THE REGULATION OF NET HEPATIC GLUCOSE UPTAKE BY NITRIC OXIDE AND SEROTONIN *IN VIVO*

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To my beloved family and friends, infinitely supportive

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CHAPTER I

INTRODUCTION

Glucose Metabolism and Homeostasis

Carbohydrates, fat, and proteins are required to make energy available to the various cells and tissues of the body. Three major sources of carbohydrates exist in the normal human diet (Chinachoti, 1995). They are sucrose, which is the disaccharide known popularly as cane sugar; lactose, which is a disaccharide found in milk; and starches, which are large polysaccharides present in almost all nonanimal foods, particularly in potatoes and the different types of grains (Chinachoti, 1995). Other carbohydrates ingested to a slight extent are amylose, glycogen, alcohol, lactic acid, pyruvic acid, pectins, dextrins, and minor quantities of carbohydrate derivatives in meats (Chinachoti, 1995). The final products of carbohydrate digestion in the alimentary tract are almost entirely glucose, fructose, and galactose—with glucose representing, on average, ~80% of these (Ivy, 1999). After absorption from the intestinal tract, much of the fructose and almost all the galactose are rapidly converted into glucose in the liver. Glucose thus becomes the final common molecule for the transport of almost all carbohydrates to cells. Glucose can be supplied by the liver as a result of release from stored glycogen (glycogenolysis) and the *de novo* hepatic synthesis of glucose through the conversion of non-carbohydrate precursors to glucose (gluconeogenesis) (Cherrington, 1999).

It is critical to maintain body glucose homeostasis. Glucose is the only nutrient that normally can be used by the brain, retina, and germinal epithelium of the gonads in sufficient quantities to supply them optimally with their required energy. Therefore, it is important to maintain the blood glucose concentration at a sufficiently high level to provide this necessary nutrition. Indeed, it is important that the pancreas secrete little insulin during fasting; otherwise, the scant supplies of glucose that are available would all go into the muscles and other peripheral tissues, leaving the central nervous system without a nutritive source. It is also important that the blood glucose concentration not rise too high: Firstly, glucose can exert a large amount of osmotic pressure in the extracellular fluid, so if the glucose concentration rises to excessive values, this can cause considerable cellular dehydration. Secondly, an excessively high level of blood glucose causes loss of glucose in the urine (glycosuria), which is in turn results in osmotic diuresis by the kidney, depleting fluids and electrolytes from body. Thirdly, long-term hyperglycemia can cause tissue damage, especially to blood vessels, nerves and kidney. For example, vascular injury, associated with uncontrolled diabetes mellitus, leads to increased risk for heart attack, stroke, end-stage renal disease, and blindness (Brownlee, 2005).

In a normal state, the blood glucose concentration is tightly controlled, usually between 80 and 90 mg/dl of blood in the over night fasted humans. This concentration increases to 120 to 140 mg/dl during the first hour or so after a meal, but the feedback systems for control of blood glucose return the glucose concentration rapidly back to the basal level, usually within 2 hours after the

absorption of carbohydrates (1969). In starvation, the glycogenolytic and gluconeogenic functions of the liver allow it to provide the glucose that is required to sustain glucose requiring tissues.

The liver functions as a pivotal blood glucose buffer system. That is, when the blood glucose rises to a high concentration after a meal and the rate of insulin secretion also increases, as much as one third of the glucose absorbed from the gut is quickly stored in the liver in the form of glycogen (Cherrington, 1999). Then, during the succeeding hours, when both the blood glucose concentration and the rate of insulin secretion fall, the liver releases the glucose back into the blood. In this way, the liver decreases the fluctuations in blood glucose concentration to about one third of what they would otherwise be. In fact, in patients with severe liver disease, it becomes almost impossible to maintain a narrow range of blood glucose concentration (Del Vecchio Blanco *et al.*, 1990).

Both insulin and glucagon function as important feedback control systems for maintaining a normal blood glucose concentration. When the glucose concentration rises, insulin is secreted; the insulin in turn causes the blood glucose concentration to decrease toward normal. Conversely, a decrease in blood glucose stimulates glucagon secretion; the glucagon then functions to cause an increase in glucose toward normal.

In severe hypoglycemia, a direct effect of low blood glucose on the hypothalamus stimulates the sympathetic nervous system (Matsuhisa *et al.*, 1997). In turn, the epinephrine secreted by the adrenal glands causes still further release of glucose from the liver. This, too, helps protect against severe

hypoglycemia. Over a period of hours and days, growth hormone and cortisol are secreted in response to prolonged hypoglycemia, and they both decrease the rate of glucose utilization by most cells of the body, converting them instead to greater amounts of fat utilization (Schwartz *et al.*, 1987). This, too, helps return the blood glucose concentration toward normal.

