to validate the RIST against other methods to provide credibility to our work and the method itself.

CHAPTER 2 – Comparison of the Rapid Insulin Sensitivity Test, Insulin Tolerance Test and Hyperinsulinemic Euglycemic Clamp, in their ability to detect HISS-dependent insulin action*

2.1 Introduction:

The Rapid Insulin Sensitivity Test (RIST) has been described in the literature (Xie et al 1996; Lautt et al 1998), but has not been directly validated against other methods used to determine insulin sensitivity. The RIST methodology has allowed for the characterization of Hepatic Insulin Sensitizing Substance (HISS), which has been reviewed earlier in this thesis. It became essential to validate the RIST method to enhance the credibility of our work, and so that other research groups will use it.

There are many tests against which to compare the RIST, but for this series the HIEC and ITT were chosen. This series provides support for the hypothesis that the RIST is comparable to the ITT, and that each test can equally identify HISS-dependent and HISS-independent insulin action. The RIST, however, is not comparable to the glucose infusion rate during the last 30 minutes of the HIEC, possibly due to the observation that use of HIEC induces HISS-dependent insulin resistance in our rat model. There is evidence, however, that the initial response during the clamp can detect some HISS-dependent insulin action, which does show a significant relationship when compared to the RIST.

---

* The experiments reported in this chapter were done by M. Reid (the author), M. Latour, D. Legare, and N. Rong.

2.2 Methods:

2.2.1 Surgical preparation

Male Sprague-Dawley (SD) rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg, Somnotol, Bimeda- MTC Animal Health Inc.). Body temperature was monitored by a rectal probe, and kept at 37.5 ± 0.5 ° C by a heated surgical table and overhead lamp. A tracheotomy was performed to allow spontaneous breathing. The femoral artery and vein were isolated and prepared for cannulation with an arterial-venous blood sampling shunt, which allows for small volume blood samples. The blood-sampling shunt is illustrated in figure 2.1. Both the femoral artery and vein were cannulated with PE60 tubing. Blood is allowed to flow from the artery to the vein via two silicon sleeves attached to a T-junction. A third line runs from the T-junction to a pressure transducer allowing the operator to monitor shunt pressure, which is an indicator of blood flow in the open shunt and arterial pressure when the venous silicon sleeve is clamped. Blood samples (25 μL) are collected by puncturing the arterial side of the shunt and drug infusions are made into the venous side of the shunt. The jugular vein was cannulated for a continuous infusion of supplemental sodium pentobarbital (2.16 mg/ml) at a rate of 0.5 ml/hr/100g body weight. A glucose infusion line was inserted into the supplemental line through a small length of silicon tubing. Animals were heparinized, 100IU/kg, to prevent clotting in the shunt. After the surgery was completed the animal was allowed to recover for 30 minutes before any blood samples are taken. Blood samples were then taken every 5 minutes and analyzed for blood glucose concentration using the glucose oxidase method (glucose analyzer by Yellow Springs Instruments, Yellow Springs, Ohio), until 3 successive stable

measurements were taken. Stable measurements are within 4 mg% of one another and cannot be following the same trend; they must be fluctuating (i.e. 116, 114, 115 mg/dL would constitute a stable animal). The mean of these 3 samples is the basal glucose level.

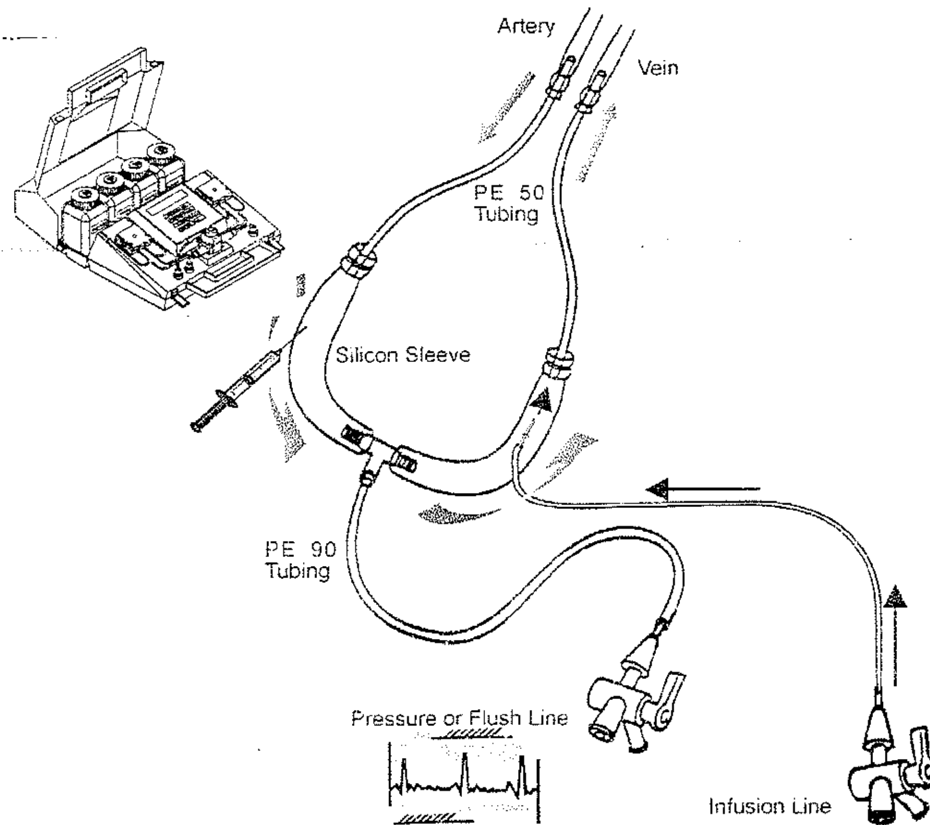

Figure 2.1: Arterial –venous blood sampling shunt. The shunt allows for blood to flow, unhindered, from the artery to the vein. Pharmacological agents can be infused into the venous side of the shunt, and blood samples can be taken from the arterial side of the shunt. Using the shunt minimizes the volume of blood lost during sampling.

2.2.2 Comparison of the RIST to the ITT

Male SD rats (281.6 ± 21.7 g) were divided into 2 groups, control (n=5) and HISS blocked (n=6). Control animals were fasted for 8 hours and fed for 2 hours prior to surgery; this prandial status is referred to as fed. Blocked animals were treated in one of three ways - either atropine (3 mg/kg i.v.) or L-NMMA (30 mg/kg i.v.) was infused over 5 minutes in fed rats, or the rats were fasted for 18 hours with no refeed. Surgery was performed as described (AV shunt, jugular cannulation, and tracheotomy). Once a basal blood glucose level was established, a RIST was performed as described by Lautt et al. (1998). Upon completion, the basal blood glucose was determined and an ITT performed.

To perform the RIST, insulin was infused into the venous side of the AV shunt using an infusion pump (Harvard Apparatus, Millis, MA) that administered a 50 mU/kg dose over 5 minutes (0.5 ml volume @ 0.1 ml/min). After 1 minute of infusion an arterial blood sample was taken and a variable glucose infusion (10%) was initiated. Blood samples were taken every 2 minutes and the glucose infusion rate was adjusted to maintain euglycemia. The RIST index is the amount of glucose infused per kilogram body weight to maintain euglycemia over the test period that terminated when no further glucose infusion was required, approximately 30 to 35 minutes.

To perform an ITT, 50 mU/kg of insulin was infused into the venous side of the AV shunt over 5 minutes (0.5 ml volume @ 0.1 ml/min). Blood samples were taken every 2 minutes, starting at 1 minute into the infusion, and analyzed for glucose concentration. The ITT index of insulin sensitivity is the nadir of the decline in blood

glucose in response to insulin, and is usually reached between 13-15 minutes. The test ended when blood glucose levels returned to a basal level or stabilized at a new level.

2.2.3 Comparison of the RIST to the HIEC

Male rats (267.7 ± 13.0 g) were divided into 2 groups, control (n=4) and HISS blocked (n=10). The blocked group consisted of animals that were fed and treated with L-NMMA (30mg/kg i.v., n=3) or atropine (3 mg/kg i.v., n=3) or fasted for 18 hours (n=4) with no refeed. Once basal blood glucose levels were determined, a RIST was performed, followed by a stabilization period and the HIEC.

To perform the HIEC, insulin was infused at 10 mU/kg/min for 180 minutes (0.01 ml/min). After one minute of insulin infusion, a variable glucose infusion (20%) was initiated and a blood sample was taken. Blood sampling continued every 2 minutes until the glucose infusion rate was relatively stable (approximately 40 to 50 minutes), then blood samples were taken every 5 minutes. The glucose infusion rate was adjusted to maintain euglycemia. Insulin infusion was terminated at 180 minutes but glucose infusion was continued until the animal could maintain euglycemia on its own, if subsequent tests were required. The HIEC's measure of insulin sensitivity was the average glucose infusion rate (GIR) during the final 30 minutes (150-180) of the test.

2.2.4 Effect of the HIEC on insulin sensitivity

In another comparison of the RIST with the HIEC, animals (265.1 ± 18.9 g) were either in a control group (n=9) or had HISS release suppressed by fasting for 18 hours (n=6). An initial RIST was performed followed by the HIEC, a second RIST, an infusion of atropine (3 mg/kg i.v.), and a third RIST. Blood samples were taken at 5-minute intervals between each test to establish basal blood glucose levels.

2.2.5 Effect of the HIEC on insulin sensitivity, using the ITT

The animals (277.4 ± 16.0g) were all tested after fasting and refeeding (n=7). Surgical preparation was as described. Once basal glucose levels were determined, an ITT was performed (50 mU/kg insulin) followed by the HIEC (10 mU/kg/min insulin), a second ITT, atropine (3 mg/kg i.v.) and a third ITT. Blood samples were taken at 5-minute intervals between each test to establish basal blood glucose levels.

2.2.6 Drugs

Human insulin (Humulin R) was purchased from Eli Lilly and Company (Toronto, Canada). Atropine and D-glucose were purchased from Sigma Chemical Co. (St. Louis, Mo.), and L-NMMA was purchased from Research Biochemicals Incorporated, (Natick, MA). All drugs were dissolved in saline.

2.2.7 Data analysis:

Data were analyzed using repeated-measures analysis of variance followed by the Tukey-Kramer multiple comparison test in each group or, when applicable, the paired and unpaired Student's t-test was used. Correlation and regression analysis were also used when necessary. The analyzed data were expressed as means ± SE throughout. Differences were accepted as statistically significant at $P<0.05$. Animals were treated according to the guidelines of the Canadian Council on Animal Care, and an ethics committee on animal care at the University of Manitoba approved all protocols.

2.3 Results

2.3.1 Comparison of the RIST to the ITT:

In this experimental series, control animals (n=5) had a mean RIST index of 225.5 ± 16.8 mg glucose/kg body weight (mg/kg) while animals with HISS release blocked (n=6) had a mean RIST index of 134.8 ± 15.5 mg/kg. Insulin sensitivity in the blocked group was 40% less than control, using the RIST. The mean ITT nadir for the control group represented a decline in arterial glucose levels of 17.2 ± 1.7 mg% and 10.2 ± 1.5 mg% for the blocked group. Insulin sensitivity in the blocked group was 41% less than control, using the ITT. A correlation of the two tests under all conditions is shown in figure 2.2 and profiles of the insulin response and HISS component of insulin action are presented in figure 2.3.

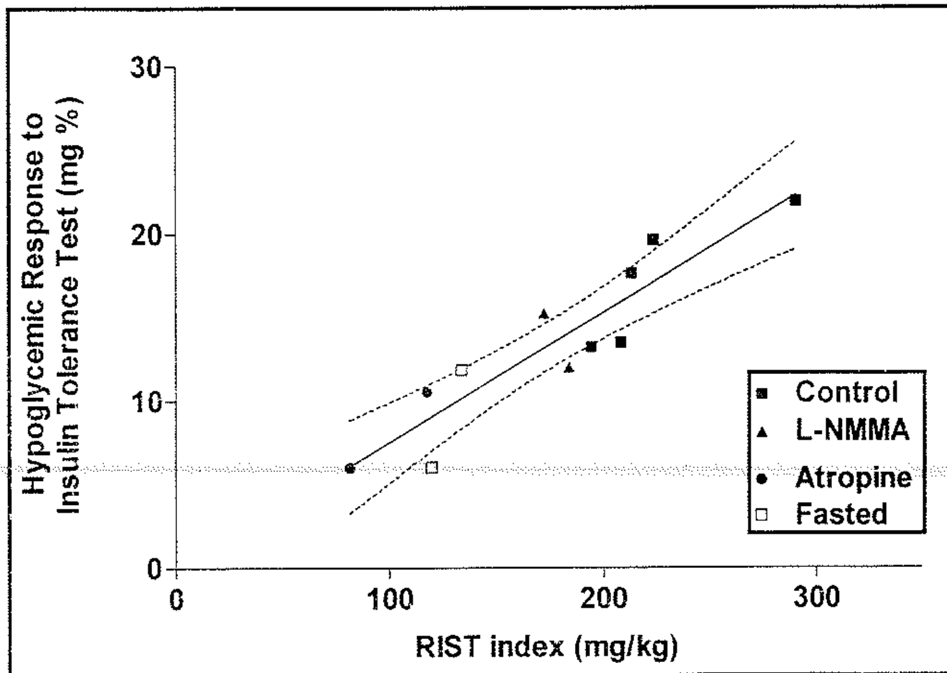

Figure 2.2: Correlation comparison of the ITT and RIST obtained in fed rats (control) and in rats with HISS action blocked by fasting, or by L-NMMA or atropine in fed rats, r^2= 0.84, P<0.0001). The RIST and ITT are comparable.

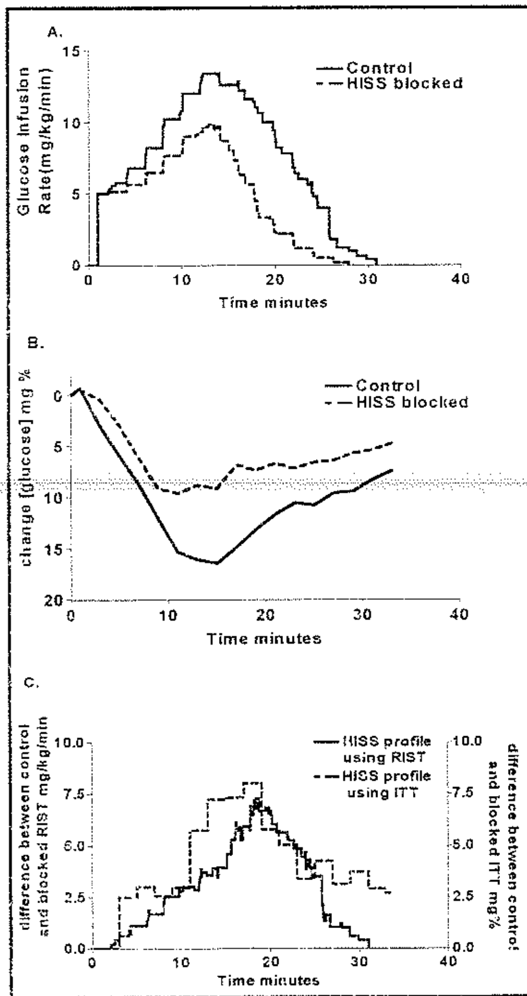

Figure 2.3: A.) Plotting the average glucose infusion rate, during the RIST, at 0.1 minute intervals in the control state reveals a dynamic curve of total insulin action (n=5). A similar plot in the HISS-blocked state reveals a dynamic curve of HISS-independent insulin action (n=6); B.) Plotting the average change in glucose concentration, during the ITT, at 2 minute intervals reveals a dynamic curve of total insulin action (n=5). A similar plot in the HISS-blocked state reveals a dynamic curve of HISS-independent insulin action; C.) Dynamic profiles of HISS-dependent insulin action are calculated by subtracting the dynamic curves in the HISS-blocked state from dynamic curves in the control state, for both the RIST and ITT.

2.3.2 Comparison of the RIST to the HIEC:

Control animals (n=4) had a mean RIST index of 222.2 ± 46.1 mg/kg and the group with HISS release blocked (n=10) had a RIST index of 105.0 ± 11.4 mg/kg. There was a 53% difference ($P<0.05$) in insulin sensitivity between the two groups when tested with the RIST. The mean GIR of the control group HIEC was 21.4 ± 1.0 mg glucose/kg body weight/min (mg/kg/min) and the blocked group HIEC had a GIR of 22.34 ± 1.2 mg/kg/min. The GIR was not significantly different between the two groups (figure 2.4). Figure 2.5 shows a correlation comparison between the RIST and HIEC under both conditions.

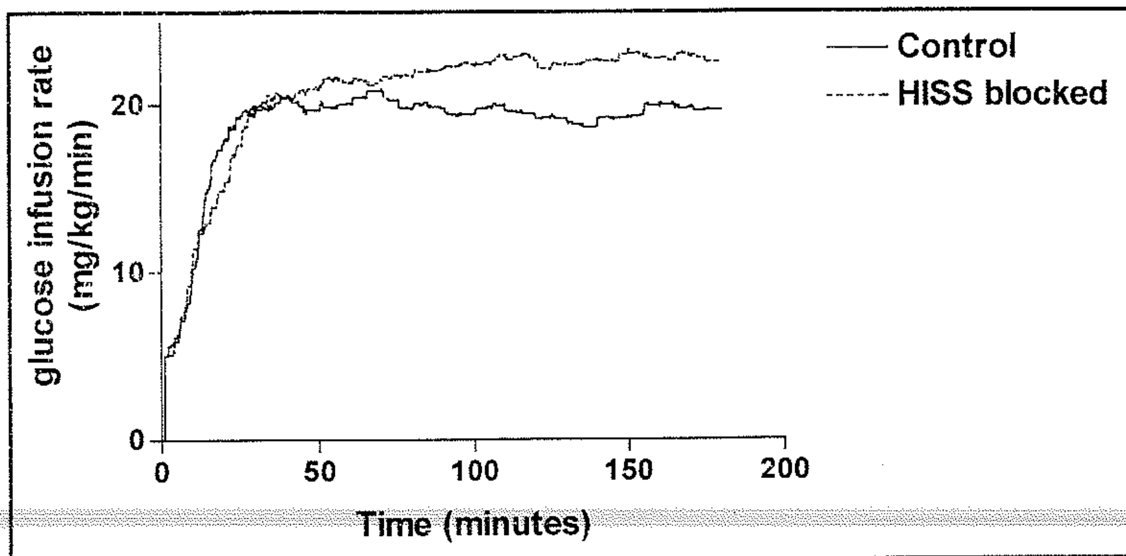

Figure 2.4: The solid line represents the average GIR during the HIEC for rats in the control condition and the dashed line represents the GIR for rats where HISS release has been blocked. There is no significant difference between the lines during the last 30 minutes of glucose infusion.

