# HISS-DEPENDENT CONTROL OF INSULIN SENSITIVITY IN HEALTH AND DISEASE

By

**Parissa Sadri**

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

**DOCTOR OF PHILOSOPHY**

Department of Pharmacology and Therapeutics
Faculty of Medicine
University of Manitoba

© January 2001

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
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.

0-612-62664-4

Canada

**THE UNIVERSITY OF MANITOBA**

**FACULTY OF GRADUATE STUDIES**

*****

**COPYRIGHT PERMISSION PAGE**

**HISS-Dependent Control of Insulin Sensitivity in Health and Disease**

**BY**

**Parissa Sadri**

**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**

**Doctor of Philosophy**

**PARISSA SADRI ©2001**

**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 Dissertations Abstracts International 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 husband, Ramin, with all my love.*

## ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Dr. Wayne Lautt for his confidence in me and for all his support, encouragement, and patience. Thank you for your guidance in science and life.

I would like to thank my committee members Dr. Frank Burczynski, Dr. John McNeil, Dr. Jerry Minuk, and Dr. Berry Rosser for their support and encouragement.

I am grateful to Dallas Legare for providing me with an excellent technical training and all his help throughout the years. I am also grateful to Karen Sanders for her assistance in applications, letters, and manuscript preparations.

I would like to thank previous and current students in the lab, Helen Wang, Chao Han, Jodi Schoen, and Maria Genovey for their friendship and support.

To my parents, Behjat Shafai and Dr. Daryoush Sadri, without you none of this would be possible, thank you.

Last, but not least, to my wonderful husband, Ramin, who has enriched my life with love and friendship, I am grateful for your endless support, encouragement and patience.

## TABLE OF CONTENTS

## LIST OF FIGURES

## ABSTRACT

It has been previously demonstrated that the hepatic parasympathetic nerves have a permissive role regulating the ability of insulin to release a hepatic insulin sensitizing substance (HISS) from the liver (Xie et al. 1993; Xie and Lautt 1995a, 1995b). This thesis work demonstrates the involvement of other permissive factors in the release of HISS from the liver in response to insulin in health and a pathological condition in rats. Furthermore, the glucose disposal effect of insulin and insulin-like growth factor-1 (IGF-1) were compared in health and a pathological condition.

To measure insulin sensitivity, we have developed a new rapid insulin sensitivity test (RIST). The RIST is a reproducible test and requires a bolus infusion of insulin (50 mU/kg) over 5 minutes. The amount of glucose infused during the RIST to maintain euglycemia is referred to as the RIST index.

The release of HISS was shown to be dependent on the production of nitric oxide (NO) and prostaglandins (PGs) in the liver. Intraportal, but not intravenous, administration of a NO synthase (NOS) antagonist and a cyclooxygenase antagonist produced significant HISS-dependent insulin resistance (HDIR). Administration of a NO donor after NOS antagonism or hepatic parasympathetic denervation reversed HDIR and restored insulin sensitivity.

IGF-1 (200 μg/kg) had a similar glucose disposal effect to insulin (50 mU/kg) but its effect did not involve the release of HISS from the liver.

In an experimental model of fetal alcohol exposure (FAE), male rats were prenatally exposed to ethanol through the maternal water supply containing 0%, 5%,

10%, 15% and 20% ethanol and female rats were prenatally exposed to 0%, 15% and 20% ethanol. FAE caused a dose-dependent increase in HDIR in both males and females in young adulthood. However, FAE did not affect the IGF-1 sensitivity.

In conclusion, the permissive role of the hepatic parasympathetic nerves to the action of insulin to release HISS from the liver is through NO and PGs production in the liver. The glucose disposal effect of IGF-1 does not involve the release of HISS from the liver. FAE causes dose-dependent HDIR in young adulthood but does not alter the glucose disposal effect of IGF-1.

# Chapter 1

# Introduction

## 1.1 Background

The research team in which I undertook my studies has previously demonstrated a series of studies consistent with the hypothesis that insulin causes release of a hepatic insulin sensitizing substance (HISS) from the liver (Fig 1). The release of HISS is also dependent on the permissive role of the hepatic parasympathetic nerves. After its release from the liver, HISS enters the blood and enhances glucose uptake at the skeletal muscle. In the absence of HISS immediate and severe insulin resistance occurs.

### 1.1.1 Involvement of the hepatic parasympathetic nerves in glucose regulation

*1.1.1.1 Hepatic parasympathetic interruption*

Xie et al. (1993) have shown that surgical denervation of the hepatic anterior plexus results in insulin resistance, which is not further worsened by denervation of the posterior nerve plexus or bilateral vagotomy. The surgical denervation of the anterior plexus eliminates parasympathetic nerves as well as some sympathetic and afferent nerves. The presence of parasympathetic nerves in this nerve bundle is shown by the rapid decrease in hepatic glucose output produced by electrical stimulation after elimination of the sympathetic nerve effects by administration of both $\alpha$- and $\beta$-adrenergic antagonists (Gardemann and Jungermann 1986) or pretreatment with 6-hydroxydopamine to destroy the sympathetic nerve terminals (Lautt and Wong 1978).

Intraportal administration of atropine, a nonselective muscarinic antagonist, resulted in insulin resistance of a similar magnitude to that produced by surgical

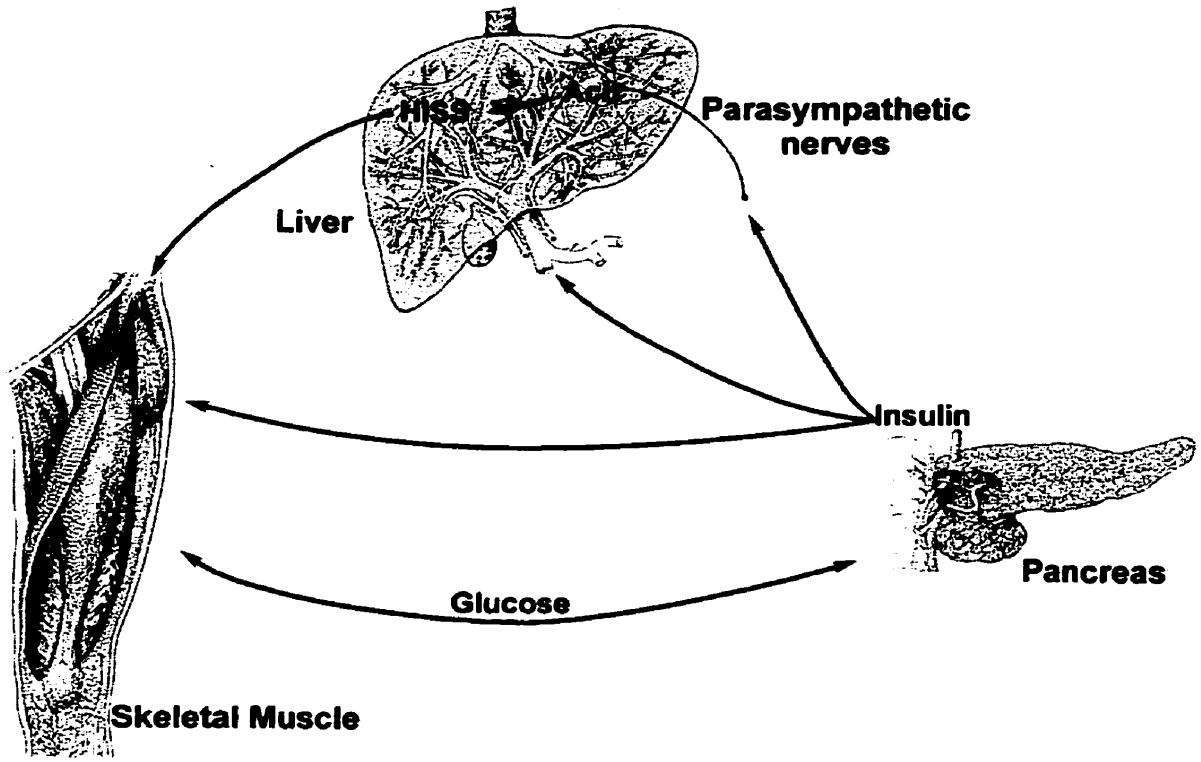



**Figure 1.** The HISS hypothesis: Following a meal, insulin is released from the pancreas and the rise in insulin concentration in the portal blood causes the release of a hepatic insulin sensitizing substance (HISS) from the liver. The hepatic parasympathetic nerve nerves, by releasing acetylcholine which acts on the muscarinic receptors in the liver, play a permissive role in allowing insulin to trigger HISS release. HISS stimulates glucose uptake in the skeletal muscle either by a synergic reaction with insulin or by an additive effect to that of insulin.

denervation of the liver (Xie and Lautt 1995*a*, 1996*a*). The response to denervation was not further potentiated by atropine nor was the response to atropine further potentiated by surgically denervating the liver. Thus, the hepatic denervation and intraportal atropine produced a similar degree of insulin resistance and the effects were not additive. The observation that, in the absence of functional hepatic parasympathetic nerves, atropine produces no additional effects suggested that the effects were produced through the same mechanism.

Intraportal administration of atropine produced a dose-related inhibition of insulin effect, reaching a maximal inhibition of insulin action at 4 μmol/kg (3 mg/kg) in cats (Xie and Lautt 1995*a*). A similar degree of insulin resistance was achieved with 100 nmol/kg of the $M_1$ muscarinic selective antagonist, pirenzepine, and with 1.0 μmol/kg of the $M_2$ muscarinic antagonist, methoctramine. The data suggest that the response may be mediated by the $M_1$ muscarinic receptor subtype (Xie and Lautt 1995*b*).

*1.1.1.2 Parasympathetic-dependent and -independent response*

To measure insulin sensitivity, our group has developed a new rapid insulin sensitivity test (RIST, explained in detail in the section on the RIST) (Xie et al. 1996; Lautt et al. 1998). The RIST index is the amount of glucose infused during the test to maintain baseline euglycemia. The magnitude of the change in RIST index after surgical denervation or atropine administration depended on the control RIST index. A high RIST index in the control response showed a large decrease in response following denervation or atropine (Xie and Lautt 1996*b*). Thus, the animals showing the greatest insulin sensitivity showed the greatest dependence on hepatic parasympathetic nerves.

Conversely, a small control RIST index resulted in minor or insignificant insulin resistance after denervation or atropine administration. This relationship was indicated by the linear regression of the control response plotted against the reduction in glucose required after denervation or atropine administration (Xie and Lautt 1996*b*). The intercept on the *x*-axis divided the insulin response into two parts, the hepatic parasympathetic nerve-independent effect and the hepatic parasympathetic nerve-dependent effect. The interpretation was that one component of insulin response is independent of the hepatic parasympathetic nerves and is quantitated by the amount of glucose required to be infused to maintain euglycemia after denervation or atropine. Insulin effectiveness greater than this level appeared to be dependent on the hepatic parasympathetic nerve function. Thus, the difference between a control RIST and the RIST index after surgical hepatic denervation or atropine is used to determine the hepatic parasympathetic component of insulin action (Xie and Lautt 1995*a*, 1996*a*).

*1.1.1.3 Site of insulin resistance*

To locate the site of insulin resistance, the arteriovenous glucose gradients were measured across the liver, hind limbs, and splanchnic organs in control state and after hepatic parasympathetic denervation or atropine administration (Xie and Lautt 1996*a*). The intestines showed no response to insulin either before or after induction of insulin resistance. Similarly, the hepatic response to insulin was not significantly changed after induction of insulin resistance. However, the glucose uptake across the hind limbs was impaired by interruption of the hepatic parasympathetic reflex (Xie and Lautt 1996*a*). This suggests that, although the liver is the target organ for induction of the insulin

resistance, the resistance occurs in peripheral tissues. Glucose uptake in response to insulin is largely dependent upon uptake in skin, bone, and skeletal muscle (Hom et al. 1984) but the skeletal muscle is believed to be the main tissue responsible for insulin-induced glucose uptake in humans and rodents (Curtis-Prior et al. 1969; Baron et al. 1988). At this time we cannot conclude which specific tissues are involved, but the large muscle mass of the hind limbs has led us to tentatively conclude that skeletal muscle is at least one of the tissues that is regulated by release of an, as yet, unidentified hepatic hormone, tentatively referred to as the hepatic insulin sensitizing substance (HISS).

*1.1.1.4 Restoring insulin sensitivity*

Administration of intraportal, but not intravenous, acetylcholine (Ach, 2.5 μg/kg/min) completely restored insulin sensitivity to normal after hepatic denervation (Xie and Lautt 1996*b*). The fact that the intraportal, but not the intravenous, dose of Ach restored insulin sensitivity suggests that the liver is the target organ. In addition, this is evidence that the hepatic parasympathetic-skeletal muscle connection that controls insulin sensitivity is hormonal and not neural. If the connection between the skeletal muscle and the liver was neural, then this connection was abolished after the denervation of the liver producing insulin resistance, and intraportal Ach infusion could have not reversed insulin resistance by a parasympathetic pathway since these nerves had been cut.

Intraportal Ach administration 15-30 min prior to insulin infusion was without notable effect on arterial glucose levels but the response to insulin was restored (Xie and Lautt 1996*b*). Thus, insulin is required for the release of HISS from the liver and providing Ach to the liver without the presence of insulin does not release HISS. We

propose that the hepatic parasympathetic nerves, by providing the background parasympathetic tone to the liver, play a permissive role in allowing insulin to trigger HISS release from the liver. Therefore, if the nerve tone is high, insulin causes greater HISS release.

Ach administration to an animal with intact parasympathetic nerves did not increase insulin sensitivity further than the control response, suggesting that full insulin activation of the release of HISS occurs with the intact parasympathetic tone (Xie and Lautt 1996*b*).

*1.1.1.5 HISS-dependent and -independent insulin action*

Following hepatic parasympathetic denervation or atropine administration the parasympathetic tone was inhibited and, therefore, the release of HISS ceased leading to HISS-dependent insulin resistance (HDIR). Thus, the difference between the control RIST index and the RIST index after interference with the hepatic parasympathetic nerves reflects the component of insulin action that is dependent on HISS (Lautt 1999). The HISS-independent component of insulin action is the RIST index remaining after blockade of HISS release. Intraportal Ach administration restored the parasympathetic-dependent tone and, thus, the release of HISS and completely restored HISS-dependent insulin action.

## 1.2 The overall objectives and hypotheses

The overall objective of this work is to further investigate the regulation of the release of HISS from the liver in health and in a diseased state. In the next chapters of this thesis I will examine different hypotheses, described below, to investigate the regulation of HISS release from the liver as assessed from the pharmacodynamic actions of HISS quantitated using the RIST.

The hypotheses tested are: (1) the hepatic parasympathetic-dependent release of HISS requires nitric oxide (NO) production in the liver (2) inhibition of prostaglandins (PGs) synthesis in the liver causes insulin resistance; (3) the hepatic parasympathetic-dependent release of HISS is not affected by the insulin-like growth factor-1 (IGF-1); (4) fetal alcohol exposure (FAE) leads to HDIR in adulthood.

The objectives are: (1) to demonstrate that NO production in the liver is essential for release of HISS from the liver; (2) to reverse the insulin resistance caused by hepatic NO inhibition by means of a NO donor; (3) to demonstrate that HISS production and release is also dependent on PGs production in the liver; (4) to demonstrate that IGF-1 does not trigger the release of HISS; (5) to demonstrate that insulin resistance in FAE is caused by interruption of the release of HISS from the liver.

### 1.3 Measurements of insulin sensitivity

Many tests have been developed to measure insulin sensitivity *in vivo*, the most widely used are: the insulin tolerance test, the oral glucose tolerance test, the minimal model with the intravenous glucose tolerance test, and the euglycemic hyperinsulinemic clamp. Our laboratory has also developed a rapid insulin sensitivity test (RIST) to measure insulin sensitivity *in vivo*.

#### 1.3.1 The Insulin-tolerance test (ITT)

One of the first methods developed to evaluate insulin sensitivity *in vivo* was the ITT, which is based on the measurement of the rate of decay of plasma glucose level after a bolus injection of regular insulin (0.1 U/kg body weight). On plotting plasma glucose concentrations measured every 5 min from 10 to 40 min after the intravenous insulin injection on a semilogarithmic scale, a reasonably linear decline is observed in most cases. The slope of this line ($k_{ITT}$ ) is used to determine insulin sensitivity. A blunted decline in the plasma glucose concentration was demonstrated in insulin-resistant subjects (Alford et al. 1971) and a significant improvement of $k_{ITT}$ was shown after a one-year hypocaloric diet in obese subjects (Beck-Nielson et al.1979). We have also shown that after denervation of the liver in cats, the hypoglycemic response to 100 mU/kg of insulin was significantly reduced in both the early (0-30 min) rate of decline and the total hypoglycemic response (Xie et al. 1993). The inhibition of the control response after denervation using ITT was of a similar magnitude to that produced using a rapid insulin sensitivity test (RIST, described below) index (Xie et al. 1996).

The ITT assumes that the glucose system is a single compartment, from which insulin accelerates the net disappearance of the substrate both by promoting its uptake into target tissues and by shutting off endogenous production (Ferrannini and Mari 1998). By assuming a glucose distribution volume (usually, 200-250 ml/kg) a clearance rate can be calculated by measuring the difference between the arterial and venous glucose concentrations and the flow rate. The $k_{ITT}$ index or the glucose clearance is found to be correlated to clamp-derived estimates of insulin sensitivity (Bonora et al. 1989). The $k_{ITT}$ value is dependent on the time interval over which it is calculated because glucose disappearance is not a mono-exponential but rather a multi-exponential process (Ferrannini and Pilo 1979). Also, the plasma insulin levels achieved with the bolus used in this test are pharmacological (ranging from 150 nmol/l soon after injection to 15 nmol/l towards the end).

The principal problem with this test is hypoglycemia, which can cause neurological and cardiovascular side effects. Also, hypoglycemic counterregulatory hormonal responses (epinephrine, glucagon, cortisol, and growth hormone) will antagonize insulin action, therefore contaminating the insulin sensitivity estimate (Tritos and Mantzoros 1997). However, since hypoglycemia reaches its nadir by 20 min and counterreglulatory hormone release occurs after that time, the method using the rate of decline in the first 15 min is useful.

### 1.3.2 The oral glucose tolerance test (OGTT)

In the 1930s, Himsworth introduced the first standard approach to measuring insulin sensitivity *in vivo* (Himsworth 1936). Two OGTTs were performed: the first with,

and the second without, a concomitant intravenous insulin injection. Insulin sensitivity was expressed as the ratio of the areas under the respective glucose tolerance curves. By using this technique it was shown type 1 diabetic subjects are more sensitive to exogenous insulin than type 2 diabetic patients.

The OGTT is now the most commonly used method to evaluate whole body glucose tolerance *in vivo*. After an overnight fast, subjects ingest 75 g of D-glucose. Blood samples are then taken at 30 min intervals for 120 min for measurements of plasma glucose and insulin concentrations. The insulin is released after ingestion of glucose and the plasma glucose and insulin levels are used to assess the adequacy of insulin secretion. Inappropriate elevated plasma insulin levels with a normal or elevated plasma glucose level during the OGTT has been accepted as evidence for insulin resistance (Reaven et al. 1983). Fasting insulin levels above 50-70 μU/ml or peak (post-OGTT) insulin levels above 350 μU/ml suggest severe insulin resistance, in contrast to the fasting serum insulin levels below 20 μU/ml or peak (post-OGTT) insulin levels below 150 μU/ml observed in normal subjects (Tritos and Mantzoros 1997).

During the OGTT, a closed-loop relationship exists between the β cells of the pancreas and the insulin-sensitive tissues (Bergman et al. 1985). Changes in insulin secretion are reflected in the blood glucose concentration; conversely, variation in glucose production or utilization cause a rapid β cell secretory compensation. In this closed loop, glucose utilization, production and insulin secretion are all variables. It is because of this closed loop relationship that it is possible only under limited conditions to interpret OGTT results to infer the physiologic status of the β cells or the extrapancreatic tissues (Bergman et al. 1985). Alterations in insulin secretion and/or clearance can alter

insulin concentrations in the absence of any change in insulin action. The OGTT is unable to verify the contributions of insulin-dependent and -independent glucose utilization and β cell responsiveness.

Another problem with OGTT is the absorption of the glucose load from the gut. The peak value of glucose level is influenced in part by gastric emptying time and the absorption rate in the intestine.

### 1.3.3 The intravenous glucose tolerance test (IVGTT) with the minimal modeling

In this test a bolus of glucose (300 mg/kg) is injected intravenously to stimulate endogenous insulin secretion. Blood samples are then collected at intervals over 3 h, for estimation of plasma insulin and glucose levels. The IVGTT has advantages over OGTT in that glucose absorption is no longer a significant variable and gut factors are not involved.

At its earliest stage, before the insulin assay had become available, insulin sensitivity was judged from the slope of the decay curve of plasma glucose concentration (plotted on a semilogarithmic graph paper) measured after an intravenous glucose injection in minutes (the glucose disappearance constant or $K_{IVGTT}$) (Lundback 1962). However, this test could not determine whether a difference in glucose disappearance rate was due to a difference in insulin sensitivity or in glucose-induced insulin response. The minimal model, proposed by Bergman *et al.* (Bergman et al. 1979), is a development of the IVGTT and it accounts for both insulin and glucose concentrations during IVGTT by using a simplified matemathical representation of the glucose-insulin relationship.

The model describes the glucose disappearance curve with two differential equations (Bergman et al. 1985). One equation represents glucose kinetics independent of the incremental insulin effect, thus assuming a single-compartment model for glucose distribution. The other equation represents the insulin effect, which is assumed to take place in a compartment remote from plasma (the effect compartment). The insulin-sensitivity index of the minimal model represents the link between insulin levels in the effect compartment and glucose disappearance from the glucose compartment. The fractional disappearance rate ($min^{-1}$) from the glucose compartment is assumed to be the linear function ($S_G+S_IZ$), where $Z$ is the increment over the basal value of insulin concentration in the effect compartment, $S_I$ (min/pmol/l) is the insulin-sensitivity index and $S_G$ ($min^{-1}$) is the glucose effectiveness (Bergman et al. 1985). By using the measured insulin concentration as the input to the model, insulin sensitivity and glucose effectiveness are estimated by least-squares fitting of the IVGTT glucose concentration profile throughout the test.

A limitation of the minimal model analysis of the IVGTT is that it requires a detectable rise in the insulin concentrations. As a result, the minimal model analysis of the IVGTT cannot be used for insulin-deficient or insulin-resistant subjects, because no effect of insulin on glucose disappearance rate is seen. In this situation, administration of exogenous insulin is necessary (Finegood et al. 1990) for an insulin response. However, even when there is a normal pancreatic function, IVGTT alone may not provide an adequate insulin response and an intravenous bolus of tolbutamide (to stimulate endogenous glucose secretion) or insulin is used to give a second peak, a greater overall insulin response and greater precision in the estimation of insulin sensitivity. Both insulin

and tolbutamide are administered 20 min after the injection of the glucose. Also, administration of a high dose of glucose (500 mg/kg) without tolbutamide has been shown to be more precise and simple (Swan et al. 1994).

There are several disadvantages with this test. First, the experiment itself is not very simple: blood is sampled frequently (22 times in the original protocol) for long periods of time (3 h) and hypoglycemia might occur late into the test and cause counter-regulatory reactions. Second, giving an injection of exogenous insulin after the glucose bolus improves the estimation of $S_I$ but modifies late-phase endogenous insulin secretion. Third, by using exogenous insulin and or tolbutamide the ability to measure β cell responsiveness to glucose is lost. Fourth, the model is unable to differentiate between the insulin-independent and -dependent mechanisms of glucose clearance to hepatic and peripheral components. Clearly, the measure of insulin sensitivity $S_I$ will reflect insulin action at both these sites, and isotopic determination is necessary to discriminate the two effects (Cobelli et al. 1986; Avogaro et al. 1989; Caumo et al. 1991). Finally, some of the assumptions about the body glucose system are not physiological (Groop et al. 1993). For example, the minimal model assumes that glucose kinetics is mono-compartmental, and that insulin action takes place in a "remote" compartment (Bergman et al. 1979). The former assumption is clearly false (Cobelli et al. 1986); the latter is true for the peripheral tissue but does not accurately describe the effect of insulin on the liver (Groop et al. 1993).

### 1.3.4 The euglycemic hyperinsulinemic clamp technique

The euglycemic hyperinsulinemic clamp methodology, the gold standard in the assessment of insulin sensitivity, was first introduced by Andres *et al.* in 1966. The clamp has been extensively studied and developed by DeFronzo *et al.* since the publication of their famous paper in 1979.

In this technique, after an overnight fast, exogenous insulin is administered as a prime followed by a constant infusion at a rate designed to maintain a pre-set hyperinsulinemic plateau (usually raising plasma insulin levels to either 100 or 1000 μU/ml); the plasma glucose concentration is clamped at the normal (5 mmol/l, euglycemic) or at the previously measured basal glucose level by means of an exogenous infusion of glucose. When a steady-state is presumably achieved (in at least 120 min), the exogenous glucose infusion rate (M) is assumed to equal the amount of glucose disposed of by peripheral tissues. Thus M is considered an indicator of insulin action at the periphery, mainly the skeletal muscle (Del Prato et al. 1986, DeFronzo et al. 1979). Even though in strict terms the glucose infusion rate never reaches a steady-state, its average value during the final 40-60 min of the study is used as an insulin sensitivity index. Usually the duration of the test is 2-3 h. The endogenous glucose release (mainly hepatic) is assumed to be suppressed, by exogenous insulin infusion, after 30-50 min of the insulin infusion. However, in most clamp studies somatostatin is administered to suppress endogenous insulin and glucagon secretions.

Arterial blood is used for glucose measurements. In human studies, arterialized venous blood is drained to measure glucose concentrations at 5-10 min intervals during

the test. This is commonly accomplished by retrograde cannulation of the wrist of the hand vein while heating the hand at 60-70 °C.

There are many disadvantages with this test. First, the conditions created with the clamp are non-physiological because insulin is infused at a constant rate for 2-3 h. Also, prolonged hyperinsulinemia may induce rate-limiting metabolic enzymes and downregulate insulin receptors and receptor affinity (Nuttall et al. 1972; Insel et al. 1980). Second, at the end of a clamp study, especially with a high insulin dose, the subject's plasma glucose level must be monitored for some time because the hypoglycemic effect extends beyond the return of plasma insulin level to its baseline value. Third, it is assumed that a steady-state is reached during the clamp, however, it has been shown that a steady-state glucose level cannot be maintained at a constant glucose infusion rate using constant insulin infusion (Deberne et al. 1981; Bergman et al. 1985). Also, It has been demonstrated that glucose utilization during the prolonged euglycemic clamp was significantly increased over time (Deberne et al. 1981), thus, the clamp cannot be used more than once in the same subject on the same day. However, insulin sensitivity does not change over time using the RIST (see the section on the RIST) as demonstrated by time controls conducted in rats and cats (Fig 4, Xie et al. 1996; Lautt et al. 1998). Finally, although similar results were obtained in two successive studies separated by 3-4 weeks (DeFronzo et al. 1979), significantly different values of glucose clearance rate were observed when two euglycemic clamp tests were performed on two separate days in the same subject (Greenfield et al. 1981).

### 1.3.5 The rapid insulin sensitivity test (RIST)

The RIST is a highly reproducible and very sensitive index of insulin action and it was first described by our laboratory in 1996 (Xie et al. 1996). Minor modifications and further technical aspects of the RIST have been recently developed, and a standard operating procedure has been described (Lautt et al. 1998). The RIST quantifies the insulin effectiveness of a bolus amount of insulin, rather than a continuous infusion of insulin, as used in the classical euglycemic clamp technique (DeFronzo et al. 1979). The RIST index is the amount of glucose needed to maintain euglycemia following administration of a pulse (5 min infusion) of insulin. The RIST is, therefore, a transient euglycemic clamp. The RIST was the primary tool used in the thesis work. The surgical procedures and the RIST methodology are explained in detail below for future references.

#### *1.3.5.1 Animal preparation*

Sprague-Dawley rats were fed standard laboratory rat food and subjected to controlled lighting (lights on 0600-1800) for at least 3 days. All experiments began at 0900. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg, Somnotol, MTC Pharmaceuticals, Mississauga, Ont.). The temperature was maintained at 37.5 ± 0.5 °C by means of a temperature controlled surgical table and a lamp over the table. The body temperature was monitored with a rectal probe thermometer (HI8857, Hanna Instruments or Fisher Scientific). The rats were heparinized with 100 IU/kg heparin.

*1.3.5.2 Surgical procedures*

The arterial-venous loop as shown in Fig 2 was primed with a heparin-saline solution (200 IU/ml). The silicone sleeve on the venous side of the loop was clamped. The right femoral artery was cannulated with the arterial side of the loop. The silicone sleeve on the arterial side of the loop was clamped, and the venous side was opened. The right femoral vein was cannulated with the venous side of the arterial-venous loop. The clamp on the arterial side was opened, allowing blood to flow through the loop. Arterial blood continuously flows through the loop to the venous side. Arterial blood samples were taken from the arterial side of the loop via puncture of the silicone sleeve. Insulin and other pharmacological agents (except glucose) were administered intravenously by puncturing the sleeve on the venous side of the loop. Arterial blood pressure was monitored via the arterial-venous loop by clamping the silicon sleeve on the venous side of the loop. One of the advantages of using this loop is that blood samples can be taken directly from a moving stream of blood with no need to wash or flush sampling catheters. To maintain anesthesia throughout the experiment, the left jugular vein was cannulated with a catheter (polyethylene tubing, PE-50) with a continuous infusion of pentobarbital solution (1.0 ml/100g of body weight/h, 1.08 mg/ml). Another catheter (polyethylene tubing, PE-50), preloaded with a D-glucose-saline solution (100 mg/ml), was inserted (with a 23 gauge needle at the delivery end) into a silicone sleeve of the anesthetic catheter. The D-glucose catheter was connected to a continuously variable infusion pump (Harvard Apparatus). Spontaneous respiration was allowed through a tracheal tube (polyethylene tubing, PE-205 or PE-240, based on the size of the animal). For any intraportal drug administration, after the laparotomy, the portal vein was cannulated with

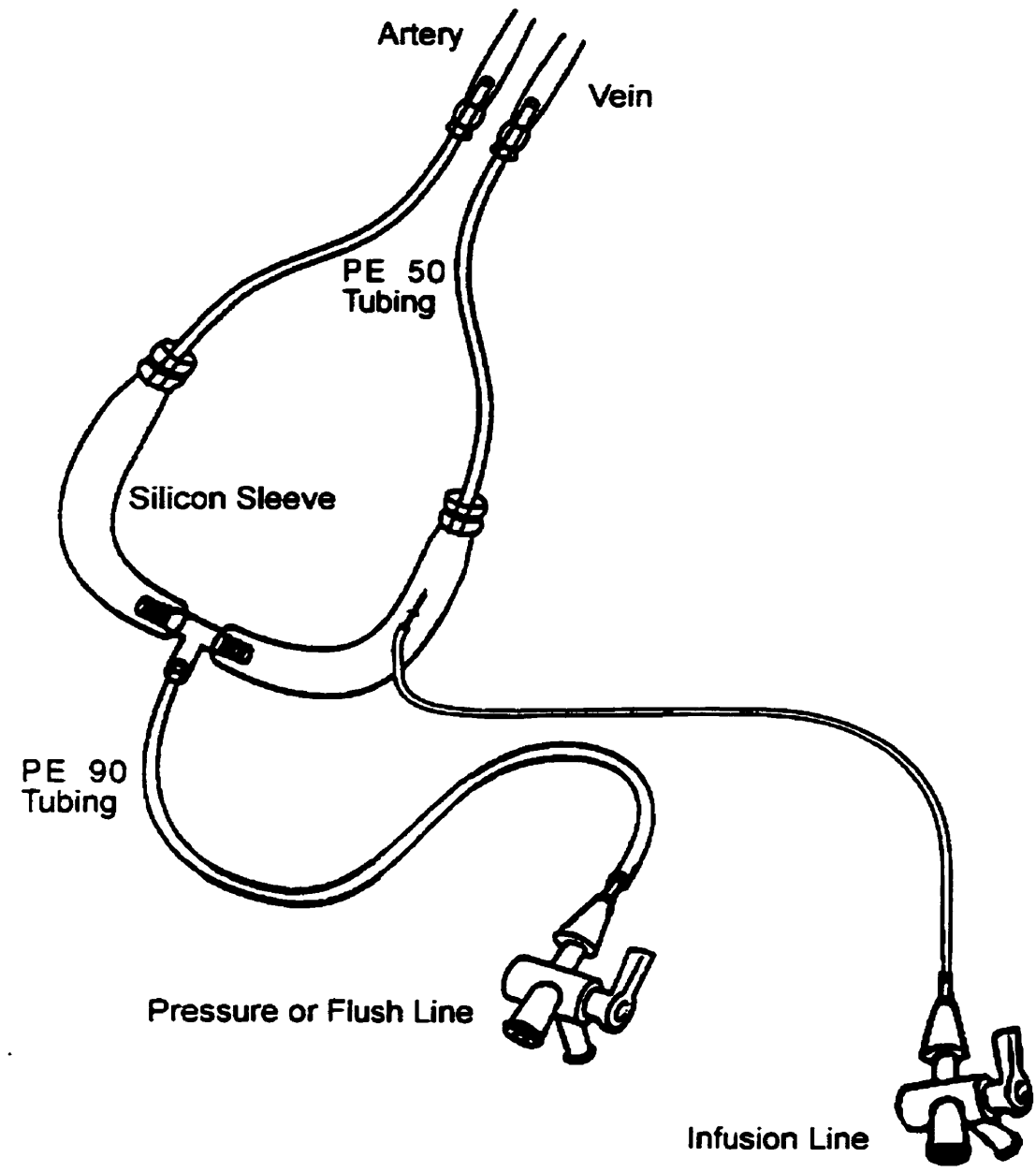



**Figure 2.** The arterial-venous loop. The loop shunts blood from the femoral artery to the femoral vein and allows multiple rapid arterial sampling and intravenous drug and fluid administration by needle puncture into the silicone sleeve. Brief occlusion of the venous outflow allows for monitoring arterial pressure.

a 24G (OPTIVA™, Johnson & Johnson Medical Inc.) intravenous catheter.

*1.3.5.3 The RIST methodology*

After surgical preparations, the animals were allowed to stabilize for at least 30 min. prior to the first arterial glucose sampling. Arterial blood samples were then taken every 5 min form the arterial side of the loop, and glucose concentrations were immediately analyzed by the oxidase method with a glucose analyzer (model 27, Yellow Springs Instruments) until three successive stable glucose concentrations were obtained. The mean of these three concentrations is referred to as the basal glucose level. After the basal glucose level was determined, insulin was infused intravenously at a dose of 50 mU/kg over a 5 min period (in 0.5 ml saline at 0.1 ml/min). To avoid hypoglycemia, the glucose infusion (5 mg/kg/min) was started 1 min after insulin infusion. On the basis of the arterial glucose concentrations measured at 2-min intervals, the infusion rate of the glucose pump was adjusted to maintain euglycemia. The amount of glucose infused over 30 min after insulin administration represents the magnitude of insulin sensitivity and is referred to as the RIST index. The time line for RIST is shown in Fig 3.

The RIST has shown to be a reproducible test (Xie et al. 1996; Lautt et al. 1998). In four rats, four consecutive RISTs (Fig. 4) were carried out with a restablization period between each test assessed by samples taken at 5 min intervals until three stable consecutive basal glucose levels were determined (Lautt et al. 1998). The RIST showed no tendency for change with time and the mean coefficient of variance was 8.8 ± 1.5%. The time between RISTs was usually 30-45 min with all four tests being completed

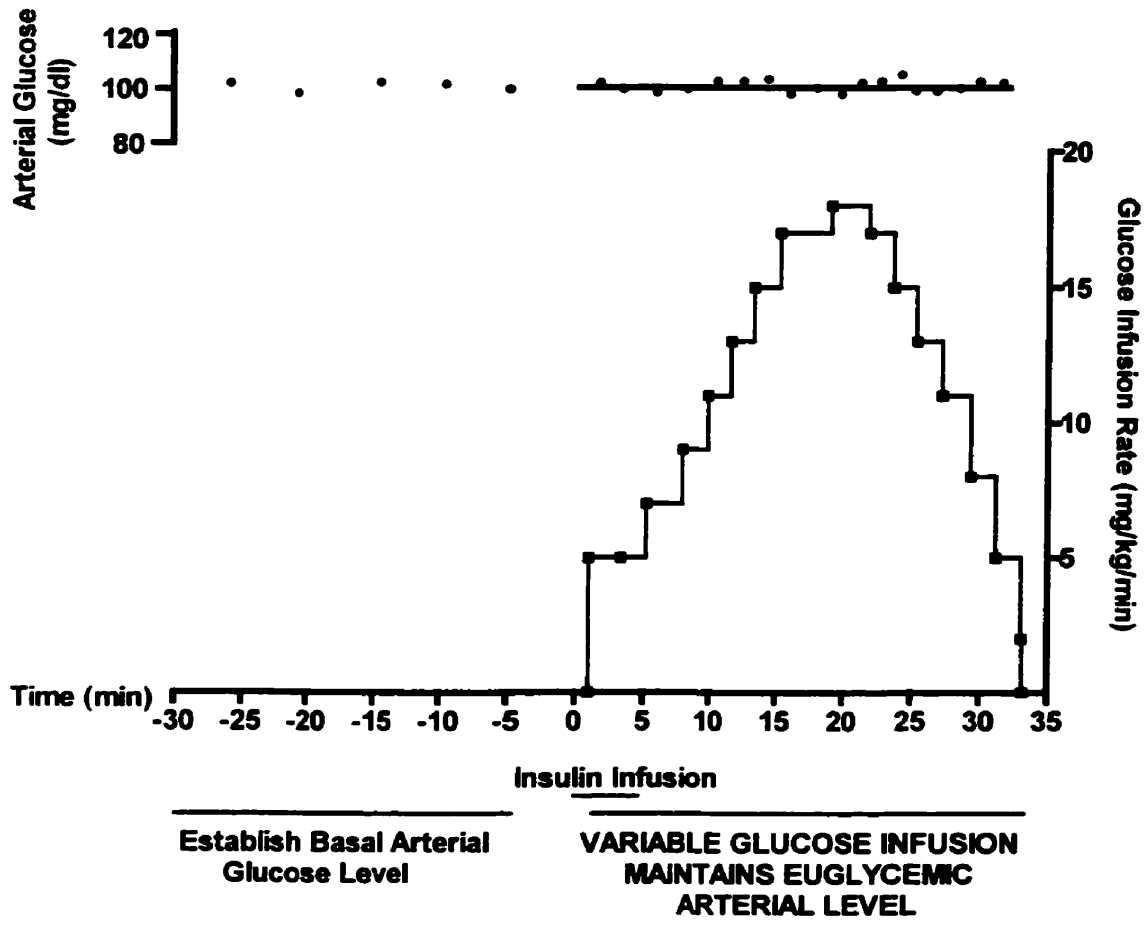



**Figure 3.** The RIST time line. Three stable arterial glucose levels taken at 5 min intervals establish the euglycemic baseline. Insulin is intravenously infused over 5 min with glucose infusion and the first arterial glucose sample commencing after 1 min of insulin infusion. A variable glucose infusion is adjusted to maintain euglycemia based on the arterial samples taken at 2 min intervals throughout the test period (30 min). The RIST index is the total amount of glucose infused during the test period of 30 min.

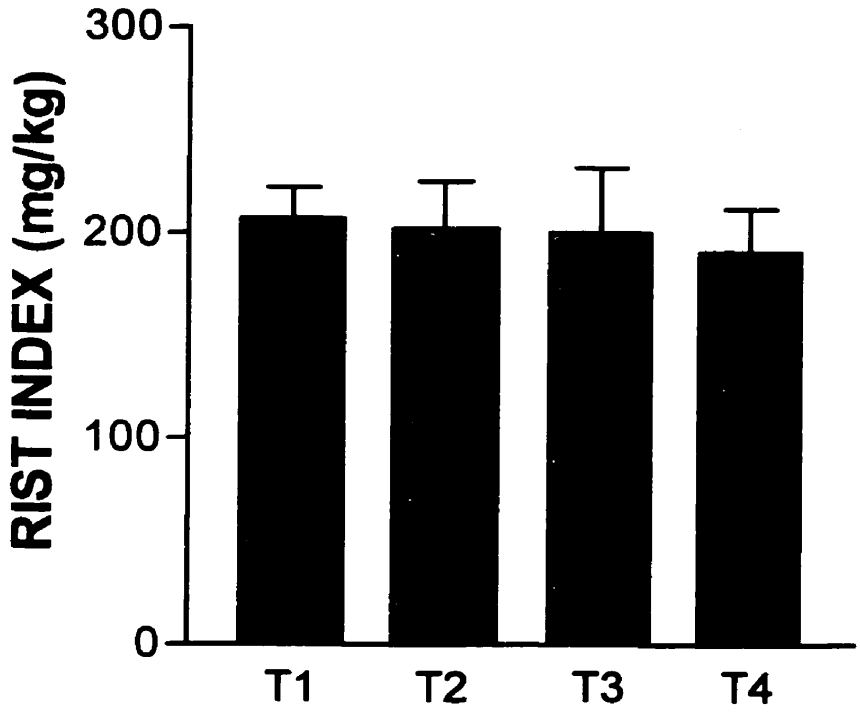



**Figure 4.** Four consecutive RISTs (50 mU/kg) in the same animal. Values are means ± SE. n=4, NS.

within 4-5 h. Basal glucose levels (mg%) stabilized between tests and were 112.2 ± 5.8, 115.5 ± 11.8, 122.4 ± 9.0, and 120.1 ± 11.4 (not significantly different) prior to each test. The RIST indices (mg/kg) did not significantly change over time and were 234.5 ± 5.0, 228.3 ± 21.4, 248.1 ± 17.0, and 220.9 ± 10.5. The blood pressure was stable throughout and between each test (98.8 ± 10.9, 93.8 ± 12.3, 90.0 ± 15.3, 93.3 ± 19.5 mmHg, respectively).

Similar time controls were also conducted in cats (Xie et al.1996). In five cats five consecutive RISTs were carried out with a mean coefficient of variance of 3.0 ± 0.5%. Arterial levels of glucose, glucagon, insulin, and catecholamines were not different between the RISTs and the level of elevation of insulin was the same during a RIST in the normal state and after blockade of the parasympathetic reflex (Xie and Lautt 1995*a*). The ability to produce multiple tests in the same animal in the same day with stable response offers clear advantages over the euglycemic clamp methodologies that utilize prolonged infusion of insulin.

# Chapter 2

## HISS release is dependent on the hepatic production of nitric oxide

### 2.1 Introduction

It was previously demonstrated that insulin-mediated release of HISS from the liver is dependent upon the permissive role of the hepatic parasympathetic nerves (chapter1, Xie and Lautt 1996*a*,*b*). Since many cholinergic effects are mediated through nitric oxide (NO) (Yamamoto et al. 1998), we tested the hypothesis that this parasympathetic-dependent control of HISS release is also mediated through NO production in the liver.