In summary, the normal human body is able to carefully maintain plasma glucose homeostasis by matching glucose utilization with endogenous glucose production and dietary glucose delivery. Liver, muscle, fat, brain and the endocrine organs work together to achieve this precise matching.

Hepatic Glucose Uptake

The liver can both provide glucose, to and remove glucose from, the circulation. A number of studies in dogs and humans have shown that the liver is responsible for ~30% of glucose disposal following glucose ingestion (Pagliassotti et al. 1994) (Meyer *et al.*, 2002), clearly demonstrating the importance of the liver under this physiological condition.

Glucose ingestion results in significant hyperglycemia, as well as marked hyperinsulinemia, with the change of glucagon being minimal. As reported by Abumrad et al. (Abumrad *et al.*, 1982), when normal conscious dogs received an intragastric glucose load of 1.6 g/kg, the arterial blood glucose concentration almost doubled within 30 minutes, the arterial plasma insulin level increased approximately 4-5 fold, and the plasma glucagon level remained unchanged. As a consequence of these signals, the liver rapidly 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 after oral glucose administration (peak rate at ~4.5 mg/kg/min). In a study performed by Hamilton et al. (Hamilton *et al.*, 1996), conscious dogs received glucose infused into the duodenum at 8 mg/kg/min for 150 min. In response to intraduodenal glucose infusion, arterial plasma glucose rapidly rose ~30%, the arterial plasma insulin increased approximately 3-4 fold, and the arterial plasma glucagon was unchanged, and this resulted in the rate of net hepatic glucose uptake ~ 2.3 mg/kg/min.

There are three main factors which impact the rate of net hepatic glucose uptake (uptake in excess of production) in response to an oral/intraduodenal glucose load: the insulin concentration in the liver, the glucose concentration (the glucose load to the liver), and a glucose gradient between the hepatic artery and portal vein. A series of studies by Cherrington et al. and others have been performed to show how these three factors work individually, or in concert with one another, to bring about the resulting net hepatic glucose uptake.

Effect of Hyperglycemia on Hepatic Glucose Uptake

It has been shown that hyperglycemia within the physiologic range (plasma glucose ~230 mg/dl) when brought about acutely by glucose infusion into a peripheral vein, in the presence of basal levels of insulin and glucagon, causes a suppression of net hepatic glucose output, but little net glucose uptake by the liver. Studies in humans have demonstrated that a plasma glucose increment of 125 mg/dl, in the presence of basal insulin levels, leads to a

decrease in net splanchnic glucose output from ~2.5 to 0.6 mg/kg/min but no net glucose uptake (DeFronzo *et al.*, 1987; DeFronzo *et al.*, 1983). It has been shown that nonhepatic net splanchnic (gut) glucose uptake makes only a small contribution (~10%) to overall net splanchnic glucose uptake, so net splanchnic glucose uptake rates in humans are very similar to net hepatic glucose uptake (Cherrington *et al.*, 1991). Similarly, doubling the blood glucose level/hepatic glucose load in the dog by infusing exogenous glucose into a peripheral vein, in the presence of basal insulin and glucagon caused a reduction in net hepatic glucose output from 2.2 to 0.9 mg/kg/min, but no evidence of net hepatic glucose uptake was observed (Cherrington *et al.*, 1987; Cherrington *et al.*, 1982). Selective hyperglycemia can result in slight net hepatic glucose uptake (up to 0.6 mg/kg/min) if hyperglycemia lasts for more than 2 hours (Pagliassotti *et al.*, 1996b). A recent study in dogs showed that the rate of net hepatic glucose could reach ~1.5 mg/kg/min after 6 hours of hyperglycemic clamp in the presence of basal levels of insulin and glucagon (Winnick *et al.*, 2009).

On the mechanistic level, an increase in blood glucose concentration per se has been shown to regulate net hepatic glucose balance by suppressing hepatic glucose production and by stimulating glucose uptake (Pagliassotti *et al.*, 1996b; Rossetti *et al.*, 1993). Transport of glucose into hepatocytes is through the GLUT2 transporter, which maintains the intracellular to extracellular glucose concentrations in equilibrium. Elevated blood glucose has been shown to increase glucose influx into hepatocytes and to activate glucokinase, resulting in an increase in the concentration of glucose 6-phosphate (G-6-P), which

modulates the phosphorylation state of downstream enzymes synergistically with glucose (Agius *et al.*, 1997). In the fasted state, glucokinase is in part sequestered in the nucleus in an inactive form, complexed to the glucokinase regulatory protein (GKRP). Glucokinase is mobilized to the cytoplasm during the postprandial state in response to elevated glucose by rapid mechanisms that involve glucose-induced dissociation from GKRP (Agius *et al.*, 1996). The elevated G-6-P concentration, consequent to glucokinase activation, has a synergistic effect with glucose, by stimulating phosphatase, to promote dephosphorylation (inactivation) of glycogen phosphorylase and induce dephosphorylation (activation) of glycogen synthase, resulting in glycogen deposition and increased hepatic glucose uptake (Ferrer *et al.*, 2003). Further, this reciprocal change of synthase and phosphorylase activities lead to an inhibition of net glycogenolysis and an suppression of hepatic glucose production (Rossetti *et al.*, 1993).