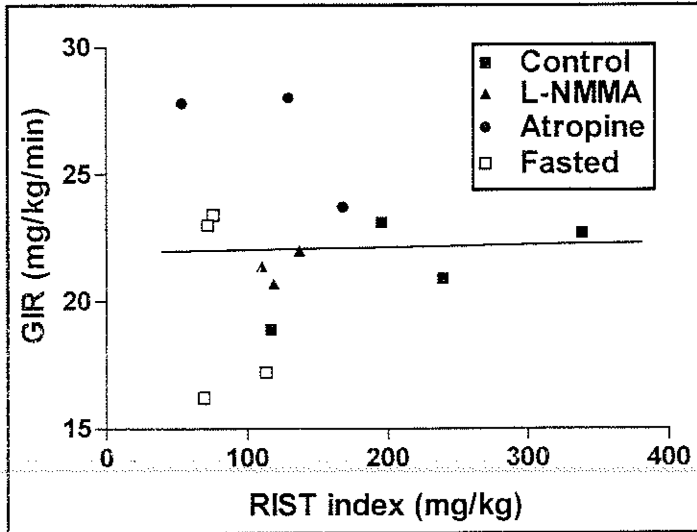

Figure 2.5: Correlation comparison of the GIR from the last 30 minutes of the HIEC to the RIST showed no relationship between the two tests. The RIST and HIEC are not comparable during this time period.

2.3.3 Effect of the HIEC on insulin sensitivity, using the RIST

In the control rats, the initial RIST was 192.2 ± 10.6 mg/kg, the post HIEC RIST was 98.7 ± 5.6 mg/kg, and the post atropine RIST was 91.5 ± 4.4 mg/kg. In the fasted rats, the initial RIST was 84.8 ± 14.7 mg/kg, the post clamp RIST was 65.4 ± 7.7 mg/kg, and the post atropine RIST was 62.5 ± 10.2 mg/kg. The mean GIR was 18.4 ± 0.9 mg/kg/min for the control group of animals and 23.4 ± 0.9 mg/kg/min for the fasted group. There is a significant, 49%, decrease in the RIST index of insulin sensitivity after the HIEC in the control group but not in the fasted group (Figure 2.6). When only the

initial RIST index from the two groups is compared, insulin sensitivity is significantly higher in the control group. The GIR does not reflect this difference, as there is no difference in the GIR between control and fasted animals, in this series.

A secondary analysis was performed where the RIST index was compared to the upslope of the GIR at the beginning of the HIEC. Specifically, we took the slope of the line from time zero to the time where the glucose infusion reached one half of the final GIR. There is a significant ($P < 0.01$) correlation between the RIST index and the upslope of the HIEC, $r^2 = 0.26$ (figure 2.7). Further, a HISS profile similar to that obtained by the RIST and ITT it seen when the HISS blocked HIEC is subtracted from the control HIEC during its first 30 minutes (Figure 2.8).

2.3.4 Effect of the HIEC on insulin sensitivity, using the ITT

In fed anesthetized rats the mean nadir for the initial ITT was 12.0 ± 0.9 mg% and the GIR was 20.6 ± 1.6 mg//kg/min. The mean nadir was 6.4 ± 1.0 mg % post HIEC, and 5.4 ± 0.7 mg % post atropine. There was a significant, 47%, decrease in insulin sensitivity after the HIEC, which was not changed by the administration of atropine (figure 2.9). The post-HIEC ITT response indicated that use of the HIEC induced insulin resistance that was not worsened by atropine.

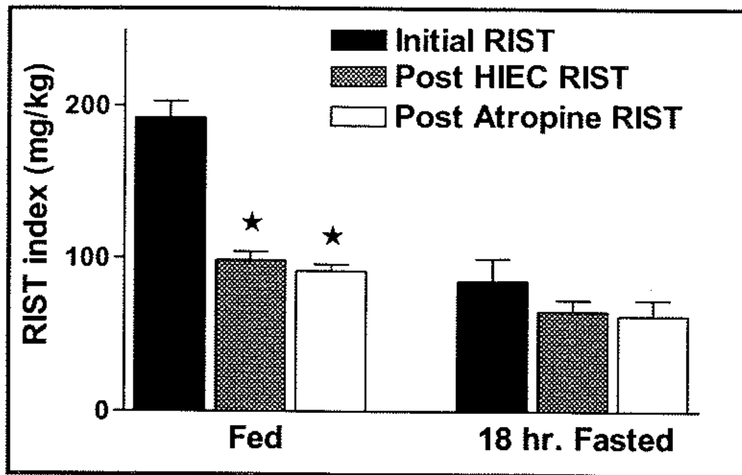

Figure 2.6: Initial RIST indexes for fed and fasted rats, after a 3 hour HIEC and after atropine. The HIEC caused a significant (P<0.001) decrease in insulin sensitivity in the fed animals that was not affected by atropine, indicating that full HISS-dependent insulin resistance (HDIR) existed after the completion of the HIEC. There was no significant impairment of insulin sensitivity by the HIEC in the fasted rat since fasting physiologically blocked HISS release, indicating that the decrease in insulin sensitivity induced by the HIEC in the control animal is HISS-dependent. The lack of additional atropine effect shows that HISS blockade was complete and that the HISS-independent component of insulin action was unaltered by either fasting or by the HIEC.

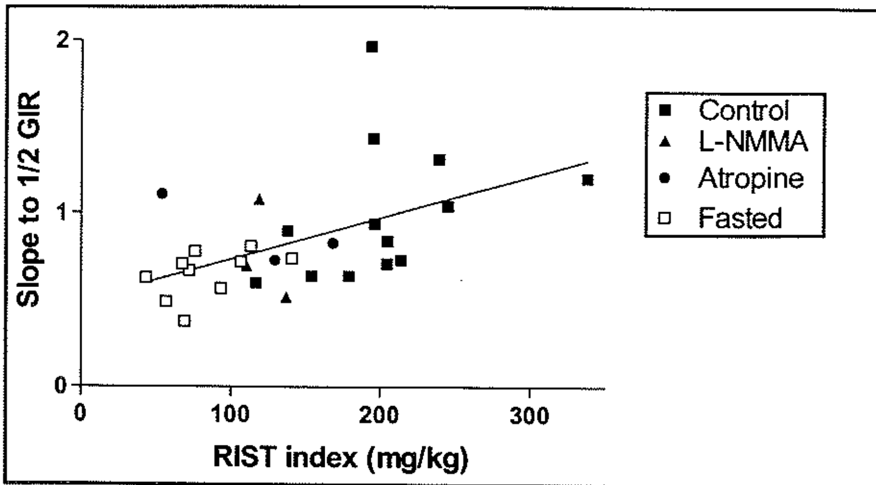

Figure 2.7: Correlation analysis of the initial rate of rise of glucose infusion during the HIEC compared to the RIST reveals a significant relationship, $r^2 = 0.26$. The RIST may be comparable to the HIEC at its onset.

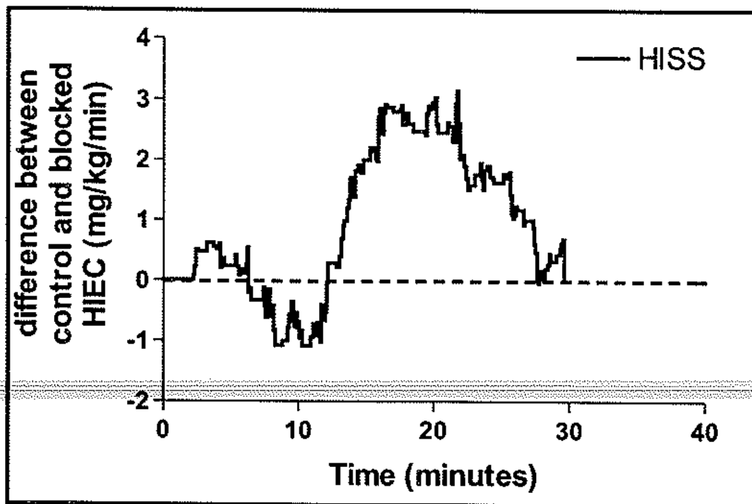

Figure 2.8: Subtracting the first 30 minutes of the HISS-blocked HIEC from the control HIEC reveals a curve that is similar in shape to the HISS curve calculated from the RIST.

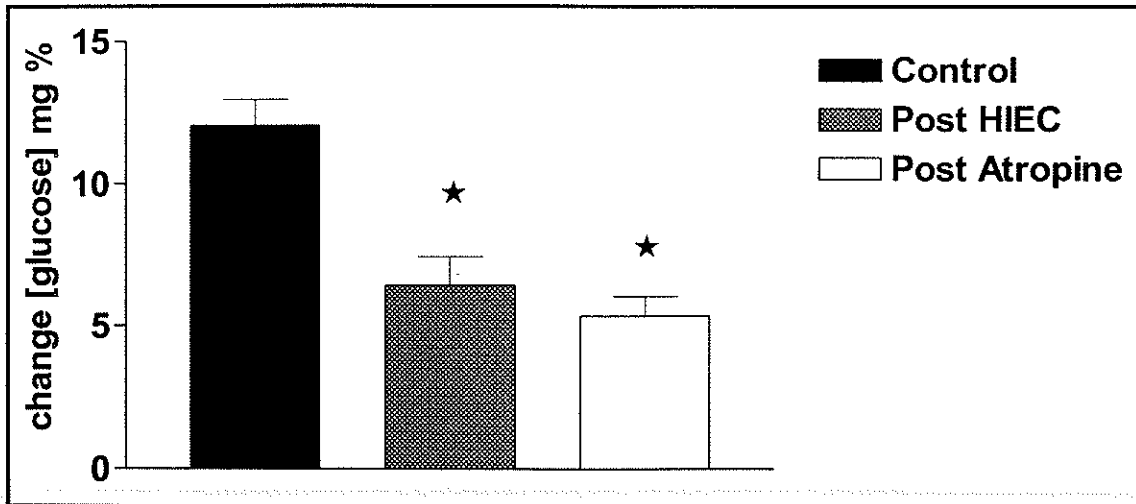

Figure 2.9: The control ITT is reduced significantly (P<0.01) after the HIEC, but not further after atropine administration. These results are similar to those produced by the RIST, indicating that the ITT can detect insulin resistance caused by use of the HIEC.

2.4 Discussion

The objective of this experimental series was to compare the ability of three insulin sensitivity tests to detect and quantify HISS-dependent insulin action in rats tested under a variety of conditions. This study was conducted to validate the RIST, which has been described earlier in this thesis and in the literature (Lautt et al 1998, Xie et al 1996). The RIST has been used to test insulin sensitivity, and has been a tool in the characterization of the physiology, pathophysiology and pharmacological manipulation of the putative hormone, hepatic insulin sensitizing substance (HISS). In a fed animal, HISS is released from the liver in response to insulin to stimulate glucose uptake by skeletal muscle (Xie and Lautt 1996a). When HISS release is blocked, the glucose disposal effect of a bolus of insulin is reduced by approximately 55%, and a state of HISS-dependent insulin resistance occurs (Lautt et al 2001). It is possible to block HISS release by fasting (Lautt et al 2001; Sadri and Lautt 2000), blocking hepatic muscarinic receptors with atropine (Takayama et al 1999, 2000; Xie and Lautt 1994, 1995a), blocking hepatic nitric oxide synthase with L-NAME or L-NMMA (Sadri and Lautt 1998, 1999), or by denervation of the liver (Xie and Lautt 1994, 1996a, 1996b; Sadri and Lautt 2000; Xie et al 1993; Latour and Lautt 2002a). Since there is a significant difference in insulin sensitivity when HISS release occurs compared to when it is blocked, any test that we compare the RIST to should be able to distinguish between the two states.

The results are consistent with the proposed hypothesis that the RIST is comparable to the ITT. This is not surprising based on the fact that the original studies in the characterization of HISS used the ITT (Xie et al 1993), and the RIST was developed

to avoid the hypoglycemia of the ITT. The same conclusion could not be drawn about the HIEC. The data suggest that the RIST may be comparable to the HIEC at its onset but not during the final glucose infusion rate, suggesting that the HIEC may be able to detect HISS action in the beginning but not at the end of the insulin infusion. Aside from its inability to detect HISS-dependent insulin action, use of the HIEC causes a maintained blockade of HISS release that can be shown using both the RIST and ITT. The mechanism for post HIEC resistance is not yet known. This work validates the RIST methodology and exposes a limitation of the HIEC.

2.4.1 Comparison of the RIST and ITT

There is a significant correlation between the RIST and the ITT (figure 2.2). The fall in blood glucose due to an insulin bolus (Norgaard and Thaysen 1929) along with its hypoglycemic dangers are well known. The ITT has been used with a conventional dose of 100 mU/kg (Bonora et al 1989), and, more recently, with a safer low dose of 50 mU/kg because it is rapid and simple (Akinmokun et al 1992; Hirst et al 1993; Gelding et al 1994). Our data show that the RIST and the ITT have a similar ability to detect insulin sensitivity, quantifying both HISS-dependent and HISS-independent insulin action. Both tests show a similar (40% vs. 41%) decrease in insulin sensitivity when HISS release is blocked, however, the RIST method has advantages over the ITT. By maintaining euglycemia during the RIST, potentially dangerous hypoglycemia and counter-regulatory responses (Garber et al 1976) are avoided. The scale on which insulin sensitivity during the RIST is measured also has advantages over that of the ITT since the range over which insulin sensitivity is measured is greater. For instance in this series the difference between the control and blocked RIST values is 90.7 mg/kg, but the difference between

the control and blocked ITT is only 7.0 mg%; it is much easier to misinterpret data when the difference between a sensitive subject and insensitive subject is small. Although the RIST and ITT are comparable, the RIST is a safer, more easily interpreted test. Figures 2.3 A and B show the average curves of the whole body response to insulin using the RIST and ITT, which serves to visualize that the insulin response pattern is similar between the tests. The RIST has the added advantage that by the termination of insulin and HISS action, about 35 minutes, a full dynamic curve of HISS action can be obtained. This is done by subtracting the glucose infusion rate of the RIST over time in the HISS-blocked test from the control test. When the change in glucose concentration in the HISS blocked ITT is subtracted from the change in glucose concentration in the control ITT a HISS pattern that is qualitatively similar to that of the RIST is evident until near the end where it deviates because extra time is required to recover from hypoglycemia. As a result, the down slope of the HISS action curve is distorted (figure 2.3C). In some instances the animal does not return to its pretest glycemia by the completion of the ITT, unlike the RIST, making it a less feasible test for numerous paired interventions in the same animal on the same experimental day.

2.4.2 Relationship between the RIST and HIEC

We were unable to show a correlation between the RIST and HIEC (figure 2.4). The HIEC is considered to be the 'gold standard' test for assessing insulin sensitivity and has been used in humans (DeFronzo et al 1979) and rats (Kraegen et al 1983). There is, however, no specific standard operating procedure for this test outlined in the literature. Factors that vary include the length of the test, amount of insulin infused, whether somatostatin is infused, if a supplemental KCl infusion is provided, and whether a

priming dose of insulin is used. Studies assessing reproducibility are lacking and those conducted were done days (Kraegen et al 1983; Soop et al 2000) or weeks (DeFronzo et al 1979; Bokemark et al 2000) apart. In comparison, the RIST is reproducible in up to four (Lautt et al 1998) or five (Xie et al 1996) consecutive tests, allowing for numerous paired interventions in the same experimental day. Since we were comparing the HIEC to the RIST, we used the same amount of insulin on a per minute basis to compare the two tests; we did not use a priming dose of insulin or somatostatin. The dose of insulin used, 10mU/kg/min, is not unusual. Many studies employ a much lower dose (1.67 mU/kg/min; 1 mU/kg/min) (Kraegen et al 1983; Moore et al 2002 respectively) or a higher dose (12 mU/kg/min) (Baron et al 1995). Since the effect of somatostatin on HISS release has not been determined it could not be used. Another point that can differ during HIEC protocols is where euglycemia is set. In the originally cited paper the protocol states that euglycemia is set at the subject's own mean glycemia (DeFronzo et al 1979), however, other studies arbitrarily pick 5 mmol/l as what euglycemia should be (Bonora et al 1989; Bokemark et al 2000). In hyperglycemic subjects plasma glucose levels must fall to this concentration before glucose infusions are even started (Bonora et al 1989).

The results indicate that there is a significant difference in insulin sensitivity between the control fed group and groups where HISS release was inhibited physiologically by fasting or pharmacologically using atropine or L-NMMA using the RIST, which agrees with previous studies (Lautt et al 2001; Takayama et al 1999; Xie and Lautt 1994; Sadri and Lautt 1998,1999). The same conclusion cannot be drawn when examining the glucose infusion rate during the HIEC since it does not differ

significantly between control and blocked conditions. Figure 2.4 presents the average dynamic curve of the HIEC in the control and HISS blocked states, and it is clear that the infusions are very similar. During the initial upslope of the curve the control HIEC curve has a higher GIR. As time progresses the blocked HIEC curve surpasses the control HIEC curve and during the last 30 minutes the GIR in the HISS blocked animals is actually higher than the GIR in the control animals. Although not significant, the infusion curves for the HIEC indicate that the HISS blocked group is slightly more sensitive than control. Since we were unable to correlate the RIST index to the HIEC GIR (figure 2.5) we concluded that the RIST index and HIEC GIR were not comparable.

Our results indicated that there was no difference in the GIR of an animal that should have full HISS action compared to one where HISS action was blocked. We designed another comparison of the two tests to examine if HISS release was possible after the HIEC. By doing a post-HIEC RIST we assessed if the animal had the same level of insulin sensitivity as it had prior to the HIEC. By administering atropine to completely block HISS release and then repeating the RIST, we assessed the extent of HISS action that remained in the post-HIEC RIST. Our results show (figure 2.6) that in a normal fed animal, insulin sensitivity is reduced 49% after the HIEC due to a full blockade of HISS release as shown by no further decline after atropine. The blocked (fasted) animals have low insulin sensitivity during the first RIST, as expected, and there was no significant decrease in insulin sensitivity after the HIEC or atropine infusion. This confirms that fasting decreased the HISS-dependent component of insulin action but not the HISS-independent (post-atropine) component. Similarly, use of the HIEC caused a blockade of HISS-release in the post-HIEC state but did not alter the HISS-independent

component of insulin action. Combined with the HIEC GIR data, which failed to show a difference between control and blocked animals, this indicates that by the end of the clamp the animal is in a state of HISS-dependent insulin resistance that extended beyond the test period.

