# THE REGULATION OF NET HEPATIC GLUCOSE UPTAKE IN VIVO

By

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**To my parents, Richard and Diane**

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# CHAPTER I

## INTRODUCTION

Glucose homeostasis reflects a delicate balance between glucose production and glucose uptake. This balance is best demonstrated during periods following food consumption and fasting when plasma glucose levels change only modestly. Glucose production and glucose uptake are tightly regulated by hormones and substrates and it has been shown that the liver plays a pivotal and unique role in glucose homeostasis. This is due to both its anatomical location (it receives a systemic and portal supply of absorbed nutrients) as well as its ability to both consume and produce glucose in a net sense (188).There have been numerous studies and much work done in the diabetes field examining hepatic glucose production but much less is known about hepatic glucose uptake. This is in part due to the inherent difficulty of measuring hepatic balance in the human (i.e, impossible to catheterize the hepatic portal vein). Thus, the following dissertation aims to further understand the post-prandial state by further clarifying the effect of insulin and the portal signal on net hepatic glucose uptake.

Following an overnight fast (in the post absorptive state), the liver is the major contributor of endogenous hepatic glucose production with the only other contributing organ being the kidney. The contribution of the kidneys to endogenous glucose production has been estimated to be 5-23% in the overnight fasted human (46, 83, 268), and 10% (178) or less (121)in the overnight fasted

dog leaving the remaining 90% to be produced by the liver. The liver is thus the principal organ which delivers glucose to both the non-insulin-sensitive (nervous, red blood cells, skin, smooth muscle) and the insulin sensitive (muscle and fat) tissues. Thus the factors which regulate hepatic glucose production are extremely important to glucoregulation.

Equally important are those mechanisms which regulate net hepatic glucose uptake in the post-prandial state. In the non-diabetic individual, the liver minimizes post-randial hyperglycemia by suppressing endogenous glucose production and by increasing glucose uptake(112). Following a modest sized oral glucose load, one third of the load is taken up by the muscle, one third is taken up by the liver, and the remaining third is taken up by non-insulin sensitive tissues such as the brain and red blood cells (215). However, if the glucose levels are elevated to an even greater extent by larger loads, the excess glucose will be taken up by the muscle and liver. In individuals with Type 2 diabetes, excessive post-prandial hyperglycemia may be due to reduced splanchnic glucose uptake, excessive (inadequately suppressed) endogenous glucose production, and reduced peripheral uptake due to defective oxidation or storage of glucose (112). Thus the liver is a critical organ in the regulation of postprandial hyperglycemia.

In the following introduction, important regulators of both glucose production and glucose uptake will be discussed. A review of basic glucose metabolism will precede two major sections titled “Hepatic Glucose Production” and “Hepatic Glucose Uptake.” In the “Hepatic Glucose Production” section, the effect of insulin on hepatic glucose production will be reviewed in light of

specific aim I, which will examine the ability of first phase insulin release to modify net hepatic glucose uptake in the post-prandial state. In addition, the effect of glucagon and catecholamines on hepatic glucose production will be briefly discussed within this section. In the "Hepatic Glucose Uptake" section, the three main factors which impact net hepatic glucose uptake (the insulin concentration at the liver, the glucose load to the liver, and the portal glucose signal) will be reviewed. The focus of this section will be on the portal glucose signal and the potential mechanisms of portal signaling. These include both the afferent and efferent parasympathetic and sympathetic nerves, and the roles of these nerves will be further clarified in specific aims II and III. Lastly, the role of other mediators, such as serotonin and nitric oxide, on net hepatic glucose uptake will be reviewed with the focus on nitric oxide since specific aim IV will further clarify the role of nitric oxide on hepatic glucose production.

## Basic Glucose Metabolism

Glucose possesses the ability to regulate its own plasma level by directly impacting both its own production and uptake by the liver (188). There has been both in-vitro and in-vivo work that has demonstrated a relationship between net hepatic glucose balance and the overall glucose concentration, independent of any changes in the plasma insulin concentration (68, 257). In the presence of basal insulin and glucagon concentrations, hyperglycemia alone can bring about a suppression of net hepatic glucose output. In the presence of modestly elevated hyperglycemia levels, minimal net hepatic glucose uptake (NHGU) can occur in

the presence of basal insulin. It is only at very high (supraphysiological) plasma glucose levels that NHGU can occur at a substantial rate if insulin is not elevated.

Glucose itself can suppress gluconeogenesis under conditions such as prolonged fasting, which results in very low and/or completely depleted glycogen stores (188). When glycogen stores are adequate, glucose autoregulates itself by suppressing glycogenolysis and by stimulating intrahepatic glucose cycling (glucose → glucose-6-phosphate (G-6-P) → glucose) (222, 236, 260).

Therefore, the size of the G-6-P pool is an important factor in regulating basic glucose metabolism as G-6-P acts as an activator of glycogen synthase (42, 93) and an inactivator of glycogen phosphorylase (8, 9). Glucose must be able to enter the cell in order to be metabolized. GLUT-2 is a membrane bound glucose transporter found in the liver and pancreatic beta cells (22, 286). It is a low affinity transporter that is not easily saturated at even high plasma glucose levels. Thus when the plasma glucose level increases, there is a rise in cellular glucose due to an increase in glucose transport by GLUT 2; this allows the beta cells, as well as the hepatocytes, to act more like glucose sensors (174) and respond accordingly. For instance, as the plasma glucose level rises, the hepatocyte decreases its hepatic glucose production. Although glucose transport into the liver is a critical step in determining the G-6-P pool, it is not a rate limiting step.

There are several other important regulators that determine the size of the G-6-P pool. Glucokinase (GK), an enzyme that phosphorylates glucose when it enters the liver, and G-6-Pase, the opposing enzyme which catalyses the last rate-determining step prior to the release of glucose by the liver are two other

important regulators. If GK is suppressed or G-6-Pase is activated, there will be a decrease in G-6-P. In the basal state, GK is bound to the glucokinase regulatory protein (GKRP) in the hepatocyte nucleus. Hyperglycemia triggers the release of glucokinase from GKRP allowing it to translocate to the cytosol where it can then phosphorylate the entering glucose (253, 277). It has also been shown that fructose or sorbitol, which are precursors of fructose-1-phosphate (F-1-P), increase F-1-P content in isolated hepatocytes and stimulate the release of GK from GKRP(6). In our laboratory, Shiota et al. (254)showed that an intraportal infusion of even a small amount of fructose (no more than 5-10% of the mass of the glucose infused) in the presence of a hyperinsulinemic, hyperglycemic clamp enhanced NHGU and hepatic glycogen storage in conscious dogs.

Having entered the cell and been phosphorylated, the glucose can then be oxidized via the Embden-Myerhof glycolytic pathway. Glycolysis will convert 1 mole of glucose into 2 moles of ATP, 2 moles of NADH, and 2 moles of pyruvate. This ATP can then be utilized for cellular energy while the NADH and pyruvate can act as substrates for other energy-producing reactions; for example, pyruvate can be converted to acetyl CoA and then enter the tricarboxylic acid cycle (TCA) where an additional 36 moles of ATP will be generated for every mole of glucose consumed.

Pyruvate, and other carbon-3 compounds such as lactate and alanine, can serve as substrates for de novo synthesis of glucose via the gluconeogenic pathway. Gluconeogenesis first involves the conversion of pyruvate to oxaloacetate and then to phosphoenolpyruvate (PEP). PEP is then converted by

phosphoenolpyruvate carboxykinase (PEPCK) to fructose-1-6 bisphosphate. It is then that another key enzyme of gluconeogenesis, fructose-1,6-bisphosphatase, converts fructose-1-6 bisphosphate to fructose-6-phosphate which is in equilibrium with G-6-P. 6-phosphofructo-1-kinase (6PF-1-K) is allosterically activated by fructose-2,6-bisphosphate (F-2,6-$P_2$) in the glycolytic pathway. F-2,6-$P_2$ stimulates glycolysis by having a positive effect on PFK-1, the enzyme that converts fructose F-6-P into F-1,6-P. It in turn, has a negative effect on F-1,6-Pase which converts F-1,6-P into F-6-P. Increasing F-2,6-$P_2$ will also suppress HGP by increasing hepatic glycolysis possibly through the stimulation of hepatic GK gene expression in an insulin dependent manner (287).The final reaction is the conversion of G-6-P to free glucose by glucose-6-phosphatase. The liver and kidney are the only two sites which are known to express G-6-Pase (235) but it has been suggested that the gut could also be gluconeogenic (184).

Glycogen synthesis and breakdown are both important regulators of net hepatic glucose balance. Glycogen phosphorylase catalyzes the first step in glycogen breakdown by cleaving a single glucose-1-phosphate. This glucose-1-phosphate is in equilibrium with G-6-P, thus glycogen phosphorylase increases G-6-P following cleavage of the glycogen molecule. This G-6-P can then be dephosphorylated by G-6-Pase and released as free glucose. Glucagon can stimulate glycogen breakdown by increasing intracellular levels of cAMP. These in turn can activate protein kinase A which can phosphorylate the phosphorylase kinase rendering it active. This, in turn, phosphorylates glycogen phosphorylase.

Insulin, on the other hand, stimulates protein phosphatase 1 which dephosphorylates glycogen phosphorylase thus inhibiting glycogen breakdown.

It has also been suggested that G-6-P promotes the stimulation of glycogen synthesis by mechanisms other than inactivation of phosphorylase, such as activation and translocation of glycogen synthase (122). Insulin will also cause the translocation of glucokinase thus allowing for a consequent increase in G-6-P. This glucokinase translocation may cause both the inactivation of phosphorylase and the activation of synthase (122). It is the synergy between these two convergent pathways that may explain why insulin has a large effect on glycogenic flux, despite its only modest effects on individual enzyme activities (122).

## Hepatic Glucose Production

### Effect of Insulin on Hepatic Glucose Production

Insulin sensitively controls hepatic glucose production. In very early studies, a pancreatic clamp was used and insulin was either left unchanged or increased fourfold by increasing the portal insulin infusion rate (144, 263). Glucagon was replaced at basal amounts in both protocols. In the dogs that received basal insulin, net hepatic glucose output (NHGO) fell only slightly by 10% as a result of a slight decrease in glycogenolysis that was not counteracted by an increase in gluconeogenesis. In those dogs that received the intraportal fourfold insulin infusion, NHGO decreased rapidly from 2.7 mg/kg/min to 1.6 mg/kg/min

and then declined more slowly. This fall was mainly due to a decrease in glycogenolysis. There was a slight decrease in gluconeogenesis compared to that seen in the control group but it was not significant. Thus, a fourfold increase in insulin has a marked inhibitory effect on hepatic glucose production.

Acute insulin deficiency has the opposite effect on glucose production (48, 51, 114, 260). In two separate groups, a pancreatic clamp was used to assess the importance of basal insulin in restraining hepatic glucose production in the overnight fasted conscious dog (51). In the control group, insulin was replaced basally throughout the experiment. In the test group, a basal insulin infusion was also given intraportally but was terminated at the onset of the experimental period, creating complete insulin deficiency. In both groups, glucagon was infused at basal rates. In the insulin deficient group, NHGO rapidly increased from 2.5 mg/kg/min to 8.7 mg/kg/min causing a significant rise in plasma glucose levels from 99 mg/dl to 288 mg/dl. Due to this increase in plasma glucose in the test group, glucose was infused peripherally in the control group to match the hepatic glucose loads between the two groups. This increase in arterial plasma glucose in the presence of basal insulin in the control group caused a decrease in NHGO. Thus the effect of insulin deficiency was the difference between the net hepatic glucose balances in the two groups and proved to be both quick and sustained. This response was primarily the result of an increase in glycogenolysis since gluconeogenesis changed only slightly.

A dose response curve can be constructed to relate the hepatic sinusoidal level to net hepatic glucose output in the fasted conscious dog. The basal hepatic

sinusoidal level (18 μU/ml) lies slightly below the half maximally effective insulin concentration; this indicates that even basal insulin levels produce greater than 50% inhibition of net hepatic glucose production. In addition, the slope of the curve is very steep, indicating that even very slight changes in plasma insulin levels will have significant effects on glucose production by the liver. This dose response curve also shows that a fourfold increase in insulin secretion will almost completely inhibit net hepatic glucose production.

The mechanism by which insulin inhibits hepatic glucose production has been a topic of much interest and investigation. It is well known that the hepatic portal insulin concentration is greater than that seen in the systemic circulation in part due to insulin's release into the portal vein. It is also well known that the liver degrades ~50% of the insulin that reaches it. It was assumed for many years that insulin's ability to inhibit net hepatic glucose production was the result of a direct interaction of the hormone with its receptor on the hepatocyte. In 1987, Prager et al. (227) suggested that peripheral insulin infusion could suppress hepatic glucose production without any change in the portal insulin concentration, thus implicating an indirect mechanism of inhibition. This concept has since been supported by many investigators (2, 25, 111, 167, 258, 259). Sindelar et al.(258) showed that a selective rise in arterial insulin of almost four fold (when portal insulin was kept at basal levels) eventually led to the inhibition of hepatic glucose production by 50%. This was the same extent of inhibition that a selective rise in portal insulin (when arterial plasma insulin levels were kept basal) caused, yet the rise in portal insulin caused a significantly more rapid decrease in HGP. Sindelar

et al. (258) went on to show that the decrease in hepatic glucose production was 30% due to a drift down in the baseline with the remaining 70% due to the selective increase in insulin concentration.

The indirect effect of the selective rise in arterial insulin on the liver appeared to alter the gluconeogenic rate and the fate of glucose within the hepatocyte rather than affecting the rate of glycogenolysis. The rise in the arterial insulin caused a reduction in the availability of gluconeogenic precursors to the liver and therefore, net hepatic gluconeogenic precursor uptake fell. Lactate production, on the other hand, increased in response to the selective rise in arterial insulin. The source of this lactate was G-6-P which underwent glycolysis within the liver rather than being exported as glucose. There was a slight decrease in the glycogenolytic rate due to a small increase in liver sinusoidal insulin which resulted from the increase in arterial plasma insulin concentration.

The increase in net hepatic lactate production correlated in time with a decline in plasma nonesterifed fatty acids (NEFA) and a fall in net hepatic NEFA uptake. Thus, the fall in lipolysis may have explained the increase in net hepatic lactate output. In order to test this hypothesis, Sindelar et al. (259) again brought about a selective rise in the arterial plasma insulin concentration. In one group, they prevented the fall in the plasma NEFA and glycerol concentrations by infusing a lipid emulsion and heparin, while in the other group, neither lipid emulsion nor heparin was infused. In the group that did not receive the NEFA infusion, the results were similar to those seen in the previous studies. In the group that did receive the lipid emulsion and heparin infusion to prevent the fall

in the plasma NEFA during the selective increase in arterial plasma insulin, the inhibition of net hepatic glucose output was reduced. The decrease in lipolysis could account for 50% of the fall in net hepatic glucose output caused by the selective rise in arterial insulin. Net hepatic lactate output did not increase when the NEFA levels were clamped. Thus the fall in net hepatic NEFA uptake triggered the re-direction of G-6-P to lactate rather than glucose. Clearly, the ability of the increase in arterial insulin concentration to suppress HGP was in part indirectly mediated by suppression of lipolysis (259).

Other investigators have also focused on this indirect mechanism of inhibition. Bergman and colleagues proposed the single gateway hypothesis by which the insulin that survives first pass degradation by the liver enters the systemic circulation where lipolysis is suppressed in the adipocyte. Their hypothesis posits that this reduction in NEFA then explains the fall in hepatic glucose production (2, 23).

Insulin can also indirectly affect net hepatic glucose production by acting on the α cell to inhibit glucagon secretion. It appears that insulin has a dose dependent effect on the suppression of glucagon which begins with a rise in arterial plasma insulin as small as 10 μU/ml (198). This is rather significant since glucagon has such a potent effect on glucose production by the liver. It has been shown in both dogs and humans that the insulin-induced suppression of α-cell function can explain a portion of the effect of a rise in systemic insulin on glucose production (110, 166).

Although these indirect mechanisms do affect net hepatic glucose production, our lab has demonstrated that insulin within the liver sinusoids also potently regulates hepatic glucose production via a direct action. First, the effect of a selective rise in the liver sinusoidal insulin level was examined. Studies were carried out in the overnight fasted conscious dog in which portal vein (and thus liver sinusoidal) insulin concentrations were increased in the absence of any change in the arterial plasma level (258). Following a 40 min control period during which the pancreatic clamp was employed, the peripheral insulin infusion was terminated and the portal insulin infusion rate was increased to four fold basal. This resulted in an increase in the portal vein insulin from 18 to 32 μU/ml without any change in the arterial insulin concentration. Euglycemia was maintained by peripheral glucose infusion and glucagon was kept basal, as mentioned previously. NHGO dropped ~40% by 30 minutes and eventually decreased by 70%. This demonstrated the liver's ability to respond quickly and sensitively to a selective rise in the liver sinusoidal insulin concentration. In addition, since there was no decrease in hepatic gluconeogenesis and no increase in net hepatic lactate output, the change in glucose production reflected a decrease in glycogenolysis.

The effects of a selective deficiency in the liver sinusoidal insulin concentration were then examined to verify the importance of the direct action of insulin on the liver (260). Again, the pancreatic clamp in the overnight fasted conscious dog was used. After establishing the clamp, the portal insulin infusion was terminated and a peripheral insulin infusion was started at half the portal

infusion rate. The portal insulin concentration decreased from 25 μU/ml to 5 μU/ml but the arterial insulin concentration remained unchanged. This selective decrease in hepatic sinusoidal insulin (Δ 15 μU/ml) was associated with a rapid (15min) increase in NHGO from ~1.5 mg/kg/min to ~5.5 mg/kg/min. NHGO declined thereafter. Gluconeogenesis by the liver did not increase during sinusoidal insulin deficiency, and net hepatic lactate output did not decrease. The rise in NHGO caused by the decline in liver sinusoidal insulin was a reflection of an increase in glycogenolysis. Thus, insulin acutely inhibits liver glucose output by directly effecting glycogenolysis and indirectly effecting glycolysis and/or gluconeogenesis. Thus in the case of endogenous insulin secretion, the direct action of the hormone is responsible for 60-85% of the hormone's inhibitory effect on the liver with the remaining percentage being due to insulin's ability to influence the liver indirectly by acting on muscle, fat, and the alpha cell (50).

Insulin also has an indirect effect on the liver through its action on the brain. It has been shown that hypothalamic insulin signaling is required for the inhibition of glucose production (211) in the rat. It has been previously shown that prolonged or chronic impairment of CNS insulin signaling leads to hyperphagia, increased plasma insulin levels, and decreased insulin sensitivity (40, 210). In a recent paper by Obici et al. (211), the infusion of insulin into the third cerebral ventricle suppressed glucose production independent of circulating levels of insulin and other glucoregulatory hormones. They also demonstrated that central antagonism of insulin signaling impairs the ability of circulating insulin to inhibit

glucose production. Thus they concluded that the hypothalamus may also be a site of action of insulin on glucose production.

The concept of insulin having both a direct and indirect effect on hepatic glucose flux is critical since the normal insulin concentration gradient between the hepatic portal and systemic circulation (with the hepatic portal circulation being higher) is reversed in diabetic patients who are treated with subcutaneously injected insulin, creating higher systemic (arterial) than portal vein insulin concentrations.

Subcutaneously administered insulin is initially absorbed into the peripheral circulation of individuals with diabetes, resulting in fat and muscle being exposed to higher insulin levels than the liver. This peripheral hyperinsulinemia predisposes the individual to hypoglycemia and is thought to be linked to weight gain and other metabolic abnormalities which in turn can lead to microvascular and macrovascular disease (148). It has been shown that agents capable of rapidly increasing the insulin concentration in response to a glucose challenge are good regulators of hepatic glucose metabolism and are more effective controllers of post-prandial glucose concentrations than peripherally delivered insulin (38).For example, studies with Lispro, an insulin analog that provides faster systemic absorption from subcutaneous depots due to its greater water solubility (219), have consistently demonstrated that a rapid onset of action can lead to improvement in post-prandial blood glucose control (124, 125, 132). One problem with such rapid-acting insulin analogs is that the duration of action

may be too short to provide optimal post-prandial control, as indicated by rising glucose levels in the postabsorptive state (59, 76).

More recently, the pulmonary delivery of insulin (Exubera, Pfizer) has been studied as an alternative method of insulin administration. It has been shown that inhaled insulin also has a rapid onset of action similar to that seen with the rapid acting insulin analogs and is considerably faster than regular insulin (230). The metabolic activity of such inhaled insulins declines more slowly than that of Lispro (230) but faster than that of Humulin. The intermediate absorption pattern may result from the pulmonary absorption of insulin being dependent on the size and dissociation rate of the particles and their aggregates (142, 143).

If insulin could be given orally, a normal portal vein/arterial insulin distribution could be restored. With the right pharmacokinetics, an oral insulin would eliminate the disproportionately high peripheral insulin levels while still delivering adequate amounts of insulin to the liver, thereby potentially being useful as a therapeutic agent.

The release of insulin in response to a glucose challenge is biphasic in nature with an early phase and a late phase. The early phase consists of two parts: a cephalic phase and a first phase. The cephalic phase is rapid (within 2 minutes after a meal) yet very small, only increasing arterial plasma insulin levels by ~5 μU/ml. The more pronounced first phase occurs 5-10 minutes postprandially and is dependent on the amount of glucose present (285). The liver responds quickly, such that the first phase insulin pulse inhibits glucose production and/or causes an increase in hepatic glucose uptake in the presence of an oral glucose load (52).

The way in which the liver responds to the first phase insulin release during a glucose challenge has been shown to affect subsequent plasma glucose responses and thus is critical in maintaining normal glycemia (75, 108). The late (second) phase of insulin release has a lower absolute peak but also plays a significant role in glucoregulation over the 2-3 hours following a meal challenge.

People with Type 2 diabetes typically demonstrate a diminished or brief first phase insulin release. They often have an enhanced second phase insulin release so as to compensate for this loss (75). Loss of first phase insulin release has many metabolic implications. It is clear that it causes a significant impairment in the suppression of hepatic glucose production post-prandially. In a study carried out by Luzi and De Fronzo (172) abolition of the first phase insulin secretion by somatostatin caused the liver to continually produce glucose despite the subjects being both hyperglycemic and hyperinsulinemic. When first phase insulin was replaced, there was a complete normalization of the suppression of hepatic glucose production. Bruce et al (38)corrected a deficiency in early prandial insulin secretion using three different approaches in individuals with type 2 diabetes. One group received insulin (1.8 U total) over the first thirty minutes to simulate “first phase” insulin release following a glucose challenge. A second group received the same amount of exogenous insulin beginning thirty minutes post glucose challenge and continuing for 30 minutes, simulating “second phase” insulin release. A third group received the same amount of insulin again over the entire duration of the study, simulating both a first phase and second phase release.

Postprandial glucose tolerance significantly improved in all individuals, even those who received only first phase insulin.

Previous experiments in our laboratory have looked at first and second phase insulin secretion in countering the action of glucagon on glucose turnover (262). Theses studies revealed that simulated first phase insulin was more effective than second phase in countering the glucagon effect.

In this context, specific aim 1 had two main intentions. The first was to examine the ability of first phase insulin release to modify net hepatic glucose uptake under conditions mimicking oral glucose loading in an insulinopenic state. The second was to explore whether a pulse of an insulin analog (hexyl-insulin monoconjugate 2, HIM2, Nobex Corp.), created to facilitate the absorption of orally delivered insulin, also has the ability to modify postprandial hepatic glucose metabolism.

## Glucagon Regulation of Hepatic Glucose Production

Hepatic glucose production is also affected by the plasma glucagon concentration. An increase in glucagon has been shown to rapidly increase net hepatic glucose output by stimulating glycogen breakdown with the effect on gluconeogenesis being rather modest (49, 56, 81, 280). A dose-response relationship between the liver sinusoidal glucagon levels and NHGO in the dog has been determined (47) and demonstrates several key features of the response to glucagon. First, basal plasma glucagon is found on the steepest part of the curve, indicating that small changes in hepatic sinusoidal glucagon levels will have

significant effects on NHGO. Secondly, the curve plateaus at a concentration eightfold basal, which will rarely be seen in physiologic conditions. Thirdly, the half-maximally effective liver sinusoidal glucagon level is 110 ng/L, which is about 2.5 fold greater than that seen after an overnight fast in the canine. Thus it is clear that glucagon is an important regulator of NHGO and that its interaction with insulin allows for precise control of hepatic glucose output.

## Hepatic Glucose Uptake

Glucose ingestion results in significant hyperglycemia, as well as marked hyperinsulinemia and modest hypoglucagonemia. In a study by Abumrad et al.(1), conscious dogs received intragastric (mimicking oral) glucose at 1.63 g/kg. The arterial blood glucose concentration almost doubled, the plasma insulin level increased approximately 5 fold, and the plasma glucagon level fell minimally. The liver switched from a state of net hepatic glucose production during the basal period (~2 mg/kg/min) to a state of net hepatic glucose uptake during the glucose infusion period (~5 mg/kg/min).

There are three main factors that have been shown to impact net hepatic glucose uptake in response to an oral glucose tolerance test: the insulin concentration at the liver, the glucose load to the liver, and the portal glucose signal. These three factors work in concert with one another to bring about the resulting net hepatic glucose uptake.

## Effect of Hyperglycemia on Hepatic Glucose Uptake

Hyperglycemia brought about by a peripheral vein glucose infusion, in the presence of fixed basal levels of insulin and glucagon, will cause very little or no net glucose uptake by the liver (213). Studies in humans have demonstrated that an increase in the plasma glucose level to 125 mg/dl with basal insulin levels causes a decrease in net splanchnic glucose output from ~2.5 to 0.6 mg/kg/min but glucose uptake will not occur (69, 70). Doubling the hepatic glucose level in the dog, in the presence of basal insulin and glucagon, has similar results to those seen in the human, with a reduction in net hepatic glucose output from 2.2 to 0.9 mg/kg/min but no evidence of net hepatic glucose uptake (53, 54). Thus hyperglycemia is able to reduce hepatic glucose production but, in the absence of an increase in insulin or oral/portal glucose delivery, it is unable to switch the liver to an uptake mode.

## Effect of Insulin on Hepatic Glucose Uptake

While the pancreas releases insulin in a pulsatile manner, many studies assessing the liver's response to insulin have used constant infusion rates which create square waves. Our lab recently undertook a study to look at whether the secretion pattern of insulin in the presence of hyperglycemia influenced net hepatic glucose uptake and whether it entrained NHGU (117). This study demonstrated that NHGU was not augmented further by pulsatile insulin delivery compared to that of square wave delivery and that there was no entrainment in hepatic glucose metabolism. The authors concluded that the loss of insulin

pulsatility likely has very little or no impact on the ability of insulin to regulate liver glucose uptake. Thus the manner by which insulin is administered, whether it be in a pulsatile fashion or a square wave, should not be a major consideration in the design of metabolic studies.

Hyperinsulinemia, under euglycemic conditions, is not very effective in promoting glucose uptake into the liver. In humans, in the presence of basal glucose levels and insulin levels greater than 100 μU/ml (8-10 times basal), net splanchnic glucose uptake was only 0.7 mg/kg/min (69). In euglycemic dogs, NHGU was 0.6 mg/kg/min in the presence of physiologic hyperinsulinemia (120 μU/ml) (53) and only pharmacological concentrations of insulin (2044 μU/ml) achieved rates of NHGU as high as 3.2 mg/kg/min (179). Thus insulin alone is able to cause the liver to take up some glucose yet it is only at high concentrations that NHGU occurs at a significant rate.

NHGU increases in the presence of the combination of hyperinsulinemia and hyperglycemia brought about by a peripheral vein glucose infusion. In humans, the combination of hyperglycemia (plasma levels of 175-225 mg/dl) and hyperinsulinemia (arterial levels of 40-55 μU/ml) resulted in rates of net splanchnic glucose uptake of 1.0 to 1.6 mg/kg/min (69-71, 239). In the dog, arterial plasma glucose levels of 160-290 mg/dl and insulin levels of 35-384 μU/ml resulted in rates of NHGU between 1.0 and 2.9 mg/kg/min (4, 19, 53, 138, 197, 199). Despite the increase in hepatic response to combined hyperglycemia and hyperinsulinemia, it is clear that these two variables cannot account for the peak rates of NHGU that are evident following glucose ingestion (up to 7.5

mg/kg/min; (1, 19, 24, 71, 92, 138, 185). Thus, it appears that both humans and dogs require some signal, in addition to an increased plasma insulin level and glucose load to the liver, to ensure adequate hepatic glucose uptake after feeding.

## The "Portal Glucose Signal" Story

In agreement with the Abumrad et al.(1) study mentioned before, a human study by DeFronzo et al. (71) demonstrated the unique effects of oral glucose. In a control group, glucose was administered through an arm vein resulting in hyperglycemia and hyperinsulinemia. There was very little net splanchnic glucose uptake in response to this treatment (1.3 mg/kg/min). If glucose was then given orally and the intravenous glucose infusion rate was reduced in order to match the arterial plasma glucose levels seen during peripheral glucose infusion alone, splanchnic uptake increased almost 5 fold (5.9 mg/kg/min). The levels of insulin following the oral glucose ingestion were higher than those seen during the peripheral glucose administration and although the arterial plasma glucose levels were matched, there was an increase in glucose load to the liver in the group which received oral glucose. These increases in the arterial insulin concentration and the glucose load, however, were not large enough to account for the total increase in splanchnic uptake.

This discrepancy seen between NHGU during peripheral glucose delivery and portal glucose delivery was initially attributed to an incretin effect associated with the oral glucose delivery (69). Several laboratories have shown since, though, that intraportal administration of glucose can mimic the results seen with

oral glucose delivery (24, 138). This enhancement of NHGU by portal glucose delivery versus peripheral glucose delivery has been attributed to a "portal glucose signal" (3).

Adkins et al. sought to determine if portal glucose delivery, which would generate a negative A-P (arterial-portal) glucose gradient, in the presence of hyperinsulinemia, would alter net hepatic glucose uptake (4). A pancreatic clamp was used, with somatostatin infused, and glucagon was replaced at basal levels while the insulin infusion rate was adjusted until plasma glucose was stabilized at a euglycemic level. At the onset of the test period, the insulin infusion rate was quadrupled. In the first group, glucose was infused peripherally to double the arterial plasma glucose during the first test period. During the second test period, glucose was infused intraportally to match that which was seen with the peripheral glucose infusion. The order of the test periods was reversed in the second group. The rate at which glucose reached the liver in response to the peripheral and portal glucose infusions was not significantly different between the two groups. NHGU was 1.4±0.7 mg/kg/min in response to peripheral glucose infusion and hyperinsulinemia but 3.5±0.8 mg/kg/min in response to portal glucose infusion in the presence of hyperinsulinemia. A similar effect of portal glucose delivery was seen in another study by Adkins et al (3) in which insulin was kept basal. In the presence of basal insulin, peripherally induced hyperglycemia did not trigger NHGU. Intraportal glucose administration in the presence of basal insulin stimulated NHGU at a rate of 1.4±0.3 mg/kg/min, almost the same change that was seen with peripheral hyperglycemia and

hyperinsulinemia. This suggests that the portal route of glucose delivery and hyperinsulinemia have an additive effect in enhancing NHGU.

## The Relationship between the Portal Glucose Signal, Hepatic Glucose Load, and Insulin

In 1991, Myers et al examined the relationship between the portal signal, the hepatic insulin level, and the hepatic glucose load more extensively (197, 199). In the first study (199), a pancreatic clamp was used and the arterial insulin level was increased by intraportal insulin infusion at two-, four-, or eight-fold basal during three 90 minute periods in two separate groups. Glucagon was kept basal throughout in both groups. In the first group, glucose was infused through a peripheral vein and was adjusted to match the arterial blood glucose concentrations across the three periods of differing insulin concentrations. In the second group, glucose was infused intraportally (~4-5 mg/kg/min) throughout the study and a peripheral glucose infusion was used to match the hepatic glucose loads in the second group to those in the first group. In the first group, NHGU was 0.6±0.3, 1.5±0.4 and 3.0±0.8 mg/kg/min, respectively, in the 3 insulin infusion periods. When a portion of the glucose was infused intraportally, NHGU was two to three fold greater than the respective peripheral glucose periods (2.0±0.5, 3.7±0.7, and 5.5±0.9 mg/kg/min) than when it was not. Thus at an arterial insulin level which was fourfold basal in the presence of hyperglycemia, insulin could account for roughly 60% of the increase in net hepatic glucose uptake with the remaining 40% attributable to the portal glucose signal.