#### *2.1.1 Synthesis and function*

NO a free radical gas, is synthesized by the enzyme, nitric oxide synthase (NOS), through incorporation of molecular oxygen into L-arginine. NOS requires cofactors such as calcium, calmodulin, tetrahydrobiopterin, NADPH, FAD, FMN, and heme for synthesis of NO (Andrews and Mayer 1999). NO acts as an intracellular messenger molecule regulating vascular tone (Vallance and Collier 1994), platelet activation (Andrews and Mayer 1999), and immune and inflammatory responses (Moilanen and Vapaatalo 1995) and acts as a neurotransmitter in the brain and in the periphery in non-adrenergic non-cholinergic (NANC) (Sanders and Ward 1992; Vallance and Collier 1994; Moilanen and Vapaatalo 1995) nerves and also in some parasympathetic (Iadecola et al. 1993; Keast 1992; Sheng et al. 1992; Vizzard et al. 1993) and sympathetic nerves (Aderson et al. 1995; Li et al. 1995*b*). NO is also synthesized in high amounts by activated macrophages and acts as a cytotoxic molecule to kill bacteria, viruses, and

protozoa as well as tumor cells (Moilanen and Vapaatalo 1995).

To evaluate the involvement of NO in the parasympathetic-dependent release of HISS, we used two NOS antagonists, N-nitro-L-arginine methyl ester (L-NAME) and N-monoethyl-L-arginine (L-NMMA). L-Arginine, the substrate for NOS, was administered to reverse the insulin resistance produced by L-NAME. 3- Morpholinosydnonimine (SIN-1), a NO donor, was infused to reverse the insulin resistance produced by L-NMMA or parasympathectomy of the liver. Insulin sensitivity was measured by using the

## 2.2 Materials and Methods

Male Sprague-Dawley rats were fed *ad-libitum* with standard laboratory rat food. Animal preparation, surgical procedures, and the RIST methodology are explained in detail in chapter 1.

*RIST in control and after L-NAME at doses 2.5 mg/kg and 5.0 mg/kg intravenously.* After the control RIST was performed, L-NAME, at a dose 2.5 mg/kg (n=12) or 5.0 mg/kg (n=17), was infused intravenously over 5 min. A stable basal arterial glucose concentration was determined, and a RIST was performed. After 30 min of restablization, basal arterial glucose concentrations were determined, and a second post L-NAME RIST was repeated to measure the duration of action of each dose.

*RIST in control, after intravenous or intraportal L-NAME infusion, and after atropine.* The RIST index was determined before and after L-NAME (1.0 mg/kg) was infused either intravenously (n=5) or intraportally (n=5) over 5 min. Atropine (3.0 mg/kg) was infused intraportally over 5 min, and the RIST was repeated.

*RIST in control and after L-NMMA infusion (n=3).* After the control RIST was performed, L-NMMA (0.73 mg/kg) was infused intraportally over 5 min. After the second RIST, the animal was allowed to restabilize for 30 min. Basal arterial glucose concentrations were determined, and another post-L-NMMA RIST was repeated to measure the duration of the action of the dose.

*RIST in control, after surgical denervation, and after L-NMMA infusion (n=3).* After the control RIST was performed, the nerve bundles around the common hepatic

artery were cut, the animal was allowed to stabilize, and the RIST was repeated. L-NMMA (0.73 mg/kg) was intravenously infused, and the RIST was performed.

*RIST in control, after L-NAME and after L-arginine infusion (n=6).* After a control RIST was performed, L-NAME (5 mg/kg) was infused intravenously over 5 min. After the second RIST, L-arginine (50 mg/kg) was infused intraportally, and the RIST was repeated.

. *RIST in control and after L-arginine infusion (n=4).* After a control RIST was performed, L-arginine (50 mg/kg) was infused intraportally, and insulin sensitivity was measured by the RIST.

*RIST in control, after L-NMMA, and after intraportal or intravenous SIN-1 infusion.* After the control RIST was performed, L-NMMA (0.73 mg/kg) was infused intraportally over 5 min. After the second RIST, SIN-1 (5.0 mg/kg) was infused either intraportally (n=5) or intravenously (n=4) over 2 min. Insulin sensitivity was measured by the RIST.

*RIST in control, after L-NMMA, and after intraportal SIN-1 infusion (n=5).* After the control RIST was performed, L-NMMA (0.73 mg/kg) was intraportally infused over 5 min. After the second RIST, SIN-1 (10.0 mg/kg) was infused intraportally over 2 min and the RIST was repeated.

*RIST in control, after surgical denervation, and after intraportal SIN-1 infusion (n=6).* After the control RIST was performed, the nerve bundles around the common hepatic artery were cut and the animal was allowed to stabilize. After the second RIST, SIN-1 (10.0 mg/kg) was infused intraportally over 2 min, and the RIST was repeated.

*RIST in control, after atropine, and after intraportal SIN-1 infusion.* After the control RIST was performed, atropine (3.0 mg/kg) was infused intraportally over 5 min. After the second RIST, SIN-1 (5.0 mg/kg, n=2 or 10.0 mg/kg, n=6) was infused intraportally over 2 min and the RIST was repeated.

*RIST in control and after intraportal (n=1) or intravenous (n=1) SIN-1 infusion.* After a control RIST was performed, SIN-1 was infused either intraportally or intravenously over 2 min, and insulin sensitivity was measured by the RIST.

*Drugs.* L-NAME, L-NMMA, L-arginine, atropine, and D-glucose were purchased from Sigma Chemical (St. Louis, MO). SIN-1 was purchased from Alexis (San Diego, CA). The human insulin was obtained from Eli Lilly (Indianapolis, IN). All the chemicals were dissolved in saline.

*Data analysis.* Data were analyzed using repeated-measures analysis of variance followed by Tukey-Kramer multiple comparison test in each group or, when applicable, paired and unpaired Student's *t*-tests. The analyzed data were expressed as means ± SE throughout. Some results were analyzed using linear regression analysis. 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 all protocols were approved by an ethics committee on animal care at the University of Manitoba.

## 2.3 Results

The index used to express insulin sensitivity is the total amount of glucose (mg/kg) infused over 30 min after insulin (50 mU/kg) administration in order to maintain euglycemia at the baseline level and is referred to as the RIST index.

*RIST after intravenous L-NAME infusion.* The control RIST index was 178.5 ± 16.5 mg/kg. L-NAME at dose 2.5 mg/kg (n=12) significantly reduced the RIST index to 78.1 ± 9.8 mg/kg and caused a 56.2 ± 6.3 % inhibition of the control response. However after 2 h when the RIST was repeated again, the amount of glucose required to maintain the euglycemia was 168.4 ± 38.7 mg/kg which was not significantly different from the control RIST (Fig. 5). The blood pressure increased after L-NAME infusion from 107.6 ± 4.7 mmHg to 133.4 ± 5.3 mmHg, but after 2 h it decreased to 110.4 ± 10.7 mmHg. The basal glucose was similar before each RIST (111.8 ± 4.2 mg/ml, 90.4 ± 5.0 mg/ml, 110.3 ± 3.0 mg/ml, respectively). In another set of animals (n=17), L-NAME at dose 5.0 mg/kg significantly reduced the control RIST index (226.9 ± 15.3 mg/kg) to 93.7 ± 8.7 mg/kg and caused a 55.3 ± 5.3% inhibition of the control response. Two hours after administration, the RIST index was 75.8 ± 16.0 mg/kg with 66.5 ± 7.5% inhibition of the control response (Fig. 5). After L-NAME infusion, the blood pressure increased from 107.6 ± 4.3 mmHg to 123.5 ± 6.0 mmHg and stayed at the same level, 120.0 ± 7.5 mmHg, after 2 h. The basal glucose was similar before each RIST (117.9 ± 3.3 mg/ml, 107.4 ± 3.4 mg/ml, 115.6 ± 5.3 mg/ml, respectively). Thus both 2.5 mg/kg and 5.0 mg/kg L-NAME produce similar insulin resistance but the duration of action is less than 2 h with the low dose but was maintained for at least 2 h for the high dose.

The change from control after L-NAME administration at 2.5 mg/kg (n=12) and 5.0 mg/kg (n=17), was plotted against the control RIST index (mg/kg) (Fig. 15, *top*). The regression line has an *x*-intercept of 79.5 and a slope of 0.94 ± 0.11. This relationship is interpreted to quantitate the HISS-dependent and HISS-independent components of insulin action. Rats showing the greatest response to insulin show the greatest HISS-dependent component of insulin action.

*RIST after intravenous verses intraportal L-NAME.* The control RIST index (n=5) of 224.1 ± 23.5 mg/kg was not significantly reduced (177.9 ± 21.2 mg/kg) after intravenous infusion of L-NAME (1.0 mg/kg). However, the intraportal administration of atropine, a non-selective muscarinic antagonist, markedly reduced the RIST index to 95.3 ± 14.6 mg/kg and caused a 56.0 ± 8.7% inhibition of the control RIST (Fig. 6). The blood pressure was constant throughout the experiment (96.0 ± 4.5 mmHg in control, 100.0 ± 11.5 mmHg after L-NAME and 93.0 ± 8.6 mmHg after atropine). In the second set of animals (n=5), the control RIST index (238.8 ± 16.4 mg/kg) was significantly reduced by intraportal L-NAME (1.0 mg/kg) administration (105.8 ± 10.8 mg/kg), causing a 54.9 ± 5.2% inhibition of the control response. However, administration of intraportal atropine caused a further significant reduction in RIST index (78.5 ± 14.2 mg/kg) (Fig. 6). The blood pressure increased from 99.0 ± 1.1 mmHg to 114.0 ± 4.5 mmHg after L-NAME, but it decreased to 104 ±8.0 mmHg after atropine, consistent with data from the 2.5 mg/kg dose, showing effects wearing off by the time of the second (atropine) test. Thus, intraportal but not intravenous L-NAME at the 1.0 mg/kg dose caused significant insulin resistance.

*RIST after L-NMMA (n=3).* Administration of intraportal L-NMMA (0.73 mg/kg) significantly reduced the RIST index from 236.8 ± 37.6 mg/kg to 123.1 ± 8.9 mg/kg (45.6 ± 12.1% inhibition of the control RIST) (Fig. 8). The blood pressure was constant throughout the experiment (96.7 ± 4.1 mmHg in control, 93.3 ± 14.3 mmHg after L-NMMA before the RIST, and 90.0 ± 9.4 mmHg before the final RIST). After 2 h, RIST was repeated again and the amount of glucose required to maintain the euglycemia was 76.1 ± 14.8 mg/kg (65.1 ± 13.0% inhibition of the control RIST). Thus, intraportal L-NMMA produces insulin resistance that is maintained for 2 h.

Administration of equimolar dose of L-NMMA (0.73 mg/kg, n=15, polled from other experiments) to L-NAME (1.0 mg/kg) produced a similar degree of inhibition of the control RIST (50.0 ± 3.4%) (Fig. 7). Thus, both L-NMMA and L-NAME cause insulin resistance by blockade of NOS in the liver.

*RIST after denervation and L-NMMA (n=3).* Surgical denervation of the hepatic anterior plexus significantly reduced the RIST index from 228.3 ± 13.8 mg/kg to 86.0 ± 7.4 mg/kg and produced 62.0 ± 4.8% inhibition (Fig. 9). Infusion of intraportal L-NMMA (0.73 mg/kg) did not cause a further significant reduction in RIST index (80.8 ± 10.5 mg/kg).

The change from control RIST index after intraportal atropine (n=6) or hepatic denervation (n=10) plotted against control RIST index (mg/kg) (Fig. 15, *bottom*) shows a $x$-intercept of 88.0 and a slope of 1.0 ± 0.1. Insulin's action has a parasympathetic-dependent and a parasympathetic-independent component, and the higher the RIST index the more the response is inhibited by atropine or hepatic parasympathetic denervation.

*RIST after L-NAME and L-arginine (n=6).* After L-NAME (5.0 mg/kg) infusion, the RIST index was significantly reduced from 237.0 ± 26.1 mg/kg to 99.0 ± 12.2 mg/kg, and a 55.4 ± 8.8% inhibition of control RIST was produced. L-arginine (50 mg/kg ipv) administration did not reverse the inhibition by L-NAME (53.8 ± 7.1%) (Fig. 10).

*RIST after L-arginine.* After the control RIST, administration of intravenous L-arginine (50 mg/kg, n=5) significantly inhibited the control response by 48.8 ± 8.2% (Fig. 10).

*RIST after L-NMMA and intraportal or intravenous SIN-1.* Intraportal infusion of L-NMMA (0.73 mg/kg, n=4) significantly reduced the RIST index from 218.4 ± 6.6 mg/kg to 88.4 ± 21.6 mg/kg (59.6 ± 9.7% inhibition of the control RIST). Intravenous administration of SIN-1 (5.0 mg/kg) did not reverse inhibition caused by L-NMMA (59.0 ± 7.2% inhibition ) (Fig. 11). In the second set of animals (n=5), the control RIST index was 236.9 ± 20.0 mg/kg. Intraportal infusion of L-NMMA (0.73 mg/kg) caused significant insulin resistance, and reduced the RIST index to 129.7 ± 14.3 mg/kg and caused 54.5 ± 2.0% inhibition (Fig. 11). Intraportal SIN-1 (5.0 mg/kg) partially reversed the inhibition caused by L-NMMA (24.0 ± 11.6%). Thus, NO production in the liver can partially reverse insulin resistance caused by NOS antagonism.

*RIST after L-NMMA and intraportal SIN-1 (n=5).* Intraportal infusion of L-NMMA (0.73 mg/kg) significantly reduced the RIST index from 221.34 ± 30.9 mg/kg to 99.3 ± 20.9 mg/kg (55.5 ± 7.0% inhibition of the control RIST). Intraportal SIN-1 (10.0 mg/kg) completely reversed the inhibition caused by L-NMMA (0.6 ± 5.8%) (Fig 12). Thus, higher NO production in the liver can completely reverse insulin resistance caused by NOS antagonism.

*RIST after denervation and intraportal SIN-1 (n=6).* Surgical denervation of the hepatic anterior plexus significantly reduced the RIST index from 208.3 ± 15.0 mg/kg to 87.7 ± 10.3 mg/kg (56.4 ± 6.7% inhibition of the control RIST). Intraportal SIN-1 (10.0 mg/kg) completely reversed the inhibition caused by denervation (3.8 ± 10.4/%) (Fig 13). Thus, NO production in the liver can reverse insulin resistance caused by surgical denervation of the liver.

*RIST after atropine and intraportal SIN-1.* Administration of intraportal atropine (3.0 mg/kg) significantly reduced the RIST index from 265.9 ± 32.8 mg/kg to 111.7 ± 51.4 mg/kg (58.9 ± 14.3% inhibition of the control RIST, n=2) in one group of rats and from 259.6 ± 31.6 mg/kg to 89.8 ± 11.0 mg/kg (63.1 ± 6.2% inhibition of the control RIST, n=6) in another group of rats. However, intraportal administration of SIN-1 at either 5.0 (n=2) or 10.0 (n=6) mg/kg did not reverse the inhibition caused by atropine (47.4 ± 1.9%, 60.2 ± 5.2%, respectively) (Fig 14). Thus, NO production in the liver cannot reverse insulin resistance caused by muscarinic receptor blockade.

*RIST after intraportal (n=1) or intravenous (n=1) SIN-1.* Intraportal (231.5 mg/kg before and 243.9 mg/kg after SIN-1) or intravenous (250.3 mg/kg before 267.0 mg/kg after SIN-1) administration of SIN-1 did not significantly change the RIST index (not shown). Thus, full parasympathetic-dependent activation of NO production occurs in response to insulin.

*HISS dynamic curves.* The average glucose infusion rate (mg/kg/min) at 0.1 min intervals throughout the test were plotted in the control RIST and after HISS blockade with either intraportal L-NMMA (0.73 mg/kg, n=23, pooled) administration, surgical denervation of the liver (n=10, pooled), or intravenous atropine (3.0 mg/kg, n=8, pooled)

administration (Fig. 16, *left graphs*). The average post-maneuver RIST curve was subtracted from the average control RIST curve in each group (Fig. 16, *right graphs*). The difference between the two curves provided a dynamic curve that is attributed to HISS action. HISS action started at 3-4 min after onset of insulin administration and continued until the end of the RIST.

The RIST curves after administration of SIN-1 (10.0 mg/kg, ipv) following the L-NMMA infusion or surgical denervation of the liver were also plotted to examine dynamics of HISS release after reversal of insulin resistance by SIN-1 (Figs. 17,18). Providing NO to the liver restored HISS release.

## 2.4 Discussion

Previous studies (chapter1, Xie and Lautt 1995*a*, 1996*a,b*) are consistent with the hypothesis that the animals respond to insulin by the hepatic parasympathetic-dependent release of HISS from the liver that enhances glucose uptake at the skeletal muscle. Surgical or pharmacological ablation of the hepatic parasympathetic nerves leads to HISS-dependent insulin resistance (HDIR). Intraportal, but not intravenous, Ach is capable of reversing the HDIR caused by denervation. This chapter demonstrates that the hepatic parasympathetic-dependent control of insulin action is mediated through hepatic NO production and that hepatic NOS antagonism and hepatic denervation produce HDIR that is reversible by providing NO to the liver using a NO donor. The hepatic parasympathetic-dependent release of HISS is concluded to be NO-mediated. Insulin sensitivity was measured by using the RIST (described in detail in chapter 1).

### *2.4.1 Nitric oxide synthase inhibition*

Administration of L-NAME, a NOS antagonist, intravenously at 2.5 mg/kg and 5.0 mg/kg caused significant and similar degrees of insulin resistance. However, the effect of the low dose of L-NAME wore off within 1 h whereas the high dose effect lasted for more than 2 h (Fig. 5).

To confirm the site of action of L-NAME, intraportal infusion of a L-NAME dose (1.0 mg/kg) was compared with intravenous infusion of the same dose. The intraportal, but not intravenous, dose caused significant insulin resistance. The observation that L-NAME caused more insulin resistance when administered intraportally (Fig. 6) shows that the site of action of L-NAME is the liver.

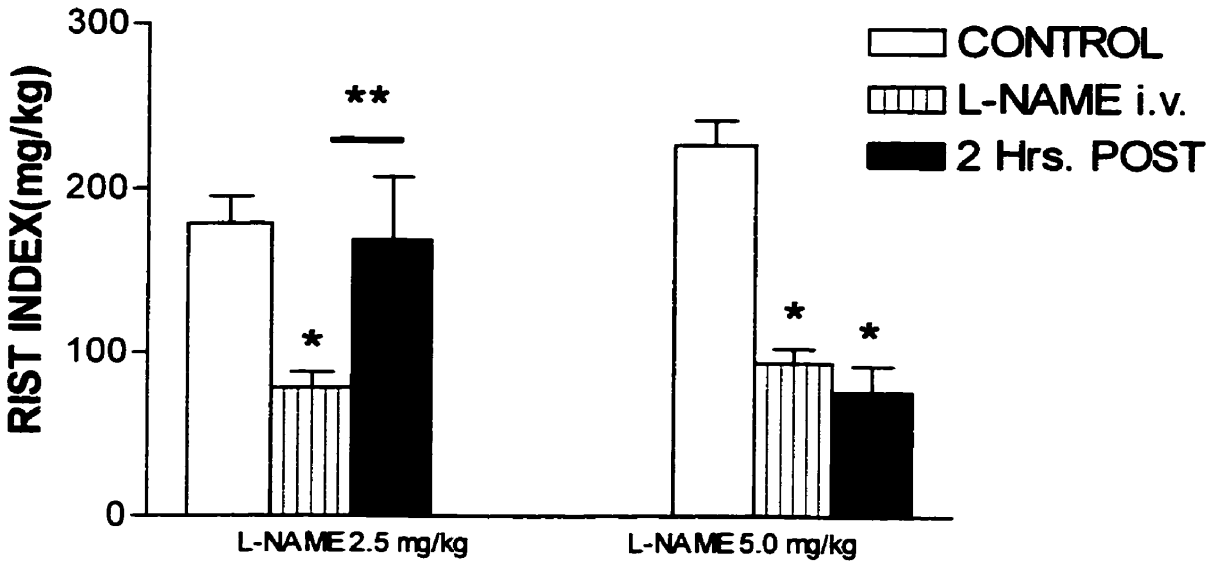



**Figure 5.** *Left*: RIST index (mg/kg) before and after intravenous L-NAME 2.5 mg/kg administration and 2 h post L-NAME. Values are means ± SE; n=12. **P*<0.001, ** *P*<0.01. *Right*: RIST index (mg/kg) in control, after intravenous L-NAME 5.0 mg/kg administration, and 2 h post L-NAME. Values are means ± SE; n=17. **P*<0.001. Insulin resistance produced by the low dose wore off by 2 h but was well maintained by the higher dose.

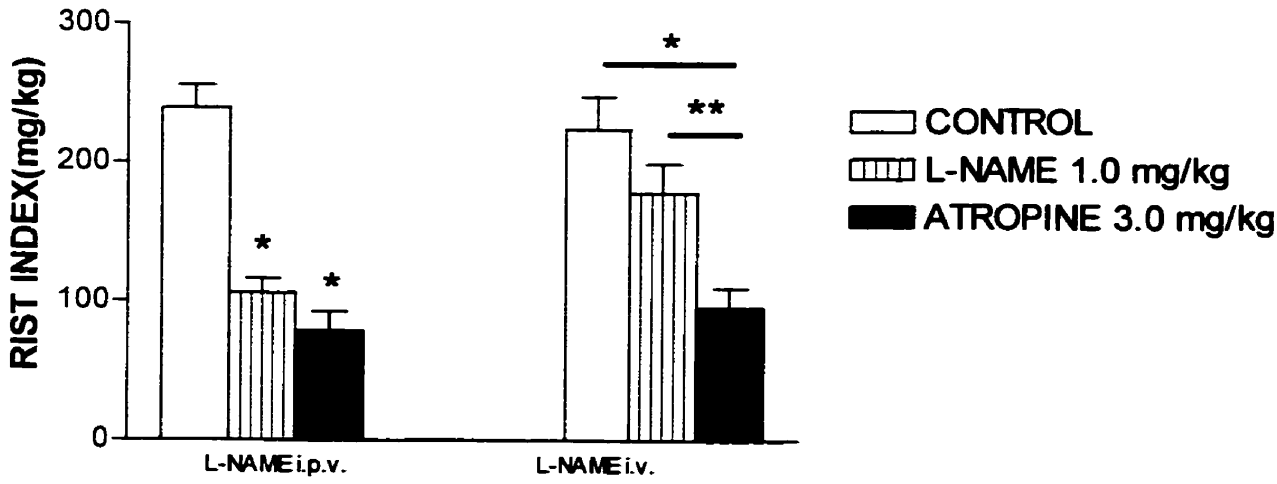



**Figure 6.** RIST index in control, after intraportal (n=5) or intravenous (n=5) L-NAME (1.0 mg/kg) administration, and after intraportal atropine (3.0 mg/kg) administration. Values are means ± SE. *$P$<0.001, ** $P$<0.05. Insulin resistance was produced by the intraportal but not intravenous route.

It had been suggested that L-NAME is both a NOS inhibitor and a muscarinic receptor antagonist (Buxton et al. 1993). To confirm that the insulin resistance we observed was a result of NOS antagonism and not muscarinic blockade, we used L-NMMA, another NOS antagonist that lacks antimuscarinic effect. L-NAME and L-NMMA have the same potency *in vitro* (Rees et al. 1990). We used an equimolar dose of L-NMMA (0.73 mg/kg) to the dose of 1.0 mg/kg L-NAME. Both L-NAME and L-NMMA produced insulin resistance to a similar degree (Fig. 7). Thus, insulin resistance produced by intraportal infusion of L-NAME appears to be only through inhibition of hepatic NOS. The insulin resistance caused by L-NMMA lasted for more than 2 h (Fig. 8), which was a duration of blockade longer than that achieved by 2.5 mg/kg L-NAME. The data do not support the idea that L-NAME has a significant additional antimuscarinic effect *in vivo*, thus, indicating that both L-NAME and L-NMMA are suitable tools for the present study.

Reports from other investigators (Baron et al. 1995) suggest that inhibition of NOS by L-NMMA causes a reduction in skeletal muscle perfusion, and this has been suggested as the mechanism of insulin resistance. In our experiments, intraportal L-NMMA (0.73 mg/kg) did not result in hypertension (arterial pressure of 90 ± 3.8 mmHg in control and 84.3 ± 4.6 mmHg after L-NMMA); however significant insulin resistance occurred (Fig. 7). Oral administration of L-NAME caused hypertension but not insulin resistance (Swislocki et al. 1995), suggesting that insulin resistance is not a result of vascular effects but of a fundamental metabolic disorder. Surgical hepatic denervation significantly reduced insulin sensitivity, and subsequent NOS inhibition with L-NMMA did not cause additional insulin resistance (Fig. 9). If the NOS antagonist effect was

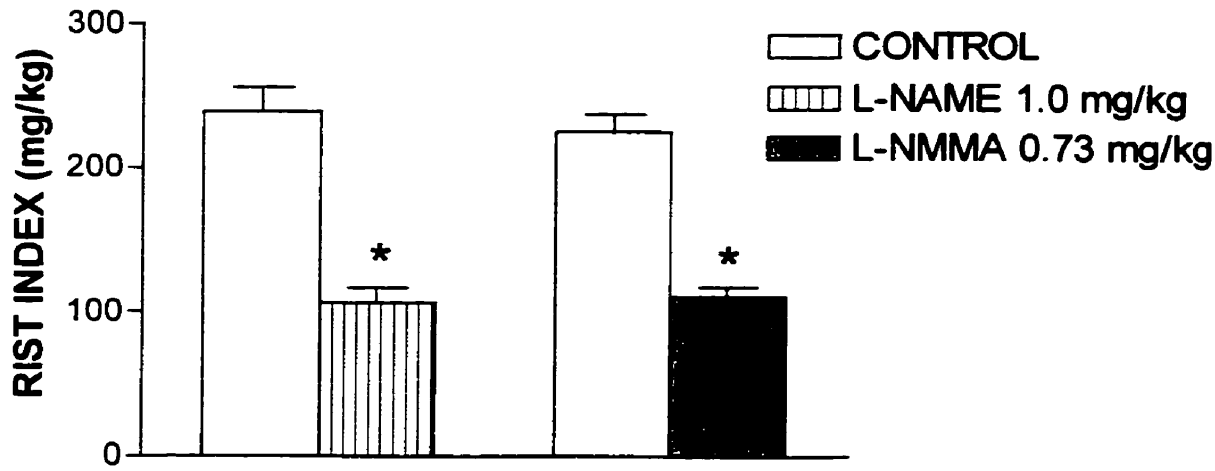



**Figure 7.** RIST index in control, after intraportal L-NAME (1.0 mg/kg, n=5) or L-NMMA (0.73 mg/kg, n=15) administration. Values are means ± SE. **P*<0.05. L-NAME and L-NMMA both produced insulin resistance through inhibition of NOS in the liver.

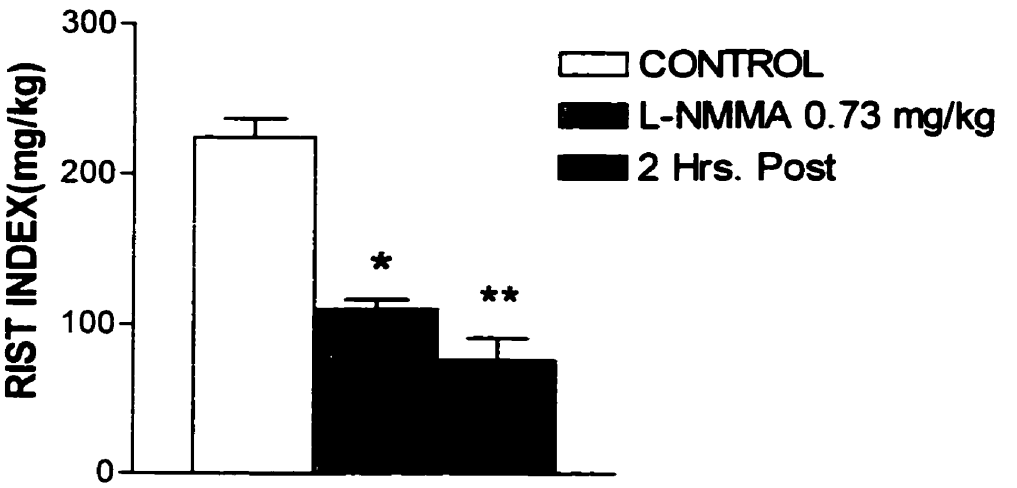



**Figure 8.** RIST index in control, after intraportal L-NMMA (0.73 mg/kg) administration, and 2 h post L-NMMA. Values are means ± SE; n=3. *$P$<0.05, ** $P$<0.01. Insulin resistance was still maintained after 2 h.

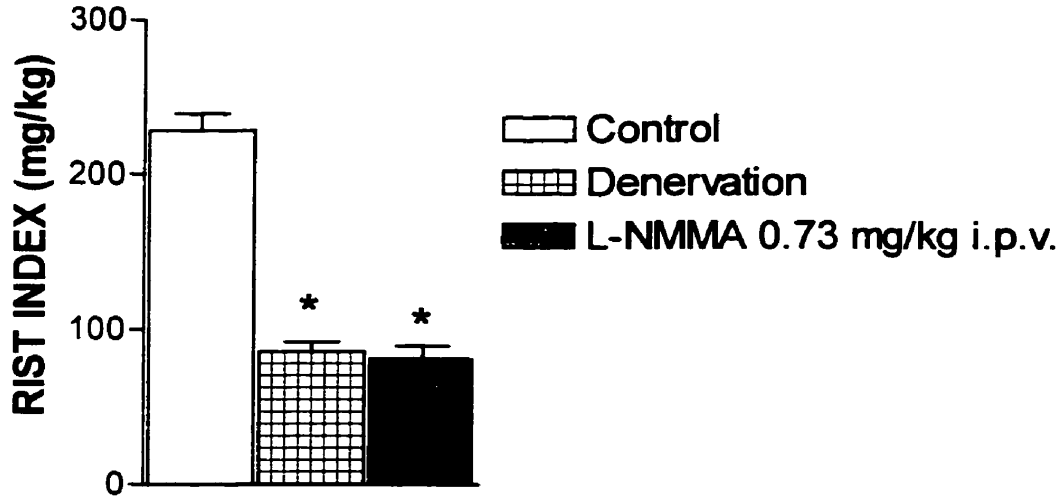



**Figure 9.** RIST index in control, after hepatic parasympathetic denervation, and after intraportal L-NMMA (0.73 mg/kg) administration. Values are means ± SE; n=3. *$P$<0.001. Insulin resistance produced by denervation was not made worse by addition of NOS antagonism.

secondary to direct peripheral effects, it should have been additive to the effects of liver denervation. This observation suggests that hepatic parasympathetic interruption by surgery or NOS inhibition in the liver caused insulin resistance by interruption of the same pathway.

We, therefore, suggest that insulin resistance caused by NOS antagonism is not a result of reduction in skeletal muscle perfusion but rather is caused by blockade of the parasympathetic-dependent release of HISS.

*2.4.2 Vasodilatory effect of insulin*

It has been proposed that insulin-mediated vasodilation, through NO release by the endothelium (Vallance and Collier 1994; Steinberg et al. 1994; Scherrer et al. 1994; Chen et al. 1996; Cardillo et al.1999), increases glucose uptake in skeletal muscle (Baron and Brechtel 1993; Pitre et al. 1996). Moreover, it has been suggested that the insulin-mediated increases in skeletal muscle blood flow are impaired in obesity (Lassko et al. 1990), type 2 diabetes (Lassko et al. 1992), and hypertension (Baron et al. 1993, 1995, and 1996) and that this defect may contribute to insulin resistance in these disease conditions. However, others have shown that insulin-mediated vasodilation, and vasodilation *per se*, is not a primary determinant of muscle glucose uptake (Scherrer et al. 1994; Mijare and Jensen 1995; Raitakari et al. 1996; Utriainen et al. 1996,1997; Natali et al. 1998). Scherrer et al. (1994) have shown that L-NMMA, when infused into one arm, reduces forearm blood flow and increases blood pressure, but does not alter the whole-body glucose uptake. Natali et al. (1998) demonstrated that increasing forearm blood flow with sodium nitroprusside in obese hypertensive patients does not improve

insulin sensitivity of forearm tissues. Mijares et al. (1995) concluded that after a mixed meal, skeletal muscle blood flow does not increase enough for blood flow to be a major contributor to glucose uptake. It has also been shown (Utriainen et al. 1997) that in type 2 diabetics cellular glucose uptake is impaired despite normal insulin effects on muscle blood flow, flow dispersion, and redirection of blood flow to glucose using-areas. The effect of insulin on blood flow is controversial. Some investigators report increased blood flow only at high physiological (DeFronzo et al. 1985; Mandarini et al. 1996; Bonadonna et al. 1996) or supraphysiological insulin concentrations (Pitre et al. 1996). Also, increases in blood flow are only seen after infusing insulin for long periods of time (Laasko et al. 1990; Baron et al. 1991; Yki-Jarvinen and Utriainen 1998). Baron has reported a 2-fold increase in leg blood flow in lean-insulin sensitive subjects after 4 h of hyperinsulinemia (Baron 1996).

Most investigators (Baron et al. 1995; Pitre et al. 1996) use the hyperinsulinemic euglycemic clamp technique (explained in detail in chapter 1) to measure insulin sensitivity. In this technique, insulin is infused at a constant rate for 2-3 hrs before steady state conditions are achieved. It is possible that infusion of insulin for long periods of time and at high concentrations results in vasodilation and increased blood flow. However, the insulin used in our experiments, given over 5 minutes, is short acting and the RIST is completed by 35 min. Baron et al. (1995) report that during the hyperinsulinemic euglycemic technique there is a fall in mean arterial pressure caused by the vasodilatory effect of insulin. In our experiments there is no significant change in blood pressure during insulin administration. Furthermore, if NOS antagonism produced insulin resistance secondary to direct blockade of dilatory responses to insulin in skeletal

muscle, the intravenous dose should have produced a greater effect than the intraportal dose, the opposite of our findings (Fig. 6). Similarly, the ability of intraportal but not intravenous NO donor to reverse L-NMMA-induced insulin resistance indicates that the drugs are acting through the liver (Fig.11). Furthermore, if NOS antagonism produced insulin resistance secondary to blocking vascular responses to insulin in skeletal muscle, the insulin resistance caused by hepatic denervation should have been made worse by the addition of this peripheral effect. Insulin resistance produced by denervation was not affected by addition of a NOS antagonist (Fig. 9). Thus, in our testing conditions the data are consistent with insulin resistance following NOS antagonism being secondary to a hepatic, rather than peripheral, effect.

*2.4.3 Reversal of insulin resistance*

L-Arginine did not produce the anticipated reversal of insulin resistance produced by L-NAME, but rather L-arginine, by itself, caused insulin resistance (48.8 ± 8.2%) (Fig. 10). Also, L-arginine does not reverse the inhibitory effects of L-NAME on the somato-vesical (bladder) parasympathetic inhibitory reflex (Morrison et al. 1996). L-NAME not only blocks NOS but also blocks arginine uptake across the hepatocyte plasma membrane (Inoue et al. 1993*b*), thus, reducing substrate available for the NOS. L-arginine is metabolized by NOS to NO, and by arginase to urea and L-ornithine (Cook et al. 1994). Since the liver has a very high arginase activity, it is possible that most L-arginine administered is converted to L-ornithine by the liver, although L-arginine can reverse the vascular effects of L-NAME in the liver (Macedo and Lautt 1996). L-arginine also causes release of growth hormone (Cyber 1994; Nakaki and Kato 1994) and

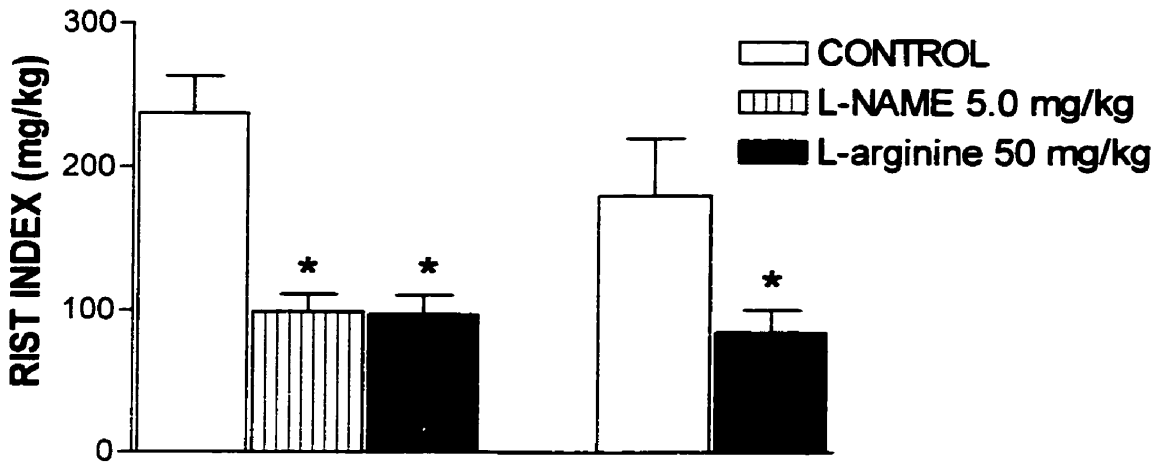



**Figure 10.** *Left*: RIST index in control, after intravenous L-NAME (5.0 mg/kg), and after intraportal L-arginine (50 mg/kg) administration. Values are means ± SE; n=6. **P*<0.001. *Right*: RIST index before and after intraportal L-arginine infusion. Values are means ± SE; n=15. **P*<0.05. L-Arginine did not reverse insulin resistance caused by NOS antagonism but rather produced insulin resistance when administered alone.

glucagon (Rocha et al. 1972); both hormones reduce insulin sensitivity. This may explain why we could not reverse insulin resistance caused by L-NAME with L-arginine and why L-arginine caused insulin resistance (Fig. 10).

Reduction in blood flow to the nerves in diabetes leads to neuropathy (Kihara and Low 1995; Stevens 1995; Cameron et al. 1995, 1996; Omawari et al. 1996) and has been suggested to result from a decrease in NO production in the vasculature (Cameron et al. 1995; Kihara and Low 1995). Administration of L-NAME in normal rats decreased nerve blood flow that was reversed by L-arginine (Kihara and Low 1995; Omawari et al. 1996). L-NAME also caused basal vasoconstriction in the intestine that was reversible by L-arginine (Macedo and Lautt 1996).These observations show that L-arginine is capable of reversing the effect of L-NAME in the vasculature. This suggests that acute insulin resistance caused by L-NAME is not secondary to effects on perfusion of hepatic nerves or peripheral blood vessels since it was not reversed with L-arginine. Further studies are required to test this interpretation.

As an alternative to using L-arginine to reverse the effect of NOS blockade, the NO donor, SIN-1, was used. SIN-1 spontaneously releases NO (Feelisch and Noack 1987; Bassenge 1994), thus, it does not utilize the NOS. Administration of intraportal, but not intravenous, SIN-1 (5.0 mg/kg) partially reversed the insulin resistance caused by L-NMMA (Fig. 11). However, administration of a higher dose of SIN-1 (10.0 mg/kg) to the liver completely reversed the insulin resistance caused by L-NMMA (Fig. 12). This indicates that insulin resistance produced after inhibition of NOS in the liver can be reversed by providing NO in the liver. Also, administration of intraportal SIN-1 after

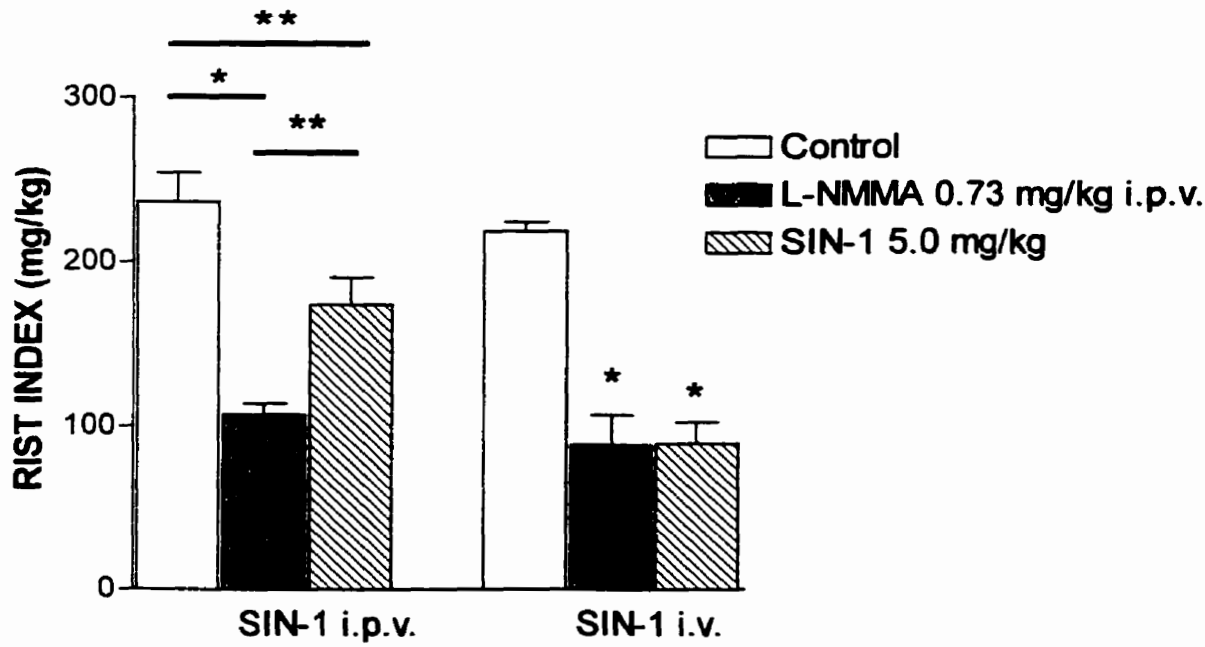



**Figure 11.** *Left*: RIST index in control, after intraportal L-NMMA (0.73 mg/kg) and after intraportal SIN-1 (5.0 mg/kg) administration. Values are means ± SE; n=5. **P*<0.001, ***P*<0.05. *Right*: RIST index in control, after intraportal L-NMMA (0.73 mg/kg), and after intravenous SIN-1 (5.0 mg/kg) administration. Values are means ± SE; n=4. **P*<0.001. The NO donor reversed insulin resistance induced by NOS antagonism only when administered directly to the liver via the portal vein.