The secondary analysis comparing the RIST to the HIEC was done under the premise that while HISS release may not occur by the end of the HIEC it should still occur at its onset, since the initial 5 minutes of the RIST are identical to that of the HIEC. The RIST index correlated with the upslope of the infusion rates obtained using the HIEC (figure 2.7), indicating that at this time HISS action was present, but by the end of the HIEC it was absent. Similar to these findings, Kingston et al (1986) found that when human subjects were given oral glucose loads (15-25 g) approximately 2 hours before an HIEC the slope of the GIR in the first 30 minutes was 1.5 times greater than persons not given the glucose. Since it is customary to analyze the HISS profile by subtracting blocked curves from control curves, this was done using the first thirty minutes of the HIEC data, since it was felt that HISS action might be present. Figure 2.8 shows a potential HISS profile that is similar in shape to that obtained by the RIST and ITT (figure 2.3), except that it has a later onset. This similar profile strengthens the idea that HISS action is present at the onset of the clamp.

Pentobarbital anesthesia has been shown to affect insulin sensitivity, and clamp studies reported in the literature often use conscious animals (Baron et al 1995; Kraegen et al 1983). Latour and Lautt (2002b) showed that there is no difference in the mean RIST index when conscious animals are compared to anesthetized animals. When the RIST and HIEC were compared in conscious animals the HIEC caused a 54% decrease in

insulin sensitivity (Reid et al 2002). The decrease in insulin sensitivity is similar to the decrease found in anesthetized animals, thus, the post HIEC resistance is not due to anesthesia.

The ITT is capable of determining HISS action (figures 2.2 and 2.3). We showed that the ITT was decreased 47% when repeated after the HIEC and atropine administration did not cause a further decrease in insulin sensitivity (figure 2.9). Both the RIST and ITT demonstrate that the HIEC produces HISS blockade resulting in HISS-dependent insulin resistance.

Contrary to the idea that use of the HIEC cannot detect HISS-dependent insulin resistance, Moore et al (2002) were able to show a 25 % decrease in insulin sensitivity caused by hepatic denervation in conscious dogs using the HIEC. The difference between our results and theirs could be due to the difference in animal model, the dose of insulin used (1 mU/kg/min in their study), or that they infused somatostatin into their dogs. It is possible that in the dog a 3-hour low dose insulin infusion is not as detrimental to insulin sensitivity as the higher dose in the rat. Since we do not know how somatostatin effects HISS action, its administration could account for the ability to detect HISS at the end of the HIEC in dogs. Baron et al (1995) was able to show a difference in the HIEC GIR between control rats and those treated with L-NMMA. However, in that study the investigators (Baron et al 1995) mixed insulin with blood from a littermate (prandial status not reported), and the basal glucose levels reported for the rats are higher than expected in fasted, unstressed rats. Either of these factors may have contributed to their results.

The mechanism by which the HIEC causes HISS-dependent insulin resistance in the rat is not yet known. Although levels of the counter-regulatory hormone norepinephrine were not measured in this study we have previously shown that there is no increase during the RIST (Xie and Lautt 1996a). Others (Anderson et al 1991; Rowe et al 1981) have shown an increase in norepinephrine during the HIEC. The effect of norepinephrine on HISS release is not known. Perhaps the nonphysiological delivery pattern of insulin blocks HISS release. Endogenous insulin release in pulsatile (Goodner et al 1977; Lang et al 1979), and while continuous insulin infusions in humans have produced insulin resistance (Marangou et al 1986), pulsatile infusions of the same duration have not (Ward et al 1990). Pulsatile insulin release and the effect of pulsatile and continuous insulin administration on HISS-dependent insulin action will be discussed further in chapters 3 and 4.

CHAPTER 3 – Introduction to Pulsatile Insulin Release

3.1 Normal physiology of pulsatile insulin release

Insulin release is pulsatile. This observation was first made in monkeys (Goodner et al 1977) and later in man (Lang et al 1979). Insulin secretion is also pulsatile in rats (Chou et al 1991). It is estimated that in the fasted state a minimum of 70% of all insulin is released in pulses (Porksen et al 1995), with a period of 10 – 15 minutes (Lang et al 1979). *In vitro* preparations of isolated human islets release insulin with a period of 6 (Song et al 2002) to 10 minutes (Marchetti et al 1994). Pulsatile insulin release allows for a 40% change in peripheral insulin concentration over 7 minutes (Matthews et al 1983b). The reported period of insulin release is not consistent within the literature. Some studies measure insulin in peripheral blood where small pulses could be missed and older studies use detection methods where sensitivity may have been low. Portal venous blood samples from dogs have 8 insulin pulses per hour (7.5 minutes/pulse) (Porksen et al 1995). Porksen et al (1997) reported a peripheral insulin interpulse interval (peak to peak) of 5 minutes when human subjects were given a low-dose glucose stimulus; they attributed their results to a high sampling frequency and more sensitive insulin assay. Song et al (2000) reported that both human portal venous and peripheral arterial blood samples have an insulin pulse frequency of 5 minutes; the insulin pulse mass and amplitude are severely dampened in the arterial samples. Reviewing the literature is difficult when measurement discrepancies exist, however, the basic principles still hold true and that is the focus of the data presented. A recent review by Porksen (2002) outlines some of the reported periods of insulin release, the methods of analysis used, and

sampling sites; he also provides an excellent discussion of pulse analysis methods which are not reviewed in this thesis.

Insulin profiles have shown that, along with high frequency pulses, insulin is released in an ultradian rhythm (Sonnenberg et al 1992). Ultradian is defined as more frequently than circadian, thus, ultradian oscillations occur more than once a day. In healthy individuals the ultradian oscillation period for insulin is 75 – 115 minutes, in both the fasted and fed state (Sonnenberg 1992). High frequency insulin pulses (5-15 minutes) are superimposed on the low frequency oscillations (75-115 minutes) (Sonnenberg et al 1992).

3.2 Control of insulin pulsatility

3.2.1 Rapid pulses (5-15 minutes)

The mechanism of pulsatile insulin release is poorly understood even though it has been greatly explored. Insulin, glucagon and somatostatin are released in bursts from the perfused pancreas (Stagner et al 1980). Isolated islet preparations also demonstrate pulsatile insulin and glucagon release (Opara and Go 1991). Blocking sodium channels within the islets with tetrodotoxin causes an increase in the frequency of insulin but not glucagon release (Opara and Go 1991). Opara and Go (1991) concluded that their results suggested an intrinsic pacemaker for insulin producing beta cells, which was different from that of alpha cells. Porksen et al (1994) demonstrated that intraportally transplanted islets could secrete insulin, but reinnervation of the islet is required for coordinated pulsatile insulin secretion. Coupling of adjacent beta cells through gap junctions perhaps allows certain cells within the islet to be pacemaker cells that initiate synchronized insulin release (Meissner 1976).

In vitro pancreatic preparations, with intact intra-pancreatic ganglia, show that insulin release occurs without a change in neural membrane potential (Sha et al 2001). However, the amplitude of insulin release increases when the nerves are stimulated (Sha et al 2001). This indicates that neural activity, while not the generator of the pulses, may serve to regulate and modify the amount of insulin released. Considering that pulsatile insulin release occurs regardless of feeding or fasting (Polonsky et al 1988), it is probable that insulin release can occur without a neural stimulus, as in fasting, but neural stimulation, during feeding (Strubbe and Steffens 1993; Benthem et al 2000), could serve to increase the pulse amplitude. Fasting has no effect on the period of insulin release but does decrease the amplitude by 50% (Juhl et al 2002). In healthy control subjects, atropine has no effect on the period or amplitude of insulin release while propranolol decreases the amplitude but does not affect the period (Matthews et al 1983b). These results suggest that, the sympathetic branch of the autonomic nervous system helps to regulate insulin release. Naloxone, phentolamine, and glucose increase the amplitude but have no effect on the period of insulin release (Matthews et al 1983b). Patients with truncal vagotomy have a pulse period of 33 minutes so even though cholinergic blockade did not affect pulsatile insulin release the vagus nerve plays an important role in insulin pulsatility (Matthews et al 1983b). Neural presence is important, but not necessary, for pulsatile insulin release.

Rapid insulin pulses lag glucose fluctuations by two minutes (Lang et al 1979), and pulsatile glucose infusions can be used to entrain insulin release in healthy subjects (Hollingdal et al 2000). Glucose is the most potent stimulant of insulin release, but others also exist, such as, arginine, other amino acids, and other sugars. One theory is

that oscillatory glucose metabolism within the beta cell is responsible for pulsatile insulin release. In a review, Tornheim (1997) explains that glycolysis within the beta cell is oscillatory, therefore, the production of ATP is oscillatory. Fluctuating ATP levels cause opening and closing of ATP gated potassium channels within the beta cell. Closing the channel causes membrane depolarization, an influx of calcium, and a release of insulin; this pathway is potassium and calcium dependent. Insulin release is pulsatile, *in vitro,* in the absence of calcium (Aizawa et al 2000), indicating that potassium channel-dependent fluctuations in calcium are not the only mediator of pulsatile insulin release. In a review by Komatsu et al (1997) potassium-dependent, calcium-independent insulin release and potassium-calcium-independent insulin release are discussed. The potassium-calcium-independent pathway of insulin release is reported to be mediated through GTP and initialized by vagal and incretin stimulation (Komatsu et al 1997). Further exploration of this pathway could shed light onto the role of neural stimulation in pulsatile insulin release.

3.2.2 Ultradian oscillations (75 – 115 minutes)

Ultradian oscillations, compared to rapid pulses, are different in control and origin. Sonnenberg et al (1992) suggested that, since ultradian insulin oscillations have a similar frequency to pituitary hormones they might use a common pulse generator; this has not been proven. Oscillations occur during fasting (Sturis et al 1992), in the prescence of enteral nutrition (Simon et al 1987), and during a glucose stimulus (Sturis et al 1993). Ultradian insulin oscillations are concomitant with C-peptide oscillations, indicating that their appearance is due to a release, rather than a clearance, pattern (Simon et al 1987). Patients with pancreatic transplant are used to demonstrate that insulin oscillations occur

without extrinsic innervation, however, the amplitude of insulin release is much higher compared to healthy control subjects (Sonnenberg et al 1992). This suggests that neural stimuli may serve to regulate ultradian insulin release. Ultradian oscillations are not related to glucagon or cortisol fluctuations but possibly to glucose fluctuations, since 85% of glucose oscillations are coupled to an insulin oscillation (Shapiro et al 1988). While insulin oscillations can be entrained by oscillatory glucose (Sturis et al 1991), both rapid pulses and slow insulin oscillations are independent of a constant glucose stimulus (Sturis et al 1993). In response to a continuous glucose stimulus the amplitude of the ultradian oscillation increases more than the amplitude of the rapid insulin pulse in the peripheral circulation (Sturis et al 1993). There is a stronger relationship between ultradian oscillations and glucose oscillations compared to rapid pulses and glucose pulses (Sturis et al 1993). These observations led to the hypothesis that rapid pulses (5-15 minutes) are, to a greater extent, cleared by the liver and are most important for controlling hepatic glucose output, whereas the ultradian oscillations may be more important for peripheral glucose disposal (Sturis et al 1993). Marsh et al (1986) showed that a constant glucose infusion, 10.18 mg/kg/min, triggered less of an insulin response than a hyperglycemic clamp, even though the target blood glucose level for the clamp (144 mg/dl) was the same as that seen with the constant glucose infusion (142 mg/dl). While total glucose uptake did not differ between the constant glucose infusion and the glucose clamp, the amount of glucose taken up per unit insulin concentration in the blood was much higher for the constant glucose infusion. Marsh et al (1986) suggested that, based on insulin and glucose profiles, the constant glucose infusion permitted endogenous insulin and circulating glucose levels to oscillate but the hyperglycemic

clamp did not. Every time glucose tended to oscillate the glucose infusion rate was increased and this prevented insulin from oscillating. They (Marsh et al 1986) concluded that insulin and glucose oscillations were necessary for efficient glucose uptake, and glucose oscillations cause negative feedback to the pancreas to prevent hyperinsulinemia.

3.3 Pulsatile insulin release and insulin sensitivity

3.3.1 People with type 2 diabetes

People with type 2 diabetes have altered pulsatile insulin release. The insulin pulse period is reduced in diabetic patients (Lang et al 1981; Zarkovic et al 1999) and the amount of insulin released in response to glucose is decreased (Hollingdal et al 2000). There is, in fact, a significant relationship between increasing pulse frequency and decreasing insulin sensitivity (Hunter et al 1996). Hunter et al (1996) proposed that an abnormal pulse frequency induces receptor and post receptor defects. This remains to be studied. Contrary to these results, Laedtke et al (2000) reported no difference in the pulse period between diabetic patients and control subjects.

Rapid insulin pulses can be entrained by pulsatile glucose infusions in healthy subjects but not in people with type 2 diabetes (Hollingdal et al 2000). Glucose pulses can be used to entrain insulin pulses in the isolated perfused pancreas from normal healthy rats but not Zucker Diabetic Fatty rats (Sturis et al 1994). Ultradian insulin oscillations become uncoupled from glucose oscillations in diabetic patients, as shown by a decrease in glucose but not insulin oscillations (Sturis et al 1993). Ultradian insulin oscillations can be entrained with oscillatory glucose (96 or 144 minute period) infusions in healthy people, but not in patients with impaired glucose tolerance or type 2 diabetes (O'Meara et al 1993).

In an attempt to clarify whether reduced beta cell mass is responsible for impaired pulsatile insulin release, Kjems et al (2001) experimentally reduced the beta cell mass, in the minipig and assessed insulin secretion. They found that the amplitude of the insulin pulse was reduced but not the frequency. Hepatic extraction of insulin was also reduced and glucagon levels were not suppressed by the ensuing hyperglycemia (Kjems et al 2001). Thus, the irregular pattern of insulin release seen in diabetic patients is not due to reduced beta cell mass.

Sulfonylureas are a class of drugs prescribed to control blood glucose levels in people with type 2 diabetes. Their mechanism of action involves blocking ATP-dependent potassium channels in the pancreas thereby causing insulin release. Whether this class of drugs affects insulin pulsatility has been examined, Matthews et al (1983b) found that in healthy subjects tolbutamide increased the amplitude of insulin release with no effect on the period. Gliclazide was shown to increase both basal and pulsatile insulin release with no effect on the period of release in people with type 2 diabetes (Juhl et al 2001). During a five week administration of gliclazide a relationship between improved glycemic control and improved insulin pattern regularity was observed (Juhl et al 2001). Sulfonylurea drugs may not be able to change the period of insulin release but they may enhance its orderliness.

3.3.2 Relatives of patients with type 2 diabetes

Are there early markers for developing type 2 diabetes? O'Rahilly et al (1986) tested normoglycemic first-degree relatives of type 2 diabetic patients and found that 20% had impaired glucose tolerance that could be related to reduced beta cell function. Fernandez-Castaner et al (1996) reported that first-degree relatives showed beta cell

dysfunction whether they were glucose tolerant or intolerant and the dysfunction progressed with age. The abnormalities observed in glucose tolerant relatives are related to the severity of family history (i.e. two diabetic parents vs. one) (Nyholm et al 2000). Superficially, the insulin pulse frequency appears normal in glucose tolerant, first-degree relatives (Nyholm et al 2000), however, the pattern of pulsatile insulin release is irregular and nonstationary (Schmitz et al 1997; Nyholm et al 2000). First-degree relatives with mild glucose intolerance do not show regular insulin pulses either (O'Rahilly et al 1988). Irregular insulin release could be an early warning sign in the development of a blatantly abnormal period of insulin release and diabetes.

3.3.3 Insulin pulsatility in relation to other conditions

Since diabetes is associated with hypertension, age, and obesity, many studies have evaluated how insulin pulsatility is affected by these conditions. Wiggam et al (2000) found that there was no significant difference in pulse frequency between hypertensive patients and healthy individuals. However, pulse frequency could be related to insulin sensitivity in healthy people but not in hypertensive patients.

Obesity is another condition that can be associated with abnormal pulsatile insulin release. Zarkovic et al (2000) found that weight loss in obese, non-diabetic subjects prolonged the interpulse interval of insulin release. Accompanying the decrease in pulse frequency was a decrease in circulating insulin and glucose, and an increase in insulin sensitivity. Obese, diabetic patients, however, have irregular insulin pulses that cannot be corrected with weight loss (Gumbiner et al 1996). Ultradian insulin oscillations appear normal and still respond to glucose entrainment in obese non-diabetic subjects (O'Meara et al 1993).

Insulin secretion and pulse regularity are reduced in healthy, non-obese, aged people compared to healthy, non-obese, young people (Meneilly et al 1999). The interpulse interval is slightly higher in the aged group, yet there is no statistical difference in rapid pulse frequency (Meneilly et al 1999). Aging is associated with a decrease in ultradian insulin oscillation frequency (Meneilly et al 1997). Thus aging, unlike diabetes, is associated with a slowing of insulin pulses and oscillations.

3.4 Efficacy and effects of pulsatile versus continuous insulin infusions

Following the observation that insulin release was pulsatile, a number of studies were performed evaluating if pulsatile, rather than continuous, insulin delivery would be advantageous. Matthews et al (1983a) found that, in the fasted state, insulin delivered in pulses had a greater hypoglycemic effect compared to a continuous delivery. The difference in glycemia, however, was seen only after 7 hours of insulin infusion. Matthews et al (1983a) did not infuse glucose to maintain euglycemia; instead they infused somatostatin to inhibit endogenous insulin and glucagon to maintain hepatic glucose output. Therefore, glycemic differences were not influenced by large, abnormal quantities of exogenous glucose or the resultant glycogen synthesis. Schmitz et al (1986) found that more glucose was required to maintain euglycemia during a 354 minute pulsatile, rather than continuous, insulin infusion. Paolisso et al (1988b) found that, in people with type 2 diabetes, the glucose infusion rate was higher by the end of a 325-minute pulsatile, compared to continuous, insulin infusion. Verdin et al (1984), in contrast, did not find a difference in glucose uptake between the insulin delivery patterns, but the test period was only 4 hours. Ward et al (1989) failed to show a difference in glucose uptake during a 2 hour continuous versus pulsatile insulin infusion.

A potential mechanism for the increased efficiency of pulsatile delivery is a greater inhibitory effect on the pancreas. Asplin et al (1981) showed that when exogenous insulin was used to inhibit endogenous insulin secretion, arginine-stimulated glucagon release was increased in healthy people and decreased in patients with type 1 diabetes. Asplin et al (1981) concluded that their results indicated that beta cells suppress alpha cells via a paracrine mechanism, and when healthy beta cells are suppressed during an exogenous insulin infusion they cannot suppress alpha cells. Paolisso et al (1988a) extended Asplin's experiment to include both continuous and pulsatile insulin infusions. In healthy subjects, pulsatile insulin infusions decreased plasma glucagon and C-peptide levels to a greater extent than continuous insulin infusions. The arginine-stimulated glucagon response was greater after a pulsatile insulin infusion indicating greater beta cell suppression (Paolisso et al 1988a). In contrast to the earlier report (Asplin et al 1981), patients with type 1 diabetes showed a decrease in arginine-stimulated glucagon release after pulsatile and not continuous insulin infusions (Paolisso et al 1988a). Paolisso et al (1988a) concluded that pulsatile insulin administration was more effective at inhibiting the alpha cell. Ward et al (1989) showed that pulsatile insulin infusions cause greater inhibition of endogenous insulin, as assessed by C-peptide levels, and prolonged suppression of glucagon, compared to continuous insulin infusion. Pulsatile insulin administration also causes greater inhibition of hepatic glucose output compared to continuous insulin administration (Paolisso et al 1991). The frequency of insulin pulses is important since pulses administered 26 minutes apart do not suppress hepatic glucose output to the same degree as pulses given 13 minutes apart (Paolisso et al 1991). Even

ultradian oscillations (120 minutes) are more effective than continuous insulin infusions in promoting glucose uptake (Sturis et al 1995).