Interestingly, in the complete absence of insulin, brought about either by pancreatectomy or by suppression of insulin secretion by somatostatin, the liver did not respond to portal glucose delivery, indicating that the augmented hepatic response to intraportal glucose delivery requires the permissive presence of insulin (216).

In a following study (197), glucagon was again kept basal while insulin was maintained at fourfold basal with the use of the pancreatic clamp. In the first group, a peripheral glucose infusion was used to increase the load of the glucose reaching the liver by ~65, 140, and 220% in three 90 minute periods. In the second group an intraportal glucose infusion was begun at the start of the first period (~10 mg/kg/min) and a peripheral glucose infusion was used to match the hepatic glucose load that was seen in the corresponding periods of group one. In the absence of the portal glucose signal, NHGU was 1.2±0.4, 2.8±0.8, and 5.1±1.2 mg/kg/min in the three periods, respectively. In the second group, which received a portal glucose infusion, NHGU was 3.8±0.4, 4.8±0.6, and 9.6±1.4 mg/kg/min during the same time periods. NHGU was positively correlated with the hepatic glucose load regardless of the route of administration. However, at any given hepatic glucose load, NHGU was higher in the presence of intraportal glucose delivery than when glucose was infused peripherally.

Pagliassotti et al. (217) then went on to examine the relationship between NHGU and the magnitude of the negative A-P glucose gradient in the presence of a four-fold increase in insulin , basal glucagon, and a doubling of the hepatic glucose load. The magnitude of the A-P gradient was maintained by keeping the

total amount of glucose infused constant but by varying the amount delivered peripherally or intraportally. When the A-P gradient was close to 0, NHGU was only 1.6 mg/kg/min. When the A-P gradient was -70 mg/dl, NHGU increased to 7.6 mg/kg/min. The relationship between net NHGU and the A-P gradient is not linear. NHGU does not linearly increase with an increase in the magnitude of the A-P gradient past and A-P gradient of -20 mg/dl. These data indicate that the liver not only responds to the presence of a negative A-P gradient but the magnitude of the response is proportional to the magnitude of the gradient itself. In addition, in order to further augment NHGU, insulin and glucose must also be increased.

## NonHepatic Effects of the Portal Glucose Signal

Portal glucose delivery has a profound effect on the liver, but the question of how the portal glucose signal would effect nonhepatic glucose uptake was unanswered. In a study by Galassetti et al. (103), insulin was elevated three-fold while glucagon was replaced in basal amounts during somatostatin infusion. Arterial plasma glucose was clamped using a peripheral glucose infusion in one group. In the second group, a small amount of glucose was infused intraportally and the peripheral glucose infusion was adjusted to maintain similar glycemic levels between the two groups. The portal signal decreased glucose uptake by nonhepatic tissues by 40%. Simultaneous measurement of net glucose balance across the hindlimb (which is primarily muscle) confirmed that muscle was the site of this inhibition. This study also indicated that the portal signal inhibited nonhepatic glucose uptake in an amount equivalent to the enhancement of NHGU.

This implies that that the portal signal does not increase whole body glucose clearance, but rather directs glucose to the liver and away from the muscle. This may be a way to ensure a balanced distribution of glucose following oral glucose consumption.

## The Portal Glucose Signal in Humans

The question arises as to the role of the portal glucose signal in the human. Much less is known regarding this phenomenon due to the inability to catheterize the portal vein in the human. This technical limitation leads the investigators to measure splanchnic uptake or whole body glucose uptake rather than net hepatic glucose uptake.

In the study of De Fronzo et al. (71) mentioned previously, a hepatic vein catheterization technique was used to determine whether the route of glucose delivery affects splanchnic glucose balance. In this study, they reported that net splanchnic balance was increased in the presence of ingested glucose despite the fact that the plasma glucose was maintained at 225 mg/dl. As previously mentioned, there was an increase in insulin concentrations following glucose ingestion as well as an increase in hepatic glucose load relative to the intravenous group. As noted earlier, the increases in insulin concentration and hepatic glucose load were not large enough to account for the entire increase in net hepatic glucose uptake seen.

In another study by Radziuk et al. (228, 229) in which a dual-isotope technique was used, no differences in hepatic glycogen storage were found

between intravenous and intraduodenal delivery of glucose. This study, however, was performed under non-steady state conditions and no attempt was made to control glycemic or insulinemic excursions.

To further clarify the role of the route of glucose delivery on splanchnic glucose uptake in the human, Fery et al. (94) explored the effects of intraduodenal and intravenous glucose infusions. Using tracer methods and indirect calorimetry, they observed that, at a constant infusion rate of 6 mg/kg/min intraduodenally or intravenously, the distribution of glucose between glycolysis, oxidation, and storage in the whole body was not affected by the route of administration. The conclusion was limited in that there were higher insulin levels and lower glucose levels during intraduodenal glucose infusion. It is also not surprising that whole body glucose metabolism was not affected since it has been previously demonstrated that the increase in net hepatic glucose uptake seen in the presence of portal glucose delivery is concomitant with a proportional decrease in nonhepatic glucose uptake thus having no effect on whole body glucose metabolism but rather a redistribution of glucose uptake (103).

Vella et al. (279)attempted to overcome the limitations of the Fery et al (94) study by using a hyperglycemic hyperinsulinemic clamp to examine the effects of the route of glucose administration. In these studies, the route of glucose administration did not appear to affect whole body glucose uptake or hepatic glycogen synthesis, as estimated by the hepatic UDP-glucose turnover. Splanchnic glucose extraction was slightly higher during intraduodenal glucose administration (16.4 vs 12.8%) than when compared to the saline. Intestinal

glucose extraction in dogs and pigs ranges from 4-10% (1, 265). Therefore, hepatic glucose extraction in this experiment could have ranged from 12.4 vs 8.8 to 6.4 vs 2.8% respectively, on the intraduodenal glucose and saline study days. Thus, the latter calculation would suggest as much as a two fold increase in hepatic glucose uptake during intraduodenal glucose administration. One limitation to these studies was that the tracer glucose was infused alone rather than with carrier glucose in the intravenous group due to the fear that a small amount of the carrier glucose could elicit an enteric signal. It is possible that the glucose tracer was metabolized in the gut in the absence of the carrier glucose, leading to an overestimation of splanchnic glucose extraction when saline was infused intraduodenally. In support of this, the ratio of splanchnic glucose extraction with intravenous infusion (intraduodenal saline) averaged 12.8%, which is significantly higher than the average of 4% previously reported by De Fronzo et al. (70). It is also possible that the infusion rate used in the study resulted in a portal signal in the human that was near a threshold value and therefore was not detected.

The most recent study comparing the effects of intraduodenal and intravenous glucose metabolism in the human was carried out by Fery et al. (95), again using a dual-isotope technique and indirect calorimetry. Unlike the initial studies done by Fery et al. (94), these studies were carried out in the presence of a hyperinsulinemic euglycemic clamp. Their data indicated that the sum of hepatic and peripheral glucose disposal is not significantly influenced by the route of glucose delivery. One drawback to this study is the use of the euglycemic clamp.

In order to mimic the postprandial situation, a hyperglycemic clamp would have been preferable but in order to control the insulin concentrations, somatostatin would have had to have been used and the authors feared that that it would inhibit splanchnic blood flow (281) and possibly delay intestinal glucose absorption (151). Another drawback is that the measurement of net first-pass splanchnic uptake is not necessarily representative of hepatic uptake. It underestimates true hepatic uptake since the enteral glucose initially taken up by the liver can be recycled after passage through G-6-P and/or glycogen. On the other hand, however, splanchnic uptake overestimates hepatic uptake by an amount equivalent to the extrahepatic splanchnic uptake, although this parameter has not been quantified in humans for technical reasons. In addition, the authors calculated first pass splanchnic uptake by subtracting the appearance of exogenous glucose from the total glucose infusion rate. This rendered a value of 0.38 mg/kg/min in the peripheral glucose group and only 0.30 mg/kg/min in the group that received intraduodenal glucose. Since the first pass splanchnic uptake is larger in the absence than in the presence of the intraduodenal infusion, the accuracy of the calculation for uptake may be questioned. Based on the large background value, it is difficult to draw any conclusion from the intraduodenal data

Thus the portal glucose signal probably exists in humans but its magnitude is still in question. Since the portal signal has been demonstrated so clearly in rats and dogs, it is possible that the drawbacks and limitations in the human studies obscure the ability to detect such a signal.

## The Effect of Other Nutrients on the Portal Glucose Signal

It is interesting to note that the concomitant intake of other nutrients modulates NHGU during portal glucose delivery. When gluconeogenic amino acids were infused simultaneously with portal glucose (190), a 50% reduction in NHGU in conscious dogs occurred, compared with portal glucose delivery alone. When these amino acids were infused peripherally rather than intraportally in the presence of portal glucose delivery, there was no effect on NHGU suggesting that there may exist competitive neural signaling or actual substrate competition when both glucose and amino acids are infused intraportally together. Peripheral infusion of a lipid emulsion in the presence of portal glucose delivery in euinsulinemic, hyperglycemic dogs also reduced NHGU by 50% (242). This effect was most likely the result of a small stimulation of hepatic glucose release and a small reduction in hepatic glucose uptake. In non-diabetic humans, when a lipid infusion was administered during oral glucose ingestion (in the presence and absence of a pancreatic clamp), net splanchnic glucose uptake was not reduced but splanchnic glucose output was stimulated and peripheral glucose uptake was reduced (233).

## Mechanism of Portal Signaling

It is clear that the route of glucose delivery, therefore, is an important regulator of net hepatic glucose uptake. It had been previously shown by Niijima and his coworkers (204) that there are glucose sensitive cells in the hepatic portal

vein. Thus, they (204) hypothesized that portal glucose delivery might generate a unique signal important in regulating net hepatic glucose uptake. The questions then arise as to how the portal glucose signal is sensed and how this signal exerts its effects.

It is known that the portal glucose level can be sensed by glucose-sensitive cells in the portal vein that signal the brain by use of vagal afferent fibers (204). A likely site for sensing of the arterial glucose level is within the hypothalamus (175). Numerous neurophysiological studies have shown the existence of neural pathways that link the brain and the liver (97, 226) and that an intact nerve supply to the liver appears to be important for the normal response to intraduodenal or intraportal glucose delivery (5, 218). These observations suggest that the brain may play an important role in the generation of the portal signal.

Hsieh et al (133) examined whether the comparison of the brain arterial glucose level with the portal glucose level initiated the stimulatory effect of portal glucose delivery on NHGU. A pancreatic clamp was used and insulin was increased 4 fold while glucagon was replaced intraportally at basal levels. Glucose was infused intraportally at ~4 mg/kg/min in both groups and a peripheral glucose infusion was begun so that the glucose load to the liver could be quickly doubled. In one group, glucose was infused into four head arteries to eliminate the glucose gradient between the arterial blood in the head and the portal vein. The peripheral glucose infusion was adjusted to fix the glucose load to the liver at twofold basal. In the second group, saline was infused into the head

instead of glucose and, again, the peripheral glucose infusion was adjusted to maintain a similar hepatic glucose load seen in the previous period.

The arterial-portal glucose gradients and the hepatic glucose loads were similar between the two groups. NHGU was ~4.3±0.7 and 4.5±0.8 mg/kg/min in the glucose and saline groups, respectively. Thus they concluded that the head arterial glucose level was not the reference standard used for comparison with the portal glucose level in the generation of the portal glucose signal.

Gardemann et al (106) and Stumpel and Jungermann (267) demonstrated that a negative glucose gradient between the hepatic artery and the portal vein could create a metabolic signal locally within the liver. Horikawa et al (131) reported that both portal vein and hepatic arterial glucose infusion stimulated NHGU in conscious dogs and that glucose sensors within the liver, rather than the portal vein, were involved in the augmentation of NHGU. Thus, attention turned towards the possibility of a reference site within the liver.

Hseih et al. (135) designed a study to clarify the role of the hepatic arterial glucose level in the generation of the portal glucose signal. Fourfold insulin and basal glucagon were replaced intraportally with the onset of a somatostatin infusion. In test period one, glucose was infused via a peripheral vein to double the hepatic glucose load in all groups. In test period two, saline was infused intraportally in the control group while two other groups received glucose intraportally at 4 mg/kg/min. In one of the portal glucose infusion groups, saline was simultaneously infused in the hepatic artery while the other portal glucose infusion group received a glucose infusion into the hepatic artery to eliminate the

negative A-P glucose gradient. The effects of the portal signal on net hepatic glucose uptake were markedly reduced by the elimination of the hepatic A-P glucose gradient in the portal glucose infusion group that also received the glucose infusion into the hepatic artery. The authors then went on to suggest that the portal signal may be sensed within the liver and that the transduction of its effect may be mediated by the autonomic nervous system outside the liver.

Since the studies performed by Hsieh et al (133, 135) supported the hepatic artery as the primary reference site for the portal signal, a follow up study was then carried out by Moore et al. (187) in which the hepatic artery was ligated. They hypothesized that ligation of the hepatic artery would result in an enhancement of NHGU during peripheral glucose delivery since the hepatic arterial glucose concentration would always be perceived as less than that in the portal vein. They also hypothesized that NHGU would not be enhanced by portal glucose delivery, in comparison with peripheral glucose delivery, after hepatic artery ligation since the maximal effects of the negative A-P gradient would be present at all times. The results demonstrated that NHGU in response to glucose delivered via a peripheral vein was identical in the dogs with the hepatic artery ligation and in dogs with intact arteries. More importantly, they demonstrated that portal delivery of glucose in dogs that had undergone the ligation still resulted in a significant increase in NHGU as well as a tendency for a suppression of nonhepatic glucose uptake. These data suggested that the portal signal could exhibit its effect on both the liver and nonhepatic tissues in dogs with hepatic artery ligation. Therefore, it appears that there may be one or more reference sites

for comparison of portal vein glucose concentrations, in addition to the hepatic artery. Thus the system appears to exhibit redundancy.

Although extensive research has been carried out relating to hepatic afferents and efferents in the euglycemic and hypoglycemic states (45, 127, 140, 141, 157, 176, 221) less is known about the hyperglycemic state and it remains unclear how the portal signal may be mediated by these nerves. Total hepatic denervation eliminates the ability of the liver to discriminate between portal and peripheral glucose delivery (5), reinforcing the notion that the response to the portal glucose signal is neurally mediated (204, 237). In dogs with denervated livers, rates of NHGU with peripheral hyperglycemia are in between the minimum rate of NHGU seen with peripheral glucose infusion and the maximum rate of NHGU seen with portal glucose infusion, in dogs with intact liver innervation. In the presence of total hepatic denervation, portal glucose delivery has no effect on NHGU suggesting the loss of a neural signaling pathway with total denervation.

These data are compatible with the idea that, in the basal state, the net effect of hepatic innervation is to inhibit NHGU. During intraportal glucose infusion, modification of neural input to the liver allows the full stimulation of NHGU. In the presence of hepatic denervation both the inhibition by the intact nerves (sympathetic input) and the stimulation by the intact nerves (parasympathetic input) are lost and the NHGU rests somewhere in between the two extremes.

It has also been shown that the portal glucose signal rapidly activates NHGU, independently of a rise in insulin. The speed of the response is consistent with neural mediation of the portal signal (214). Pagliassotti et al. (214) examined the time course of effects of the portal glucose signal on the liver. In this protocol, glucagon and insulin were kept at basal values using the pancreatic clamp in both the control and the experimental groups. In both groups, there was an initial hyperglycemic period to set NHGU to near zero followed by a period in which various signals occurred. This allowed the authors to look at the time course of induction of NHGU. In the control group, the pre-existing conditions were continued. In the first experimental group, glucose was infused intraportally while the peripheral glucose infusion was adjusted to match the glucose loads to the liver seen in the control group. In the absence of the portal signal, the liver took up a small amount of glucose (0.4 mg/kg/min). In the experimental group, when the portal signal was generated under the same experimental conditions, NHGU rapidly increased (within 15 minutes) to almost 3 mg/kg/min. In the second experimental group, the insulin level was increased four fold in the absence of a portal signal. The ability of fourfold basal insulin to increase NHGU to 3 mg/kg/min took almost 5 times the amount of time (90 minutes) seen with the portal glucose signal (214). In the third experimental group, in which insulin was increased fourfold in the presence of a portal glucose signal, NHGU reached a maximum of ~4.3 mg/kg/min after 60 minutes. Thus the portal signal activates the liver much more quickly than would otherwise be the case. It has also been shown that the effect of the portal signal on liver and muscle can also be turned off very

rapidly with the cessation of portal glucose infusion, indicating that uncoupling is very efficient (134, 136).

Since it has been suggested that the portal signal may be neurally mediated, the parasympathetic and sympathetic nerves that innervate the liver are of interest. Glucose-sensitive neurons in the portal vein (204) have a discharge rate that is inversely correlated with portal vein glucose concentration. The change in afferent firing is accompanied by an increase in efferent firing in the pancreatic branch of the vagus nerve and decreases in the efferent firing of the hepatic branch of the splanchnic nerve and the adrenal nerve (200).

In the 1960's, Shimazu et al (251, 252) showed that the autonomic nerves from the hypothalamus control glycogen metabolism. Fluro gold and transsynaptic tracer pseudorabies virus (PRV) techniques have confirmed the presence of both parasympathetic and sympathetic nerve terminals within the liver, as well as direct connections between hypothalamic centers and the liver (41, 77, 154). These autonomic nerves innervate the liver along three routes: the portal vein, the hepatic artery, and the bile ducts. Efferent innervation by both parasympathetic and sympathetic nerve fibers has been shown to be responsible for hepatic hemodynamics, bile flow regulation, and control of carbohydrate and lipid metabolism (275).

Sympathetic nerve fibers reach the liver through the celiac ganglia, celiac plexus, and the splanchnic nerves (134, 136, 247-249). The sympathetic fibers form an anterior plexus around the hepatic artery. Alexander showed that the hepatic artery receives only sympathetic fibers (12). The sympathetic efferents

penetrate into the acinus, where they end with varicosities in the space of Disse close to the hepatocytes (232) and the hepatic stellate cells (31). Stimulation of the sympathetic efferents results in an increase in glucose output by the liver through rapid activation of glycogen phosphorylase (247-249, 275) as well as an increase in PEPCK activity, thus stimulating glycogenolysis and gluconeogenesis (209).

Norepinephrine (NE), the major sympathetic neurotransmitter, affects glucose metabolism at the hepatocytes via $\alpha_1$- adrenergic receptors (16, 104, 271). Epinephrine exerts most of its glucoregulatory effects in both dogs and humans (28, 55, 74, 234) via the $\beta_2$-adrenergic receptor (55) which is the predominate adrenergic receptor subtype found in the canine liver (164, 180). In vitro (61, 128), norepinephrine has a very low affinity for this $\beta_2$-adrenergic receptor (7% that of epinephrine) Likewise, in vitro data has shown the NE has a high affinity for $\alpha_1$- adrenergic receptors and it is this α-adrenergic receptor subtype that is found in the canine liver (61, 128, 164).

The liver and the gut extract a large percentage of infused norepinephrine (approximately 86-93% and 45-55%, respectively) (57). High levels of plasma catecholamines increase NE spillover from both the liver and the gut, suggesting that the percentage of NE released from the presynaptic neurons that can possibly escape the synaptic cleft is increased in the presence of high circulating catecholamine levels.

Besides NE, sympathetic nerves can release other neuropeptides such as NPY and galanin. These have both been implicated in the regulation of hepatic

glucose metabolism (196, 270). Intraportally delivered galanin, in contrast to NPY, potentiates the NE-mediated glucose output from hepatocytes without having the effect on hepatic blood flow that is seen when NE is infused into the hepatic artery of the anesthetized dog (195). Peripherally infused galanin does not potentiate glucose output, while NPY inhibits the NE-elicited hepatic glucose output (171).

Specific aim II in this thesis addresses the importance of the sympathetic nerves and their possible role in mediating the effects of the portal glucose delivery. The hypothesis is that the sympathetic nervous system exerts a restraining effect on hepatic glucose uptake that can be reversed by the entry of glucose into the portal vein. Since stimulation of the sympathetic nerves is able to increase glucose output, they may act as inhibitors to glucose uptake. Thus by eliminating the sympathetic nerves using selective hepatic sympathectomy (more information on this procedure can be found in chapter II, as well as in the experimental design and discussion of chapter IV), we expected an increase in NHGU in response to peripheral glucose infusion and a reduction in the augmentation of net hepatic glucose uptake in response to the portal glucose signal.

The parasympathetic fibers, on the other hand, form a posterior plexus around the portal vein. The postganglionic parasympathetic nerves are derived from ganglia located at the hepatic hilus and within the portal spaces (100). These parasympathetic fibers are separate from any aminergic nerves found in the area (14, 62, 181, 231, 261, 269). Stimulation of the parasympathetic efferents results

in an increase in glycogen synthesis due to an activation of glycogen synthase and a decrease or complete inactivation of PEPCK activity (247-250). Therefore signals from the hypothalamus down regulate gluconeogenesis in the liver. There are only two reports that actually document intrahepatic parasympathetic ganglia. Retrograde tracing experiments and Fluro gold staining experiments, which will discriminate the autonomic ganglia and neurons, do not show any existence of intrahepatic ganglia (225). Therefore, significant direct vagal cholinergic innervation of the liver is doubtful. Rather, it is more likely that the vagal nerves indirectly control functions of the liver by affecting celiac ganglia or microganglia near the celiac artery (26, 27). Vagal preganglionic terminals have been found in the celiac ganglia as well as microganglia near the celiac artery (276).

Afferent signals also emanate from the liver. Afferent fibers constitute 90% of the fibers in the hepatic vagus nerve (267). They have been shown to convey information regarding plasma glucose (241) and other nutrients, as well as to be responsible for osmoregulation, ionoreception, and baroreception (276). It is likely that net glucose uptake by the liver is the result of an equilibrium between inhibitory sympathetic input and stimulatory parasympathetic input. These contrasting inputs appear to be simultaneously present in the post-absorptive state. The stimulation of NHGU should therefore result in a reduction in sympathetic tone and an increase in parasympathetic tone, or both.

Several studies have examined the role of the parasympathetic nerves in bringing about the effect of the portal glucose signal. Stumpel and Jungermann (267) showed in the perfused rat liver that the effect of the portal signal could be

abolished by the addition of atropine but mimicked by an infusion of acetylcholine into the portal vein, leading to the conclusion that the effect was mediated by the parasympathetic nerves. Shiota et al. showed that an adrenergic blockade (portal vein phentolamine and propanolol infusion) and coincident cholinergic stimulation (portal vein acetylcholine infusion), brought about in the presence of hyperinsulinemic, hyperglycemic conditions, increased NHGU by 1.8 mg/kg/min when compared to controls (255). These results were inconclusive, however, because portal vein administration of acetylcholine caused an increase in hepatic artery blood flow, leading to a rise in the glucose and insulin loads to the liver. Thus the increase in NHGU may have been in part load dependent. The possibility also exists that the mediator which increased the hepatic blood flow may have also increased NHGU directly. Since increased hepatic artery flow does not occur in response to portal glucose delivery, this raises a question as to the relevance of the above finding.

Recently, Cardin et al (44) showed that in the presence of hyperinsulinemia and hyperglycemia brought about by portal glucose infusion, vagal blockade (using cooling coils), which interrupts parasympathetic firing (101), had no effect on NHGU. This result can be interpreted in several different ways. First, since glucose was given intraportally, afferent vagal firing may have been maximally decreased before the vagal cooling took place. This would imply that the vagal efferents are not involved in the response to the portal glucose signal because efferent vagal firing would also have been eliminated and if present, may have caused a decrease in NHGU. It is also possible that the vagus

nerve does not play any role in the mediation of the portal glucose signal and thus vagal cooling would have no effect on NHGU. Thus specific aim III was undertaken to further examine the role of the vagus nerves in the absence of the portal signal. Assuming that the vagus nerve does play a role in mediating the portal glucose signal, we hypothesized that in the absence of the portal glucose signal, decreasing vagal firing using the vagal cooling technique, would increase net hepatic glucose uptake in a similar manner to that seen with portal glucose delivery.

Although very little is known in respect to the mechanism by which the portal glucose signal affects glucose uptake by nonhepatic tissues, two major hypotheses have been proposed (183, 272, 289, 290). The first hypothesis involves the mediation by a neural reflex while the second hypothesis involves the release of a hepatic humoral factor. Minokoshi et al. (183) and Takahashi et al. (272) have shown that stimulation of the ventromedial hypothalamus alters skeletal and cardiac muscle glucose uptake. If the afferent fibers that reach the brain are stimulated and therefore there is a change in their firing rates, the efferent nerves, those which emanate away from the brain, will respond accordingly. The sympathetic fibers that are present in the ventromedial hypothalamus may represent one of the efferent pathways stemming from the brain after apparent afferent stimulation.

The humoral factor hypothesis is supported by the finding that, in the cat, surgical liver denervation decreases insulin sensitivity in the skeletal muscle (289, 290) suggesting that there may exist a factor that is released from the liver that

directly affects insulin sensitivity at the muscle. It has also been shown that muscle insulin sensitivity can be restored by intraportal (but not peripheral) infusion of acetylcholine, suggesting that hepatic sympathetic stimulation may also have a distant effect on skeletal muscle, presumably via a humoral factor released by the liver. This hypothesized humoral factor that may mediate these effects will be discussed more thoroughly later in this introduction.

## The Mechanism of Action of the Portal Glucose Signal

The portal glucose signal causes a rapid and large increase in net hepatic glucose uptake and, subsequently, enhances the intrahepatic level of glucose-6-phosphate in the hepatocyte (47). This increase in glucose-6-phosphate may be due to an enhancement of glucokinase translocation in the liver by the portal glucose signal (47). In the unstimulated state, glucokinase is sequestered in the hepatocyte nucleus where it is bound to its regulatory protein (GKRP) (7, 278). Treatment with fructose leads to a rise in intracellular fructose-1-phosphate which in turn causes the dissociation of GK from GKRP. This dissociation then allows GK to exit the nucleus and enter the cytosol where it can catalyze the phosphorylation of glucose, thus promoting glucose entry into the hepatocyte. This in turn would cause a rise in G-6-P levels that could then activate glycogen synthase; these changes would then result in an increase in glucose uptake and glycogen storage, two characteristics of the portal glucose signal.

Pagliasssotti et al. (214) has also shown that both insulin and the portal signal have the ability to increase glycogen deposition by increasing glycogen

synthase activity. Initially, when glucose uptake is increased in the presence of the portal signal and hyperinsulinemia, there is a spike in lactate due to the increase in G-6-P (possibly brought about by the activation of GK by the portal signal) and increased flux down the glycolytic pathway. This increase in lactate wanes with time as the G-6-P pool increases due to the continuous increase in glucose uptake. Once this G-6-P pool is large enough and the portal glucose signal and insulin have activated glycogen synthase, an increase in glycogen deposition can occur. It has been suggested that ~75% of the glucose that enters the portal vein is directed into glycogen in the presence of the portal glucose signal while the remainder appears to leave the liver as lactate (214).

## Other Mediators of Net Hepatic Glucose Uptake

Other mechanisms for regulating NHGU also exist. The effect of the portal signal may have a centrally neurally mediated component but it may also be composed of an intrahepatic reflex which could involve other neurotransmitters such as serotonin or nitric oxide.

### *Serotonin*

Peptidergic innervation of the liver includes both aminergic and cholinergic neurons which contain such neuropeptides as NPY, substance P, vasoactive intestinal peptide, glucagon-like peptide, somatostatin, and serotonin (10, 84, 266). These nerves are typically found associated with the branches of the portal vein, hepatic artery, bile duct in the connective tissue of portal tracts (177). Serotonin is localized in the enterochromaffin cells of the gastrointestinal mucosa

and within neurons in the enteric nervous system (212). It can be released into the blood or into the lumen of the gut and thus eventually reach the hepatic portal vein. The 5-$HT_{2B}$ receptor for serotonin is known to be expressed in the liver and kidney in humans (36), implying that serotonin may have a direct effect on hepatocytes. It has also been shown that an intraportal injection of 5-HT resulted in a decrease in the afferent firing of the hepatic branch of the vagus nerve and a stimulation of the efferent firing in the pancreatic branch of the vagus (207), similar to the effect of intraportal glucose injection (201), suggesting that 5-HT may elicit a neural signal that could enhance NHGU.

The effect of serotonin (5-HT) on NHGU has been previously examined in our laboratory. Intraportal infusion of 5-HT enhanced NHGU and blunted nonhepatic glucose uptake under hyperglycemic, hyperinsulinemic conditions (191) but also caused an increase in circulating levels of catecholamines and cortisol, evidence of gastrointestinal distress. To more clearly demonstrate that the enhancement of NHGU was serotonin dependent, a selective serotonin re-uptake inhibitor (fluvoxamine) was administered intraportally (192). Similar studies were also carried out by infusing 5-hydroxytryptophan (5-HTP), a serotonin precursor, into the portal vein (192). Both 5-HTP and fluvoxamine enhanced NHGU without elevating circulating serotonin or catecholamine levels.

### *Nitric Oxide*

Nitric oxide, an important biological mediator, is synthesized during the catabolism of the amino acid L-arginine to L-citrulline by nitric oxide synthase (NOS). Three isoforms of NOS have been elucidated. Endothelial NOS (eNOS)

and neuronal NOS (nNOS) are both constitutive isoforms that are dependent on $Ca^{2+}$/calmodulin binding and activation. Inducible NOS (iNOS), which is not dependent on $Ca^{2+}$/calmodulin binding and activation, appears ubiquitously in the body in response to immunologic or inflammatory stimuli.

NO influences numerous physiological processes in multiple organs and tissues. NO effects energy substrate balance (including fatty acids and glucose) in adipocytes, skeletal muscle, heart and the whole body (102). Since NO can modulate peripheral glucose metabolism as well as lipolysis, it is not surprising that NO alterations may play an important role in the evolution of insulin resistance and type 2 diabetes (186). Although high circulating levels of NO have been found in individuals with type 2 diabetes in comparison to those seen in healthy volunteers (18, 186), it appears that individuals with type 2 diabetes may be less sensitive to nitric oxide than healthy volunteers. It has also been postulated that eNOS gene polymorphisms may be susceptibility factors for hyperinsulinemia, insulin resistance and type 2 diabetes (186). It is of interest that dietary administration of L-arginine, the substrate required for NO production, reduced body weight, increased lipolysis, and increased oxidation of glucose in abdominal and epididymal adipose tissue in Zucker diabetic fatty rats (102). It also improved peripheral insulin sensitivity in patients with Type 2 diabetes (223). Intrahepatic nerves containing the neuroeffector nitric oxide (NO) have been identified in a variety of mammalian species (87-89, 220). Hepatocytes can be exposed to NO derived from neighboring Kupffer cells, endothelial and Ito cells as well as autogenously-derived NO (274). The anterior hepatic plexus is

comprised of several capsaicin-sensitive sensory fibers (86) including nitrergic neurons (246).

NO clearly plays a role in the regulation of glucose uptake at the muscle. Both nNOS and eNOS are expressed in skeletal muscle, and NO is released from the muscle at rest and release is increased with the onset of exercise (149). NO potentiates insulin-stimulated muscle blood flow therefore increasing the glucose availability to the muscle and indirectly allowing for greater muscle glucose uptake (63). In vitro studies have suggested that NO may stimulate muscle glucose transport directly by increasing cell surface GLUT4 protein levels (90). More recently, Higaki et al. (129), showed that NO can increase muscle glucose uptake through a mechanism which is clearly distinct from both the insulin and contraction signaling pathways; this NO-stimulated glucose uptake may be associated with an activation of the α1 catalytic subunit of AMPK.