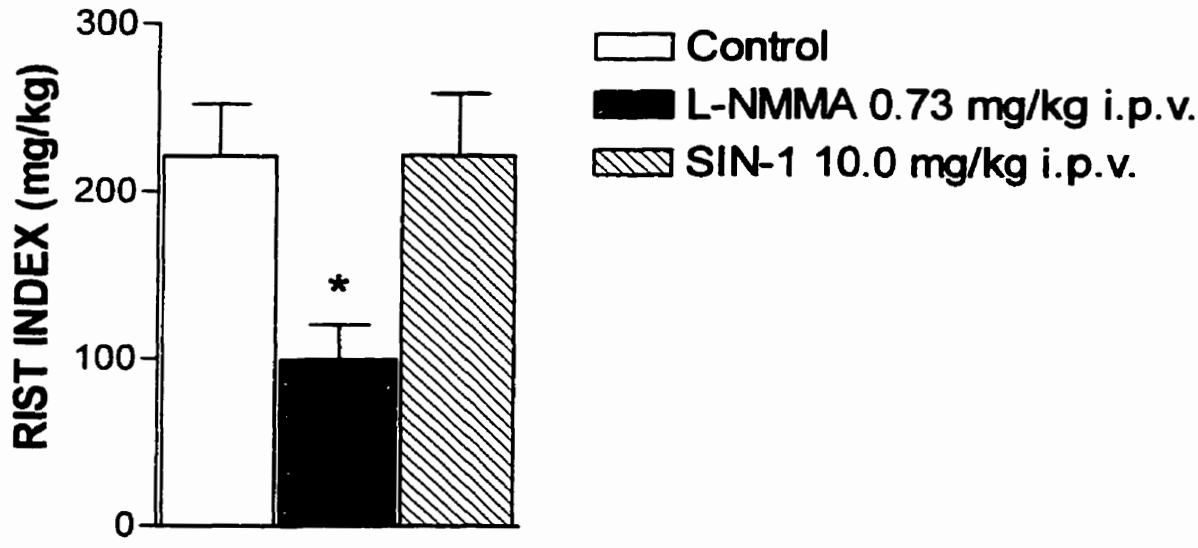



**Figure 12.** RIST index in control, after intraportal L-NMMA (0.73 mg/kg) and after intraportal SIN-1 (10.0 mg/kg) administration. Values are means ± SE; n=5. *$P<0.05$. Insulin resistance produced by NOS antagonism was completely reversed by providing higher amount of NO to the liver.

denervation of the liver completely restored insulin sensitivity (Fig. 13). Thus, NO production in the liver is confirmed to be essential for insulin sensitivity.

It is possible that insulin also directly stimulates the production of NO in liver, since SIN-1 reversed the insulin resistance after the hepatic parasympathetic nerves were cut. However, administration of SIN-1 (either 5.0 or 10.0 mg/kg) did not reverse the insulin resistance produced by atropine (Fig. 14). Atropine is a non-selective muscarinic antagonist, it is likely that atropine also blocks another possible regulator (e.g. prandial state) in the release of HISS (see chapter 3) that does not involve the NO.

Administration of SIN-1 (5.0 mg/kg) intraportal or intravenously without any prior interventions did not affect insulin sensitivity, suggesting that full parasympathetic-dependent activation of NO production occurs in response to the bolus of insulin.

Reversal of denervation-induced insulin resistance by SIN-1 is additional evidence that the parasympathetic tone involves a hormonal pathway. If there was a neural connection between the liver and skeletal muscle that was controlling insulin sensitivity, then this connection had been severed in order to produce the insulin resistance. Administration of SIN-1 into the portal vein cannot restore the response by a parasympathetic-dependent pathway since the nerves have been cut. Thus, the hepatic parasympathetic nerves and the hepatic NO production provide the background tone to the liver and have a permissive role to the action of insulin to release HISS from the liver.

Administration of SIN-1 (10.0 mg/kg, ipv) 30-45 min before insulin infusion did not effect the arterial glucose levels but restored insulin sensitivity after denervation. Thus, insulin is required for the release of HISS from the liver and providing NO to the liver without any insulin administration does not reverse insulin resistance.

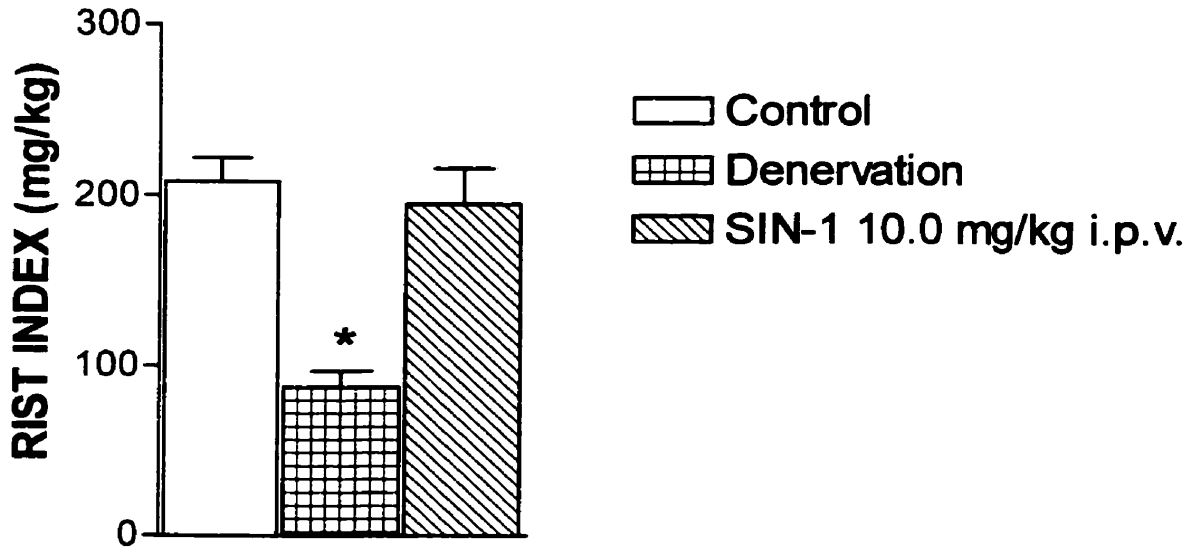



**Figure 13.** RIST index in control, after hepatic parasympathetic denervation, and after intraportal SIN-1 (10.0 mg/kg) administration. Values are means ± SE; n=6. *$P$<0.001. Providing NO to the denervated liver completely restored insulin sensitivity.

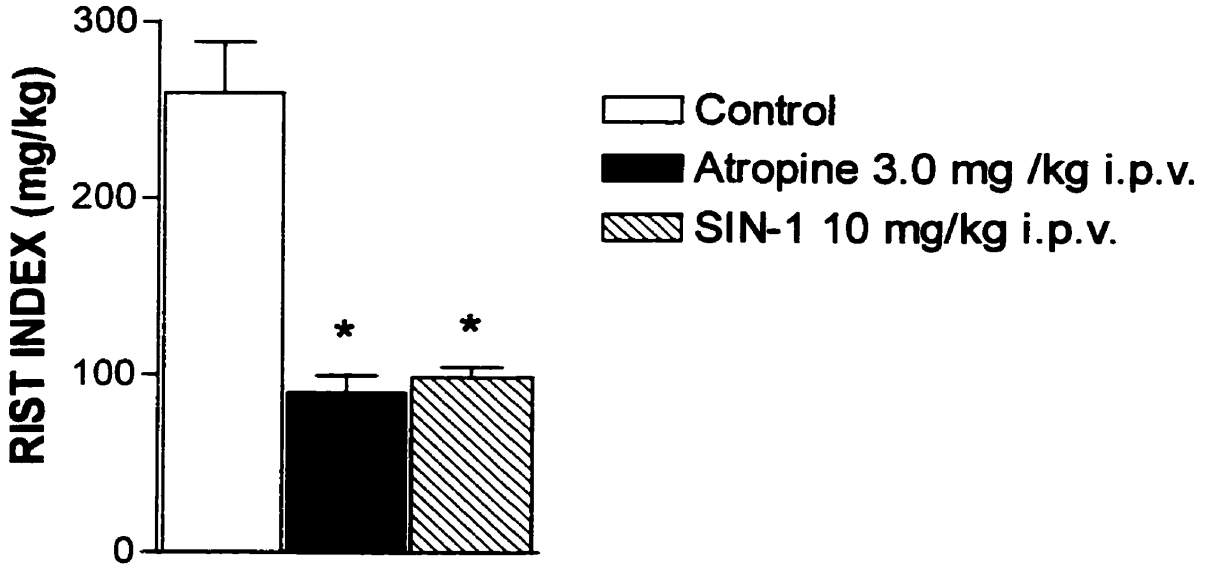
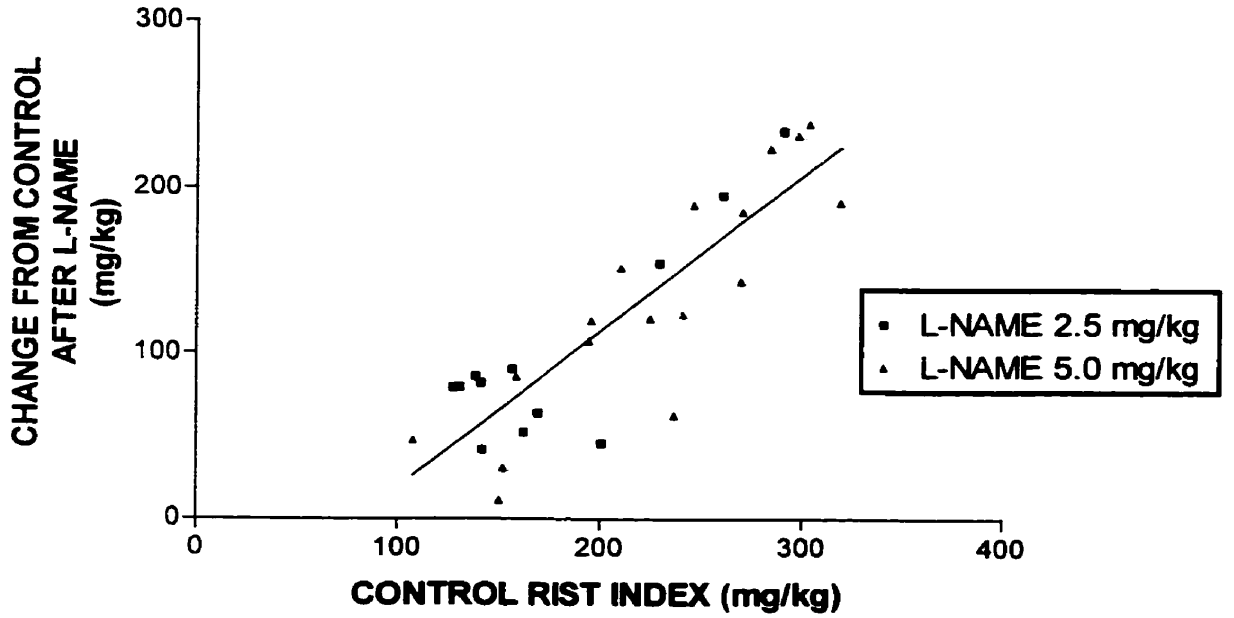



**Figure 14.** The RIST index in control, after intraportal atropine (3.0 mg/kg), and after intraportal SIN-1 (10.0 mg/kg) administration. Values are means ± SE; n=6. **P*<0.001. Providing NO after muscarinic blockade does not restored insulin sensitivity.

*2.4.4 HISS-dependent and –independent effect*

The RIST index in control responses and the reduction in control RIST index after atropine or denervation was examined by linear regression as previously reported (Xie and Lautt, 1996*b*). The rats showing the highest control RIST index had the greatest reduction in response after atropine or denervation, and rats showing the lowest control RIST index had the smallest decrease in control RIST index (Fig 15, *bottom*). The decrease in the RIST after denervation or atropine represents the HISS-dependent component of insulin action. This shows a parasympathetic-dependent component (to the right of the *x*-intercept) and a parasympathetic-independent component (the *x*-intercept) of insulin action. A similar relationship is observed after L-NAME administration. After L-NAME, the rats showing high control RIST indexes had large decreases in the RIST index, and the rats showing small control RIST indexes had small decreases in the RIST index (Fig 15, *top*). This suggests a hepatic NO-dependent component and a NO-independent component involved in insulin action. The regression analysis is not significantly different in slope or intercept using the combined atropine and denervation data compared to the NOS blockade data. It appears that there is a parasympathetic-dependent and -independent and also a NO-dependent and -independent component involved in insulin responsiveness; we propose that both, the NO and the parasympathetic nerve-dependent, components act through the same pathway. This pathway is suggested to consist of a hepatic parasympathetic tone, acting through muscarinic receptors, resulting in production of NO in the liver, leading to release of the putative hormone, HISS, that enhances glucose uptake at the skeletal. Interruption of this NO-mediated pathway inhibits HISS release from the liver and HDIR follows.

**Figure 15**


CHANGE FROM CONTROL AFTER L-NAME (mg/kg)
300
200
100
0
0
100
200
300
400
CONTROL RIST INDEX (mg/kg)
L-NAME 2.5 mg/kg
L-NAME 5.0 mg/kg

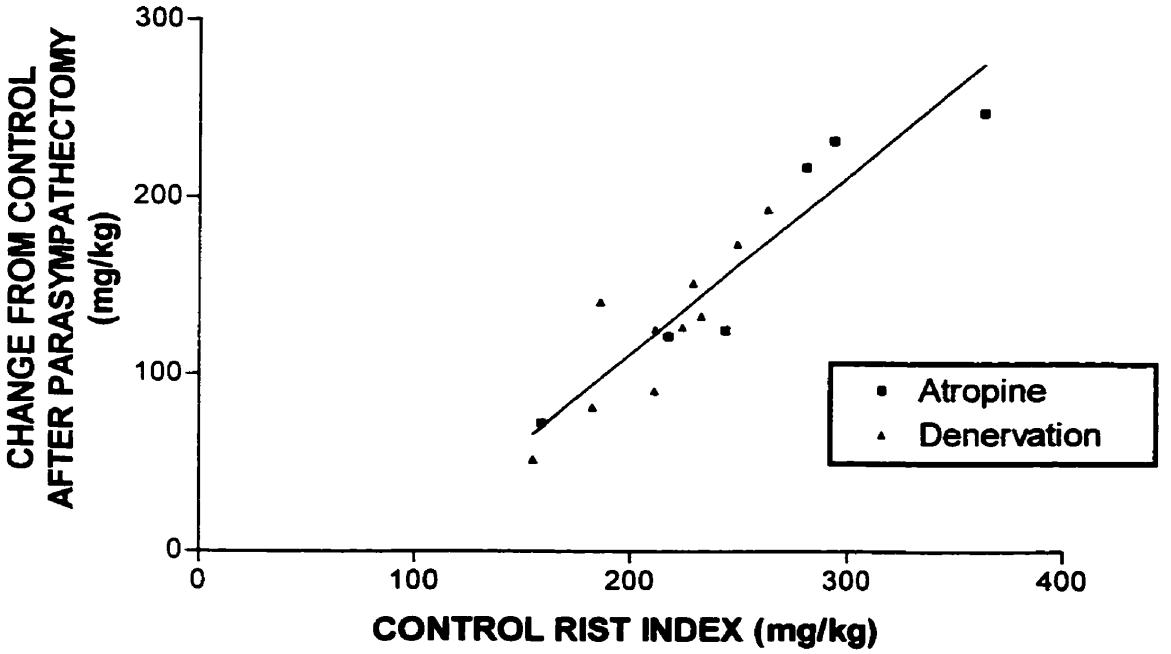

CHANGE FROM CONTROL AFTER PARASYMPATHECTOMY (mg/kg)
300
200
100
0
0
100
200
300
400
CONTROL RIST INDEX (mg/kg)
Atropine
Denervation

**Figure 15.** *Top*: linear regression of RIST index (mg/kg) in control against reduced RIST index (mg/kg) after 2.5 mg/kg (n=12) and 5.0 mg/kg (n=17) intravenous L-NAME administration. The slope is 0.94 ± 0.11; intercept on *x*-axis is 79.5 (mg/kg); $r^2$=0.75 ($P$=0.0001). *Bottom*: linear regression of RIST index (mg/kg) in control against reduced RIST index (mg/kg) after hepatic parasympathetic denervation (n=10) and intraportal atropine administration (n=6). The slope is 1.0 ± 0.1; intercept on *x*-axis is 88.0 (mg/kg); $r^2$=0.86 ($P$=0.0001). The *y*-axis represents the difference in RIST index between control and after NOS blockade or parasympathetic blockade and is interpreted as the HISS-dependent component of insulin action. The HISS-independent component of insulin action is determined either from the intercept on the *x*-axis or the RIST index after NOS or nerve blockade.

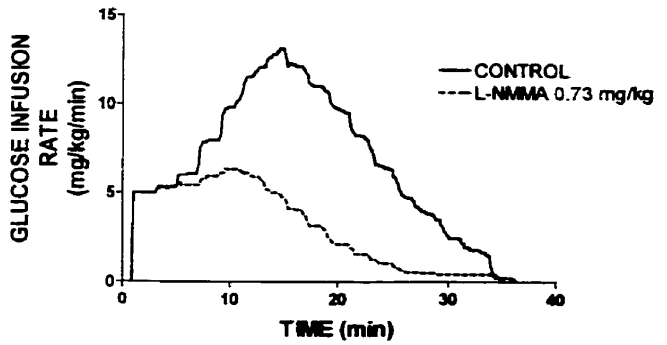
*2.4.5 Dynamics of HISS action*

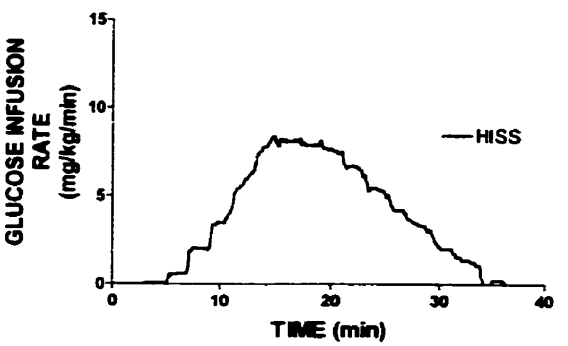
Although the chemical identity of HISS is unknown, the dynamics of HISS action can be described by examination of the shape of the RIST curve. For this purpose RIST curves in control and after intraportal L-NMMA (0.73 mg/kg) administration, surgical denervation of the liver, or intravenous atropine (3.0 mg/kg,) administration were used. All these interventions produced HDIR by interruption of HISS release from the liver (above). The control RIST curves were significantly inhibited after L-NMMA, surgical denervation of the liver, and atropine (Fig. 16, *left graphs*). The difference between the two curves provided a dynamic curve that is attributed to HISS action and, thus, represented the HISS-dependent action of insulin (Fig 16, *right graphs*). HISS release appeared to begin after 3-4 min from the onset of insulin action and to continue for about 9 min after the direct effect of insulin was no longer seen. This may suggest that HISS has an additive, rather than a synergistic, insulin-like action. Calculated from the decline in HISS action from the peak level, the half-life of HISS action is about 9 min.

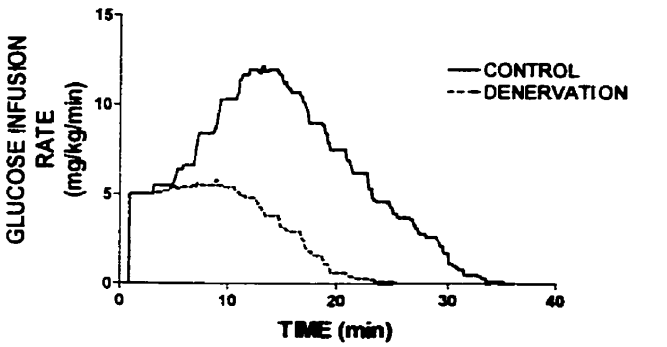
The HISS release inhibited by L-NMMA or denervation were completely restored after administration of SIN-1 (Fig, 17, 18, *top*). The HISS curves calculated from the difference between the control RIST curve and the RIST curve after L-NMMA or denervation were similar to the HISS curves calculated from the difference between L-NMMA or denervation RIST curve and SIN-1 RIST curve (Fig. 17, 18, *bottom*).

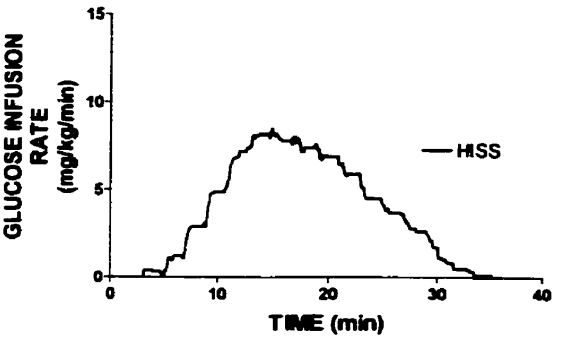
This indicates that inhibition of NO in the liver or surgical denervation of the liver interrupted the release of HISS from the liver and produced HDIR without any effect on the HISS-independent component of insulin action. However, providing NO to the liver restored the release of HISS from the liver and reversed HDIR.

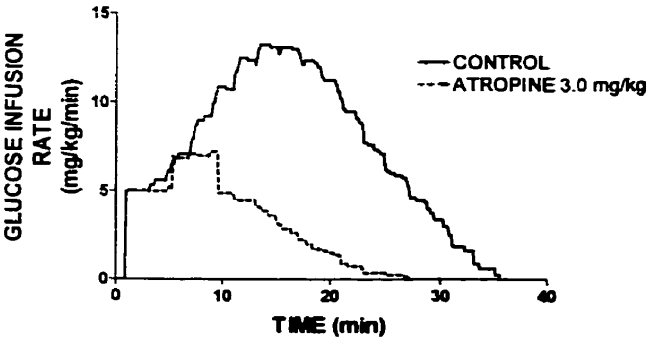
**Figure 16**


GLUCOSE INFUSION RATE (mg/kg/min)
CONTROL
L-NMMA 0.73 mg/kg
TIME (min)


GLUCOSE INFUSION RATE (mg/kg/min)
HISS
TIME (min)


GLUCOSE INFUSION RATE (mg/kg/min)
CONTROL
DENERVATION
TIME (min)


GLUCOSE INFUSION RATE (mg/kg/min)
HISS
TIME (min)


GLUCOSE INFUSION RATE (mg/kg/min)
CONTROL
ATROPINE 3.0 mg/kg
TIME (min)

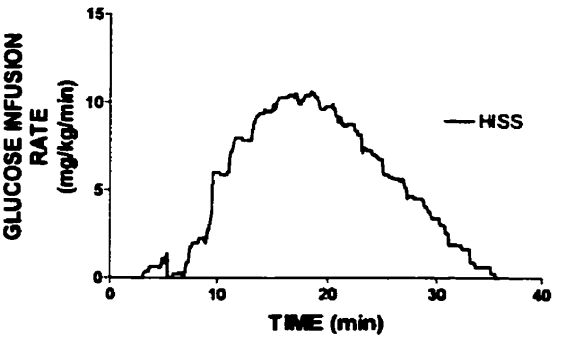
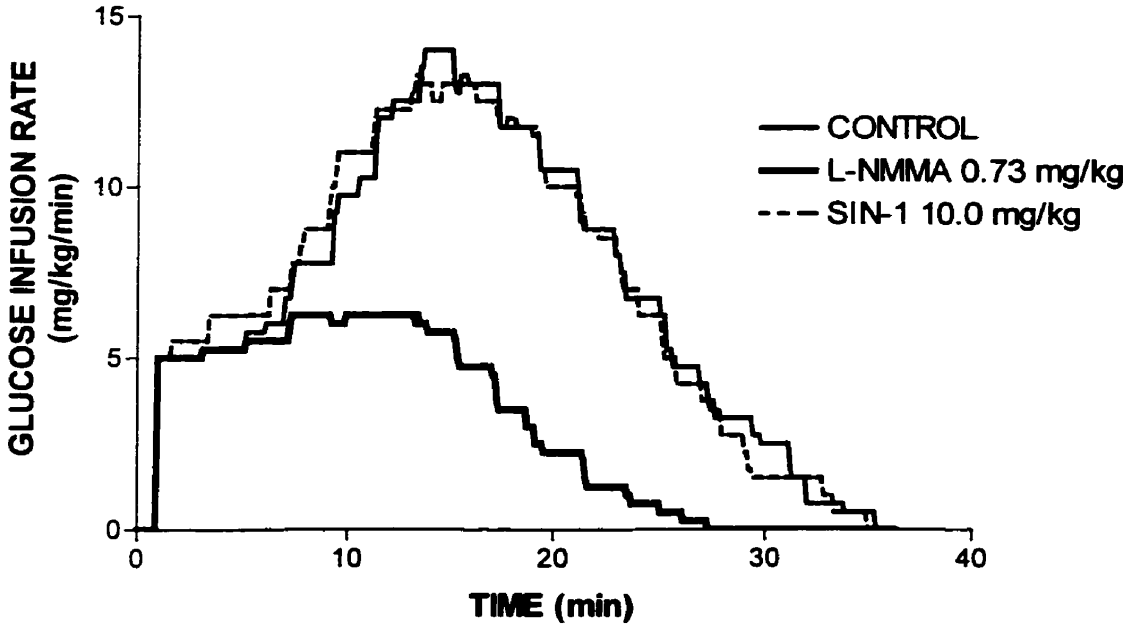

GLUCOSE INFUSION RATE (mg/kg/min)
HISS
TIME (min)

**Figure 16.** A plot of the average glucose infusion rate (mg/kg/min) as determined at 0.1 min intervals. *Left.* Control RISTs (solid line) and the RIST after HISS blockade (broken line) with intraportal L-NMMA (n=23, pooled), surgical denervation of the liver (n=10, pooled), and intravenous atropine (n=8, pooled). *Right.* The HISS-dependent component of insulin action that was calculated from the difference between the control curve and the curve after blockade of HISS. HISS action started at 3-4 min after the onset of insulin administration and continued until the end of the RIST.

**Figure 17**


GLUCOSE INFUSION RATE
(mg/kg/min)
15
10
5
0
0
10
20
30
40
TIME (min)
CONTROL
L-NMMA 0.73 mg/kg
SIN-1 10.0 mg/kg

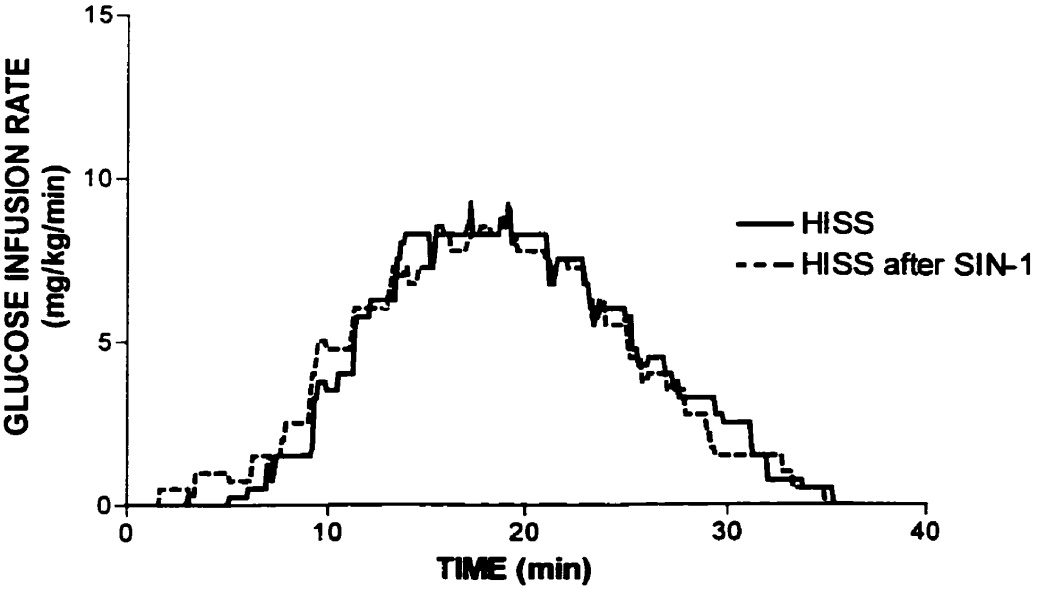
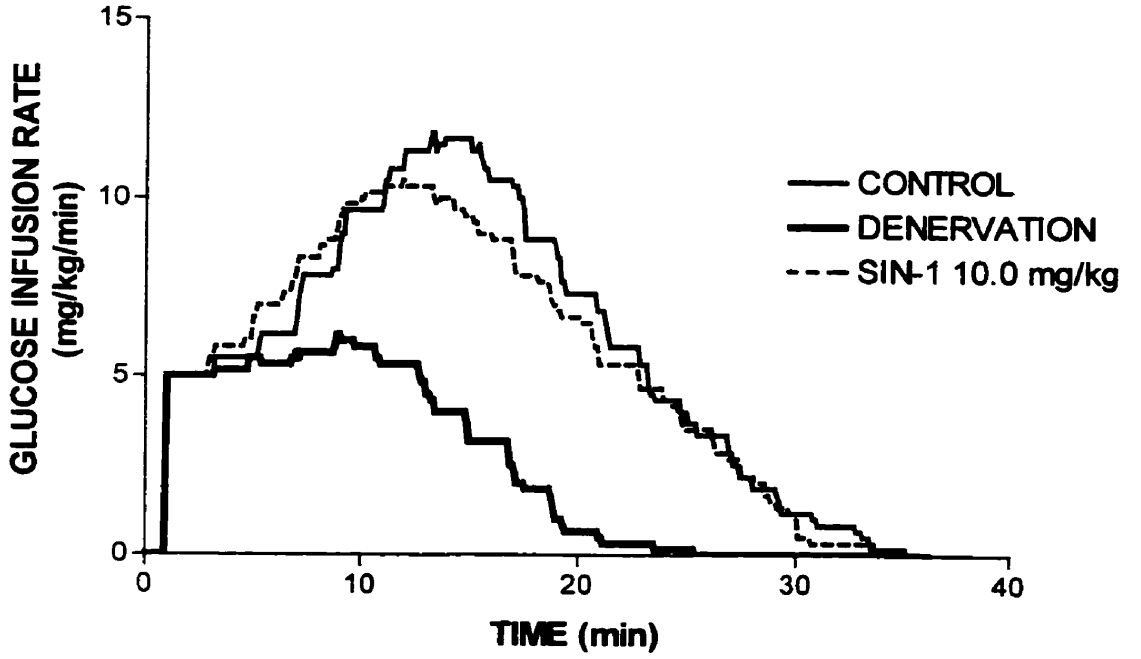

GLUCOSE INFUSION RATE
(mg/kg/min)
15
10
5
0
0
10
20
30
40
TIME (min)
HISS
HISS after SIN-1

**Figure 17.** *Top.* The average glucose infusion rate (mg/kg/min) during the control RIST, the RIST after intraportal L-NMMA and the RIST after intraportal SIN-1 (n=5). *Bottom.* The HISS-dependent component of insulin action which was calculated from the difference between the control curve and the curve after L-NMMA (solid line) and the difference between the curve after L-NMMA and the curve after SIN-1 (broken line). HISS release was eliminated by L-NMMA but it was restored to similar levels after SIN-1 infusion.

**Figure 18**


GLUCOSE INFUSION RATE
(mg/kg/min)
15
10
5
0
0
10
20
30
40
TIME (min)
CONTROL
DENERVATION
SIN-1 10.0 mg/kg

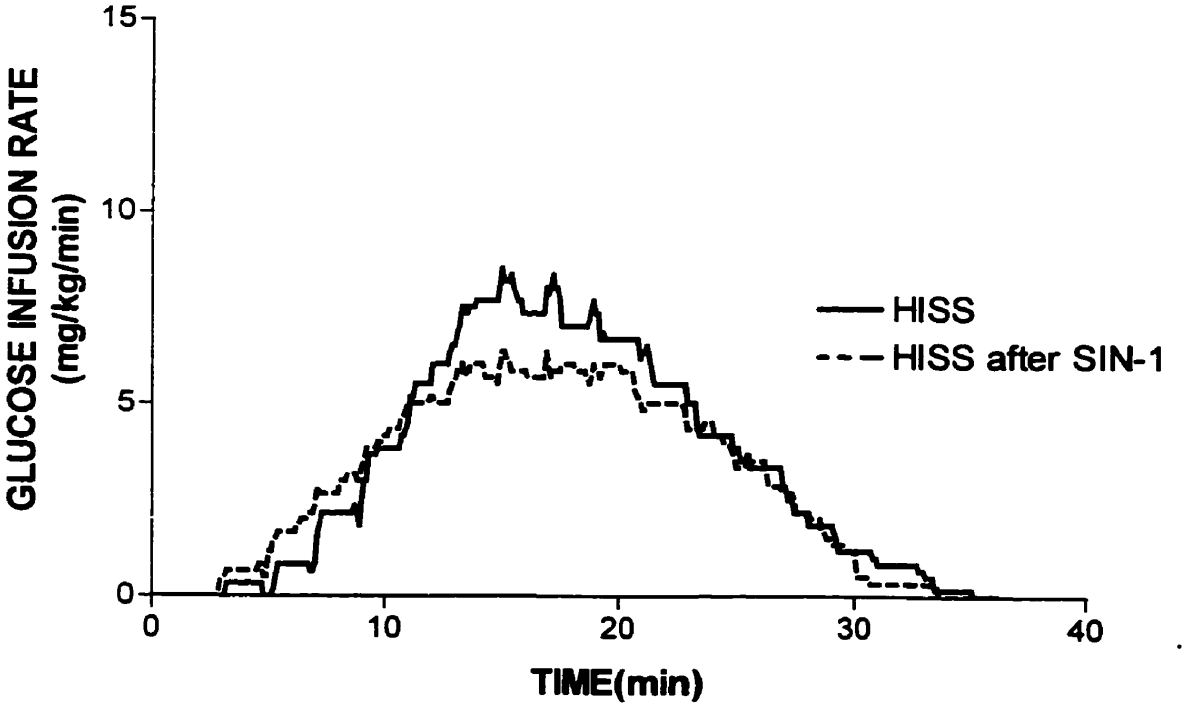

GLUCOSE INFUSION RATE
(mg/kg/min)
15
10
5
0
0
10
20
30
40
TIME(min)
HISS
HISS after SIN-1

**Figure 18.** *Top.* The average glucose infusion rate (mg/kg/min) during the control RIST, the RIST after surgical denervation of the liver and the RIST after intraportal SIN-1 (n=7). *Bottom.* The HISS-dependent component of insulin action which was calculated from the difference between the control curve and the curve after denervation (solid line) and the difference between the curve after denervation and the curve after SIN-1 (broken line). The release of HISS was eliminated by surgical denervation of the liver but it was restored to similar levels after SIN-1 infusion.

*2.4.6 Isoforms of NOS involved in the release of HISS*

Three NOS isoforms have been characterized. The constitutive neural (nNOS) and endothelial (eNOS) isoforms are regulated by intracellular calcium. The nNOS is expressed in the brain and in peripheral neurons and the eNOS is expressed in endothelial cells, platelets, and the heart (endocardium and myocardium) (Vallance and Collier 1995). The inducible (iNOS) isoform is calcium-independent and it is expressed only after activation of cells by products of infection, including bacterial endotoxins or exotoxins, or cytokines. The iNOS is expressed in most types of vascular cells, including endothelial cells, smooth muscle, cardiac myocytes, gut, immune cells and macrophage (Vallance and Collier 1995). All three isoforms of NOS are found in the liver (Knowles et al. 1990; Curran et al. 1990; Geller et al. 1993; Esteban et al. 1997; Clemens 1998).

The iNOS is expressed by most cells of the liver (Clemens 1999). NO synthesis by iNOS is delayed for the several hours required for synthesis of iNOS. Once the induction has occurred, iNOS produces large amounts of NO for prolonged periods (Moilanen and Vapaatalo 1995). However, HISS is released within minutes of insulin infusion and it does not require to be released for long periods of time. Also iNOS is virtually absent in the normal liver but markedly increased in response to inflammation and a variety of oxidative stresses (Clemens 1999), thus, it is unlikely that iNOS is involved in the release of HISS.

On the other hand, eNOS produces small amounts of NO rapidly and transiently (seconds) in response to agonists such as Ach or bradykinin (Moilanen and Vapaatalo 1995). It has also been shown that mice with targeted disruption of the gene encoding the eNOS have marked metabolic insulin resistance (Satori and Scherrer 1999). Thus, it is

possible that eNOS is involved in the parasympathetic-dependent release of HISS from the liver.

Another possibility for NO involvement in the release of the HISS from the liver is that NO acts as a neurotransmitter of the hepatic parasympathetic tone. nNOS has been identified in parasympathetic neurons, many of them cholinergic, innervating cerebral vessels, the penis, airways, the uterus, the bladder, and the tongue (Iadecola et al. 1993; Keast 1992; Sheng et al. 1992; Vizzard et al. 1993). These nerves are believed to serve a vasodilator function, releasing NO among other vasodilator neurotransmitters. nNOS has been shown to be present in the nerve fibers in both supply vessels of the hepatic hilus, the interlobular portal vein and the interlobular hepatic artery, although thicker bundles of nitrergic fibers were found along the interlobular hepatic artery (Esteban et al. 1997). In addition, a rich plexus of nerve fibers containing nNOS was detected around the wall and in the muscular layer of the interlobular hepatic artery. These nitrergic fibers are suggested to be involve in the control of the global liver blood flow. NO is also released as a co-transmitter with Ach in sudomotor and in vasodilator neurons (Anderson et al. 1995). Thus, it is possible that NO is co-released with Ach by the parasympathetic nerves. Administration of either Ach or SIN-1 after parasympathetic denervation of the liver restored insulin sensitivity, thus, either of the two neurotransmitters is required to trigger the parasympathetic release of HISS. The involvement of central nitrergic neuronal pathways has been recently suggested in maintaining the glucose homeostasis (Shankar et al. 1998). Inhibition of NOS (possibly nNOS) in the brain caused peripheral insulin resistance, hyperglycemia, defective insulin secretion, and hypertension in rats. Thus, it is possible that production of NO in the brain, activates the parasympathetic

nerves and these nerves, with the rise of insulin in portal blood, cause the release of HISS from the liver. It is also conceivable that both the parasympathetic and the nitrergic neurons in the brain, and these neurons project from the brain to the liver and modulate the release of HISS.

At this point we do not know which isoform of NOS is involved in the hepatic parasympathetic-dependent release of HISS from the liver, however, we can speculate that the constitutive forms of NOS, either eNOS or nNOS, are more likely to be involved.

*2.4.7 NO as a second messenger*

NO mainly acts as a second messenger stimulating the soluble guanylate cyclase (sGC) thus increasing cGMP which in turn modulates a variety of biological functions. It has been also shown that SIN-1, via release of NO, inhibits platelet aggregation and relaxes vascular smooth muscle by activation of sGC and increases in intracellular cGMP (Nishikawa et al. 1982; Gerzer et al. 1988; Noack and Feelisch 1989; Bassenge and Mulsch 1989). Although we have no direct evidence, we can speculate that NO causes HISS release by activation of sGC and increase in cGMP in the liver. Further experiments are required to test this hypothesis.

In conclusion, there is a strong relationship between inhibition of NOS in the liver and insulin resistance. Inhibition of NO production in the liver produced HDIR with no effect on the HISS-independent component of insulin action. Providing NO to the liver reversed this HDIR by restoring the HISS-dependent component of insulin action. Thus,

we propose that inhibition of the NOS in the liver interrupts the hepatic parasympathetic-dependent HISS release and, because HISS is needed to sensitize the skeletal muscle response to insulin, HDIR occurs.

# Chapter 3

# Hepatic parasympathetic interruption causes insulin, but not IGF-1, resistance

## 3.1 Introduction

Insulin-like growth factor-1 (IGF-1) is a single chain polypeptide characterized by high structural homology with insulin (Sara and Hall 1990). IGF-1 has many insulin-like activities including production of hypoglycemia by the enhancement of glucose uptake in skeletal muscle (Guler et al. 1987; Jacob et al. 1989; Borg and Sherwin 1995). We have demonstrated in chapter 1 that the action of insulin to increase glucose uptake at the skeletal muscle is mediated through the hepatic parasympathetic-dependent release of HISS. The release of HISS in response to insulin occurs in the immediate post-prandial state and decreases progressively with the duration of fasting (Macedo et al. 1998). The involvement of the hepatic parasympathetic nerves in glucose uptake stimulated by IGF-1 is unknown. Liver disease results in insulin but not IGF-1 resistance (Petersen et al. 1997), and spontaneously diabetic rats become insulin but not IGF-1 resistant (Jacob et al.1991). Based on these observations we hypothesize that the hepatic parasympathetic reflex is triggered by insulin but not by IGF-1. Accordingly insulin, but not IGF-1, action should be inhibited by atropine, liver denervation, and fasting.

### *3.1.1 Synthesis and function*

IGF-1 has 48% amino acid sequence homology to proinsulin, the A and B domains have 60-70% homology, but there is no homology with the C domain (Simpson et al. 1998). In contrast to insulin, IGF-1 has many sites of production. Although the liver

is the major source of circulating IGF-1 (Schwander et al. 1983; Giacca et al. 1990; Borg and Sherwin 1995; Simpson et al. 1998), it is also locally produced in smaller quantity in many other tissues of the body such as the bone, the adipose tissue, the kidney, the muscle (Matthews et al. 1986; Roberts et al. 1987; Daughaday and Rotwein 1989). Serum concentrations of IGF-1 in man are regulated by growth hormone, insulin, age, and nutritional state (Simpson et al. 1998). Growth hormone and insulin are the main regulators of hepatic IGF-1 production (Schwander et al. 1983). Infusion of IGF-1 suppresses circulating insulin and glucagon levels, inhibits hepatic glucose output, increases glucose uptake, and decreases circulating free fatty acids and amino acids (Bach 1999). The hypoglycemic action of IGF-1 is only about 6% of that of insulin on a molar basis (Guler et al. 1987; Boulware et al. 1994), however, it circulates at about 1000 times the concentration of insulin in plasma, being highly protein bound. IGF-1 exerts its physiological actions in two ways: as a classical endocrine hormone and also as an auto-paracrine factor.

*3.1.2 Receptors*

There are two major IGF receptors on cells: the type I and type II. The type I receptor is structurally and functionally very similar to the insulin receptor and it is present on virtually all cell types (Massague and Czech 1982). Large numbers are present in muscle (Florini 1987), with fewer in the human liver (Caro et al. 1988). Its post-receptor signaling mechanisms are also similar to the insulin receptors. IGF-1 can bind to the insulin receptor, but with only 1-5% affinity compared to insulin (Guler et al. 1987). The affinity of insulin for binding to the IGF type I receptor is 5-10% of that of IGF-1.

The type II receptor is structurally identical to the mannose-6-phosphate receptor and has no structural homology to the type I or the insulin receptor (Morgan et al. 1987). Under normal physiological conditions it is thought that IGF-1 acts through the type I receptor. However, under high concentrations of IGF-1, there is likely to be cross-activation with the insulin receptor. Some of the insulin-like effects of IGF-1 are mediated by a cross-reaction of the IGF-1 with insulin receptors (Froesch et al. 1996). Hybrid IGF-1/insulin receptors have been well-documented and sequenced, but their role is unclear (Soos and Siddle 1989). Almost all known *in vitro* and *in vivo* effects of IGF-1 so far described are mediated through the type I receptors. IGF-1 appears more potent at stimulating glucose uptake by muscle and less effective at inhibiting glucose production by liver and free fatty acid production by adipocytes compared to insulin (Zapf et al. 1978; Jacob et al. 1989). It has been suggested that it acts through its own receptors in muscle but mediates its effects via the insulin receptor in adipocytes (Zapf et al. 1981).