Aside from glycemic effects, pulsatile insulin infusions may have other advantages. Paolisso et al (1988b) showed that in people with type 2 diabetes, pulsatile insulin delivery caused a significant decrease in plasma triglycerides and very-low-density lipoproteins and an increase in high-density lipoprotein. Considering that dyslipidemia is often associated with diabetes (Goldberg 2001), an alteration in pulsatile insulin release, as seen in diabetic subjects, could be associated with some of the lipid abnormalities. The progression of nephropathy is also decreased when patients with type 1 diabetes are given weekly, 1-hour pulsatile insulin infusion treatments, along with their normal intensive insulin therapy (Dailey et al 2000).

A study by Koopmans et al (1996) tested, in streptozotocin treated rats, whether physiological pulsatile insulin infusion had any advantages over continuous insulin infusion. They monitored insulin secretion in response to feeding and fasting before streptozotocin treatment. Following treatment, they simulated normal insulin concentrations for 2 weeks by administering insulin in pulses or continuously using programmable insulin pumps. They (Koopmans et al 1996) found that plasma glucose levels were 50% lower in the rats given insulin in pulses, and glucose loss in the urine was $1/5^{th}$ that of the rats given a continuous insulin infusion. After the 2 week period they assessed insulin sensitivity with a four-step HIEC and found that the rats given insulin continuously showed insulin resistance two clamp levels (3 and 48mU/kg/min). The rats given insulin in pulses only showed resistance in the low dose (3 mU/kg/min) HIEC. This indicates that pulsatile insulin administration is more effective than

continuous, and programmable pumps should not use continuous delivery systems, since it appears that continuous insulin infusions result in reduced insulin sensitivity.

3.5 Hypothesis tested and rationale

Considering that normal insulin release appears to be pulsatile, we hypothesized that the insulin resistance observed after the use of the HIEC (as described in chapter 2) was due to the continuous delivery during the test, and that pulsatile insulin delivery would prevent HISS-dependent insulin resistance from developing. Further, we hypothesized that, since insulin release is pulsatile, HISS would be more responsive to a pulsatile, rather than continuous insulin delivery pattern, and that glucose uptake would be greater in response to a pulsatile, rather than continuous insulin infusion.

CHAPTER 4 – The Effect of Insulin Delivery Pattern on Glucose Uptake and Insulin Sensitivity

4.1 Introduction:

Endogenous insulin release is pulsatile (Goodner et al 1977; Lang et al 1979). Pulsatile hormone secretion is not unique to the pancreas, since many hormones, including gonadotropin-releasing hormone (GnRH), are released in pulses. When GnRH is administered continuously, rather than in pulses, it is ineffective at stimulating leutenizing hormone (LH) and follicle stimulating hormone (FSH) (Belchetz et al 1978). Pulsatile insulin secretion, likewise, is required for proper glucose homeostasis. Diabetic subjects and their close relatives, although asymptomatic, show aberrations in insulin release patterns (Zarkovic et al 1999; Schmitz et al 1997; O'Rahilly et al 1988). Thus, the loss of proper pulsatile insulin release could contribute to the progression of type 2 diabetes.

Previous experiments have shown that use of the HIEC causes HISS-dependent insulin resistance (Chapter 2), possibly because it employs a continuous rather than pulsatile insulin infusion. We, therefore, hypothesized that pulsatile insulin delivery would cause greater glucose uptake and HISS release compared to a continuous delivery, and continuous, but not pulsatile or bolus insulin infusions, would induce HISS-dependent insulin resistance. The results from the current study failed to show a difference in glucose uptake between continuous and pulsatile insulin infusions. The results did, however, show that a continuous insulin infusion caused full HISS-dependent insulin resistance while pulsatile and bolus infusions did not.

4.2 Methods:

4.2.1 Comparison of the efficacy of three delivery methods

Male SD rats were fasted for 8 hours and fed for 2 hours prior to surgical preparation to standardize prandial status and ensure a high degree of HISS release (Lautt et al 2001). Animals underwent surgical preparation as previously described and were allowed to stabilize for 30 minutes. Glycemia was determined using a glucose analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). Once basal glycemia was established the animals were treated in one of three ways. One treatment involved a 3-pulse infusion of insulin where 20 mU/kg of insulin was delivered over 1 minute (0.136 ml/min) at times 0, 20 and 40 minutes (n=8). Another treatment involved a 6-pulse infusion of insulin where 10 mU/kg of insulin was delivered over 1 minute (0.136 ml/min) at times 0, 10, 20, 30, 40 and 50 minutes (n=8). The third treatment was a continuous 1 mU/kg/min insulin infusion for 60 minutes (n=8). In each case, the animal received 60 mU/kg of insulin during the test. One minute into the test a variable glucose infusion was initiated (5 mg/kg/min for the 3 and 6 pulse tests and 2 mg/kg/min for the continuous test) and a blood sample was taken and analyzed for blood glucose concentration. Blood samples were continued every 2 minutes and the glucose infusion was adjusted to maintain euglycemia. When all the insulin was infused and the animal no longer needed the glucose infusion to maintain euglycemia the test was complete. All animals then received atropine (3 mg/kg i.v. over 5 minutes). After a brief rest period the basal glucose levels were established and the test was repeated.

4.2.2 Effect of a continuous insulin infusion on insulin sensitivity

Rats in this series (n=6) were fasted for 8 hours and fed for 2 hours prior to surgical preparation. Each animal was prepared surgically, as previously described, and allowed to rest for 30 minutes. The basal glycemia was determined and a RIST (50 mU/kg insulin, as previously described) was performed to establish insulin sensitivity. The animal was stabilized and underwent a continuous (1 mU/kg/min) insulin infusion for 60 minutes. Blood samples were taken to monitor glucose levels and a variable glucose infusion was adjusted to maintain euglycemia. After the test the animal was allowed a brief rest period and basal glycemia was determined. A second RIST was performed to assess the effect of the continuous infusion on insulin sensitivity, followed by an atropine infusion (3 mg/kg i.v. over 5 minutes). A third RIST was conducted to assess insulin sensitivity after atropine.

4.2.3 Effect of three insulin pulses given 20 minutes apart on insulin sensitivity

In this series, rats (n=7) were fasted for 8 hours, fed for 2 hours, and prepared surgically as previously described. Basal glycemia was established and a control RIST was performed to assess insulin sensitivity. The animal was restabilized and underwent a 3-pulse infusion where 20 mU/kg bolus of insulin was infused over 1 minute every 20 minutes (0, 20, 40), until three pulses were given. The animal received 60 mU/kg of insulin in total. Euglycemia was assessed by blood samples taken every 2 minutes and maintained with a variable glucose infusion. A second RIST was performed to assess the effect of the pulsatile insulin infusion on insulin sensitivity, followed by atropine (3 mg/kg i.v. over 5 minutes) and a post-atropine RIST.

4.2.4 Effect of three insulin pulses given according to glucose uptake on insulin sensitivity

In this series, rats (n=7) were fasted for 8 hours and fed for 2 hours and prepared surgically. Basal glycemia was established and a RIST was done to assess insulin sensitivity. The second test was a 3-pulse insulin infusion similar to the test previously described, but instead of the boluses being given 20 minutes apart the boluses were given when the action of the previous bolus had ceased. Once the glucose infusion could be held at 0 mg/kg/min for 4 minutes, the second and third boluses were administered. Thus, no arbitrary time pattern was followed. A second RIST was conducted, followed by atropine (3 mg/kg i.v. over 5 minutes) and a post atropine RIST.

4.2.5 Effect of a bolus insulin infusion on insulin sensitivity

Animals (n=6) were fasted for 8 hours and fed for 2 prior to surgical preparation. A RIST was performed to assess initial insulin sensitivity in the animal. In the second test 60mU/kg of insulin was infused over 6 minutes, and euglycemia was maintained with a variable glucose infusion. After a rest period and stabilization a second RIST was performed, followed by atropine (3 mg/kg i.v. over 5 minutes). A third RIST was done once the basal glycemia was determined.

4.2.6 Effect of various durations of continuous insulin infusion on insulin sensitivity

In this series, animals (n=15) were fasted for 8 hours, fed for 2 hours and prepared surgically. Basal glycemia was established and a RIST was conducted to assess insulin sensitivity. In the second test, insulin was infused, 1 mU/kg/min, for 10, 15, 20, 25, or 30 minutes while euglycemia was maintained with a variable glucose infusion. The

animal was stabilized and a second RIST was conducted to assess how the infusion affected insulin sensitivity. Atropine was then infused (3 mg/kg i.v.) and a post atropine RIST was conducted after the animal had stabilized.

4.2.7 Drugs

Human insulin (Humulin R) was purchased from Eli Lilly and Company (Toronto, Canada). Atropine and D-glucose were purchased from Sigma chemical Co. (St. Louis, Mo). All drugs were dissolved in saline.

4.2.8 Data analysis

Data were analyzed using regular and repeated-measures analysis of variance followed by the Tukey-Kramer multiple comparison test or, when applicable, the paired Student's t-test was used. Correlation and regression analysis were also used. The analyzed data were expressed as means and ± SE throughout. Differences were accepted as significantly different at $P<0.05$. Animals were treated according to the guidelines of the Canadian Council on Animal Care, and an ethics committee on animal care at the University of Manitoba approved all protocols.

4.3 Results

4.3.1 Efficacy of different delivery methods

The mean glucose uptake, for animals (275.7 ± 6.0 g) given three 20 mU/kg pulses of insulin (n=8), was 458.0 ± 34.4 mg/kg before atropine and 162.2 ± 15.6 mg/kg after atropine, a 64.6 % difference. Dynamic profiles of glucose uptake are created by plotting the glucose infusion rate, at 0.1-minute intervals, in response to insulin. A dynamic profile of glucose uptake for the two tests is presented in figure 4.1. The mean glucose uptake, for the animals (274.6 ± 7.1 g) given six 10 mU/kg pulses of insulin

(n=8), was 374.1 ± 31.2 mg/kg before atropine and 180.8 ± 22.6 mg/kg after atropine, a 51.7% difference. A dynamic curve of the glucose infusion is presented in figure 4.2. The mean glucose uptake, for the animals (269.1 ± 5.2 g) given a continuous insulin infusion (n=8), was 433.6 ± 52.8 mg/kg before atropine and 190.3 ± 29.7 mg/kg after atropine, a 56.1% difference. A dynamic curve of the glucose infusion is presented in figure 4.3. The difference in glucose uptake between the three groups in the control state was not significant. While each test was significantly higher in the control test compared to post-atropine test, there were no significant differences when post-atropine tests were compared to each other. Subtracting the HISS blocked glucose infusion from the control glucose infusion gives a dynamic profile of HISS action, which is presented in figure 4.4. There was no difference in the quantity of HISS-dependent insulin action among the tests.

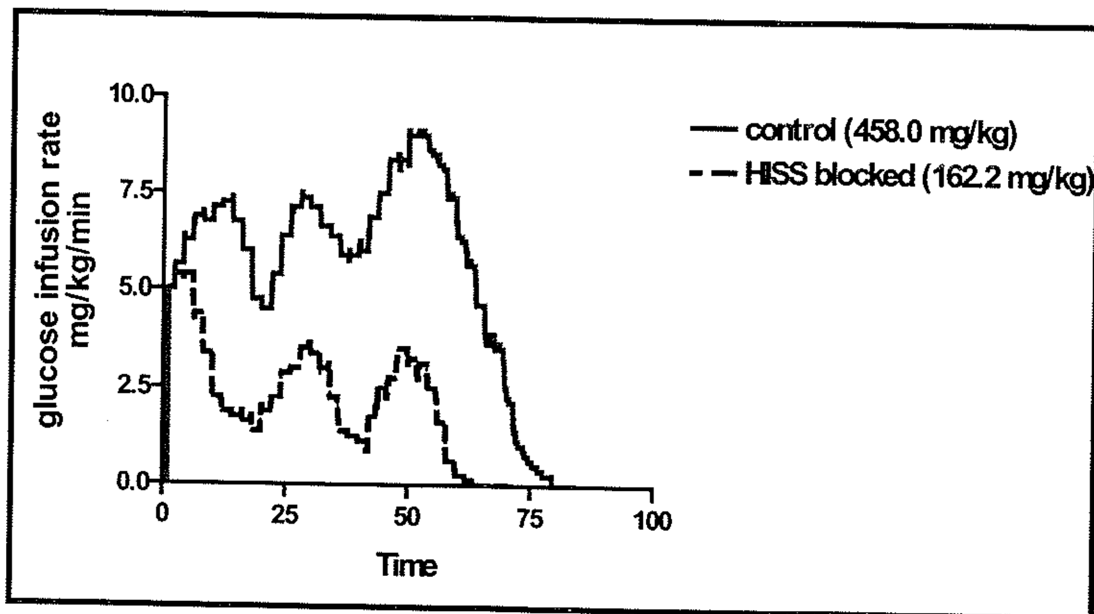

Figure 4.1: Glucose uptake, plotted in 0.1-minute intervals, for three pulses of insulin administered in the control and HISS blocked condition.

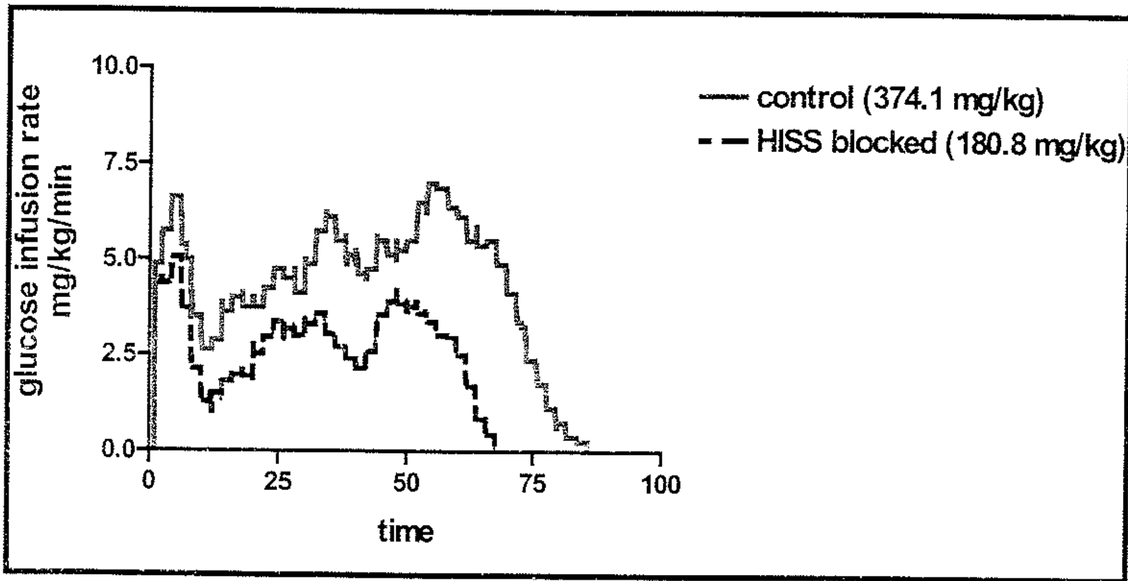

Figure 4.2: Glucose uptake, plotted in 0.1-minute intervals, for 6 pulses of insulin in the control and HISS blocked condition.

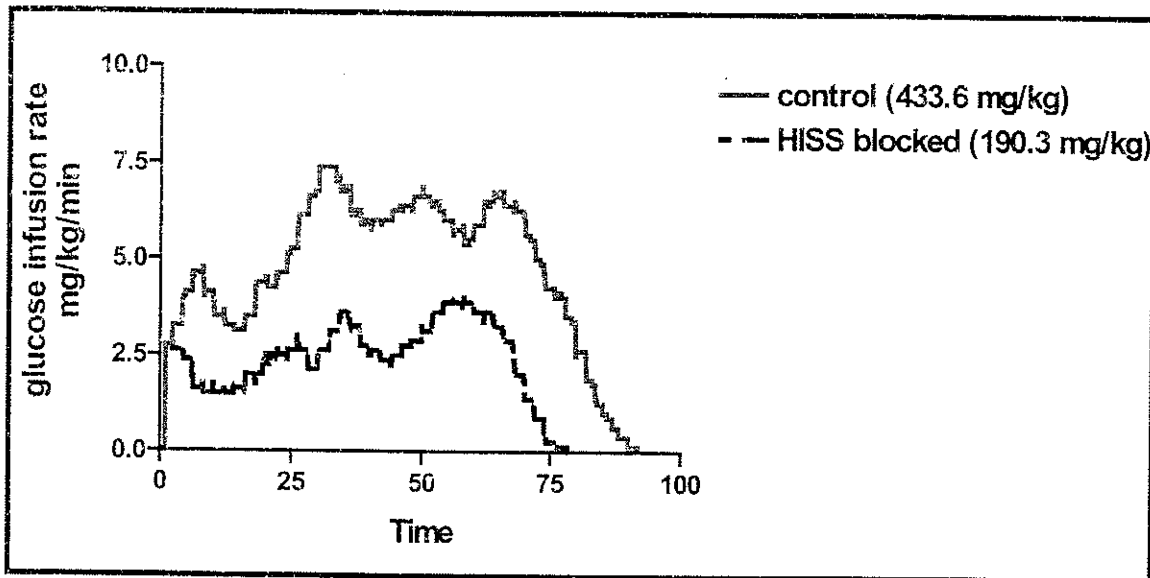

Figure 4.3: Glucose uptake, plotted in 0.1-minute intervals, during a continuous insulin infusion in both the control and HISS blocked condition.