Lautt et al. (158-161) have recently described a novel neurohumoral mechanism by which hepatic sympathetic nerves, through permissive release of a putative hepatic insulin sensitizing substance (HISS), regulate the glucose disposal resulting from a bolus of insulin. According to this theory, immediately following a meal, the hepatic parasympathetic nerves increase NO concentrations which increase the release of HISS from the liver. HISS then stimulates peripheral glucose uptake and accounts for 50-60% of the glucose disposal that is seen following a bolus of insulin. HISS does not act as a true sensitizer but rather potentiates the glucose disposal induced by insulin administration.

The parasympathetic signal involves the release of acetylcholine which in turn acts on hepatic muscarinic receptors, resulting in the production of NO and the release of HISS (120, 240, 291). Sadri et al.(240) have shown that intraportal, but not systemic administration, of L-NAME, a nonselective NO synthase inhibitor, will cause significant insulin resistance at the peripheral tissues. Alternatively, intraportal, but not intravenous, administration of SIN-1 (3-morphonylosydnonimide), a non-enzymatic NO donor, will restore insulin sensitivity in insulin resistant animal models (240). Following hepatic denervation, which results in a decrease in muscle insulin sensitivity, insulin responsiveness can be restored by intraportal administration of NO donors (224), indicating that there is a role for hepatically derived NO in insulin-mediated glucose disposal at the periphery. It has also been shown that hepatic glutathione levels, which are decreased by fasting and rapidly replenished during feeding, play an integral part of the HISS regulatory pathway (118) as does hepatic guanylyl cyclase (64).

Our earlier studies have exhibited a reciprocity between peripheral glucose uptake and hepatic glucose uptake in the presence of portal glucose delivery (3, 134, 214). Since hepatically derived NO appears to have dramatic effects on peripheral glucose uptake, it is possible that NO may also demonstrate glucose regulatory ability at the liver. Both iNOS and eNOS have been found in hepatocytes while iNOS has also been localized to the hepatic Kupffer cells and Ito cells (11, 168). Under normal conditions, only eNOS can be found in the liver but iNOS can be rapidly upregulated in the liver under such conditions as

endotoxemia, sepsis, infection, and liver regeneration (168). NO can elicit changes in hepatic metabolism either by exerting a direct effect on hepatic mechanisms such as uptake, storage, and clearance of glucose (123) or by exerting an indirect effect via the modulation of hepatic vascular tone(11). It is clear that NO may modulate both portal vein and hepatic artery resistance to hepatic blood flow via vasodilation (11) but little is known about NO's ability to act directly on the liver.

Since NO is clearly an important mediator in insulin sensitivity at the muscle and in turn glucose uptake at the muscle, we hypothesized that NO would also have dramatic effects at the liver. Due to the reciprocity between hepatic and muscle glucose uptake, we hypothesized that NO may act as an inhibitor of hepatic glucose uptake. Thus in specific aim IV, a portal infusion of a nitric oxide donor, SIN-1, in the presence of the portal glucose signal, was used to increase NO levels at the liver and hepatic substrate balance was calculated in the presence and absence of this donor.

# CHAPTER II

# MATERIALS AND METHODS

## Animals and Surgical Procedures

### Animal Care

Experiments were conducted on 18h fasted conscious mongrel dogs (20-28 kg) of either sex that had been fed once daily a meat (Kal Kan; Vernon, CA) and chow diet (Purina Lab Canine Diet No. 5006; Purina Mills, St Louis, MO) comprised of 52% carbohydrate, 31% protein, 11% fat, and 6% fiber, based on dry weight. Water was available *ad libitum.* Each dog was only used for one experiment. The surgical facility met the standards published by the American Association for the Accreditation of Laboratory Animal Care, and the Vanderbilt University Medical Center Animal Care Committee approved the protocols.

### Surgical Procedures

Approximately 16 days prior to the study, a laparotomy was performed under general anesthesia (0.01 mg/kg buprenophine HCl and prop, presurgery and 1% isoflurane inhalation anesthetic during surgery) by making a midline incision 1.5 cm caudal to the xyphoid process through the skin, subcutaneous layers and linea alba and extending caudally about 10 cm. A portion of the jejunum was exposed and a branch of a jejunal vein was selected for cannulation. The vein was

separated from surrounding connective tissue and ligated with 4-0 silk (Ethicon, Inc., Sommerville, NJ). A silastic infusion catheter (0.03 in ID; Dow Corning Corp., Midland, MI) was inserted into the vessel through a small incision and passed anterograde until the catheter tip was at the next major jejunal vein junction. The catheter was secured in place with three ties of 4-0 silk. The jejunum was replaced in the abdomen and the spleen was exteriorized. One of the branches of the common splenic vein was cannulated in a similar manner to the jejunal vein and the spleen was then replaced in the abdomen. The jejunal and splenic catheters were used for intraportal infusion of pancreatic hormones (insulin and glucagon).

The liver was retracted, the left lateral lobe of the liver caudally and the central lobe cephalically. The left common hepatic vein and the left branch of the portal vein were exposed. A 14 gauge Angiocath (Benton Dickinson Vascular Access; Sandy, UT) was inserted in the left branch of the portal vein 2 cm from the central liver lobe. A silastic catheter (0.04 in ID) for blood sampling was inserted, advanced retrograde about 4 cm into the portal vein and secured with three ties of 4-0 silk through the adventitia of the vessel and around the catheter. An angiocath was inserted into the left common hepatic vein 2 cm from its exit from the left lateral lobe. A silastic sampling catheter (0.04 in ID) was inserted into the hole and passed retrograde 2 cm and secured into place with three ties of 4-0 silk. These catheters were used for obtaining blood samples from the portal vein and hepatic vein, respectively.

An arterial sampling catheter was inserted into the left femoral artery following a cut-down in the left inguinal region. A 2 cm incision was made parallel to the vessel. The femoral artery was isolated and ligated distally. The sampling catheter (0.04 in ID) was inserted and advanced 16 cm in order to place the tip of the catheter in the abdominal aorta. Again, like all afore mentioned catheters, the catheter was secured, filled with heparinized saline (1U/ml; Abbott Laboratories, North Chicago, IL), knotted and placed in a subcutaneous pocket prior to closure of the skin. This catheter was used to obtain arterial blood.

For specific aim I, the duodenum was exposed, and a small incision was made through the duodenal mucose, 3-4 cm below the pylorus. A 0.08 in ID silastic catheter was then inserted through the incision and secured to the duodenum with a purse string stitch. This catheter was used for the infusion of glucose.

For specific aim II, selective sympathetic denervation or sham denervation occurred at the celiac nerve bundle near the common hepatic artery. The nerve fibers that heavily encased the common hepatic artery were resected from 1-2 cm distal to the origin of the hepatic artery at the celiac trunk to several cm past the hepatic branches of the common hepatic artery. The hepatic arterial branches themselves were also skeletonized and the gastroduodenal artery was stripped of nerves. All nerves surrounding the portal vein and its branches, as well as the vagus nerve, were left intact.

For specific aim III, hollow coils were placed around the vagus nerves in order to cool each vagus nerve thus eliminating signal transmission or for sham

cooling. A ventral midline incision was made 3 cm superior to the manubrium of the sternum that extended 8 cm rostrally through the sternocephalicus muscle. Blunt dissection bilaterally through the anterior fascia and between the sternocleidomastoid and sternohyoid muscles provided exposure of the carotid sheaths. Each vagus nerve was carefully isolated from the carotid artery over a length of 4 cm. It was then elevated with umbilical tape to facilitate placement of the coil. Hollow stainless steel coils of ~1.5 cm in length and five complete revolutions (ID 0.04 in., OD 0.625 in) were placed around each vagus nerve (194). This length of coil was great enough to prevent saltation of nerve transmission over the block. Silastic tubing (ID 0.04 in., OD 0.085 in.) was secured to each end of each coil. The coils were then insulated with tygon tubing (ID 0.375 in., OD 0.129 in.) to prevent cooling of the surrounding tissue and carotid blood. The sternocephalicus muscle was sutured to the sternothyroideus muscle to create a wall between the nerve and carotid artery at the level of the coil on both sides of the neck. The end of the Silastic tubes were placed in a subcutaneous pocket and the incision was closed. The morning of the experiment, the Silastic tubes connected to the cooling coils were exteriorized from their subcutaneous pockets under local anesthesia (2% lidocaine: Abbott Laboratories, North Chicago, IL). The ends of the Silastic tubes connected to the vagal cooling coils were either connected to inflowing lines (ID 0.125 in., OD 0.25 in.) from the cooling bath or to outflowing lines to the collection reservoir. The vagus nerves were cooled by perfusing the previously implanted coils with a cold solution (50% methanol: 50% saline). A reservoir temperature in an isotemp refrigerated

circulator bath (Digital Temperature Controller, PolyScience) of -10°C was associated with an exiting neck temperature of ~0°C and a bath return temperature of ~3°C and resulted in a vagal blockade. Effective cooling was confirmed by observation of a doubling in heart rate and bilateral Horner's syndrome (44, 58, 165, 243).

Sections of the portal vein and hepatic artery were exposed by retracting the duodenum laterally. A small section of the portal vein was exposed by blunt dissection taking care not to disturb the nerve bundle located on the vessel. A 6 or 8 mm ID ultrasonic flow probe (Transonic Systems Inc, Ithaca, NY) was placed around the vessel. A small portion of the common hepatic artery was also carefully exposed and a 3 mm ID ultrasonic flow probe was secured around the vessel. The flow probes were used to determine portal vein and hepatic artery blood flow during experiments. The gastroduodenal vein was isolated and ligated to prevent blood from entering the portal vein beyond the site of the flow probe. Blood that would normally flow through the gastroduodenal vein was shunted through the caudal pancreatoduodenal vein draining the tail of the pancreas. The ultrasonic flow probe leads were positioned in the abdominal cavity and secured with the ends of the catheters to the abdominal wall.

The subcutaneous layer was closed with a continuous suture of 2-0 chromic gut (Ethicon, Inc.). The skin was closed with horizontal mattress sutures of 3-0 Dermalon (Ethicon, Inc.). The dogs received penicillin G (Procaine; Anthony Products, Irwindale, CA) intramuscularly ($10^6$ U) immediately after surgery to minimize the possibility of infection. Flunixin (Meglumine 50 mg/ml;

Phoenix Scientific, Inc., St. Joseph, MO) was injected intramuscularly (1 mg/kg body weight) after wound closure for acute pain relief. Animals awoke from surgery within 2 h, were active, and ate normally approximately 8 h after surgery. They also received 500 mg ampicillin (Principen; Bristol-Myers Squibb, Princeton, NJ) orally twice a day for 3 days post-operatively or as needed.

All dogs studied had: 1) leukocyte count <18,000/$mm^3$, 2) a hematocrit >35%, 3) a good appetite, and 4) normal stools at the time of study. On the morning of the study, the free ends of the catheters and ultrasonic leads were removed from their subcutaneous pockets under local anesthesia (2% lidocaine; Abbott Laboratories, North Chicago, IL). The contents of each catheter were aspirated, and they were flushed with saline. Blunt needles (18 gauge; Monoject, St. Louis, MO) were inserted into the catheter ends and stopcocks (Medex, Inc, Hilliard, OH) were attached to prevent the backflow of blood between sampling times. Twenty gauge Angiocaths (Beckton Dickson) were inserted percutaneously into the left and right cephalic veins and into a saphenous vein for the infusion of tracers, dye and glucose, when appropriate. A continuous infusion of heparinized saline was started via the femoral artery at a rate to prevent any clotting in the line. Animals were allowed to rest quietly in a Pavlov harness for 30 min before the experiments started. At the end of the experiment, dogs were euthanized with an intravenous injection of Euthansia-5 (Veterinary Laboratories, Inc., Lenexa, KS) and the position of the catheter tips was confirmed upon autopsy.

## Collection and Processing of Samples

### Blood Samples

Arterial and portal blood samples were taken simultaneously approximately 30 s before collection of the hepatic venous samples to compensate for the transit time of glucose the liver (115). Prior to sampling, a catheter was cleared of saline by withdrawing 5 ml of blood into a syringe. The blood sample was then drawn into a separate, pre-labeled syringes that had been flushed with heparinized saline (1U/ml; Abbott Laboratories, North Chicago, IL). After sampling, the blood taken during the clearing process was re-infused into the animal, and the catheter was then flushed with heparinized saline (1U/ml; Abbott Laboratories, North Chicago, IL). Before the experiment started, a blood sample was drawn and centrifuged. The plasma from this blood sample was used for the preparation of hormone infusions, various recovery standards, and the indocyanine green standard curve. After onset of the experiment, samples were taken at various time points depending on the specific protocol. If a glucose clamp was performed, small arterial samples of 0.3 ml were taken every 5 minutes for the measurement of the plasma glucose concentration. For all studies, no more than 20% of the animal's total blood volume was withdrawn during the study, and two volumes of saline (0.9% sodium chloride; Baxter Healthcare Co., Deerfield, IL) were given for each volume of blood withdrawn.

Immediately after each sample was obtained, the blood was processed. A small arterial aliquot (20 μl) was used immediately for determination of the

hematocrit in duplicate using capillary tubes (0.4 mm ID; Drummond Scientific Co., Broomall, PA). One ml of the collected blood was placed in a tube containing 20 ul of 0.2M glutathione (Sigma Chemical Co.) and 1.8 mg EGTA (Sigma Chemical Co.) for catecholamine measurement. This tube was vortexed, centrifuged at 3000 rpm for 7 minutes, and the supernatant was stored in a separate tube for later analysis. The remaining blood was placed in a tube containing potassium EDTA (1.6 mg EDTA/ml; Sarstedt, Newton, NC). After gentle mixing, 1 ml of whole blood containing EDTA was pipetted into a tube containing 3 ml of 4% perchloric acid (PCA; Fisher Scientific, Fair Lawn, New Jersey). The tube was vortexed, centrifuged, and the supernatant was stored in a separate tube for later analysis of metabolite levels. A portion of this sample was used for the measurement of lactate in all of the specific aims and glycerol in specific aims I, II, and IV. The remainder of the whole blood containing EDTA was also centrifuged, to obtain plasma.

The plasma samples were used for all other measurements. Glucose concentrations were immediately determined on at least four 10 µl aliquots of plasma using the glucose oxidase method with a glucose analyzer (Beckman Instruments, Inc., Fullerton CA or Analox Instruments; Lunenburg, MA). Insulin, non-esterified fatty acids (NEFA), and cortisol were measured from aliquots of plasma (1.0, 1.0, and 0.5 ml respectively). Glucagon was processed for future measurement; 1 ml aliquot of plasma was added to 50 µl of 10,000 KIU/ml aprotinin (Trasylol; FBA Pharmaceuticals, New York, NY) a protease inhibitor.

Plasma for measurement of NEFA was frozen immediately in dry ice to inhibit lipase activity, while the remainder of the samples remained on ice throughout the experiment. The arterial and hepatic insulin samples were used for measurement of indocyanine green, as will be described later, and then frozen at -70ºC until insulin was measured. All solutions were placed at -70ºC after the experiment until assays were completed.

### Tissue Samples

After completion of the experiment in all of the protocols, the animal was euthanized with pentobarbital (390 mg/ml Fatal-Plus; Vortech Pharmceutical Inc., Dearborn, MI) at 1ml/5kg. In specific aim II, the laparotomy incision was reopened and samples from each of the seven lobes of the liver were taken from all 9 dogs in each group for analysis of liver norepinephrine content by HPLC. This was done to ensure complete sympathetic denervation. In specific aim III, the liver was examined for signs of ischemia and damage due to the presence of the vagal cooling coils.

## Sample Analysis

### Plasma Glucose

Plasma glucose levels were determine during the experiment using the glucose oxidase method (145) with a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). The reaction sequence was as follows:

glucose oxidase

α-D-glucose + $O_2$ ----------→ gluconic acid and $H_2O_2$ (1)

catalase

$H_2O_2$ + ethanol ----------→ acetaldehyde + $H_20$ (2)

molybdate

$H_2O_2 + 2H^+ + 2I^-$ ----------→ $I_2$ +2 $H_20$ (3)

The plasma glucose concentration is proportional to the rate of oxygen ($O_2$) consumption, and the glucose level in the unknown plasma samples is determined by comparison with the rate of oxygen consumption in a standard solution. The second and third reactions quickly remove all hydrogen peroxide ($H_2O_2$), so that there is no end-product inhibition of the process. Glucose was measured a minimum of 4 times at each sampling time point for each vessel and a minimum of 2 times for samples drawn to clamp glucose.

## Plasma Non-esterified Fatty Acid (NEFA)

Plasma NEFA levels were determined spectrophotometrically using the Packard Multi Probe Robotic Liquid Handling System (Perkin Elmer; Shelton, CT) and a kit from Wako Chemicals (Richmond, VA). In essence, coenzyme A (CoA) is acylated by fatty acids in the presence of added acyl-CoA synthetase.

The acyl-CoA that results is oxidized by acyl-CoA oxidase, leaving $H_2O_2$ as a byproduct. Subsequent addition of peroxidase, in the presence of $H_2O_2$, allows for oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple colored adduct. The purple color adduct optical density is measured at 550 nm and is proportional to the NEFA concentration in the sample. The NEFA values are obtained from a calibration curve with known amounts of oleic acid. The reactions were run at 37ºC. The specific reactions were as follows:

$$\text{NEFA} + \text{ATP} + \text{CoA} \xrightarrow[\text{Acyl-CoA synthetase}]{} \text{Acyl-CoA} + \text{AMP} + \text{Ppi} \qquad (4)$$

$$\text{Acyl-CoA} + O_2 \xrightarrow[\text{Acyl-CoA oxidase}]{} \text{2,3-trans-enoyl-CoA} + H_2O_2 \qquad (5)$$

$$2\ H_2O_2 + \text{3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline} + \text{4- aminoantipyrine} \xrightarrow[\text{Peroxidase}]{} \text{Purple color adduct} \qquad (6)$$

Whole Blood Metabolites: Lactate and Glycerol

Whole blood concentrations of lactate and glycerol were measured according to the method of Llyod et al (170) adapted to the Packard Multi Probe

Robotic Liquid Handling System (Perkin Elmer; Shelton, CT). Enzymes and coenzymes for these metabolic assays were purchased from Sigma Chemical Co and Boehringer-Mannheim Biochemicals (Germany). The general reaction for the procedure involves the addition of an excess amount of NAD and an enzyme to the metabolite samples. NAD becomes reduced to NADH upon oxidation of the metabolite, and NADH has a native fluorescence that the oxidized form lacks. A fluorometer in the system detects changes in the florescence resulting from changes in NADH levels. The concentration of the metabolite present is proportional to the NADH produced.

Metabolites were measured from the PCA treated blood samples described under Sample Processing. A standard curve was constructed for each metabolite using known concentrations diluted in 3% PCA. Finally, each sample value was corrected for the dilution with PCA that had occurred during processing.
The lactate assay involved the following reaction:

$$\text{Lactate} + NAD^+ \xrightarrow{\text{Lactate deyhdrogenase}} \text{Pyruvate} + NADH + H^+ \quad (7)$$

The enzyme buffer used was 0.24 M glycine, 0.25 M hydrazine dihydrochloride, and 7 mM EDTA, pH 9.6. The amount of $NAD^+$ added was 4.6 mg and the amount of lactate dehydrogenase added was 0.1 U (to 10 ml of buffer).

The glycerol assays involved the following reaction:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{L-glycerol-1phosphate} + \text{ADP} \quad (8)$$

$$\text{L-glycerol-1 phosphate} + \text{NAD}^+ \xrightarrow{\text{Glycerol-3phosphate dehydrogenase}} \text{Dihydroxyacetone phosphate} + \text{NADH} + \text{H}^+ \quad (9)$$

The enzyme buffer used was 0.09 M glycine, 1 mM hydrazine, and 0.01 mM $MgCl_2$, pH 9.5. The amount of ATP added was 15.4 mg, the amount of Glycerokinase added was 0.3U, the amount of $NAD^+$ added was 15.4 mg, and the amount of glycerol-3-phosphate dehydrogenase added was 0.6U (to 10 ml buffer).

## Hormones

The plasma levels of insulin, glucagon, and cortisol were measured using radioimmunoassay (RIA) techniques (284)**.** In general, a sample containing an unknown amount of hormone was incubated with an antibody specific for that hormone. A known amount of radiolabeled hormone was added to the mixture to compete with the antibody binding sites. The sample was then treated so as to separate unbound hormone from the antibody-hormone complexes, generally by utilizing a double antibody procedure (single antibody procedure for cortisol) which caused precipitation of the bound complex. The radioactivity of the precipitate was measured via a Cobra II Gamma Counter (Packard Instrument Co,

Meriden, CT). The binding of the radiolabeled hormone was inversely proportional to the amount of unlabeled hormone present, and a standard curve was constructed using known concentrations of the unlabelled hormone.

*Insulin*

Immunoreactive plasma insulin was measured using a double antibody RIA as described previously (193). Insulin antibodies and $^{125}$I tracers were obtained from Linco Research Inc (St. Charles, MO). A 100 µl aliquot of the plasma sample was incubated for 18 h at 4ºC with 200 µl of $^{125}$I- labeled insulin and 100 µl of a guinea pig specific antibody to insulin. Next, the samples was incubated with 100 µl goat anti-guinea pig IgG (2$^{nd}$ antibody) and 100 µl IgG carrier for 30 min at 4ºC. One ml of wash buffer was added, and tubes were centrifuged at 3000 rpm. The liquid portion of the samples was decanted and the remaining pellet containing the total radioactivity bound to the antibody was counted in a Cobra II Gamma Counter.

The log of the amount of hormone in the samples was inversely proportional to the log of (bound label/fee label). The insulin concentration in each samples was determined by comparison to a standard curve obtained using known amounts of unlabeled hormone. The samples were corrected for non-specific binding, and the sample detection range was 1-150 µU/ml. The antibody is specific to porcine, canine, and human insulin, but cross-reacts with bovine insulin (90%), human proinsulin (38%), and the split proinsulin products Des 31,32 (47%) and Des 64,65 (72%). Overall, less than 15% of the basal insulin level is due to non-insulin material. There is no cross-reactivity with glucagon,

pancreatic polypeptide, C-peptide, or somatostatin. The recovery for the assay was between 90-100% (based on spiking samples with known amounts of insulin), and the interassay coefficient of variation (CV) was approximately 7-8% for the entire range of the dose response curve.

HIM-2 in specific aim 1 was measured in the exact same manner as described above. A comparison of standard curves was performed using Humulin and HIM2. The two curves were similar but not identical, having slightly different $ED_{50}$'s. When human insulin standards were used, the assay slightly underestimated (5%) HIM2 values at the high end of the standard curve (~150 uU/mL) and slightly overestimated (20%) them at the lower end of the curve (~10 uU/mL). At the mid points of the curves, the values were similar. Samples for insulin and HIM-2 were diluted when necessary to achieve a value near the mid point of the curve.

*Glucagon*

Immunoreactive plasma glucagon was also measured using a double antibody RIA (Linco) (85). The protocol was modified by utilizing primary and secondary antibodies specific for glucagon (kit with glucagon antibodies and $^{125}I$ tracers from Linco). A 100 µl aliquot of the samples was incubated for 24 h at 4ºC with 100 µl of guinea pig specific antibody to glucagon. Next, 100 µl $^{125}I$-labelled glucagon was added, and the solution was incubated for an additional 24 h at 4ºC. The samples was then incubated with 100 µl goat anti-guinea pig IgG ($2^{nd}$ antibody) and 100 µl IgG carrier for 2 h at 4ºC. One ml of wash buffer was added, and the tubes were centrifuged at 3000 rpm. The samples were decanted and the

portion of total radioactivity bound to the antibody (pellet) was counted in a Cobra II Gamma Counter.

The log amount of hormone in the samples was inversely proportional to the log of (bound label/free label). The glucagon concentration in each sample was determined by comparison to a standard curve using known amounts of unlabeled hormone. The samples were corrected for non-specific binding, and the sample detection range was 20-400 pg/ml. The antibody is 100% specific for glucagon, with only slight cross reactivity with oxytomodulin (0.01%), and no cross reactivity with human insulin, human proinsulin, human C-peptide, somatostatin, pancreatic polypeptide, or glucagon like peptide-1. A protein effect in the assay causes zero glucagon to read as 15-20 pg/ml. This represents a stable, constant background in all samples. The recovery for the assay was between 80-100%, and the interassay CV was approximately 6-10% for the entire range of the dose response curve.

*Cortisol*

Immunoreactive plasma cortisol was measured with a single antibody technique (98) using a gamma coat RIA from Diagnostic Products Corporation (Los Angeles, CA). A 25 μl aliquot of plasma and 1 ml of $^{125}$I-labeled cortisol were pipetted into a cortisol specific antibody-coated tube with the antibody immobilized on the lower inner wall of the tube. They were incubated for 2 hours in a 31ºC water bath. Next, the tubes were decanted and rinsed with deionized water. The tubes were allowed to dry, then counted in a Cobra II Gamma Counter for 4 min.

The log of the amount of hormone in the samples was inversely proportional to the log of (bound label/free label). The cortisol concentration in each sample was determined by comparison to a standard curve using known amounts of unlabeled hormone. The sample detection range was 0.5-50 ug/dl. The antibody is 100% specific for cortisol, with only slight cross-reactivity with 11-deoxycortisol (6%) and 17-hydroxyprogesterone (1%), and no cross reactivity with corticosterone, aldosterone, progesterone, deoxycorticosterone, and tetrahydrocortisone. The recovery for the assays was > 90%, and the interassay CV was approximately 8-10% for the entire range of the dose response curve.

*Catecholamines*

The catecholamines were the only hormones not measured by RIA. Instead, a high-performance liquid chromatography (HPLC) method was used to determine epinephrine and norepinephrine levels as previously described by Goldstein et al (113). Four hundred microliters of the plasma samples were partially purified by absorption to 10 mg of acid-washed alumina (Bioanalytical Systems, West Lafayette, IN) in 600 µl of Tris/EDTA, pH 8.6, and 50 µl of an internal standard (final concentration 500 pg/ml dihydroxybenzylamine (DHBA); Sigma Chemical Co.). Samples were shaken for 15 min, centrifuged for 4 min, and aspirated. The alumina pellet was rinsed with 2 ml water, then the solution was vortexed, centrifuged, and aspirated, and the process was repeated twice. Next, the catecholamines were eluted with 200 µl 0.1M perchloric acid (PCA) according to Anton and Sayre (15).

Samples were then injected onto an HR-80, reverse phase, 3 μm octadecylsilane column. The mobile phase was composed of 14.2 g of disodium phosphate, 440 mg of sodium octyl sulfate, 37 mg of sodium EDTA, pH 3.4, and 43 ml of methanol. The system utilized a Coulchem II Detector, Model 5021 Conditioning Cell, and Model 5011 Analytical Cell (all obtained from ESA, Bedford, MA). Samples were measured against a linear calibration curve that was comprised of 5 standards(ranging from 50-1000 pg/ml) prepared from epinephrine bitartrate and (-)-arterenol bitartrate (norepinephrine) salts (Sigma Chemical Co.). In addition, a known amount of epinephrine and norepinephrine were added to the sample taken at the start of each experiment to ensure precise identification of the peaks, and to measure recovery.

Data reduction was performed with ESA 500 Chromatograph and data station software to identify peaks. The ratio of the peak height of the internal standard to the catecholamine was calculated, and the concentration of catecholamine was determined by comparison with the standard curve. The limit of detection of the assay was 20 pg/ml and 5 pg ml for epinephrine and norepinephrine, respectively. Recovery was between 80-100% for both hormones. The interassay CVs was 3-11% for epinephrine; the low and high ends of the curve resulted in large variances. The interassay CVs was 4-6% for norepinephrine for the entire range of the dose response curve.

To measure tissues catecholamines (liver), frozen tissue samples were ground into powder using mortars and pestles chilled in liquid nitrogen. Next, 5 mM glutathione in 0.4 N perchloric acid was added to achieve 10% w:v extract.

The solution was homogenized, the sample was centrifuged at 3000 rpm for 20 min, and the supernatant was decanted and frozen at -70ºC. Epinephrine and norepinephrine were then measured by HPLC as described for the plasma catecholamine levels, and the concentrations were corrected for the dilution of samples.

## Blood Flow

Blood flow was determined directly in the hepatic artery and portal vein with the use of ultrasonic flow probes implanted during surgery. Total hepatic blood flow was also assessed using the indocyanine green (ICG) dye extraction method, according to Leevy et al (163). The results presented in this thesis were calculated using ultrasonic-determined flow, as this flow does not require an assumption about the distribution of arterial versus portal flow. ICG-determined flow was used as a backup measurement. However, the same conclusion were drawn when the ICG flows were used to calculate the data.

Ultrasonic flow probes allowed for instantaneous measurement of variation in velocity and provided blood flow in individual vessels. Each probe worked by determining the mean transit time of an ultrasonic signal passed back and forth between two transducers within the probe that were located upstream and downstream of the direction of blood flow in the vessel. The two transducers were piezoelectric, a material which is capable of both receiving and transmitting the ultrasonic signal. The downstream transducer first emitted an ultrasonic pulse into the blood vessel that was received upstream by a second transducer. After the

upstream transducer received the ultrasonic signal, it re-emitted the ultrasonic pulse signal back to the downstream transducer. The transit time of each ultrasonic beam as measured by the upstream and downstream transducers ($\Delta T_{up}$ and $\Delta T_{down}$, respectively) was defined by the following relationships:

$$\Delta T_{up} = D / (v_o - v_x) \quad (10)$$

$$\Delta T_{down} = D / (v_o + v_x) \quad (11)$$

where D was the distance traveled by the ultrasonic beam within the acoustic window of the probe, $v_o$ was the phase velocity, or the speed of sound, in blood, and $v_x$ was the component of fluid velocity that was parallel or antiparallel to the phase velocity. The parallel component augmented the phase velocity when the signal was traveling in the same direction of blood flow, while the antiparallel component subtracted from phase velocity if the ultrasonic signal was moving against the flow of blood in the vessel. Combining the two expressions for transit time yielded the following equation:

$$\Delta T_{up} - \Delta T_{down} = (D / (v_o - v_x)) - (D / (v_o + v_x)) \quad (12)$$

Since the transit times measured by both transducers, the distance traveled by the beam, and the speed of sound in blood were all known quantities, this equation

was used to calculate $v_x$. Once $v_x$ was attained, the transit velocity (V) of blood traveling through the vessel could be found according to the following equation:

$$V \cos \theta = v_x \quad (13)$$

where θ was the angle between the centerline of the vessel and the ultrasonic beam axis. Finally, blood flow was determined as the product of the transit velocity and the cross-sectional area of the vessel. The cross-sectional area of the vessel was pre-determined by the size of the acoustic window according to the probe model. Since transit time was sampled at all points across the diameter of the vessel, volume flow was independent of the flow velocity profile. If a flow probe failed during the experiment, the missing values were estimated by one of two methods: either the mean blood flow for that vessel in a given protocol was used, or the values from the functional flow probes were subtracted from the corresponding ICG values (for example, values from the arterial flow probe were subtracted from the ICG vales to yield estimates of the portal vein flow).

The ICG method is based on the Fick principle, according to which the net balance of a substrate across an organ is equal to the concentration difference of the substrate across the organ multiplied by the blood flow through the organ. The equation can be rearranged to calculate hepatic blood flow from the ratio of hepatic ICG balance divided by the arteriovenous difference of ICG across the liver. Because the liver is assumed to be the only site of ICG clearance, hepatic ICG uptake is equal to the ICG infusion rate in steady state conditions. The

extraction of ICG across the liver remains constant for brief infusions. However, if ICG is infused for a longer time (> 4 h), the dye level in the plasma gradually increases, resulting in a slight overestimation (5-10%) of hepatic blood flow (126).