### *3.1.3 Binding proteins*

Bio-availability of IGF-1 is determined by its binding proteins (IGFBPs). To date, six binding proteins have been fully characterized and sequenced (Rechler and Brown 1992; Oh et al. 1996), although recent evidence suggests that there may, in fact, be as many as ten binding proteins (Simpson et al. 1998). The IGFBPs bind IGF-1 with high affinity, forming complexes that prevent the IGF-1 from binding to the IGF-1 receptor (Clemmons 1997), although it has been shown that some IGFBPs also potentiate IGF's action (Rechler and Clemmons 1998). Most of the IGFBPs are relatively stable in serum, showing minimal changes in response to physiological perturbation. The

exception is IGFBP-1, the circulating concentration of which is influenced by nutritional status. Its production is suppressed by glucose ingestion (Busby et al. 1988; Yeoh and Baxter 1988), or intravenous infusion (Snyder and Clemmons 1990), and increased in response to fasting (Busby et al. 1988; Yeoh and Baxter 1988; Hall et al. 1988; Snyder and Clemmons 1990) and in type 2 diabetes (Brismar et al. 1988; Suikkari et al. 1988). Serum IGFBP-1 levels are reduced in the immediate postprandial period and it has been shown that its production is suppressed by insulin. IGFBP-1 is inversely related to the insulin levels (Cotterill et al.1989) and has a diurnal variation with the highest levels being overnight when insulin levels are lowest (Holly et al. 1988). IGFBP-1 may inhibit or sometimes also enhance the activity of IGF-1 (Froesch et al. 1996). Acute administration of IGFBP-1 in rodents can increase blood glucose (Lewitt et al. 1992) and transgenic mice overexpressing IGFBP-1 develop hyperglycemia (Rajkumar et al. 1995).

The majority of IGF-1 (~85%) is bound in a 150 kDa complex with IGFBP-3 and an acid labile subunit (ALS) that is synthesized in the liver (Baxter and Martin 1989). This large molecule is unable to pass through vessel walls, so acts as an intravascular reservoir of inactive IGF-1. Most remaining circulating IGF-1s are bound to other IGFBPs (50 kDa complexes) , and less than 1% of the IGF-1 in circulation is free (the biologically active form) (Bach 1999). The low molecular weight IGFBPs found in the bloodstream can cross endothelial barriers and transport IGF-1 from the circulation to peripheral tissue. Thus, in addition to their potential role as a storage reservoir for IGF-1, the IGFBPs may function to deliver IGF-1 to their cell-surface receptors. Non-phosphorylated IGFBP-1, proteolytic fragments of IGFBP-3, and IGFBP-5, which bind IGF-1 with low affinity, potentiate the actions of IGF-1 *in vitro* (Rechler and

Clemmons1998). This is believed to be mediated through enhanced delivery of ligand to receptor. The half-life of IGF-1 in the complex with IGFBP-3 and ALS is 12-15 h compared with 20 min for IGF-1 bound to IGFBP-1, -2, -3 and with 10-12 min for free IGF-1 (Bach and Rechler 1995).

To test the hypothesis that the IGF-1 action is not through the release of HISS from the liver, we used the RIST to measure IGF-1, and insulin, sensitivity. Different doses of IGF-1 were compared to the standard dose of insulin (50 mU/kg) used in our laboratory. Insulin and IGF-1 sensitivities were measured after the interruption of the parasympathetic release of HISS either by surgical denervation of the liver or by atropine or after 16 h of fasting.

### 3.2 Materials and methods

Male Sprague-Dawley rats (274.8 ± 6.7 g) were fasted overnight (8 h) and were fed standard laboratory rat food for 2 h before the start of any surgical procedures. Animal preparation, surgical procedures, and the RIST methodology are explained in detail in chapter 1.

*IGF-1 sensitivity Test.* To measure IGF-1 sensitivity the RIST approach was used as described in chapter 1, however, instead of insulin the IGF-1 was infused over 5 min. After the control RIST with insulin was performed, the rats were allowed to stabilize for at least 15 min. The basal arterial glucose levels were determined and the RIST was repeated, however, instead of insulin, IGF-1 at doses 25 μg/kg (n=4), 100 μg/kg (n=4), and 200 μg/kg (n=10) was administered over 5 min to measure IGF-1 sensitivity. In some rats, after at least 15 min of stabilization, the basal arterial glucose level was determined and a second RIST with insulin and IGF-1 was repeated.

*RIST in control with insulin and IGF-1 and after SIN-1 (n=2) or Ach (n=2) infusion.* After a control RIST with insulin, the RIST was repeated again with IGF-1. After at least 15 min of stabilization, either SIN-1 (10.0 mg/kg) or Ach (2.5 μg/kg/min) was infused intraportally. A stable basal arterial glucose level was established and the RIST was repeated with insulin.

*RIST in control and after atropine infusion (n=5).* After the control RIST with insulin, atropine (1.0 mg/kg) was infused intravenously over 5 min. After the stable arterial glucose level was determined, the RIST was performed using insulin. The rats were allowed to stabilize for at least 15 min and the RIST was repeated with IGF-1.

*RIST with IGF-1 in control and after atropine (n=3) or SIN-1 (n=2).* The control RIST was performed with IGF-1. Atropine (1.0 mg/kg, iv) or SIN-1 (5.0 mg/kg, ipv) was infused over 5 min. After stabilization the RIST was repeated again with IGF-1.

*RIST in control and after surgical denervation (n=5).* After the control RIST with insulin, the nerve bundles around the common hepatic artery were cut and the animal was allowed to stabilize and the RIST was repeated. After the rats were allowed to stabilize, another RIST was performed using IGF-1.

*RIST in control and after atropine in 16 hr fasted rats (n=5).* After the rats were fasted for 6 h and then fed for 2 h, they were fasted again for 16 h. This was done to ensure that the rats were not fasted for longer than 16 hr. After the rats were fasted for 16 hr, the control RIST was performed with insulin. Atropine (1.0 mg/kg) was infused intravenously over 5 min. A stable basal arterial glucose was established and the RIST was repeated. After stabilization the IGF-1 sensitivity was measured using the RIST.

*Drugs.* The human insulin was purchased from Eli Lilly & Company (Indianapolis, IN). rhIGF-1 was donated by Genentech Inc. (San Francisco, CA). Atropine, Ach, and D-glucose were purchased from Sigma Chemical (St. Louis, MO). SIN-1 was purchased from Alexis (San Diego, CA). All the chemicals were dissolved in saline.

*Data analysis.* Data were analyzed using repeated-measures analysis of variance followed by Tukey-Kramer multiple comparison test in each group or, when applicable, paired and unpaired Student's t tests. 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 all

protocols were approved by an ethics committee on animal care at the University of Manitoba.

### 3.3 Results

The index used to express insulin, or IGF-1, sensitivity is the total amount of glucose (mg/kg) infused after insulin or IGF-1 administration in order to maintain euglycemia at the baseline level and is referred to as the RIST index. The RIST is completed after 30 min for the standard dose of insulin (50 mU/kg) or IGF-1 dose of up to 200 μg/kg. There was no statistically significant difference in arterial pressure throughout the experiments between the groups.

*RIST with insulin and with different doses of IGF-1.* In the first set of rats (n=4), the RIST index with insulin was 239.4 ± 14.6 mg/kg before and 71.0 ± 17.0 mg/kg after the RIST with IGF-1 (Fig 19, *top left*). IGF-1 at dose 25 μg/kg had a significantly lower RIST index (66.6 ± 19.3 mg/kg) than the first RIST with insulin. The blood pressure was constant throughout the experiment (100.0 ± 6.2 mmHg, 101.3 ± 7.6 mmHg, and 90.0 ± 4.7 mmHg, respectively). In the second set of rats (n=4), the RIST index with insulin was 235.6 ± 20.6 mg/kg before and 94.3 ± 27.3 mg/kg after the RIST with IGF-1 (Fig 19, *top right*). IGF-1 at dose 100 μg/kg had a RIST index of 184.7 ± 41.2 mg/kg. The blood pressure was constant throughout the experiment (84.3 ± 4.3 mmHg, 94.5 ± 7.1 mmHg, and 81.7 ± 8.2 mmHg, respectively). In the third set (n=10), the RIST index with insulin was 255.3 ± 16.5 mg/kg and of 250.3 ± 18.5 mg/kg with IGF-1 at dose 200 μg/kg (Fig. 20, *top*). The average glucose infusion rat (mg/kg/min) as determined at 0.1 min intervals was plotted after both insulin and IGF-1 infusions (Fig. 20, *bottom*). In the RIST with insulin, the glucose infusion rate showed a sharp increase and reach a plateau from about 11-16 min, and then rapidly decreased to a stable level around 35 min after the start of the test. In the RIST with IGF-1, the glucose infusion rate also showed a sharp increase and

reach a peak at about 16 min, and then rapidly decreased to a stable level around 33 min. In six of the rats a second RIST with insulin was repeated after IGF-1 infusion and the RIST index was 114.6 ± 35.1 mg/kg. In the same rats a second RIST with IGF-1 was repeated and the RIST index was 226.3 ± 19.9 mg/kg (Fig 19, *bottom left*). The blood pressure was constant throughout the experiment (87.5 ± 2.7 mmHg, 86.7 ± 3.4 mmHg, and 81.7 ± 2.7 mmHg) but decreased to 70.8 ± 3.3 mmHg by the time the second IGF-1 RIST was performed. Thus, IGF-1 at any dose administered caused insulin resistance. Dose 200 μg/kg of IGF-1 was selected as the standard dose for the remainder of the experiments.

*RIST in control with insulin and IGF-1 and after SIN-1 (n=2) or Ach (n=2) infusion.* In one group of rats, the intraportal administration of SIN-1 (10.0 mg/kg) following the IGF-1 RIST did not reverse the insulin resistance caused by IGF-1 (194.1 ± 0.2 mg/kg before and 122.6 ± 30.6 mg/kg after the IGF-1 RIST). In another group of rats, the intraportal administration of Ach (2.5 μg/kg/min) following the IGF-1 RIST did not reverse the insulin resistance caused by IGF-1 (252.2 ± 41.4 mg/kg before and 92.5 ± 32.4 mg/kg after IGF-1 RIST). Thus, either SIN-1 or Ach cannot reverse the inhibitory effects of IGF-1 on insulin-mediated glucose uptake.

*RIST in control and after atropine infusion (n=5).* After intravenous atropine (1.0 mg/kg) infusion, the control RIST index with insulin was significantly reduced from 292.1 ± 39.6 mg/kg to 114.5 ± 17.5 mg/kg, and a 58.1 ± 5.9% inhibition of the control RIST was produced. However, the RIST index was not inhibited by atropine when IGF-1 was used (256.7 ± 30.1 mg/kg) (Fig. 21, *top*). The blood pressure was stable (83.6 ± 3.7,

81.0 ± 4.1, and 76.0 ± 4.1 mmHg, respectively) throughout the experiment. Thus atropine infusion caused insulin, but not IGF-1, resistance.

*RIST with IGF-1 in control and after atropine (n=3).* After the control RIST with IGF-1 (256.1 ± 23.5 mg/kg) administration of intravenous atropine (1.0 mg/kg) did not affect IGF-1 sensitivity (258.2 ± 38.2 mg/kg) (Fig. 21, *bottom*).

*RIST in control and after surgical denervation (n=5).* Surgical denervation of the liver significantly reduced the control RIST index with insulin from 230.7 ± 7.6 mg/kg to 106.0 ± 27.7 mg/kg, and produced a 54.8 ± 10.8% inhibition of the control RIST. However, the RIST index was not reduced when the RIST was repeated with IGF-1 (256.3 ± 10.6 mg/kg) (Fig. 22). The blood pressure was constant throughout the experiment (88.0 ± 5.5, 95.0 ± 5.6, and 89.0 ± 7.6 mmHg, respectively). Thus surgical denervation of the liver produces insulin, but not IGF-1, resistance.

*RIST in control and after atropine in 16 hr fasted rats (n=5).* There was no significant difference between basal arterial glucose levels in all fed groups (108.7 ± 2.4 mg/dl, n=27) and in the 16 h fasted group (109.5 ± 3.5 mg/dl). After 16 h of fasting the RIST index using insulin was 104.1 ± 18.7 mg/kg. Atropine administration did not significantly reduce the RIST index (78.9 ± 14.1 mg/kg). The RIST index was significantly higher when IGF-1 sensitivity was measured (225.9 ± 95.0 mg/kg) (Fig. 23). The response to IGF-1 was not significantly different in fed and fasted rats. The blood pressure was stable (90.0 ± 13.1, 90.0 ± 14.3, and 93.6 ± 12.3 mmHg, respectively) throughout the experiment. Therefore, a 16 hr fast causes insulin, but not IGF-1, resistance.

*RIST with IGF-1 in control and after SIN-1 (n=2).* After the control RIST with IGF-1 (213.2 ± 34.8 mg/kg) administration of intraportal SIN-1 (5.0 mg/kg) did not affect IGF-1 sensitivity (214.4 ± 36.8 mg/kg) (not shown).

### 3.4 Discussion

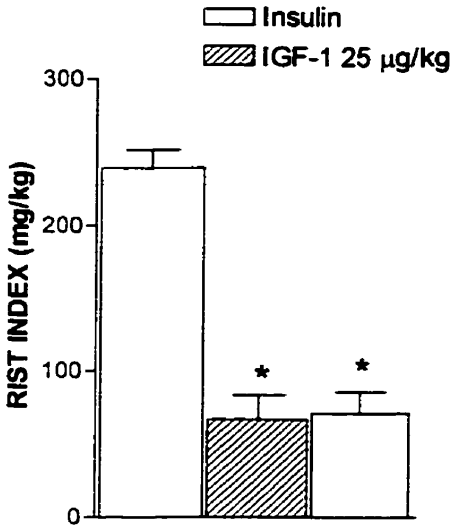
We have confirmed previous observations that IGF-1 and insulin have similar glucose disposal effects (Guler et al. 1987; Jacob et al. 1989; Giacca et al. 1990; Borg and Sherwin 1995; Simpson et al. 1998). However, it appears that these two hormones act through different mechanisms. Insulin action is mediated through the permissive role of the hepatic parasympathetic-dependent release of HISS from the liver (chapter 1). HISS enhances glucose uptake at the skeletal muscle (chapter 1). Based on our current results, the effect of IGF-1 to induce glucose uptake does not involve the parasympathetic-dependent release of HISS.

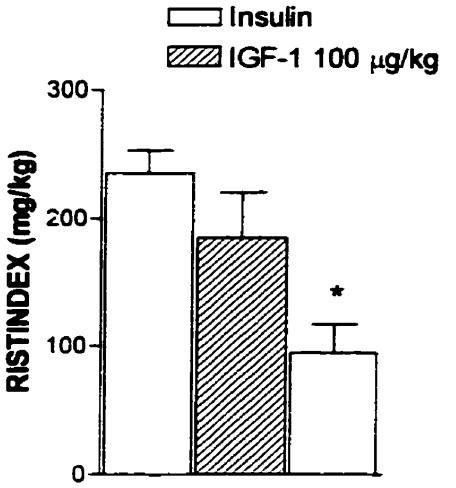
*Technical considerations.* The RIST (described in detail in chapter 1) was used to measure insulin sensitivity. The IGF-1 sensitivity was also measured using the RIST, however, instead of insulin a bolus of IGF-1 was used. The utility of the RIST method was shown for quantification of IGF-1 action.

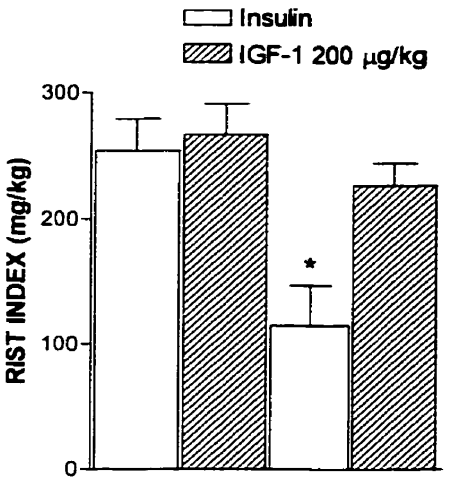
HISS release in response to insulin is blocked by surgical denervation of the liver and muscarinic antagonists (chapters 1, 2). Fasting decreased the HISS-dependent component of insulin action but did not affect the HISS-independent component (post-atropine or denervation) (Macedo et al. 1998). The present study used three methods to reduce HISS release to determine that insulin action but not IGF-1 action was mediated to a large extent (50-60%) by HISS.

Different doses of IGF-1 were examined to detect a dose that had a similar effect to 50 mU/kg of insulin (standard dose of insulin used in all our experiments) (Fig. 19, *bottom right*). The dose of 200 μg/kg of IGF-1 showed the most similarity in glucose uptake to our standard dose of insulin (50 mU/kg) and it was used as the standard IGF-1

**Figure 19**


Insulin
IGF-1 25 µg/kg
RIST INDEX (mg/kg)
300
200
100
0
*
*


Insulin
IGF-1 100 µg/kg
RISTINDEX (mg/kg)
300
200
100
0
*


Insulin
IGF-1 200 µg/kg
RIST INDEX (mg/kg)
300
200
100
0
*

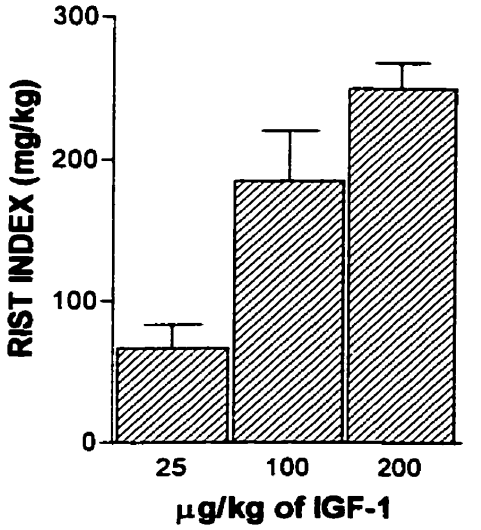

RIST INDEX (mg/kg)
300
200
100
0
25
100
200
µg/kg of IGF-1

**Figure 19.** *Top left.* The RIST index with insulin and with dose 25 μg/kg of IGF-1. Values are means ± SE; n=4. *$P$<0.001. *Top right.* The RIST index with insulin and with dose 100 μg/kg of IGF-1. Values are means ± SE; n=4. *$P$<0.05. *Bottom left.* The RIST index with insulin and with dose 200 μg/kg of IGF-1. Values are means ± SE; n=6. *$P$<0.05. The response to insulin was inhibited after administration of any dose of IGF-1. *Bottom right.* The RIST index (mg/kg) in response to 25 (n=4), 100 (n=4), 200 (n=10) μg/kg of IGF-1. Values are means ± SE. The RIST index used for testing insulin action is also useful for assessing IGF-1 action as demonstrated by the ability to show dose-response relations with a 30 min test period.

dose in the rest of the experiments (Fig. 20). However, IGF-1 appears to inhibit insulin's action, since the RIST with insulin was significantly reduced after IGF-1 infusion even with the lowest dose of IGF-1 used (25 μg/kg) (Fig. 19). As the result we decided to perform all of the RISTs with insulin before the infusion of IGF-1. At this point we do not know the mechanism by which IGF-1 causes insulin resistance, but we can speculate that IGF-1 may bind to the insulin receptors and antagonize the insulin binding to its receptor at the skeletal muscle or at the liver and prevent HISS release. It has been also shown that IGFBP-7 has low affinity for IGF-1 but binds insulin with very high affinity (Yamanaka et al. 1997). IGFBP-7 blocks insulin binding to its receptors and therefore inhibiting the earliest steps in insulin action (Yamanaka et al. 1997). It is possible that after administration of exogenous IGF-1, the production of IGFBPs increase and when insulin is infused its action is inhibited by binding to the IGFBP-7. It is also possible that IGF-1 causes insulin resistance by disruption of HISS release from liver. However, administration of intraportal Ach and SIN-1 did not reverse the inhibitory effects of IGF-1 on insulin's action. Considering that denervation-induced (chapter 2), but not IGF-1-induced, insulin resistance was reversed by Ach and SIN-1 administration, we suggest that IGF-1 does not cause insulin resistance through inhibition of either Ach release or NO production.

Administration of intraportal SIN-1 (5.0 mg/kg), without any other interventions, did not significantly change the IGF-1 RIST index. Thus, providing NO to the liver does not affect IGF-1 sensitivity.

**Figure 20**

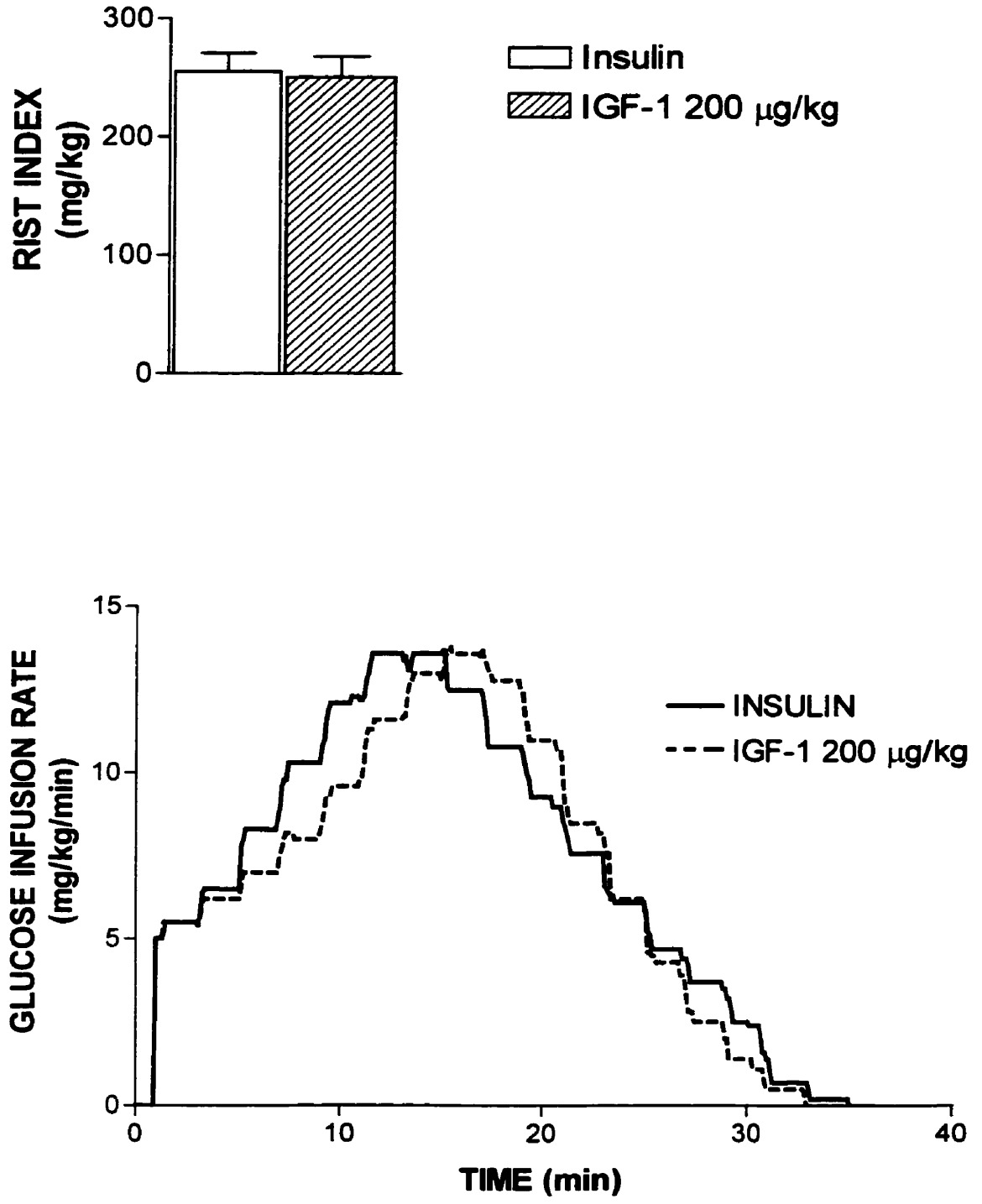

RIST INDEX (mg/kg)
300
200
100
0
Insulin
IGF-1 200 µg/kg
GLUCOSE INFUSION RATE (mg/kg/min)
15
10
5
0
INSULIN
IGF-1 200 µg/kg
0
10
20
30
40
TIME (min)

**Figure 20.** *Top.* The RIST index with insulin (50 mU/kg) and with IGF-1 (200 μg/kg). Values are means ± SE; n=10. $P>0.05$. ***Bottom.*** A plot of the average glucose infusion rate (mg/kg/min) as determined at 0.1 min intervals during the same RISTs on top with insulin (solid line) and IGF-1 (broken line). Insulin and IGF-1, at these doses, have similar RIST indexes and similar dynamic curves.

### *3.4.1 Parasympathetic reflex inhibition*

Interruption of the parasympathetic-dependent release of HISS, by either atropine administration (Fig. 21, *top*) or surgical denervation of the liver (Fig. 22), resulted in severe and immediate insulin resistance. The stimulation of glucose uptake was decreased by 58.1 ± 5.9% after atropine and 54.8 ± 10.8% after hepatic denervation. However, the glucose uptake response to IGF-1 was not affected by the disruption of this parasympathetic tone (Fig. 21, 22). Thus, IGF-1 acts through a different mechanism, independent of HISS release, to increase glucose uptake.

### *3.4.2 Effect of fasting on insulin and IGF-1 action*

We have previously reported that HISS release is also dependent upon the prandial state of the animal (Macedo et al. 1998). After feeding, the HISS release in response to insulin leads to an increase in glucose uptake by the insulin sensitive tissues. However, in the fasted state HISS is not released in response to insulin, thus the hypoglycemic action of insulin is very low. We suggest that this regulatory system controls nutrition partitioning so that when food is being absorbed, glucose is selectively stored as glycogen in the skeletal muscle. In the fasted state, the response to insulin is severely blunted. The HISS-dependent component of insulin action can be quantitated by the decrease in RIST index after atropine, denervation, or hepatic nitric oxide synthase inhibition (chapters 1, 2). The response seen after blockade of HISS release is due to the direct action of insulin, independent of HISS. Fasting reduces the HISS-dependent, but not the HISS-independent component of insulin action. Sixteen hours of fasting in rats produced a reduced insulin response and further atropine administration did not

**Figure 21**

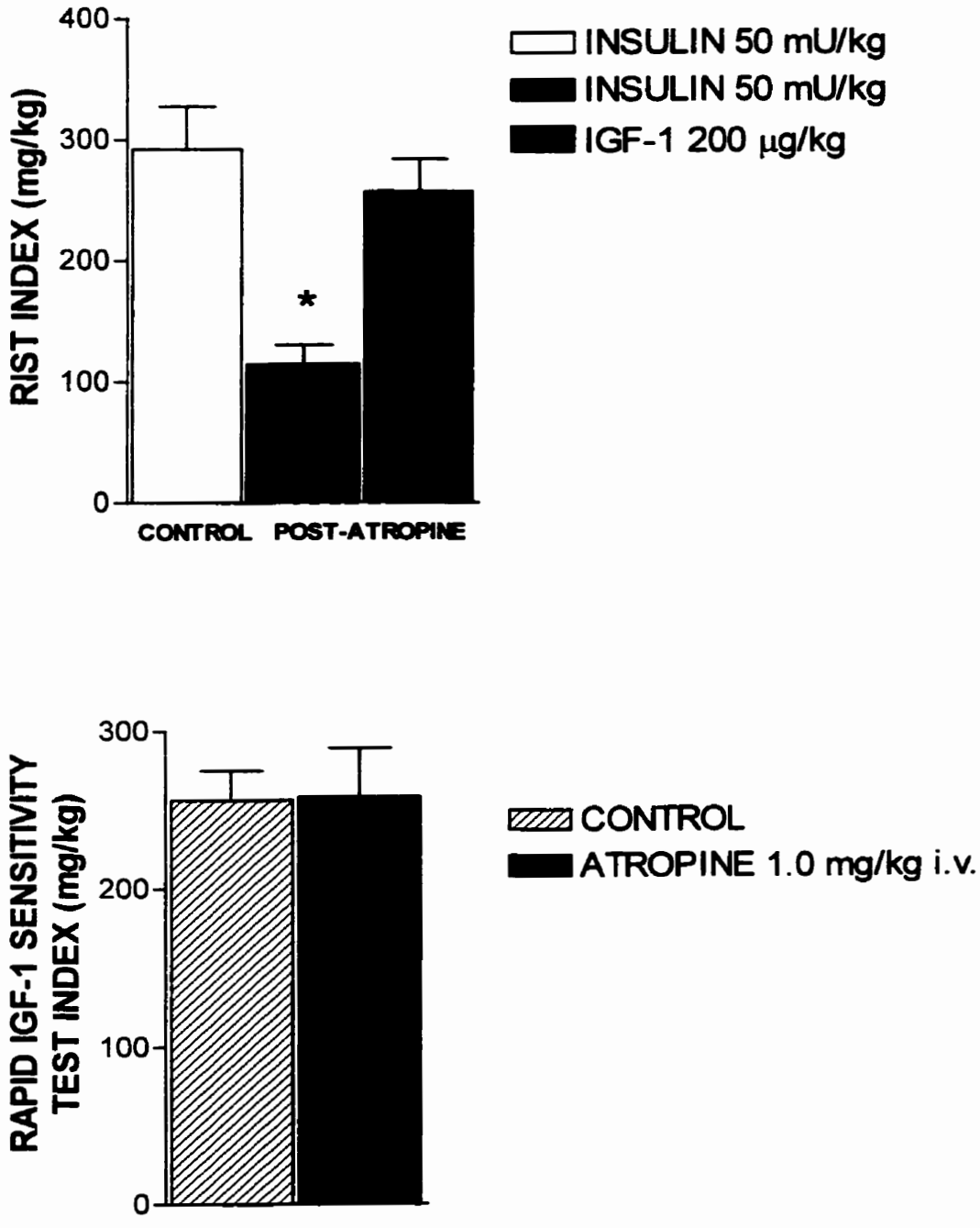

400
300
200
100
0
RIST INDEX (mg/kg)
*
CONTROL
POST-ATROPINE
INSULIN 50 mU/kg
INSULIN 50 mU/kg
IGF-1 200 µg/kg
300
200
100
0
RAPID IGF-1 SENSITIVITY TEST INDEX (mg/kg)
CONTROL
ATROPINE 1.0 mg/kg i.v.

**Figure 21.** *Top.* The RIST index with insulin in control and with insulin and IGF-1 after intravenous atropine (1.0 mg/kg) administration. Values are means ± SE; n=5. **P*<0.01. Atropine causes insulin, but not IGF-1, resistance. ***Bottom.*** The RIST index with IGF-1 before and after intravenous atropine (1.0 mg/kg) administration. Values are means ± SE; n=3. *P*>0.5. Blockade of parasympathetic nerves does not affect the hypoglycemic action of IGF-1.

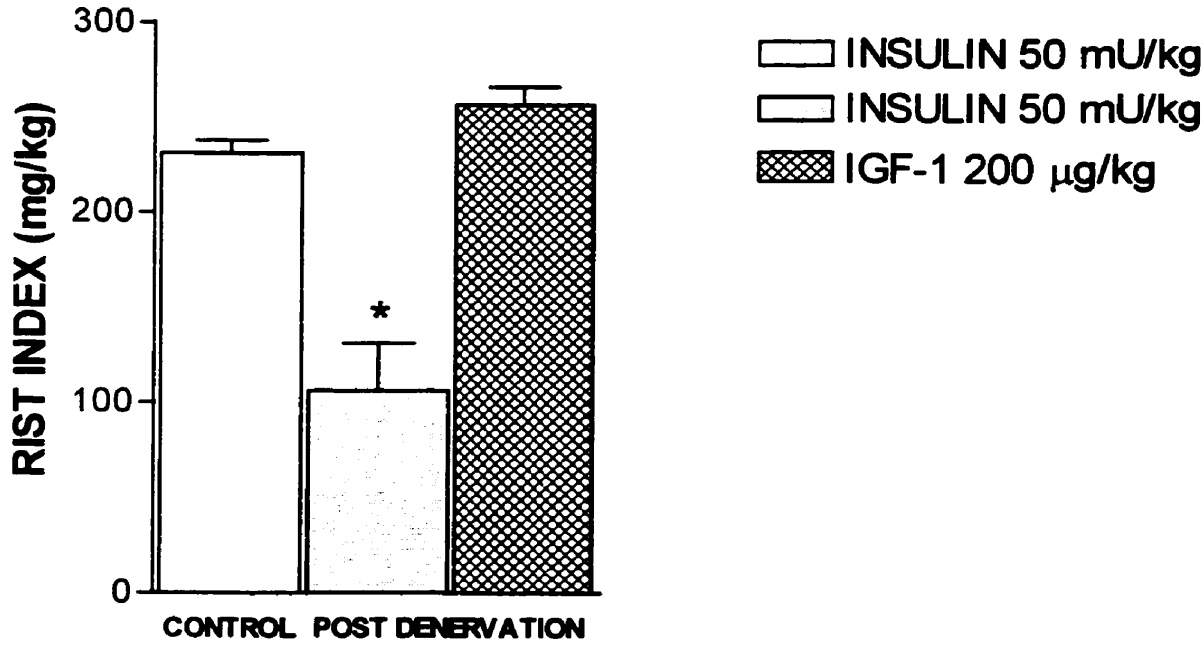



**Figure 22.** The RIST index with insulin in control and with insulin and IGF-1 after parasympathetic denervation of the liver. Values are means ± SE; n=5. **P*<0.001. Hepatic parasympathetic denervation results in insulin, but not IGF-1, resistance.

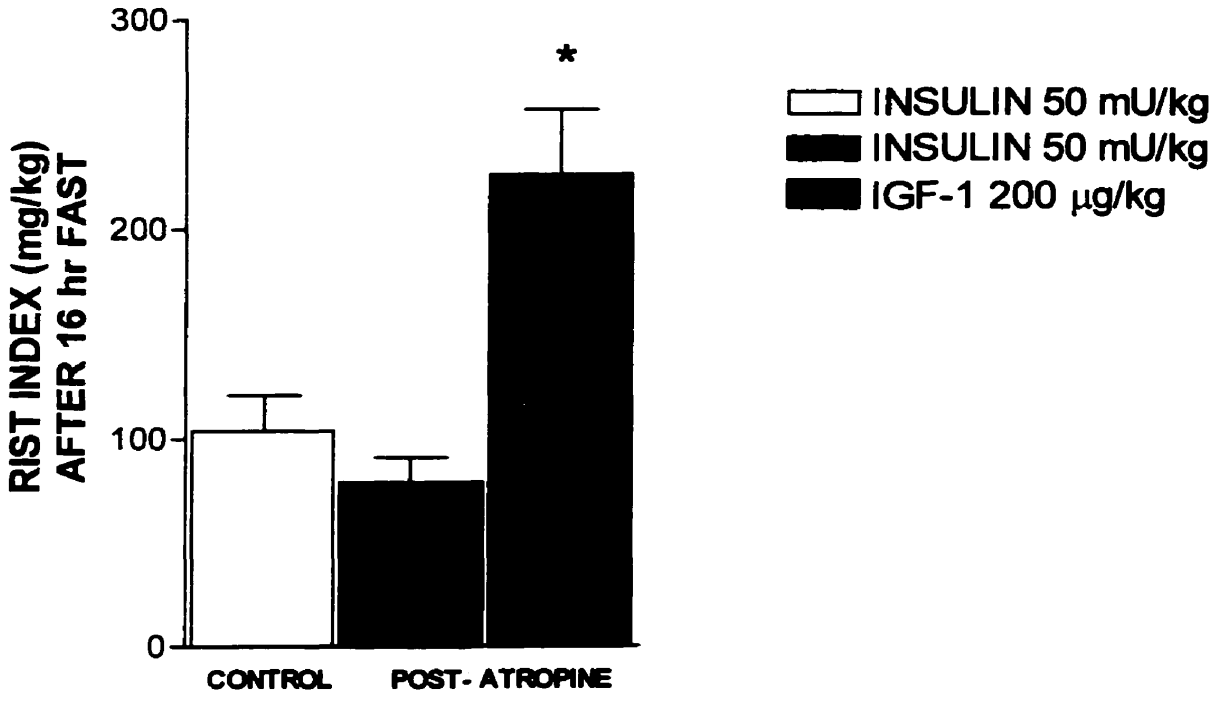



**Figure 23.** The RIST index with insulin in control and with insulin and IGF-1 after intravenous atropine (1.0 mg/kg) administration in 16 hour fasted rats. Values are means ± SE; n=5. *$P<0.01$. HISS release, assessed from the difference in insulin action between control and post-atropine RIST index, was insignificant after the 16 h fast. 16 hours of fasting results in insulin, but not IGF-1, resistance.

significantly inhibit the response to insulin (Fig. 23). Thus, the HISS-dependent component of the insulin response was insignificant after a 16 h fast. However, the IGF-1 response was not affected after fasting, indicating that the hypoglycemic effect of IGF-1 was not regulated by the prandial state and confirming that IGF-1 action was not dependant on the parasympathetic-induced release of HISS.

IGFBP-1 has an inhibitory effect on the action of IGF-1 and it has been shown to increase during fasting (Busby et al. 1988; Yeoh and Baxter 1988; Hall et al. 1988; Snyder and Clemmons 1990). However, the IGF-1 RIST indexes after feeding (266.8 ± 26.6 mg/kg) or fasting (225.9 ± 35.0 mg/kg) were not significantly different. Thus, the increase in IGFBP-1 after fasting did not inhibit the IGF-1's action in these experiments. Also, it has been shown that intravenous administration of glucose suppresses IGFBP-1 levels (Snyder and Clemmons 1990) and since we have already infused glucose during the first RIST with insulin, then at the time of the RIST with IGF-1 the plasma levels of IGFBP-1 would be anticipated to be already suppressed. Thus, IGFBP-1 was unlikely to have an effect on glucose uptake during the RIST with IGF-1.

In conclusion, insulin and IGF-1 have similar effects on glucose disposal as assessed by the RIST and their dynamic curves (Fig 20). However, insulin acts by mediating the release of HISS from the liver. HISS sensitizes the skeletal muscle response to insulin and accounts for 50-60% of insulin action. Stimulation of glucose uptake by IGF-1 does not depend upon HISS action. Type 2 diabetics and people with chronic liver disease are highly insulin resistant (Proietto et al. 1980; Iversen et al. 1984; Simpson et al. 1998) and it has been shown that IGF-1 improves glycemic control in both

disease conditions (Jacob et al. 1991; Rossetti et al. 1991; Zenobi et al. 1992; Moses et al. 1996; Simpson et al. 1998) which are associated with insulin but not IGF-1 resistance. We have proposed that insulin resistance produced in type 2 diabetes and chronic liver disease is caused by a hepatic parasympathetic neuropathy leading to cessation of HISS release from the liver (Lautt 1999). Since the glucose disposal effect of IGF-1 does not involve the hepatic parasympathetic nerves, IGF-1 sensitivity in these conditions is not affected. However, the ability of IGF-1 to cause insulin resistance in our setting raises concern about the possibility of the same response occurring in the clinical situation. This concern may be somewhat modified by the fact that only those who already have severe insulin resistance would be considered to receive IGF-1.

# Chapter 4

## Blockade of hepatic cyclooxygenase causes insulin resistance

### 4.1 Introduction

We have demonstrated in chapter 2 that the permissive role of the hepatic parasympathetic-dependent release of HISS involves the production of nitric oxide (NO) in the liver. In many physiological and pathological events NO and Prostaglandins (PGs) are co-released and/or NO action is mediated through production of PGs (below). In addition, indomethacin, a cyclooxygenase inhibitor, has been shown to produce insulin resistance (Syvalahti 1974; Kilbom and Wennmalm 1976; Cavagnini et al. 1977; Widstrom 1977; Dietze et al. 1978; Chen and Robertson 1979; Wasner et al. 1994). Thus, we hypothesized that the release of HISS from the liver is also mediated through the hepatic production of PGs.

*4.1.1 Synthesis*

PGs are among the most potent naturally occurring autacoids and are recognized as critically important cell regulatory substances. Prostaglandin H synthase (PGHS) is a bifunctional glycoprotein which catalyzes the biosynthesis of $PGH_2$, a precursor for prostaglandins ($PGE_2\alpha$, $PGF_2\alpha$, and $PGD_2$), prostacyclin ($PGI_2$), and thromboxane $A_2$ (Wu 1995). PGHS contains two enzymic activities: 1) cyclooxygenase (COX) which adds two molecules of oxygen to arachidonic acid to form $PGG_2$ and, 2) peroxidase which reduces $PGG_2$ to $PGH_2$ (Smith and Marnett 1990). Both enzymic activities require heme. Two isoforms of COX have been identified; one is constitutively expressed (COX-

1), whereas the other is induced (COX-2) during an inflammatory insult (DeWitt 1991; Seibert and Masferre 1994). COX-1 is present in almost all cells and tissues and is involved in the regulation of physiological functions (Smith 1989; Vane 1994). COX-2 is expressed primarily in macrophages, endothelial cells, fibroblasts, and smooth muscle cells after stimulation with endotoxins, certain cytokines, or mitogens ( Maier et al. 1990; Xie et al. 1992; Lee et al. 1992; Wu 1995). COX is inhibited by non-steroidal anti-inflammatory drugs such as aspirin and indomethacin (Ferreira and Vane 1974), however, they are more potent inhibitors of COX-1 than COX-2 (Meade et al. 1993; Mitchell et al. 1993). On the other hand, glucocorticoids inhibit the induction of COX-2 without affecting the activity of COX-1 (Fu et al. 1990; Masferrer et al. 1990, 1992).

*4.1.2 Functions*

The biologically active metabolites produced by PGHS play important roles in a wide variety of physiological and pathological functions. For example, $PGI_2$ produced by vascular endothelial and smooth muscle cells strongly inhibits platelet aggregation and relaxes smooth muscle (Vane et al. 1990; Hecker et al.1995). These actions of $PGI_2$ are through activation of adenylate cyclase leading to increased intracellular cAMP levels which eventually causes a decrease in the free intracellular calcium levels (Hardman 1984; Vane et al.1990; Hecker et al. 1995). Other physiological roles of PGs include increase in body temperature, induction of sleep, inhibition of release of norepinephrine, and stimulation of secretion of some hormones (e.g., growth hormone, thyroid-stimulating hormone, follicle-stimulating hormone, luteininzing hormone, and prolactin) (Hecker et al. 1995). PGs can have opposite effects depending on the PG produced and

on the target tissue and organ. For example, in contrast to vasodilatory effects of $PGI_2$ in vasculature, $PGF_2\alpha$ and $TXA_2$ cause vasoconstriction, especially in veins (Hecker et al. 1995). In addition, longitudinal smooth muscles of the gastrointestinal tract are contracted by $PGE_2$ and $PGF_2\alpha$, while circular muscle is contracted by $PGI_2$ and $PGF_2\alpha$ but relaxed by $PGE_2$ (Hecker et al. 1995). PGs have been shown to be released and to participate in the inflammatory response (Wu 1995; Hecker et al. 1995). In experimental acute and chronic inflammation animal models, enhanced COX-2 expression parallels the degree of tissue inflammation. COX-2 in the inflammatory tissues can be induced in a number of cell types such as fibroblast, endothelial cells, and chondrocytes by inflammatory cytokines and growth factors (Maier et al. 1990; Xie et al. 1992; Lee et al. 1992; Wu 1995). However, macrophages are the only principal class of the immune system that can synthesis all PGs (Hecker et al.1995). $PGE_2$ and $PGI_2$ affect T cell proliferation. They inhibit T cell clonal expression by inhibiting IL-1 and -2 and class II antigen expression on macrophages or other antigen presenting cells (Hecker et al. 1995). $PGE_2$ inhibits both antigen-driven and mitogen-induced B lymphocyte proliferation and differentiation to plasma cells, resulting in inhibition of immunoglobulin M (IgM) synthesis (Hecker et al. 1995).