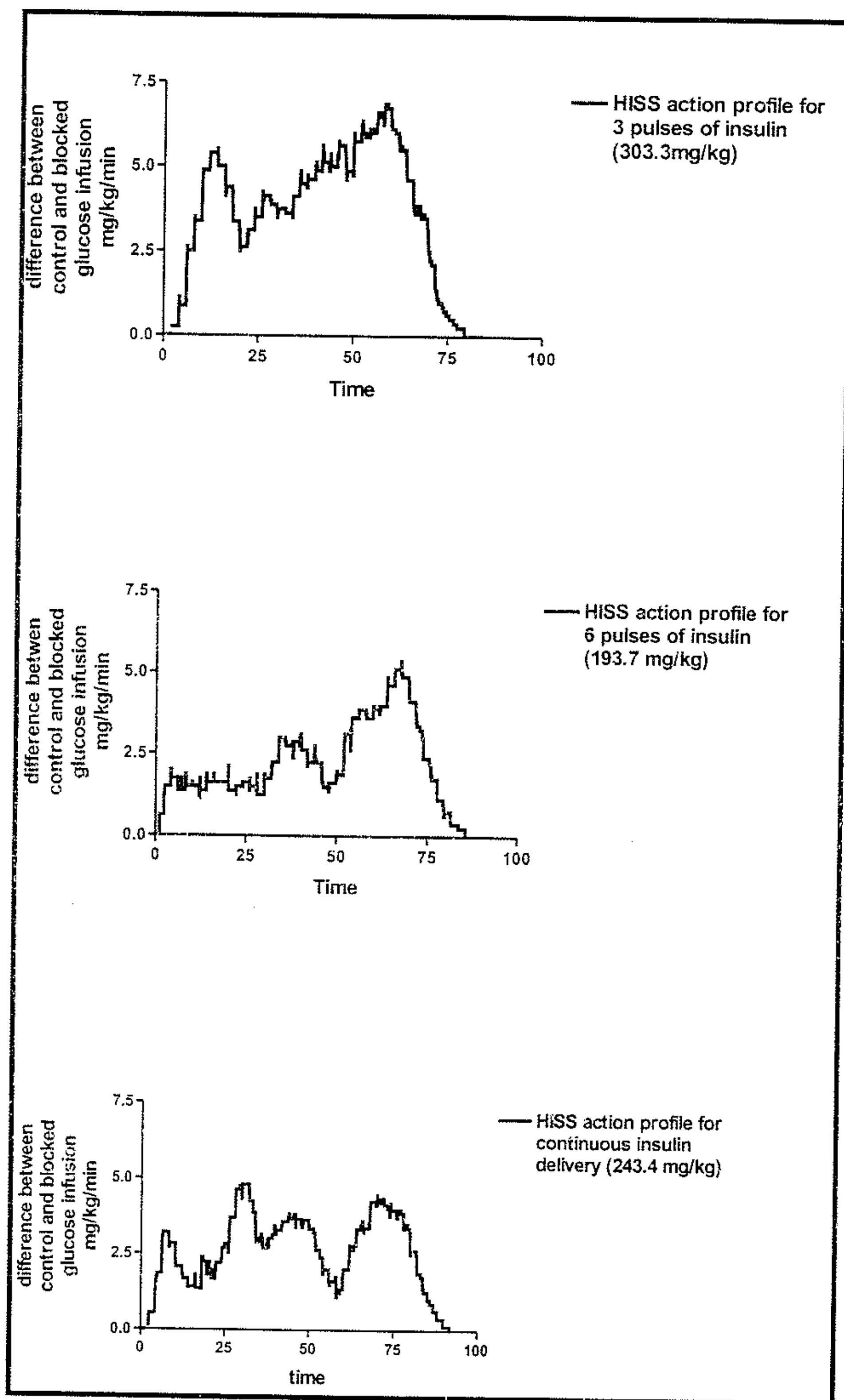

Figure 4.4: HISS action profiles, calculated by subtracting glucose uptake in the blocked condition from control, in response to different insulin delivery patterns.

4.3.2 Effect of continuous insulin delivery on insulin sensitivity

Animals (302.3 ± 9.9 g; n=6) in this series had an initial RIST index of 247.9 ± 23.3 mg/kg, a post-continuous infusion RIST index of 73.2 ± 5.0 mg/kg and a post-atropine RIST of 72.8 ± 4.5 mg/kg. The decrease, 70.5%, in insulin sensitivity between the control and post-continuous infusion RIST is significant (P<0.001), and is represented graphically in figure 4.5. The continuous infusion, 1 mU/kg/min for 60 minutes, yielded a glucose uptake of 351.0 ± 42.8 mg/kg, in 77.2 ± 1.7 minutes.

4.3.3 Effect of three insulin pulses given 20 minutes apart on insulin sensitivity

Animals (276.3 ± 3.4 g; n=7) in this series had an initial RIST index of 219.1 ± 22.9 mg/kg, a post-pulse RIST index of 145.9 ± 7.3 mg/kg and a post-atropine RIST of 71.0 ± 4.4 mg/kg. The decrease, 33.4%, in insulin sensitivity between the initial and post-pulse RIST is significant (P<0.01). The 51.3% decrease in insulin sensitivity between the post-pulse and post-atropine RIST is also significant (P<0.001). This data is represented in figure 4.6. The three pulse insulin infusion, with pulses given 20 minutes apart, produced a glucose uptake of 287.9 ± 14.5 mg/kg, in 66.1 ± 2.0 minutes.

4.3.4 Effect of three insulin pulses given according to glucose uptake on insulin sensitivity

Animals (284.7 ±6.6 g; n=7) in this series had an initial RIST index of 211.6 ± 7.8 mg/kg, a post-pulse RIST index of 191.1 ± 8.9 mg/kg and a post-atropine RIST index of 78.1 ± 3.9 mg/kg. There is no significant difference between the first two RISTs, only after atropine is there a significant (P<0.001) decrease of 63% in insulin sensitivity. Figure 4.7 shows these results graphically. The three pulse insulin infusion, where each

pulse was given after the glucose infusion rate could be maintained at 0 mg/kg/min, caused a glucose uptake of 297.4 ± 17.1 mg/kg, in 71.42 ± 1.6 minutes.

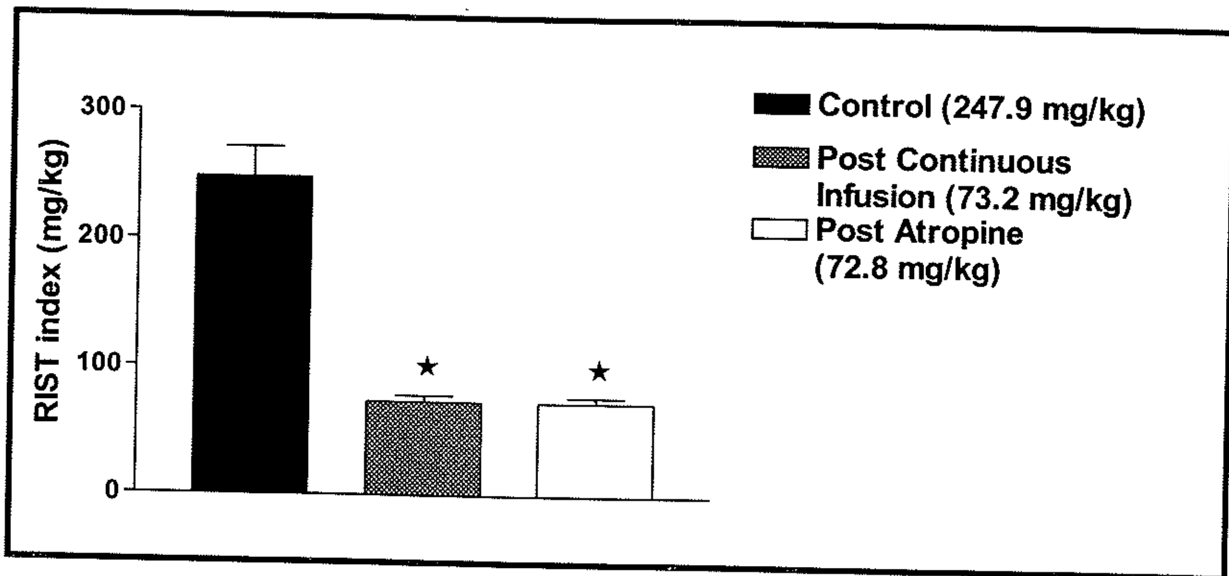

Figure 4.5: Effect of a continuous insulin infusion on insulin sensitivity. There is a 70.5 % decrease (P<0.001) in insulin sensitivity after the continuous insulin infusion that is not changed by atropine. The results indicate that a 60-minute continuous infusion of insulin causes full HISS-dependent insulin resistance.

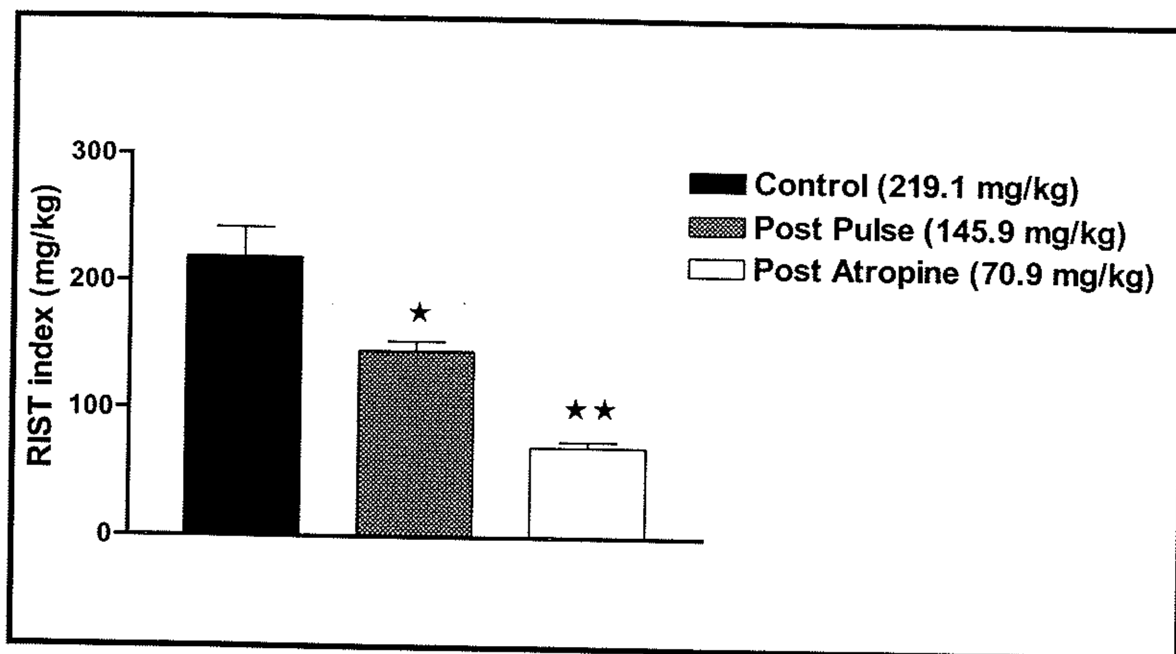

Figure 4.6: Effect of three pulses of insulin, given 20 minutes apart, on insulin sensitivity. There is a 33.4 % inhibition (P<0.01) in insulin sensitivity after the three pulse insulin infusion and a further 51.3% decrease (P<0.001) in insulin sensitivity after atropine. The results indicate that the three pulses delivered 20 minutes apart cause partial blockade of HISS release, but not full HISS-dependent insulin resistance.

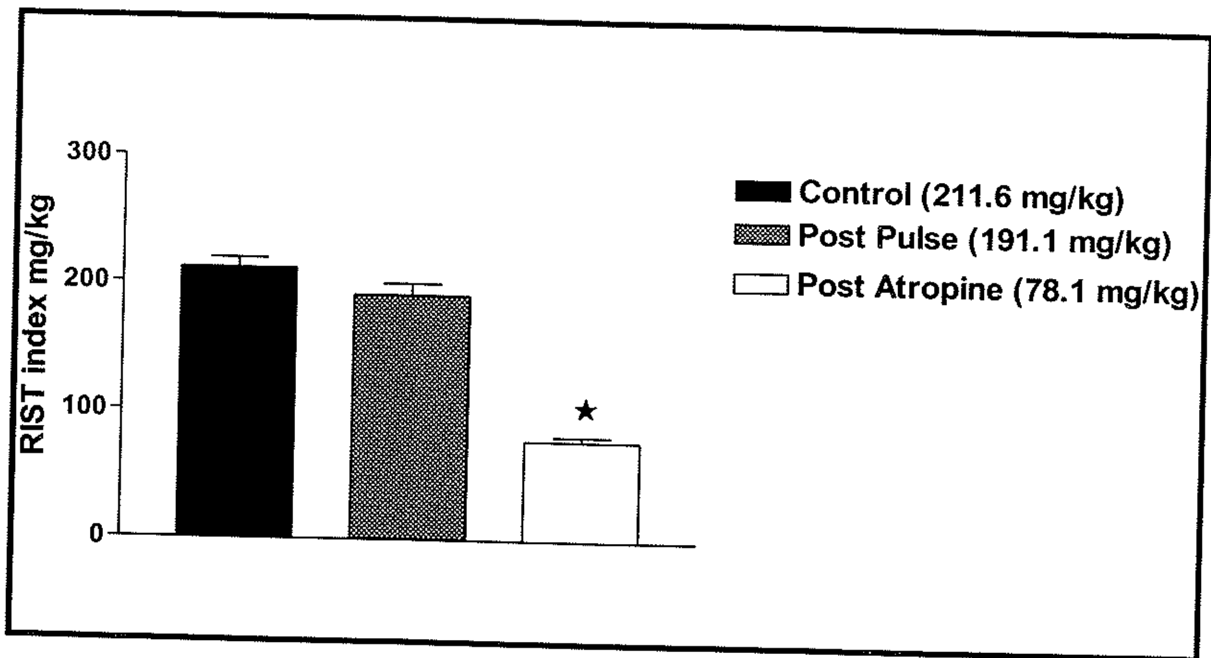

Figure 4.7: Effect of three pulses of insulin, given after the previous pulse's action is finished, on insulin sensitivity. There is no change in insulin sensitivity when insulin is applied after the previous pulse has finished acting. Only after atropine is there a 63% decrease ($P<0.001$) in insulin sensitivity. The results indicate that there is no blockade of HISS release when insulin pulses are delivered after the previous insulin pulse has finished acting.

4.3.5 Effect of a bolus insulin infusion

Animals (290.2 ± 2.9 g) in this series had an initial RIST index of 205.8 ± 8.2 mg/kg, a post-bolus RIST of 189.9 ± 7.8 mg/kg, and a post-atropine RIST of 71.9 ± 4.0 mg/kg. There is no difference in insulin sensitivity between the first two RISTs, only after atropine is there a significant 65% decrease (P<0.001) in insulin sensitivity. This data is presented graphically in figure 4.8. The 60 mU/kg bolus of insulin caused a glucose uptake of 233.4 ± 8.9 mg/kg, in 32.1 ± 1.4 minutes.

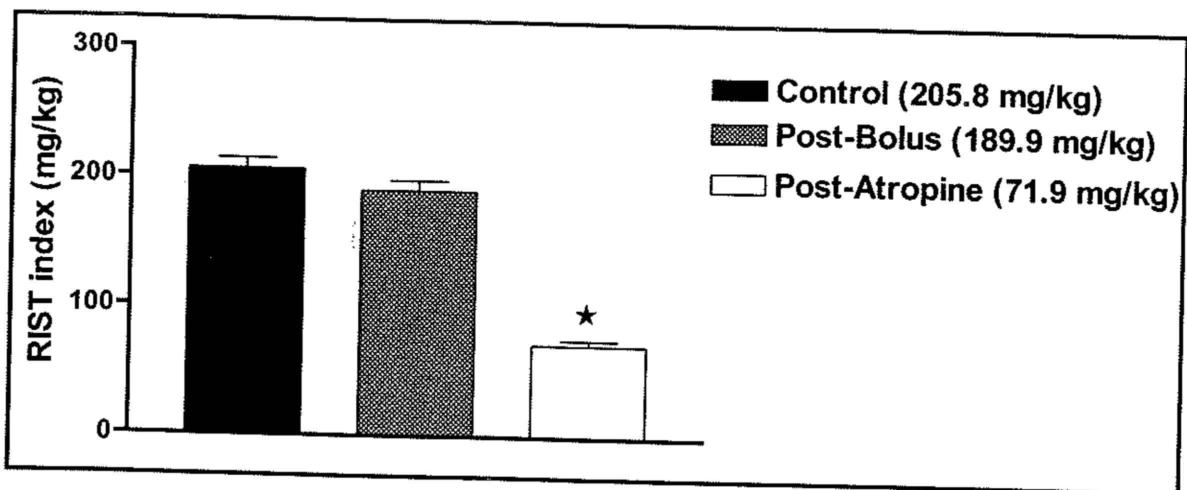

Figure 4.8: Effect of a bolus of insulin on insulin sensitivity. The RIST index is not different before and after the 60 mU/kg insulin bolus, only after atropine is insulin sensitivity reduced (65%) significantly (P<0.001). A bolus of insulin similar to that given during the RIST does not induce HDIR.

4.3.6 Effect of different durations of continuous insulin infusion on insulin sensitivity

The mean initial RIST index, for all animals (272.3 ± 4.0 g; n=15) in this series, was 184.1 ± 4.6 mg/kg and the mean post-atropine RIST index was 71.5 ± 2.0 mg/kg. Table 4.1 shows the mean post-continuous infusion RIST index for duration of continuous insulin infusion and the glucose infused in response to each of the durations. Significant differences exist between the mean control RIST and post 10-minute infusion RIST, and between the mean post-atropine RIST and post 25-minute infusion RIST. There is no difference between the mean post-atropine RIST and post 30-minute infusion RIST. These results indicate that after a 10-minute continuous infusion a slight, but significant, inhibition in HISS release occurred. Full HISS-dependent insulin resistance is not evident until after the 30-minute continuous infusion. Significant differences exist between some of the other durations but are not reported. Figure 4.9 shows the percent inhibition from control caused by the continuous infusion.

Table 4.1 Data from various durations of continuous (1 mU/kg/min) insulin infusion. The amount of glucose infused to maintain euglycemia in response to insulin, the length of that glucose infusion, the control RIST index, the post-continuous insulin infusion RIST index and the inhibition from the control RIST index that it represents, and the post-atropine RIST index for each group.

Duration of continuous (1mU/mg/min) insulin infusion (minutes) (n=3)	Glucose infused in response to continuous insulin infusion (mg/kg)	Length of glucose infusion (minutes)	Control RIST index (mg/kg)	Post-continuous infusion RIST index (mg/kg)	% Inhibition after continuous insulin infusion	Post-atropine RIST index (mg/kg)
10	47.4 ± 2.6	18.9 ± 0.7	171.0 ± 1.3	158.0 ± 6.7 * Φ	7.6 ± 2.1	76.4 ± 3.6
15	67.5 ± 5.3	23.3 ± 1.2	185.6 ± 9.9	140.3 ± 9.5 # Φ	24.5 ± 2.3	68.7 ± 5.7
20	81.6 ± 1.5	28.2 ± 1.7	186.6 ± 17.3	130.9 ± 5.2 # Φ	29.1 ± 4.3	72.7 ± 5.8
25	126.1 ± 13.8	36.5 ± 1.0	202.0 ± 7.1	108.3 ± 5.9 # ¶	46.5 ± 1.4	68.9 ± 2.7
30	125.0 ± 11.4	40.2 ± 0.6	175.1 ± 2.6	75.2 ± 5.7 #	57.1 ± 2.8	71.9 ± 5.1

* $P<0.05$, compared to mean control; # $P<0.001$, compared to mean control; ¶ $P<0.01$, compared to mean post-atropine; Φ $P<0.001$, compared to mean post-atropine.