Thus, acute hyperglycemia resulting from a peripheral glucose infusion is able to reduce net hepatic glucose output, but cannot independently switch the liver, in a net sense, from glucose output to uptake in the short term.

Effect of Insulin on Hepatic Glucose Uptake

In humans, in the presence of euglycemia and arterial plasma insulin levels which were maintained at about 100 μU/ml (8-10 times basal levels) using the pancreatic clamp technique, net splanchnic glucose uptake was only 0.7 mg/kg/min (DeFronzo *et al.*, 1987). In dogs, NHGU was 0.6 mg/kg/min in the

presence of euglycemia and physiologic hyperinsulinemia (120 μU/ml), and even pharmacological levels of insulin (2044 μU/ml) were only able to achieve rates of 2.9 mg/kg/min (McGuinness *et al.*, 1990). Thus high physiologic levels of insulin alone are only able to trigger hepatic glucose uptake to a very limited extent. Thus, hyperinsulinemia, without elevated glucose, is not very effective means of causing glycogen storage in the liver.

It has been shown that the augmentation of NHGU in response to elevated insulin occurs through both an increase in hepatic glucose uptake and suppression of hepatic glucose production (Pagliassotti *et al.*, 1996b). These changes were due to an increase in glycogen synthesis and an inhibition in glycogen breakdown (Cherrington, 1999), which was mediated by promoting dephosphorylation of glycogen phosphorylase (inactivation) and glycogen synthase (activation). The change of phosphorylation state of these two enzymes is regulated by activation of protein phosphatase-1 and inhibition of GSK-3, resulting from activation of PKB/Akt in response to elevated insulin (Cheatham *et al.*, 1995; Taniguchi *et al.*, 2006).

Selective glucagon deficiency (i.e. usually kept basal), which was brought about using the pancreatic clamp in the presence of euglycemia, reduced hepatic glucose output but failed to cause net glucose uptake by the liver in dogs and humans (Liljenquist *et al.*, 1977; Shulman *et al.*, 1978). Further, hypoglucagonemia, even when brought about in the presence of hyperglycemia and hyperinsulinemia did not result in any measurable enhancement of NHGU (Holste et al., 1997).

Therefore, physiologic changes in glucose, insulin or glucagon individually cannot explain the significant net hepatic glucose uptake evident following oral glucose intake.

Effect of Combined Hyperglycemia and Hyperinsulinemia on Hepatic Glucose Uptake

Elevated insulin, when brought about in the presence of basal glucagon, and hyperglycemia resulting from the infusion of glucose into a peripheral vein can result in significant net hepatic glucose uptake. In humans, the combination of hyperglycemia (arterial plasma levels of 175-225 mg/dl) and hyperinsulinemia (arterial blood levels of 40-55 μU/ml) caused rates of net splanchnic glucose uptake of 1.0 to 1.6 mg/kg/min (DeFronzo *et al.*, 1987; DeFronzo *et al.*, 1983; Sacca *et al.*, 1982). In dogs, arterial plasma glucose levels of 160-290 mg/dl and insulin levels of 35-384 μU/ml caused rates of NHGU of 1.0 to 2.9 mg/kg/min (Adkins-Marshall *et al.*, 1990; Barrett *et al.*, 1985; Cherrington *et al.*, 1987; Ishida *et al.*, 1983; Myers *et al.*, 1991a; Myers *et al.*, 1991b). Despite the increase in NHGU in response to combined hyperglycemia and hyperinsulinemia, it is clear that these two factors at physiological levels (arterial plasma glucose levels <225 mg/dl and arterial blood insulin levels <100 μU/ml) cannot result in the peak rates of NHGU that are seen following a moderate oral glucose load. Thus, one can postulate that there must be other factors, in addition to increased insulin and glucose, which are involved in the regulation of NHGU, to ensure normal glucose uptake by the liver after a carbohydrate meal.