*4.1.3 Involvement of NO*

It has been suggested that NO regulates both physiological and pathological events through direct activation of COX leading to an increase in production of PGs (Salvemini et al. 1993, 1995, 1996; Davidge et al. 1995; Di Rosa et al. 1996; Janabi et al. 1996; Maccarrone et al. 1997; Failli et al. 1998). The COX is believed to be a target for

NO because it contains an iron-heme center at its active site (De Groot et al. 1975; Greenwald et al. 1980; Kalyanaranman et al. 1982; Davidge et al. 1995) and the vast majority of effects of NO are a consequence of its interaction with iron or iron-containing enzymes. For example, the ability of NO to inhibit platelet aggregation and to relax vascular smooth muscle is the result of NO binding to the heme-$Fe^{2+}$ prosthetic group of the soluble guanylate cyclase leading to its stimulation and subsequent increase in the levels of cGMP (Mellion et al. 1981; Ignarro 1991). In the same manner, NO interacts with hemoglobin (Kanner et al. 1992) or can exert its cytotoxic effects by interacting with iron-sulfur centers in the key enzymes of the respiratory cycle and DNA synthesis (Nathan 1992), thus raising the possibility that NO modulates the activity of COX. iNOS and COX-2 are not normally expressed but they are induced following appropriate stimulation with pro-inflammatory agents such as *E. coli* lipopolysaccride (LPS) (Fu et al. 1990; Masferrer et al. 1990, 1992; Moncada et al. 1991). Inhibition of NO production in LPS-induced macrophages *in vitro* and *in vivo* has been shown to result in an attenuation of PGs release (Salvemini et al. 1993, 1995). This stimulatory action of NO on the COX pathway has been confirmed in other cell systems including hypothalamic slices (Rettori et al. 1992), smooth muscle cells (Inoue et al. 1993*a*), islet cells (Corbett et al. 1993), endothelial cells (Davidge et al. 1995) the microcirculation of rat (Koller et al. 1993), and in rat perfused kidney (Salvemini et al. 1994).

In several physiological and pathological conditions, NO and PGs have been shown to work synergistically. For example, NO and $PGI_2$ act synergistically via cGMP and cAMP pathway , respectively, to inhibit platelet activation and aggregation and relax vascular tone thus maintaining blood fluidity and normal vascular tone (Radomski et al.

1987; Gryglewski et al. 1989; Maurice and Haslam 1990; Kaley and Koller 1995; Koller and Huang, 1995; Salvemini et al. 1993,1996). Moreover, LPS and many inflammatory cytokines have been found to induce both iNOS and COX-2 in several cell types. The co-expression iNOS and COX-2, induced by LPS, TNF-$\alpha$, IFN-$\gamma$, and IL-1$\beta$, has been documented in macrophages (Sthuer and Marletta 1987; Drapier et al. 1988; Gaillard et al. 1992; Riese et al. 1994; Arias-Negrete et al. 1995), endothelial cells (Radomski et al. 1990; Kilbourn and Belloni 1990; Akarasereenont et al. 1995), vascular smooth muscle cells (Inoue et al. 1993*a*), rat mesangial cells (Tetsuka et al. 1994), and rat islets (Corbett et al. 1993). In addition, it has been also shown that NO and PGs function synergistically after LPS insult to maintain hepatocellular integrity (Harbrecht et al.1994).

*4.1.4 Involvement in glucose homeostasis*

The involvement of PGs in glucose regulation has been well documented. *In vivo* studies using PG infusions or PG synthesis inhibitors have generally supported a hyperglycemic effect of E-series PGs (Bergstrom et al. 1966; Sacca et al. 1974; Miller et al. 1983), resulting from increased hepatic glucose output. In contrast, *in vitro* studies demonstrate no effects (Levine 1974; Sweat and Yamashita 1978; Sweat et al. 1983) or inhibition (Wheeler and Epand 1975; Levine and Schwartzel 1980; Brass et al. 1984; Brass and Garrity 1985) by PGE of hepatic glucose production. These discrepancies can be explained by recognizing that *in vivo* PGE can alter circulating hormone levels, such as inhibition of insulin secretion (Robertson and Chen 1977; Hedqvist 1977; Luyckx and LeFebvre 1978) or stimulation of glucagon secretion (Pek et al. 1975), and/or stimulation of the sympathetic nervous system (Miller et al. 1985).

Administration of $PGE_2$ in humans has been shown to inhibit glucose-stimulated insulin release and to impair glucose tolerance as a result of insulin resistance (Robertson et al. 1974; Robertson and Chen 1977; Konturek et al. 1978; Newman and Brodows 1982). It has been suggested that the insulin resistance effect of $PGE_2$ is mediated through activation of the adrenergic system, since plasma levels of both epinephrine and norepinephrine significantly increased during $PGE_2$ infusion (Newman and Brodows 1982). Contrary to these studies, *in vitro* administration of $PGE_2$ was shown to enhance insulin-mediated glucose transport in adipocytes (Vaughan 1967). On the other hand, $PGE_1$ has been shown to stimulate peripheral glucose uptake in the rat *in vivo* (Sacca et al. 1974). In addition, Iloprost, a chemically stable derivative of $PGI_2$, has been shown to improve insulin action and non-oxidative glucose metabolism in healthy subjects (National Diabetes Data Group 1979) and in hypertensive patients, despite a similar skeletal muscle blood flow to controls (Paolisso et al. 1995).Thus, PGs appear to be involved in glucose homeostasis but the significance and regulatory roles remain unclear.

It has been well documented that indomethacin causes marked insulin resistance (Syvalahti 1974; Kilbom and Wennmalm 1976; Cavagnini et al. 1977; Dietze et al. 1978). Dietze et al. (1978) have shown that indomethacin administration significantly decreases insulin's action to increase glucose uptake at the skeletal muscle. They suggested that this action of indomethacin can be explained if PGs increased the sensitivity of muscle to the effects of insulin (Dietze et al. 1978).

Acetylsalicylic acid (ASA), another COX inhibitor, has also been shown to produce insulin resistance in healthy (Giugliano et al. 1982; Newman and Brodows 1983; Bratusch-Marrain et al. 1985) and type 2 diabetic patients (Bratusch-Marrain et al. 1985).

ASA causes a rise of basal insulin (Robertson and Chen 1977; Giugliano et al. 1982) and glucose-stimulated insulin concentrations in normal subjects (Field et al. 1967; Micossi et al. 1978; Robertson and Chen 1977; Chen and Robertson 1979; Newman and Brodows 1983) and in type 2 diabetic patients (Field et al. 1967; Micossi et al. 1978, Vierhapper et al. 1983). It has been suggested that ASA-induced hyperinsulinemia is a result of reduced clearance of insulin since there is a lack of associated change in plasma C-peptide levels (Giugliano et al. 1982). Several studies have demonstrated that salicylate or ASA lowers plasma glucose concentrations in normal subjects (Field et al. 1967; Micossi et al. 1978; Giugliano et al. 1978) and in type 2 diabetic patients (Field et al. 1967; Micossi et al. 1978). This can be explained by the fact that ASA increases insulin levels leading to reduction in the hepatic glucose production (Giugliano et al. 1982), thus, reducing the plasma glucose levels.

To evaluate the involvement of PGs in the hepatic release of HISS, we used indomethacin to inhibit PGs synthesis. The intravenous and the intraportal infusion of the same dose of indomethacin were compared to determine the location of PGs inhibition leading to insulin resistance. Ach and 3- Morpholinosydnonimine (SIN-1), a NO donor, were administered to reverse the insulin resistance produced by indomethacin.

## 4.2 Materials and methods

Male Sprague-Dawley rats (278.3 ± 5.4 g) were fasted overnight (8 h) and were fed standard laboratory rat food for 2 h before the start of any surgical procedures. Animal preparation, surgical procedures, and the RIST methodology are explained in detail in chapter 1.

*RIST in control and after indomethacin infusion (n=15).* After the control RIST was performed, indomethacin (8.0 mg/kg) was intraportally infused over 2 min. The rats were then allowed to stabilize for 30 min. A stable basal arterial glucose concentration was established and another RIST was performed. Some of the rats (n=3) were allowed to stabilize for another 30 min, and after determination of basal arterial glucose concentration a second post-indomethacin RIST was repeated to measure the duration of action of the drug.

*RIST in control, after intravenous or intraportal indomethacin infusion, and after atropine.* After the control RIST, indomethacin (4.0 mg/kg) was infused either intravenously (n=6) or intraportally (n=6) over 2 min. The animals were then allowed to stabilize for at least 30 min and another RIST was performed. Atropine (3.0 mg/kg) was infused intraportally over 5 min in both groups, and the RIST was repeated.

*RIST in control, after indomethacin, after intraportal Ach infusion, and after atropine (n=4).* After the control RIST, indomethacin (8.0 mg/kg) was intraportally infused over 2 min. The animals were then allowed to stabilize for at least 30 min. After the second RIST, Ach (2.5 mg/kg/min) was infused intraportally and the RIST was repeated. Atropine (3.0 mg/g) was then administered intravenously and a fourth RIST was performed.

*RIST in control, after indomethacin, and after intraportal SIN-1 infusion (n=5).* After the control RIST was performed, indomethacin (8.0 mg/kg) was intraportally infused over 2 min. The animals were then allowed to stabilize for at least 30 min. After the second RIST, SIN-1 (10.0 mg/kg) was infused intraportally over 2 min and the RIST was repeated.

*Drugs.* Atropine, Ach, D-glucose, and indomethacin were purchased from Sigma Chemicals (St. Louis, MO). SIN-1 was purchased from Alexis (San Diego, CA). The human insulin was obtained from Eli Lilly (Indianapolis, IN). All the chemicals, except indomethacin, were dissolved in saline. Indomethacin was dissolved in 5% sodium bicarbonate (Fisher Scientific, Fair Lawn, NJ).

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

## 4.3 Results

The index used to express insulin sensitivity is the total amount of glucose (mg/kg) infused over 30 min after insulin (50 mU/kg) administration in order to maintain euglycemia at the baseline level and is referred to as the RIST index.

*RIST after indomethacin infusion (n=15).* Administration of indomethacin significantly reduced the control RIST index from 241.1 ± 11.3 mg/kg to 110.2 ± 10.3 mg/kg and caused a 54.5 ± 3.5% inhibition of the control response (Fig. 24). The blood pressure was 104.8 ± 2.8 mmHg before the control RIST, but it was significantly reduced to 89.6 ± 6.6 mmHg after the indomethacin administration. The basal glucose concentration (122.5 ± 1.7 mg/dl) was significantly reduced from the control after indomethacin administration (97.9 ± 4.9 mg/dl). Two hours after indomethacin administration the RIST was repeated again in three of the rats and the RIST index was 97.8 ± 29.1 mg/kg with 48.9 ± 16.9% inhibition of the control response (Fig. 25). Thus, intraportal administration of indomethacin (8.0 mg/kg) produced insulin resistance that was maintained for at least 2 h.

*RIST in control, after intravenous or intraportal indomethacin infusion, and after atropine.* The control RIST index (n=6) of 200.2 ± 10.9 mg/kg was not significantly reduced (162.1 ± 18.1 mg/kg) after intravenous indomethacin (4.0 mg/kg) administration. However, administration of intravenous atropine (3.0 mg/kg), a non-selective muscarinic antagonist, markedly reduced the RIST index to 81.0 ± 4.5 mg/kg and caused a 58.8 ± 2.6% inhibition of the control RIST (Fig. 26). The blood pressure was 104.7 ± 8.3 mmHg before the control RIST, but it was significantly reduced to 75.0 ± 3.7 mmHg after indomethacin administration and it remained low at 74.2 ± 3.8 mmHg after atropine

administration. The basal glucose concentrations before each RIST were not significantly different (114.2 ± 5.5 mg/dl before the control RIST, 105.2 ± 7.2 mg/dl before the indomethacin RIST, and 108.1 ± 2.4 mg/dl before the atropine RIST). In the second set of animals (n=6), the control RIST index (227.4 ± 12.2 mg/kg) was significantly reduced by intraportal infusion of the same dose of indomethacin (RIST index = 82.2 ± 11.8 mg/kg), causing a 64.3 ± 5.1% inhibition of the control response. Administration of intravenous atropine (3.0 mg/kg) did not cause a further significant reduction in RIST index (50.1 ± 7.7 mg/kg) (Fig. 26). The blood pressure was 97.2 ± 6.7 mmHg before the control RIST and 79.0 ± 5.8 mmHg after indomethacin but it was significantly reduced to 74.2 ± 3.8 mmHg after atropine administration. The basal glucose concentrations was 124.4 ± 5.1 mg/dl before the control RIST but it was significantly reduced to 106.6 ± 4.4 mg/dl and to 107.4 ± 4.0 mg/dl after indomethacin and atropine administrations, respectively. Thus, intraportal but not intravenous indomethacin at the 4.0 mg/kg dose produced significant insulin resistance.

*RIST after indomethacin, after intraportal Ach, and after intravenous atropine infusion (n=4).* Administration of intraportal of indomethacin (8.0 mg/kg) significantly reduced the RIST index from 246.5 ± 31.2 mg/kg to 87.4 ± 11.9 mg/kg and caused a 64.0 ± 4.4% inhibition of the control response. Intraportal administration of Ach (2.5 mg/kg/min) did not reverse the inhibition caused by indomethacin (RIST index = 85.8 ± 14.3 mg/kg) (Fig. 27). Administration of intravenous atropine (3.0 mg/kg) did not produced further significant insulin resistance (RIST index= 95.1 ± 14.6 mg/kg). Thus, Ach production in the liver cannot reverse the insulin resistance produced by COX inhibition.

*RIST after indomethacin and after intraportal SIN-1 infusion (n=5).* Intraportal infusion of indomethacin (8.0 mg/kg) significantly reduced the RIST index from 257.1 ± 9.8 mg/kg to 142.4 ± 102 mg/kg and caused a 44.5 ± 3.9% inhibition of the control response. Intraportal administration of SIN-1 (10.0 mg/kg) did not reverse the inhibition caused by indomethacin (RIST index = 131.6 ± 32.9 mg/kg) (Fig. 28). Thus, NO production in the liver cannot reverse the insulin resistance produced by COX inhibition.

## 4.4 Discussion

In chapter 2 we demonstrated that the permissive role of the hepatic parasympathetic-dependent release of HISS was mediated through the production of NO in the liver. Since many physiological and pathological actions of NO are mediated through PGs and/or NO and PGs are co-released, we hypothesized that the hepatic release of HISS is also mediated through PGs production in the liver. The RIST (described in detail in chapter 1) was used to measure insulin sensitivity in all experiments.

### *4.4.1 COX inhibition*

Administration of intraportal indomethacin (8.0 mg/kg), a COX inhibitor, produced significant insulin resistance that was maintained for more than 2 h (Figs. 24, 25).

To confirm the site of action of indomethacin, intraportal infusion of a submaximal indomethacin dose (4.0 mg/kg) was compared with intravenous infusion of the same dose. The intraportal, but not intravenous, dose caused significant insulin resistance (Fig. 26). Atropine, a non-selective muscarinic receptor antagonist, has been shown to produce HISS-dependent insulin resistance while leaving the HISS-independent component of insulin action unchanged (Xie and Lautt 1995*a*, chapter 2). Administration of atropine (3.0 mg/kg, iv) after intraportal indomethacin (4.0 mg/kg) did not produce further significant insulin resistance (Fig. 26). Thus, COX inhibition with intraportal indomethacin administration completely blocked the HISS release from the liver, resulting in insulin resistance. However, administration of atropine after intravenous indomethacin administration of the same dose produced significant insulin

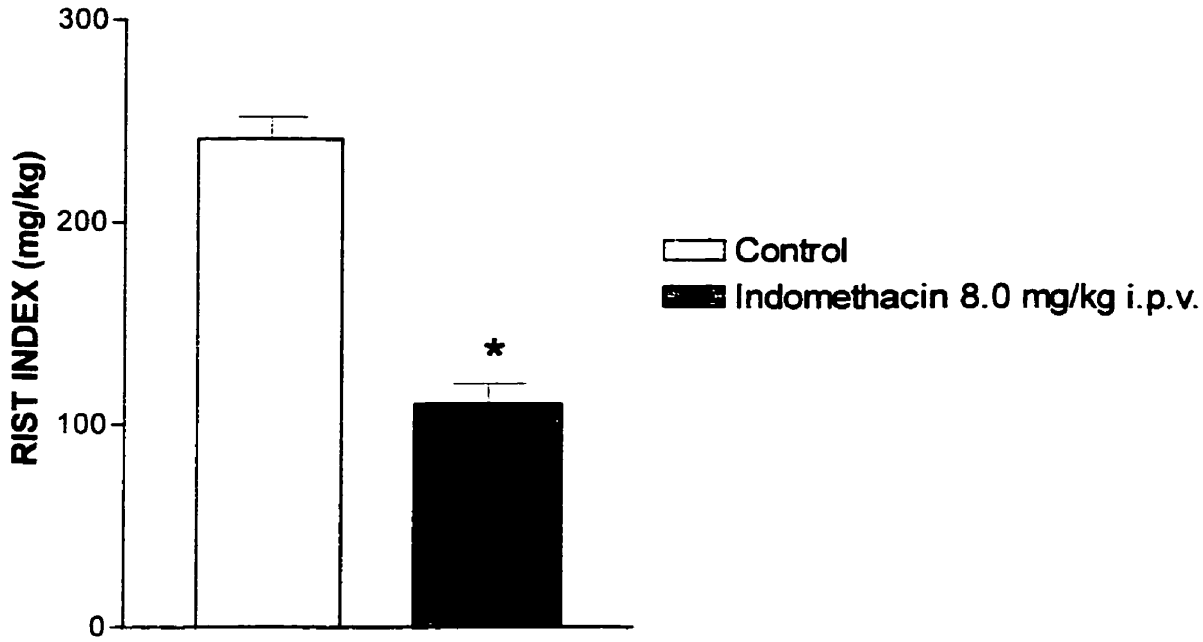



**Figure 24.** The RIST index in control and after intraportal indomethacin (8.0 mg/kg) administration. Values are means ± SE; n=15. **P*<0.0001. COX inhibition produced insulin resistance.


**Figure 25.** The RIST index in control, after intraportal indomethacin (8.0 mg/kg) administration, and 2 h post-indomethacin. Values are means ± SE; n=3. **P*<0.05. The insulin resistance produced by indomethacin lasted for more than 2h.

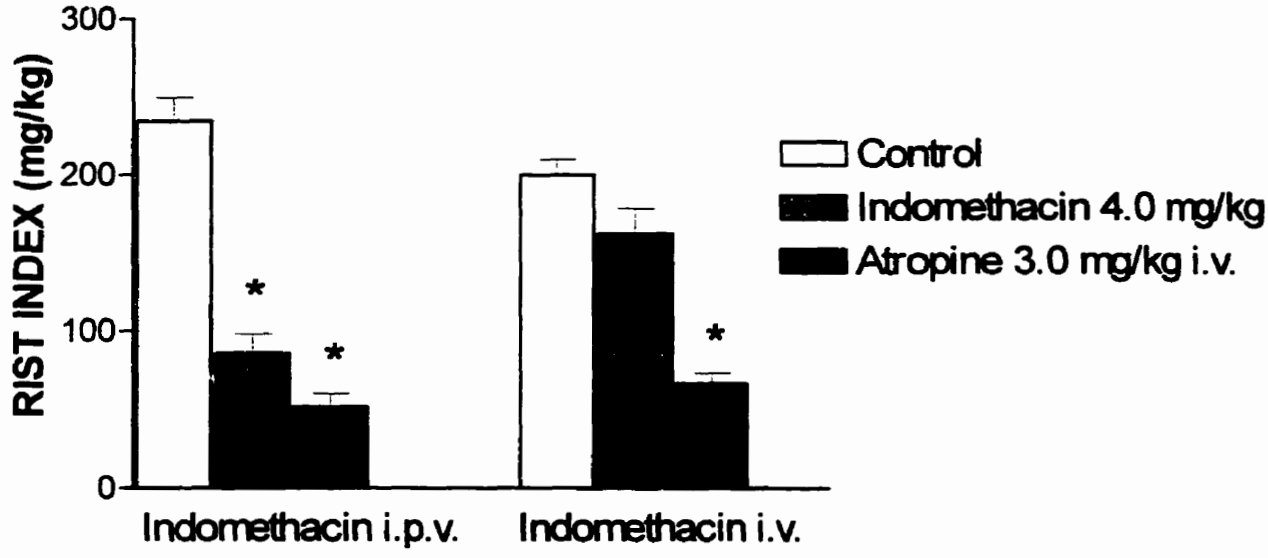



**Figure 26.** The RIST index in control, after intraportal (n=6) or intravenous (n=6) indomethacin (8.0 mg/kg), and after intravenous atropine (3.0 mg/kg) administration. Values are means ± SE. **P*<0.001. Insulin resistance was produced by the intraportal but not the intravenous route.

resistance (Fig. 26). This suggests that administration of intravenous indomethacin did not effectively inhibit the release of HISS from the liver. Thus, the fact that indomethacin produced significant HISS-dependent insulin resistance when administered intraportally, but not intravenously, demonstrates that the site of action of indomethacin is the liver.

It has been shown that indomethacin increases the release of insulin from the pancreatic β cells when the cells are stimulated with glucose (Wasner et al. 1994). In addition, plasma insulin levels rose to significantly higher levels during glucose tolerance tests in subjects treated with indomethacin compared to controls (Wasner et al. 1994). Although we did not measure the insulin concentration before and after indomethacin, we can assume that the plasma concentration of insulin may have increased, since the basal glucose concentration was significantly reduced after indomethacin (4.0 and 8.0 mg/kg, ipv) administration. However, the basal glucose concentration did not significantly change before and after administration of the intravenous lower dose of indomethacin (4.0 mg/kg). The indomethacin-stimulated insulin release does not seem to affect the insulin resistance produced by indomethacin, because the lower dose of intraportal indomethacin produced the same degree of insulin resistance compared to the higher dose (Figs. 24, 26).

*4.4.2 Reversal of insulin resistance*

Ach (2.5 μg/kg/min, ipv) and SIN-1 (10.0 mg/kg, ipv) administration did not reverse the insulin resistance produced by indomethacin (Figs. 27, 28). Ach and SIN-1 at these doses have been shown to reverse insulin resistance produced by denervation of the liver and intraportal L-NMMA administration (chapters 1, 2). But, after hepatic COX

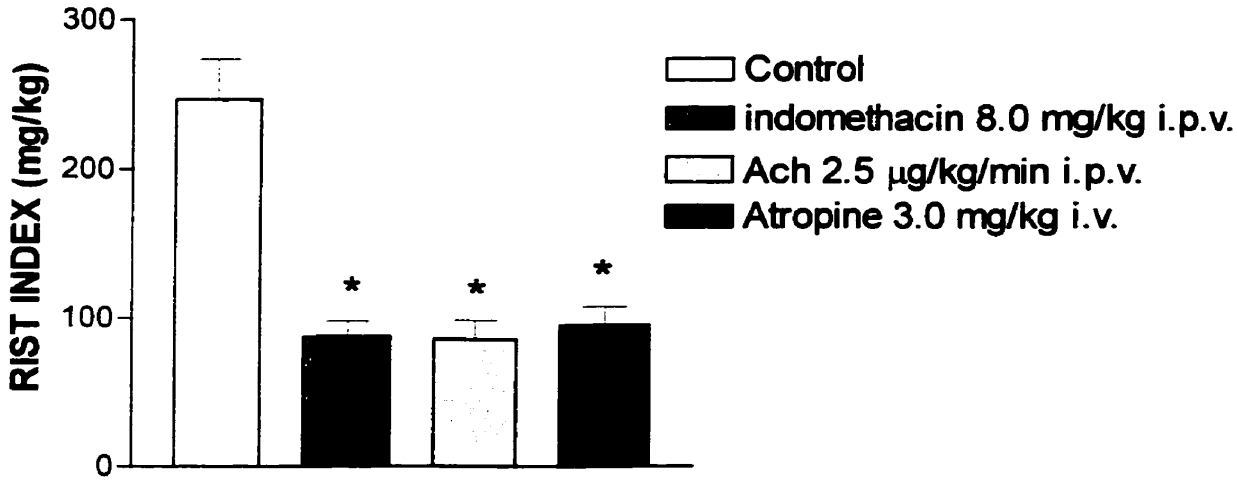



**Figure 27.** The RIST index in control, after indomethacin (8.0 mg/kg, ipv), after Ach (2.5 μg/kg/min, ipv), and after atropine (3.0 mg/kg, iv) administration. Values are means ± SE; n=4. **P*<0.001. Ach did not reverse insulin resistance produced by COX inhibition.

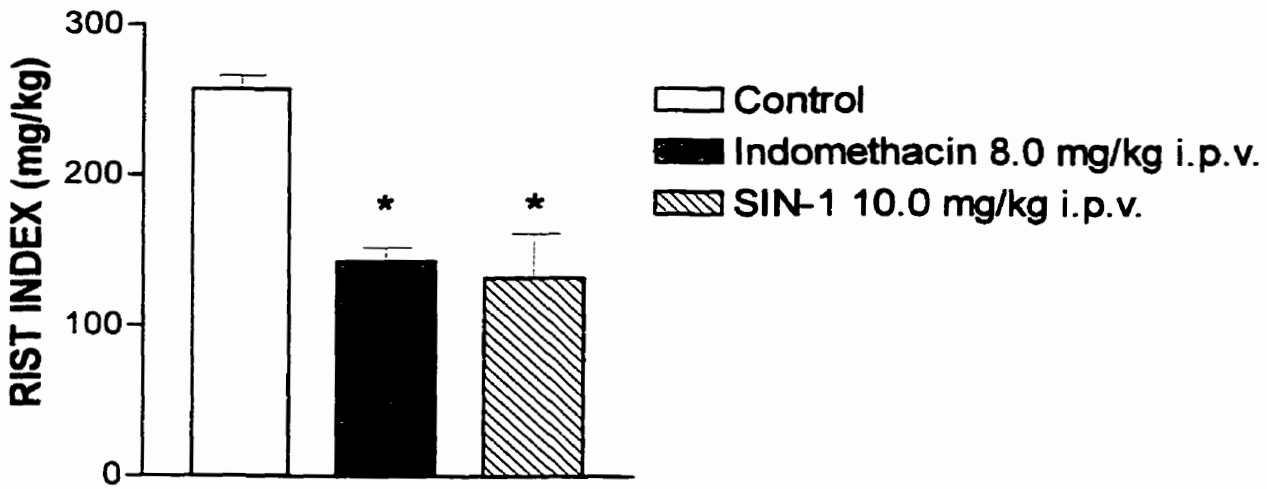



**Figure 28.** The RIST index in control, after indomethacin (8.0 mg/kg, ipv), and after SIN-1 (10.0 mg/kg, ipv) administration. Values are means ± SE; n=5. *$P$<0.01. Providing NO to the liver after COX antagonism did not restore insulin sensitivity.

blockade, providing a muscarinic agonist or NO to the liver did not restore insulin sensitivity. We can speculate that PGs synthesis occurs after activation of muscarinic receptors by Ach and also after NO production because Ach and SIN-1 did not reverse the insulin resistance produced by indomethacin. If PGs were produced before either muscarinic receptor activation or NO production, then Ach or SIN-1 should have restored insulin sensitivity.

Administration of atropine (3.0 mg/kg, iv) after Ach did not produce further significant insulin resistance (Fig. 27), thus, indomethacin (8.0 mg/kg, ipv) produced full blockade of HISS release.

*4.4.3 Isoform of COX involved*

At this point we do not know which hepatic isoform of COX is involved in the release of HISS from the liver, because indomethacin inhibits both isoforms of the enzyme. However, we can speculate that possibly the hepatic COX-1 is involved, since it is present in almost all cells and it is involved in the regulation of physiological functions (Smith 1989; Vane 1994). In contrast, COX-2 is expressed primarily in cells of the immune system and it is induced after stimulation with endotoxins, certain cytokines, or mitogens (Maier et al. 1990; Xie et al. 1992; Lee et al. 1992; Wu 1995).

In conclusion, there is a strong relation between inhibition of COX in the liver and insulin resistance. Inhibition of COX in the liver interrupts the hepatic parasympathetic-dependent release of HISS and results in HDIR with no effect on the HISS-independent component of insulin action.

# Chapter 5

## Fetal ethanol exposure causes HDIR in adulthood

### 5.1 Introduction

There is an epidemically high incidence of fetal alcohol syndrome (FAS) (Abel 1995) and type 2 diabetes (Zimmet et al. 1997) in socioeconomic disadvantaged populations around the world. FAS can result in polyneuropathies (Hannigan 1996), endocrine dysfunctions (Weinberg 1993), and insulin resistance (Castells et al. 1981). We have demonstrated that insulin action to increase glucose uptake at the skeletal muscle is mediated by the permissive role of the hepatic parasympathetic release of HISS from the liver (chapter 1, Xie and Lautt 1996*a*,*b*). Interruption of the hepatic parasympathetic-dependent release of HISS resulted in HDIR (chapters 1-4). Based on these observations we hypothesized that FAS can lead to type 2 diabetes (HDIR) secondary to hepatic parasympathetic neuropathy.

The effects of drinking alcohol during pregnancy on children of alcoholic mothers was first reported in 1968 by Lemoine and his colleagues (Lemoine et al. 1968). They reported a retrospective analysis of 127 children born to alcoholic parents and described four common characteristics: distinctive facial features, growth retardation, high frequency of malformations, and psychomotor disturbances. However, this observation received little medical and scientific attention. In 1973 a description of a common pattern of birth defects observed in eight children born to alcoholic mothers was published (Jones and Smith 1973). Another report from the same group labeled the unique cluster of symptoms, FAS (Jones et al. 1973). The patterns of birth defects were nearly identical to that reported by Lemoine (1968).

Fetal alcohol exposure is now associated with a wide variety of effects ranging from fetal or neonatal death and FAS at one extreme, and to partial FAS and more devastating defects at the other, such as behavioral disorders in the absence of physical anomalies (Abel 1985). The diagnosis of FAS, which has changed little since 1978, consists of pre- and/or postnatal growth retardation, morphological anomalies, and central nervous system (CNS) dysfunction (Rosett 1980; Streissguth 1986; Day and Richardson 1991). FAS is now recognized as a leading non-genetic cause of mental retardation (Jones and Smith 1973; Abel and Sokol 1986) and other serious physical and cognitive anomalies (Abel and Hannigan 1995). Prenatal alcohol exposure may also give rise to other alcohol-related birth defects including spontaneous abortion, heightened stress reactivity, decreased immune function, attention problems, hearing impairment, delayed development, altered play behavior, and a wide variety of other anomalies (Abel and Hannigan 1995).

The incidence of FAS in the western world, based on 29 prospective epidemiological studies, is reported to be 1.02 cases per 1000 live births (Abel 1995). The estimated incidence of FAS among women who drink "heavily" (consumption of 5 or more drinks per occasion, an average of 2 or more drinks per day, or a clinical diagnosis) is about 4.3% of all live births (Abel 1995). Thus, not all children prenataly exposed to high concentrations of alcohol develop FAS. This low rate of occurrence among high risk groups suggests that "FAS is not an equal opportunity birth defect" (Abel 1995). There seems to be other factors in addition to alcohol consumption during pregnancy that can affect the expression of FAS (Abel and Hannigan 1995). Abel and Hannigan (1995) have proposed two categorical types of factors involved in the

development of FAS: permissive and provocative. The permissive factors are behavioral, social, or environmental characteristics such as alcohol consumption patterns, smoking, low socioeconomic status, and culture that can produce certain biological conditions that enhance the chance for development of FAS. The provocative factors are the biological conditions such as high blood alcohol levels and decreased antioxidant status resulting from permissive factors, which create the internal environment responsible for the increased fetal vulnerability to alcohol at the cellular level.

### *5.1.1 Blood alcohol level*

Both the amount and the pattern of alcohol consumption are important in the development of FAS. The more alcohol consumed, and the more quickly it is consumed, the higher the blood alcohol level. The higher the blood alcohol level, the more likely it is that a fetus can be affected by the alcohol. A very high level of alcohol consumption during a single drinking occasion, such as bingeing, results in higher peak blood alcohol levels than sustained alcohol when similar total amounts of alcohol are consumed (Abel and Hannigan 1995). It has been suggested that it is the number of drinks per occasion and the high peak blood alcohol level, rather than a relatively constant lower blood alcohol level, that is a major risk factor for alcohol related birth defects (Pierce and West 1986*a,b*; Bonthius et al. 1988; Sampson et al. 1989; Streissguth et al. 1989, 1994). For example, it was shown that a critical factor in alcohol-induced CNS damage in rats exposed during a developmental period equivalent to the third trimester brain growth spurt in human, is the peak blood alcohol level, rather than total daily amount of alcohol consumption (Pierce and West 1986*a,b*; Bonthius et al. 1988). In addition, a recent

analysis of seven major medical research studies involving over 130,000 pregnancies suggested that consuming 2 to14 drinks per week does not increase the risk of FAS or malformations (Polygenis et al. 1998).

*5.1.2 Nutrition*

The mothers involved in all cases of FAS reported in the literature (Abel and Sokol 1986, 1991; Hannigan et al. 1992; Abel 1995) were malnourished, reflected by low pre-pregnancy weight or poor maternal weight gain during pregnancy. Heavy alcohol consumption itself can cause both primary and secondary malnutrition (Abel and Hannigan 1995). Primary malnutrition occurs because alcohol has a high energy content (providing 7.1 kcal/g) and replaces other energy sources in diet (Weinberg 1984). For example, an alcoholic could consume one third to one half of her daily energy requirements as alcohol (Weinberg 1984), and thus have a significantly less demand for food to fulfill her caloric needs. Weinberg referred to calories in alcohol as "empty" calories because they are not associated with vitamins, minerals, proteins or other essential nutrients (1984). The intake of these "empty" calories can result in nutrient deficiencies which is especially critical for pregnant and lactating females whose nutritional needs are even greater (Weinberg 1984). Thus, alcohol can reduce nutrient availability for both mother and fetus. Secondary malnutrition occurs as a result of alcohol-related gastrointestinal dysfunction such as inhibition of nutrient absorption from the gut, inhibition of placental transport of nutrients essential to the fetal growth and metabolic activity, and impairment of energy-dependent mechanisms in nutrient utilization (Henderson et al. 1980, 1982; Fisher et al. 1981, 1983; Fisher 1988).

In addition, nutrient delivery to the fetus is also reduced because alcohol impairs placental blood flow (Mukherjee and Hodgen 1982; Altura et al. 1983; Yang et al. 1986; Savoy-Moore et al. 1989; Falconer 1990) which can also lead to hypoxia in the fetus. It has been shown that hypoxia causes an increase in the rate of anaerobic breakdown of glucose to pyruvic and lactic acids within the brain cells (Pratt 1980). During pregnancy, an excess of lactic acid could cause a lactic acidosis in the fetus, and thus increase the risk of osmotic damage to the fetal brain in any hypoxic episode suffered by the mother (Weinberg 1984). Furthermore, decreased blood flow to the fetus by alcohol or maternal hypoglycemia (caused by period of heavy drinking) could reduce transport of glucose to the fetus which could affect brain development (Pratt 1980).

*5.1.3 Metabolic and mitogenic changes in FAS*

Whether FAS results from the direct action of ethanol in utero or from nutritional deprivation is not clear. It has been showed that ethanol can interfere with the maternal transfer of nutrients such as amino acids (above, Lin 1981). Furthermore, because ethanol can cross the placenta freely (Kaufman and Wollam 1981), it may produce metabolic changes in the fetus. Several investigators have shown that ethanol impairs protein synthesis in the fetus and neonates (Jarlstedt and Hamberger 1972; Schreiber et al. 1972; Morland and Bessesen 1977; Rawat 1979). Decreased protein synthesis has been considered a major factor in growth retardation associated with FAS (Henderson et al. 1981). In addition, it has been suggested that ethanol suppresses the rate of cell division in embryonic tissue resulting in fewer cells/embryo for a given time of gestation (Pennington et al. 1981). Other studies have demonstrated that ethanol exposure will

decrease the DNA synthesis of the developing embryonic cells (Guerri et al. 1990; Adickes et al. 1993; Weston et al. 1994). Litter survival and fetal body weight has been shown to decrease as a result of in utero exposure to ethanol (Singh and Snyder 1982, Singh et al. 1984).

Fetal glucose levels have been demonstrated to be a significant factor in normal embryonic growth (Shibley and Pennington 1997). The rate of transfer of glucose across the placenta increases during embryonic growth spurts (Rosso 1975). Prolonged maternal hypoglycemia induced in rats has been shown to result in intrauterine growth retardation (Gruppuso et al. 1981; Nitzan 1981) with a concomitant decrease in embryonic glucose levels. Thus, the limitation of fetal glucose appears to be a cause of intrauterine growth retardation. Chronic alcoholic mothers suffer from undernutrition and therefore would be expected to experience impaired glucose levels which in turn can lower fetal glucose levels. However, Singh et al. (1986) have shown that in utero exposure to ethanol in rats resulted in significantly lower blood glucose levels in the fetuses but not in the mother. This suggests that ethanol may have a direct effect on glucose uptake in fetal tissue. The effect of ethanol may intensify the decreased fetal glucose levels caused by ethanol-induced maternal undernutrition. Several studies have reported that maternal ethanol exposure inhibits the uptake of glucose by fetal tissue (Tanaka et al. 1982; Singh et al., 1989, 1992; Pennington et al. 1995). Furthermore, it has been suggested that in utero exposure to ethanol results in a resistance of the embryonic tissue to the action of insulin and therefore disrupts the molecular pathway for the growth of the embryo (Sandstrom et al. 1993).

*5.1.4 Mechanisms*

Abel and Hannigan (1995) have suggested that the cause of birth defects and FAS arises from a combination of alcohol-induced fetal hypoxia and alcohol-induced free radical formation.

*5.1.4.1 Hypoxia.* Hypoxia is the most common cause of all cellular damage (Cotran et al. 1989). Hypoxia has been implicated in the pathogenesis of FAS (Abel and Hannigan 1995). Umbilical blood flow is linearly related to oxygen delivery to the fetus (Itskovitz et al. 1983) and ischemia of umbilical vessels can occur even at relatively low blood alcohol levels (e.g. 10 mg/dl) (Altura et al. 1983). Low levels of alcohol exposure constrict human umbilical cord arteries (Savoy-Moore et al. 1989). Very high blood alcohol levels, e.g. bingeing, can disrupt or completely collapse umbilical cord arteries (Mukherjee and Hodgen 1982; Yang et al. 1986). In addition, the oxygen content of blood delivered to the fetus can also be reduced by alcohol because considerable oxygen is removed during the hepatic metabolism of alcohol by the mother (Israel et al. 1977; Thurman et al. 1984; Lieber 1991). The standard markers for hypoxia such as blood lactate concentrations and/or the lactate-pyruvate ratio are both elevated by prenatal alcohol exposure (Peeters et al. 1979; Sheldon et al. 1979; Morin and Weiss 1992).

*5.1.4.2 Free-radical oxidative stress.* The problems associated with alcohol related birth defects and FAS may also arise from excess generation of short-lived reactive oxygenated free radicals (De Groot and Littauer 1989; Bondy 1992; Dargel 1992; Nordmann et al. 1992). These molecules are highly unstable and reactive, they become more stable by either removing an electron from or donating their unpaired electrons to other molecules. In the course of normal metabolism in cells free radicals are

constantly produced (Forman and Boveris 1982) and they are normally scavenged by the endogenous antioxidative enzymes (De Groot and Littauer 1989; Bondy 1992; Dargel 1992; Nordmann et al. 1992). Increased production of reactive oxygen radicals or decreased levels of endogenous cellular defense protection, as the result of alcohol ingestion, can alter the balance of free radicals and the antioxidant system and could be the cause of cellular damage (Harris 1990; Reyes et al. 1993). Any alteration in favor of the former causes oxidative stress (Nordmann et al. 1992). Fetal cells have lower levels of free radical scavengers and antioxidants and, thus, may be more sensitive to oxidative stress (Davis et al. 1990).

*In vitro* studies have shown that neural crest cells, which do not have superoxide dismutase (an endogenous antioxidant), are particularly sensitive to alcohol exposure (Davis et al. 1990). This sensitivity could account for both the facial and visceral malformations associated with FAS, because craniofacial and visceral structures derive from neural crest cells (Davis et al. 1990).

### *5.1.5 CNS defects in FAS*

FAS leads to CNS anomalies which may manifest as learning and memory deficits, lowered IQ, attention deficit, mental retardation and in some cases, microcephaly (Mitchell et al. 1998). It has been shown that ethanol exposure during embryogenesis can result in changes in fetal cerebral metabolism (Abel and Hannigan 1995; Abel 1996). For example, reduction in fetal rat cerebral uptake of glucose and oxygen has been shown to be a result of maternal ethanol exposure (Abel 1996). Significant reductions in cerebral metabolism, caused by ischemia, have also been shown in the fetal lamb after maternal

infusion of ethanol (Richardson et al. 1985). These alterations in cerebral metabolism can contribute to disruptions of CNS structure and function in FAS. In addition, Balduini et al. (1994) have shown that administration of ethanol to developing rats during the brain growth spurt selectively decreases muscarinic receptor-induced proliferation of glial cells that may lead to microencephaly.

*5.1.6 Insulin sensitivity in FAS*

Human and animals studies have described many endocrine and metabolic systems that are affected by prenatal ethanol exposure (Thadani 1981; Anderson 1982; Ludena et al. 1983; Schweistal and Gingerich 1985). In the sheep, acute ethanol exposure in the mother enhances the insulin response to glucose load in the fetus (Castro et al. 1981). It has been shown that chronic ethanol exposure in the rat during pregnancy produces a high insulin response to glucose load in newborns up to three days after birth (Villarroya and Mampel 1985) and in 30 days and 90 days old adult rats (Lopez-Tejero et al. 1989). In addition, Castells et al. (1981) have shown an enhanced insulin pancreatic response and a peripheral insulin resistance in FAS children. In these FAS children fasting TSH, T4, T3, FSH, and LH were all normal. Their plasma levels of prolactin and cortisol were also normal before and after stimulation with chlorpromazine and insulin-induced hypoglycemia, respectively. Thus, insulin sensitivity appears to be reduced in offspring of alcohol fed mothers.

To test our hypothesis that FAS leads to type 2 diabetes and HDIR, we used a range of doses of alcohol (5%, 10%, 15%, and 20%) provided through the drinking water

to rats prior to and throughout the pregnancy and to the time of weaning. After weaning, the offspring received no further exposure to alcohol. Insulin sensitivity was evaluated using the RIST (described in detail in chapter 1) in both male and female pups when they were young adults. Atropine, a muscarinic receptor antagonist, was administered to determine the HISS-dependent and the HISS-independent component of insulin action.

We have shown in chapter 3 that insulin and IGF-1 have similar effects on glucose disposal as assessed by the RIST. Insulin acts through the hepatic parasympathetic-dependent release of HISS from the liver. HISS enhances glucose uptake at the skeletal muscle and accounts for 50-60% of insulin action. However, stimulation of glucose uptake by IGF-1 does not depend upon HISS action (chapter 3). We hypothesized that FAS causes hepatic parasympathetic neuropathy that results in insulin resistance, but not IGF-1, resistance. To test this hypothesis we performed the RIST using IGF-1 (200 μg/kg) in some of the males in the 0%, 5%, and 15% ethanol groups.