Arterial and corresponding hepatic vein plasma samples were centrifuged at 3000 rpm for 30 min without the brake to pellet particulate matter. Optical density was then measured on a Spectronic spectrophotometer at 810 nm. The procedure was then repeated, and the values obtained for each sample were averaged. A standard curve was constructed by adding successive 5 μl aliquots of diluted dye (1:10 dilution) to 1 ml of plasma obtained from the animal before the dye infusion commenced. Hepatic plasma flow (HPF) was then calculated as follows:

$$HPF = (IR \times 10 \times SCMD)/(\text{dog weight (kg)} \times (0.005) \times (A-H)) \quad (14)$$

where IR is the ICG infusion rate (ml/min), SCMD is the standard curve mean difference per 5 μl increments and A-H is the difference in absorbance between the arterial and the hepatic venous sample. The value of 10 was used to correct for the dilution of ICG used in the standard curve, and 0.005 was the volume in ml used as increments in the standard curve. Hepatic blood flow (HBF) was derived from HPF:

$$HBF = HPF/(1-\text{hematocrit}) \quad (15)$$

Because this technique measured total hepatic blood flow only, the distribution of blood flow in the vessels supplying the liver was assumed. The normal distribution of flow was assumed to be 20% artery and 80% portal vein at baseline. However, flow distribution was altered during somatostatin infusion, since the latter decreases portal flow modestly. Flow distribution was therefore assumed to be 28% artery and 72% portal in the presence of somatostatin (116).

## Glucose Mixing in the Portal Vein

When glucose is infused in the slow, laminar flow of the portal vein, mixing of the glucose in the blood can be problematic. The paraaminohippuric acid (PAH) method was used in specific aims III and IV in this thesis to assess whether good mixing of glucose had occurred in the portal vein during intraportal glucose infusion.

The liver PAH method is based on the principle that this substance, not being extracted by the liver or erythrocytes, should maintain a constant concentration across the liver. PAH is mixed with the glucose that is infused in the portal vein, so that the PAH infusion rate is 0.4 mg/kg/min. The concentration of PAH is then measured on whole blood samples from arterial, portal venous, and hepatic venous blood (39). The assay involves a 1:5 dilution of the blood sample in a reagent solution (10 g p-dimethyamino-benzaldeyhyde, 600 ml 95% ethanol, 40 ml 2N HCl, deionized $H_2O$ up to 1000 ml). Light absorbance of the diluted samples is then measured on a spectrophotometer at 465 nm, and

compared with a standard curve built with increasing PAH concentrations in blood drawn from the animal before the start of the PAH infusion. The ratio between the recovery of PAH in portal and hepatic veins, and the actual intraportal PAH infusion rate is then used as an index of mixing of the intraportal infusate with the blood entering and exiting the liver. A ratio of 1.0 would represent perfect mixing.

Because of the magnitude of the CV for assessing mixing, samples were considered unmixed if the hepatic vein PAH recovery was >140% or <60% of the actual amount of PAH infused. Animals were excluded from the study if poor mixing, as defined above, occurred in more than two out of the five time points of the intraportal glucose infusion period.

### Blood Pressure and Heart Rate

For specific aims III and IV, systolic and diastolic blood pressure and heart rate were determined throughout the experiments at each sampling time point using a Digi-Med Blood Pressure Analyzer (Micro-Med, Inc., Louisville, KY).

## Calculations

### Net Hepatic Substrate Balance

Both ICG and ultrasonic flow probes were used to estimate total hepatic blood flow in these studies via the arterio-venous difference technique. The net

hepatic balances and net hepatic fractional extraction of blood glucose, lactate, glycerol, and plasma NEFA were calculated using both ultrasonic-determined and ICG-determined flow. As previously mentioned, the data shown are those calculated using ultrasonic-determined flow as this flow does not require an assumption about the distribution of arterial versus portal flow.

For both the indirect and direct method of calculation, the net balance of a substrate across an organ, otherwise known as the A-V difference technique, utilized the Fick Principle as described for ICG blood flow. In short, net hepatic substrate balance (NHSB) was calculated directly as the difference between the substrate load exiting the liver ($Load_{out}$) and the substrate load entering the liver ($Load_{in}$), as shown in the following equation:

$$NHSB = Load_{out} - Load_{in} \text{ (D)} \quad (16)$$

The $Load_{out}$ was calculated from the ultrasonic data according to the equation:

$$Load_{out} = [S]_H \times HBF \quad (17)$$

where $[S]_H$ is the substrate concentration in the hepatic vein and HBF is the total hepatic blood flow, as determined by adding the arterial flow to the portal vein flow. The hepatic artery supplies blood directly to the liver and the portal vein drains the digestive oranges and the spleen, and then supplies blood to the liver.

Thus, $Load_{in}$ (D) calculated from the ultrasonic data was the sum of the loads in the two vessels as calculated according to the following equation:

$$Load_{in}\ (D) = ([S]_A \times ABF) + ([S]_P \times PBF) \quad (18)$$

where $[S]_A$ and $[S]_P$ are substrate concentration sin the hepatic artery and the portal vein, respectively, and ABF and PBF are blood flow in the hepatic artery and the portal vein, respectively. For all glucose balance calculations, glucose concentrations were converted from plasma to blood values by using previously determined correction factors (CF: the mean of the ratio of the blood value to the plasma concentration for each period and for each blood vessel). Mean CFs during basal and experimental period were 0.74 and 0.74 for the artery, 0.74 and 0.73 for the portal vein and 0.73 and 0.73 for the hepatic vein, respectively. (134, 214). For NEFA calculations, plasma substrate concentrations and plasma flow were used rather than blood concentrations and blood flow. Plasma flow was determined by multiplying blood flow by (1-hematocrit). Positive numbers for net hepatic substrate balance indicate net production while negative numbers indicate net uptake. When the data were plotted as net hepatic uptake, positive values were used.

To circumvent any potential errors arising from incomplete mixing of glucose in the circulation during intraportal glucose infusion, a second, indirect method was also used for the calculation of the NHGU. This method differs from

the direct calculation described above in that $Load_{in}$ is determined using the equation:

$$Load_{in}\ (I) = (G_A \text{ X } HBF) + GIR_{PO} - GUG \qquad (19)$$

where $G_A$ is the arterial blood glucose concentration, $GIR_{PO}$ is the portal glucose infusion rate, and GUG is the uptake of glucose by the gastrointestinal tract. GUG is measured in each animal in the absence of intraportal glucose infusion, and this value is then corrected for differences in glucose load reaching the gut during intraportal infusion. The glucose balance data presented in the results sections of specific aims II, III, and IV of this thesis were determined by use of the indirect method; there was no statistical difference between using the indirect method versus the direct method.

Net hepatic fractional extraction (FE) was also calculated using ultrasonic-determined blood flow according to the following equation:

$$FE = NHSU / Load_{in} \qquad (20)$$

where NHSU is net hepatic substrate uptake and could represent NHGB.

Net hepatic substrate balances and net hepatic fractional extractions were also calculated using the ICG blood flow data. These calculations were performed to verify the ultrasonic results, and to ensure that conclusions drawn from either

method were similar, as explained earlier. The only difference in the ICG method is that $Load_{in}$ and $Load_{out}$ were calculated difference as shown below:

$$Load_{out} = [S]_H \times HBF \quad (21)$$

$$Load_{in} = (0.28 \times [S]_A + 0.72 \times [S]_P) \times HBF \quad (22)$$

where HBF is total hepatic blood flow calculated using the ICG method, and 0.28 and 0.72 are estimates of the normal distribution of flow in the artery and portal vein during somatostatin infusion (116) as explained earlier.

Limitations of the arterio-venous difference technique include: 1) variability of vascular anatomy and heterogeneity of tissue structure and function, 2) imprecision in measurement of local blood flow, 3) measurement of net rather than absolute flux across the organ, and 4) access to the portal vein is required, making this procedure only useful in animals. In addition, transit time through the organ must be taken into account. Because arterio- venous difference represents net flux across an organ, it is most valid during steady state conditions.

## Nonhepatic Glucose Uptake and Clearance

Nonhepatic glucose uptake (nonHGU) was calculated over time intervals by the following overall equation:

NonHGU = average total glucose infusion between T1 and T2 + ($T1_{NHGB}$ + $T2_{NHGB}$)/2 –glucose mass change in the pool (23)

where T1 and T2 represent the two time points for which the parameter is being measured over. Note that the (($T1_{NHGB}$ + $T2_{NHGB}$)/2) term will be a negative number in the presence of NHGU. The glucose mass change in the pool is calculated using the following equation:

Glucose mass change in the pool = ((($[G_A]_{T2}$ - $[G_A]_{T1}$) / 100) * ((0.22 * body wt in kg * 1000 *0.65) / body wt in kg)) / (T2-T1) (24)

where $[G_A]$ is the blood glucose concentration, T1 and T2 are the two end time points of the interval, 0.22 represents the volume of extracellular fluid (the volume of distribution) or 22% of the dog's weight (13), and 0.65 represents the pool fraction (65).

Nonhepatic glucose clearance was calculated using the following equation:

Nonhepatic glucose clearance = NonHGU / (($[G_A]_{T1}$ + $[G_A]_{T2}$) /2 / 100) (25)

where $[G_A]$ is the blood glucose concentration and T1 and T2 are the two end time points of the interval.

### Sinusoidal Hormone Concentrations

Because the liver is supplied by blood flow from both the hepatic artery and the portal vein, neither represents the true inflowing hepatic blood flow supply. For this reason, hepatic sinusoidal hormone levels (of insulin and glucagon) were calculated as follows:

$$\text{Hepatic Sinusoidal Hormone Level} = [S]_A \times (APF/TPF) + [S]_P \times (PPF/TPF) \quad (26)$$

where A and P are arterial and portal vein plasma substrate concentration; APF and PPF are the arterial and portal vein plasma flow measured by the ultrasonic flow probes; TPF (total hepatic plasma flow) = APF + PPF. Note this calculation represent the average inflowing hepatic sinusoidal hormone level, rather than the average sinusoidal level.

## Statistical Analysis

Data are expressed as means ± standard error of mean. The specific statistical tests used in the studies are described in the experimental design sections of Chapters III-VI.

# CHAPTER III

## SIMULATED FIRST-PHASE INSULIN RELEASE USING HUMULIN OR HIM2 IS ASSOCIATED WITH PROLONGED IMPROVEMENT IN POST PRANDIAL GLYCEMIA

### Aim

The goals of specific aim 1 were two-fold. The first was to examine the ability of first phase insulin release to modify NHGU under conditions mimicking oral glucose loading in an insulinopenic state. The second was to explore whether a pulse of an insulin analog (hexyl-insulin monoconjugate 2, HIM2, Nobex Corp.), created to facilitate the absorption of orally delivered insulin, also has the ability to modify postprandial hepatic glucose metabolism.

### Experimental design

Each experiment consisted of a 100-min equilibration period (-140 to –40 min), a 40-min basal period (-40 to 0 min), and a 270-min experimental period (0 to 270 min). In all experiments, a constant infusion of indocyanine green dye (0.076mg/min) was initiated at –140 min. At 0 min, a constant infusion of somatostatin (SRIF) (0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was begun to suppress endogenous insulin and glucagon secretion, and glucagon (0.55 $ng \cdot kg^{-1} \cdot min^{-1}$) and human insulin (0.25$mU \cdot kg^{-1} \cdot min^{-1}$) were replaced intraportally to maintain basal levels in each protocol. The rate of SRIF infusion was lower than that used in past studies so that the inhibitory effect of the peptide on gastric emptying seen with higher SRIF doses could be eliminated. This rate of SRIF infusion did not affect gastric

motility as indicated by the fact that 98% of the intraduodenal glucose that was infused was absorbed and yet both the insulin and glucagon levels were successfully clamped. Thus a somatostatin rate of 0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ was adequate to clamp the pancreatic hormones while not impeding glucose absorption.

In the first group, insulin was infused into the portal vein at a basal rate of 0.25 $mU \cdot kg^{-1} \cdot min^{-1}$ throughout the study (BI, n=6). In the second group, a pulse of human insulin was given into the portal vein from 0 to 5 minutes at an infusion rate of 10 $mU \cdot kg^{-1} \cdot min^{-1}$ to mimic first phase insulin release postprandially (HI, n=6) followed by basal insulin infusion as above. In the third group HIM2 (an orally active modified insulin created by the Nobex Corp. Ltd, North Carolina) was infused into the portal vein at 10 $mU \cdot kg^{-1} \cdot min^{-1}$ (HIM2, n=6) over five minutes followed by a basal infusion of human insulin as above. Glucose (50% dextrose) was infused peripherally at $5mg \cdot kg^{-1} \cdot min^{-1}$ from 5 to 10 min and then at $10mg \cdot kg^{-1} \cdot min^{-1}$ from 10 to 15 minutes to prevent hypoglycemia from occurring. At 15 minutes, the duodenal glucose (20 % dextrose) infusion was started and continued at $5mg \cdot kg^{-1} \cdot min^{-1}$ for the duration of the experiment. The purpose of the 15 minute delay in starting the duodenal glucose infusion was to simulate insulin dosing prior to a meal. Femoral artery, portal vein, and hepatic vein blood samples were taken every 20 min during the basal period (-40 to 0 min) and every 30 min after t=30 minutes.

## Statistical Analysis

All data are presented as means ± SE. Time course data were analyzed with repeated-measures analysis of variance. Independent *t*-tests were used for any comparisons of mean data. Statistical significance was accepted at $p<0.05$

## Results

**Plasma glucagon and insulin concentrations:** Arterial and liver sinusoidal plasma glucagon levels were basal throughout the study in all three groups (Table 3.1). The arterial and liver sinusoidal plasma insulin levels in the basal insulin group remained basal (6 ± 1 and 18 ± 2 uU/ml respectively) throughout the experiment (Figure 3.1). The arterial plasma insulin level in the HI group rose rapidly and peaked at 52 ± 15 uU/ml while the liver sinusoidal insulin level peaked at 171 ± 66 uU/ml; these levels returned to baseline by 30 minutes. The arterial and liver sinusoidal plasma insulin levels in the HIM2 group peaked at 164 ± 44 and 427 ±185 uU/ml respectively. These values did not return to baseline until ~ 1 hr.

**Net gut balance, glucose absorption, and arterial glucose level:** Net gut glucose uptake was similar in all three groups during the control period and averaged 0.50 ± 0.05 mg/kg/min. The switch from net gut glucose uptake to net glucose output occurred about 10 minutes after the initiation of the intraduodenal

glucose infusion in all three groups. Net gut glucose output averaged 3.7 ± 0.4 mg/kg/min over the study period (Figure 3.2).

Glucose absorption occurred at a steady state (4.9 ± 0.8 mg/kg/min) in the basal insulin group and caused the plasma glucose level to rise to a plateau of 265 ± 20 mg/dl (Figure 3.2) during the last hour of the study. At steady state, glucose absorption accounted for 98% of the infused glucose; the remaining 2% was presumed to be metabolized by the gut.

In the group given a pulse of human insulin, glucose absorption was similar (4.8 ± 0.5 mg/kg/min) to that seen in the basal insulin group. In this case, however, the arterial plasma glucose rose less, reaching only 214± 15 mg/dl by the last hour of the study. Glucose absorption in the group given the pulse of the insulin analogue (HIM2) was identical to that in the other two groups (4.9 ± 0.5 mg/kg/min) but the glycemic profile was markedly different. The rise in arterial plasma glucose occurred more slowly, eventually reaching a plateau of only 193 ± 14 mg/dl during the last hour of the study. Despite very similar glucose absorption rates, the glycemic profiles in the three groups were markedly different indicating that the insulin spike augmented disposition of the absorbed glucose and resulted in glycemic improvement even 4.5 hours later.

**Hepatic glucose load, net hepatic glucose balance (NHGB), and hepatic fractional glucose extraction*:*** Average hepatic blood flows were similar among the three groups. In the BI group, the total blood flow was 33 ± 3 ml/kg/min while in the HI and HIM2 groups it was 36 ± 3 and 31 ± 4 ml/kg/min,

respectively. At the end of the experiment, the hepatic glucose loads were 70 ±5, 60 ± 1, and 50± 7 mg/kg/min in the BI, HI, and HIM2 groups, respectively ( $p < 0.05$ in BI vs. HIM2, Figure 3.3).

During the basal period net hepatic glucose output was similar in all three groups (~1.7± 0.1 mg/kg/min). In the basal insulin group, it did not decrease significantly until 90 minutes after the initiation of glucose absorption. By the last hour of the study however, net hepatic glucose uptake (NHGU) occurred at the rate of 2.7 ± 0.7 mg/kg/min. Net hepatic glucose output (NGHO) declined more rapidly in the HI group, ceasing by 60 minutes and most rapidly in the HIM2 group in which it ceased at 30 minutes. By the last hour of the study, NHGU was 2.7 ± 0.6 and 2.0 ± 0.3 mg/kg/min in the HI and HIM2 groups respectively (Figure 3.3). At that time, the fractional extraction of glucose by the liver was approximately 3-5% in all three groups (Table 3.1).

**NEFA and glycerol:** In the basal insulin group, glycerol levels and net hepatic glycerol uptake fell slightly and eventually plateaued (Figure 3.4). In the HI group, the glycerol levels and net hepatic glycerol uptake fell rapidly in the first 15 minutes then rose slightly and then drifted down. In the HIM2 group, the glycerol levels and the net hepatic glycerol uptake fell rapidly over the first 30 minutes, reached a minimum at 60 minutes and then drifted up slightly. By the end of the experiment, there were no significant differences in glycerol levels or net hepatic glycerol uptake among the three groups. In general, the plasma NEFA

levels (Figure 3.5) and net hepatic NEFA uptakes paralleled the changes in glycerol.

**Lactate levels and hepatic balance:** The liver exhibited net lactate uptake in the basal periods in all three groups and hyperglycemia resulted in a switch to net hepatic lactate output (Figure 3.6). At the end of the experiment, net hepatic lactate output was 11.2 ± 4.4, 8.8 ± 1.4, and 5.4 ± 3.2 umol/kg/min in the BI, HI, and HIM2 groups respectively. The arterial blood lactate levels steadily rose in all three groups due to the switch in net hepatic lactate balance and reached an average value of 1500 umol/L by the end of the experiment.

**Nonhepatic glucose uptake and nonhepatic glucose clearance:** By the end of the experiment, the HIM2 pulse was associated with a significantly greater nonhepatic glucose clearance (1.61 ± 0.29 ml/kg/min) than basal insulin (BI= 0.62 ± 0.11 ml/kg/min) or the Humulin pulse (HI= 0.76 ± 0.26 ml/kg/min) (Fig 3.7). These differences in nonhepatic glucose clearances are also reflected in the nonhepatic glucose uptake values (Table 3.1).

## Discussion

These data indicate that simulation of first phase insulin release during an intraduodenal glucose infusion, given on the background of a pancreatic clamp, resulted in a significant improvement of the glycemic profile for as long as four hours. We also showed that a modified insulin (HIM2) molecule improved

glycemia even more than Humulin, probably as a result of its modified pharmokinetics. It would appear that a pulse of insulin improves postprandial glycemia by two different mechanisms. The early blunting of the glycemic rise is mediated by a quick increase in NHGU while the later improvement is associated with an increase in nonhepatic glucose clearance.

Following the 5 minute pulse of human insulin, arterial plasma insulin levels rose rapidly and remained elevated for ~30 minutes. Administration of HIM2 caused an elevation in plasma insulin levels that lasted almost 60 minutes. Despite infusion of the same amount of insulin in both groups, the HIM2 spike created a significantly higher arterial plasma insulin level than did the HI spike. The AUC for HIM2 was almost 3 times greater than that seen in the HI group. These data suggest that clearance of the insulin analog was significantly reduced relative to that of Humulin. HIM2 clearance was undoubtedly altered as a result of the structural modification of the molecule. Its structure is composed of a single ampiphilic oligomer of low molecular weight covalently linked to a free amino group on the Lys-B29 residue of recombinant human insulin via an amide bond. It is readily absorbed due to its amphiphilic nature and has enhanced resistance to enzymatic degradation by enzymes, such as insulin protease(82), which is unable to reach its sites of action due to steric interference (264). It remains unclear whether the biological activity of HIM2 insulin was reduced relative to Humulin but since the HIM2 spike brought about an improvement in glycemia relative to the Humulin spike, any decrease in its biological action cannot have been in proportion to its altered clearance. It has also been suggested that HIM2 may have

an increased binding affinity and/or capacity with the insulin receptor in such tissues as the liver and muscle; this increased binding affinity may differ from that which is seen with native insulin, thus leading to a more prolonged activation of the receptor itself (282).

Clearly any improvements in glycemia seen in response to the pulse of insulin cannot be due to the difference in glucose delivery since all three groups received glucose intraduodenally at the same rate. Furthermore, the calculated glucose absorption rate was identical in all three groups. Therefore, the difference in the arterial plasma glucose levels must be attributed to changes in glucose utilization. In the early part of the response (15-120 minutes), the liver ceased glucose production and began to take up glucose most rapidly in the HIM2 group. There are three major factors which control NHGU: the glucose load to the liver, the insulin level, and the portal signal (i.e. generated when the plasma glucose concentration in the portal vein is higher than the arterial plasma glucose concentration). In these experiments, the portal signal was the same in all three groups and thus the differences in NHGU must be related to the glucose load and/or the insulin concentration (214). The increased hepatic response in the Humulin group must have been attributable to the increase in insulin since the hepatic glucose loads over the first 2 hours in the BI and HI groups were equal. In the HIM2 group, the hepatic glucose load was less than that in the basal insulin group, but the insulin spike was much larger. Clearly the latter therefore was responsible for the more rapid increase in NHGU seen in this group.

At the end of the experiment, there were minimal differences between the three groups in either net hepatic glucose balance or hepatic fractional glucose extraction. The rate of NHGU was slightly less with HIM2 as a result of the decreased hepatic glucose load. Nevertheless, differences in NHGU cannot explain the prolonged improvement that the insulin spikes had on the arterial plasma glucose level. Instead it appears to be attributable to the effect of insulin on nonhepatic tissues. This is somewhat surprising given that the plasma insulin levels returned to baseline no later than 60 min after dosing even in the HIM2 group. One possible explanation for the finding, however, is that the insulin levels in the interstitial fluid may still be elevated at these latter time points as suggested by Getty et al. (109). Additionally, altered insulin kinetics in the interstitial fluid may have prolonged the effect of HIM2 on nonhepatic tissues even further. In agreement with this, nonhepatic glucose clearance in the HIM2 group was significantly greater than it was in the BI group. Nonhepatic glucose clearance in the HI group, on the other hand, was only slightly greater than that seen in the basal group.

Free fatty acids are known to alter muscle and liver glucose metabolism. There exists an inverse relationship between plasma free fatty acid concentrations and insulin sensitivity (256). In normal individuals, an increase in free fatty acids has been shown to cause hepatic insulin resistance by interfering with the normal suppression of glycogenolysis by insulin (34). Insulin resistance in skeletal muscle can also be attributed to an increase in fatty acids. Type 2 diabetics who are given nicotinic acid in order to induce a *decrease* in plasma FFA levels have a

decrease in gluconeogenesis with no compensatory increase in glycogenolysis; this leads to an overall decrease in endogenous glucose production(33). It has been shown (259) that hepatic glucose production can be suppressed by an increase in peripheral insulin partly due to an inhibition of lipolysis. We have shown in the dog that FFA levels control the glycolytic flux in the liver(242). A fall in FFA levels and as a result in the net hepatic uptake of FFA, directs intrahepatic carbon into glycolysis eventually giving rise to lactate (259). Given that the FFAs fall most dramatically in the HIM2 group and substantially in the Humulin group, the early differences in net hepatic glucose output might in part be attributable to changes in plasma free fatty acid levels. Wajcberg et al. (282) have suggested that HIM2's prolonged biological action may be attributed to a persistent suppressive effect on FFA. There may be a prolongation of antilipolysis due to an enhancement of HIM2 binding to the insulin receptors in the adipocytes. Unless these early changes in free fatty acids had a prolonged effect on glucose uptake by muscle, it seems unlikely that they were affecting the glucose profile towards the end of the experiments since the plasma FFA levels were similar in all three groups by then.

In summary, a brief 5 minute pulse of insulin infused intraportally at a rate of 10 mU/kg/min simulated the first phase insulin release seen postprandially, increased plasma insulin levels for 30 minutes, and eliminated net hepatic glucose production by 60 minutes. It still had a significant effect on plasma glucose levels 4.5 hours later. HIM2 was cleared less efficiently than Humulin, resulting in higher plasma insulin levels at both the liver and the periphery. This pulse

increased plasma insulin levels for almost 60 minutes, eliminated net hepatic glucose production by 30 minutes, and continued to have a significant effect on plasma glucose levels even at the end of the experiment (4.5 hours). These data demonstrate the ability of a brief burst of insulin secretion to result in prolonged glycemic improvement and speak to the importance of first phase insulin release in postprandial glucose homeostasis. They also point to the need to further evaluate the pharmacokinetic and pharmacodynamic properties of the HIM2 insulin analogue. Subsequent comparison studies will be done using HIM2 and Humulin and matching the concentrations of insulin by adjusting the rates of infusion. Several other studies have also more recently examined oral doses of HIM2 in individuals with Type I diabetes, Type II diabetes, and nondiabetic subjects (60, 148, 282) and found them to be effective. Thus it should be possible to administer HIM2 via the oral route in future studies.

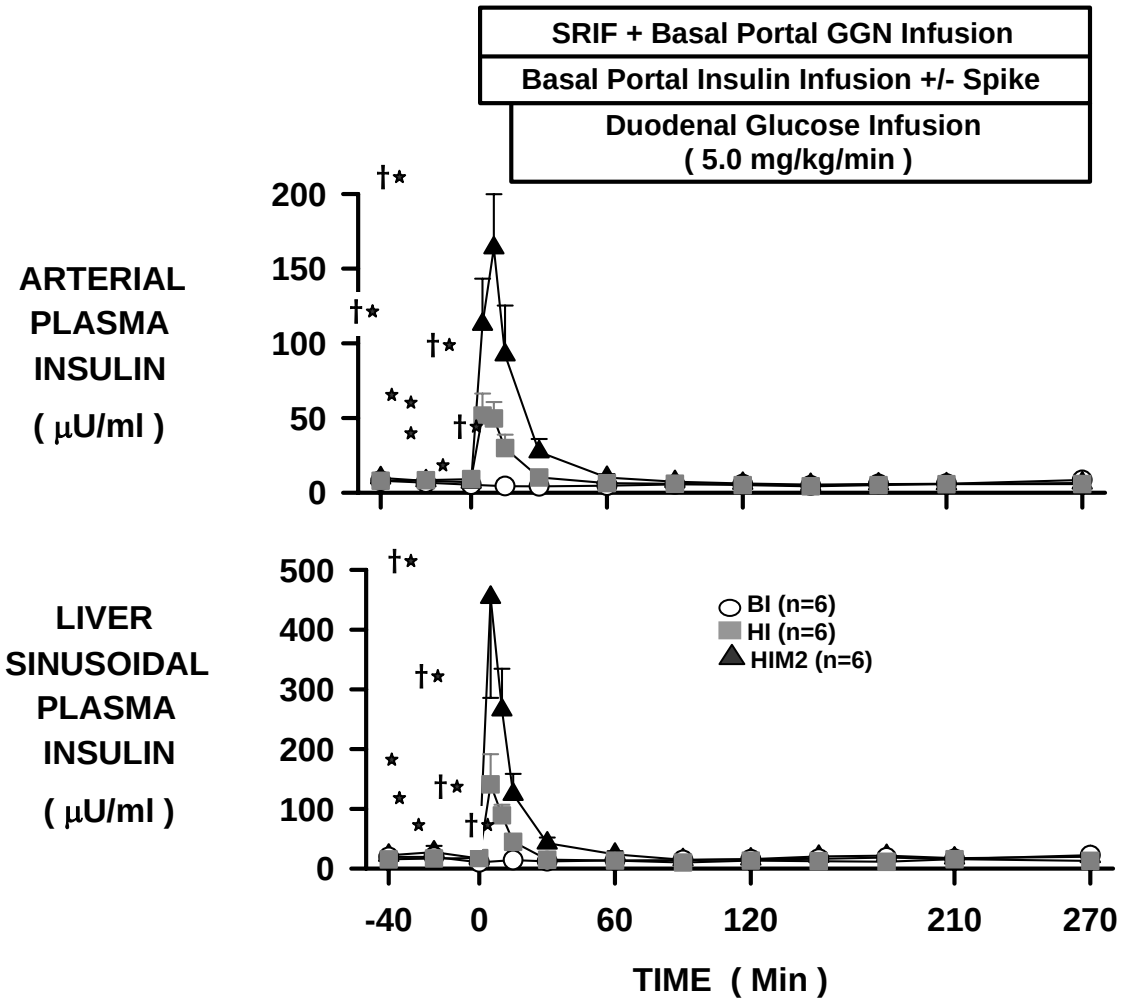


**Figure 3.1**: Arterial plasma insulin (top) and liver sinusoidal plasma insulin (bottom) in 42-h-fasted conscious dogs maintained on a pancreatic clamp. During the experimental period, insulin was kept basal (BI), or Humulin spike (HI), and HIM2 spikes (HIM2) were created on the background of basal insulin (n =6/group). SRIF, somatostatin; GGN, glucagon. Data are mean ± SE. ★ = P<0.05 from BI † = P<0.05 from HI.