The "Portal Glucose Signal" Theory

In contrast to the hyperglycemic hyperinsulinemic data above, it has been shown that in conscious dogs receiving an intragastric glucose load of 1.6 g/kg, that by 75 min after feeding, when the plasma glucose concentrations in the artery, hepatic portal vein, and hepatic vein were 190, 230, and 220 mg/dl, respectively, with elevated plasma insulin at ~5X basal, and the plasma glucagon level at basal, net hepatic glucose uptake peaked at ~4.5 mg/kg/min (Abumrad et al., 1982). In line with this work in dogs, a human study conducted by DeFronzo (DeFronzo et al., 1978) demonstrated that the route of glucose administration could be another signal in regulating hepatic glucose uptake. They showed that infusion of glucose through an antecubital vein resulted in hyperglycemia (arterial plasma glucose ~220 mg/dl) and hyperinsulinemia, but there was little net splanchnic glucose uptake in response to this treatment (~1.0 mg/kg/min). When glucose was given orally (1.2 g/kg), and the intravenous glucose infusion rate was decreased to achieve a similar arterial glucose level (plasma glucose ~220 mg/dl) to that seen during peripheral glucose infusion, net splanchnic glucose uptake increased almost 6 fold (5.9 mg/kg/min). As expected, insulin levels following the oral glucose ingestion were higher than those as observed during the peripheral glucose administration even though the arterial glucose levels were equivalent. However, it is unlikely that this explains the difference in net splanchnic glucose uptake, since the rate of net splanchnic glucose uptake was only ~1.1 mg/kg/min when the insulin levels went even higher than those seen following the oral glucose ingestion and arterial plasma glucose levels were

similar (~220 mg/dl) in the presence of intravenous insulin and glucose infusion. It also needs to be noted that in these experiments, although the arterial plasma glucose levels (~220 mg/dl) were matched, there was an increase in glucose load to the splanchnic bed in the group giving oral glucose load. This increase of the glucose load was probably not large enough to account for the increase in splanchnic glucose uptake. The discrepancy between NHGU during oral glucose delivery and peripheral glucose delivery was initially attributed to effects of incretins associated with oral glucose delivery (DeFronzo *et al.*, 1978; Lickley *et al.*, 1975). Several laboratories have since shown in conscious dogs, however, that intraportal administration of glucose can bring about the same response of net hepatic glucose uptake as oral glucose delivery (Bergman *et al.*, 1982; Ishida *et al.*, 1983; Ogihara *et al.*, 2003). In a key study, Adkins et al. determined that when plasma insulin and glucagon were clamped at basal levels in conscious dogs, and glucose was infused into either a peripheral vein or the hepatic portal vein such that the hepatic glucose load (the mass of glucose reaching the liver per unit of time) was doubled, the liver showed a trivial glucose output (~0.1 mg/kg/min) during peripheral glucose delivery, but significant glucose uptake (~1.4 mg/kg/min) during portal glucose delivery (Adkins *et al.*, 1987). This enhancement of NHGU by portal versus peripheral glucose delivery has been attributed to the “portal glucose signal”.

In another work performed by Adkins et al. in 18h and 36h fasted dogs, glucose was given either via a peripheral vein or the hepatic portal vein to double the hepatic glucose load in the presence of four fold elevated insulin and basal

glucagon. Despite a similar hormonal milieu and hepatic glucose loads, NHGU was 1.4±0.7 mg/kg/min in response to peripheral glucose infusion but 3.5±0.8 mg/kg/min in response to portal glucose infusion in 18h fasted dogs (Adkins-Marshall et al., 1990). Similar to these data, NHGU was 1.6±0.4 mg/kg/min and 4.0±0.4 mg/kg/min during the peripheral and intraportal glucose infusions, respectively, in the presence of hyperinsulinemia and hyperglycemia in 36h fasted dogs (Adkins-Marshall et al., 1990).

Further exploration of the effects of the portal signal in the conscious dog revealed that when hormone concentrations and the hepatic glucose load were fixed and equivalent, delivery of a portion of the glucose into the hepatic portal vein enhanced NHGU ~2-3 fold, compared with the rate when all of the glucose was infused through a peripheral vein (Myers *et al.*, 1991a; Myers *et al.*, 1991b; Pagliassotti *et al.*, 1996b).

The Relationships between the Portal Glucose Signal, Hepatic Glucose Load, Insulin and NHGU