## 5.2 Material and methods

*Administration of ethanol*

Female Sprague-Dawley rats (219.5 ± g) underwent a training period to accustom them to the taste of ethanol in the water. The dams were divided into five groups: 0% (no ethanol in the drinking water), 5%, 10%, 15%, and 20% ethanol in the drinking water. Water and food (standard laboratory rat food) intake were monitored for 4 days prior to introduction of ethanol 5% v/v as the sole source of liquid intake. Food and water consumption were monitored throughout the entire period of ethanol administration. After 2 days or until food and water consumption returned to normal levels or stabilized, ethanol content was increased to 10% in the second group of rats. The same procedure was followed for administration of concentrations of 15% and 20% ethanol in the third and fourth groups. When food and water consumptions were stabilized, the male rat was introduced to the female and the date of conception was noted. Control (0%) dams were treated in the same manner but ethanol was not included in the drinking water.

At birth, the litter composition, mortality, and birth weights were determined. To minimize nutritional deficiencies, all litters were culled to twelve and the pups were nursed by the dam. The nursing dam continued to receive ethanol through the drinking water and as the pups became mobile, the water bottle was raised to a level to prevent the pups from reaching the water. The dam was sacrificed at the time of weaning and the pups were raised in a normal manner until the time of testing for insulin sensitivity at age of 43-75 days.

Insulin sensitivity was measured in all the male pups of all the groups, however, it was only measured in the female pups of the 0%, 15% and 20% ethanol groups.

*Determination of insulin sensitivity.* The rats were fasted overnight (8 h) and were fed standard laboratory rat food for 2 h before the start of any surgical procedures. Animal preparation, surgical procedures, and the RIST methodology are explained in detail in chapter 1.

*RIST in control and after atropine.* A control RIST was performed on the adult male rats of the 0% (n=28), 5% (n=10), 10% (n=6), 15% (n=27), and 20% (n=18) ethanol groups, and on the adult female rats of the 0% (n=12), 15% (n=6), and 20% (n=4) ethanol groups. After the control RIST, atropine (3.0 mg/kg) was intravenously administered over 5 min. Basal glucose concentration was determined and another RIST was performed.

*Determination of IGF-1 sensitivity.* In some of the male rats from the 0% (n=4), 5% (n=6), and 15% (n=7) ethanol groups IGF-1 sensitivity was measured using the RIST with IGF-1 (200 μg/kg) as described in chapter 4. The IGF-1 sensitivity was measured either after the control RIST with insulin or after the control RIST with insulin and atropine administration. We have shown in chapter 4 that atropine administration does not affect IGF-1 sensitivity.

*Determination of the basal insulin concentrations.* Basal insulin concentrations were determined in some of the male rats in the 0% (n=15), 15% (n=8), and 20% (n=6) ethanol groups. Arterial blood samples (50 μl) were taken after the rats were stabilized from the surgical preparations and before the control RIST was preformed. The blood samples were analyzed for insulin concentrations by a rat insulin ELISA kit.

*Drugs.* The human insulin was purchased from Eli Lilly & Company (Indianapolis, IN). The 95% ethanol, atropine and D-glucose were purchased from Sigma Chemical (St. Louis, MO). rhIGF-1 was donated by Genentech Inc. (San Francisco, CA).

All the chemicals were dissolved in saline. The rat insulin ELISA kit was purchased from Alpco (Windham, NH).

*Data analysis.* Data were analyzed using repeated-measures analysis of variance followed by Tukey-Kramer multiple comparison test in each group or, when applicable, paired and unpaired Student's t tests. Some results were analyzed using linear regression analysis. 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 all protocols were approved by an ethics committee on animal care at the University of Manitoba.

## 5.3 Results

### *5.3.1 Dams*

The dams had similar body weights in the 0% (n=11), 5% (n=3), 10% (n=3), 15% (n=11), and 20% (n=5) ethanol groups before the breeding (281.4 ± 10.5, 316.7 ± 29.4, 242.8 ± 18.7, 265.6 ± 16.0, and 269.6 ± 18.8 g, respectively) and just before giving birth (417.9 ± 12.2, 441.3 ± 33.7, 327.5 ± 38.9, 380.3 ± 16.0, and 380.5 ± 25.1 g, respectively). However, the pre-weaning body weights of the dams in the 15% and the 20% ethanol groups (259.6 ± 11.7 and 236.6 ± 18.2 g, respectively, $P<0.001$) were significantly lower than the body weight of the dams in the 0% and the 5% ethanol group (344.3 ± 6.9 and 361.3 ± 14.8 g, respectively).

The average fluid consumption significantly increased during gestation in the 0% (from 29.6 ± 1.1 to 39.3 ± 1.6 ml, $P<0.001$) and 5% (from 36.3 ± 1.7 to 50.7 ± 1.0 ml, $P<0.001$) dams but not in the 10% (from 22.7 ± 3.1 to 27.5 ± 1.0 ml), 15% (from 24.6 ± 2.0 to 26.3 ± 1.4 ml), and 20% (from 31.0 ± 3.2 to 30.9 ± 4.5 ml) dams. In addition, after giving birth to the time of weaning the dams' fluid intake significantly increased in the 0% (71.8 ± 4.3 ml, $P<0.001$) group but not in the 5% (64.7 ± 8.9 ml), 10% (39.8 ± 0.7 ml), 15% (31.6 ± 1.7 ml), and 20% (29.8 ± 1.9 ml) groups.

### *5.3.2 Litter demographics*

There was no significant difference between the mean number of pups delivered by the dams in any of the groups (13.6 ± 1.2 pups in the 0%, 15.0 ± 1.2 pups in the 5%, 12.3 ± 0.8 pups in the 10%, 13.2 ± 0.6 pups in the 15%, and 12.0 ± 2.2 pups in the 20%).

The mean litter weights (107.4 ± 8.2, 114.0 ± 8.2, 102.5 ± 4.7, 102.2 ± 6.1, and 75.4 ± 21.1 g, respectively) and the mean pup weights (8.1 ± 0.4, 7.7 ± 0.7, 8.3 ± 0.4, 8.1 ± 0.5, and 6.9 ± 0.7 g, respectively) were similar in all groups.

The ethanol showed a dose-dependent increase in mortality in the pups before weaning. The pup mortality rate before weaning was 0.67% for the 0% (1 in 149 pups), 4.4% for the 5% (2 in 45 pups), 0% for the 10% (0 in 37 pups), 6.2% for the 15% (9 in 145 pups), and 11.7% for the 20% (7 in 60 pups) ethanol group. The number of days to wean was similar in the 0% (20.2 ± 0.5 days), 5% (17.7 ± 0.4 days), 10% (19.3 ± 0.4 days), and 15% (19.6 ± 0.6 days) ethanol groups. However, it took the 20% pups significantly longer to wean (24.3 ± 1.0 days) compared to the other groups ($P<0.01$).

*5.3.3 Males*

The male pups from the 0% (n=28), 5% (n=10), 10% (n=6), 15% (n=27), and 20% (n=18) ethanol groups were taken for experiments at age 43 to 75 days old. There was no significant difference in age between the 0% (57.8 ± 1.7 days), 5% (52.0 ± 0.8 days), 10% (54.0 ± 0.9 days), and 15% (59.3 ± 1.9 days) ethanol groups, however, the 20% group (62.0 ± 1.6 days) was significantly older than the 5% group ($P<0.05$). The body weights in the pups from the 0% (332.3 ± 13.5 g), 5% (279.7 ± 12.2 g), 10% (262.3 ± 7.1 g), and 15% (297.7 ± 13.2 g) ethanol groups were similar, however, the 20% ethanol group (263.0 ± 6.2 g) was significantly lighter in weight than the 0% group ($P<0.01$). There were no significant differences in the mean arterial pressures (97.9 ± 3.3, 98.5 ± 5.2, 94.2 ± 5.2, 98.7 ± 3.1, and 104.0 ± 3.7 mmHg, respectively), or the basal glucose concentrations (120.5 ± 2.6, 129.7 ± 7.2, 116.7 ± 5.0, 116.7 ± 2.7, and 118.0 ±

3.0 mg/dl, respectively), between the groups. Basal insulin levels were analyzed in the some of the males of the 0% (n=15), 15% (n=8), and 20% (n=6) ethanol groups and there was no significant difference in the insulin levels between the three groups (6.2 ± 1.8, 4.0 ± 1.1, and 18.6 ± 13.4 ηg /ml, respectively). There was also no correlation between the basal glucose and the basal insulin levels in the same three groups.

The index used to express insulin, or IGF-1, sensitivity is the total amount of glucose (mg/kg) infused over 30-35 min after insulin (50 mU/kg), or IGF-1 (200 μg/kg), administration in order to maintain euglycemia at the baseline level and is referred to as the RIST index.

*Control RISTs.* There were no significant differences between the control RIST indexes in the 0%, 5% and 10% ethanol groups (189.7 ± 5.5, 152.0 ± 17.8, and 157.7 ± 14.7 mg/kg, respectively) however, there were significant differences between the control RIST indexes of the 0% and 15% (136.9 ± 8.3 mg/kg, $P<0.001$) ethanol groups and the 0% and the 20% (142.0 ± 11.4 mg/kg, $P<0.01$) ethanol groups (Fig. 29). There were no significant differences between the control RIST indexes of any other groups. Thus, in utero exposure to 15% and 20%, but not 5% and 10%, ethanol produced significant insulin resistance in male adult rats.

There was no correlation between the control RIST indexes and the ages, the body weights, the mean arterial pressures, the basal glucose concentrations, and the glucose/insulin ratios of the 0%, 15%, and the 20% ethanol groups (the groups that showed significant differences in RIST indexes). However, there was a correlation between the control RIST index, the basal insulin (slope= 2.17 ± 0.85), and

insulin/glucose ratio (slope=301.6 ± 108.0) in the 0% but not in the 15% and the 20% ethanol groups. Thus, the higher the basal insulin concentrations the higher the control RIST index in the normal non-ethanol exposed rats.

*RISTs after atropine.* After administration of atropine (3.0 mg/kg, iv) the control RIST index was significantly reduced to 82.1 ± 3.9 mg/kg ($P<0.001$) in the 0% (56.0 ± 2.4% inhibition), 72.8 ± 12.5 mg/kg ($P<0.001$) in the 5% (51.6 ± 5.8% inhibition), 79.1 ± 14.5 mg/kg ($P<0.05$) in the 10% (47.0 ± 10.6% inhibition), 82.5 ± 7.6 mg/kg ($P<0.001$) in the 15% (37.5 ± 4.9% inhibition), and 83.0 ± 10.6 mg/kg ($P<0.001$) in the 20% (40.0 ± 6.2% inhibition) ethanol groups (Fig. 29). The males in the 15% and 20% ethanol groups were insulin resistant, some portion of the HISS-dependent insulin action was still intact.

*RIST in control with insulin and with IGF-1.* There was no significant difference between the control RIST index with insulin (50 mU/kg) of the 0% (n=10) and 5% (n=6) ethanol groups (235.0 ± 19.0 and 152.2 ± 23.6 mg/kg, respectively), but there was a significant difference between the control RIST index with insulin of the 0% and the 15% (127.3 ± 6.4 mg/kg, n=7, $P<0.05$) ethanol groups (Fig. 31). The IGF-1 (200 μg/kg) RIST index was similar between the groups (254.1 ± 19.8 mg/kg in the 0%, 252.6 ± 28.6 mg/kg in the 5%, and 255.9 ± 22.6 mg/kg in the 20% ethanol groups). The IGF-1 RIST indexes of all the groups were compared to IGF-1 (200 μg/kg) RIST index (266.8 ± 26.2 mg/kg) in the chapter 4 and there were no significant differences between them. The RIST indexes with insulin and with IGF-1 were similar in the 0% ethanol group. But, there was a significant difference between the RIST index with insulin and the RIST index with IGF-1 in the 5% ($P<0.05$) and 15% ($P<0.01$) ethanol groups (Figure 31). Thus, in utero exposure to 15% ethanol causes insulin, but not IGF-1, resistance in adult rats.

*5.3.4 Females*

The female pups from the 0% (n=12), 15% (n=6), and 20% (n=4) ethanol groups were taken for experiments at age 54 to 91 days old. There were no significant differences in age (74.2 ± 2.4, 67.7 ± 5.8, and 81.0 ± 1.9 days, respectively) and in body weights (233.8 ± 11.3, 241.0 ± 14.2, and 233.3 ± 4.1 g, respectively) between the groups. The mean arterial pressures (84.4 ± 3.0, 78.3 ± 5.6, and 87.8 ± 6.7 mmHg, respectively) and the basal glucose concentrations (103.4 ± 1.5, 102.3 ± 6.5, and 111.5 ± 4.3 mg/dl, respectively) were also similar between the groups.

*Control RISTs.* The control RIST indexes in the 15% (134.1 ± 16.1 mg/kg, $P<0.05$) and the 20% (98.7 ± 9.7 mg/kg, $P<0.01$) ethanol groups were significantly lower than the RIST index in the 0% (220.9 ± 27.6 mg/kg) ethanol group (Fig. 31). However, the control RIST indexes of the 15% and the 20% group were not significantly different from each other. Thus, in utero exposure to 15% and 20% ethanol produced significant insulin resistance in female adult rats.

There was no correlation between the control RIST indexes and the ages, the body weights, the mean arterial pressures, and the basal glucose concentrations in any of the groups.

The control RISTs of the males and females were compared. The 0% males had significantly lower RIST indexes than the 0% females (189.7 ± 5.5 mg/kg in males and 220.0 ± 27.6 mg/kg in females, $P<0.001$). The 15% males and females had similar RIST indexes, but the 20% females had significantly lower RIST indexes than 20% males ($P<0.001$). Thus, the prenatal exposure to 20% ethanol produced a more severe insulin

resistance in the females.

*RISTs after atropine.* After administration of atropine (3.0 mg/kg, iv) the control RIST index was significantly reduced to 77.7 ± 9.5 mg/kg ($P$<0.001) in the 0% (59.3 ± 7.2% inhibition) ethanol group. However, atropine did not significantly reduce the control RIST index in the 15% (82.9 ± 14.5 mg/kg, 32.5 ± 14.3% inhibition) and the 20% (83.8 ± 20.5 mg/kg, 7.0 ± 33.8% inhibition) ethanol groups (Fig. 30). Thus, the HISS-dependent insulin action was essentially eliminated in the females as a result of prenatal exposure to 15% and 20% ethanol.

## 5.4 Discussion

Based on high prevalence of FAS (Abel 1995) and type 2 diabetes (Zimmet et al. 1997) in socioeconomic disadvantage groups and the fact that in both diseases there is high incidence of polyneuropathies, we hypothesized that FAS leads to hepatic parasympathetic neuropathy that may result in type 2 diabetes (HDIR). To test our hypothesis insulin sensitivity was measured in prenataly ethanol exposed pups using the RIST (described in detail in chapter 1).

*Technical considerations.* Different concentrations of ethanol (5%, 10%, 15%, 20%) were provided in the dams' drinking water. The dams were on the ethanol before breeding, throughout pregnancy and until the pups were weaned. Male pups from all ethanol exposed dams and the female pups from the 15% and 20% ethanol exposed dams were tested for insulin sensitivity when they were young adults. Since we could not observe any facial or other visual deformities associated with FAS in any of the pups we refer to their condition from here on as fetal alcohol exposure (FAE) and not FAS.

### *5.4.1 Insulin sensitivity in FAE*

Control RIST indexes were compared in all ethanol exposed male and female pups. There were no significant differences between the control RIST indexes of the 0%, 5%, and 10% ethanol groups in the males. However, prenatal exposure to 15% and 20% ethanol produced significant insulin resistance in both male and female pups and the effects of prenatal exposure to ethanol appears to be dose related (Figs. 29, 30).

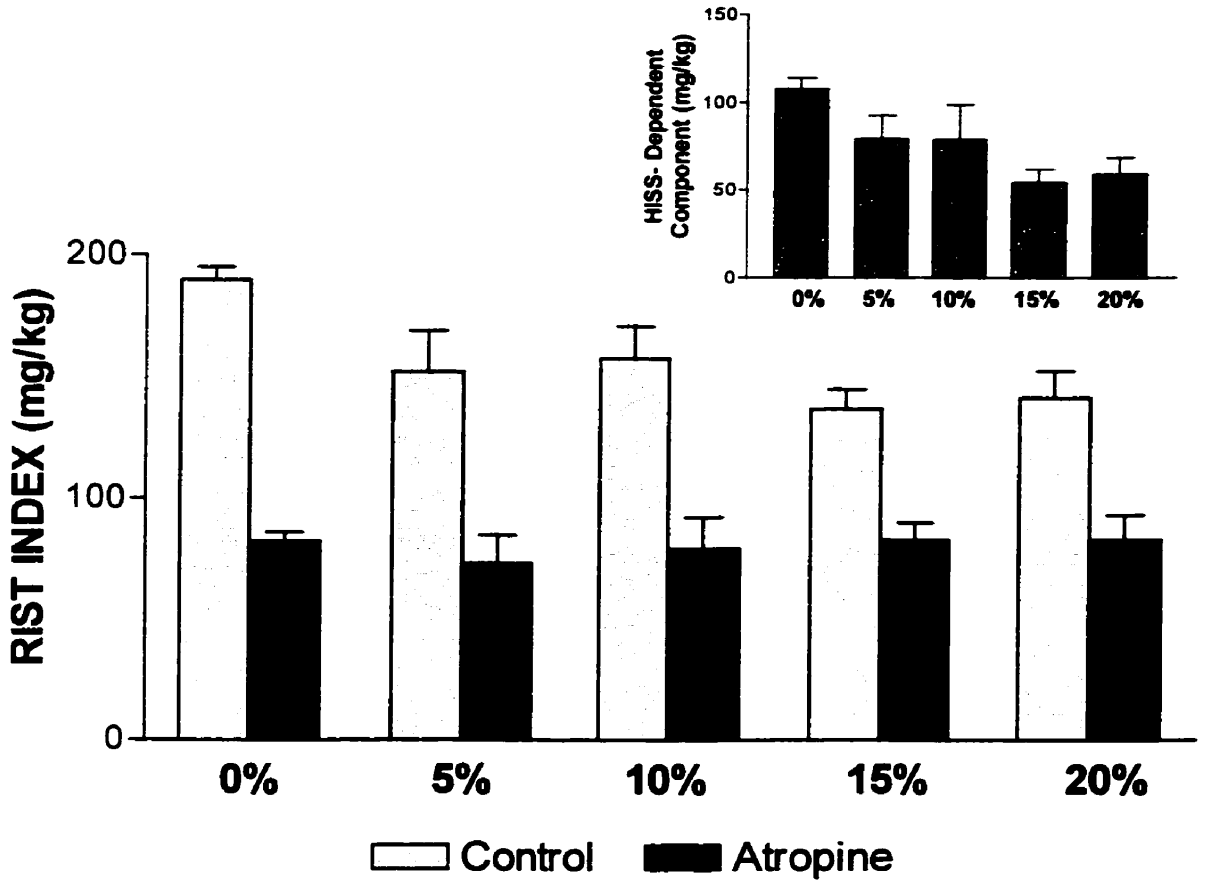



**Figure 29.** The RIST index in control and after intravenous atropine (3.0 mg/kg) administration in the males of all groups. Values are means ± SE. Parasympathetic inhibition caused significant insulin resistance in all groups. *Insert*. The HISS-dependent component of the insulin action in all groups. Values are means ± SE. Prenatal exposure to ethanol produced significant dose-dependent insulin resistance through inhibition of the HISS-dependent component of insulin action, although considerable amount of the HISS-dependent component was still intact.

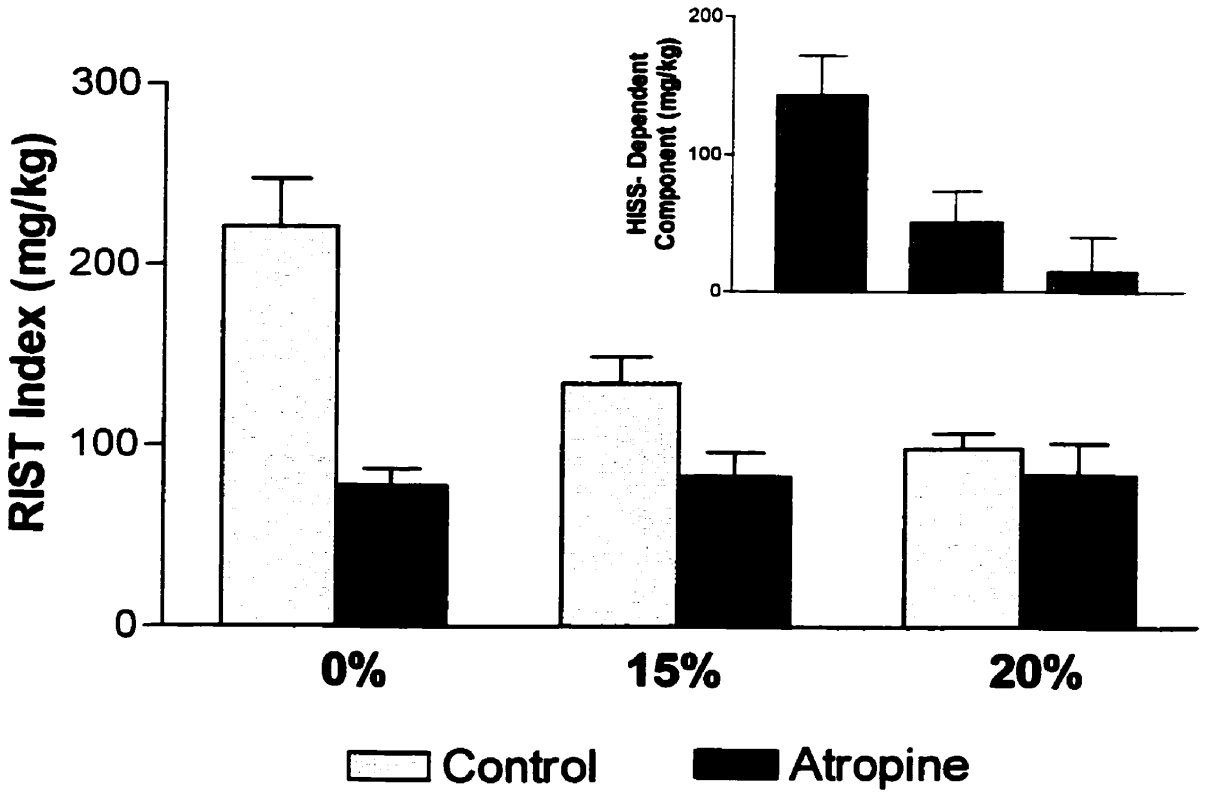



**Figure 30.** The RIST index in control and after intravenous atropine (3.0 mg/kg) administration in the females of all groups. Values are means ± SE. Parasympathetic inhibition caused significant insulin resistance in the 0%, but not in the 15% and 20%, ethanol group. *Insert.* The HISS-dependent component of the insulin action in all groups. Values are means ± SE. Prenatal exposure to 15% and 20% ethanol produced significant insulin resistance through inhibition of the HISS-dependent component of insulin action with no effect on the HISS-independent component.

The control RIST indexes of the males and the females were compared in each group. The 0% females were significantly more sensitive to insulin compared to the 0% males but the 15% females and males showed similar insulin sensitivity. However, the 20% ethanol exposed females were significantly more insulin resistant than the 20% ethanol exposed males. Thus, the prenatal exposure to 20% ethanol had a greater effect in the females than in the males.

*5.4.2 Parasympathetic inhibition*

It has been shown in other chapters that atropine blocks the HISS-dependent component of insulin action. Administration of intravenous atropine (3.0 mg/kg) produced significant HDIR in all of the prenataly ethanol exposed male groups (Fig. 29). From the total insulin action in the male groups the HISS-dependent component of insulin action blocked by atropine accounted for 56.0 ± 2.4% of the 0%, 51.6 ± 5.8% of the 5%, 47.0 ± 10.6% of the 10%, 37.5 ± 4.9% of the 15%, and 40.0 ± 6.2% of the 20% ethanol group (Fig. 29, *insert*). This indicates that even though prenatal exposure to 15% and 20% ethanol produced insulin resistance in male pups, there was still some portion of the hepatic parasympathetic tone intact that was further blocked by atropine.

Administration of the same dose of atropine in the 0% group in females also produced HDIR, however, atropine did not produce significant additional HDIR in the 15% and 20% ethanol exposed females (Fig. 30). From the total insulin action in the female groups the HISS-dependent component of insulin action blocked by atropine accounted for 59.3 ± 7.2% in the 0% group but only 32.5 ± 14.3% in the 15% and 7.0 ± 33.8% in the 20% female groups (Fig. 30, *insert*). This indicates that prenatal exposure to

15% and 20% ethanol significantly blocked the HISS-dependent component of the insulin action in both males and females but the males retained a higher HISS-dependent insulin action than did the females.

The RIST indexes after atropine administrations were similar in all male and female ethanol exposed groups. This indicates that the HISS-independent component of insulin action was similar in all groups. Since the control RIST indexes of both the male and female 15% and 20% ethanol groups were significantly lower than the 0% groups but the post-atropine response was similar, the insulin resistance produced by FAE was entirely accounted for by reduction in the HISS-dependent component of the insulin action while the HISS-independent component (post-atropine) was not altered.

*5.4.3 Nutritional factors*

FAS (or FAE) has been associated with malnutrition of the mother (Abel and Sokol 1986, 1991; Hannigan et al. 1992). Weinberg (1984) indicated that alcohol consumption may alter metabolism, transport, utilization, activation, and storage of almost every essential nutrient. Furthermore, chronic alcohol consumption decreases blood flow to the placenta and reduces placental glucose transport to the fetus as well as producing reduced glucose absorption from the intestine of the dam. Thus, some of the toxic effects of FAE may have been through nutritional interference.

All the dams' weights just before giving birth were similar in all groups. However, after giving birth and up to the time of weaning the 15% and the 20% dams were undernourished according to their small increase in body weight during that time. In addition, the average fluid consumption was not significantly increased during gestation

in the 10%, 15%, and the 20% ethanol groups. Furthermore, after giving birth to the time of weaning, the fluid intake was almost doubled in the 0% dams but it was not significantly increased in the 5%, 10%, 15%, and 20% dams. This indicates that during gestation the 10%, 15%, and 20% dams and, during nursing the 5%, 10%, 15%, and 20% dams, were dehydrated. The undernourishment and dehydration of the dams during gestation or nursing could have had severe effects on their pups. The 5% and 10% ethanol exposed pups did not exhibit any significant reduction in insulin sensitivity but the 15% and the 20% ethanol exposed pups were insulin resistant. Others (Singh and Snyder 1982) have shown that pair-fed control dams (0% ethanol) were underweight but their pups were not affected by the undernourishment of their mother. However, the ethanol-exposed dams in their study were underweight and their pups were severely affected by FAS. In addition, there is the possibility that malnutrition and dehydration secondary to ethanol consumption could have enhanced the severity of the FAE defects.

It has been shown that fetal exposure to ethanol can result in decreased litter size, survival, and weight (Singh and Snyder 1982), however, in our study there was no significant difference between litter size and pups weight in any of the groups. The ethanol showed a dose-dependent increase in mortality in the pups from birth to weaning. It appears that the pups that were affected the most by the FAE died before they were tested for insulin sensitivity. It is possible that the pups that had the highest degree of hepatic parasympathetic neuropathy and insulin resistance did not survive, thus the degree of insulin resistance by FAE may have been more severe.

The FAE in our study clearly caused insulin resistance secondary to impairment of the hepatic parasympathetic release of HISS in response to insulin. Whether this

neuropathy was caused solely by the toxic effects of ethanol or whether malnutrition and dehydration secondary to ethanol ingestion had additive roles, cannot be determined at this point.

Our study does not determine when the hepatic parasympathetic neuropathy leading to insulin resistance occurs in FAE. Alterations in glucose metabolism have been shown in fetuses (Tanka et al. 1982) and neonates (Singh et al. 1986), and oral glucose tolerance tests showed elevated glucose and insulin levels at day 30 but normal insulin levels at day 90 indicating that insulin responsiveness was reduced at both time points (Lopez-Tejero et al. 1989). The observation that abnormalities are seen in the fetuses, the neonates and in adult offspring that were nursed by dams not exposed to alcohol strongly suggests that the damage occurred in utero (Lopez-Tejero et al. 1989). The human fetus is more sensitive to FAS in the third trimester during which the rapid burst of brain growth occurs (Balduini et al. 1994). Since our animals were exposed to ethanol through the entire gestation and nursing period we cannot comment on the period of susceptibility to FAE.

*5.4.4 IGF-1 sensitivity*

In chapter 3 we demonstrated that insulin and IGF-1 have similar effects on glucose disposal as assessed by the RIST. However, it was determined that insulin, but not IGF-1, action was through the hepatic parasympathetic dependent release of HISS from the liver (chapter 3). Based on these observations we hypothesized that FAE causes hepatic parasympathetic neuropathy that results in insulin resistance, but not IGF-1,

resistance.

IGF-1 sensitivity was tested using the RIST in some of the males in the 0%, 5%, and 15% ethanol groups. The 0% group showed similar insulin and IGF-1 sensitivity (Fig. 31). However, prenatal exposure to 15% ethanol resulted in insulin, but not IGF-1, resistance (Fig. 31). The insulin sensitivity in the 5% group was not significantly different from the 0% group, their IGF-1 sensitivity was significantly higher. Thus, in utero exposure to 15% ethanol results in insulin, but not IGF-1, resistance. IGF-1 sensitivity was not affected by FAE since it does not involve the release of HISS from the liver. Comparison of insulin and IGF-1 responses was a sensitive index of insulin resistance as the insulin action of the 5% group was significantly reduced compared to the paired IGF-1 response but not when compared with the unpaired 0% insulin response.

In conclusion, prenatal exposure to ethanol produced insulin resistance through inhibition of the HISS-dependent component of insulin action. The HISS-independent component of insulin action was not affected by FAE. The high prevalence of FAS and type 2 diabetes in the world may be in part explained by the fact that prenatal exposure to ethanol inhibited the hepatic parasympathetic-dependent release of HISS from the liver leading to HDIR.

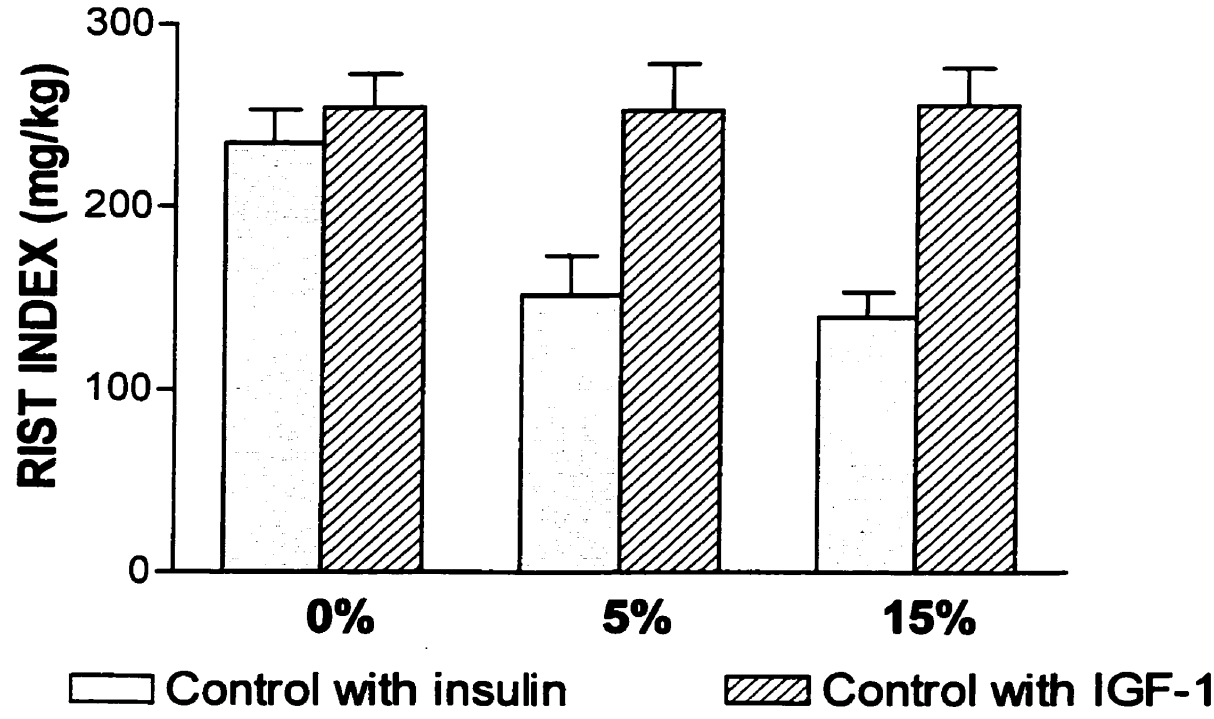



**Figure 31.** Insulin and IGF-1 RIST indexes in the 0%, 5%, and 15% prenatal ethanol exposed males. Values are means ± SE. Prenatal exposure to 15% ethanol produced insulin, but not IGF-1, resistance.

# Chapter 6

# Conclusions and Speculations

## 6.1 Conclusions

It has been previously shown that insulin causes the release of a hepatic insulin sensitizing substance (HISS) from the liver. The hepatic parasympathetic nerves were shown to play a permissive role in allowing insulin to trigger HISS release and, thus, they are essential in the release of HISS (Xie et al. 1993; Xie and Lautt 1995*a*, 1996*a*). The release of HISS was blocked by denervation of the hepatic anterior plexus (chapter 2), by pharmacological antagonism of muscarinic receptors by atropine (chapter 2), pharmacological antagonism of nitric oxide synthase (NOS) by L-NAME and L-NMMA (chapter 2), and pharmacological antagonism of cyclooxygenase (COX) by indomethacin (chapter 4). These results confirmed the importance of the permissive role of the hepatic parasympathetic nerves in the release of HISS and demonstrated that the hepatic parasympathetic-dependent release of HISS is through the production of NO and prostaglandins (PGs) in the liver. Since all these interventions produced insulin resistance by blocking the release of HISS from the liver, the portion of the response that was blocked is called the HISS-dependent component and the portion of the response that was not blocked is called the HISS-independent component of insulin action. The insulin resistance produced after blockade of HISS release is referred to as HISS-dependent insulin resistance (HDIR).

### *6.1.1 Measurement of insulin sensitivity*

To measure insulin sensitivity, we have developed a new rapid insulin sensitivity test (RIST, chapter 1) (Xie et al. 1996; Lautt et al. 1998). After establishment of the baseline euglycemia, a bolus of insulin (50 mU/kg) is infused over five minutes and euglycemia is maintained during the test by a variable glucose infusion pump. The RIST index is the amount of glucose infused during the test, in response to insulin, to maintain baseline euglycemia. The RIST has been shown to be comparable to the insulin tolerance test but not to the euglycemic hyperinsulinemic clamp technique (the gold standard). The euglycemic hyperinsulinemic clamp has several disadvantages which are explained in chapter 1 but the main problems with this test include the non-physiological nature of the test, since the insulin is infused at a constant rate for 2-3 h, and also it has been demonstrated that glucose utilization during the prolonged euglycemic clamp was significantly increased over time (Deberne et al. 1981), thus, the clamp cannot be used more than once in the same subject on the same day. However, insulin sensitivity does not change over time using the RIST (chapter 1).

### *6.1.2 Site of action*

Measurement of the arteriovenous glucose gradients across the liver, hind limbs, and splanchnic organs in control state and after hepatic parasympathetic denervation or atropine administration showed impairment of the glucose uptake only across the hind limbs (Xie and Lautt 1996*a*). This led us to believe that the skeletal muscle of the hind limbs is at least one of the tissues that are regulated by HISS.

*6.1.3 Involvement of NO*

The release of HISS from the liver was shown to be also dependent on the production of NO in the liver (chapter 2). Inhibition of NO in the liver with L-NAME, a NOS antagonist, significantly decreased insulin sensitivity and produced HDIR that was not further inhibited by atropine administration. However, the intravenous administration of the same dose of L-NAME did not significantly decrease insulin sensitivity, but further administration of atropine produced significant HDIR. Thus, NO inhibition in the liver, and not the periphery, completely blocked the release of HISS from the liver and produced significant HDIR. Intraportal, but not intravenous, administration of a NO donor (SIN-1) partially reversed the HDIR after NO inhibition with L-NMMA, another NOS antagonist. Intraportal administration of higher dose of SIN-1 completely restored insulin sensitivity after L-NMMA and denervation of the liver. Thus, NO production in the liver, and not the periphery, is important for the parasympathetic-dependent release of HISS from the liver.

We do not know the chemical identity of HISS, however, an analysis of the shape of the glucose infusion curve during the RIST, compared before and after atropine, denervation and L-NMMA, reveals the HISS-dependent component with an onset of action 3-5 minutes after the onset of insulin action and the HISS-dependent component that continues for approximately 9 minutes after the HISS-independent component of insulin action has terminated. This analysis revealed the hormonal nature of HISS (chapter 2).

*6.1.4 Involvement of PGs*

PGs production in the liver was shown to be also required for the release of HISS from the liver (chapter 4). Intraportal, but not intravenous, administration of indomethacin, a COX inhibitor, produced significant insulin resistance that was not further worsened by atropine suggesting that PGs are also involved in the release of HISS from the liver. However, the HDIR produced by indomethacin was not reversed by either Ach or SIN-1 suggesting that PGs may be released after Ach and NO productions.

*6.1.5 Involvement of the prandial state*

The HISS release is also dependent upon the prandial state of the animal (Macedo et al. 1998). After feeding, the HISS release in response to insulin leads to an increase in glucose uptake by the insulin sensitive tissues. However, in the fasted state HISS is not released in response to insulin, thus the hypoglycemic action of insulin is very low. Fasting reduces the HISS-dependent, but not the HISS-independent component of insulin action. Sixteen hours of fasting in rats produced a reduced insulin response and further atropine administration did not significantly inhibit the response to insulin. Thus, there appears to be a feeding signal that controls the hepatic parasympathetic-dependent release of HISS and the amount of HISS release, depending on the prandial state, controls insulin sensitivity.

*6.1.6 Involvement of IGF-1*

IGF-1 (200 μg/kg) had a similar glucose disposal effect to insulin (50 mU/kg) (chapter 3). However, inhibition of the hepatic parasympathetic reflex by denervation,

atropine administration, or fasting produced significant insulin resistance, but not IGF-1 resistance. This suggests that the hepatic parasympathetic pathway is not involved in the glucose disposal action of IGF-1 and IGF-1 acts through a different pathway.

### *6.1.7 HISS release in fetal alcohol exposure*

The hepatic parasympathetic-dependent release of HISS was evaluated in an experimental model of fetal alcohol exposure (FAE) (chapter 5). Adult male offspring of dams that were exposed to different amounts of ethanol (0%, 5%, 10%, 15%, and 20%) during pregnancy and throughout nursing were tested for insulin sensitivity. The 0%, 5% and 10% male group had similar insulin sensitivity. However, insulin sensitivity was significantly reduced in the 15% and 20% male groups but it was further worsened by atropine administration. The effects of ethanol on insulin sensitivity seemed to be dose-related and to be more severe with the higher doses. IGF-1 sensitivity was tested in some of the males in the 0%, 5%, and 15% ethanol groups. Prenatal exposure to different amounts of ethanol did not affect the IGF-1 sensitivity. Adult female offspring of dams that were exposed to 0%, 15%, and 20% ethanol during pregnancy and nursing were also tested. Insulin sensitivity was significantly reduced in the 15% and 20% female group in a dose-related manner, compared to the 0% group, and it was further worsened by atropine administration. Thus, prenatal exposure to 15% and 20% ethanol produced HDIR in both male and female offspring without affecting the HISS-independent component of insulin action.

At this point the chemical identity of the HISS is not known to us but based on our experiments, we know that it is required to increase glucose uptake at the skeletal muscle. We do not know how the HISS is actually functioning at the skeletal muscle level. On the next pages I have described the cellular insulin action on glucose uptake from the receptor activation to glucose transporter mechanism and I have also speculated on where or how HISS can interact with this pathway.

## 6.2 Speculations

### *6.2.1 Insulin receptor*

All of the pleiotropic cellular responses to insulin, including increase in glucose uptake, are mediated by the insulin receptor. The insulin receptor is a large heterotetrameric transmembrane glycoprotein that is expressed in nearly all mammalian tissues, although the number of receptors varies, with the highest concentration being found on insulin's major target sites: the adipose tissue and the liver (Khan et al. 1981). The skeletal muscle, which is the main tissue responsible for insulin-induced glucose uptake in humans and rodents (Curtis-Prior et al. 1969; Baron et al. 1988) has a relatively lower concentration of insulin receptors (Cheatham and Kahn 1995). Thus, we can speculate that since skeletal muscle has a lower concentration of insulin receptors compared to the liver and the adipose tissue but the highest glucose uptake effect then there may be other factors or components involved in its glucose disposal action (e.g. HISS).

The insulin receptor is composed of two $\alpha$-subunits and two $\beta$-subunits covalently linked through disulfide bonds to form $\alpha_2\beta_2$-heterotetramer (Cheatham and Kahn 1995).

The α-subunit is located entirely at the extracellular face of plasma membrane and contains the insulin-binding site (Yip et al. 1978; Jacobs et al. 1979). The β-subunit is a transmembrane peptide and contains an insulin-regulated tyrosine kinase domain in its intracellular site (Kasuga et al. 1982; Rosen 1987). Tyrosine kinases catalyze the transport of phosphate from ATP to hydroxyl groups of tyrosine residues on intracellular proteins, thus regulating their activity and function (Handberg 1995). After insulin binding to the α-subunit, the β-subunit undergoes autophosphorylation on tyrosine residues in the intracellular juxtamembrane domain (Ullrich et al. 1985), the regulatory region within the tyrosine kinase domain, and the carboxyl-terminus (Kahn and Folli 1993; White and Kahn 1994; Lee and Pilch 1994). The autophosphorylation of the tyrosine residues in the regulatory region enhances the activity of the receptor tyrosine kinase 10 to 20-fold, leading to greatly increased tyrosine phosphorylation of intracellular proteins, such as insulin-receptor substrate-1 (IRS-1) (White et al. 1988). The intracellular juxtamembrane domain has also been shown to be involved in tyrosine phosphorylation of IRS-1 (Yonezawa et al. 1994). The carboxyl-terminus has been shown not to be essential for signaling to glucose transport, but it may be important for activation of other intracellular signals (Holman and Kasuga 1997).

HISS might facilitate binding of insulin to the α-subunit of the receptor or it might be involved in transmitting a signal from the α-subunit, after its stimulation by insulin, to the β-subunit of the receptor. HISS might also stimulate autophosphorylation of tyrosine residues on the β-subunit of the insulin receptor and in this manner increase the action of insulin.

*6.2.3 IRS-1*

IRS-1 tyrosine residues are phosphorylated in response to tyrosine phosphorylation of insulin receptor. cDNA cloning has shown that IRS-1 contains 22 potential tyrosine phosphorylation sites that serve as specific recognition sites for cellular substrates containing *src*-homology 2 (SH2) domains (Sun et al. 1991; Keller et al. 1993). SH2 domains are present in many intracellular signaling molecules, and bind to specific phosphotyrosine motifs, thus allowing protein-protein interaction within the cell (Cheatham and Kahn 1995). IRS-1 also has a specific site (pleckstrin homology domain) that is important for IRS-1 association with the insulin receptor (Yenush et al. 1996).