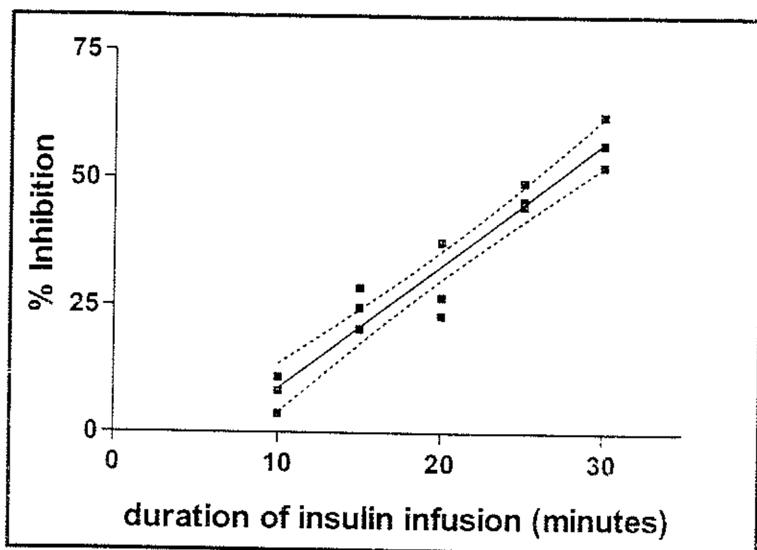

Figure 4.9: Percent inhibition of insulin sensitivity after continuous insulin infusion compared to the duration of continuous (1 mU/kg/min) insulin infusion, $r^2 = 0.93$, n=3 for each time point. Insulin sensitivity decreases progressively with the duration of the continuous infusion. Full HDIR is present after a 30-minute continuous insulin infusion.

4.4 Discussion:

Hepatic Insulin Sensitizing Substance (HISS) action accounts for 55% of insulin action (Lautt et al 2001). We previously demonstrated that use of the HIEC blocked HISS action thereby causing HISS-dependent insulin resistance in the rat. Since endogenous insulin release is pulsatile (Goodner et al 1979; Lang et al 1979), we hypothesized that the insulin resistance observed after use of the HIEC was due to the continuous insulin infusion, and resistance would be prevented if exogenous insulin was applied in pulses or as a bolus. We tested this hypothesis by assessing insulin sensitivity, with the RIST, before and after bolus, continuous or pulsatile insulin infusions. We hypothesized that HISS release, and therefore glucose uptake, is greater in response to a pulsatile rather than continuous insulin infusion. The results do not support this hypothesis as there were no differences between continuous, 3-pulse, or 6-pulse insulin infusions, in terms of their HISS-dependent, HISS-independent or total insulin action. The results do indicate, however, that continuous, but not pulsatile or bolus, insulin infusions can induce full HISS-dependent insulin resistance, in as little time as 30 minutes in the rat. These results can perhaps explain why, but not how, use of the HIEC causes HISS-dependent insulin resistance.

4.4.1 Efficacy of Continuous versus Pulsatile Insulin Delivery

This series was conducted under the premise that pulsatile insulin infusions would cause greater glucose uptake compared to continuous insulin infusions, because HISS release would be a greater. Other studies (Verdin et al 1984; Ward et al 1989) failed to show increased efficacy when using pulsatile insulin infusions for short periods of time, however, these studies were done in the fasted state where HISS release is blocked (Lautt

et al 2001; Sadri and Lautt 2000). The results may have been different, from previous studies, since we used fed animals. The results do not support our hypothesis because the amount of glucose infused to maintain euglycemia was similar among the pulsatile and continuous insulin infusion groups (figures 4.1, 4.2 and 4.3). Glucose uptake is significantly higher in the control rather than post atropine condition in each test, but the HISS-dependent (figure 4.4) and HISS-independent components of insulin action do not differ between the pulsatile and continuous groups. Matthews et al (1983a) found that differences in glucose uptake between pulsatile and continuous insulin infusions were evident only after 7 hours of insulin infusion. If our insulin infusion had been for a longer time period our results may have been different. It is difficult to say whether we would have seen a divergence in insulin effectiveness before 7 hours of insulin infusion.

The HISS action profiles (figure 4.4) and glucose uptake profiles, before and after atropine (Figures 4.1, 4.2, and 4.3), differed greatly for the pulsatile and continuous insulin infusions. When insulin was given in three pulses, three distinct glucose peaks were evident (figure 4.1), and the HISS profile (Figure 4.4) showed two distinct peaks.. The six-pulse insulin infusion did not display 6 discrete glucose peaks (Figure 4.2). For this reason, and because it did not differ in total glucose uptake, the six-pulse infusion was not included in further studies. During the six-pulse infusion the peaks appeared to blend together, probably because the amount of insulin given in each of the pulses was too great for the frequency of administration. The continuous insulin infusion caused the appearance of an oscillatory pattern in glucose uptake (Figure 4.3) and HISS action (figure 4.4). Two-minute, instead of five-minute, blood samples during the continuous insulin infusion could have contributed to the shape of the glucose curve, since this

allows for frequent adjustments to the glucose infusion. In fact, the oscillations are very small in magnitude and only appear pronounced because the glucose infusion rate scale is small. Whether or not HISS release is pulsatile deserves further investigation.

The HISS action profiles (figure 4.4) reveal that HISS release occurs throughout each of the insulin infusions in the control condition, including the continuous infusion. Previous results indicated that at the end of the 3-hour HIEC there was no difference in the glucose infusion rate between the control and HISS blocked condition (Chapter 2). The discrepancy could exist for many reasons, including the lower (1 mU/kg/min) dose of insulin used in the present study as well as the shorter duration of insulin infusion. Perhaps if the insulin infusion had been extended past 1 hour there would have been a point where the control and HISS blocked curves of insulin action converged. Depending on the species this could occur after 180 minutes. Moore et al (2002) showed, in dogs, that the 3-hour HIEC (1 mU/kg/min) could distinguish between the HISS blocked and control state. However, the difference between the control and HISS blocked state was only 25%, in their study. With the RIST Lautt et al (2001) have reported a 55% difference between the control and HISS blocked state, this indicates that the animals, in the study by Moore et al (2002), may have been partially insulin resistant. It was necessary to investigate whether HISS action is present after the 60-minute continuous and pulsatile insulin infusions since it was not present in the post-HIEC RIST (Chapter 2).

4.4.2 Effect of Continuous and Pulsatile Insulin Infusions on Insulin Sensitivity

The rats tested exhibited full HISS-dependent insulin resistance following the continuous, but not the pulsatile or bolus insulin infusions. The bolus insulin infusion for these tests was only 10 mU/kg greater than the insulin bolus administered during the RIST. Since the RIST is reproducible in 4 (Lautt et al 1998) or 5 (Xie et al 1996) consecutive tests the 60mU/kg bolus was not expected to affect insulin sensitivity. The fact that a decrease in insulin sensitivity was not seen, after the 60mU/kg bolus of insulin, shows that the dose of insulin is not directly responsible for initiating insulin resistance (figure 4.8). The amount of glucose infused in response to the pulsatile, bolus and continuous insulin infusions is very similar; the only statistical difference is between the continuous and bolus infusions. Thus, it is unlikely that the amount of glucose used to maintain euglycemia, in these studies, could be implicated as the cause of insulin resistance, as it has in *in vitro* studies (Richter et al 1988). The duration of glucose infusion does not differ between the tests where insulin was given continuously or as 3-pulses where the glucose infusion came to 0 mg/kg/min between the pulses. It is unlikely that the duration of glucose infusion could be implicated in inducing insulin resistance. The cause of insulin resistance appears to lie in the pattern and timing of insulin infusion.

Both 20 and 40-hour continuous insulin infusions have been shown to induce insulin resistance in healthy humans (Marangou et al 1986; Rizza et al 1985). In contrast, a 20-hour pulsatile insulin infusion did not induce insulin resistance in humans (Ward et al 1990). Our results allowed us to draw similar conclusions in a much shorter time period. The 60-minute continuous insulin infusion induced full HISS-dependent insulin

resistance, as assessed by a post-continuous infusion RIST that was not affected by atropine administration (figure 4.5). This agrees with previous results that demonstrate full HISS-dependent insulin resistance after use of the HIEC (Chapter 2). Since HISS release is not possible in the post-continuous insulin infusion RIST it is not known why HISS action was detectable during the hour long continuous insulin infusion, as identified by the HISS action profile (figure 4.4).

Three pulses of insulin did not initiate insulin resistance when the glucose infusion rate was allowed to come to 0 mg/kg/min between pulses (figure 4.7), but caused a partial inhibition (33.4%) of HISS release when given 20 minutes apart without allowing re-establishment of the previous baseline of insulin action (figure 4.6). Pulsatile insulin infusion is better at maintaining HISS release compared to continuous insulin infusion, but the timing of the pulses is obviously important as well. This is not surprising considering that diabetic subjects have irregular and more frequent insulin bursts compared to healthy people (Lang et al 1981; Zarkovic et al 1999), and insulin sensitivity decreases as the number of insulin pulses in a given period increases (Hunter et al 1996). Normally, insulin pulses lag glucose pulses by 2 minutes (Lang et al 1979) and glucose pulses can be used to entrain insulin pulses in healthy individuals but not diabetic subjects (Hollingdal et al 2000). Insulin pulses occur more frequently and are uncoupled from glucose pulses in people with type 2 diabetes. A study by Marsh et al (1986) concluded that to maintain insulin sensitivity and normal feedback mechanisms glucose had to oscillate, so that as it rose it stimulated insulin secretion and as it fell it inhibited insulin secretion thereby preventing hyperinsulinemia. It is possible that administering more insulin while circulating insulin is still causing glucose uptake, as

done in the experiments where insulin was given every 20 minutes regardless of glucose uptake, interferes with normal feedback responses and causes a partial HISS blockade. Or maybe, HISS release is blocked when circulating insulin levels do not fall below a particular circulating concentration. A limitation of relating the work presented by Marsh et al (1986) to our own is that they were looking at slow oscillations, and not rapid pulses. There are no reports, within the literature, stating that rapid insulin pulses are just as important as the slow insulin oscillations for maintaining the insulin-glucose negative feedback cycle. One could speculate that this is because the rapid pulses serve to control the HISS-insulin feedback cycle.

4.4.3 Effect of Different Durations of Continuous Insulin Infusion

The results from this series indicate that a 30-minute continuous insulin infusion (1 mU/kg/min) can induce a full blockade of HISS action in the rat. Slight inhibition of HISS action occurs after a 10 minute insulin infusion and appears to progress with the duration of insulin infusion (figure 4.9). During the 30-minute continuous insulin infusion only 30 mU/kg of insulin was administered; it is unlikely that this amount of insulin has any direct effect on insulin sensitivity, considering the 60 mU/kg bolus did not affect insulin sensitivity. Both the duration of glucose infusion and quantity of glucose infused (table 4.1) are similar to, or less than, that seen during the RIST and probably had little effect on insulin sensitivity. It is possible that the continuous insulin delivery caused changes in insulin receptor binding. Goodner et al (1988) showed, in isolated hepatocytes, that surface insulin receptors recycled within 15 minutes of exposure to a pulse or continuous, 60-minute administration of insulin with no change in affinity. Mandarino et al (1984) found, in adipocytes, that there was no change in insulin

receptor affinity when people were exposed to a continuous six-hour insulin infusion. There was, however, a decrease in glucose transport. Whether HISS-dependent insulin resistance would develop sooner if the dose of insulin were greater than 1mU/kg/min was not tested. The duration of continuous insulin infusion that induces HISS-dependent insulin resistance could differ between species, and if we had used a different animal model there may have been a delay in the onset of insulin resistance.

To date, HISS has not been identified, only characterized. For this reason, it is impossible to know if the HISS-dependent insulin resistance that develops in response to a continuous insulin infusion is due to a loss of HISS release or HISS action. Considering that continuous GnRH ablates LH and FSH release (Belchetz et al 1978), one could speculate that, in a similar way, continuous insulin ablates HISS release. However, until HISS and its receptor are identified we cannot definitively say whether HISS release or HISS action is blocked.

CHAPTER 5 – Final Remarks

5.1 Conclusions

The RIST has been used to quantify HISS-dependent and HISS-independent insulin action in a series of studies that suggests that this novel pathway is of major physiological, pathological and therapeutic importance. While both the ITT and RIST are equally able to detect HISS action, the ensuing hypoglycemia during the ITT requires a longer recovery time and is complicated by counter-regulatory hormones. For these reasons it is more difficult to obtain a profile of HISS action during the ITT and repeating the ITT is less feasible. In contrast to the ITT and RIST, the HIEC can only detect HISS action, in the rat, during the first 30 minutes of glucose infusion. By its conclusion HISS action cannot be detected. Further, HISS action cannot be detected in post HIEC tests using either the RIST or the ITT indicating that use of the clamp induces HISS-dependent insulin resistance. Therefore, in any study attempting to evaluate HISS action the method being used must first be shown to be capable of detecting HISS-dependent and HISS-independent insulin action.

Studies indicate that normal insulin release is pulsatile (Goodner et al 1977; Lang et al 1979). Continuous and pulsatile insulin infusions stimulate glucose uptake to a similar extent. Both the HISS-dependent and HISS-independent components of insulin action are similar during a pulsatile or continuous 60-minute insulin infusion. However, our results show that, continuous insulin infusions induced HISS-dependent insulin resistance in the rat, while bolus and pulsatile infusions of the same dose did not. These observations may explain why HISS release was not possible during post-HIEC tests. This finding lends support to the idea that continuous insulin infusions should be used

with caution during both the HIEC and insulin treatment because they induce HISS-dependent insulin resistance.

5.2 Speculations

Why does continuous insulin infusion block HISS release? We know that HISS release is blocked during fasting (Lautt et al 2001), presumably to prevent hypoglycemia. Continuous insulin infusions maintain circulating insulin levels at a constant level but pulsatile insulin infusions cause peaks and troughs in the concentration. Perhaps, when the body cannot sense insulin levels falling, or they do not fall past a particular circulating concentration, it responds by blocking HISS release. HISS release would be blocked to prevent hypoglycemia.

To date, the feeding signal that allows HISS release has not been elucidated. Placing food in the stomach of a fasted, anesthetized rat can increase the RIST index, but the exact mechanism is unknown (Lautt et al 2001). Based on the importance of insulin pulsatility, and its ability to effect HISS release, an increase in insulin pulse amplitude could serve as part of the feeding signal. Earlier in this thesis, I speculated that rapid insulin pulses worked in a negative feedback loop with HISS. Because insulin release is pulsatile during fasting and feeding there must be a way to distinguish the two conditions; an increase in pulse amplitude may accomplish this and be part of the trigger. Alternatively, the signal that increases the insulin pulse amplitude works in a parallel pathway to initiate HISS release. In this instance the insulin pulsations could serve to modify HISS release.

The mechanism by which HISS causes glucose uptake has not been determined. A recent paper by Manchem et al (2001) describes a small molecule that sensitizes the

insulin receptor. When insulin is administered the small molecule causes increased autophosphorylation of the insulin receptor and glucose uptake. It is possible that HISS could act through a similar mechanism to enhance intracellular signaling and resultant glucose uptake. Whether or not HISS binds to the insulin receptor, and what the mechanism for increased glucose uptake is, is unknown. Once HISS is identified these questions can be directly addressed.

5.3 Future direction

Clearly, this is an interesting and exciting area of research. A major goal should be to identify HISS and its receptor. The identification of HISS would allow the many questions its characterization has uncovered to be answered. Once HISS has been identified, new therapies for treating type 2 diabetes can be developed. Until HISS is identified there are other questions that can be answered.

Other research groups use somatostatin to inhibit endogenous insulin release while doing their HIEC studies. Our inability to show a difference between a control and blocked HIEC, as Moore et al (2002) did, could be because we did not use somatostatin. The effect of somatostatin on HISS release and action needs to be determined. Until we know that somatostatin does not block HISS release it cannot be incorporated into our experimental protocols.

There are numerous experiments that could follow the series that investigated the effect of pulsatile and continuous insulin delivery on HISS release. Whether or not HISS release is pulsatile should be determined. Other experiments would be to extend the 1 mU/kg/min continuous infusion past 1 hour and determine at what duration of insulin infusion do differences in glucose uptake between the control and HISS blocked

conditions occur. Others (Matthews et al 1983a) determined, in the fasted state, that the differences in blood glucose level were evident only after 7 hours of pulsatile vs. continuous insulin infusion. Whether or not a divergence in glycemia will occur sooner in the fed state should be determined. Determining the time point at which resistance develops in different species could further the interpretation of the present results.

The duration of insulin resistance caused by continuous insulin delivery has not been tested. Thus, the duration of rest that is needed for full HISS action to return should be determined. The final question, which was unanswered by this thesis, is why HISS-dependent insulin resistance is evident after just 30 minutes during the 10 mU/kg/min HIEC and not during, but after, the 1 mU/kg/min insulin infusion. Understanding the mechanism by which continuous insulin infusions permit HISS release during the 1 mU/kg/min insulin infusion but result in a post-infusion block would further the interpretation of the present results and the understanding of HISS regulation.

References

Aizawa, T., T. Kaneko, H. Yajima, S. Yamada, Y. Sato, Y. Kanda, S. Kanda, M. Noda, T. Kadowaki, M. Nagai, K. Yamauchi, M. Komatsu, and K. Hashizume. Rapid oscillation of insulin release by the rat pancreatic islets under stringent Ca^{2+}- free conditions. J. Endocrinol. 166: 545-551, 2000.

Akinmokun, A., P. L. Selby, K. Ramaiya, and K. G. M. M. Alberti. The short insulin tolerance test for determination of insulin sensitivity: a comparison with the euglycemic clamp. Diabetic Med. 9: 432-437, 1992.

Anderson, E. A., R. P. Hoffman, T. W. Balon, C. A. Sinkey, and A. L. Mark. Hyperinsulinemia produces both sympathetic and neural activation and vasodilation in normal humans. J. Clin. Invest. 87: 2246-2252, 1991.

Asplin, C. M., T. L. Paquette, and J. P. Palmer. In vivo inhibition of glucagon secretion by paracrine beta cell activity in man. J. Clin. Invest. 68: 314-318, 1981.

Avogaro, A., P. Vicini, A. Valerio, A. Caumo, and C. Cobelli. The hot and not the cold minimal model allows precise assessment of insulin sensitivity in NIDDM subjects. Am. J. Physiol. 270: E532-E540, 1996.