Myers et al. (Myers *et al.*, 1991b) examined the relationship between the insulin level and NHGU, and the effects of portal glucose delivery on that relationship. A pancreatic clamp was used in 42h fasted conscious dogs and the arterial plasma insulin level was increased by intraportal insulin infusion at two-, four-, or eight-fold basal (each rate lasted for 90 min) in two sets of experiments (with or without portal glucose delivery) in the presence of basal glucagon. In one set of experiments, the arterial blood glucose concentration was clamped at ~175 mg/kg/min by glucose infusion exclusively through a peripheral vein, and NHGU

was 0.6±0.3, 1.5±0.4 and 3.0±0.8 mg/kg/min, respectively, in response to the three different levels of insulin. In the second set of experiments, glucose was infused intraportally (4-5 mg/kg/min) throughout the study and peripheral glucose infusion was used to match the hepatic glucose loads to those observed in the first protocol. NHGU was 2.0±0.5, 3.7±0.7, and 5.5±0.9 mg/kg/min, respectively, in the three different insulin infusion periods. One can calculate that when arterial plasma insulin levels were 4X basal in the presence of hyperglycemia (2X basal), insulin could account for approximately half of the increase in NHGU with the rest being attributable to the portal glucose signal. These results suggested that NHGU is dependent on both insulin levels and the route of glucose delivery, and under physiological conditions the "portal" signal is at least as important as insulin in the regulation of postprandial NHGU.

In another work by Myers et al (Myers et al., 1991a), similar metabolic clamp techniques were used in 42h fasted conscious dogs to keep plasma glucagon at basal, to elevate the arterial plasma insulin fourfold, and to examine the effect of three different hepatic glucose loads (2, 3 and 4X basal) on NHGU in the presence or absence of portal glucose delivery. In the absence of portal glucose delivery, increasing the hepatic glucose load increased NHGU by 1.2±0.4, 2.8±0.8, and 5.1±1.2 mg/kg/min in the three periods, respectively. In the presence of portal glucose delivery, NHGU was 3.8±0.4, 4.8±0.6, and 9.6±1.4 mg/kg/min in response to the three increments in the hepatic glucose load. These results indicated that NHGU was positively correlated with the hepatic glucose load regardless of the route of glucose administration and it did not

reach saturation even when the hepatic glucose load was elevated four times basal. In addition, at any given rate of hepatic glucose load in this study, NHGU was greater when portal glucose delivery was present than when glucose was exclusively infused through a peripheral vein. It was also apparent that portal glucose delivery lowered the threshold at which the liver switched from net glucose output to net glucose uptake from ~180 to ~100 mg/dl.

As stated above, it has been well established that physiologic elevations in insulin or glucose do not individually lead to significant net hepatic glucose uptake in humans and dogs (DeFronzo *et al.*, 1987; McGuinness *et al.*, 1990). In the absence of insulin, brought about either by pancreatectomy or by suppression of insulin secretion by somatostatin infusion, the liver did not respond to portal glucose delivery, even when hyperglycemia was present. These results suggested that the augmentation of NHGU in response to intraportal glucose delivery requires the presence of insulin (Pagliassotti et al., 1992b). On the other hand, Galassetti (Galassetti et al., 1999) showed that under euglycemic conditions, the portal signal is still effective in activating NHGU in the presence of hyperinsulinemia.

Pagliassotti et al. (Pagliassotti *et al.*, 1991) determined the relationship between the magnitude of the portal glucose signal and NHGU in the presence of three to four-fold elevation in insulin, basal glucagon, and a doubling of the hepatic glucose load in 42h fasted dogs. The magnitude of the portal signal (the glucose gradient between hepatic artery and hepatic vein, A-P gradient) was achieved by varying the portion of the glucose delivered intraportally vs.

peripherally. When the A-P gradient was -7 mg/dl, NHGU was ~2.1 mg/kg/min, whereas when the A-P gradient was increased to -15 mg/dl, NHGU was ~5.3 mg/kg/min, and when the A-P gradient was increased to -75 mg/dl, NHGU increased to ~7.6 mg/kg/min. This suggests that the relationship between the A-P gradient and NHGU is such that NHGU does not linearly increase with the increase in the magnitude of the A-P gradient. These results indicated that the liver can sense the magnitude of the negative A-P glucose gradient under physiologic conditions.

Combination of hyperinsulinemia and hyperglycemia in the presence of the portal glucose signal re-produces rates of NHGU that approach those observed after glucose ingestion. It is clear that the rate of NHGU is determined by dynamic interaction between the levels of pancreatic hormones, glucose, and the route of glucose delivery. Thus, these factors act efficiently in concert under physiological conditions to ensure adequate net removal of glucose by the liver, without requiring excessive excursions of insulin or glucose.