*6.2.4 PI 3-kinase*

Specific phosphorylated tyrosines in IRS-1 bind strongly to the SH2 domain of the α-p85 subunit of phosphatidylinositol (PI) 3-kinase (Holman and Kasuga 1997). The association of PI 3-kinase and IRS-1 appears to activate the enzyme (Backer et al. 1992). PI 3-kinase is a heterodimeric enzyme composed of a regulatory subunit (p85) and a catalytic subunit (p110) (Cheatham and kahn 1995). The p85 subunit contains two SH2 domains and a SH3 domain. PI 3-kinase catalyses the phosphorylation of PI, PI-4-phosphate (PI-4-P), and PI-4,5-diphosphate (PI-4,5-$P_2$) on the D-3 position of the inositol ring to produce PI-3-P, PI-3,4-$P_2$, and PI-3,4,5-triphosphate (PI-3,4,5-$P_3$), respectively (Whitman et al. 1988; Escobedo et al. 1991; Skolnik et al. 1991; Otsu et al. 1991; Cantley et al. 1991).

After PI 3-kinase activation, the glucose transporter 4 (GLUT 4) is translocated from an intracellular pool to the plasma membrane. Inhibition of insulin-stimulated PI 3-

kinase blocks both glucose uptake and GLUT 4 translocation (Cheatham et al. 1994; Okada et al. 1994). Although not all the intracellular events have been identified, the PI 3-kinase activation has been suggested to enhance exocytosis of the GLUT 4 by increasing the budding of GLUT 4 from an intracellular located tubulo-vesicular system or facilitate the movement or docking of vesicles with plasma membrane (Holman and Kasuga 1997).

The HISS might be involved in any of the intracellular events, from PI 3-kinase activation to GLUT4 translocation and fusion with the plasma membrane and thus facilitate the action of insulin in glucose uptake.

PIP3 is thought to be the physiologically important product of PI 3-kinase (Holman and Kasuga 1997). The PIP3 may interact with downstream signaling molecules and thus transmit the PI 3-kinase-dependent signaling processes. There is evidence that PIP3 can interact with protein kinase B (PKB) and protein kinase C (PKC) isoforms (Nakanishi et al. 1993; Toker et al. 1994). Translocation of PI 3-kinase and some of the PKC isoforms to the plasma membrane has been shown in response to insulin (Yamada et al.1995). The involvement of PKC has been implicated in the glucose transport, although there are still speculations of its importance. Direct stimulation of PKC with phorbal - esters causes 2-3 fold elevations in both GLUT 4 and GLUT 1 at the cell surface (Holman et al. 1990; Gibbs et al. 1991) while insulin produces 10-20 fold elevation of GLUT 4. Other signaling molecules implicated in the GLUT 4 translocation downstream to PI 3-kinase activation are PKB (Kohn et al. 1996), G-proteins (Vannucci et al. 1992; Cormont et al. 1993; Clarke et al. 1994; Uphues et al. 1994; Li et al. 1995*a*; Moxham and Malbon 1996), and 1,2-diacylglycerol (Standaert et al. 1988; Farese et al. 1993). Thus,

the HISS may be involved in the stimulation of many or any of these intracellular molecules and facilitate the translocation of GLUT 4 to the plasma membrane.

As mentioned above not all the intracellular events concerning GLUT 4 translocation have been identified but the link between PI 3-kinase activation and other intracellular molecules involved in translocation have been suggested. Further experiments are required to identify the specific molecules and steps involved.

*6.2.5 Glucose transporters*

One of the most important roles of insulin is the rapid stimulation of glucose transport across muscle and adipose cells plasma membrane. Glucose uptake into tissues is accomplished by the facilitative glucose transporters. Five different facilitative glucose transporters have been identified and cloned and are referred to as GLUT 1-5 (Bell et al. 1990). GLUT 1 is present in placenta, brain, kidney, and colon and is present in lower amounts in adipose and muscle. In the skeletal muscle GLUT 1 is believed to be responsible for the basal glucose uptake (Handberg 1995). GLUT 2 is found mainly in liver and pancreatic β-cells. GLUT 3 is present in brain, placenta, and kidney. GLUT 5 is present predominantly in the small intestine. GLUT 4 is the only glucose transporter that has been show to be regulated by insulin and is found in insulin-sensitive tissues, which include skeletal and cardiac muscle and adipose tissue (Birnbaum 1992). In the absence of insulin, almost all of GLUT 4 is found in an intracellular pool (Cheatham and Kahn 1995). In response to insulin, a rapid translocation of the intracellular GLUT 4 to the plasma membrane occurs which results in a 20 to 30-fold increase in the rate of glucose uptake (Cushman and Wardzala 1980; Birnbaum 1992). However, the amount of

translocated GLUT 4 (~10-fold increase) does not account for the 20 to 30-fold increase in glucose uptake suggesting that other mechanisms may be involved in glucose uptake. Thus, HISS may be involved in enhancing glucose uptake by GLUT 4.

### *6.2.6 Intracellular trafficking of GLUT4*

There is an intracellular pool of GLUT 4-containing vesicles within the insulin sensitive cells. These vesicles also contain other associated accessory proteins such as secretory carrier membrane proteins (SCAMPs), Vesicule-associated membrane proteins (VAMPs), a Rab4 protein, and potentially small GTP-binding proteins which appear to be involved in the translocation process (Holman and Kasuga 1997). The SCAMPs and VAMPs are widely distributed among different cell types and are associated with a general mechanism of secretion and endocytosis (Cain et al. 1992; Laurie et al.1993). After treatment with insulin, both SCAMPs and VAMPs colocalize with GLUT 4 from the intracellular pool to the cell periphery and ~ 40% of the VAMPs (similar to GLUT 4) fuse with the plasma membrane (Holman and Kasuga 1997). Only a very minor portion of the SCAMPs become associated with the plasma membrane; they appear to remain closely associated with either secretory or endocytic vesicles (Holman and Kasuga 1997). Fusion of the GLUT 4 vesicles with the plasma membrane translocates the GLUT 4 from an intracellular location to the cell surface, an event that is necessary for glucose transport to the cell. Other molecules such as GTP-binding proteins have also been shown to be involved in GLUT 4 vesicles trafficking. These proteins participate in vesicular trafficking through secretory and endocytotic pathways (Chardin 1991). After termination

of the glucose uptake through GLUT 4, the GLUT 4 containing vesicles are recycled back to the intracellular pool (Cheatham and Kahn 1995).

Using a bis-manose photolabel in kinetic studies has suggested that insulin's main effect on GLUT 4 trafficking is to stimulate, by 8-10 fold, the exocytosis limb of the recycling pathway and some inhibition (30-50%) of endocytosis (Czech and Buxton 1993; Yang and Holman 1993; Yang et al. 1996). The involvement of insulin in GLUT4 trafficking is mediated through the activation of PI 3-kinase (Holman and Kasuga 1997). The mechanism of PI 3-kinase actions in GLUT 4 translocation is unclear at present but PI 3-kinase is required for GLUT 4 exocytosis from a subcellular compartment. PI 3-kinase may be involved with the budding and fission of GLUT 4 vesicles from an intracellular tubulo-vesicular system or with the fusion of the vesicle with the plasma membrane (Holman and Kasuga 1997). The HISS may be involved in one or all steps of PI 3-kinase activation to GLUT 4 exocytosis.

At this point we do not know how the HISS functions at the skeletal muscle to increase glucose uptake. HISS may function independently from insulin and its effects may be additive to that of insulin or it may function in synergy with insulin. HISS may have its own unique receptors and enter the skeletal muscle cell and interact with the insulin intracellular pathway. HISS may be involved in one or many of the insulin pathways from insulin binding to the $\alpha$-subunit of the insulin receptor to PI 3-kinase activation and GLUT 4 translocation and exocytosis. Future experiments are required to discover the site, or sites, of interaction of HISS with the insulin intracellular pathway.

### 6.3 Future directions

The future direction of this work will be to determine the chemical identity of HISS. It is important to identify the chemical nature of the HISS because its mechanism of release and action can be further investigated. For this purpose, first the most sensitive organ (probably the skeletal muscle) to the action of HISS will be identified using radioactive glucose, then that organ will be used to develop a bio-assay to isolate the HISS. Another direction for this work will be to identify different muscarinic receptor agonists to stimulate the release of HISS from the liver. This is important in the development of new pharmacological agents for the treatment of type 2 diabetes.

## REFERENCES

**Abel, E.** Prenatal effects of alcohol on growth: a brief review. Federation Proc. 44: 2318-2322, 1985.

**Abel, E.** An update on incidence of FAS: FAS is not an equal opportunity birth defect. Teratol. 17(4): 437-443, 1995.

**Abel, E.** Alcohol induced changes in blood gases, glucose, and lactate in pregnant and nonpregnant rats. Alcohol 13: 281-285, 1996.

**Abel, E., and J. H. Hannigan** Maternal risk factors in fetal alcohol syndrome: Provocative and permissive influences. Neurotoxicol. Teratol. 17(4): 445-462, 1995.

**Abel, E., and R. J. Sokol.** Fetal alcohol syndrome is now leading cause of mental retardation. Lancet 2: 1222, 1986.

**Abel, E., and R. J. Sokol.** A revised conservative estimate of the incidence of FAS and its economic impact. Alcohol. Clin. Exp. Res. 15: 514-524, 1991.

**Adickes, E. D., T. J. Mollner, and M. C. Makoid.** Teratogenic effects of ethanol during hyperplastic growth in cardiac myocyte cultures. Alcohol. Clin. Exp. Res. 17(5): 988-992, 1993.

**Akarasereenont, P., E. Hide, P. Ney, C. Thiemermann, and J. R. Vane.** The induction of cyclooxygenase-2 elicited by endotoxin in endothelial cells and macrophages is inhibited by prostaglandin $E_1$ and 13,14-dihydro-prostaglandin $E_1$. Agents Actions 45: 59-64, 1995.

**Alford, F. P., F. I. R. Martin, and M. J. Pearson.** The significance and interpretation of mildly abnormal oral glucose tolerance. Diabetologia 7: 173-180, 1971.

**Altura, B. M., B. T. Altura, A. Carella, M. Chatterjee, S. Halevy, and N. Tejani.** Alcohol produces spasm of human umbilical blood vessels: relationship to fetal alcohol syndrome (FAS). Eur. J. Pharmacol. 86: 311-312, 1983.

**Anderson, C. R., R. M. McAllen, and S. L. Edwards.** Nitric oxide synthase and chemical coding in cat sympathetic post-ganglionic neurons. Neurosci. 68: 255-264, 1995.

**Anderson, R. A.** Endocrine balance as a factor in the etiology of the fetal alcohol syndrome. Neurobehav. Toxicol. Teratol. 3: 89-104,1982.

**Andres, R., R. Swerdlof, T. Pozefsky, and D. Coleman.** Manual feedback technique for the control of blood glucose concentration. In: Automation in analytical chemistry. Ed. Skeggs, L. T. Jr. Mediad, New York. pp. 486-491, 1966.

**Andrews, P. J., and B. Mayer.** Enzymic function of nitric oxide synthases. Cardiovasc. Res. 43: 521-531, 1999.

**Arias-Negrete, S., K. Keller, and K. Chadee.** Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophage. Biochem. Biophys. Res. Commun. 208: 582-589, 1995.

**Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby, and L. C. Cantley.** PDGF-dependent tyrosine phosphorylation stimulates production of novel phosphoinositides in intact cells. Cell 57: 167-175, 1989.

**Avogaro, A., J. D. Bristow, D. M. Bier, C. Cobelli, and G. Toffolo.** Stable-label intravenous glucose tolerance test minimal model. Diabetes 38: 1048-1055, 1989.

**Bach, L. A.** The insulin-like growth factor system: basic and clinical aspects. Aust.NZ J. Med. 29: 355-361, 1999.

**Bach, L.A., and M. M. Rechler.** Insulin-like growth factor proteins. Diabetes Rev. 3: 38-61, 1995.

**Baker, J. M., M. G. Mayers, S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White.** The phosphatidylinisitol 3-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. 11: 3469-3479, 1992.

**Balduini, W., F. Reno, L. G. Costa, and F. Cattabeni.** Developmental neurotoxicity of ethanol: further evidence for an involvement of muscarinic receptor-stimulated phosphoinositide hydrolysis. Eur. J. Pharmacol. 266: 283-289, 1994.

**Baron, A.D.** Insulin and the vasculature-old actors, new roles. J Invest Med. 44: 406-412, 1996.

**Baron, A. D., and G. Brechtel.** Insulin differentially regulates systemic and skeletal muscle vascular resistance. Am. J. Physiol. 265: E61-E67, 1993.

**Baron, A. D., G. Brechtel-Hook, A. Johnson, J. Cronin, R. Leaming, and H. O. Steinberg.** Effect of perfusion rate on the time course of insulin-mediated skeletal muscle glucose uptake. Am. J. Physiol. 271: E1067-E1072, 1996.

**Baron, A. D., G. Brechtel-Hook, A. Johnson, and D. Hardin.** Skeletal muscle blood flow: a possible link between insulin resistance and blood flow. Hypertension. 21: 129-135, 1993.

**Baron, A. D., G. Brechtel, P. Wallance, and S. V. Edelman.** Rates and tissue sites of non-insulin- and insulin-mediated glucose uptake in humans. Am. J. Physiol. 255: E769-E774, 1988.

**Baron, A. D., M. Lassko, G. Brechtel, and S.V. Edelman.** Reduced capacity and affinity of skeletal muscle for insulin-mediated glucose uptake in non-insulin-dependent diabetes mellitus. J. Clin. Invest. 87: 1186-1194, 1991.

**Baron, A. D., J. S. 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:E709-E715, 1995.

**Bassenge, E.** Coronary vasomotor responses: role of endothelium and nitrovasodilators. Cardiovasc. Drugs Ther. 8: 601-610, 1994.

**Bassenge, E., and A. Mulsch.** Anti-ischemic actions of molsidomine by venous and large coronary dilatation in combination with antiplatelet effects. J. Cardiovasc. Pharmacol. 14: S23-S28, 1989.

**Baxter, R.C., and J. L. Martin.** Binding proteins for the insulin-like growth factors: structure, regulation and function. Progr. Growth Factor Res. 1: 49-68, 1989.

**Beck-Nielson, H., O. Pedersen, and H. O. Lindskow.** Normalization of the insulin sensitivity and the cellular insulin binding during treatment of obese diabetics for one year. Acta Endocrinol. 90: 103-112, 1979.

**Bell, G. I., T. Kayano, J. B. Buse, C. F. Burant, J. Takeda, D. Lin, H. Fukumoto, and S. Seino.** Molecular biology of mammalian glucose transporters. Diabetes Care 13: 198-208, 1990.

**Bergman, R. N., D. T. Finegood, and M. Ader.** Assessment of insulin sensitivity *in vivo*. Endocrine Rev. 6: 45-86, 1985.

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

**Bergstrom, S., L. A. Carlson, and L. Oro.** Effects of different doses of prostaglandin E on free fatty acids of plasma, blood glucose and heart rate in the non anesthetized dog. Prostaglandin and related factors 53. Acta Physiol. Scand. 7:185-193, 1966.

**Birnbaum, M. J.** The insulin-sensitive glucose transporter. Int. Rev. Cytol. 137: 239-297, 1992.

**Bonadonna, R. C., S. Del Prato, E. Bonora, M. P. Saccomani, G. Gulli, A. Natali, S. Frascerra, N. Pecori, E. Ferrannini, D. Bier, C. Cobelli, and R. A. DeFronzo.** Roles of glucose transport and glucose phosphorylation in muscle insulin resistance of NIDDM. Diabetes. 45: 915-925, 1996.

**Bondy, S. C.** Ethanol toxicity and oxidative stress. Toxicol. Lett. 63: 231-241, 1992.

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

**Bonthius, D. J., C. R. Goodlett, and J. R. West.** Blood alcohol concentrations and severity of microencephaly in neonatal rats depend on the pattern of alcohol administration. Alcohol 5: 209-214, 1988.

**Borg, W. P., and R. S. Sherwin.** 3 Metabolic effects of insulin-like growth factor 1. Diabetes Ann. 9: 57-70,1995.

**Boulware, S. D., W. V. Tamborlane, N. J. Rennert, N. Gesundhi, and R. S. Sherwin.** Comparison of metabolic effects of recombinant human IGF-1 and insulin. J. Clin. Invest. 93: 1131-1139, 1994.

**Brass, E. P., and M. J. Garrity.** Effects of E-series prostaglandin on cyclic AMP-dependent and -independent hormone-stimulated glycogenolysis in hepatocytes. Diabetes 34: 291-294, 1985.

**Brass, E. P., M. J. Garrity, and R. P. Robertson.** Inhibition of glucagon-stimulated hepatic glycogenolysis by E-series prostaglandins. FEBS 162(2): 293-296, 1984.

**Bratusch-Marrian, P. R., H. Vierhapper, M. Komjati, and W. K. Waldhausl.** Acetyl-salicylic acid impairs insulin-mediated glucose utilization and reduces insulin clearance in healthy and non-insulin-dependent diabetic man. Diabetologia 28: 671-676, 1985.

**Brismar, K., M. Gutniak, G. Povoa, S. Werner, and K. Hall.** Insulin regulates the 35 kDa IGF binding protein in patients with diabetes mellitus. J. Endocrinol. Invest. 11: 599-602, 1988.

**Busby, W. H., D. G. Klapper, and D. R. Clemmons.** Purification of a 31000 dalton insulin-like growth factor binding protein from human amniotic fluid. J. Biol. Chem. 263: 14203-14210, 1988.

**Buxton, I. L. O., D. J. Cheek, D. Eckman, D. P. Westfall, K. M. Sanders, and K. D. Keef.** N-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. Circ. research. 72: 387-395, 1993.

**Cain, C. C., W. S. Trimble, and G. E. Lienhard.** Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes. J. Biol. Chem. 267: 11681-11684, 1992.

**Cameron, N. E., and M. A. Cotter.** Effects of chronic treatment with a nitric oxide donor on nerve conduction abnormalities and endoneurial blood flow in streptozotocin-diabetes rats. Eur. J. Clin. Invest. 25: 19-24, 1995.

**Cameron, N. E., M. A. Cotter, and T. C. Hohman.** Interactions between essential fatty acids, prostanoid, polyol pathways and nitric oxide mechanisms in the neurovascular deficit of diabetic rats. Diabetologia. 39: 172-182, 1996.

**Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. kapeller, and S. Soltoff.** Oncogenes and signal transduction. Cell 64: 281-302, 1991.

**Cardillo, C., S. S. Nambi, C. M. Kilcoyne, W. K. Choucair, A. Katz M. J. Quon, and J. A. Panza.** Insulin stimulates both endothelin and nitric oxide activity in the human forearm. Circulation. 100: 820-825, 1999.

**Caro, J. F., J. Poulos, O. Ittoop, W. J. Pories, E. G. Flickinger, and M. K. Sinha.** IGF-I binding in hepatocytes from human liver, human hepatoma and normal regenerating, and fetal rat liver. J. Clin. Invest. 81: 976-981, 1988.

**Castells, S., E. Mark, F. Abaci, and E. Schwartz.** Growth retardation in fetal alcohol syndrome. Dev. Pharmacol. Ther. 3: 232-241, 1981.

**Castro, M. I., D. R. Koritnik, and J. C. Rose.** Fetal plasma insulin and thyroid hormone levels during in utero ethanol exposure in a maternal-fetal sheep model. Endocrinology 118: 1735-1742, 1981.

**Caumo, A., A. Giacca, M. Morgese, G. Pozza, P. Micossi, and C. Cobelli.** Minimal models of glucose disappearance: lessons from labeled IVGTT. Diabet. Med. 8: 822-832, 1991.

**Cavagnini, F., A. Di-landro, C. Invitti, U. Raggi, P. Alessandrini, M. Pinto, G. Girotti, and P. Vigo.** Effects of acetylsalicylic acid and indomethacin on growth hormone secretion in man. Metabolism 26:193-200, 1977.

**Chardin, P.** Small GTP-binding proteins of the ras family: a conserved functional mechanism? Cancer Cells 3: 117-126, 1991.

**Cheatham, B., and C. R. Kahn.** Insulin action and the insulin signaling network. Endocrine Rev. 16 (2): 117-142, 1995.

**Cheatham, B., C. J. Vlahos, L. Cheatham, L. Wang, J. Blenis, and C. R. Kahn.** Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis and glucose transporter translocation. Mol. Cell. Biol. 14: 4902-4911, 1994.

**Chen Y. L., and E. J. Messina.** Dilation of isolated skeletal muscle arterioles by insulin is endothelium dependent and nitric oxide mediated. Am. J. Physiol. 270: H2120-H2124, 1996.

**Chen, M., and R. P. Robertson.** Effects of prostaglandin synthesis inhibitors on human insulin secretion and carbohydrate tolerance. Prostaglandins 18: 557-567, 1979.

**Clarke, J. F., P. W. Young, K. Yonezawa, M. Kasuga, and G. D. Holman.** Inhibition of the translocation of GLUT 1 and GLUT 4 in 3T3-L1 cells by the PI 3-kinase inhibitor, wortmannin. Biochem J. 300: 631-635, 1994.

**Clemens, M. G.** Does altered regulation of ecNOS in sinusoidal endothelial cells determine increased intrahepatic resistance leading to portal hypertension? Hepatology 27: 1745-1747, 1998.

**Clemens, M. G.** Nitric oxide in liver injury. Hepatology 30: 1-5, 1999.

**Clemmons, D. R.** insulin-like growth factor binding protein and their role in controlling IGF actions. Cytokine Growth Factor Rev. 8: 45-62, 1997.

**Cobelli, C., G. Pacini, G. Toffolo, and L. Sacca.** Estimation of insulin sensitivity and glucose clearance from minimal model : new insights from labeled IVGTT. Am. J. Physiol. 250: E591-E598, 1986.

**Cook, H. T., A. Jansen, S. Lewis, P. Largen, M. O'Donnell, D. Reaveley, and V. Cattell.** Arginine metabolism in experimental glomerulonephritis: interaction between nitric oxide synthase and arginase. Am. J. Physiol. 267: F646-F653, 1994.

**Corbett, J. A., G. Kwon, J. Turk, and M. J. McDaniel.** IL-1β induces coexpression of both nitric oxide synthase and cyclooxygenase by islets of langerhans: activation of cyclooxygenase by nitric oxide. Biochem. 32:13767-13770, 1993.

**Cormont, M., J. F. Tanti, A. Zahraoui, E. Van Obberghen, A. Tavitian, and Y. Le Marchand-Brustel.** Insulin and okadaic acid induced Rab4 redistribution in adipocytes. J. Biol. Chem. 268: 19491-19497, 1993.

**Cotran, R. S., V. Kumar, and S. L. Robbins.** Cell function and injury. In: Robbins' pathologic basis of disease. W. B. Saunders, Philadelphia. pp. 1-87, 1989.

**Cotterill A. M., C. J. Cowell, and M. Silink.** Insulin and variation in glucose levels modify the secretion rates of growth hormone independent insulin-like growth factor-1 in the human hepatoblastoma cell line Hep G2. Endocrinology 123: R17, 1989.

**Curran, R. D., T. R. Billiar, D. J. Stuehr, J. B. Ochoa, B. G. Harbrecht, S. G. Flint, and R. L. Simmons.** Multiple cytokines are required to induce hepatocyte nitric oxide production and inhibit total protein synthesis. Ann. Surg. 212: 462-469, 1990.

**Curtis-Prior, P. B., J. Trethewey, G. A. Stewart, and T. Hanley.** The contribution of different organs and tissues of the rat to assimilation of glucose. Diabetologia 5: 384-391, 1969.

**Cushman, S. W., and L. J. Wardzala.** Potential mechanism of insulin action of glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. J. Biol. Chem. 255: 4758-4762, 1980.

**Cyber, C.** Can arginine and ornithine support gut function? Gut (supplement). 1: S42-S45, 1994.

**Czech, M. P., and J. M. Buxton.** Insulin action on the internalization of the GLUT 4 glucose transporter in isolated rat adipocytes. J. Biol. Chem. 268: 9187-9191, 1993.

**Dargel, R.** Lipid peroxidation-A common pathogenetic mechanism? Exp. Toxic. Pathol. 44: 169-181, 1992.

**Daughady, W. H., and P. Rotwein.** IGF-I and II, peptide, mRNA and gene structures, serum and tissue concentrations. Endocrin. Rev. 10: 168-191, 1989.

**Davidge, S. T., P. N. Baker, M. K. McLaughlin, and J. M. Roberts.** Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. Cir. Res. 77: 274-283, 1995.

**Davis, W. L., L. A. Crawford, O. J. Cooper, G. R. Farmer, D. L. Thomas, B. L. Freeman.** Ethanol induces the generation of reactive free radicals by neural crest in vitro. J. Craniofac. Genet. Devel. Biol. 10: 277-293, 1990.

**Day, N. L., and G. A. Richardson.** Prenatal alcohol exposure: A continuum of effects. Seminars Perinatology 15: 271-279, 1991.

**Deberne, L., M. S. Greenfield, B. Schulz, and G. M. Reaven.** Enhanced glucose utilization during prolonged glucose clamp studies. Diabetes 30: 829-835, 1981.

**De Groot, H., and A. Littauer.** Hypoxia, reactive oxygen, and cell injury. Free Radial Biol. Med. 6: 451-551, 1989.

**De Groot, J. J., G. A. Veldink, J. F. G. Vliegenthart, J. Boldingh, R. Wever, and B. F. Van Gelder.** Demonstration by EPR spectroscopy of the functional role of iron in soybean lipoxygenase-1. Biochem. Biophys. Acta 377: 71-79, 1975.

**DeFronzo, R. A., R. Gunnarsson, O. Bjorkman, M. Olsson, and J. Wahren.** Effects of insulin on peripheral and splanchnic glucose metabolism in non-insulin-dependent (type II) diabetes mellitus. J. Clin. Invest. 76: 149-155, 1985.

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

**Del Prato, S., E. Ferrannini, and R. A. De Fronzo.** Evaluation of insulin sensitivity in man. In: Methods in Diabetes Research. Eds. Clark, W. L., J. Larner, and S. L. Pohl. Wiley, New York. pp. 36-76, 1986.

**DeWitt, D. L.** Prostaglandin endoperoxide synthase: regulation of enzyme expression. Biochem. Biophys. Acta. 1083: 121-134, 1991.

**Di Rosa, M., A. Latenti, A. Ianaro, and L. Sautebin.** Interaction between nitric oxide and cyclooxygenase pathways. Prostaglandins Leukot. Essent. Fatty Acids. 54 (4): 229-238, 1996.

**Dietze, G., M. Wicklmayr, I. Bottger, and L. Mayer.** Insulin action on glucose uptake into skeletal muscle. FEBS Let. 92: 294-298, 1978.

**Drapier, J. C., J. Wietzerbin, and J. B. Jr. Hibbs.** Interferon-γ and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. Eur. J. Immunol. 18:1587-1592, 1988.

**Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams.** cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI 3-kinase to the PDGF β-receptor. Cell 65: 75-82, 1991.

**Esteban F. J., J. A. Pedrosa, A. Jimenez, A. P. Fernandez, M. L. Bentura, R. Martinez-Murillo, J. Rodrigo, and M. A. Peinada.** Distribution of neuronal nitric oxide synthase in the rate liver. Neurosci. Lett. 226: 99-102, 1997.

**Esteban, F. J., J. A. Pedrosa, A. Jimenez, A. P. Fernandez, M. L. Bentura, R. Murillo, J. Rodrigo, and M. A. Peinado.** Distribution of neural nitric oxide synthase in the rat liver. Neurosci. Let. 226: 99-102, 1997.

**Failli, P., E. Cecchi, C. Tosti-Guerra, A. Mugelli, G. Laffi, L. Zilletti, and A. Giotti.** Effects of some cyclooxygenase inhibitors on the increase in guanosine 3':5'-cyclic monophosphate induced by NO-donors in human whole platelets. Br. J. Pharmacol. 123: 1457-1463, 1998.

**Falconer, J.** The effect of maternal ethanol infusion on placental blood flow and fetal glucose metabolism in sheep. Alcohol. Alc. 25: 413-416, 1990.

**Farese, R. V., M. Standaert, B. Yu, H. Hernandez, and D. R. Cooper.** 2-hydroxypropyl-β-cyclodextrin enhances phorbol ester effects on glucose transport and/or protein kinase C-β translocation to the plasma membrane in rat adipocytes and soleus muscle. J. Biol. Chem. 268:19949-19955, 1993.

**Feelisch, M., and E. Noack.** Nitric oxide (NO) formation from nitrovasodilators occurs independently of hemoglobin or non-heme iron. Eur. J. Pharmacol. 142: 465-469, 1987.

**Ferrannini, E., and A. Mari.** How to measure insulin sensitivity. J. Hypertens. 16: 895-906, 1998.

**Ferrannini, E., and A. Pilo.** Pattern of insulin delivery after intravenous glucose injection, and its relation to plasma glucose disappearance. J. Clin. Invest. 64: 243-250, 1979.

**Ferreira, S. H., and J. R. Vane.** New aspects of the mode of action of non-steroid anti-inflammatory drugs. Annu. Rev. Pharmacol. 14: 57-73, 1974.

**Field, J. B., C. Boyle, and A. Remer.** Effect of salicylate infusion on plasma-insulin and glucose tolerance in healthy person and mild diabetics. Lancet 1: 1191-1194, 1967.

**Finegood, D. T., I. M. Hramiak, J. Dupre.** A modified protocol for estimation of insulin sensitivity with the minimal model of glucose kinetics in patients with insulin-dependent diabetes. J. Clin. Endocrinol. Metab. 70: 1538-1549, 1990.

**Fisher, S. E.** Selective fetal malnutrition: The fetal alcohol syndrome. J. Amer. Coll. Nutr. 7: 101-106, 1988.

**Fisher, S. E., M. Atkinson, S. Jacobson, P. Sehgal, J. Burnap, E. Holmes, S. Teichberg, E. Kahn, R. Jaffe, and D. H. Van Thiel.** Selective fetal malnutrition: The effects of in vivo ethanol exposure upon in vitro placental uptake of amino acids in the nonhuman primate. Pediatr. Res. 17: 704-707, 1983.

**Fisher, S. E., M. A. Barnicle, B. Steis, I. Holzman, and D. H. Van Thiel.** Effects of acute ethanol exposure upon in vivo leucine uptake and protein synthesis in the fetal rat. Pediatr. Res. 15: 335-339, 1981.

**Florini, J. R.** Hormonal control of muscle growth. Muscle and Nerve 10: 577-598,1987.

**Forman, H. J., and A. Boveris**. Superoxide radical and hydrogen peroxide in mitochondria. In: Free radicals in biology. Ed. Pryor, W. A. Academic Press, New York. pp. 65-90, 1982.

**Froesh, E.R., M. Hussain, C. Schmid, and J. Zapf.** Insulin-like growth factor I: physiology, metabolic effects and clinical uses. Diabetes Metab. Rev. 12: 195-215, 1996.

**Fu, J. Y., J. L. Masferre, K. Seibert, A. Raz, and P. Needleman.** The induction and suppression of prostaglandin $H_2$ synthase (cyclooxygenase) in human monocytes. J. Biol. Chem. 265: 16737-16740, 1990.

**Fu, J. Y., J. L. Masferrer, K. Seibert, A. Raz, and P. Needleman.** The induction and suppression of prostaglandin $H_2$ synthase (cyclooxygenase) in human monocytes. J. Biol. Chem. 265: 16737-16740, 1990.

**Gaillard, T., A. Mulsch, H. Klein, and K. Decker.** Regulation by prostaglandin $E_2$ of cytokine-elicited nitric oxide synthesis in rat liver macrophages. Biol. Chem. 373: 897-902, 1992.

**Gardemann, A., and K. Jungermann.** Control of glucose balance in the perfused rat liver by the parasympathetic innervation. Biol. Chem. Hoppe-Seyler 367: 559-566, 1986.

**Geller, D. A., C. J. Lowenstein, R. A. Shapiro, A. K. Nussler, M. Di Silvio, S. C. Wang, D. K. Nakayama.** Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. Proc. Natl. Acad. Sci. 90: 3491-3495, 1993.

**Gerzer, R., B. Karrenbrock, W. Siess, and J. M. Heim.** Direct comparison of the effects of nitroprusside, SIN-1, and various nitrates on platelet aggregation and soluble guanylate cyclase activity. Thromb. Res. 52: 11-21, 1988.

**Giacca, A., R. Gupta, S. Efendic, K. Hall, A. Skottner, L. Lickley, and M. Vranic.** Differential effects of IGF-1 and insulin on glucoregulation and fat metabolism in depancreatized dogs. Diabetes. 39: 340-47,1990.

**Gibbs. E. M., D. M. Calderhead, G. D. Holman, and G. W. Gould.** Phorbol ester only partially mimics the effects of insulin on glucose transport and glucose-transporter distribution in 3T3-L1 adipocytes. Biochem. J. 275: 145-150, 1991.

**Giugliano, D., L. Sacca, G. Scognamiglio, B. Ungaro, and R. Torella.** Influence of acetylsalicylic acid on glucose turnover in normal man. Diab Metab. 8: 279-282, 1982.

**Giugliano, D., R. Torella, N. Siniscalchi, L. Impronta, and F. D' Onofrio.** The effect of acetylsalicylic acid on insulin response to glucose and arginine in normal man. Diabetologia 14: 359-362, 1978.

**Greenfield, M. S., L. Deberne, F. Kraemer, T. Tobey, and G. Reaven.** Assessment of insulin resistance with insulin suppression test and the euglycemic clamp. Diabetes 30: 387-392, 1981.

**Greenwald, J. E., M. S. Alexander, R. H. Fertel, C. A. Beach, L. K. Wong. And J. R. Bianchine.** Role of ferric iron in platelet lipoxygenase activity. Biochem. Biophys. Res. Commun. 96: 817-822, 1980.

**Groop, L. C., E. Widen, and E. Ferrannini.** Insulin resistance and insulin deficiency in the pathogenesis of type 2 (non-insulin-dependent) diabetes mellitus: errors of metabolism or of methods? Diabetologia 36: 1326-1331, 1993.

**Gruppuso, P. A., R. Miqliori, J. B. Susa, and R. Schwartz.** Chronic maternal hyperinsulinemia and hypoglycemia. A model for experimental intrauterine growth retardation. Biol. Neonate. 40: 113-120, 1981.

**Gryglewski, R. J., R. Korbutr, E. Trabka-Janik, A. Zembowicz, and M. Trybutec.** Interaction between NO donors and iloprost in human vascular smooth muscle, platelets, and leukocytes. J. Cardiovasc. Pharmacol. 14: S124-S128, 1989.

**Guerri, C., R. Saez, M. Sancho Tello, E. Martin de Aquilera, and J. Renau Piqueras.** Ethanol alters astrocyte development: a study of critical periods using primary cultures. Neurochem. Res. 15 (5): 559-565, 1990.

**Guler, H. P., J. Zapf, and E. R. Froesch.** Short-term metabolic effects of recombinant human insulin-like growth factor I in healthy adults. N. Engl. J. Med. 317: 137-140, 1987.

**Hall, K., G. Lundin, and G. Povoa.** Serum levels of the low molecular form of insulin-like growth factor binding protein in healthy subjects and in patients with growth hormone deficiency, acromegaly, and anorexia nervosa. Acta endocrinol. 118: 321-326, 1988.

**Handberg, A.** Glucose transporters and insulin receptors in skeletal muscle. Danish Med. Bull. 42(3): 211-226, 1995.

**Hannigan, J. H.** What research with animals is telling us about alcohol-related neurodevelopmental disorder. Pharmacol. Biochem. Behav. 55(4): 489-499, 1996.

**Hannigan, J. H., R. A. Welch, and R. A. Sokol.** Recognition of fetal alcohol syndrome and alcohol-related birth defects. In: Medical diagnosis and treatment of alcoholism. Eds. Mendelson, J., and N. Mello. McGraw-Hill, New York. pp. 639-667, 1992.

**Harbrecht, B. G., J. Stadler, A. J. Demetris, R. L. Simmons, and T. R. Billiar.** Nitric oxide and prostaglandin interact to prevent hepatic damage during murine endotoxemia. Am. J. Physiol. 266: G1004-G1010, 1994.

**Hardman, J. G.** Cyclic nucleotides and regulation of vascular smooth muscle. J. Cardiovasc. Pharmacol. 6: S639-S645, 1984.

**Harris, J. E.** Hepatic glutathione, metallothionein and zinc in the rat on gestational day 19 during chronic ethanol administration. J. Nutri. 120: 1080-1086, 1990.

**Hecker, M., M. L. Foegh, and P W. Ramwell.** The eicosanoids: prostaglandins thromboxanes, Leukotrinenes, and related compounds. In: Basic and clinical pharmacology. Appleton Lange, Norwalk, Connecticut. 290-304, 1995.

**Hedqvist, P.** Basic mechanisms of prostaglandin action on autonomic neurotransmission. Annu. Rev. Pharmacol. Toxicol. 17: 259-279, 1977.

**Henderson, G. I., A. M. Hoyumpa, and M. A. Rothschild.** Effects of ethanol and ethanol-induced hypothermia on protein synthesis in pregnant and fetal rats. Alcohol. Clin. Exp. Res. 4: 165-177, 1980.

**Henderson, G. I., R. V. Patwardhan, A. M. Jr. Hoyumpa, and S. Schenker.** Fetal alcohol syndrome: overview of pathogenesis. Neurobehav. Toxicol. Teratol. 3: 73-80, 1981.

**Henderson, G. I., R. V. Patwardhan, S. McLeroy, and S. Schenker.** Inhibition of placental amino acid uptake in rats following acute and chronic ethanol. Alcohol. Clin. Exp. Res. 6: 495-505, 1982.

**Himsworth, H. P.** Diabetes mellitus. Its differentiation into insulin-sensitive and insulin-insensitive types. Lancet 1: 127-130, 1936.

**Holly, J. M. P., R. A. Biddlecombe, D. B. Dunger, J. A. Edge, S. A. Amiel, R. Howel, T. Chard, L. H. Rees, and J. A. Wass.** Circadian variations of GH-independent IGF-binding protein in diabetes mellitus and its relationship to insulin. A new role for insulin? Clin. Endocrinol. 29: 667-675, 1988.

**Holman, G. D., and M. Kasuga.** From receptor to transport: insulin signaling to glucose transport. Diabetologia 40: 991-1003, 1997.

**Holman, G. D., I. J. Kozka, A. E. Clark, C. J. Flower, J. Saltis, A. D. Habberfield, I. A. Simpson, and S. W. Cushman.** Cell-surface labeling of glucose transporter isoform GLUT 4 by bis-mannose photolabel: correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. J. Biol. Chem. 265: 18172-18179, 1990.

**Hom, F. G., C. J. Goodner, and M. A. Berrie.** A ($^3$H)2-deoxyglucose method for comparing rates of glucose metabolism and insulin responses among rat tissues *in vivo*: validation of the model and the absence of an insulin effect on brain. Diabetes 33: 141-152, 1984.

**Iadecola, C., A. J. Beitz, W. Renno, X. Xu, B. Mayer, and F. Zhang.** Nitric oxide synthase-containing neural processes in large cerebral arteries and cerebral microvessels. Brain. Res. 606:148-155, 1993.

**Ignarro, L. J.** Signal transduction mechanisms involving nitric oxide. Biochem. Pharmacol. 41: 485-490, 1991.

**Inoue, T., K. Fujuo, S. Morimoto, E. Koh, and T. Ogihara.** Nitric oxide mediates interleukin-1-induced prostaglandin $E_2$ production by vascular smooth muscle cells. Biochem Biophys. Res. Commun. 194: 420-424, 1993*a*.

**Inoue, Y., B. P. Bode, D. J. Beck. A. P. Li, K. I. Bland, and W. W. Souba.** Arginine transport in human liver. Ann. Surgery. 218(3): 350-363,1993*b*.

**Insel, J. R., O. G. Kolterman, M. Saekow, and M. Olefsky.** Short-term regulation of insulin receptor affinity in man, Diabetes 29: 132-139, 1980.

**Israel, Y., H. Kalant, J. M. Khanna, H. Orrego, M. J. Phillips, and D. J. Stewart.** Ethanol metabolism, oxygen availability and alcohol induced liver damage. In: Alcohol intoxication and withdrawal. Ed. Gross, M. M. Plenum Press, New York. pp 343-358, 1977.

**Itskovitz, J., E. F. LaGamma, and A. M. Rudolph.** The effect of reducing umbilical blood flow on fetal oxygenation. Am. J. Obstet. Gynecol. 145: 813-818, 1983.

**Iversen, J., H. Vilstrup, and N. Tygstrup.** Kinetics of glucose metabolism in relation to insulin concentrations in patients with alcoholic cirrhosis and in healthy persons. Gastroenterology 87: 1138-1143, 1984.

**Jacob, R., E. Barrett, G. Plewe, K. D. Fagin, and R. S. Sherwin.** Acute effects of insulin-like growth factor 1 on glucose and amino acid metabolism in the awake fasted rat. J. Clin. Inves. 83: 1717-1723,1989.

**Jacob, R., E. Barrett, R. S. Sherwin, L. Bowen, D. Fryburg, K. D. Fagin, W. V. Tamborlane, and G. I. Shulman.** Metabolic effects of IGF-1 and insulin in spontaneously diabetic BB/w rats. AM. J. Physiol. 260(23): E262-E268, 1991.

**Jacobs, S., E. Hazum, Y. Shechter, and P. Cuatreasas.** Insulin receptor: covalent labeling and identification of subunits. Natl. Acad. Sci. USA 76: 4918-4926, 1979.

**Janabi, N., S. Chabrier, and M. Tardieu.** Endogenous nitric oxide activates prostaglandin $F_{2\alpha}$ production in human microglial cells but not in astrocytes. J. Immun. 157: 2129-2135, 1996.

**Jarlstedt, J., and A. Hamberger.** Experimental alcoholism in rats. Effect of acute ethanol intoxication on the in vitro incorporation of ( 3 H) leucine into neuronal and glial proteins. J. Neurochem. 19: 2299-2309, 1972.

**Jones, K. L., and D. W. Smith.** Recognition of the fetal alcohol syndrome in early infancy. Lancet 2: 999-1001, 1973.

**Jones, K. L., D. W. Smith, C. N. Ulleland, and A. P. Streissguth.** Patterns of malformation in offspring of chronic alcoholic mothers. Lancet 1: 1267-1271, 1973.

**Kahn, C. R., K. L. Baird, J. S. Flier, C. Grunfeld, J. T. Harmon, L. C. Harrison, F. A. Karlsson, M. Kasuga, G. L. King, U. Lang, J. M. Podskalny, and E. Van Obberghen.** Insulin receptors, receptor antibodies, and the mechanism of insulin action. Recent Prog. Horm. Res. 37: 447-538, 1981.

**Kahn, C. R., and F. Folli.** Molecular determinants of insulin action. Horm. Res. 39: 93-98, 1993.

**Kaley, G., and A. Koller.** Prostaglandin-nitric oxide interactions in the microcirculation. Adv. Prost. Throm. Leuk. Res. 23: 485-490, 1995.

**Kalyanaraman, B., R. P. Mason, B. Trainer, and T. E. Eling.** The free radical formed during the hydroperoxide-mediated deactivation of ram seminal vesicle is hemoprotein-derived. J. Biol. Chem. 257: 4764-4768, 1982.

**Kanner, J., S. Harel, and R. Granit.** Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase, and hemoglobin. Lipids 27: 46-49, 1992.