Bagdade, J. D., E. L. Bierman, and D. Porte Jr. The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. J. Clin. Invest. 46(10): 1549-1557, 1967.

Barnett, A. H., C. Eff, R. D. G. Leslie, and D. A. Pyke. Diabetes in identical twins. Diabetologia. 20: 87-93, 1981.

Baron, A. D., J. Zhu, S. Marshall, O. Irsula, G. Brechtel, and C. Keech. Insulin resistance after hypertension induced by the nitric oxide synthesis inhibitor L-NMMA in rats. Am. J. Physiol. 269: E702-E715, 1995.

Belchetz, P. E., T. M. Plant, Y. Nakai, E. J. Keogh and E. Knobil. Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. Science 202 (10): 631-633, 1978.

Bergman, R. N., Y. Z. Ider, C. R. Bowden, and C. Cobelli. Quantitative estimation of insulin sensitivity. Am. J. Physiol. 236: E667-E677, 1979.

Benthem, L., T. O. Mundinger, and G. J. Taborsky, Jr. Meal-induced insulin secretion in dogs is mediated by both branches of the autonomic nervous system. Am. J. Physiol. 278: E603-E610, 2000.

Bjornholm, M., Y. Kawano, M. Lehtihet, and J. R. Zierath. Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. Diabetes. 46: 524-527, 1997.

Bokemark, L., A. Froden, S. Attvall, J. Wikstrand, and B. Fagerberg. The euglycemic hyperinsulinemic clamp examination: variability and reproducibility. Scand. J. Clin. Lab. Invest. 60: 27-36, 2000.

Bonora, E., P. Moghetti, C. Zancanaro, M. Cigolini, M. Querena, V. Cacciatori, A. Corgnati, and M. Muggeo. Estimates of *in vivo* insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. J. Clin. Endocrinol. Metab. 68: 374-378, 1989.

Bourn, D. M., J. I. Mann, B. J. McSkimming, M.A. Waldron, and J. D. Wishart. Impaired glucose tolerance and NIDDM: does a lifestyle intervention program have an effect? Diabetes Care 17(11): 1311-1319, 1994.

Chan, J. M., E. B. Rimm, G. A. Colditz, M. J. Stampfer, and W. C. Willett. Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. Diabetes Care 17(9): 961-969, 1994.

Chou, H., R. McGivern, N. Berman, and E. Ipp. Oscillations of circulating plasma insulin concentrations in the rat. Life Sciences. 48: 1463-1469, 1991.

Cockburn, B. N., D. M. Ostrega, J. Sturis, C. Kubstrup, K. S. Polonsky, and G. I. Bell. Changes in pancreatic islet glucokinase and hexokinase activities with increasing age, obesity, and the onset of diabetes. Diabetes 46: 1434-1439, 1997.

Dailey, G. E., G. H. Boden, R. H. Creech, D.G. Johnson, R. E. Gleason, F. P. Kennedy, L. A. Weinrauch, M. Weir, and J. A. D'Elia. Effects of pulsatile intravenous insulin therapy on the progression of diabetic nephropathy. Metabolism. 49(11): 1491-1495, 2000.

Daniel, S., M. Noda, S. G. Straub, and G. W. G. Sharp. Identification of the docked granule pool responsible for the first phase of glucose-stimulated insulin secretion. Diabetes. 48: 1686-1690, 1999.

DeFronzo, R. A., J. D. Tobin, and R. Andres. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am. J. Physiol. 237(3): E214-E223, 1979.

DeFronzo, R. A., R. C, Bonadonna, and E. Ferrannini. Pathogenesis of NIDDM. Diabetes Care 15: 318-368, 1992.

Efanova, I. B., S. V. Zaitsev, B. Zhivotovsky, M. Kohler, S. Efendic, S. Orrenius, and P. Berggren. Glucose and tolbutamide induce apoptosis in pancreatic β-cells. J. Biol. Chem. 273(50): 33501-33507, 1998.

Elahi, D. In praise of the hyperglycemic clamp. Diabetes Care. 19(3): 278-286, 1996.

Fernandez-Castaner, M., J. Biarnes, I. Camps, J. Ripolles, N. Gomez, and J. Soler. Beta-cell dysfunction in first-degree relatives of patients with non-insulin-dependent diabetes mellitus. Diabetic Med. 13: 953-959, 1996.

Garber, A. J., P. E. Cryer, J. V. Santiago, M. W. Haymond, A. S. Pagliara, and D. M. Kipnis. The role of adrenergic mechanisms in the substrate and hormonal response to insulin-induced hypoglycemia in man. J. Clin. Invest. 58: 7-15, 1976.

Garvey, W. T., L. Maianu, J. Zhu, G. Brechtel-Hook, P. Wallace, and A. D. Baron. Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. J. Clin. Invest. 101: 2377-2386, 1998.

Gelding, S. V., S. Robinson, S. Lowe, R. Niththyananthan, and D. G. Johnston. Validation of the low dose short insulin tolerance test for evaluation of insulin sensitivity. Clin. Endocrinol. 40: 611-615, 1994.

Goldberg, I. J. Diabetic dyslipidemia: causes and consequences. J. Clin. Endocrinol. Metab. 86(3): 965-971, 2001.

Goodner, C. J., B. C. Walike, D. J. Koerker, J. W. Ensinck, A. C. Brown, E. W. Chideckel, J. Palmer and L. Kalnasy. Insulin, glucagon and glucose exhibit synchronous sustained oscillations in fasting monkeys. Science. 195: 177-179, 1977.

Goodner, C. J., I. R. Sweet, and H. C. Harrison. Rapid reduction and return of surface insulin receptors after exposure to brief pulses of insulin in perfused rat hepatocytes. Diabetes. 37: 1316-1323, 1988.

Grundleger, M. L., and S. W. Thenen. Decreased insulin binding, glucose transport, and glucose metabolism in soleus muscle of rats fed a high fat diet. Diabetes 31: 232-237, 1982.

Gumbiner, B., E. Van Cauter, W. F. Beltz, T. M. Ditzler, K. Griver, K. S. Polonsky, and R. R. Henry. Abnormalities of insulin pulsatility and glucose oscillations during meals in obese noninsulin-dependent diabetic patients: Effects of weight reduction. J. Clin. Endocrinol. Metab. 81: 2061-2068. 1996.

Henquin, J-C. Cell biology of insulin secretion. *In* Joslin's Diabetes Mellitus. *Edited by* C. R. Kahn and G. C. Weir. Lea & Febiger, Philadelphia. p56.

Hirst, S., D. I. W. Phillips, S.K. Vines, P. M. Clark, and C. N. Hales. Reproducibility of the short insulin tolerance test. Diabetic Med. 10: 839-842, 1993.

Holligdal, M., C. B. Juhl, S. M. Pincus, J. Sturis, J. D. Veldhuis, K. S. Polonsky, N. Porksen, and O. Schmitz. Failure of physiological plasma glucose excursions to entrain high-frequency pulsatile insulin secretion in type 2 diabetes. Diabetes. 49: 1334-1340, 2000.

Hou, X., Z. Ling, E. Quartier, A. Foriers, F. Schuit, D. Pipeleers, and C. Van Schravendijk. Prolonged exposure of pancreatic beta cells to raised glucose concentrations results in increased cellular content of islet amyloid polypeptide precursors. Diabetologia. 42: 188-194, 1999.

Hribal, M. L., M. Federici, O. Porzio, D. Lauro, P. Borboni, D. Accili, R. Lauro, and G. Sesti. The Gly-Arg^{942} amino acid polymorphism in insulin receptor substrate-1 affects glucose metabolism in skeletal muscle cells. J. Clin. Endocrinol. Metab. 85: 2004-2013, 2000.

Hunter, S. J., A. B. Atkinson, C. N. Ennis, B. Sheridan, and P. M. Bell. Association between insulin secretory pulse frequency and peripheral insulin action in NIDDM and normal subjects. Diabetes. 45: 683-686, 1996.

Iozzo, P., T. Pratipanawatr, H. Pijl, C. Vogt, V. Kumar, R. Pipek, M. Matsuda, L. J. Mandarino, K. J. Cusi, and R. A. DeFronzo. Physiological hyperinsulinemia impairs insulin-stimulated glycogen synthase activity and glycogen synthesis. Am. J. Physiol. 280: E712-E719, 2001.

Janson, J., R. H. Ashley, D. Harrison, S. McIntyre, and P. C. Butler. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. Diabetes. 48: 491-498, 1999.

Johnson, J. H., A. Ogawa, L. Chen, L Orci, C. B. Newgard, T. Alam, and R. H. Unger. Underexpression of β-cell high K_m glucose transporters in noninsulin-dependent diabetes. Science. 250: 546-549, 1990.

Johnson, K. H., T. D. O'Brien, C. Betsholtz, and P. Westermark. Islet amyloid, islet amyloid polypeptide, and diabetes mellitus. N. Engl. J. Med. 321(8): 513-518, 1989.

Juhl, C. B., N. Porksen, S. M. Pincus, A. P. Hansen, J. D. Veldhuis, and O. Schmitz. Acute and short-term administration of slufonylurea (Gliclazide) increases pulsatile insulin secretion in Type 2 diabetes. Diabetes 50: 1778-1784, 2001.

Juhl, C., T. Grofe, P. C. Butler, J. D. Veldhuis, O. Schmitz, and N. Porksen. Effects of fasting on physiologically pulsatile insulin release in healthy humans. Diabetes 51 (Suppl. 1): S255-S257, 2002.

Kahn, S. E. The importance of β-cell failure in the development and progression of type 2 diabetes. J. Clin. Endocrinol. Metab. 86(9): 4047-4058, 2001.

Kahn, S. E., J. C. Beard, M. W. Schwartz, W. K. Ward, H. L. Ding, R. N. Bergman, G. J. Taborsky Jr., and D. Porte Jr. Increased β-cell secretory capacity as mechanism for islet adaptation to nicotinic acid-induced insulin resistance. Diabetes. 38: 562-568, 1989.

Kaiyala, K. J., R. L. Prigeon, S. E. Kahn, S. C. Woods, D. Porte Jr., and M. W. Schwartz. Reduced β-cell function contributes to impaired glucose tolerance in dogs made obese by high-fat feeding. Am. J. Physiol. 277: E659-E667, 1999.

Kingston, W. J., J. N. Livingston, and R. T. Moxley III. Enhancement of insulin action after oral glucose ingestion. J. Clin. Invest. 77: 1153-1162, 1986.

Kjems, L. L., B. M. Kirby, E. M. Welsh, J. D. Veldhuis, M. Straume, S. S. McIntyre, D. Yang, P. Lefebvre, and P. C. Butler. Decrease in β-cell mass leads to impaired pulsatile insulin secretion, reduced postprandial hepatic insulin clearance, and relative hyperglucagonemia in the minipig. Diabetes 50: 2001-2012, 2001.

Komatsu, M., T. Schermerhorn, M. Noda, S. G. Straub, T. Aizawa, and G. W. G. Sharp. Augmentation of insulin release by glucose in the absence of extracellular Ca^{2+}. Diabetes 46: 1928-1938, 1997.

Koopmans, S. J., H. C. M. Sips, H. M. J. Krans, and J. K. Radder. Pulsatile intravenous insulin replacement in streptozotocin-diabetic rats is more efficient than continuous delivery: effects on glycaemic control, insulin-mediated glucose metabolism and lipolysis. Diabetologia. 39: 391-400, 1996.

Kraegen, E. W., D. E. James, S. P. Bennett, and D. J. Chisholm. In vivo insulin sensitivity in the rat determined by euglycemic clamp. Am. J. Physiol. 245: E1-E7, 1983.

Krook, A., R. A. Roth, X. J. Jiang, J. R. Zierath, and H. Wallberg-Henriksson. Insulin stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. Diabetes. 47: 1281-1286, 1998.

Laedtke, T., L. Kjems, N. Porksen, O. Schmitz, J. Veldhuis, P.C. Kao, and P.C. Butler. Overnight inhibition of insulin secretion restores pulsatility and proinsulin/insulin ratio in type 2 diabetes. Am. J. Physiol. 279: E520-E528, 2000.

Lang, D. A., D. R. Matthews, J. Peto, and R. C. Turner. Cyclic oscillations of basal plasma glucose and insulin concentrations in human beings. N. Engl. J. Med. 301: 1023-1027, 1979.

Lang, D. A., D. R. Matthews, M. Burnett, and R. C. Turner. Brief, irregular oscillations of basal plasma insulin and glucose concentrations in diabetic man. Diabetes 30: 435-439, 1981.

Latour, M. G., and W. W. Lautt. The hepatic vagus nerve in the control of insulin sensitivity in the rat. Autonomic Neurosci. 95: 125-130, 2002a.

Latour, M. G., and W. W. Lautt. Insulin sensitivity regulated by feeding in the conscious unrestrained rat. Can. J. Physiol. Pharmacol. 80: 8-12, 2002b.

Lautt, W.W., and H. Xie. Intraportal acetylcholine reverses insulin resistance caused by chronic bile duct ligation. Proc. West. Pharmacol. Soc. 41: 35-36, 1998.

Lautt, W. W., M. P. Macedo, P. Sadri, S. Takayama, F. D. Ramos, and D. J. Legare. Hepatic parasympathetic (HISS) control of insulin sensitivity determined by feeding and fasting. Am. J. Physiol. Gastrointest. Liver Physiol. 281: G29-G36, 2001.

Lautt, W. W., X. Wang, P. Sadri, D. J. Legare, and M. P. Macedo. Rapid Insulin Sensitivity Test (RIST). Can. J. Physiol. Pharmacol. 76: 1080-1086, 1998.

Lee, Y., H. Hirose, M. Ohneda, J. H. Johnson, J. D. McGarry, and R. H. Unger. B-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-β-cell relationships. Proc. Natl. Acad. Sci. USA. 91: 10878-10882, 1994.

Livingston, J. N., B. J. Purvis, and D. H. Lockwood. Insulin induced changes in insulin binding and insulin-sensitivity of adipocytes. Metabolism 27(Suppl 2): 2009-2014, 1978.

Maedler, K., G. A. Spinas, D. Dyntar, W. Moritz, N. Kaiser, and M. Y. Donath. Distinct effects of saturated and monounsaturated fatty acids on β-cell turnover and function. Diabetes 50: 69-76, 2001.

Maegawa, H., Y. Shigeta, K. Egawa, and M. Kobayashi. Impaired autophosphorylation of insulin receptors from abdominal skeletal muscles from nonobese subjects with NIDDM. Diabetes. 40: 815-819, 1991.

Manchem, V. P., I. D. Goldfine, R. A. Kohanski, C. P. Cristobal, R. T. Lum, S. R. Schow, S. Shi, W. R. Spevak, E. Laborde, D. K. Toavs, H. O. Villar, M. M. Wick, and M. R. Kozlowski. A novel small molecule receptor that directly sensitizes the insulin receptor in vitro and in vivo. Diabetes 50: 824-830, 2001.

Mandarino, L., B. Baker, R. Rizza, J. Genest, and J. Gerich. Infusion of insulin impairs human adipocyte glucose metabolism in vitro without decreasing adipocyte insulin receptor binding. Diabetologia 27: 358-363, 1984.

Marangou, A. G., K. M. Weber, R.C. Boston, P. M. Aitken, J. C. P. Heggie, R. L. G. Kirsner, J. D. Best and F. P. Alford. Metabolic consequences of prolonged hyperinsulinemia in humans evidence for induction of insulin insensitivity. Diabetes. 35: 1383-1389, 1986.

Marchetti, P., D. W. Sharp, M. Mclear, R. Gingerich, E. Finke, B. Olack, C. Swanson, R. Giannarelli, R. Navalesi, and P. E. Lacy. Pulsatile insulin secretion from isolated human pancreatic islets. Diabetes 43: 827-830, 1994.

Marsh, B. D., D. J. Marsh, and R. N. Bergman. Oscillations enhance the efficiency and stability of glucose disposal. Am. J. Physiol. 250: E576-E582, 1986.

Matthews, D. R., B. A. Naylor, R. G. Jones, G. M. Ward and R. C. Turner. Pulsatile insulin has greater hypoglycemic effect than continuous delivery. Diabetes. 32: 617-621, 1983a.

Matthews, D. R., D. A. Lang, M. A. Burnett, and R. C. Turner. Control of pulsatile insulin secretion in man. Diabetologia. 24: 231-237, 1983b.

Meissner, H. P. Electrophysiological evidence for coupling between β cells of pancreatic islets. Nature 262: 502-504, 1976.

Meneilly, G. S., A. S. Ryan, J. D. Veldhuis, and D. Elahi. Increased disorderliness of basal insulin release, attenuated insulin secretory burst mass, and reduced ultradian rythmicity of insulin secretion in older individuals. J. Clin. Endocrinol. Metab. 82: 4088-4093, 1997.

Meneilly, G. S., J. D. Veldhuis, and D. Elahi. Disruption of the pulsatile and entropic modes of insulin release during an unvarying glucose stimulus in elderly individuals. J. Clin. Endocrinol. Metab. 84: 1938-1943, 1999.

Merriam-Webster's Collegiate Dictionary [online] Merriam-Webster Incorporated ©2002. Available at http://www.m-w.com/dictionary [cited 24, April, 2002]

Meyer, C., M. Stumvoll, V. Nadkarni, J. Dostou, A. Mitrakou, and J. Gerich. Abnormal renal and hepatic glucose metabolism in type 2 diabetes mellitus. J. Clin. Invest. 102(3): 619-624, 1998.

Mitrakou, A., H. Vuorinen-Markkola, G. Raptis, I. Toft, M. Mokan, P. Strumph, W. Pimenta, T. Veneman, T. Jenssen, G. Bolli, M. Korytkowski, H. Yki-Jarvinen, and J. Gerich. Simultaneous assessment of insulin secretion and insulin sensitivity using a hyperglycemic clamp. J. Clin. Endocrinol. Metab. 75: 379-382, 1992.

Moore, M.C., S. Satake, B. Baranowski, P-S. Hsieh, D. W. Neal, and A. D. Cherrington. Effect of hepatic denervation on peripheral insulin sensitivity in conscious dogs. Am. J. Physiol. 282: E286-E296, 2002.

Neel, J. V. Diabetes Mellitus: a "thrifty" genotype rendered detrimental by "progress"? Am. J. Hum. Gen. 14: 353-362, 1962.

Norgaard, A., and Th. E. H. Thaysen. Clinical investigations into the effect of intravenous injection of insulin. Acta. Med. Scand. 72: 492-510, 1929.

Nyholm, B., N. Porksen, C. B. Juhl, C. H. Gravholt, P.C. Butler, J. Weeke, J. D. Veldhuis, S. Pincus, and O.Schmitz. Assessment of insulin secretion in relatives of patients with type 2 (non-insulin-dependent) diabetes mellitus: evidence of early β-cell dysfunction. Metabolism. 49(7): 896-905, 2000.