A further study by Pagliassotti et al. (Pagliassotti *et al.*, 1996b) extended the temporal response of the liver glucose uptake to insulin and/or portal glucose delivery in the dog. The experiments were carried out under hyperglycemic (2X basal) conditions with basal glucagon in 42-h-fasted conscious dogs, when insulin levels were kept at basal or elevated four fold, in the presence or absence of portal glucose delivery. NHGU was minimal in the presence of basal insulin and hyperglycemia (brought about by peripheral glucose infusion) in the absence of portal glucose delivery. In response to four-fold insulin and hyperglycemia

without portal glucose delivery, NHGU was 0.7±0.6 mg/kg/min at 15 min and gradually rose to the maximum rate (~2.9±1.2 mg/kg/min) until 90 min. In contrast, NHGU quickly reached a maximum of 2.3±0.6 mg/kg/min in only 15 min in response to portal glucose delivery in the presence of basal insulin and hyperglycemia. NHGU was 2.7±0.5 mg/kg/min at 15 min and reached a maximum as 4.3±0.6 mg/kg/min at 60 min in the presence of both elevated insulin and portal glucose delivery. Therefore, intraportal insulin could enhance NHGU minimally in the first 15 min after the rise in glucose, while the portal signal produced a rapid (15 min) and significant stimulation of NHGU. These results demonstrated that portal glucose delivery generates a signal that is associated with meal ingestion to activate NHGU rapidly and significantly. Interestingly, Hsieh et al. (Hsieh *et al.*, 1998) demonstrated that in the presence of basal insulin and glucagon, the effect of the portal signal on NHGU was rapidly reversed when portal glucose delivery was stopped. Taken together, it seems that the ‘‘on’’ and ‘‘off’’ times of the effect are similar and both very rapid. The quick response of the liver to the portal signal allows the tight matching of the meal associated rise in plasma glucose to hepatic glucose uptake in order to minimize perturbations in the circulating blood glucose levels.

Effects of the Portal Glucose Signal on Other Tissues

In addition to its effects on the liver, portal glucose delivery suppresses glucose uptake in non-hepatic tissues, especially skeletal muscle (Galassetti *et al.*, 1999; Galassetti *et al.*, 1998). Several studies (Adkins *et al.*, 1987;

Pagliassotti *et al.*, 1996b) showed that although NHGU increases in response to portal glucose delivery, the change of whole body glucose clearance was minimal. Therefore, in response to intraportal glucose delivery, a reduction in net glucose uptake by non-hepatic tissues must occur in parallel with the increase in NHGU.

In a study by Galassetti et al. (Galassetti *et al.*, 1998), insulin was elevated three-fold while glucagon was replaced at basal levels. In one set of the experiments, arterial blood glucose (2-fold basal) was clamped by glucose infusion into a peripheral vein, and in another set, glucose was given intraportally at 5 mg/kg/min along with the peripheral glucose infusion to maintain similar blood glucose levels. Despite comparable glucose loads and insulin levels, the portal signal blunted the increase of glucose uptake by nonhepatic tissues in response to hyperglycemia and hyperinsulinemia by ~40%. Simultaneous measurement of net glucose balance across the hindlimb, which is primarily muscle, showed that virtually all nonhepatic glucose uptake could be accounted for by muscle glucose uptake. This study concluded that muscle and hepatic glucose uptake are altered reciprocally in response to portal glucose delivery, suggesting that the portal signal does not increase whole body glucose clearance, but rather directs glucose to the liver and away from the muscle to ensure a balanced distribution of glucose and rapid accumulation of hepatic glycogen reserves following glucose ingestion. Interestingly, like the temporal response of NHGU to portal glucose delivery, the suppression of nonhepatic glucose uptake in the presence of portal glucose signal can also be quickly reversed once the

signal is removed (Hsieh *et al.*, 1998).

Another study (Galassetti *et al.*, 1999) showed that the portal signal is as effective in increasing NHGU under euglycemic conditions as it is during hyperglycemia. The inhibition of glucose uptake by nonhepatic tissues, on the other hand, is minimal under euglycemic as opposed to hyperglycemic conditions.

The above results clearly show that the portal signal enhances glucose uptake by the liver but reduces muscle glucose uptake in the presence of fixed hormone concentrations and hyperglycemia. Further, even when hormones are free to change, Moore et al. (Moore et al., 2000) found that in the absence of somatostatin, when the arterial blood glucose concentrations during glucose infusion were at 120-125 mg/dl, the portal glucose delivery alters partitioning of glucose among the tissues, enhancing NHGU and reducing glucose uptake by peripheral tissues.

In the study above, Moore et al. (Moore et al., 2000) also observed that when 42-h-fasted dogs received identical (10 mg/kg/min for 3 hours) infusions of glucose via the hepatic portal or a peripheral vein, arterial plasma insulin levels tended to be higher during the first hour, particularly at 60 min in dogs receiving intraportal glucose infusion (~50% greater, but not significant due to high variabilities). This suggests that the portal signal might also stimulate pancreatic insulin secretion. Dunning et al. (Dunning *et al.*, 2002) then went on to determine the effects of the portal signal on insulin secretion by direct measurement of the pancreatic venous effluent using the superior pancreaticoduodenal vein catheter. In that study, hyperglycemia (arterial plasma glucose ~155 mg/dL) was