**Kasuga, M., F. A. Karlsson, and C. R. Kahn.** Insulin stimulates the phosphorylation of the 95,000 Dalton subunit of its own receptor. Science 215: 185-187, 1982.

**Kaufman, M. H., and D. M. H. Wollam.** The passage to the fetus and liquor amnii of ethanol administered orally to the pregnant mouse. Br. J. Exp. Pathol. 62: 357-361, 1981.

**Keast, J. R.** A possible source of nitric oxide in the rat penis. Neurosci. Lett. 149: 69-73, 1992.

**Keller, S. R., R. Aebersold, C. W. Garner, and G. E. Lienhard.** The insulin elicited 160 kDa phophotyrosine protein in mouse adipocytes is an insulin receptor substrate-1-identification by cloning. Biochem. Biophys. Acta. 1172: 323-326, 1993.

**Kihara, M., and P. A. Low.** Impaired vasoreactivity to nitric oxide in experimental diabetic neuropathy, Exp. Neurol. 132:180-185, 1995.

**Kilbom, A., and A. Wennmalm.** Endogenous prostaglandins as local regulators of blood flow in man: effect of indomethacin on reactive and functional hyperaemia. J. Physiol. 257: 109-121, 1976.

**Kilbourn, R, G., and P. Belloni.** Endothelial cell production of nitrogen oxides in response to interferon-gamma in combination with tumor necrosis factor, interleukin or endotoxin. J. Natl. Cancer Inst. 82: 772-776, 1990.

**Knowles, R. G., M. Merret, M. Salter, and S. Moncada.** Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. Biochem J. 270: 833-836, 1990.

**Kohn, A. D., S. A. Summers, M. J. Birnbaum, and R. A. Roth.** Expression of a constitutively active Akt ser/thr kinase in 3T3-L1 adipocytes stimulates glucose transport and glucose transporter-4 translocation. J. Biol. Chem. 271: 31372-31378, 1996.

**Koller, A., and A. Huang.** Shear-induced dilation is attenuated in skeletal muscle arterioles of hypertensive rats. hypertension 25: 758-763, 1995.

**Koller, A., D. Sun, and G. Kaley.** Role of shear stress and endothelial prostaglandins in flow- and viscosity-induced dilation of arterioles in vitro. Cir. Res. 72: 1276-1284, 1993.

**Konturek, S. J., E. M. Mikos, R. Drol, Z. Wierzicki, and M. Dobrzanska.** Effect of methylated prostaglandin $E_2$ analogue on insulin secretion in man. Prostaglandins 15: 591-596, 1978.

**Laakso, M., S. Edelman, G. Brechtel, and A. D. Baron.** Decreased effect of insulin to stimulate skeletal blood flow in obese man. J. Clin. Invest. 85:1844-1852, 1990.

**Laakso, M., S. Edelman, G. Brechtel, and A. D. Baron.** Impaired insulin mediated skeletal muscle flow in patients with NIDDM. Diabetes. 41: 1076-1083, 1992.

**Laurie, S. M., C. C. Cain, G. E. Lienhard, and J. D. Castle.** The glucose transporter GLUT 4 and secretory carrier membrane proteins (SCAMPs) colocalize in rat adipocytes and partially segregate during insulin stimulation. J. Biol. Chem. 268: 19110-19117, 1993.

**Lautt, W. W.** Hepatic parasympathetic neuropathy as cause of maturity-onset diabetes? Gen. Pharmacol. 11:343-345, 1980.

**Lautt, W. W.** The HISS story overview: a novel hepatic neurohumoral regulation of peripheral insulin sensitivity in health and diabetes. Can. J. Physiol. Pharmacol. 77; 553-562, 1999.

**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.

**Lautt, W. W., and C. Wong.** Hepatic parasympathetic neural effect on glucose balance in the intact liver. Can. J. Physiol. Pharmacol. 56: 679-682, 1978.

**Lee, J., and P. F. Pilch.** The insulin receptor: structure, function and signaling. Am. J. Physiol. 266: C319-329, 1994.

**Lee, S. H., E. Soyoola, P. Chanmugarn, S. Hart, W. Sun, H. Zhong, S. Liou, D. Simmons, and D. Hwang.** Selective expression of mitogen-inducible cyclooxygenase in macrophage stimulated with lipopolysacchride. J. Biol. Chem. 267: 25934-25938, 1992.

**Lemoine, P., H. Harousseau, J. P. Borteyru, and J. C. Menuet.** Les enfants des parents alcooliques: anomalies observées a propos de 127 cas. Quest Med. 477-482, 1968.

**Levine, R. A.** Effect of prostaglandin $E_1$ on hepatic cyclic AMP activity, carbohydrate and lipid metabolism. Prostaglandins 6:509-521, 1974.

**Lewitt, M. S., H. Saunders, and R. C. Baxter.** Regulation of rat insulin-like growth factor-binding protein-1: the effect of insulin-induced hypoglycemia. Endocrinology 131: 2357-2364, 1992.

**Levine, R. A., and E. H. Schwartzel.** Prostaglandin $E_2$ inhibition of glucagon-induced hepatic gluconeogenesis and cyclic adenosine 3',5'-monophosphate accumulation. Biochem. Pharmacol. 29: 681-684, 1980.

**Li, G., C. D'Souza-Schorey, M. A. Barbiere, R. L. Roberts, A. Klippel, L. T. Williams, and P. D. Stahl.** Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. Proc. Natl. Acad. Sci. USA 92: 10207-10211, 1995*a*.

**Li, W. M., A. Santo, and A. Suzuki.** The inhibitory role of nitric oxide (NO) in the somatocardiac sympathetic C-reflex in anesthetized rats. Neurosci. Res. 22:375-380, 1995*b*.

**Lieber, C. S.** Alcohol, liver, and nutrition. J. Am. Coll. Nutr. 10: 602-632, 1991.

**Lin, G. W. J.** Effect of ethanol feeding during pregnancy on placental transfer of alpha aminoisobutyric acid in rat. Life Sci. 28: 595-601, 1981.

**Lopez-Tejero, D., M. Liobera, and E. Herrera.** Permanent abnormal response to a glucose load after prenatal ethanol exposure in rats. Alcohol 6(6): 469-473, 1989.

**Ludena, M. C., M. A. Mena, M. Salinas, and E. Herrera.** Effects of alcohol ingestion in the pregnant rat on daily food intake, offspring growth and metabolic parameters. Gen. Pharmacol. 14: 327-332, 1983.

**Lundbaek, K.** Intravenous glucose tolerance as a tool in definition and diagnosis of diabetes mellitus. Br. Med. J. 1: 1507-1513, 1962.

**Luyckx, A. S., and P. J. LeFebvre.** Possible role of endogenous prostaglandins in glucagon secretion by isolated guinea-pig islets. Diabetologia 15: 411-416, 1978.

**Maccarrone, M, S. Putti, A. F. Agro.** Nitric oxide donors activate the cyclooxygenase and peroxidase activities of prostaglandin H synthase. FEBS Let. 410: 470-476, 1997.

**Macedo, M. P., and W. W. Lautt.** Autoregulatory capacity in the superior mesenteric artery is attenuated by nitric oxide. AM. J. Physiol. 271: G400-G404, 1996.

**Macedo, M. P., D. J. Legare, and W. W. Lautt.** Insulin sensitivity is reduced by fasting and elevated by feeding. N. S. Arch. Pharmacol. 358(1)S2: R558, 1998.

**Maier, J. A. M., T. Hla, and T. Machag.** cyclooxygenase is an immediate early gene induced by interleukin-1 in human endothelial cells. J. Biol. Chem. 265: 10805-10809, 1990.

**Mandarini, L. J., A. Consoli, A. Jain, and D. E. Kelley.** Interaction of carbohydrate and fat fuels in human skeletal muscle: impact of obesity and NIDDM. Am. J. Physiol. 270: E463-E470, 1996.

**Masferrer, J. L., K. Seibert, B. Zweifel, and P. Needleman.** Endogenous glucocorticoids regulates an inducible cyclooxygenase enzyme. Proc. Natl. Acad. Sci. USA 89: 3917-3921, 1992.

**Masferrer, J. L., B. Zweifel, K. Seibert, and P. Needleman.** Selective regulation of cellular cyclooxygenase by dexamethasone and endotoxin in mice. J. Clin. Invest. 86: 1375-1379, 1990.

**Massague, J., and M. P. Czech.** The subunit structures of two distinct receptors for insulin-like growth factor I and II and their relationship to the insulin receptor. J. Biol. Chem. 257: 5038-5045, 1982.

**Matthews, L. S., G. Norstedt, and P. D. Palmiter.** Regulation of insulin-like growth factor 1 gene expression by growth hormone. Proc. Natl. Acad. Sci. USA 83: 9343-9347, 1986.

**Maurice, D. H., and R. J. Haslam.** Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. Mol. Pharmacol. 37: 671-676, 1990.

**Meade, E. A., E. L. Smith, and D. L. DeWitt.** Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isoenzymes by aspirin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 268: 6610-6614, 1993.

**Mellion, B. T., L. J. Ignarro, E. H. Ohlstein, E. G. Pontecorvo, A. L. Hyma, and P. J. Kadowitz.** Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced platelet aggregation in the presence of nitric oxide and related vasodilators. Blood 57: 946-955, 1981.

**Micossi, P., A. E. Pontiroli, S. H. Baron, R. C. Tamayo, F. Lengel, M. Bevilacqua, U. Raggi, G. Norbiato, and P. P. Foa.** Aspirin stimulates insulin and glucagon secretion and increases glucose tolerance in normal and diabetic subjects. Diabetes 27: 1196-1204, 1987.

**Mijare, A. H., and M. D. Jensen.** Contribution of blood flow to leg glucose uptake during a mixed meal. Diabetes. 44: 1165-1169,1995.

**Miller, J. D., S. Ganguli, R. Artal, and M. A. Sperling.** Indomethacin and salicylate decrease epinephrine-induced glycogenolysis. Metabolism 34: 148-153, 1985.

**Miller, J. D., S. Ganguli, and M. A. Sperling.** Prostaglandin synthesis inhibitors impair hepatic glucose production in response to glucagon and epinephrine stimulation. Diabetes 23:439-444, 1983.

**Mitchell, J. A., P. Akarasereenont, C. Thiemermann, R. G. Flower, and J. R. Vane.** Selectivity of non-steroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc. Natl. Acad. Sci. USA 90: 11693-11697, 1993.

**Mitchell, J. J., M. Paiva, D. Blaine Moore, and D. W. Walker.** A comparative study of ethanol, hypoglycemia, hypoxia, and neurotrophic factor interactions with fetal rat hippocampus neurons: a multi-factor in vitro model for developmental ethanol effects. Dev. Brain. Res. 105: 241-250, 1998.

**Moilanen, E., and H. Vapaatalo.** Nitric oxide in inflammation and immune response. Ann. Med. 27: 359-367, 1995.

**Moncada, S., R. M. J. Palmer, and E. A. Higgs.** Nitric oxide: physiology, pathology and pharmacology. Pharmacol. Rev. 43:109-141, 1991.

**Morgan, D. O., J. C. Edman, D. N. Standring, V. A. Fried, M. C. Smith, R. A. Roth, and W. J. Rutter.** Insulin-like growth factor II receptor as a multifunctioning binding protein. Nature 329: 301-303, 1987.

**Morin, F. C., and K. I. Weiss.** Response of the fetal circulation to stress. In:. Fetal and neonatal physiology. Eds. Polin, R. A., and W. W. Fox. W. B. Sanders, Philadelphia. pp.620-629, 1992.

**Morland, J., and A. Bessesen.** Inhibition of protein synthesis by ethanol in isolated rat liver parenchymal cell. Biochim. Biophys. Acta 474: 312-320, 1977.

**Morrison, J. F. B., A. Sato, Y. Sato, and A. Suzuki.** The nitric oxide synthase inhibitor L-NAME reduces inhibitory components of somato-vesical parasympathetic reflexes in the rat. Nurosci. Res. 24: 195-199, 1996.

**Moses, A. C., S. C. J. Young, L. A. Morrow, M. O'Brian, and D. R. Clemmons.** Recombinant human insulin-like growth factor-I increases insulin sensitivity and improves glycemic control in type II diabetics. Diabetes 45: 91-100, 1996.

**Moxham, C. M., and C. C. Malbon.** Insulin action impaired by deficiency of the G-protein subunit G ($I\alpha_2$). Nature 379: 840-844, 1996.

**Mukherjee, A. B., and G. D. Hodgen.** Maternal ethanol exposure induces transient impairment of umbilical circulation and fetal hypoxia in monkeys. Science. 218: 700-701, 1982.

**Nakaki, T., and R. Kato.** Beneficial circulatory effects of L-arginine. Jpn. J. Pharmacol. 66:167-171,1994.

**Nakanishi, H., K. A. Brewer, and J. H. Exton.** Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-triphosphate. J. Biol. Chem. 268: 13-18, 1993.

**Natali, A.** Skeletal muscle blood flow and insulin action. Nutr. Metab. Cardio. Dis. 7: 105-109, 1997.

**Natali, A., A. Q. Galvan, N. Pecorri, G. Sanna, E. Toschi, and E. Ferrannini.** Vasodilation with sodium nitroprusside does not improve insulin action in essential hypertension. Hypertension. 31: 632-636, 1998.

**Nathan, C.** Nitric oxide as a secretory product of mammalian cells. FASEB J. 6:3051-3064, 1992.

**National Diabetes Data Group.** Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 28: 1039-1957, 1979.

**Newman, W. P., and R. G. Brodows.** Metabolic effects of prostaglandin $E_2$ infusion in man: possible adrenergic mediation. J. Clin. Endocrinol. Meta. 55: 496-501, 1982.

**Newman, W. P., and R. G. Brodows.** Aspirin causes tissue insensitivity to insulin in normal man. J. Clin. Endocrinol. Meta. 57: 1102-1106, 1983.

**Nishikawa, M., M. Kanamori, and H. Hidaka.** Inhibition of platelet aggregation and stimulation of guanylate cyclase by an antianginal agent, molsidomine, and its metabolites. J. Pharmacol. Exp. Ther. 220: 183-190, 1982.

**Nitzan, M.** Relation between maternal and fetal blood glucose levels in experimentally intrauterine growth retardation. Isr. J. Med. Sci. 17: 378-380, 1981.

**Noack, E., and M. Feelisch.** Molecular aspects underlying the vasodilator action of molsidomine. J. Cardiovasc. Pharmacol. 14: S1-S5, 1989.

**Nordmann, R., C. Ribiere, and H. Rouach.** Implication of free radical mechanisms in ethanol-induced cellular injury. Free Radial Biol. Med. 12: 219-239, 1992.

**Nuttall, F. Q., M. C. Gannon, and J. Larner,** oral glucose effect on glycogen synthase and phosphorylase in heart, muscle and liver. Physiol. Chem. Phys. 4: 497-515, 1972.

**Oh, Y., S. R. Nagalla, Y. Yamanaka, H. S. Kim, E. Wilson, and R. G. Rosenfeld.** Synthesis and characterization of insulin-like growth factor binding protein (IGFBP)-7: recombinant human mac25 protein specifically binds IGF-I and IGF-II. J. Biol. Chem. 271: 322-325, 1996.

**Okada, T., L. Sakuma, Y. Fukui, O. Hazeki, and M. Ui.** Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. J. Biol. Chem. 269: 3568-3573, 1994.

**Omawari, N., M. Dewhurst, P. Vo, S. Mahmood, E. Stevens, and D. R. Tomlinson.** Deficient nitric oxide responsible for reduced nerve blood flow in diabetes rats: effects of L-Name, L-arginine, sodium nitroprusside, and evening primrose oil. Br. J. Pharmacol. 118:186-190, 1996.

**Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruis-Larrea, G. Panayotou, A. Thompon, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Courtneidge, P. J. Parker, M. D. Waterfield.** Characterization of two 85kD proteins that associate with receptor tyrosine kinases, middle-T/pp60$^{c\text{-}src}$ complexes and PI3-kinase. Cell 65: 91-104, 1991.

**Paolisso, G., A. Gambardella, F. Saccomanno, G. Varricchio, A. D'Amore, and M. Varricchio.** Low-dose Iloprost infusion improves insulin action and non-oxidative glucose metabolism in hypertensive patients. Eur. J. Clin. Pharmacol. 48: 333-338, 1995.

**Peeters, L. L. H., R. E. Sheldon, M. D. Jones, E. L. Makowski, and G. Meschia.** Blood flow to fetal organs as a function of arterial oxygen content. Am. J. Obstet. Gynecol. 135: 637-646, 1979.

**Pek, S., T. Y. Tai, A. Elster, and S. S. Fajans.** Stimulation by prostaglandin E2 of glucagon and insulin release from isolated rat pancreas. Prostaglandins 10: 493-502, 1975.

**Pennington, S. N., I. A. Shibley, M. D. Gavigan, J. M. Monaghan, L. P. Sandstrom, and J. L. Morgan.** Insulin signaling in chick embryo exposed to alcohol. Alcohol. Clin. Exp. Res. 19: 701-707, 1995.

**Pennington, S. N., J. W. Boyd, G. W. Kalmus and R. W. Wilson.** The molecular mechanism of fetal alcohol syndrome (FAS). Ethanol-induced growth suppression. Neurobehav. Toxicol. Teratol. 5(2): 259-262, 1981.

**Petersen, K. F., R. Jacob, A. B. West, R. S. Sherwin, and G. I. Shulman.** Effects on insulin-like growth factor I on glucose metabolism in rats with liver cirrhosis. Am. J. Physiol. 273: E1189-E1193, 1997.

**Pierce, D. R., and J. R. West.** Alcohol-induced microencephaly during the third trimester equivalent: relationship to dose and blood alcohol concentration. Alcohol 3: 185-191, 1986*a*.

**Pierce, D. R., and J. R. West.** Blood alcohol concentration: a critical factor for producing fetal alcohol effects. Alcohol 3: 269-272, 1986*b*.

**Pitre, M., A. Nadeau, and H. Bachelard.** Insulin sensitivity and hemodynamic responses to insulin in wistar-kyoto and spontaneously hypertensive rats. Am J. Physiol. 271:E658-E668, 1996.

**Polygenis, D., S. Wharton, C. Malmberg, N. Sherman, D. Kennedy, G. Koren, and T. R. Einarson.** Moderate alcohol consumption during pregnancy and the incidence of fetal malformations: a meta-analysis. Neurotoxicol. Teratol. 20: 61- 67, 1998.

**Porter, J. P., I. G. Joshua, D. Kabith, and H. S. Bokil.** Vasodilator effect of insulin on the microcirculation of the rat cremaster muscle. Life Sciences. 61(7): 673-687,1997.

**Pratt, O. E.** The fetal alcohol syndrome: transport of nutrients and transfer of alcohol and acetaldehyde from mother to fetus. In: Psychopharmacology of Alcohol. Ed. Sandler, M. Raven Press, New York. pp. 229-256, 1980.

**Proietto, J., F. P. Alford, and F. J. Dudley.** The mechanism of the carbohydrate intolerance of cirrhosis. J. Clin. Endocrinol. Metab. 51: 1030-1036, 1980.

**Radomski, M. W., P. M. J. Palmer, S. Moncada.** The antiaggreating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br. J. Pharmac. 92: 639-646, 1987.

**Radomski, M., R. M. J. Palmer, and S. Moncada.** Glucocorticoids inhibit the expression of an inducible, but not the constitutive nitric oxide synthase in vascular endothelial cells. Proc. Natl. Acad. Sci. USA 87: 10043-10047, 1990.

**Raitakari, M., P. Nuutila, U. Ruotsalainen, H. Lain, M. Teras, H. Iida, S. Makimattila, T. Utriainen, V. Oikonen, H. Sipila, M. Haaparanta, O. Solin, U. Wegelius, J. Knuuti, and H. Yki-Jarvinen.** Evidence for dissociation of insulin stimulation of blood flow and glucose uptake in human skeletal muscle. Diabetes. 45:1471-1477, 1996.

**Rajkumar, K., D. Barron, M. S. Lewitt, and L. J. Murphy.** Growth regulation and hyperglycemia in insulin-like growth factor binding protein-1 transgenic mice. Endocrinology 136: 4029-4034, 1995.

**Rawat, A. K.** Derangement in cardiac protein metabolism in fetal alcohol syndrome. Res. Commun. Chem. Pathol. Pharmacol. 25: 365-375, 1979.

**Reaven, G. M., J. Moore, and M. Greenfield.** Quantification of insulin secretion and *in vivo* insulin action in non-obese and moderately obese individuals with normal glucose tolerance. Diabetes 32: 600-604, 1983.

**Rechler, M. M., and A. L. Brown.** IGF binding proteins: gene structure and expression. Growth Reg. 2: 55-68, 1992.

**Rechler, M. M., and D. R. Clemmons.** Regulatory actions of insulin-like growth factor-binding proteins. Trends Endocrin, Metab. 9: 176-183, 1998.

**Rees, D. D., R. M. J. Palmer, R. Schulz, H. F. Hodson, and S. Moncada.** Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. Br. J. Pharmacol. 101:746-752, 1990.

**Rettori, V., M. Gimeno, K. Lyson, and S. M. McCann.** Nitric oxide mediates norepinephrine-induced prostaglandin $E_2$ release from the hypothalamus. Proc. Natl. Acad. Sci. USA 89: 11543-11546,1992.

**Reyes, E., S. Oh, and B. Robinson.** Effects of in vitro administration of alcohol on glutathione levels in brain and liver. Alcohol. Clin. Exp. Res. 17: 887-881, 1993.

**Richardson, B., J. Patrick, J. Bousquet, J. Homan, and J. Brien.** Cerebral metabolism in fetal lamb after maternal infusion of ethanol. Am. J. Physiol. 249: R505-R509, 1985.

**Riese, J., T. Hoff, A. Nordhoff, D. L. DeWitt, K. Resch, and V. Kaever.** Transient expression of prostaglandin endoperoxide synthase-2 during mouse macrophage activation. J. Leukot. Biol. 55:476-482, 1994.

**Roberts, C. T., S. R. Lasky, W. L. Lowe, W.T. Seaman, and D. LeRoith.** Molecular cloning of rat IGF-1 cDNA: differential mRNA processing and regulation by GH in extrahepatic tissues. Mol. Endocrinol. 1: 243-248, 1987.

**Robertson, R. P., and M. Chen.** A role for prostaglandin E in defective insulin secretion and carbohydrate intolerance in diabetes mellitus. J. Clin. Invest. 60: 747-753, 1977.

**Robertson, R. P., D. J. Gavareski, and D. Port.** Inhibition of *in vivo* insulin secretion by prostaglandin $E_1$. J. Clin. Invest. 54: 310-315,1974.

**Rocha, D. M., G. R. Faloona, and R. H. Unger.** Glucagon-stimulating activity of 20 amino acids in dogs. J. Clin. Invest. 51: 2346-2351, 1972.

**Rosen, O. M.** After insulin binds. Science 237: 1452-1458, 1987.

**Rosett, H. L.** A clinical perspective of the fetal alcohol syndrome. Alcohol. Clin. Exp. Res. 4: 119-122, 1980.

**Rossetti, L., S. Frontoni, R. Dimarchi, R. A. Defronzo, and A. Giaccari.** Metabolic effects of IGF-1 in diabetic rats. Diabetes 40: 444-448, 1991.

**Rosso, P.** Changes in the transfer of nutrients across the placenta during normal gestation in the rat. Am. J. Obstet. Gynecol. 122: 761-766, 1975.

**Sacca, L., G. Perez, G. Raisz F. Rengo, and M. Condovelli.** Effects of different prostaglandins on glucose kinetics in the rat. Diabetes 23: 532-535, 1974.

**Sadri, P., and W. W. Lautt.** Blockade of nitric oxide production in the liver causes insulin resistance. Proc. West. Pharmacol. Soc. 1998.

**Salvemini, D., K. Seibert, J. L. Masferrer, T. P. Misko, M. G. Currie, and P. Needleman.** Endogenous nitric oxide enhances prostaglandin production in a model or renal infiammation. J. Clin. Invest. 93: 1940-1947, 1994.

**Salvemini, D., M. G. Currie, and V. Mollance.** Nitric oxide-mediated cyclooxygenase activation. J. Clin. Invest. 97: 2562-2568, 1996.

**Salvemini, D., S. L. Settle, J. L. Masferrer, K. Seibert, M. G. Currie, and P. Needleman.** Regulation of prostaglandin production by nitric oxide; an in vivo analysis. Br. J. Pharmacol. 114: 1171-1178, 1995.

**Salvemini, D., T. P. Misko, J. L. Masferrer, K. Seibert, M. G. Currie, and P. Needleman.** Nitric oxide activates cyclooxygenase enzymes. Proc. Natl. Acad. Sci. USA 90: 7240-7244, 1993.

**Sampson P. D., A. P. Streissguth, H. M. Barr, and F. L. Brookstein.** Neurobehavioral effects of prenatal alcohol. Part II. Partial least squares analysis. Neurotoxicol. Teratol. 11: 477- 491, 1989.

**Sanders, K. M., and S. M. Ward.** Nitric oxide as a mediator of non-adrenergic non-cholinergic neurotransmission. Am. J. Physiol. 262: G379-G392, 1992.

**Sandstrom, L. P., P. A. Sandstrom, and S. N. Pennington.** Ethanol-induced insulin resistance suppresses the expression of embryonic ornithine decarboxylase activity. Alcohol 10: 1-8, 1993.

**Sara, V. R., and K. Hall.** The insulin-like growth factors and their binding proteins. Physiol. Rev. 70: 591-614, 1990.

**Satori, C., and U. Scherrer.** Insulin, nitric oxide and the sympathetic nervous system: at the crossroads of metabolic and cardiovascular regulation. J. Hyper. 17: 1517-1525, 1999.

**Savoy-Moore, R. T., M. P. Dombrowski, A. Cheng, E. Abel, and R. J. Sokol.** Low dose alcohol contracts the human umbilical artery in vitro. Alcohol. Clin. Exp. Res. 13: 40-42, 1989.

**Scherrer, U., D. Randin, P. Vollenweider, L. Vollenweider, and P. Nicod.** Nitric oxide release accounts for insulin's vascular effects in humans. J. Clin. Invest. 94: 2511-2515, 1994.

**Schreiber, S. S., K. Briden, M. Oratz, and M. A. Rothschild.** Ethanol, acetaldehyde, and myocardial protein synthesis. J. Clin. Invest. 51: 2820- 2826, 1972.

**Schwander, J. C., C. Hauri, J. Zapf, and E. R. Froesch.** Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. Endocrinology 113: 297-305, 1983.

**Schweistal, M. R. and R. L. Gingerich.** The effects of ethanol diet on insulin release in maternal, fetal and neonate rats. Nutr. Rep. Int. 32: 863-871, 1985.

**Seibert, K., and J. Masferre.** Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor 94: 17-23, 1994.

**Shankar, R., J. Zhu, B. Ladd, D. Henry, H. Shen, and A. D. Baron.** Central nervous system nitric oxide synthase activity regulates insulin secretion and insulin action. J. Clin. Invest. 102: 1403-1412, 1998.

**Sheldon R. E., L. L. Peeters, M. D. Jones, E. L. Makowski, and G. Meschia.** Redistribution of cardiac output and oxygen delivery in the hypoxemic fetal lamb. Am. J. Obstet. Gynecol. 135: 1071-1078, 1979.

**Sheng H., H. H. W. Schmidt, M. Nakone, J. A. Nitchell, J. S. Pollock, U. Forstermann, and F. Murad.** Characterization and localization of nitric oxide synthase in non-adrenergic noncholinergic nerves from bovine retractor penis muscles. Br. J. Pharmac. 106: 768-773, 1992.

**Shibley, I. A., and S. N. Pennington.** Metabolic and mitotic changes associated with the fetal alcohol syndrome. Alcohol Alcohol. 32: 423-434, 1997.

**Simpson, H. l., A. M. Umpleby, and D. L. Russell-Jones.** Insulin-like growth factor-1 and diabetes. A review. Growth Hormone and IGF Res. 8: 83-95, 1998.

**Singh, S. P., G. L. Pullen, K. S. Srivenugopal, X. H. Yuan, and A. K. Snyder.** Decreased glucose transporter 1 gene expression and glucose uptake in fetal brain exposed to ethanol. Life Sci. 51: 527-536, 1992.

**Singh, S. P., and A. K. Snyder.** Ethanol ingestion during pregnancy: effects on pregnant rats and their offspring. J. Nutr. 112: 98-103, 1982.

**Singh, S. P., A. K. Snyder, and G. L. Pullen.** Fetal alcohol syndrome: glucose and liver metabolism in term rat fetus and neonate. Alcohol. Clin. Exp. Res. 10: 54-58, 1986.

**Singh, S. P., A. K. Snyder, and G. L. Pullen.** Maternal alcohol ingestion inhibits fetal glucose uptake and growth. Neurotoxicol. Teratol. 11: 215-219, 1989.

**Singh, S. P., A. K. Snyder, and S. K. Singh.** Effects of ethanol ingestion on maternal and fetal glucose homeostasis. J. Lab. Clin. Med. 104: 176-184, 1984.

**Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger.** Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell 65: 83-90, 1991.

**Smith, W. L.** The eicosanoids and their biochemical mechanisms of action. Biochem. J. 259: 315-329, 1989.

**Smith, W. L., and L. J. Marnett.** Prostaglandin endoperoxide synthase: structure and catalysis. Biochem. Biophys. Acta. 1083:1-17, 1990.

**Snyder, A. K., F. Jiang, and S. P. Singh.** Effects of ethanol on glucose utilization by cultured mammalian embryos. Alcohol. Clin. Exp. Res. 16: 466-470, 1992.

**Soos, M. A., and K. Siddle.** Immunological relationships between receptors for insulin and insulin-like growth factor 1. Evidence for structural heterogeneity of insulin-like growth factor 1 receptors involving hybrids with insulin receptors. Biochem. J. 263: 553-563, 1989.

**Standaert, M. L., R. V. Farese, D. R. Cooper, and R. J. Pollet.** Insulin-induced glycerolipid mediators and the stimulation of glucose transport in BCC3H-1 myocytes. J. Biol. Chem. 263: 8696-8705, 1988.

**Steinberg, H. O., G. Brechtel, A. Johnson, N. Fineberg, and A. d. Baron.** Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. J. Clin. Invest. 94:1172-1179,1994.

**Stevens, M. J.** Nitric oxide as a potential bridge between the metabolic and vascular hypotheses of diabetic neuropathy. Diabetic Med. 12: 292-295, 1995.

**Sthuer, D. J., M. A. Marletta.** Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon-γ. J. Immunol. 139: 518-525, 1987.

**Streissguth, A. P.** The behavioral teratology of alcohol: Performance, behavioral and intellectual deficit in prenatally exposed children. In: Alcohol and brain development. Ed. West, J. R. Oxford Press, New York. pp. 3-44, 1986.

**Streissguth, A. P., F. L. Brookstein, P. D. Sampson, and H. M. Barr.** Neurobehavioral effects of prenatal alcohol. Part III. PLS analysis of neuropsychological tests. Neurotoxicol. Teratol. 11: 493- 507, 1989.

**Streissguth, A. P., P. D. Sampson, H. C. Olson, F. L. Brookstein, , H. M. Barr M. Scott, J. Feldman, and A. F. Mirsky.** Maternal drinking during pregnancy: attention and short-term memory in 14-year-old offspring. A longitudinal prospective study. Alcohol. Clin. Exp. Res. 18: 202-218, 1994.

**Suikkari, A. M., V. A. Koivisto, E. M. Rutanen, H. Yki-Jarvinen, S. L. Karonen, and M. Seppala.** Insulin regulates the serum levels of low molecular weight insulin-like growth factor-binding protein. J. Clin. Endocrinol. Metab. 66: 266-272, 1988.

**Sun, X. J., P. Rothenberg, C. R. Kahn, J. M. Baker, E. Araki, P. A. Wilder, D. A. Cahill, B. J. Goldstein, and M. F. White.** Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature 352: 13-77, 1991.

**Swan, J. W., C. Walton, and I. F. Godsland.** Assessment of insulin sensitivity in man: a comparison of minimal model- and euglycemic clamp-derived measures in health and heart failure. Clin. Sci. 86: 317-322, 1994.

**Sweat, F. W., and L. Yamashita.** Prostaglandin $E_1$ effects on cyclic AMP and glycogen metabolism in rat liver. Biochem. Biophys. Res. Commun. 82:879-886, 1978.

**Sweat, F. W., L. Yamashita, and W. Jubiz.** Dissociation of prostaglandin E effects on liver glycogenolysis and cyclic AMP levels. Mol. Cell. Endocrinol. 32: 131-142, 1983.

**Swislocki, A., T. Eason, and G. A. Kayen.** Oral administration of nitric oxide biosynthesis inhibitor, N-Nitro-L-Arginine Methyl Ester (L-NAME), causes hypertension, but not glucose intolerance or insulin resistance, in rats. AJH 8:1009-1014, 1995.

**Synder, D. K., and D. R. Clemmons.** Insulin-dependent regulation of insulin-like growth factor-binding protein-1. J. Clin. Endocrinol. Metab. 71: 1632-1636, 1990.

**Syvalahti, E. K.** The effects of indomethacin on serum growth hormone, immunoreactive insulin, and blood glucose levels of young adult males. Int. J. Clin. Pharmacol. 10: 111-116, 1974.

**Tanka, H., N. Suzuki, and M. Arima.** Hypoglycemia in the fetal alcohol syndrome. Brain Dev. 4: 97-103, 1982.

**Tetsuka, T., D. Daphna-Iken, S. K. Srivastava, L. D. Baier, J. DuMaine, and A. R. Morrison.** Cross-talk between cyclooxygenase and nitric oxide pathways: prostaglandin $E_2$ negatively modulates induction of nitric oxide synthase by interleukin1. Proc. Natl. Acad. Sci. USA 91: 12168-12172, 1994.

**Thadani, P. V.** Fetal alcohol syndrome: neurochemical and endocrinological abnormalities. Prog. Biochem. Pharmacol. 18: 83-98, 1981.

**Thurman, R. G., S. Ji, T. Matsumura, and J. J. Lemasters.** Is hypoxia involved in the mechanism of alcohol-induced liver injury? Fund. Appl. Toxicol. 4: 125-133, 1984.

**Toker, A., M. Meyer, K. K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas, and L. C. Cantley.** Activation of protein kinase C family members by the novel phosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. J. Biol. Chem. 269: 32358-32367, 1994.

**Tritos, N. A., and C. S. Mantzoros.** Leptin: its role in obesity and beyond. Diabetologia 40: 1371-1379, 1997.

**Ullrich, A., J. R. Bell, E. Y. Chen, R. Herrera, L. M. Petruzzelli, T. J. Dull, A. Gray, L. Coussens, Y. C. Liao, M. Tsubokawa, A. Mason, P. H. Seeburg, C. Grunfeld, O. M. Rosen, and J. Ramachandran.** Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature 313: 756-761, 1985.

**Uphues, I., T. Kolter, B. Goud, and J. Eckel.** Insulin-induced translocation of the glucose transporter GLUT 4 in cardiac muscle. Studies on the role of small molecular mass GTP-binding proteins. Biochem. J. 302: 177-182, 1994.

**Utriainen, T., S. Makimattila, A. Virkamaki, R. Bergholm, and H. Yki-Jarvinen.** Dissociation between insulin sensitivity of glucose uptake and endothelial function in normal subjects. Diabetologia. 39: 1477-1482, 1996.

**Utriainen, T., P. Nuutila, T. Takala, P. Vicini, U. Ruotsalainen, T. Ronnemaa, T. Tolvanen, M. Raitakari, M. Haaparanta, O. Kirvela, C. Cobelli, and H. Yki-Jarvinen.** Intact insulin stimulation of skeletal muscle blood flow, its heterogeneity and redistribution, but not of glucose uptake in non-insulin-dependent diabetes mellitus. J. Clin. Invest. 100: 777-785, 1997.

**Vallance, P., and J. Collier.** Biology and clinical relevance of nitric oxide. BMJ 309: 453-457, 1994.

**Vane, J.** Towards a better aspirin. Nature 367: 215-216, 1994.

**Vane, J. R., E. E. A. Anggard, and R. M. Botting.** Regulatory functions of the vascular endothelium. N. Engl. J. Med. 323: 27-36, 1990.

**Vannucci, S. J., H. Nishimura, S. Satoh, S. W. Cushman, G. D. Holman, and I. A. Simpson.** Changes in cell-surface accessibility of GLUT 4 glucose transporter: effect of insulin, isoproterenol and adenosine. Biochem. J. 288: 325-330, 1992.

**Vaughan, M.** An effect of prostaglandin $E_1$ on glucose metabolism in rat adipose tissue. In: Prostaglandins. Proceedings of the Nobel Symposium. Eds. Bergstrom, S., and B. Samulson. Almquist and Wiksele, Stockholm. p. 139, 1967.

**Vierhapper, H., P. Bratusch-Marrain, W. Waldhausl, P. Nowotny, and S. Panzer.** Increased secretion of insulin but unchanged secretion of growth hormone in hyperglycemic type II diabetics treated with acetyl-salicylic acid. Clin Endocrinol. 18: 613-620, 1983.

**Villarroya, F., and T. Mampel.** Glucose tolerance and insulin response in offspring of ethanol-treated pregnant rats. Gen. Phamacol. 16: 415-417, 1985.

**Vizzard, M. A., S. L. Erdman. and W. C. De Groat.** Localization of NADPH diaphorase in bladder afferent and post-ganglionic efferent neurons of the rat. J. Auton. Nerv. Syst. 44: 85-90, 1993.

**Wasner, H. K., S. Weber, H. J. Partke, and H. Amini-Hadi-Kiashar.** Indomethacin treatment causes loss of insulin action in rats: involvement of prostaglandins in the mechanism of insulin action. Acta Diabetol. 31; 175-182, 1994.

**Weinberg, J.** Nutritional issues in prenatal alcohol exposure. Neurobehav. Toxicol. Teratol. 6: 261-269, 1984.

**Weinberg, J.** Neuroendocrine effects of prenatal alcohol exposure. Ann. N Y Acad. Sci. 697: 86-96, 1993.

**Weston, W. M., R. M. Greene, M. Uberti, and M. M. Pisano.** Ethanol effects on embryonic craniofacial growth and development: implications for study of the fetal alcohol syndrome. Alcohol. Clin. Exp. Res. 18(1): 177-182, 1994.

**Wheeler, G. E., and R. M. Epand.** Prostaglandin $E_1$: anomalous effects on glucose production in rat liver. Mol. Pharmacol. 11: 335-339, 1975.

**White, M. F., and C. R. Kahn**. The insulin signaling system. J. Biol. Chem. 269: 1-16,1994.

**White, M. F., S. E. Shoelson, H. Keutmann, and C. R. Kahn.** A cascade of tyrosine autophosphorylation in the β-subunit activates the insulin receptor. J. Biol. Chem. 263: 2969-2980, 1988.

**Whitman, M., C. P. Downes, M. Keeler, T. Keeler, and L. Cantley.** Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. Nature 332: 644-646, 1988.

**Widstrom, A.** Influence of indomethacin on glucose-induced insulin response in normal man: role of prostaglandins in the rapid insulin release? Horm. Metab. Res. 9: 172-175, 1977.

**Wu, K. K.** Inducible cyclooxygenase and nitric oxide synthase. In: Advances in pharmacology. Academic Press. Inc., New York. pp. 179-207, 1995.

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

**Xie, H., and W.W. Lautt.** M1 muscarinic receptor blockade causes insulin resistance in the cat. Proc. West. Pharmacol. Soc. 38: 83-84, 1995*b*.

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

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

**Xie, W., D. L. Robertson, and D. L. Simmons.** Mitogen-inducible prostaglandin G\H synthase: a new target for non-steroidal anti-inflammatory drugs. Drug Dev. Res. 25: 249-265, 1992.

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

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

**Yamada, K. A. Avignon, M. L. Standaert, D. R. Cooper, B. Spencer, and R. V. Farese.** Effects of insulin on the translocation of protein kinase C theta and other protein kinase C isoforms in rat skeletal muscle. Biochem. J. 308: 177-180, 1995.

**Yamamoto, S., A. Miyamoto, S. Kawana, A. Namiki, and H. Ohshika.** Role of nitric oxide production through $M_2$-cholinergic receptors in cultured rat ventricular myocytes. Biochem. Biophys. Res. commun. 251:791-795, 1998.

**Yamanaka, Y., E. M. Wilson, R. G. Rosenfeld, and Y. Oh.** Inhibition of insulin receptor activation by insulin-growth factor binding proteins. J. Biol. Chem. 272: 30729-30734, 1997.

**Yang, J., J. F. Clarke, C. J. Ester, P. W. Young, M. Kasuga, and G. D. Holman.** Phosphatidylinositol-3-kinase acts at an intracellular membrane site to enhance GLUT 4 exocytosis in 3T3-L1 cells. Biochem. J. 313: 125-131, 1996.

**Yang, J., and G. D. Holman.** Comparison of GLUT 4 and GLUT 1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells. J. Biol. Chem. 268: 4600-4603, 1993.

**Yang, H. Y., A. Y. C. Shum, H. T. Ng, and C. F. Chen.** Effects of ethanol on human umbilical artery and vein in vitro. Gynecol. Obstet. Invest. 21: 131-135, 1986.

**Yenush, L., K. J. Makati, J. Smith-Hall, O. Ishibashi, M. G. Mayers, and M.F. White.** The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1. J. Biol. Chem. 271: 24300-24306, 1996.

**Yeoh, S. I., R. C. Baxter.** Metabolic regulation of the growth hormone independent insulin-like growth factor binding protein in human plasma. Acta Endocrinol. 119: 465-473, 1988.

**Yip, C. C., C. W. T. Yeung, and M. L. Moule.** Photoaffinity labeling of insulin receptor of rat adipocyte plasma membrane. J. Biol. Chem. 253: 1743-1747, 1978.

**Yki-Jarvinen, H., and T. Utriainen.** Insulin-induced vasodilation: physiology or pharmacology? Diabetologia. 41: 369-379, 1998.

**Yonezawa, K., A. Ando, Y. Kaburagi, R. Yamamoto-Honda, T. Kitamura, K. Hara, M. Nakafuku, Y. Okabayashi, T. Kadowaki, and Y. Kaziro.** Signal transduction pathways from insulin receptors to Ras; analysis by mutant insulin receptors. J. Biol. Chem. 269: 4634-4640, 1994.

**Zapf, J., E. R. Froesch, and R. E. Humbel.** The insulin-like growth factors (GF) of human serum: chemical and biological characterization and aspects of their possible physiological role. Curr. Top. Cell. Regul. 19: 257-309, 1981.

**Zapf, J., E. Schoenle, and E. R. Froesch.** Insulin-like growth factors I and II. Some biological actions and receptor binding characteristics of two purified constituents of nonsuppressible insulin-like activity of human serum. Eur. J. Biochem. 87: 285-296, 1978.

**Zenobi, P. D. , S. Jaeggi-Grossman, W. F. Riesen, M. E. Roder, and E. R. Froesh.** Insulin-like growth factor I improves glucose and lipid metabolism in type 2 diabetes mellitus. J. Clin. Invest. 90: 2234-2241, 1992.

**Zimmet, P. Z., D. J. McCarty, and M. P. de Courten.** The global epidemiology of non-insulin-dependent diabetes mellitus and the metabolic syndrome. J. Diabetes Complic. 11: 60-68, 1997.