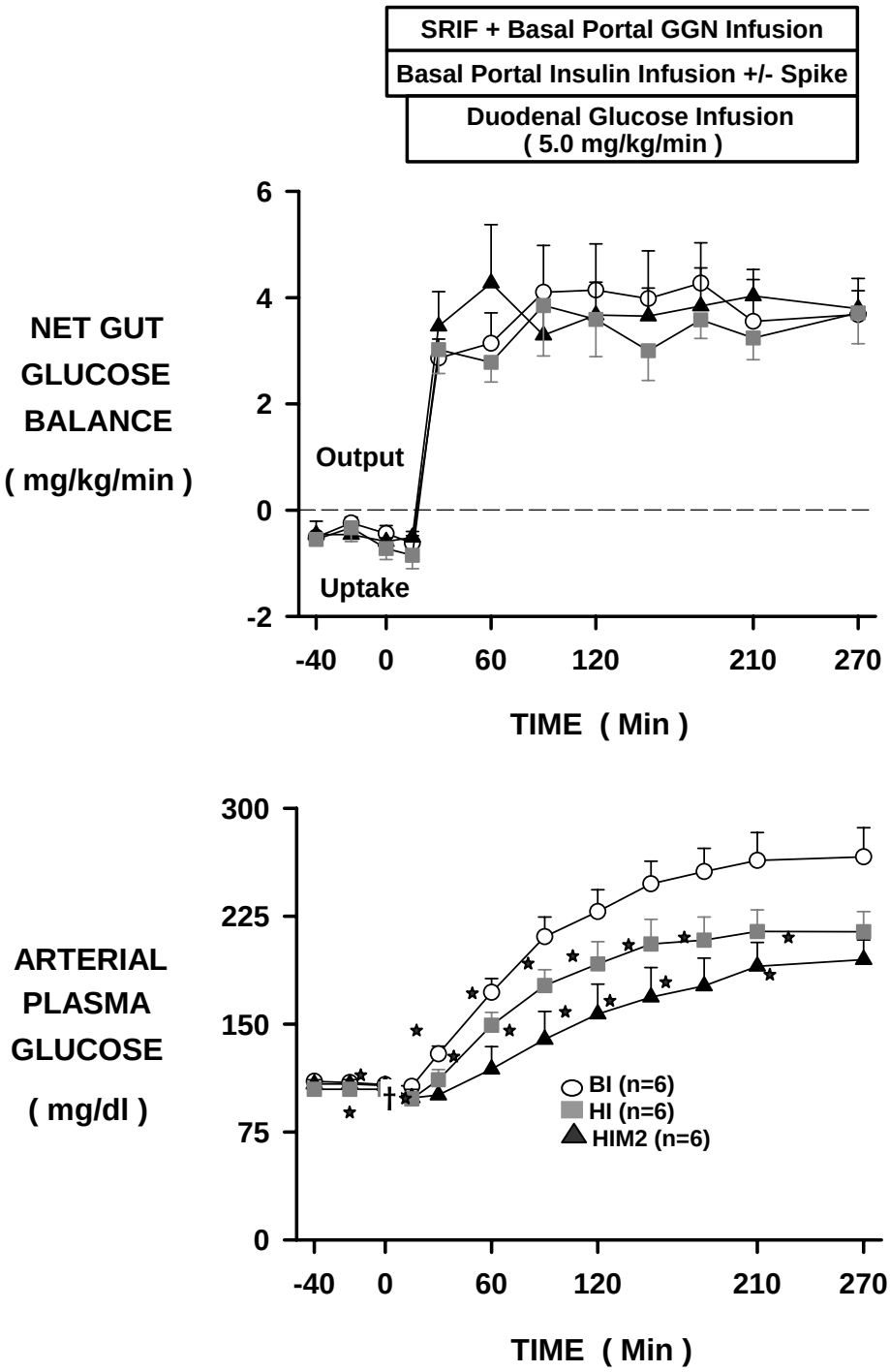


**Figure 3.2**: Net gut glucose balance (top) and arterial plasma glucose levels (bottom) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in the BI, HI, and HIM2 groups (n = 6/group). Data are mean ± SE. ★ = P<0.05 from BI † = P<0.05 from HI

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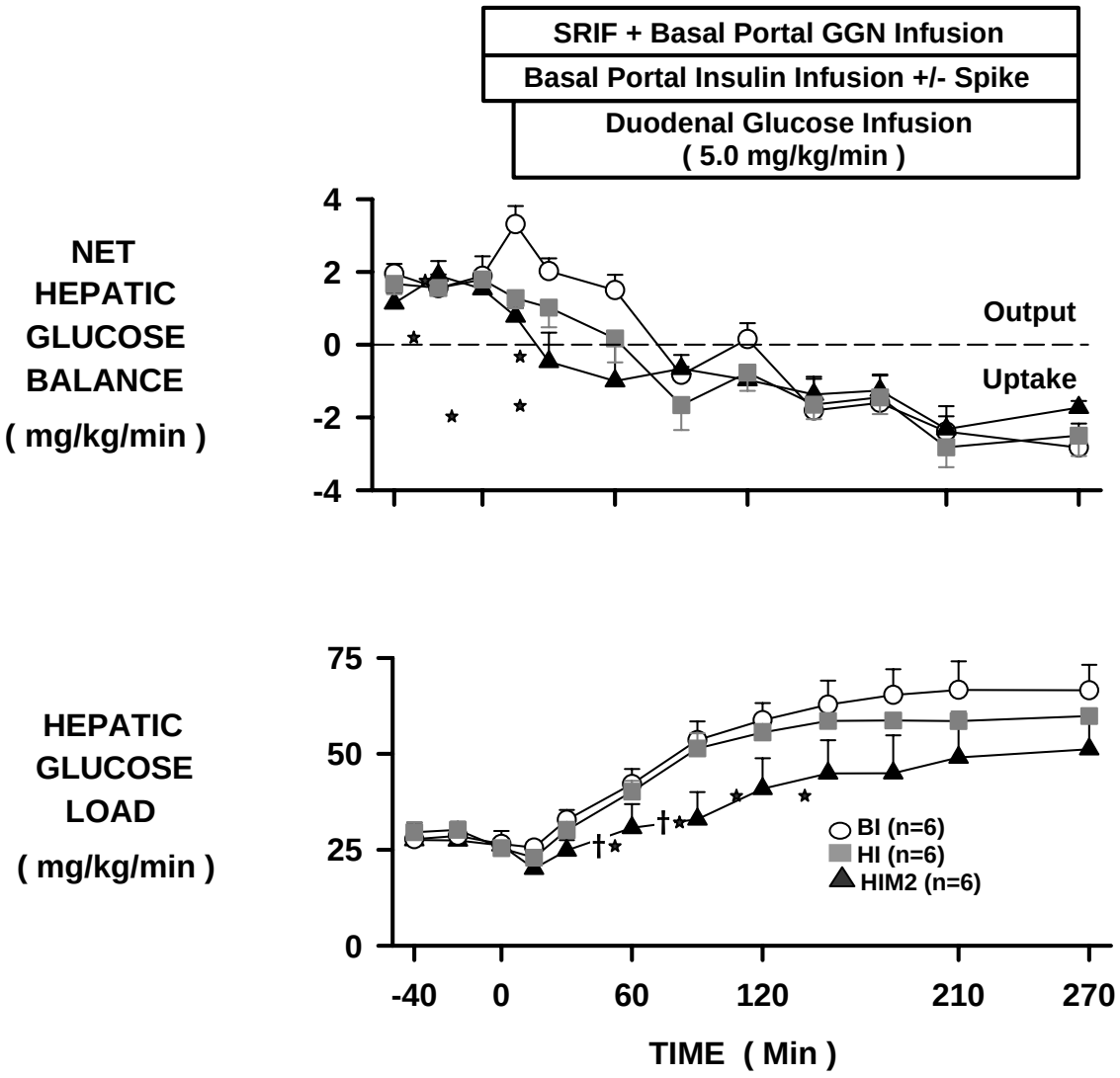


**Figure 3.3**: Net hepatic glucose balance (top) and hepatic glucose load (bottom) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in the BI, HI, and HIM2 groups (n = 6/group). Data are mean ± SE. ★ = P<0.05 from BI † = P<0.05 from HI

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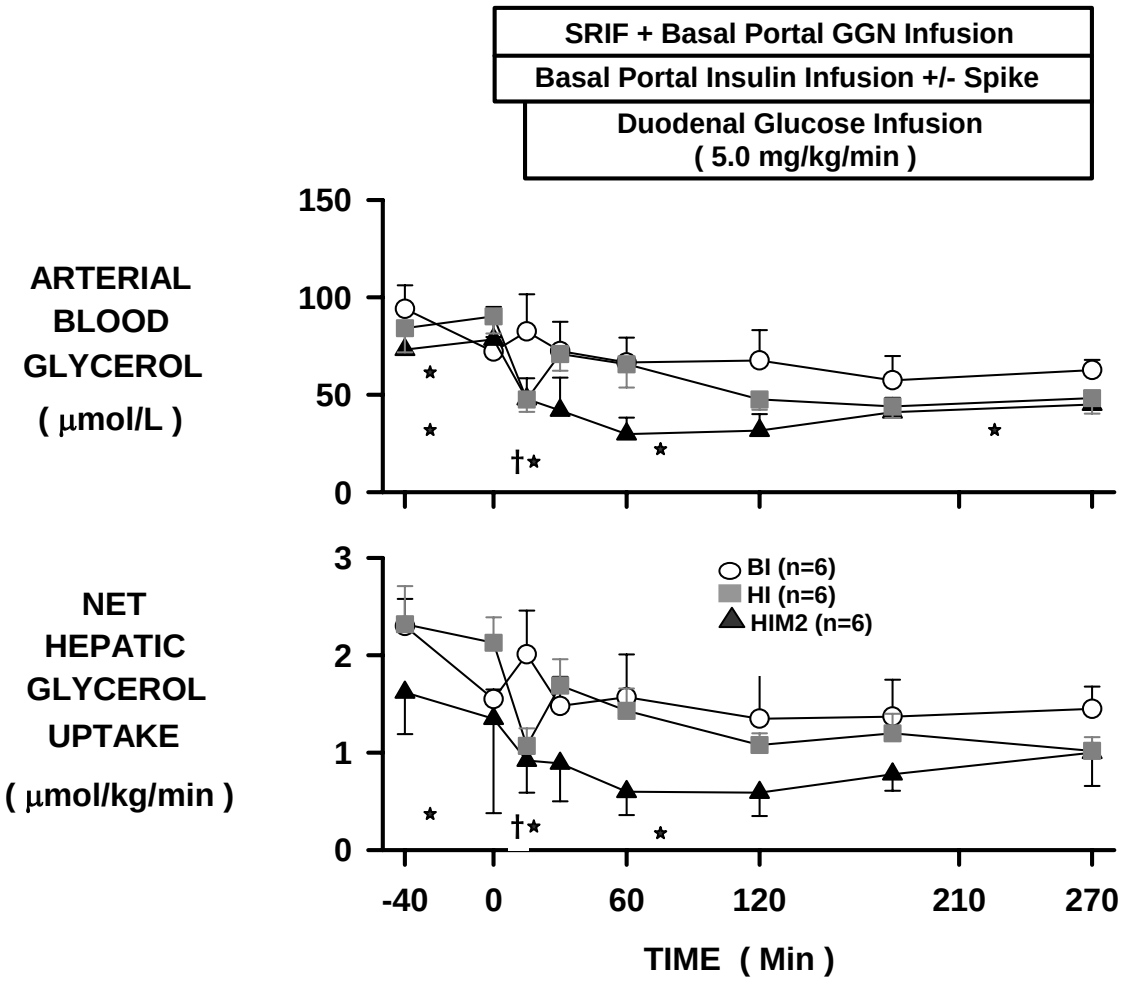


**Figure 3.4**: Arterial blood glycerol levels (top) and net hepatic glycerol uptake (bottom) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in the BI, HI, and HIM2 groups (n = 6/group). Data are mean ± SE. ★ = P<0.05 from BI † = P<0.05 from HI.

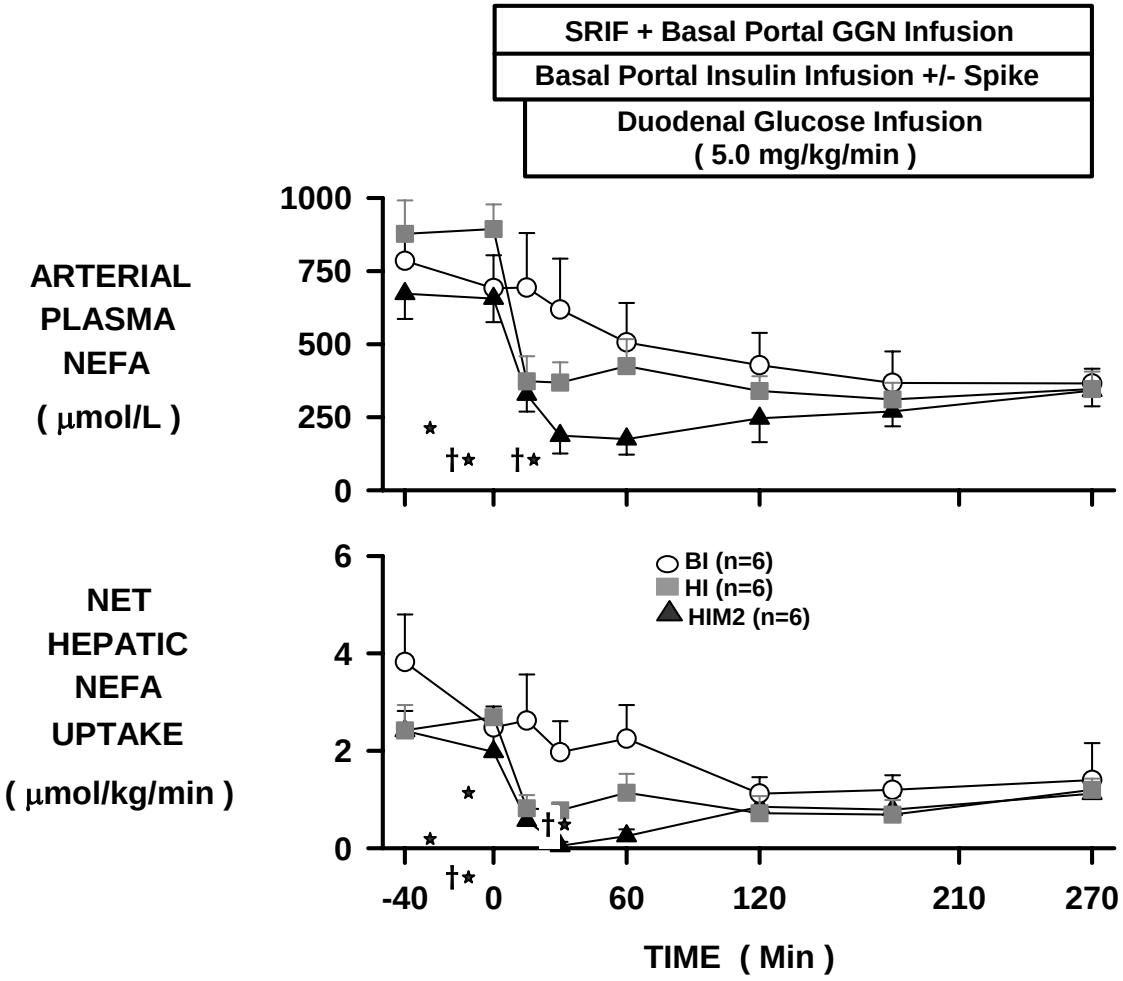


**Figure 3.5**: Arterial plasma non-esterified fatty acid (NEFA) levels (top) and net hepatic NEFA uptake (bottom) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in the BI, HI, and HIM2 groups (n = 6/group). Data are mean ± SE. ★ = P<0.05 from BI † = P<0.05 from HI.

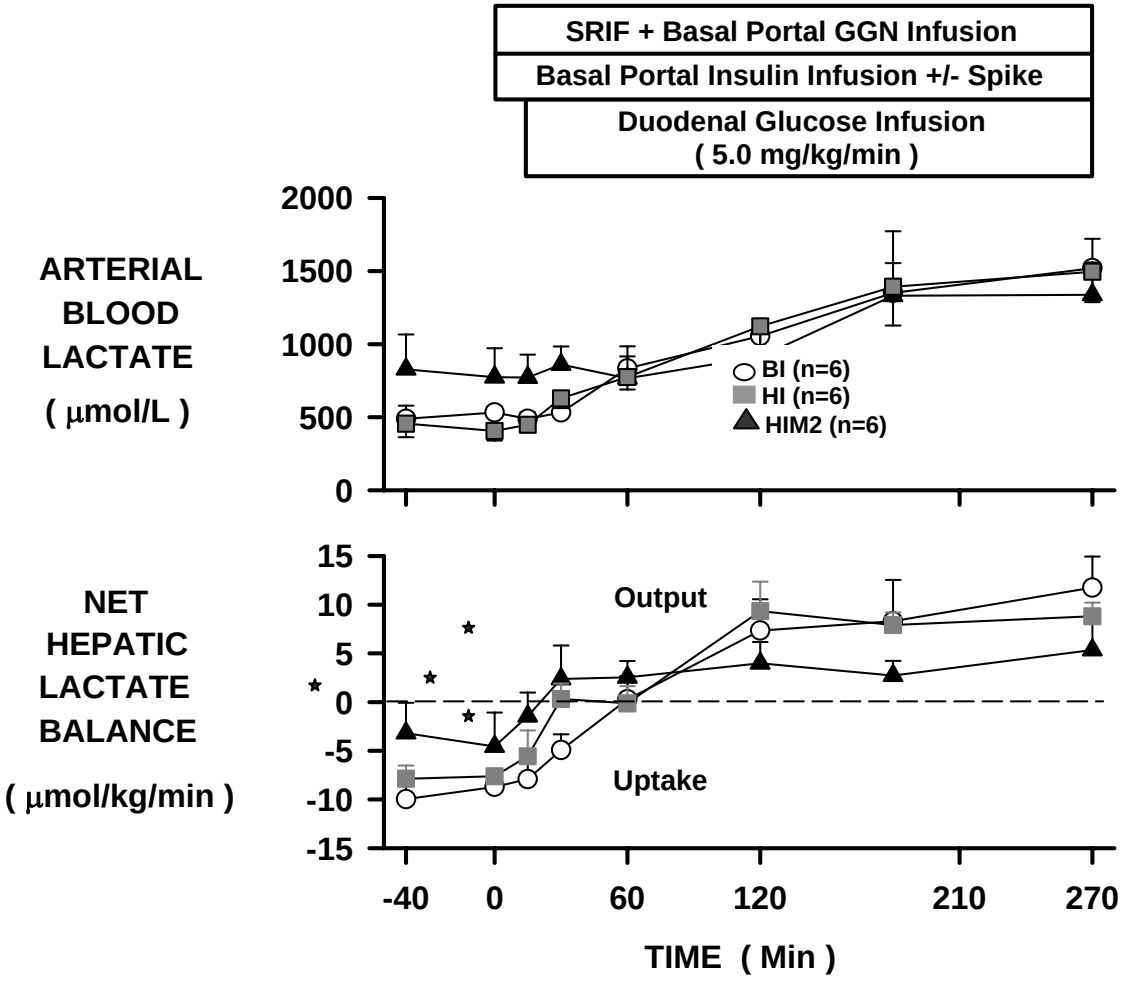


**Figure 3.6**: Arterial blood lactate levels (top) and net hepatic lactate balance (bottom) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in the BI, HI, and HIM2 groups (n = 6/group). Data are mean ± SE.★ = P<0.05 from BI † = P<0.05 from HI.

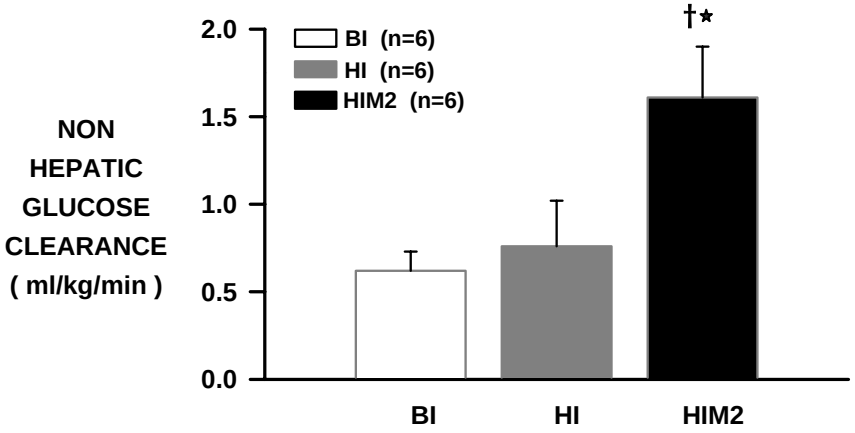


**Figure 3.7**: Non hepatic glucose clearance in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the experimental period in the BI, HI, and HIM2 groups (n = 6/group). Data are mean ± SE.. * = P<0.05 from BI † = P<0.05 from HI

# CHAPTER IV

## THE ROLE OF THE HEPATIC SYMPATHETIC NERVES IN THE REGULATION OF NET HEPATIC GLUCOSE UPTAKE AND THE MEDIATION OF THE PORTAL GLUCOSE SIGNAL

### Aim

Specific aim II was to examine the role of the sympathetic nerves in mediating the effects of the portal glucose delivery. We hypothesized that the sympathetic nervous system exerts a restraining effect on hepatic glucose uptake that can be reversed by the entry of glucose into the portal vein. We suggested that eliminating the sympathetic nerves using selective hepatic sympathectomy would result in an increase in NHGU in response to peripheral glucose infusion and a reduction in the augmentation of NHGU in response to the portal glucose signal.

### Experimental Design

Each experiment consisted of a 100-min equilibration period (-140 to –40 min), a 40-min basal period (-40 to 0 min), and a 270-min experimental period (0 to 270 min) which was divided into three 90 minute periods denoted as P1, P2, and P3. In all experiments, a constant infusion of indocyanine green dye (0.076mg/min) was initiated at –140 min. At 0 min, a constant infusion of somatostatin (0.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was begun to suppress endogenous insulin and

glucagon secretion. Glucagon (0.57 $ng \cdot kg^{-1} \cdot min^{-1}$) and insulin (0.3$mU \cdot kg^{-1} \cdot min^{-1}$) were then replaced intraportally at basal rates. In addition, a primed continuous peripheral infusion of 50% dextrose was begun at time 0 so that the blood glucose could quickly be clamped at the desired hyperglycemic level (~ 235 mg/dl). During P1 (t=0 to t=90), glucose was infused peripherally to double the hepatic glucose load (HGL) in both groups. During P2 (t=90 to t= 180), a portal glucose infusion of 20% dextrose at (3-4 mg/kg/min) was initiated to activate the portal signal, and the peripheral glucose infusion was decreased so that the same glucose load to the liver seen in P1 was maintained in P2. During P3 (t=180 to t=270), the portal glucose infusion was terminated and the peripheral glucose infusion was again adjusted to match the HGLs across the three periods. The peripheral glucose infusion rate was adjusted based on the plasma glucose levels and the hepatic blood flow in each individual dog. PAH was added to the portal glucose infusate to assess proper mixing with the blood in the portal and hepatic veins. In each group, 13 dogs were studied and 3 dogs were not included due to poor mixing as defined previously in the Methods section. In the control and denervated animals that were retained, the ratio of PAH recovery in the portal vein to the PAH infusion rate was 1.0±0.1 and 1.0±0.1 whereas the ratio of PAH recovery in the hepatic vein to the PAH infusion rate was 0.9±0.03 and 1.0±0.04, respectively.

Femoral artery, portal vein, and hepatic vein blood samples were taken every 20 min during the basal period (-40 to 0 min) and every 15 minutes for the last half hour of each experimental period (P1, P2, and P3). Arterial blood

samples were also taken every 5 min from 0 to 270 minutes of the experimental period to allow changes to be made in the glucose infusion rate as necessary.

## Statistical Analysis

Time course data were analyzed with repeated-measures analysis of variance. Independent *t*-tests were used for any comparisons of mean data. Statistical significance was accepted at $p<0.05$.

## Results

**Hepatic sympathetic denervation:** Liver norepinephrine levels (Table 4.1) were assessed using HPLC. The average norepinephrine level in the CON group was 658 ± 68 ng/g liver while in the DEN group, it was only 10 ± 4 ng/g liver. These values indicate a >98% denervation.

**Arterial and portal plasma glucose levels:** The arterial plasma glucose levels in the control group was 238±3, 236±7, and 242±3 mg/dl during P1, P2, and P3 respectively while the portal vein plasma glucose levels were 235±3, 255±8, and 239±3 mg/dl respectively (Figure 4.1a). The arterial plasma glucose levels (236±3, 223 ±3, 235±2 mg/dl) in the DEN group were similar to those seen in the control group as were the portal vein glucose levels (234±3, 243±4, and 233±2 mg/dl) during P1, P2, and P3 respectively (Figure 4.1b).

**Arterial plasma and liver sinusoidal insulin and glucagon:** Arterial and liver sinusoidal plasma insulin and glucagon levels were maintained at basal values throughout the experiment in both groups (Figure 4.2).

**Hepatic blood flow and glucose load:** Hepatic blood flow (ml/kg/min) was similar in all three periods (P1, P2, and P3) in CON (32±2, 30±2, 32±2) and DEN (31±2, 32±2, 33±2) respectively (Table 4.2). Since the glucose levels and hepatic blood flows were similar in the two groups, there were no differences in the hepatic glucose load across time or between treatments (Table 4.2). The average HGLs (mg/kg/min) in the CON and DEN groups were 53±3 and 54±4 in P1, 55± 3 and 55± 4 in P2, and 57± 3and 55± 5 in P3, respectively. There were no statistical differences in the total glucose infusion rates between the two groups across the three periods. The total glucose infusion rates (mg/kg/min) in P1, P2, and P3 were 3.5±0.4, 4.1±0.2, and 4.1±0.2 in the CON group, respectively. The total glucose infusion rates in the DEN group were 4.3±0.4, 4.8±0.5, and 4.6±0.3 in P1, P2, and P3, respectively.

**Blood levels and net hepatic balance of lactate and glycerol:** Both groups exhibited net lactate uptake in the basal period. Hyperglycemia resulted in a switch to net hepatic lactate output that was similar in both groups and constant over time. The arterial blood lactate levels rose steadily over the three periods in both groups due to net hepatic lactate output (Table 4.3).

Arterial glycerol levels and net hepatic glycerol uptake fell rapidly in response to hyperglycemia in both groups and eventually plateaued at an average of ~50 μmol/L and ~1 μmol/kg/min (Table 4.3) respectively (no differences between groups).

**Net hepatic glucose balance and non-hepatic glucose uptake**: Basal net hepatic glucose output was not statistically different between the two groups (1.8±0.3 and 1.9±0.2 mg/kg/min in the CON and DEN groups, respectively). Due to the drift that is typically seen in NHGU over time (Table 4.4), P1 and P3 can be averaged to obtain the mean NHGU response to peripheral hyperglycemia. Thus, the average NHGU during peripheral glucose infusion in the CON group was 1.7±0.3 mg/kg/min. NHGU in response to peripheral glucose infusion was significantly greater ($P<0.05$) in the DEN group (2.9±0.4 mg/kg/min; Figure 4.3a). NHGU significantly increased during P2 (portal glucose infusion) in the CON group, increasing to 2.9±0.3 (Δ1.2) mg/kg/min, but did not change significantly in the DEN group (3.2±0.2 (Δ0.3) mg/kg/min).

Net hepatic fractional glucose extraction was 3.1% and 5.4% in response to peripheral glucose infusion in the CON and DEN groups, respectively. It increased in response to portal glucose infusion in CON group (5.4%) ($P<0.05$) but failed to increase significantly (6.5%) in the DEN group (Table 4.4).

The average non hepatic glucose uptake rate seen in response to peripheral glucose infusion was 2.2±0.1 mg/kg/min in the CON group and 1.6±0.4 mg/kg/min in the DEN group (Table 4.4). Non hepatic glucose uptake decreased

significantly ($P<0.05$) during portal glucose infusion (P2) in the CON group to 1.2±0.4 (Δ1.0) mg/kg/min but it did not change during the same time period in the DEN group (1.6±0.4 (Δ0.0) mg/kg/min; Figure 4.3b).

## Discussion

In this study we tested the hypothesis that a basal inhibitory sympathetic tone to the liver blocks NHGU and that this inhibition is relieved by entry of glucose into the portal vein. Our findings support this concept. There was almost a doubling of NHGU during peripheral glucose infusion in dogs that had undergone selective hepatic sympathectomy compared to those that had not. Furthermore, when the liver was sympathetically denervated, the response to portal glucose delivery was reduced by ≈75%. The data regarding nonhepatic glucose uptake are less clear. Although nonhepatic glucose uptake was lower during peripheral glucose infusion in DEN and it did not change in response to portal glucose infusion, the study lacked the statistical power to allow definitive conclusions to be drawn.

Sympathetic nerve fibers reach the liver through the celiac ganglia, celiac plexus, and the splanchnic nerves (134, 136, 247-249). The sympathetic fibers form an anterior plexus around the hepatic artery and Alexander showed that the hepatic artery receives only sympathetic fibers (12). The parasympathetic fibers form a posterior plexus around the portal vein. The postganglionic parasympathetic nerves are derived from ganglia located at the hepatic hilus and within the portal spaces (100). These parasympathetic fibers are separate from any

aminergic nerves found in the area (14, 62, 181, 231, 261, 269). In order to selectively eliminate sympathetic input to the liver, we removed the nerves associated with the hepatic arterial circulation. The success of the denervation was confirmed by a reduction of norepinephrine in all seven lobes of the liver which averaged 98%. Neural regeneration and hepatic reinnervation of the dog liver requires 3 months (139) and thus the denervation was still virtually complete two weeks post surgery. It is possible that some vagal innervation may have been compromised during surgery, but given the association of parasympathetic nerves with the portal vein, we believe that most of the parasympathetic fibers remained intact. They were thus still able to exert any regulatory effects which they might have on NHGU and nonhepatic glucose uptake. It should be noted that there were no differences in body weight, food consumption, or basal NHGU between the two groups, suggesting that the chronic decrease in hepatic sympathetic tone did not have any demonstrable baseline effect on the animals. It is important to note that we did not directly measure hepatic sympathetic tone during these experiments but assumed that the level of norepinephrine in the liver reflected an elimination of sympathetic input to the liver.

Several variables that affect NHGU were minimized in order to accurately assess the effect of the selective sympathetic denervation on NHGU. Arterial insulin was maintained at a basal level in both groups in order to avoid the variability seen in the response of NHGU to a rise in insulin. Likewise, the arterial plasma glucagon concentrations were kept constant and basal. The arterial plasma glucose concentration was doubled and clamped across the three periods in both

groups. Since there was no change in hepatic blood flow, the hepatic glucose load to the liver was the same throughout the three experimental periods and was equal in both groups. Thus, the differences in NHGU between the two groups cannot be explained by differences in plasma insulin or glucagon levels or the glucose load to the liver.

Prior work in our laboratory has examined the effect of portal glucose delivery on NHGU. In hyperglycemic experiments performed by both Pagliassotti et al. (214) and Hseih et al. (134) in which both insulin and glucagon were kept basal while plasma glucose was doubled, peripheral glucose delivery resulted in average NHGUs (mg/kg/min) of 0.4±0.3 and 1.5±0.3, respectively. Thus the NHGU of 1.7±0.3 mg/kg/min which we observed in response to peripheral glucose administration in the control animals of the present study is consistent with previous data. In our earlier studies (134, 214), when glucose was given intraportally at ~3.5 mg/kg/min and the hepatic glucose loads were matched to those seen during peripheral glucose administration, NHGU (mg/kg/min) increased to 2.3±0.7 and 3.4±0.5, respectively. Thus we have shown previously that the portal signal, on the background of hyperglycemia, basal insulin, and basal glucagon, increases NHGU an average of ~1.9 mg/kg/min. In the present control experiments, the increase in NHGU during portal vs. peripheral glucose delivery was 1.2±0.2 mg/kg/min, in line with the increase we have observed previously.

The dogs with sympathetically denervated livers responded to hyperglycemia quite differently from the normal animals. The average NHGU

during the peripheral glucose infusion period was 2.9±0.4 mg/kg/min, a value almost double ($P<0.05$) that seen under the same conditions in the dogs with innervated livers. With portal glucose delivery, NHGU increased to 3.2±0.2 mg/kg/min in the DEN group, which is a similar value to that seen in the control animals (2.9±0.3 mg/kg/min), yet only ~0.3 mg/kg/min greater than that seen during average peripheral glucose administration. This limited increase is not due to a saturation of the hepatic response since these rates of NHGU are significantly lower than rates we have previously shown to occur during portal glucose infusion (254) and lower than the peak rates observed during oral glucose delivery (1). Thus we conclude that the sympathetic efferents play an important role in the regulation of NHGU by exerting a basal inhibitory tone which limits glucose uptake in response to hyperglycemia of peripheral origin. This is consistent with previous work from our laboratory (45) which showed that cooling the vagus nerves (decreasing afferent vagal firing) in the presence of euglycemia and euinsulinemia decreased net hepatic glucose output, presumably by reflexively decreasing the efferent sympathetic outflow to the liver. It is interesting to note that we saw no differences in basal net hepatic glucose output between the two groups in this current study despite hepatic denervation. There are several possible explanations for the discrepancy between our two studies. First it is possible that vagal cooling results in changes in addition to reduced sympathetic input to the liver. Alternatively, there may be a difference between acute and chronic sympathectomy.

The question arises as to whether cutting the sympathetic efferents changed NHGU in response to *glucose* per se(i.e. regardless of the site of delivery) or if, in addition, it blocked the effect of the portal glucose signal. If the response was to glucose per se, then the portal glucose signal should have caused a change of ~1.2 mg/kg/min when glucose was delivered intraportally in the hepatic denervated dogs. Since the change was only 0.3 mg/kg/min, these data suggest that reduced sympathetic tone to the liver is responsible for a significant portion of the increase in NHGU attributed to portal glucose delivery. It must be remembered, however, that we chronically removed sympathetic efferents to the liver. Chronic removal of sympathetic input may result in changes which increase the ability of glucose to increase NHGU which might not occur in response to the acute removal of sympathetic input associated with portal glucose delivery. It is also possible that sympathetic denervation resulted in a saturation of the response to the portal glucose signal, thus masking any further input (i.e., positive stimuli) generated by portal glucose delivery. Thus while our data suggest that the portal glucose signal increases NHGU by causing a decrease in sympathetic tone, further proof of this concept is still required.

Based on our data, we cannot draw a definitive conclusion about the additional involvement of a stimulatory signal in the response to portal glucose administration. In the control dogs, the response to portal glucose infusion was ~1.2 mg/kg/min while in the denervated animals it was ~0.3 mg/kg/min. Taken at face value, this would suggests that 75% of the response to portal glucose delivery is caused by removal of an inhibitory tone and 25% is due to the activation of a

stimulatory signal. In the presence of the portal signal, there is an increase in glycogen synthase activation which could act as the stimulatory factor (214). It has also been suggested that the portal signal may cause the translocation of glucokinase thus also contributing a stimulatory input (47). Unfortunately, the present study lacks the power needed to determine the quantitative contribution of any putative stimulatory signal to the overall response, so that equal contribution by inhibitory and stimulatory factors remains possible.