maintained in 2 groups of 42h-fasted conscious dogs by glucose infusion into the hepatic portal vein or a leg vein. Arterial plasma insulin levels increased by 28±5 and 16±3 mU/mL, and pancreatic insulin output increased by 10±3 and 7±2 mU/min in response to portal or peripheral glucose administration, respectively. Pancreatic polypeptide output and norepinephrine spillover were measured as indices of pancreatic parasympathetic and sympathetic neural activity, respectively. The pancreatic polypeptide output to norepinephrine spillover ratio decreased by 65% in response to peripheral glucose infusion, while the ratio did not change significantly in response to portal glucose infusion. Thus, the portal glucose signal appears to inhibit the shift toward sympathetic dominance that would otherwise result in stimulation of insulin secretion.

The Portal Glucose Signal in Humans

The portal signal is less well explored in the human than in animal models, largely due to the difficulty in accessing the hepatic and portal veins. This technical limitation leads investigators to use the oral/intraduodenal glucose delivery rather than intraportal glucose infusion, and measure splanchnic glucose uptake or even whole body glucose disposal rather than net hepatic glucose uptake. It has been shown that the extrahepatic portion of net splanchnic glucose uptake makes only a small contribution (~10%) to overall net splanchnic glucose uptake, so the net splanchnic glucose uptake rate in humans is a reasonable approximation of net hepatic glucose uptake (Cherrington *et al.*, 1991).

As discussed previously, DeFronzo et al. (DeFronzo *et al.*, 1978) reported

that the increase in net splanchnic balance of glucose was much greater in response to ingested glucose than in response to intravenous glucose and insulin, suggesting that the route of glucose delivery was an important determinant of splanchnic/hepatic glucose uptake.

In a recent study carried out by Vella et al. (Vella et al., 2002) in non-diabetic humans under hyperglycemic hyperinsulinemic conditions, glucose was delivered via a peripheral vein or intraduodenal infusion. Somatostatin was used to inhibit pancreatic endocrine secretion, plasma insulin concentrations were clamped at ~75 µU/mL by infusion into a leg vein (creating a two to three fold rise in hepatic sinusoidal insulin), and plasma glucose concentrations were maintained at ~150 mg/dL with the two treatments. [^{3}H] glucose was mixed with the intraduodenal infusate (i.e. saline during peripheral glucose delivery and glucose during intraduodenal glucose delivery). In that investigation, initial splanchnic glucose extraction was modestly but not significantly greater in response to duodenal vs. peripheral glucose delivery (16.4 vs. 12.8%). The latter rate was very likely an overestimate, however, considering that the systemic appearance of duodenally delivered [^{3}H] glucose was probably underestimated due to the fact that duodenally delivered [^{3}H] glucose was infused duodenally without cold glucose carrier. Furthermore, studies in dogs and pigs have showed that intestinal glucose extraction ranges from 4-10% (Abumrad *et al.*, 1982; Stoll *et al.*, 1999). Thus, if one corrects the data in the vella's study for that, it becomes apparent that a 40-130% increase in hepatic glucose uptake occurred in response to intraduodenal glucose administration despite the modest A-P

glucose gradient and the limited hyperglycemia. Therefore, these findings supported the concept that hepatic glucose extraction is enhanced by duodenal versus peripheral glucose infusion. It should be noted that the route of glucose delivery did not appear to affect whole body glucose uptake. This is not surprising since it has been previously showed that the increase in net hepatic glucose uptake in response to portal glucose delivery is concomitant with a proportional decrease in nonhepatic glucose uptake, thus the whole body glucose disposal remains unchanged (Galassetti *et al.*, 1999).

Other investigators using tracer methods and indirect calorimetry rather than direct measurement, observed that when glucose was infused intraduodenally or intravenously at 6 mg/kg/min into healthy humans for 180 min, neither hepatic nor whole body glucose storage was affected by the route of administration (Fery et al., 2001). However, this study was limited in that it was not a clamp study, thus there were higher insulin levels and lower glucose levels following intraduodenal glucose infusion, and no direct data regarding hepatic glucose uptake were presented.

Another study comparing the metabolic responses to intraduodenal and intravenous glucose delivery in the humans also concluded that hepatic and peripheral glucose disposal is not significantly influenced by the route of glucose delivery (Fery *et al.*, 2004). These authors used dual-isotope techniques and indirect calorimetry, in the presence of a hyperinsulinemic euglycemic clamp. They found whole body glucose uptake, glycolysis, oxidation, and storage were similar whether glucose was administered enterally or via a peripheral vein. A

major drawback to this study is the fact that it was carried out under euglycemic conditions, when the role of the liver in glucose disposition is less important and the role of muscle is dominant (Galassetti *et al.*, 1999). Another drawback is that net first-pass splanchnic uptake, which was estimated by subtracting the appearance of exogenous glucose from the total glucose infusion rate, did not coincide with the value of hepatic glucose uptake. This may be due to the recycling of the enteral glucose initially taken up by the liver through glucose 6-phosphate and/or glycogen, and this estimation omits the portion of glucose taken up by extrahepatic splanchnic tissues.