O'Meara, N. M., J. Sturis, E. Van Cauter, and K. S. Polonsky. Lack of control by glucose of ultradian insulin secretory oscillations in impaired glucose tolerance and in non-insulin-dependent diabetes mellitus. J. Clin. Invest. 92: 262-271, 1993.

Opara, E. C., and V. L. W. Go. Effect of nerve blockade on pulsatile insulin and glucagon secretion in vitro. Pancreas. 6(6): 653-658, 1991.

O'Rahilly, S. P., Z. Nugent, A. S. Rudenski, J. P. Hosker, M. A. Burnett, P. Darling, and R. C. Turner. Beta-cell dysfunction, rather than insulin insensitivity is the primary defect in familial type 2 diabetes. Lancet. 2(8503): 360-364, 1986.

O'Rahilly, S., R. C. Turner, and D. R. Matthews. Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes. N. Engl. J. Med. 318: 12285-1330, 1988.

Ostenson, C. G., The pathophysiology of type 2 diabetes mellitus: and overview. Acta. Physiol. Scand. 171: 241-247, 2001.

Pan, X., G. Li, Y. Hu, J. Wang, W. Yang, Z. An, Z. Hu, J. Lin, J. Xiao, H. Cao, P. Liu, X. Jiang, Y. Jiang, J. Wang, H. Zheng, H. Zhang, P.H. Bennett, and B. V. Howard. Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. Diabetes Care. 20(4): 537-544, 1997.

Paolisso, G., A. J. Scheen, D. Giugliano, S. Sgambato, A. Albert, M. Varricchio, F. D'Onofrio, and P. J. Lefebvre. Pulsatile insulin delivery has greater metabolic effects than continuous hormone administration in man: Importance of pulse frequency. Clin. Endocrinol. Metab. 72: 607-615, 1991.

Paolisso, G., S. Sgambato, R. Torella, M Varricchio, A. Scheen, F. D'Onofrio, and P. J. Lefebvre. Pulsatile insulin delivery is more efficient than continuous infusion in modulating islet cell function in normal subjects and patients with type 1 diabetes. J. Clin. Endocrinol. Metab. 66: 1220-1226, 1988a.

Paolisso, G., S. Sgambato, S.Gentile, P. Memoli, D. Giugliano, M. Varricchio, and F. D'Onofrio. Advantageous metabolic effects of pulsatile insulin delivery in noninsulin-dependent diabetic patients. J. Clin. Endocrinol. Metab. 67:1005-1010, 1988b.

Perely, M. J., and D. M. Kipnis. Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects. J. Clin. Invest. 46(12): 1954-1962, 1967.

Pigon, J., A. Giacca, C-G. Ostenson, L. Lam, M. Vranic, and S. Efendic. Normal hepatic insulin sensitivity in lean, mild noninsulin-dependent diabetic patients. J. Clin. Endocrinol. Metab. 81: 3702-3708, 1996.

Polonsky, K. S., B. D. Given, and E. Van Cauter. Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. J. Clin. Invest. 81: 442-448, 1988.

Porksen, N. The in vivo regulation of pulsatile insulin secretion. Diabetologia 45: 3-20, 2002.

Porksen, N., B. Nyholm, J. D. Veldhuis, P. C. Butler, and O. Schmitz. In humans at least 75% of insulin secretion arises from punctuated insulin secretory bursts. Am. J. Physiol. 273: E908-E914, 1997.

Porksen, N., S. Munn, D. Ferguson, T. O'Brein, J. Veldhuis, and P. Butler. Coordinate pulsatile insulin secretion by chronic intraportally transplanted islets in the isolated perfused rat liver. J. Clin. Invest. 94: 219-227, 1994.

Porksen, N., S. Munn, J. Steers, S. Vore, J. Veldhuis, and P. Butler. Pulsatile insulin secretion accounts for 70% of total insulin secretion during fasting. Am. J. Physiol. 269: E478-E488, 1995.

Pratipanawatr, T., K. Cusi, P. Ngo, W. Pratipanawatr, L. J. Mandarino, and R. A. DeFronzo. Normalization of plasma glucose concentration by insulin therapy improves insulin-stimulates glycogen synthesis in type 2 diabetes. Diabetes. 51: 462-468, 2002.

Ravussin, E., M. E. Valencia, L. Esparza, P. H. Bennett, and L. O. Shulz. Effects of a traditional lifestyle on obesity in Pima Indians. Diabetes Care 17(9): 1067-1074, 1994.

Reid, M. A. G., M. G. Latour, D. J. Legare, N. Rong, and W. W. Lautt. Comparison of the rapid insulin sensitivity test (RIST), insulin tolerance test (ITT), hyperinsulinemic euglycemic clamp (HIEC) to measure insulin action in rats. Can. J. Physiol. Pharmacol. 80: in press.

Ribeiro, R. T., F. Duarte-Ramos, and M. P. Macedo. Effect of the hepatic insulin sensitizing substance in the spontaneously hypertensive rat. Proc. West. Pharmacol. Soc. 44: 27-28, 2001a.

Ribeiro, R. T., F. Duarte-Ramos, and M. P. Macedo. The fatty Zucker rat fa/fa shows a dysfunction of the HISS-dependent and –independent components of insulin action. Proc. West. Pharmacol. Soc. 44: 29-30, 2001b.

Ribeiro, R. T., F. Duarte-Ramos, and M. P. Macedo. The action of hepatic insulin sensitizing substance is decreased in rats on a high-sucrose diet. Proc. West. Pharmacol. Soc. 44: 31-32, 2001c.

Richter, E. A., B. F. Hansen, and S. A. Hansen. Glucose-induced insulin resistance of skeletal-muscle glucose transport and uptake. Biochem. J. 252: 733-737, 1988.

Rizza, R. A., L. J. Mandarino, J. Genest, B. A. Baker, and J.E. Gerich. Production of insulin resistance by hyperinsulinemia in man. Diabetologia. 28: 70-75, 1985.

Robertson, R. P., L. K. Olson, and H. Zhang. Differentiating glucose toxicity from glucose desensitization: a new message from the insulin gene. Diabetes. 43: 1085-1089, 1994.

Rossetti, L., A. Giaccari, R. A. DeFronzo. Glucose Toxicity. Diabetes Care. 13(6): 610-630, 1990.

Rowe, J. W., J. B. Young, K. L. Minaker, A. L. Stevens, J. Pallotta, and L. Landsberg. Effect of insulin and glucose infusions on sympathetic nervous system activity in normal man. Diabetes. 30: 219-225, 1981.

Sadri, P., and W. W. Lautt. Blockade of nitric oxide production in the liver causes insulin resistance. Proc. West. Pharmacol. Soc. 41: 37-38, 1998.

Sadri, P., and W. W. Lautt. Blockade of hepatic nitric oxide synthase causes insulin resistance. Am. J. Physiol. 277: G101-G108, 1999.

Sadri, P., and W. W. Lautt. Glucose disposal by insulin, but not IGF-1, is dependant on the hepatic parasympathetic nerves. Can. J. Physiol. Pharmacol. 78: 807-812, 2000.

Sadri, P., D. J. Legare, and W. W. Lautt. Insulin resistance caused by nitric oxide synthase inhibition. Proc. West. Pharmacol. Soc. 40: 19-20, 1997.

Sadri, P., D. J. Legare, S. Takayama, and W. W. Lautt. Fetal ethanol exposure causes HISS-dependent insulin resistance: Submitted.

Scheen, A. J., N. Paquot, M. J. Castillo, and P. J. Lefebvre. How to measure insulin action *in vivo*. Diabetes Metab. Rev. 10(2): 151-188, 1994.

Schmitz, O., J Arnfred, O. H. Nielsen, H. Beck-Nielsen, and H. Orskov. Glucose uptake and pulsatile infusion: euglycemic clamp and [3-^{3}H] glucose studies in healthy subjects. Acta. Endocrinol.(Copenh). 113: 559-563, 1986.

Schmitz, O., N. Porksen, B Nyholm, C. Skj□r□k, P. C. Butler, J. D. Veldhuis, and S. M. Pincus. Disorderly and nonstationary insulin secretion in relatives of patients with NIDDM. Am. J. Physiol. 272:E218-E226, 1997.

Sha, L., J. Westerlund, J. H. Szurszewski, and P. Bergsten. Amplitude modulation of pulsatile insulin secretion by intrapancreatic ganglion neurons. Diabetes. 50: 51-55, 2001.

Shao, J., H. Yamashita, L. Qiao, and J. E. Friedman. Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Lepr$^{db/db}$ mice. J. Endocrinol. 167: 107-115, 2000.

Shapiro, E. T., H. Tillil, K. S. Polonsky, V. S. Fang, A. H. Rubenstein, and E. Van Cauter. Oscillations in insulin secretion during constant glucose infusion in normal man: relationship to changes in plasma glucose. J. Clin. Endocrinol. Metab. 67: 307-314, 1988.

Shimbukuro, M., Y-T. Zhou, M. Levi, and R. H. Unger. Fatty acid-induced β cell apoptosis: a link between obesity and diabetes. Proc. Natl. Acad. Sci. USA 95: 2498-2502, 1998.

Shulman, G. I. Cellular mechanisms of insulin resistance. J. Clin. Invest. 106: 171-176, 2000.

Simon, C., G. Brandenberger, and M. Follenius. Ultradian oscillations of plasma glucose, insulin, and C-peptide in man during continuous enteral nutrition. J. Clin. Endocrinol. Metab. 64: 669-674, 1987.

Song, S. H., L. Kjems, R. Ritzel, S. M. McIntyre, M. L. Johnson, J. D. Veldhuis, and P. C. Butler. Pulsatile insulin secretion by human pancreatic islets. J. Clin. Endocrinol. Metab. 87: 213-221, 2002.

Song, S. H., S. S. McIntyre, H. Shah, J. D. Veldhuis, P. C. Hayes, and P. C. Butler. Direct measurement of pulsatile insulin secretion from the portal vein in human subjects. J. Clin. Endocrinol. Metab. 85: 4491-4499, 2000.

Sonnenberg, G. E., R. G. Hoffmann, C. P. Johnson, and A. H. Kissenbah. Low- and high-frequency insulin secretion pulses in normal subjects and pancreas transplant recipients: Role of extrinsic innervation. J. Clin. Invest. 90: 545-553, 1992.

Soop, M., J. Nygren, K. Brismar, A. Thorell, and O. Ljungqvist. The hyperinsulinaemic-euglycaemic glucose clamp: reproducibility and metabolic effects of prolonged insulin infusion in healthy subjects. Clin. Sci.98: 367-374, 2000.

Stagner, J. I., E. Samols, and G. C. Weir. Sustained oscillations of insulin, glucagon, and somatostatin from the isolated canine pancreas during exposure to a constant glucose concentration. J. Clin. Invest. 65: 939-942, 1980.

Strubbe, J. H., and A. B. Steffens. Neural control of insulin secretion. Horm. Metab. Res. 25: 507-512, 1993.

Sturis, J., A. J. Scheen, R. Leproult, K. S. Polonsky, and E. Van Cauter. 24-hour glucose profiles during continuous or oscillatory insulin infusion. J. Clin. Invest. 95: 1464-1471, 1995.

Sturis, J., E. Van Cauter, J. D. Blackman, and K. S. Polonsky. Entrainment of pulsatile insulin secretion by oscillatory glucose infusion. J. Clin. Invest. 87: 439-445, 1991.

Sturis, J., K. S. Polonsky, E. T. Shapiro, J. D. Blackman, N. M. O'Meara, and E. Van Cauter. Abnormalities in the ultradian oscillations of insulin secretion and glucose levels in Type 2 (non-insulin-dependent) diabetic patients. Diabetalogia. 35: 681-689, 1992.

Sturis, J., N. M. O'Meara, E. T. Shapiro, J. D. Blackman, H. Tallil, K. S. Polonsky, and E. Van Cauter. Differential effects of glucose stimulation upon rapid pulses and ultradian oscillations of insulin secretion. J. Clin. Endocrinol. Metab. 76: 895-901, 1993.

Sturis, J., W. L. Pugh, J. Tang, D. M. Ostrega, J. S. Polonsky, and K. S. Polonsky. Alterations in pulsatile insulin secretion in the Zucker diabetic fatty rat. Am. J. Physiol. 267: E250-E259, 1994.

Sweeny, G., J. Keen, R. Somwar, D. Konrad, R. Garg, and A. Klip. High leptin levels acutely inhibit insulin-stimulated glucose uptake without affecting glucose transporter 4 translocation in L6 rat skeletal muscle cells. Endocrinology 142:4806-4812, 2001.

Takayama, S., D. J. Legare, and W. W. Lautt. Dynamic control of the release of a hepatic insulin-sensitizing substance. Proc. West. Pharmacol. Soc. 42: 63-64, 1999.

Takayama, S., D. J. Legare, and W. W. Lautt. Dose-related atropine-induced insulin resistance: comparing intraportal versus intravenous administration. Proc. West. Pharmacol. Soc. 43: 33-34, 2000.

Thorburn, A. W., B. Gumbiner, F. Bulacan, P. Wallace, and R. R. Henry. Intracellular glucose oxidation and glycogen synthase activity are reduced in non-insulin-dependent (type II) diabetes independent of impaired of glucose uptake. J. Clin. Invest. 85: 522-529, 1990.

Thorens, B., G. C. Weir, J. L. Leahy, H. F. Lodish, and S. Bonner-Weir. Reduced expression of the liver/beta-cell glucose transporter isoform in glucose-insensitive pancreatic beta cells of diabetic rats. Proc. Natl. Acad. Sci. USA. 87: 6492-6496, 1990.

Tornheim, K. Are metabolic oscillations responsible for normal oscillatory insulin secretion? Diabetes. 46: 1375-1380, 1997.

Tremblay, F., C. Lavigne, H. Jacques, and A. Marette. Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (ζ/λ) activities. Diabetes 50: 1901-1910, 2001.

Ueki, K., T. Yamauchi, H. Tamemoto, K. Tobe, R. Yamamoto-Honda, Y. Kaburagi, Y. Akanuma, Y. Yazaki, S. Aizawa, R. Nagai, and T. Kadowaki. Restored insulin-sensitivity in IRS-1-deficient mice treated by adenovirus mediated gene therapy. J. Clin. Invest. 105(10): 1437-1445, 2000.

Verdin, E., M. Castillo, A. S. Luyckx, and P. J. Lefebvre. Similar metabolic effects of pulsatile versus continuous human insulin delivery during euglycemic, hyperinsulinemic glucose clamp in normal man. Diabetes 33: 1169-1174, 1984.

Ward, G.M., A. G. Marangou, J. D. Best, P.M. Aitken, and F.P. Alford. Effects of short term pulsatile and continuous insulin delivery on glucagon secretion and insulin secretion and action. Metabolism. 38(4): 297-302, 1989.

Ward, G. M., J. M. Walters, P. M. Aitken, J. D. Best, and F. P. Alford. Effects of prolonged pulsatile hyperinsulinemia in humans: Enhancement of insulin sensitivity. Diabetes. 39: 501-507, 1990.

Ward, W. K., D. C. Bolgiano, B. McKnight, J. B. Halter, and D. Porte Jr. Diminished B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. J. Clin. Invest. 74: 1318-1328, 1984.

Ward, W. K., E. C. LaCava, T. L. Paquette, J. C. Beard, B. J. Wallum, and D. Porte Jr. Disproportionate elevation of immunoreactive proinsulin in type 2 (non-insulin-dependent) diabetes mellitus and experimental insulin resistance. Diabetalogia. 30:698-702, 1987.

Westermark, P., and E. Wilander. The influence of amyloid deposits on the islet volume in maturity onset diabetes mellitus. Diabetologia. 15: 417-421, 1978.

Wiggam, M. I., S. J. Hunter, V. L. Armstrong, C. N. Ennis, B. Sheridan, A. B. Atkinson, and P. M. Bell. Insulin resistance and insulin pulsatility in essential hypertension. J. Hypertens. 18: 743-748, 2000.

World Health Organization. Definition, diagnosis, and classification of diabetes mellitus and its complications [online]. World Health Organization Department of Noncommunicable Diseases Surveillance, Geneva. © 1999. Available from http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf [cited 25 April, 2002]

Xie, H., and W. W. Lautt. Insulin resistance produced by hepatic denervation or muscarinic cholinergic blockade. Proc. West. Pharmacol. Soc. 37: 39-40, 1994.

Xie, H., and W. W. Lautt. Induction of insulin resistance by cholinergic blockade with atropine in the cat. J. Auton. Pharmacol. 15: 361-369, 1995a.

Xie, H., and W. W. Lautt. M_1 muscarinic receptor blockade causes insulin resistance in the cat. Proc. West. Pharmacol. Soc. 38: 83-84, 1995b.

Xie, H., and W. W. Lautt. Insulin resistance of skeletal muscle produced by hepatic parasympathetic interruption. Am. J. Physiol. 270:E858-E863, 1996a.

Xie, H., and W. W. Lautt. Insulin resistance caused by hepatic cholinergic interruption and reversed by acetylcholine administration. Am. J. Physiol. 271: E587-E592, 1996b.

Xie, H., L. Zhu, L. Zhang, D. J. Legare, and W. W. Lautt. Insulin sensitivity tested with a modified euglycemic technique in cats and rats. J. Pharmacol. Toxicol. Meth. 35: 77-82, 1996.

Xie, H., V. A. Tsybenko, M. V. Johnson, and W. W. Lautt. Insulin resistance of glucose response produced by hepatic denervations. Can. J. Physiol. Pharmacol. 71: 175-178, 1993.

Yoshikawa, H., Y. Tajiri, Y. Sako, T. Hashimoto, F. Umeda, and H. Nawata. Effects of free fatty acids on β-cell functions: a possible involvement of peroxisome proliferator-activated receptors α or pancreatic/duodenal homeobox. Metabolism. 50(5): 613-618, 2001.

Yoshioka, N., T. Kuzuya, A. Matsuda, M. Taniguchi, and Y. Iwamoto. Serum proinsulin levels at fasting and after oral glucose load in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia. 31: 355-360, 1988.

Zarkovic, M., J. Ciric, M. Stojanovic, Z. Penezic, B. Trbojevic, M. Drezgic, and M. Nesovic. Effect of insulin sensitivity on pulsatile insulin secretion. Eur. J. Endocrinol. 141: 494-501, 1999.

Zarkovic, M., J. Ciric, Z. Penezic, B. Trbojevic, and M. Drezgic. Effect of weight loss on the pulsatile insulin secretion. J. Clin. Endocrinol. Metab. 85: 3673-3677, 2000.

Zierath, J. R., A. Krook, and H. Wallberg-Henriksson. Insulin action and insulin resistance in human skeletal muscle. Diabetologia. 43:821-835, 2000.

Zierath, J. R., K. L. Houseknecht, L. Gnudi, and B. B. Kahn. High-fat feeding impairs insulin-stimulated GLUT4 recruitment via an early-insulin signaling defect. Diabetes. 46: 215-223, 1997.