Previous work in our laboratory by Shiota et al. (255) showed that an intraportal infusion of α- and β-adrenergic receptor antagonists (phentolamine and propanolol, respectively) did not enhance NHGU during hyperinsulinemia and hyperglycemia (achieved via peripheral glucose infusion). This is in apparent conflict with our current findings but several issues should be noted. Intraportal infusion of the adrenergic blockers would have caused only a partial blockade of sympathetic tone to the liver if the dose of the blockers used was inadequate. Likewise, it is not clear if blockers delivered via the portal vein can reach the synapse and block endogenously released norepinephrine effectively. Additionally, phentolamine has been shown to stimulate postsynaptic ($\alpha_1$) receptors while inhibiting presynaptic ($\alpha_2$) receptors, resulting in an enhanced output of norepinephrine (283). It has also been shown that propanolol can have an intrinsic (partial agonist) effect on β-adrenergic receptors in the liver (55). The partial agonistic properties of these adrenergic antagonists may thus have counteracted any effects attributable to adrenergic blockade. Another way to reconcile our current observations and our previous findings would be to conclude

that, in the present study, we removed afferent nerve fibers that travel with the efferent sympathetic nerves. For that to be the case, the afferent fibers would initiate an inhibitory signal to the liver through non-adrenergic mechanisms which would be blocked by sympathetic nerve section. This seems unlikely since it has been shown that glucose sensitive afferent fibers originating in the liver travel with the vagus nerves.

It has been shown that portal glucose delivery not only leads to an increase in hepatic glucose uptake but also to a decrease in nonhepatic glucose uptake (3, 134, 214). Under conditions similar to those seen in this experiment, Hsieh showed an average nonHGU of 4.3±0.5 mg/kg/min during peripheral glucose administration, which decreased to 1.0±0.3 mg/kg/min during portal glucose administration and then returned to 4.6±0.7 mg/kg/min at the termination of the portal glucose signal. In the control animals of the current study, the average nonHGU during the first peripheral glucose infusion period was 2.1±0.2 mg/kg/min, decreasing significantly to 1.2±0.4 mg/kg/min with portal glucose delivery. At the termination of the portal glucose administration, nonHGU returned to 2.3±0.2 mg/kg/min. Thus the response to portal glucose delivery in the control animals was consistent with previous data although the nonHGU was lower in P1 and P3 than in earlier studies for reasons which are not clear. In the animals with denervated livers, nonHGU was only slightly less in response to peripheral glucose infusion (1.6±0.4 mg/kg/min) than in the control dogs. There was no change in nonhepatic glucose balance in response to portal glucose infusion in the DEN group. Given the subtle difference noted above, however one

cannot draw a definitive conclusion regarding the role of sympathetic nerves in the mediation of the change in nonHGU.

In summary, the sympathetic nerves exert an inhibitory tone on liver glucose uptake. Removal of this inhibition by selective hepatic sympathetic denervation leads to an increase in NHGU in response to hyperglycemia induced by peripheral glucose infusion. It also results in a blunting of the increase in NHGU seen in response to intraportal glucose delivery. The portal signal, therefore, leads to the removal of an inhibitory tone to the liver which in turn allows NHGU to increase. Whether hepatic sympathetic nerves act in concert with an additional stimulatory signal still needs to be determined.

**Table 4.1**: *Hepatic norepinephrine content in each of the seven lobes of 42 hr fasted dogs with innervated (CON) or denervated (DEN) livers*

| Lobe | Norepinephrine Content ng/g liver | | % NE Remaining |
|---|---|---|---|
| | **CON** | **DEN** | |
| Caudate | 476±83 | 2.5±2.7 | 0.5 |
| Left Central | 524±126 | 4.3±3.8 | 0.8 |
| Left Lateral | 545±90 | 3.8±3.2 | 0.6 |
| Left Posterior | 939±202 | 3.8±2.3 | 0.4 |
| Quadrate | 582±82 | 29.1±21.4 | 5.0 |
| Right Lateral | 546±94 | 27.6±12.6 | 5.0 |
| Right Central | 716±149 | 4.4±3.6 | 0.6 |

*Data are mean ± SE. n=10 group*

**Table 4.2:** *Total hepatic blood flow and hepatic glucose load during the experimental periods in experiments on 42 hr fasted dogs with innervated (CON) or denervated (DEN) livers*

**Total Hepatic Blood Flow (ml/kg/min)**

| | Period 1 | | | | Period 2 | | | | | Period 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time (min)** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** | **195** | **210** | **240** | **255** | **270** |
| **CON** | 30±2 | 31±2 | 32±2 | 32±3 | 31±2 | 30±2 | 30±2 | 30±2 | 30±3 | 31±3 | 31±2 | 32±2 | 31±3 | 32±2 |
| **DEN** | 31±2 | 32±2 | 31±2 | 31±2 | 32±2 | 32±2 | 32±3 | 31±2 | 32±2 | 33±2 | 32±2 | 33±2 | 34±2 | 34±2 |

**Hepatic Glucose Load (mg/kg/min)**

| | Period 1 | | | | Period 2 | | | | | Period 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time (min)** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** | **195** | **210** | **240** | **255** | **270** |
| **CON** | 52±3 | 54±4 | 55±4 | 55±5 | 56±4 | 56±5 | 54±4 | 56±4 | 55±5 | 52±5 | 56±5 | 56±4 | 55±5 | 56±5 |
| **DEN** | 52±4 | 54±4 | 53±4 | 53±3 | 56±3 | 55±3 | 56±4 | 55±3 | 56±3 | 53±3 | 55±3 | 56±3 | 58±4 | 58±3 |

*Data are mean ± SE. n=10 group  *= Significant statistical difference (P<0.05) from CON*

**Table 4.3:** *Arterial blood lactate and glycerol and net hepatic balance of lactate and glycerol during the basal and experimental periods in experiments on 42 hr fasted dogs with innervated (CON) or denervated (DEN) livers*

**Arterial Blood Lactate (umol/L)**

| | Basal | P1 | P2 | P3 |
|---|---|---|---|---|
| **CON** | 384±52 | 845±104 | 1064±78 | 1036±107 |
| **DEN** | 532±114 | 1195±153* | 1318±131* | 1331±128* |

**Net Hepatic Lactate Balance (umol/kg/min)**

| | Basal | P1 | P2 | P3 |
|---|---|---|---|---|
| **CON** | -5.9±0.8 | 5.8±2.4 | 6.9±1.5 | 5.4±1.6 |
| **DEN** | -7.0±1.5 | 8.3±2.7 | 7.3±1.9 | 7.7±1.8 |

**Arterial Blood Glycerol (umol/L)**

| | Basal | P1 | P2 | P3 |
|---|---|---|---|---|
| **CON** | 84±10 | 53±10 | 47±8 | 48±6 |
| **DEN** | 84±10 | 56±7 | 50±7 | 49±7 |

**Net Hepatic Glycerol Uptake (umol/kg/min)**

| | Basal | P1 | P2 | P3 |
|---|---|---|---|---|
| **CON** | 1.8±0.2 | 1.1±0.2 | 1.0±0.2 | 1.0±0.2 |
| **DEN** | 2.1±0.3 | 1.2±0.2 | 1.1±0.2 | 1.0±0.2 |

*Data are mean ± SE. n=10 group *= Significant statistical difference (P<0.05) from CON (-) indicates uptake*

**Table 4.4***: Net hepatic glucose uptake, net hepatic glucose fractional extraction, and non hepatic glucose uptake during the experimental periods in experiments on 42 hr fasted dogs with innervated (CON) or denervated (DEN) livers.*

**Net Hepatic Glucose Uptake (mg/kg/min)**

| | Basal | P1 | P2 | P3 |
|---|---|---|---|---|
| **CON** | 1.8±0.3 | 1.4±0.4 | 2.9±0.3§ | 2.0±0.3 |
| **DEN** | 1.9±0.2 | 2.5±0.4* | 3.2±0.2 | 3.3±0.5* |

**Net Hepatic Glucose Fractional Extraction (%)**

| | | | | |
|---|---|---|---|---|
| **CON** | | 2.6±0.6 | 5.4±0.4§ | 3.6±0.3 |
| **DEN** | | 4.9±0.5* | 6.5±0.7 | 6.1±0.5* |

**Non Hepatic Glucose Uptake (mg/kg/min)**

| | | | | |
|---|---|---|---|---|
| **CON** | | 2.1±0.2 | 1.2±0.4§ | 2.3±0.2 |
| **DEN** | | 1.9±0.2 | 1.6±0.4 | 1.4±0.5 |

*Data are mean ± SE. n=10 group  *= Significant statistical difference (P<0.05) from CON*
*§= Significant statistical difference (P<0.05) from P1 and P3*

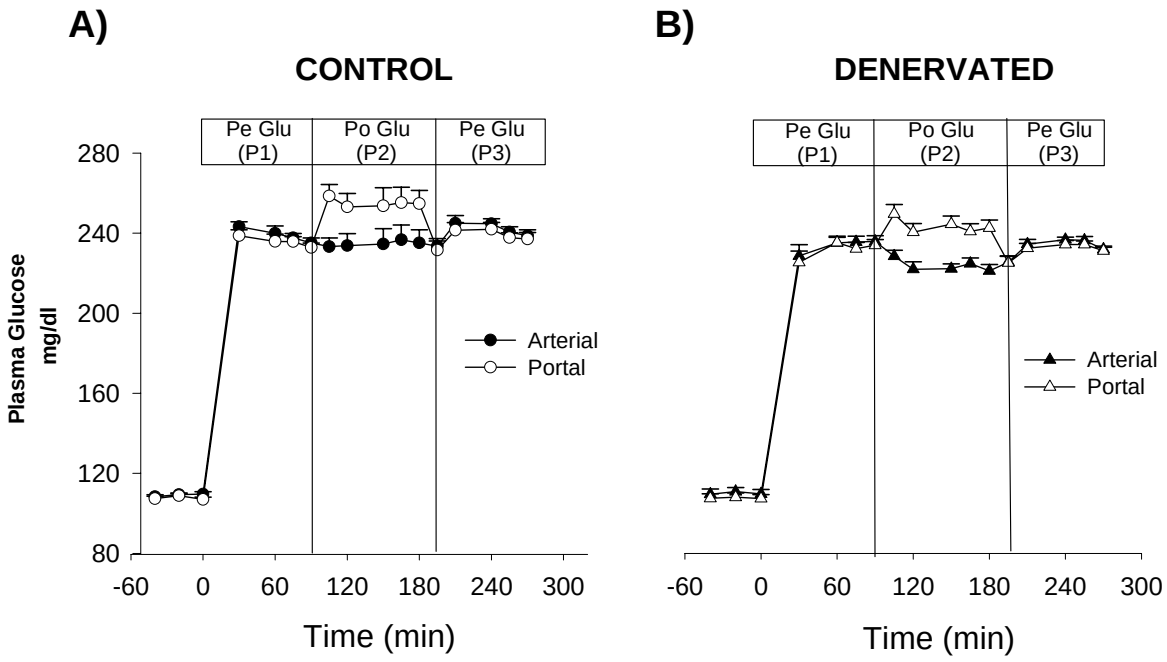


**Figure 4.1**: Arterial and portal plasma glucose levels in control (a) and hepatic denervated (b) 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods (n=10/group). Data are mean ± SE.

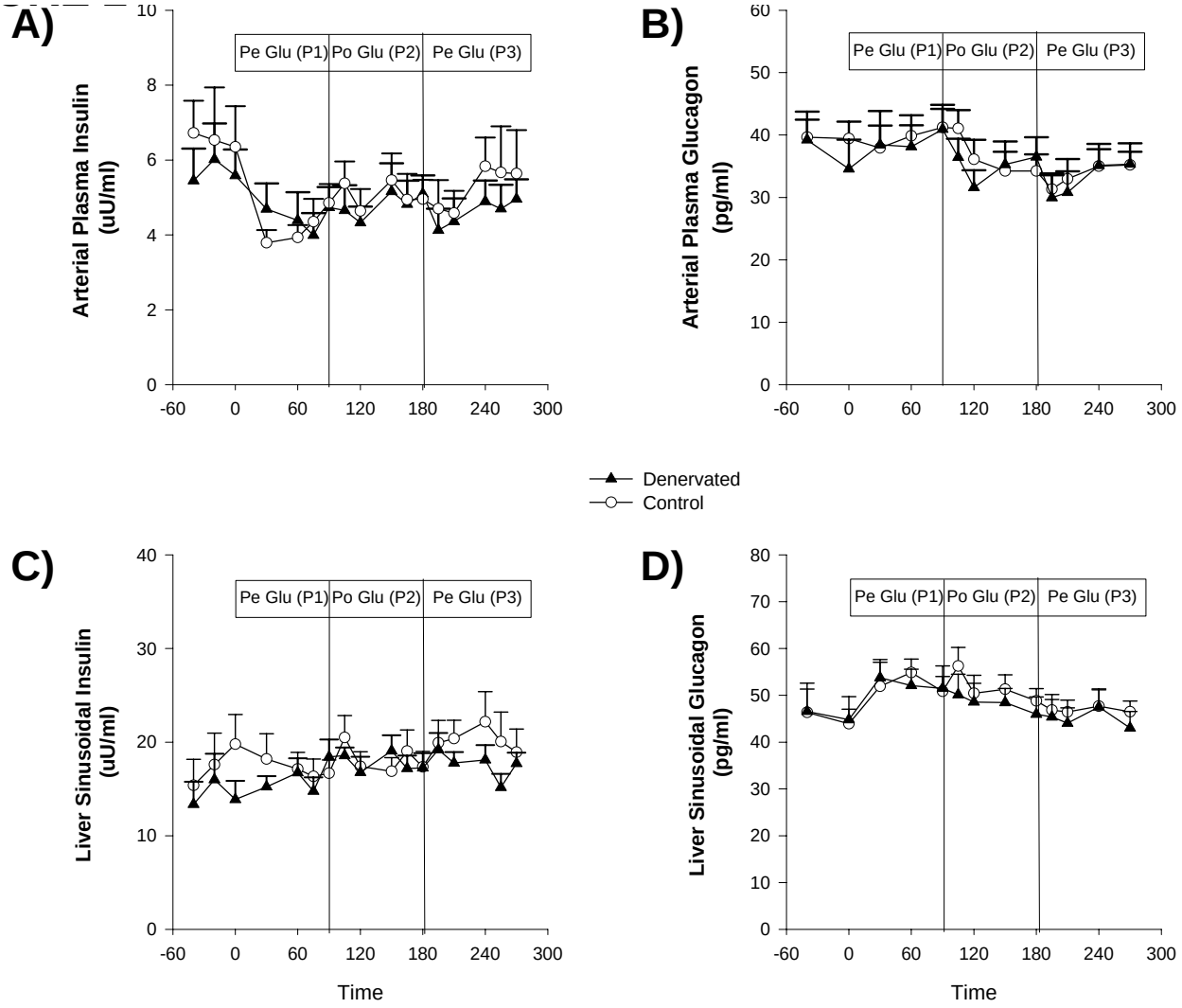


**Figure 4.2:** a) arterial plasma insulin, b) arterial plasma glucagon, c)liver sinusoidal insulin, and d) liver sinusoidal glucagon levels in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in both the CON and DEN groups (n=10/group). Data are mean ± SE

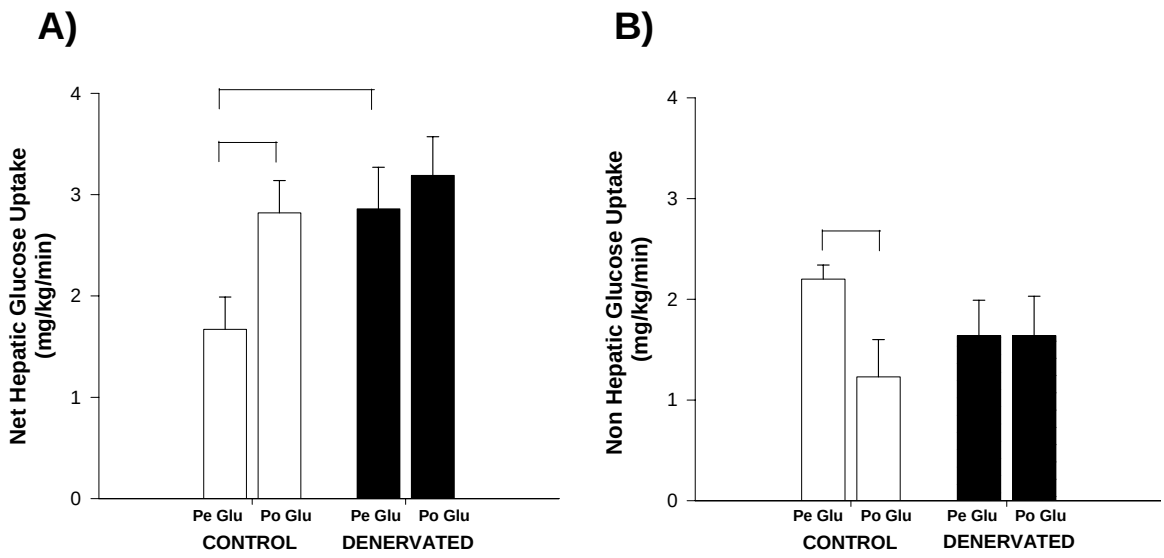


**Figure 4.3:** a) net hepatic glucose uptake and b) non-hepatic glucose uptake in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the peripheral and portal glucose infusion periods in both the CON and DEN groups (n=10/group). Data are mean ± SE. ∗= P<0.05 compared to CON group

# CHAPTER V

## THE EFFECT OF SIMULATING THE PORTAL GLUCOSE SIGNAL BY VAGAL COOLING ON CANINE NET HEPATIC GLUCOSE METABOLISM IN THE PRESENCE OF PERIPHERAL HYPERGLYCEMIA

### Aim

Specific aim III used the vagal cooling method to simulate the decrease in afferent firing seen after glucose delivery to the portal vein to further elucidate the role of those nerves involved in the mediation of the effect of the portal glucose signal.

### Experimental Design

Each experiment consisted of a 100-min equilibration period (-140 to –40 min), a 40-min basal period (-40 to 0 min), and a 180-min experimental period (0 to 180 min) which was subsequently divided into two 90 minutes periods. In all experiments, a constant infusion of indocyanine green dye (0.076mg/min) was initiated at –140 min. At 0 min, a constant infusion of somatostatin (0.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was begun to suppress endogenous insulin and glucagon secretion, and glucagon (0.57 $ng \cdot kg^{-1} \cdot min^{-1}$) and insulin (1.2$mU \cdot kg^{-1} \cdot min^{-1}$) were replaced intraportally. In addition, a primed continuous peripheral infusion of 50% dextrose was begun at time 0 so that the blood glucose could quickly be clamped at the desired hyperglycemic level (~ 235 mg/dl). Glucose was infused

peripherally to double the hepatic glucose load (HGL) during the entire experimental period. At t=90, the vagal coils were infused with a 50/50 methanol/saline solution at body temperature (37°C) in the SHAM dogs (n=6) or at ~ -10°C in the COOL dogs (n=6).

Femoral artery, portal vein, and hepatic vein blood samples were taken every 20 min during the basal period (-40 to 0 min) and every 15 minutes for the last half hour of each experimental period. Arterial blood samples were also taken every 5 min from 0 to 180 minutes of the experimental period to monitor the glucose level.

## Statistical Analysis

Time course data were analyzed with repeated-measures analysis of variance. Independent *t*-tests were used for any comparisons of mean data. Statistical significance was accepted at $p<0.05$.

## Results

**Cardiovascular parameters and hepatic blood flow (Fig. 5.1):** Blockade of vagal transmission during the cooling period was confirmed by the presence of a significantly greater heart rate in the COOL (183±3 bpm) group when compared to the SHAM (102±5 bpm) group. In addition, all COOL dogs exhibited prolapse of the 3$^{rd}$ eyelid (Horner's sign) and a deepening and slowing of respirations

during the cooling period. There were no significant changes in blood pressure during the basal or experimental periods in either group.

As expected, with the onset of somatostatin infusion arterial blood flow increased slightly while portal blood flow decreased modestly. Hepatic blood flow (Table 5.1) was initially similar in both groups and, as a result of the above changes, fell slightly but not significantly during the first experimental period in both groups. It increased significantly during the cooling period as the result of a significant rise in arterial blood flow (9±1 to ~13±2 ml/kg/min). Sham cooling was without effect on arterial blood flow.

**Arterial and portal plasma glucose levels (Fig 5.2):** The average arterial plasma glucose level (mg/dl) in the COOL dogs prior to vagal cooling was similar to that seen in the SHAM dogs (230±2 and 231±2, respectively). The average glucose infusion rates (mg/kg/min) were 7.9±1.6 and 6.8±0.4 in the SHAM and COOL dogs, respectively. During nerve cooling, the arterial plasma glucose concentration was allowed to fall to an average of 194±4 mg/dl in order to hold the hepatic glucose load constant in the presence of the increase in arterial blood flow. During coil perfusion, the average glucose infusion rates were 10.0±1.5 vs 7.5±0.9 mg/kg/min in the SHAM and COOL dogs, respectively. It was slightly less in the latter group as a result of the need to lower the arterial glucose levels to compensate for the increase in hepatic blood flow while clamping the hepatic glucose load. As expected the portal vein glucose levels were slightly lower than the arterial plasma glucose levels in both groups throughout the study.

**Arterial plasma and liver sinusoidal insulin and glucagon, cortisols, and catecholamines (Table 5.2):** Arterial and liver sinusoidal plasma insulin levels were maintained at ~3-4X basal throughout the experimental periods in both the SHAM and COOL groups. The arterial plasma and liver sinusoidal glucagon levels, on the other hand, were maintained at basal values throughout the experimental periods in both groups.

Cortisol (Table 5.2) and catecholamine (data not shown) levels did not change from basal in either the SHAM group or the COOL group and were not statistically different from one another at any time point during the experiment.

**Hepatic glucose load, net hepatic glucose balance, and net hepatic fractional extraction (Fig 5.3):** By adjusting the peripheral glucose infusion rate to compensate for the increase in total hepatic blood flow which occurred in response to cooling, the hepatic glucose load was kept constant overtime in the two experimental periods and was not significantly different in the two groups. The average hepatic glucose load throughout the entire experiment was 46±1 and 50±2 mg/kg/min in the SHAM and COOL groups, respectively.

With the onset of peripheral hyperglycemia, net hepatic glucose balance switched from output to uptake. Net hepatic glucose uptake (NHGU), in the 90 minutes prior to the cooling period, averaged 2.2±0.5 and 2.9±0.8 mg/kg/min in the SHAM and COOL dogs, respectively. During cooling, the average NHGU was 3.0±0.5 and 3.4±0.6 in the SHAM and COOL dogs, respectively. Thus

NHGU was not affected by vagal cooling. Likewise, the fractional extraction of glucose by the liver was not altered by the decrease in parasympathetic signaling.

**Blood levels and net hepatic balance of lactate (Table 5.3):** The liver exhibited net lactate uptake in the basal period in both groups. Hyperglycemia resulted in a switch to net hepatic lactate output that was similar in both groups and waned over time. The arterial blood lactate levels initially rose due to the change in net hepatic lactate balance and then fell slightly over time. Given our ability to measure net hepatic glucose and lactate balance, we were able to indirectly calculate the average glycogen synthetic rate. The hepatic glycogen synthetic rates prior to cooling (1.4±0.5 and 2.2±0.5 mg/kg/min) and during sham cooling or cooling (2.5±0.5 and 3.1±0.6 mg/kg/min) were not significantly different in the SHAM and COOL dogs, respectively.

**Nonhepatic glucose uptake and clearance (Figure 5.4):** There was a slight drift up in nonhepatic glucose uptake over time in each group. Vagal cooling had no effect on this parameter in either group. Likewise, no changes were apparent in nonhepatic glucose clearance.

## Discussion

In the current studies NHGU was stimulated by hyperinsulinemia and hyperglycemia brought about in the absence of portal glucose delivery. Vagal nerve activity was then blocked using a nerve cooling technique to simulate the

decrease in afferent firing seen after glucose delivery into the portal vein (202-205, 208). The efficacy of cooling was evident from the increase in heart rate and the 3rd eyelid response. Blocking vagal nerve activity had no effect on either net hepatic or nonhepatic glucose uptake.

There have been several studies that have explored the role of the hepatic nerves in the mediation of the portal glucose signal. In a recent study by Cardin et al (44), NHGU was stimulated by hyperinsulinemia, hyperglycemia, and the portal glucose signal. When vagus nerve activity was blocked using the same method as employed in the present study (vagal cooling), NHGU did not change. In an earlier study by Shiota et al. (255), adrenergic blockade (portal vein phentolamine and propanolol infusion) and coincident cholinergic stimulation (portal vein acetylcholine infusion) brought about in the presence of hyperinsulinemia and hyperglycemia (produced by peripheral glucose infusion) *increased* NHGU by 1.8 mg/kg/min when compared to controls (255). These results were inconclusive, however, because portal vein administration of acetylcholine caused an increase in hepatic artery blood flow, leading to a rise in the glucose and insulin loads to the liver. Other studies have shown that delivery of glucose into the hepatic portal vein results in a fall in the firing rate of efferent fibers in the hepatic branch of the splanchnic nerve (206). More recently we have shown that selective sympathetic denervation results in a greater increase in NHGU in response to hyperglycemia of peripheral origin than would otherwise be the case, suggesting that the sympathetic nerves exert a tonic inhibition of NHGU (78). This raises the possibility that the portal glucose signal may bring

about its effect in part at least by causing a diminution in this inhibitory sympathetic tone.

There are three possible interpretations of the Cardin et al. (44) data. First, afferent signaling via the vagus nerve may have been maximally suppressed by portal glucose delivery, and thus vagal cooling would have caused no further reduction in afferent firing and would have had no effect on efferent sympathetic nerve activity to the liver. Since NHGU did not change, this would also indicate that inhibiting parasympathetic efferent activity was without effect. Thus one would conclude that parasympathetic nerve input is not involved in the response of the liver to the portal glucose signal. Second, it is possible that a further decrease in afferent firing did occur and that it led to a further reduction in sympathetic input to the liver which would have increased NHGU. Since NHGU did not change, however, this leads to the conclusion that elimination of efferent parasympathetic input must have resulted in an offsetting decrease in NHGU. The third possibility is that the vagus nerves do not play any role (afferent or efferent) in the transmission of the portal glucose signal, thus explaining why vagal cooling had no effect on NHGU.

The current study was undertaken in order to further elucidate the role of the vagus nerves in the mediation of the portal glucose signal. More specifically, we examined the role of the vagus nerves by creating hyperglycemia in the presence of increased insulin but in the absence of the portal glucose signal. Our hope was that by examining the data from the present study and that from the study of Cardin et al. (44) together, that we could clarify the role of the vagus

nerves in control of hepatic glucose uptake. If the first interpretation of the Cardin et al (44) data were correct, NHGU should have increased in the current study in the presence of vagal cooling since there would have been a significant reduction in sympathetic tone (the latter being present in the absence of portal glucose delivery) and no consequences of efferent parasympathetic inhibition. This was not the case. In regard to the second possible interpretation, in the current study decreasing the afferent firing associated with the vagus nerves should have again inhibited sympathetic efferent firing leading to an increase in NHGU. Inhibition of parasympathetic efferent firing, on the other hand, would have had no effect if basal parasympathetic tone plays no role in augmenting NHGU in the presence of peripheral glucose. To the extent that it does exert an effect under these conditions, its removal would mitigate the rise in NHGU resulting from removal of the sympathetic tone. Total denervation of the liver results in an increase in NHGU compared to the totally intact liver (5) but not as large as seen with a selective sympathetic denervation (78) suggesting that the input from the sympathetic efferents plays a more important role in control of NHGU than that from the parasympathetic efferents. Thus, an increase in NHGU should again have been seen when vagal cooling occurred and this did not occur.

Based on the aforementioned data, it, thus, appears that the vagus nerves do not play a role in the mediation of the portal glucose signal. However, several caveats to this conclusion must be considered. First, it is possible the afferent signal is transmitted through the vagal parasympathetic nerves, but that the nerves were not adequately cooled. This seems unlikely, given that the heart rate

increased significantly during cooling and Horner's sign was present. In addition, it has been shown previously that this vagal cooling method is sufficient to halt vagal signaling in the dog (141) and cat (99) and that an injection of atropine is not able to increase the rate over the effect of cooling at this temperature (141). The second caveat relates to the possibility that the increase in NHGU is due to a local reflex involving the parasympathetic nerves within the hepatoportal region as previously suggested (44, 267). This does not rule out a centrally mediated component of the portal signal (5, 44) but rather suggests that the two mechanisms (a centrally mediated (non-vagal) and a locally mediated (vagal) reflex) could work in concert with one another to elicit a full response. Additionally, the rise in arterial blood flow caused by vagal cooling, albeit small (50%), might be associated with a signal which could itself alter NHGU, thus complicating data interpretation.

It should be remembered that other neurotransmitters might be involved in the response of NHGU to portal glucose delivery. The effect of serotonin (5-HT) on NHGU has been previously examined in this laboratory. Intraportal infusion of 5-HT enhanced NHGU and blunted nonhepatic glucose uptake under hyperglycemic, hyperinsulinemic conditions (191) but also caused an increase in circulating levels of catecholamines and cortisol that caused gastrointestinal distress. To demonstrate that the response seen on NHGU was serotonin and not catecholamine dependent, a selective serotonin re-uptake inhibitor (fluvoxamine) was administered intraportally (189). Similar studies were also done by infusing 5-hydroxytryptophan (5-HTP), a serotonin precursor, into the portal vein to

eliminate the gastrointestinal side effects (192). Both 5-HTP and fluvoxamine enhanced NHGU without elevating circulating serotonin or catecholamine levels suggesting that hepatic targeted serotonin delivery may be effective in reducing postprandial hyperglycemia and that serotonin may be involved in the response to the portal glucose signal.

It is interesting to note that we observed a statistically significant effect of vagal blockade on hepatic arterial blood flow and, as a result, total hepatic blood flow. The former increased by 50% in response to vagal cooling. Studies of the effect of the hepatic vagal nerves on hepatic blood flow (HBF) are limited and the results are controversial; Bobbioni et al (32) showed that there was an increase in HBF in rats when the vagus nerve was electrically stimulated. Most other studies, however, have reported no change in HBF after vagal nerve stimulation (105, 150, 152, 162) or hepatic vagotomy (273). It is unclear why there was an increase in hepatic arterial blood flow in response to vagal cooling in the present study since we did not observe such an effect in our earlier "cooling" studies (43-45).

In summary, vagal cooling to halt electrical transmission in the vagus nerves had no effect on NHGU under hyperglycemic and hyperinsulinemic conditions. Such is the case whether glucose was given via a peripheral or the portal vein. This makes it less likely that the vagus nerves are involved in the mediation of the portal glucose signal.

**Table 5.1:** *Arterial, Portal, and Total Hepatic Blood Flow in the basal and experimental periods in both sham cooled (SHAM) and vagally cooled (COOL) 42-hr fasted conscious dogs.*

**Arterial Blood Flow (ml/kg/min)**

| | basal | | | exp+ saline | | | | exp +/- COOL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **-20** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** |
| **SHAM** | 5±1 | 5±1 | 5±1 | 7±1 | 7±1 | 8±1 | 7±1 | 7±2 | 7±2 | 7±2 | 8±2 | 8±1 |
| **COOL** | 6±1 | 6±1 | 7±1 | 8±1 | 9±1 | 9±1 | 9±1 | 12±2* | 14±2*¥ | 14±2*¥ | 13±2*¥ | 13±2*¥ |

**Portal Blood Flow (ml/kg/min)**

| | basal | | | exp+ saline | | | | exp +/- COOL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **-20** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** |
| **SHAM** | 27±3 | 27±2 | 27±2 | 21±1 | 21±1 | 20±2 | 21±2 | 20±1 | 21±2 | 20±1 | 20±1 | 21±1 |
| **COOL** | 27±4 | 28±4 | 26±3 | 21±3 | 20±3 | 21±3 | 21±3 | 22±3 | 22±3 | 22±3 | 23±3 | 23±3 |

**Total Hepatic Blood Flow (ml/kg/min)**

| | basal | | | exp+ saline | | | | exp +/- COOL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **-20** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** |
| **SHAM** | 31±3 | 31±2 | 32±2 | 27±2 | 28±2 | 27±2 | 28±3 | 27±2 | 28±2 | 27±2 | 27±1 | 28±2 |
| **COOL** | 33±4 | 34±4 | 33±3 | 29±3 | 29±3 | 30±3 | 29±3 | 34±4 | 36±4* | 36±4* | 36±4* | 36±4* |

*n=6 in each group * = P<0.05 vs SHAM dogs ¥= P<0.05 vs exp+saline period in respective group*
*exp denotes experimental period*

**Table 5.2:** *Arterial plasma and hepatic sinusoidal insulin and glucagon levels during the basal and experimental periods in both sham cooled (SHAM) and vagally cooled (COOL) 42-hr fasted conscious dogs.*

**Arterial Plasma Insulin (uU/ml)**

| | basal | | | exp+ saline | | | | exp +/- COOL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **-20** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** |
| **SHAM** | 9±1 | 8±1 | 8±1 | 22±1 | 22±1 | 22±2 | 22±1 | 23±1 | 21±2 | 23±1 | 23±2 | 24±1 |
| **COOL** | 6±1 | 6±1 | 8±2 | 24±2 | 21±2 | 22±3 | 23±3 | 26±3 | 25±2 | 24±3 | 25±3 | 27±3 |

**Hepatic Sinusoidal Insulin (uU/ml)**

| | basal | | | exp+ saline | | | | exp +/- COOL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **-20** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** |
| **SHAM** | 22±4 | 13±3 | 19±5 | 76±6 | 87±10 | 81±7 | 78±6 | 82±11 | 95±6 | 77±7 | 95±4 | 75±8 |
| **COOL** | 17±4 | 18±5 | 23±7 | 94±20 | 71±12 | 84±13 | 87±15 | 96±8 | 82±7 | 83±8 | 96±9 | 97±8* |

**Arterial Plasma Glucagon (pg/ml)**

| | basal | | exp+ saline | | | exp +/- COOL | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **0** | **30** | **60** | **90** | **105** | **120** | **150** | **180** |
| **SHAM** | 36±7 | 35±6 | 34±5 | 40±6 | 41±4 | 39±5 | 37±5 | 35±4 | 33±6 |
| **COOL** | 37±4 | 35±6 | 44±5 | 43±4 | 40±5 | 42±4 | 37±2 | 40±3 | 40±2 |

**Hepatic Sinusoidal Glucagon (pg/ml)**

| | basal | | exp+ saline | | | exp +/- COOL | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **0** | **30** | **60** | **90** | **105** | **120** | **150** | **180** |
| **SHAM** | 41±5 | 46±6 | 53±7 | 59±10 | 56±8 | 52±6 | 55±6 | 48±5 | 47±6 |
| **COOL** | 42±6 | 43±7 | 65±7 | 59±5 | 62±4 | 59±3 | 52±5 | 55±2 | 56±4 |

**Plasma Cortisol (ug/dl)**

| | basal | exp+ saline | exp+/- COOL |
|---|---|---|---|
| | **-20** | **75** | **165** |
| **SHAM** | 2.8±0.9 | 3.4±0.4 | 2.0±0.5 |
| **COOL** | 4.2±1.0 | 5.4±1.2 | 5.9±2.5 |

*n=6 in each group * = P<0.05 vs SHAM dogs Note: There were no significant differences between exp+/- cool and exp+saline periods in each respective group in any of the parameters above. Exp denotes experimental period*

**Table 5.3:** *Arterial blood lactate levels and net hepatic lactate balance during the basal and experimental periods in both sham cooled (SHAM) and vagally cooled (COOL) 42-hr fasted conscious dogs.*

**Arterial Blood Lactate (umol/L)**

| | basal | | | exp+ saline | | | | exp +/- COOL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **-20** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** |
| **SHAM** | 365±37 | 346±43 | 324±44 | 604±55 | 871±92 | 846±85 | 784±69 | 838±73 | 845±86 | 804±85 | 779±92 | 796±132 |
| **COOL** | 403±112 | 375±104 | 400±108 | 746±148 | 1038±147 | 1057±139 | 1001±92* | 964±56 | 910±68 | 707±165 | 658±145¥ | 698±141¥ |

**Net Hepatic Lactate Balance (umol/kg/min)**

| | basal | | | exp+ saline | | | | exp +/- COOL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **-40** | **-20** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** |
| **SHAM** | -7.0±1.5 | -5.9±1.4 | -6.0±1.0 | 7.6±2.4 | 10.8±2.2 | 6.5±1.5 | 5.6±1.5 | 4.5±1.6 | 4.6±1.0 | 3.5±2.3 | 2.8±1.6 | 4.5±2.4 |
| **COOL** | -4.2±0.4 | -3.6±0.6 | -4.0±0.5 | 6.9±3.5 | 6.6±2.5 | 5.5±2.3 | 3.6±1.5 | 1.8±2.8 | 1.6±1.2* | 1.7±1.1 | 1.2±1.1¥ | 0.7±1.2¥ |

*n=6 in each group * = P<0.05 vs SHAM dogs ¥= P<0.05 vs exp+saline period in respective group*
*exp denotes experimental period*

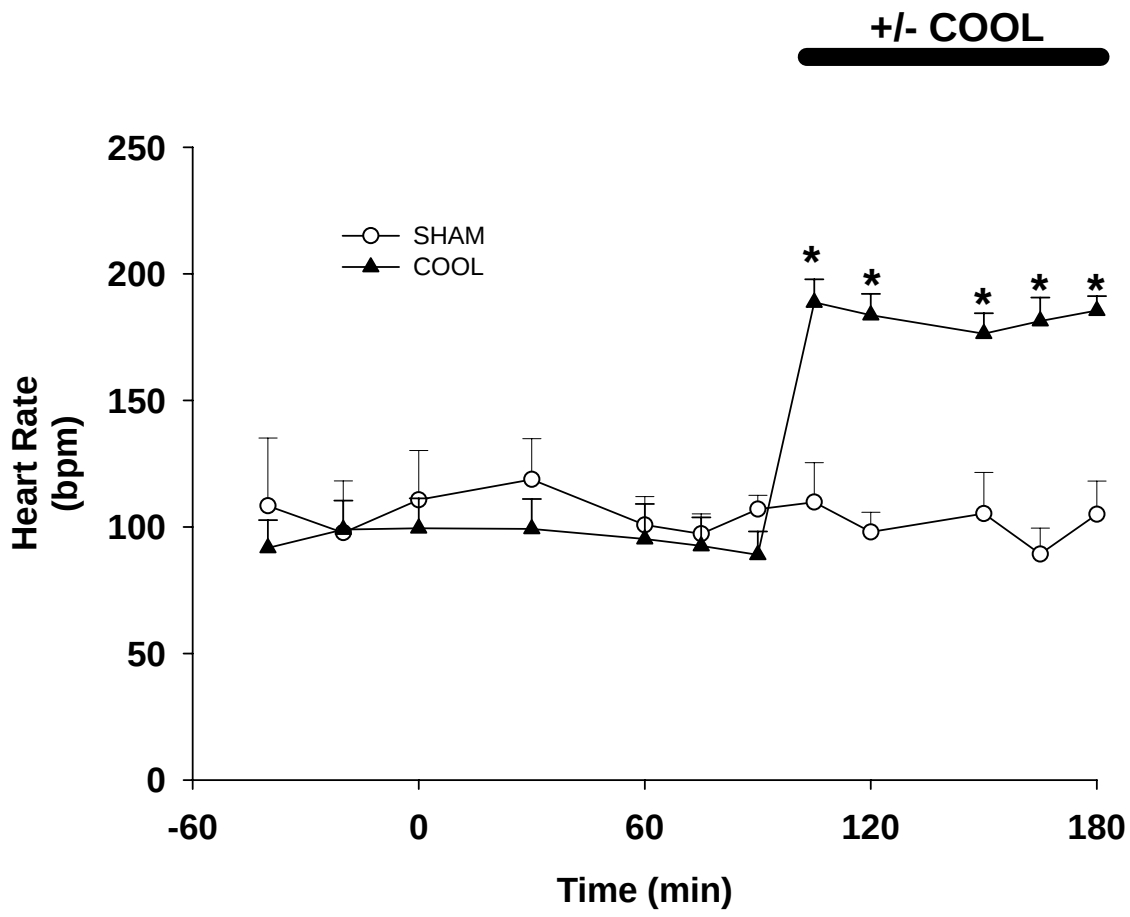


**Figure 5.1**: Heart rate (bpm) in both sham cooled (SHAM) and vagally cooled (COOL) 42-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods (n=6/group). Data are mean ± SEM. * = P<0.05

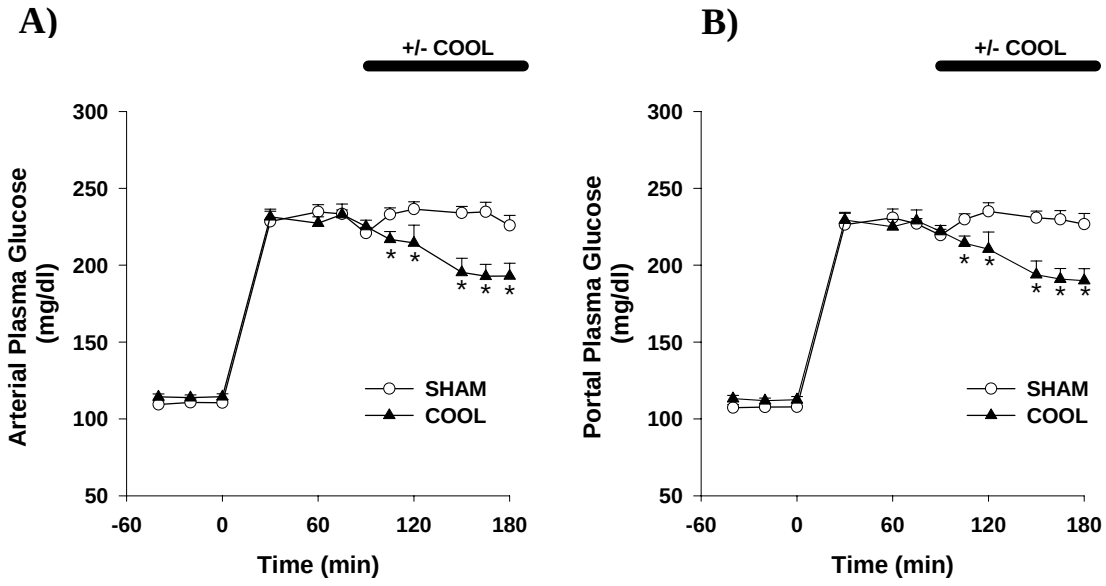


**Figure 5.2:** Arterial (a) and portal (b) plasma glucose levels in sham cooled (SHAM) and vagally cooled (COOL) 42-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods (n=6/group). Data are mean ± SEM. * = P<0.05

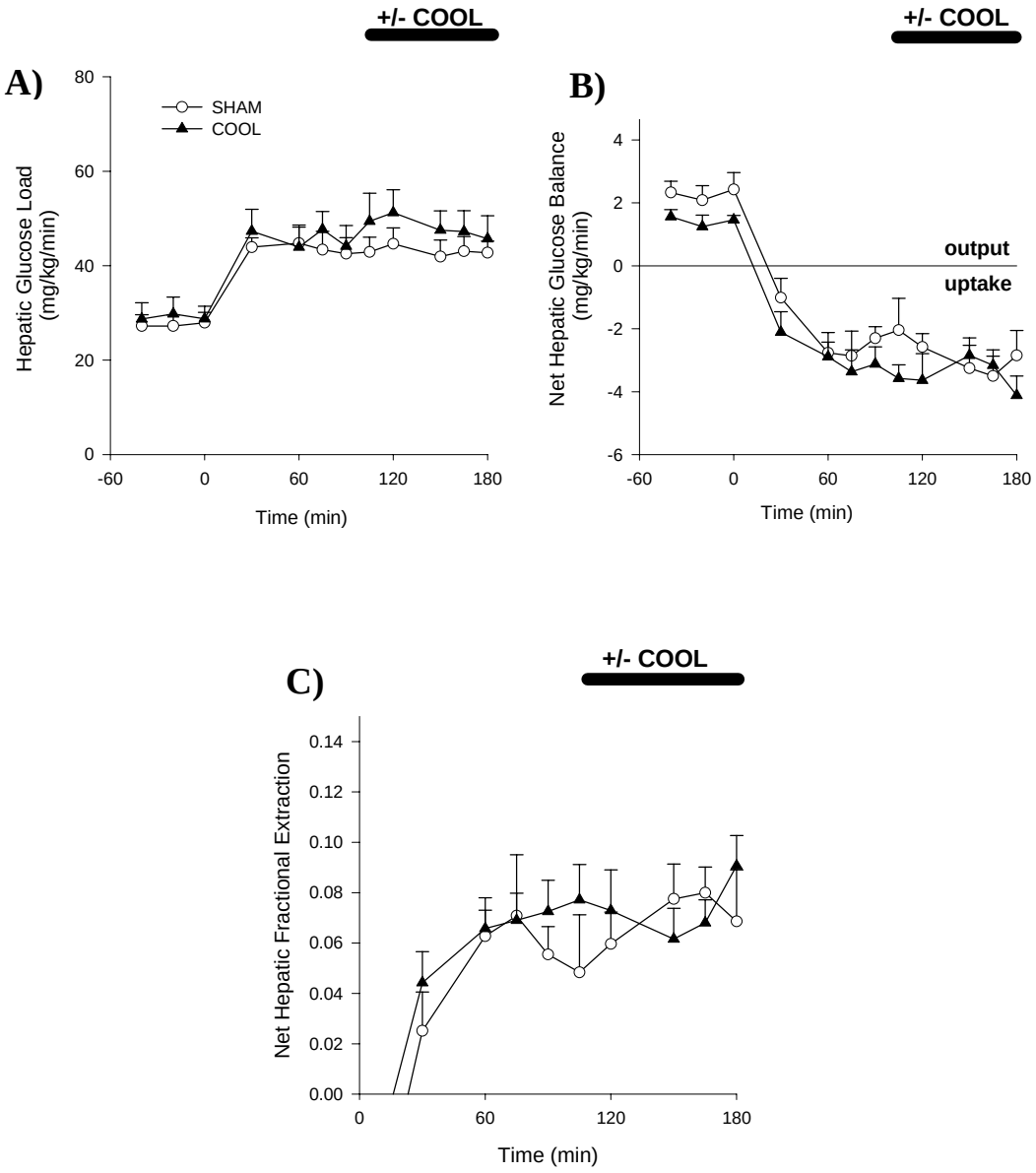


**Figure 5.3:** a) hepatic glucose load, b) net hepatic glucose balance, and c) net hepatic fractional extraction in sham cooled (SHAM) and vagally cooled (COOL) 42-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods (n=6/group). Data are mean ± SEM.

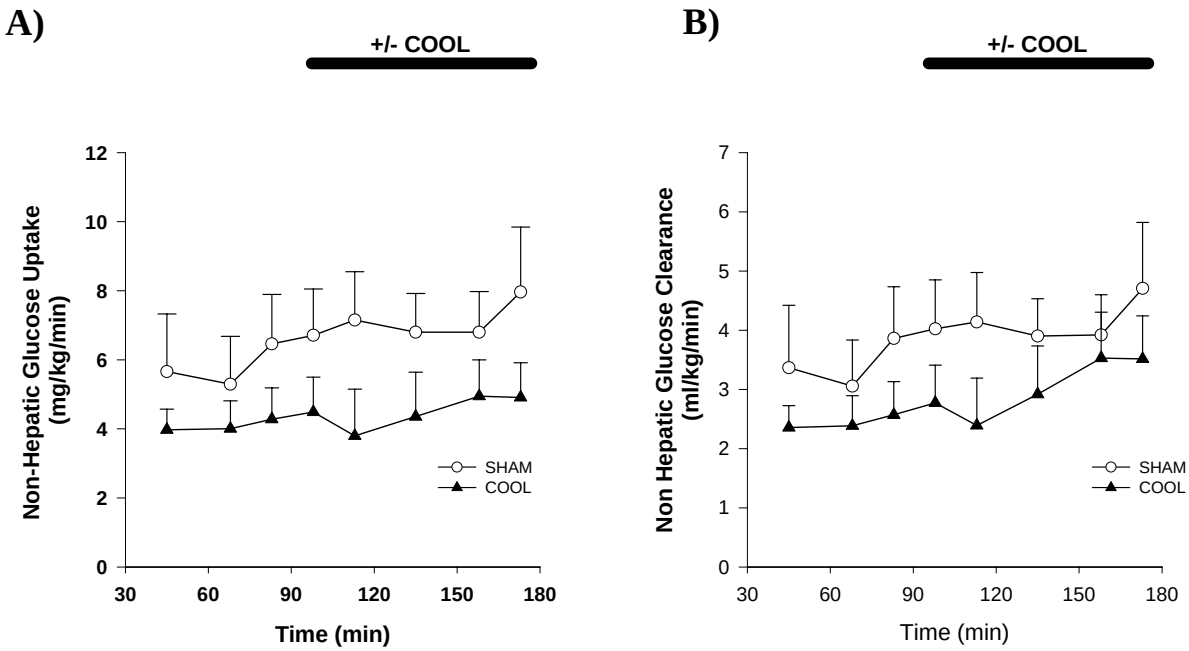


**Figure 5.4:** a) nonhepatic glucose uptake and b) nonhepatic glucose clearance in sham cooled (SHAM) and vagally cooled (COOL) 42-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods (n=6/group). Data are mean ± SEM.

# CHAPTER VI

## THE EFFECT OF AN INTRAPORTAL NITRIC OXIDE (NO) DONOR (SIN-1) ON CANINE NET HEPATIC GLUCOSE METABOLISM IN THE PRESENCE OF THE PORTAL GLUCOSE SIGNAL

### Aim

Nitric oxide (NO) is clearly an important mediator in insulin sensitivity at the muscle. Due to the reciprocity between hepatic and muscle glucose uptake, we hypothesized that NO would also have dramatic effects at the liver by inhibiting hepatic glucose uptake. In specific aim IV, a portal infusion of the nitric oxide donor, SIN-1, in the presence of the portal glucose signal, was used to increase NO levels at the liver and hepatic substrate balance was calculated in the presence and the absence of this donor.

### Experimental Design

Each experiment consisted of a 100-min equilibration period (-120 to –20 min), a 20-min basal period (-20 to 0 min), and a 240-min experimental period (0 to 240 min) which was divided into one 90 minute period (P1) followed by a 150 minute period (P2). In all experiments, a constant infusion of indocyanine green dye (0.076mg/min) was initiated at –120 min. At 0 min, a constant infusion of somatostatin (0.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was begun to suppress endogenous insulin and glucagon secretion and basal glucagon (0.57 $ng \cdot kg^{-1} \cdot min^{-1}$) and 4-fold basal

insulin (1.2mU·kg$^{-1}$·min$^{-1}$) were replaced intraportally. Glucose (20% dextrose) was delivered intraportally at 4mg/kg/min. In addition, a primed continuous peripheral infusion of 50% dextrose was begun at time 0 so that the blood glucose could quickly be clamped at the desired hyperglycemic level (~ 235 mg/dl). From t=90 to t=240, normal saline (CON, n=9) or SIN-1 (SIN-1 4ug/kg/min, n=9) were delivered intraportally. The peripheral glucose infusion rate was adjusted to maintain a similar hepatic glucose load to that which was seen during the first 90 minutes of the experimental period (P1). PAH was added to the portal glucose infusate to assess mixing of the infusate with the blood in the portal and hepatic veins as previously described (214). n the CON group, 9 dogs were studied and all were included due to adequate mixing as defined previously (197); 11 dogs were studied in the SIN-1 group and 9 were included using the same standards. In the control and SIN-1 animals that were retained, the ratio of PAH recovery in the portal vein to the PAH infusion rate was 0.9±0.1 and 0.9±0.1 whereas the ratio of PAH recovery in the hepatic vein to the PAH infusion rate was 0.9±0.1 and 0.9±0.1, respectively (a ratio of 1.0 would represent perfect mixing).

Femoral artery, portal vein, and hepatic vein blood samples were taken every 20 min during the basal period (-20 to 0 min) and at pre-determined time points throughout the experimental periods. Arterial blood samples were also taken every 5 min from 0 to 240 minutes of the experimental period to monitor the glucose level and facilitate the clamp.

## Statistical analysis

Time course data were analyzed with repeated-measures analysis of variance. Independent *t*-tests were used for any comparisons of mean data. Statistical significance was accepted at $p<0.05$.

## Results

**Heart rate and systolic and diastolic blood pressure (Table 6.1):** The average heart rates during the basal period tended to be lower in the CON group (88±5 bpm) than in the SIN-1 group (100±6) but there were no statistically significant changes over time in either group.

The average systolic blood pressure during the basal period in the CON group was 172±6 mmHg and did not change significantly during P1 (167±5 mmHg) or P2 (166±4 mmHg). The average systolic blood pressure in the SIN-1 group was similar to that seen in the CON group in the basal period (162±4 mmHg) and P1 (162±4) but fell significantly during P2 (112±7 mmHg). The diastolic blood pressure followed a similar pattern to the systolic blood pressure.

**Hepatic sinusoidal plasma insulin and glucagon (Figure 6.1):** Hepatic sinusoidal plasma insulin was maintained at elevated levels as expected in both the CON (75±7 uU/ml) and SIN-1 (78±8) groups throughout P1. Neither SIN-1 nor saline infusion had any effect on the plasma insulin level. The hepatic sinusoidal plasma insulin levels during P2 were unchanged (79±4 and 72±2 uU/ml) in the CON and SIN groups, respectively.

Hepatic sinusoidal plasma glucagon levels (Figure 6.1b) were basal and did not differ significantly between the CON and SIN-1 groups at any point in the study.

**Arterial plasma glucose levels, hepatic glucose load, net hepatic glucose balance, fractional glucose extraction, nonhepatic glucose uptake and clearance:** The arterial plasma glucose levels were doubled from basal values of 113±2 and 114±2 mg/dl, in the CON and SIN-1 groups respectively, to average levels of 235±4 and 249±3 mg/dl, respectively in P1. During the SIN-1 infusion (P2), the arterial plasma glucose level was decreased to an average of 220±6 mg/dl in order to hold the hepatic glucose load constant in the face of a rise in hepatic blood flow (22%). The arterial plasma glucose level was not changed appreciably during saline infusion (237±3 mg/dl) since total hepatic blood flow did not change (8%) (Figure 6.2a).

The hepatic glucose load in the control group (Figure 6.2b) was not significantly different in P1 and P2 (49±3 and 53±4 ml/kg/min, respectively). In the SIN-1 group, the average hepatic glucose loads were also similar in P1 and P2 (43±3 and 46±3 ml/kg/min) and although they tended to be slightly lower than those seen in the CON group, this difference was not significant.

Net hepatic glucose output was similar during the basal periods in both the CON and SIN-1 groups (1.7±0.3 vs 1.5±0.1 mg/kg/min, respectively). With the onset of hyperglycemia and the portal glucose signal, net hepatic balance switched from output to uptake in both groups (Figure 6.3). Likewise, net hepatic glucose uptake (NHGU) was similar in P1 in both groups with an average uptake of 4.1±0.4 and

4.0±0.7 in the CON and SIN-1 groups, respectively. NHGU averaged 4.8±0.4 mg/kg/min during P2 in the CON group. In the presence of the SIN-1 infusion, NHGU decreased significantly within 15 minutes and averaged 3.1±0.4 mg/kg/min in P2 ($P<0.05$ vs CON). These differences were also reflected in the fractional extraction of glucose by the liver in that during P2, the liver extracted 9.3% of the glucose it received in the control group and 7.0% in the SIN-1 group ($P<0.05$).

There was no change in non-hepatic glucose uptake or clearance in the presence of SIN-1 or saline in either group over time. There were also no significant differences between the two groups in either non hepatic glucose uptake or non hepatic glucose clearance (Figure 6.4).

**Blood levels and net hepatic balance of lactate (Figure 6.5):** Both groups exhibited net lactate uptake in the basal period. The combination of increased insulin, hyperglycemia, and the portal glucose signal resulted in a switch to net hepatic lactate output (NHLO) and a rise in the arterial blood lactate levels rose. In response to saline infusion, NHLO drifted down over time causing a slow fall in the blood lactate level. Such a fall was not seen during SIN-1 infusion although NHLO at 90 minutes was only half that in the saline group.

**Plasma NEFA levels, net hepatic NEFA balance, arterial blood glycerol levels, and net hepatic glycerol balance (Figure 6.6):**Arterial plasma NEFA levels fell as expected at the start of the experimental period and remained

constant in both groups throughout the experimental period even in the presence of SIN-1. Likewise, net hepatic NEFA uptake fell to a very low rate in both groups.

Arterial blood glycerol levels and net hepatic glycerol uptake also fell rapidly in response to the combination of an increase insulin and hyperglycemia. However, with the onset of the SIN-1 infusion, arterial blood glycerol levels doubled to an average of 47±2 μmol/L during the last hour of the experiment. This was associated with an increase in net hepatic glycerol uptake.

**Hepatic blood flow (Table 6.2):** Hepatic arterial blood flow increased slightly from basal, as expected, with the start of the somatostatin infusion (t=0) in both groups while portal vein blood flow decreased modestly, as expected. In P1, the average hepatic portal blood flow was 17.8±1.2 and 15.9±1.4 ml/kg/min in the CON and SIN-1 groups, respectively. In the CON group, it did not change significantly (18.7±1.4 ml/kg/min). In the SIN-1 group there was a rise in average hepatic portal vein blood flow to 19.6±1.4 ml/kg/min ($P<0.05$) during P2. The average hepatic portal blood flows were not significantly different between the two groups at any point.

The average total blood flow during P1 was 27±2 ml/kg/min and 22±2 ml/kg/min in the CON and SIN-1 groups, respectively. In P2, the average total blood flow in the control group increased non-significantly to 29±2 ml/kg/min while in the SIN-1 group, it increased significantly to 27±2 ml/kg/min. The

average total blood flow was significantly different between the two groups during P1 but not during P2.

## Discussion

In this study we tested the hypothesis that nitric oxide exerts an inhibitory effect on NHGU. In the presence of hyperinsulinemia, hyperglycemia, and the portal glucose signal, a portal infusion of the NO donor SIN-1 caused a 35% decrease in NHGU. There were no significant effects on non-hepatic glucose uptake or clearance.

The effect of intraportal SIN-1 infusion on NHGU was rapid and significant. In the control group, NHGU increased slightly over time to an average of 4.8±0.4 mg/kg/min. Over the same time period, NHGU averaged 3.2±0.5 mg/kg/min in the SIN-1 group. This decrease remained evident throughout. Thus, intraportal SIN-1 administration decreased NHGU by ~35% ($P<0.05$) compared to that seen in the control group. Intraportal SIN-1 infusion had no effect on non hepatic glucose uptake or clearance. Several variables that affect NHGU were controlled in order to accurately assess the effect of intraportal nitric oxide delivery on NHGU. The arterial plasma glucose concentration was doubled and the glucose load to the liver was kept constant over time in both groups. The hepatic sinusoidal plasma glucagon concentrations were kept basal while the hepatic sinusoidal plasma insulin concentrations were increased four fold in both groups. Thus the response of the liver cannot be ascribed to changes in insulin, glucagon, or the hepatic glucose load.

Our lab has previously shown that portal glucose delivery and hyperinsulinemia have additive effects in enhancing glucose uptake by the liver. In hyperglycemic experiments by both Adkins et al. (3, 4) and Pagliassotti et al. (214) in which glucagon was kept basal and insulin was either basal or elevated four fold, portal glucose delivery enhanced NHGU compared to that which was seen with peripheral glucose delivery. In the present study, the average NHGU during the first experimental period in the control and SIN-1 groups was 4.1±0.4 and 4.1±0.7 mg/kg/min, respectively. These rates are consistent with previous data (4, 5, 214) obtained under similar experimental conditions.

The question then arises as to how this effect of intraportal SIN-1 infusion was brought about. Did it occur because of a direct action of NO on the liver or through an indirect action mediated by some other means? One such indirect mechanism could involve an increase in sympathetic drive to the liver which resulted from the decreases in blood pressure caused by the intraportal infusion of SIN-1. There are two observations that support this hypothesis. These include an increase in the heart rate and an increase in lipolytic activity. The intraportal administration of SIN-1 had significant cardiovascular effects. Both systolic and diastolic pressure decreased significantly, although this was not surprising as orally absorbed SIN-1 has previously been shown to rapidly lower blood pressure (17). This in turn was associated with a slight increase in heart rate. Besides the cardiovascular effects seen with the intraportal administration of SIN-1, there was also an increase in the arterial blood glycerol level. An increase in norepinephrine and epinephrine have been shown to activate lipolysis via the $B_1$, $B_2$, and $B_{3-}$,

adrenoceptors and these sympathetic neurotransmitters are important lipolytic regulators in vivo (155, 156). An increase in lipolysis will in turn cause an increase in glycerol concentration due to the break down of the triglycerides to free fatty acids and glycerol. Thus the increase in arterial blood glycerol concentrations in the SIN-1 treated group suggests that SIN-1 may have increased sympathetic drive to fat and in turn activated lipolysis.

In further support of the concept that NO may be having an indirect effect via sympathetic nerve activation is our recent finding that removal of sympathetic input to the liver results in an increase in NHGU suggesting that the sympathetic nerves exert a tonic inhibition on NHGU (79). If the intraportal SIN-1 infusion increased sympathetic drive to the liver, it may well have resulted in a decrease in NHGU.

It is also possible that SIN-1 may be acting directly on both the hepatocyte and the adipocyte in eliciting its effects. The effect of endogenously gnerated NO has been shown to stimulates lipolysis in white adipose tissue and increase fatty acid oxidation in hepatocytes (101, 107, 147, 153). More recently, Fu et al. (102), showed that dietary L-arginine supplementation (a precursor of NO synthesis) in Zucker diabetic fatty rats increased NO synthesis and lipolysis. Thus the increase in arterial blood glycerol concentrations seen in the current study may have been due to the direct action of SIN-1 on NO release in adipose tissue.

The exact source of the endogenous hepatic NO is not clear. In addition, the direct mechanism by which NO decreases NHGU is not completely clear. It is known that cGMP can stimulate the hydrolysis of cAMP through the stimulation

of cAMP phosphodiesterase (244). Others have shown that hepatocytes (29, 66)and neighboring Kupffer cells (30) are also able to produce NO from L-arginine in an inducible manner (involving iNOS, a $Ca^{2+}$- independent NO synthase) which can in turn stimulate soluble guanylate cyclase and increase cGMP levels. Both iNOS and eNOS have been found in hepatocytes and in addition, iNOS has been localized to both the hepatic Kupffer cells as well as the Ito cells (11, 168). Only eNOS can be found in the liver under normal conditions but iNOS can be rapidly upregulated in the liver due to endotoxemia, sepsis, infection, and liver regeneration (168). Thus both isoforms of NOS may be involved with the direct action of NO on the liver. In the perfused rat liver, infusion of NO (34 μmol/L) greatly increased the rate of glucose output and therefore increased glucose concentrations on average two fold (37). Lactate production also increased. The fact that both glucose and lactate output were increased suggested that glycogen was the source of the additional glucose output. The mechanism by which NO increases glycogenolysis in the perfused rat liver was suggested to be activation of glycogen phosphorylase. This requirement for the activation of phosphorylase was confirmed by the ineffectiveness of NO in phosphorylase-kinase deficient livers of gsd/gsd rats (37).

There are several NO donors (GEA 3162, V-PYRRO, SNAP, DEA/NO) but we specifically chose to use SIN-1. SIN-1 decomposes non-enzymatically in a two step reaction with the second step yielding NO and $O_2^{\cdot -}$ (35, 91)which will readily form peroxynitrite (137). The NO and $O_2^{\cdot -}$ can also lead to the formation of GSNO. This formation of GSNO outcompetes the formation of peroxynitrite as

well as $O_2^{\cdot -}$ scavenging by superoxide dismutase (245). This makes SIN-1 the most favorable NO donor to use in these experiments due to its propensity to form the GSNO complex over the peroxynitrite radical. Although we did not measure NO or SIN-1 concentrations across the liver, it would appear that some SIN-1 did pass the liver since it had potent cardiovascular effects.

More recently, a liver-selective nitric oxide donor, V-PYRRO/NO has been developed which dissociates to NO rapidly (169, 238). It has a high first pass effect through the liver with a blood half-life of approximately 12 minutes. Once the V-PYRRO/NO is activated, the local NO half-life is very short. It might be of interest in the future to repeat these current studies using this liver-selective NO donor to eliminate or reduce the cardiovascular effects that were present although it is unclear whether V-PYRRO/NO has the ability to react with GSH.

The effect of NO on non-hepatic glucose metabolism, primarily muscle glucose uptake, has been explored more extensively than the effects of NO on hepatic glucose metabolism. Xie et al (288, 291)has suggested that insulin sensitivity may be mediated by a neurohumoral mechanism through which the liver releases a putative hormone referred to as hepatic insulin-sensitizing substances (HISS). This HISS then enters the blood and can sensitize the skeletal muscle to insulin action, possibly accounting for as much as~55% of total insulin action (159, 161). It has been shown that HISS release is dependent on the activation of hepatic muscarinic receptors by acetylcholine (presumably released from the parasympathetic nerves) and is clearly mediated by NO that is produced in the liver (120, 240, 291). In a study performed by Sadri et al. (240), intraportal,

but not intravenous, administration of L-NAME (a nitric oxide synthase antagonist) significantly reduced insulin sensitivity as measured using the rapid insulin sensitivity test (RIST) in rats. This suggests that NOS inhibition at the liver causes insulin resistance. The authors then concluded that this insulin resistance caused by NOS antagonism was not due to a reduction in skeletal muscle perfusion but rather due to the blockade of the parasympathetic reflex release of HISS that occurred in response to insulin.

In these same experiments, the authors administered SIN-1 both intraportally and intravenously and only saw a reversal of the insulin resistance caused by the L-NAME in those rats that received the SIN-1 intraportally. Thus, the insulin resistance that was produced after the inhibition of NOS in the liver was reversed by providing NO to the liver. They also went on to show that intraportal SIN-1 delivery could completely restore insulin sensitivity in rats that had undergone hepatic denervation. These data suggest that there is an insulin-induced hepatic parasympathetic reflex, which acts through the binding of acetylcholine to muscarinic receptors, resulting in production of NO in the liver, leading to the release of the putative hormone (HISS), which in turn sensitizes the skeletal muscle to the action of insulin.

It is interesting to note that in another complementary set of experiments, Guarino et al. (119) showed that whereas SIN-1 can restore HISS action after inhibition of NOS by L-NAME, acetylcholine (ACh) cannot. This suggests that NOS is one of the targets for the hepatic muscarinic receptors in the HISS

pathway. Thus the release of endogenous NO in the liver is required for the HISS secretion in response to insulin.

Based on the above data, we expected to see an increase in non-hepatic glucose uptake when we infused SIN-1 to the liver. According to the theories proposed by several investigators (118, 159, 224), this increase of NO at the liver should have stimulated the release of HISS and increased insulin sensitivity at the muscle. We did not see an effect of SIN-1 on either non hepatic glucose balance or non hepatic glucose clearance. There are several possible explanations as to why this was the case. First, it could be solely a species phenomenon and thus not apparent in the dog. Second, it has been previously shown that HISS synthesis may be controlled by the prandial status with its synthesis being maximal in the post-prandial state and minimal in the fasted state (146). Most of the experiments that have been conducted on animals looking at HISS have been carried out under euglycemic conditions in animals that have been fasted and refed. In this study, 42-h fasted dogs were used and a hyperglycemic clamp was maintained, and either or both of these conditions may have prevented us from seeing any effects of HISS on non hepatic glucose metabolism.

Another important consideration regarding our failure to see a peripheral action of portal SIN-1 involves the role of glutathione (GSH) on the action of the hepatic insulin-sensitizing substance. Glutathione levels in the liver are significantly lower following an overnight fast than seen after feeding. In experiments performed by Guarino et al.(119) in which they depleted hepatic GSH using the GSH synthesis inhibitor 1-buthionine-[S,R]-sulfoximine (BSO),

SIN-1 did not reverse the insulin resistance brought about by the depletion of GSH in combination with the administration of L-NAME, suggesting that both NO and GSH are both essential for HISS-dependent insulin action. Nitrosylated derivatives of GSH, including S-nitrosoglutathione (GSNO), can act as endogenous NO reservoirs (120), since this compound is a very stable molecule and has a biologically active NO adduct (130). It has yet to be determined whether the GSNO compound is only a stable NO pool that in turn allows for HISS secretion or if it also has the ability to activate key enzymes and pathways. GSNO may activate other key enzymes by S-nitrosylation or S-thiolation. Thus we may not have seen the effect of NO on non hepatic glucose uptake due to the depletion of GSH following the 42-h fast.

In summary, an intraportal NO donor (SIN-1) decreased NHGU and net hepatic glucose fractional extraction while having no effect on non- hepatic glucose uptake. The presence of the NO donor also increased arterial blood glycerol concentrations and impeded the fall of arterial blood lactate levels over time suggesting that NO may play a role in the regulation of glycogen synthesis. It is also possible that intraportal administration of SIN-1 triggers a sympathetic response secondary to hypotension which can then account for the changes seen in hepatic glucose metabolism as well as lipolysis. Future studies need to elucidate the exact mechanism by which NO is exerting its effects and clarify the relationship between hepatic and non hepatic glucose metabolism in the light of HISS and NO.

*Table 6.1: Heart rate and systolic and diastolic blood pressure in 42-h fasted conscious dogs during the basal and experimental periods in the control and SIN-1 groups*

**Heart Rate (bpm)**

| | Basal | | Experimental | | | | Experimental +/- SIN-1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time (min)** | **-30** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** | **195** | **210** | **225** | **240** |
| **Control** | 87±8 | 88±6 | 90±6 | 98±5 | 96±6 | 93±5 | 84±5 | 93±11 | 86±6 | 88±7 | 91±6 | 88±10 | 102±8 | 103±8 | 107±9 |
| **SIN-1** | 104±7 | 97±7 | 94±6 | 100±7 | 92±8 | 95±5 | 98±4* | 101±6 | 106±8* | 109±8* | 105±8 | 106±8 | 105±9 | 108±10 | 107±10 |

**Systolic Blood Pressure (mmHg)**

| | Basal | | Experimental | | | | Experimental +/- SIN-1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time (min)** | **-30** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** | **195** | **210** | **225** | **240** |
| **Control** | 171±7 | 174±6 | 167±7 | 165±7 | 164±3 | 171±5 | 169±7 | 171±7 | 161±4 | 167±5 | 162±5 | 166±5 | 167±8 | 162±6 | 165±6 |
| **SIN-1** | 160±4 | 164±6 | 162±5 | 158±5 | 162±4 | 166±7 | 127±6* | 122±6* | 117±9* | 114±7* | 111±8* | 109±6* | 106±7* | 103±8* | 103±9* |

**Diastolic Blood Pressure (mmHg)**

| | Basal | | Experimental | | | | Experimental +/- SIN-1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time (min)** | **-30** | **0** | **30** | **60** | **75** | **90** | **105** | **120** | **150** | **165** | **180** | **195** | **210** | **225** | **240** |
| **Control** | 89±4 | 94±4 | 91±5 | 89±4 | 89±3 | 95±5 | 89±6 | 90±6 | 93±5 | 92±5 | 91±4 | 92±6 | 98±6 | 92±5 | 91±5 |
| **SIN-1** | 87±2 | 85±3 | 86±3 | 90±4 | 85±3 | 85±3 | 83±3 | 87±3 | 77±5* | 76±5* | 74±5* | 74±4* | 71±6* | 70±6* | 68±7* |

*Data are mean+SE n=9/group *= Significant statistical difference (P<0.05) from control group*

*Table 6.2: Average hepatic arterial, portal, and total blood flow in 42-h fasted conscious dogs during the basal and experimental periods in the control and SIN-1 groups*

| | Basal | Experimental | Experimental +/- SIN-1 |
|---|---|---|---|
| **Average Hepatic Arterial Blood Flow (ml/kg/min)** | | | |
| **Control** | 6.0±0.4 | 9.0±0.8§ | 10.3±1.1§ |
| **SIN-1** | 4.9±0.4* | 6.0±0.5*§ | 7.2±0.6*§ |
| **Average Hepatic Portal Blood Flow (ml/kg/min)** | | | |
| **Control** | 22.4±1.7 | 17.8±1.2§ | 18.7±1.4§ |
| **SIN-1** | 22.1±1.8 | 15.9±1.4§ | 19.6±1.4¥ |
| **Average Total Hepatic Blood Flow (ml/kg/min)** | | | |
| **Control** | 28.4±1.9 | 26.8±1.9 | 29.0±2.1 |
| **SIN-1** | 26.9±1.8 | 21.9±1.7*§ | 26.8±1.7 |

*Data are mean+SE n=9/group*
*§ = Significant statistical difference (P<0.05) from basal period within the group*
*¥ = Significant statistical difference (P<0.05) from period within the group*
** = Significant statistical difference (P<0.05) from control group*

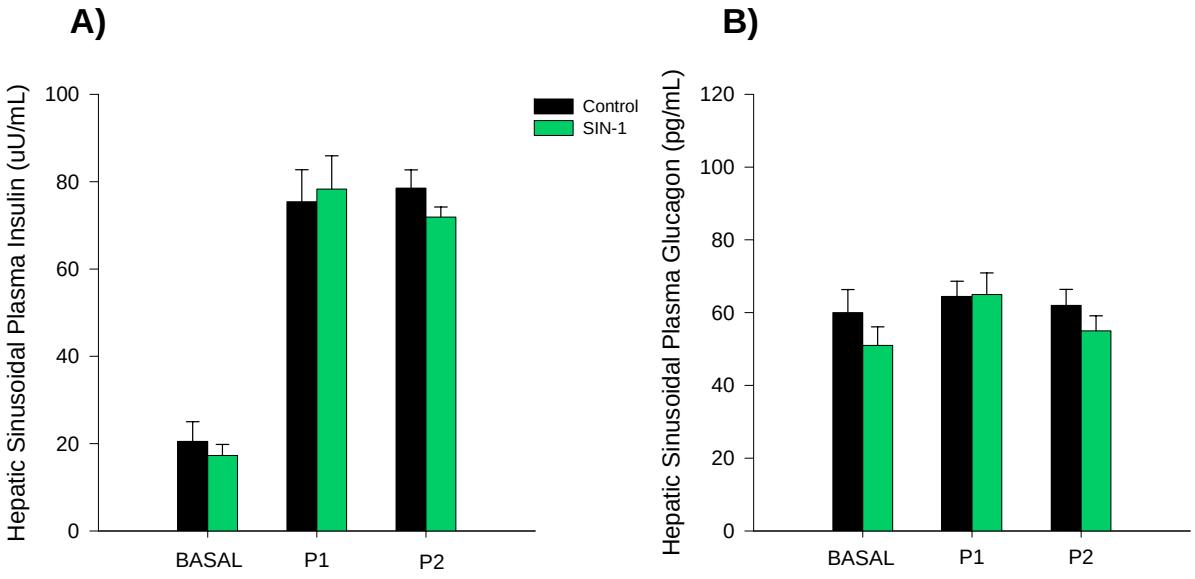


**Figure 6.1**: Hepatic sinusoidal plasma insulin (a) and glucagon (b) levels in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in both the control and SIN-1 treated groups (n=9/group). Data are mean ± SE.

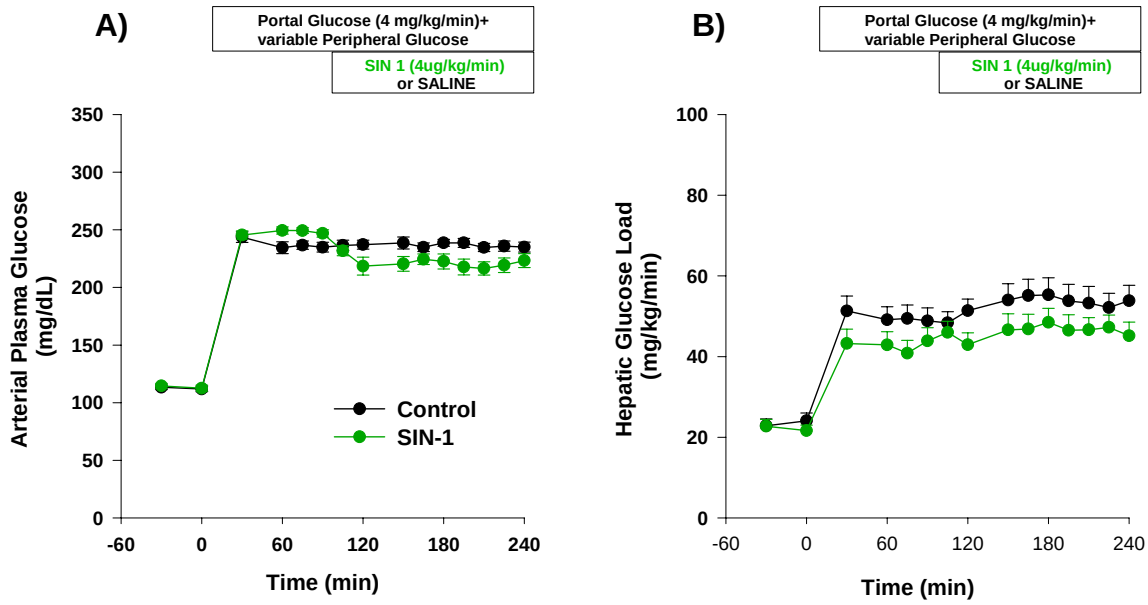


**Figure 6.2:** Arterial plasma glucose levels (a) and hepatic glucose load (b) in control and SIN-1treated 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods (n=9/group). Data are mean ± SE.

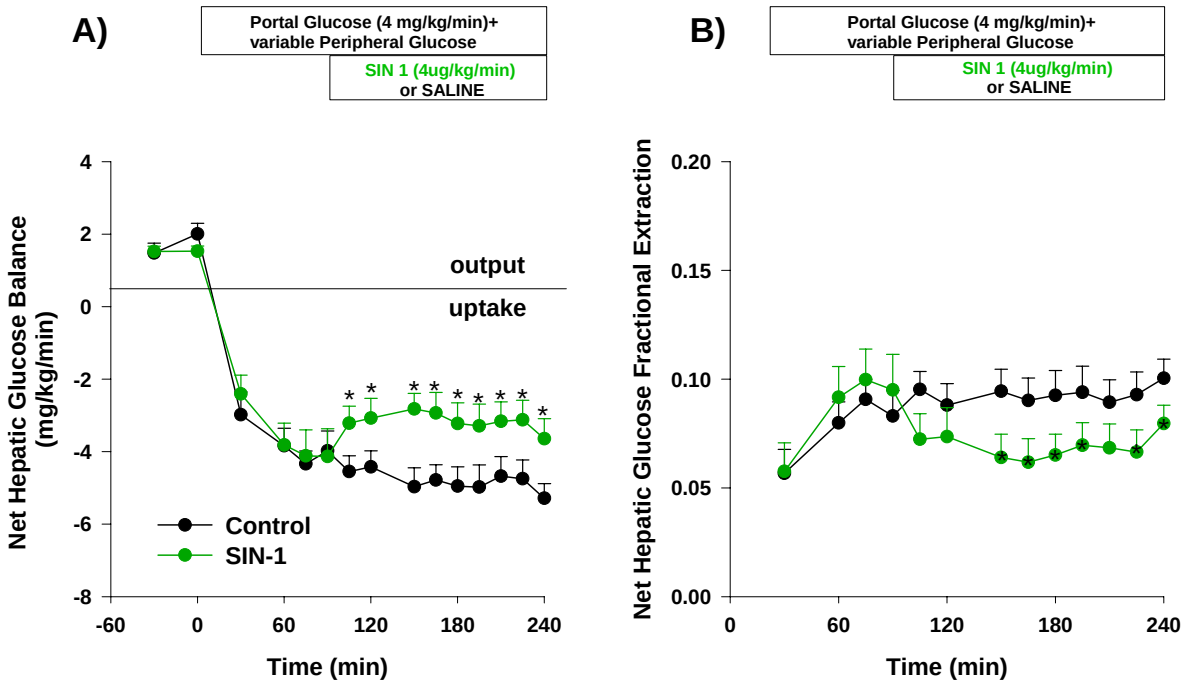


**Figure 6.3**: Net hepatic glucose balance (a) and net hepatic glucose fractional extraction (b) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in both the control and SIN-1 treated groups (n=9/group). Data are mean ± SE ∗= P<0.05 compared to control group

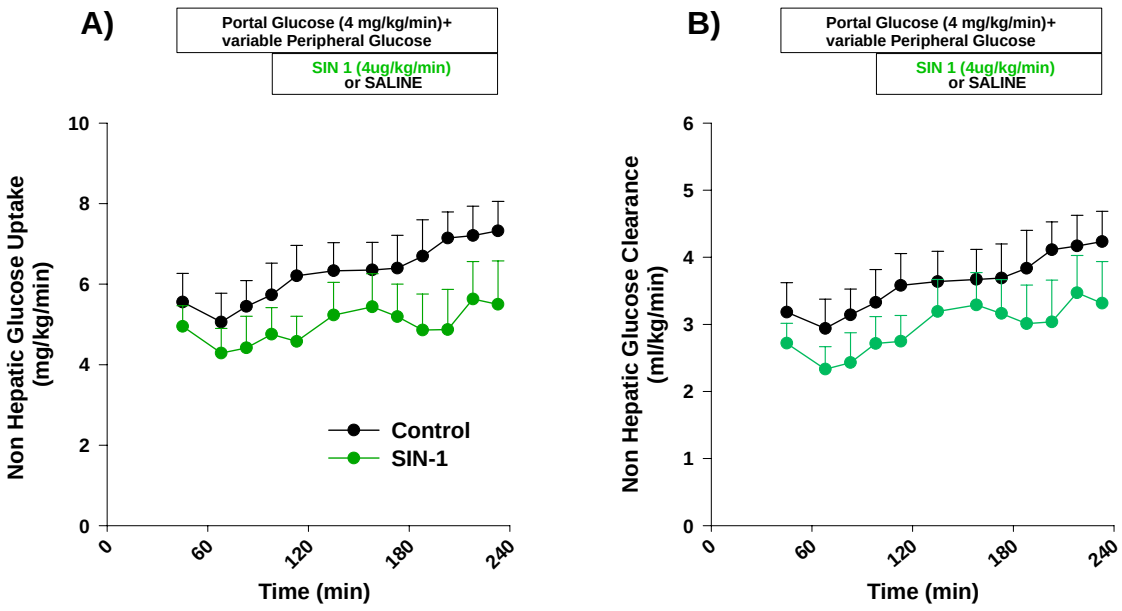


**Figure 6.4:** Non hepatic glucose uptake (a) and non hepatic glucose clearance (b) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in both the control and SIN-1 treated groups (n=9/group).

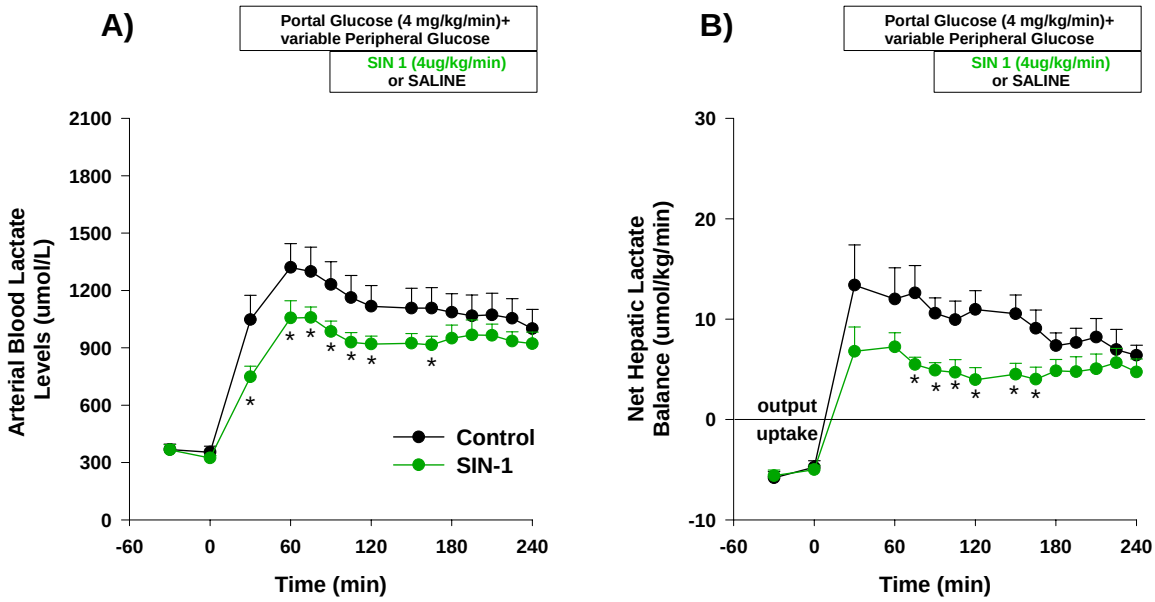


**Figure 6.5:** Arterial blood lactate (a) and net hepatic lactate balance (b) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in both the control and SIN-1 treated groups (n=9/group). Data are mean ± SE. ∗= P<0.05 compared to control group

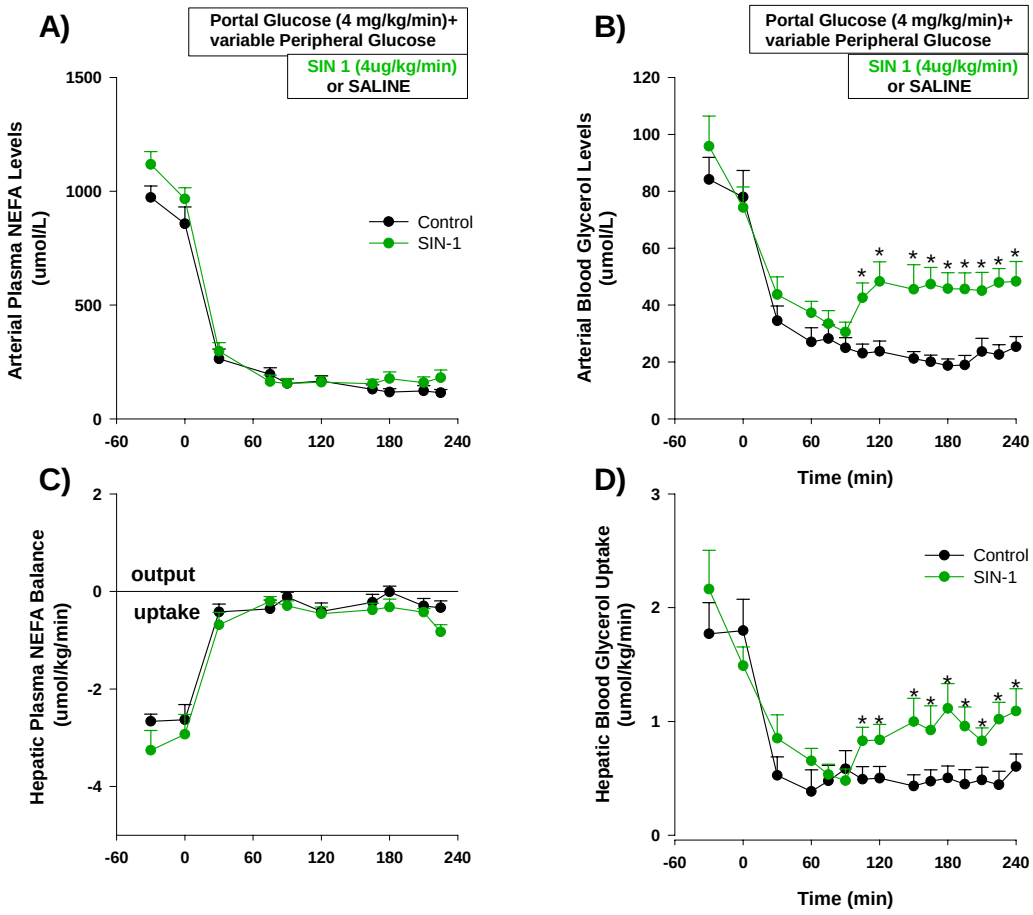


**Figure 6.6:** Arterial plasma NEFA levels (a), arterial blood glycerol levels (b), hepatic plasma NEFA balance (c) and hepatic blood glycerol uptake (d) in 42-h-fasted conscious dogs maintained on a pancreatic clamp during the basal and experimental periods in both the control and SIN-1 treated groups (n=9/group). Data are mean ± SE. *= P< 0.05 compared to control group

# CHAPTER VII

## SUMMARY AND CONCLUSIONS

Type II diabetes is characterized by both fasting and postprandial hyperglycemia (72, 80, 92, 96). In both of these situations, the extent of the hyperglycemia is determined by the net balance of glucose, i.e the amount of glucose entering and leaving the circulation. Following an overnight fast, the liver and kidney are primary sites of glucose production (46, 182). Glucose released from the liver is derived from both glycogenolysis as well as gluconeogenesis while the glucose released from the kidney is solely due to gluconeogenesis (83, 182). This situation becomes more complex following carbohydrate ingestion (71, 73, 80, 92). Since hepatic glycogenolysis is not fully suppressed following food ingestion (67, 173), the amount of glucose that is released into the systemic circulation from the splanchnic tissues represents the interplay between the rate of glucose absorption, the rate of splanchnic (gut and hepatic) glucose extraction, and the rate of hepatic glucose production (20).

It is well known that the arterial plasma insulin/glucagon level, the hepatic glucose load, and the route of glucose delivery are the three major determinants of NHGU (47). Recent studies have reported that the ability of insulin and glucose to stimulate splanchnic glucose uptake is impaired in individuals with type 2 diabetes (21). Thus, in specific aim I of this thesis we explored the ability of first phase insulin release to control the glycemic excursion on the background of basal

insulin and a duodenal glucose infusion. The pulse of insulin acutely affected net hepatic glucose production and increased NHGU while it still had significant effects on plasma glucose levels 4.5 hours later due to its ability to affect non hepatic glucose clearance. An analogue of insulin developed for oral administration (HIM2) was cleared from the body less efficiently than Humulin, resulting in higher plasma insulin levels at both the liver and the periphery. This pulse increased plasma insulin levels for almost 60 minutes, eliminated net hepatic glucose production by 30 minutes, and continued to have a significant effect on plasma glucose levels even at the end of the experiment (4.5 hours). With the focus in the pharmaceutical market turning towards new insulin therapies, this study demonstrated the ability of a brief burst of insulin secretion to result in prolonged glycemic improvement and speaks to the importance of first phase insulin release in postprandial glucose homeostasis. Unfortunately, the biologic activity of HIM-2 was only half that of regular insulin rendering it unsuitable for further clinical development. Future efforts will be directed towards the development of new insulin molecules which might allow oral insulin to be a treatment modality of the future.

The role of the portal signal in the regulation of NHGU has been an area of research for many years. It is clear that when the portal vein glucose concentration is greater than that seen in the artery, i.e, what is seen following an oral glucose load, that there is an increase in NHGU. This increase in NHGU in response to the portal glucose signal is also affected by both the insulin concentration as well as the hepatic glucose load presented to the liver. It has been suggested that the

portal signal may be impaired in diabetic individuals, resulting in a defect in splanchnic glucose uptake when glucose is given orally. Thus, it is critical to understand how NHGU is regulated.

Although it has been demonstrated that the portal glucose signal is an important regulator of NHGU, the mechanism of action by which it exerts its effects is not completely understood. Prior to the work completed in this thesis it was demonstrated that total hepatic denervation could eliminate the ability of the liver to increase NHGU in response to the portal glucose signal suggesting that neural input was important in regulating NHGU. The relative importance of the sympathetic and parasympathetic nerves, however, was not known. Other investigators had suggested that the portal signal may exert its effects through an intrahepatic reflex involving other mediators and neurotransmitters. Thus the second aim of this thesis was to further elucidate the potential regulators of the portal glucose signal and NHGU.

Our attention first turned towards the role of the sympathetic nerves in the regulation of NHGU. The data from specific aim II suggest that the sympathetic nerves exert a tonic inhibition on NHGU in the presence of hyperglycemia, and that removal of these nerves, results in an increase in NHGU. While our data suggest that the portal glucose signal increases NHGU, in part at least, by causing a decrease in sympathetic tone, further understanding of this event is still required. For example, it still remains to be determined whether there is additional involvement of a stimulatory signal in the response to portal glucose administration.

Our conclusions from specific aim II led us to further investigate the vagus nerve and the role of the vagal afferents in mediating the portal glucose signal. The data from specific aim III suggested that the vagus nerve does not play a role in the regulation of NHGU suggesting that the afferent nerves found in the vagus are not the transmitters of the portal glucose signal.

The data from specific aims II and III suggested that the sympathetic nerves exerted a dominant effect on NHGU while the input from the vagus nerve is not essential for the regulation of the portal glucose signal. Thus, the possibility exists that a local effect of the portal signal might be occurring via an intrahepatic reflex. With the idea that other signals and mediators may regulate NHGU, our work turned towards the role of nitric oxide in the regulation of NHGU. It is clear that the intraportal administration of the nitric oxide donor SIN-1 significantly decreased NHGU in the presence of the portal signal. As discussed in chapter VI, this may be due to either a direct or indirect effect on hepatic glucose metabolism. It is possible that the vasodilation caused by NO resulted in a decrease in blood pressure which in turn resulted in an increase in the response of the sympathetic nervous system. This may have caused a decrease in NHGU, an increase in lipolysis, and an increase in the heart rate. Alternatively, it is possible that the NO generated from the SIN-1 had a direct effect on the liver itself by affecting the glycogenoyltic pathway and in addition may have had a direct effect on the adipocyte to increase lipolysis.

Thus the work presented in this dissertation demonstrates that NHGU is regulated by both neural mechanisms, primarily the sympathetic nerves, as well as other

mediators which may be acting through neural pathways or having direct actions of their own. It now becomes important to determine whether the NO is decreasing NHGU indirectly by stimulating the sympathetic nervous system or directly by affecting the hepatocytes. In future experiments SIN-1 could be delivered peripherally to determine its effects. If SIN-1 still decreases NHGU, this suggests that the site of the SIN-1 administration is not important and that the effects seen may be due to the indirect mechanism. If, on the other hand, peripheral SIN-1 administration does not decrease NHGU then this suggests that the intraportal SIN-1 administration may be due to a direct effect of SIN-1 on the hepatocytes. Another way to examine the question of whether NO is exerting its effect via an indirect or direct mechanism would be to administer SIN-1 intraportally in a denervated animal.

Studies are currently underway to examine the role of L-NAME, a potent NOS antagonist, on NHGU. We hypothesize that the intraportal infusion of L-NAME will increase NHGU, opposite to that seen with the intraportal infusion of SIN-1.

In summary, all studies described in this dissertation have clinical applications. Because post-prandial hyperglycemia is thought to predispose an individual to the development of complications, it is critical to understand the regulators that control this state. Insulin is a clear regulator of NHGU in the post-prandial state and we have demonstrate that “restoring” first phase insulin release can have a significant effect on the hepatic glycemic profile even 4.5 hours following a 5 minute pulse of insulin. We have also suggested the importance of the sympathetic nerves in the regulation of NHGU and the possible regulatory role of

NO in mediating NHGU. If it is found that the portal signal is not as effective in increasing NHGU in individuals with type 2 diabetes, then those factors which affect NHGU, including both neural input as well as other mediators such as NO, may become important in the treatment of the disease.

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