As mentioned previously, one of the major limitations in exploration of hepatic glucose uptake in response to route of glucose administration in the human is an ability to access the hepatic portal vein. As a consequence, splanchnic glucose uptake is commonly used as the index of hepatic glucose uptake. Tracer kinetics technique could be an alternative method to measure hepatic glucose uptake, but its accuracy depends heavily upon the experimental conditions. In addition, the effects of factors released from the gut, such as incretins, following an oral/enternal glucose load will make it more difficult to differentiate the effects of portal glucose signal on hepatic glucose uptake. Therefore, whereas the portal signal has been demonstrated to exist in the human, its relative importance in human glucose metabolism remains to be completely elucidated.

Mechanism of Portal Signaling

The mechanism by which the portal glucose signal exerts its effects has been also investigated. Niijima and his coworkers (Niijima, 1982) showed the existence of glucose sensitive cells in the portal vein, therefore it is possible that portal glucose delivery somehow generates a unique signal important in regulating hepatic glucose uptake via portal glucose sensors.

It has been shown that neural involvement is important in the regulation of glucose homeostasis. The hypothalamus is one of the key sites for sensing of the glucose level to regulate both energy and nutrient homeostasis (Borg *et al.*, 1997; Lam *et al.*, 2007; Lam *et al.*, 2005; Matsuhisa *et al.*, 1997). Furthermore, it has been shown that the activation of hypothalamic adenosine triphosphate (ATP)-sensitive potassium channels is required for hypothalamic glucose sensing (Lam *et al.*, 2007). Recent research updated the hypothalamocentric model of glucose sensing with one emphasizing a widespread neural network involving numerous aspects of the central nervous system and peripheral sensory inputs. Thus, in addition to the hypothalamus, the area postrema, the nucleus of the solitary tract, and the dorsal motor nucleus have now all been shown to be important (Levin *et al.*, 2004; Marty *et al.*, 2007). Key peripheral glucose sensors have been identified in the oral cavity (Marty *et al.*, 2007), carotid bodies (Koyama *et al.*, 2000), gastrointestinal tract (Berthoud, 2008), and portal-mesenteric vein (Donovan, 2002). The question then is how the portal glucose signal is generated. It is conceivable that when oral/portal glucose delivery is present, the portal glucose level is compared with the arterial glucose level at some as-yet

undetermined reference site to initiate the response.

Since as noted above, the brain plays an important role in sensing glucose, Hsieh et al (Hsieh *et al.*, 1999a) determined whether the comparison of the brain arterial glucose level with the portal glucose level initiated the stimulatory effect of portal signal on NHGU. The study was performed under hyperglycemic and hyperinsulinemic conditions with basal glucagon levels. Glucose was infused into the hepatic portal vein at ~4 mg/kg/min and a peripheral vein at a variable rate to double the hepatic glucose load. In one group, glucose was infused into both vertebral and carotid arteries to eliminate the glucose gradient between the arterial blood in the head and the portal vein, while in another group, saline was infused into the head. The arterial-portal glucose gradients and the hepatic glucose loads were similar between groups. NHGU was 4.3±0.7 and 4.5±0.8 mg/kg/min in the glucose and saline infusion groups, respectively, in the present of elevated insulin and glucose. Therefore, the head arterial glucose level is not the reference standard used for comparison with the portal glucose level in the generation of the portal glucose signal.

The carotid body had been proposed as a possible arterial glucose sensing site in cats (Alvarez-Buylla *et al.*, 1988), rats (Alvarez-Buylla *et al.*, 1994), and dogs (Koyama *et al.*, 2000). However, Hsieh's results (Hsieh *et al.*, 1999a) do not support the involvement of carotid body in the initiation of the portal signal, since the rate of NHGU was not different when the glucose gradient between the arterial body and the portal vein was eliminated by giving glucose infusion into carotid arteries (infused glucose flowed over carotid sinus region).

Research using isolated perfused rat livers demonstrated that a negative glucose gradient between the hepatic artery and the portal vein could create a metabolic signal locally within the liver (Gardemann et al., 1986; Stumpel et al., 1997). Horikawa et al (Horikawa et al., 1998) reported that both portal vein and hepatic arterial glucose infusion stimulated NHGU in conscious dogs. These data suggested that glucose sensors within the liver, rather than the portal vein, might be involved in the enhancement of NHGU in response to the portal glucose delivery.

Hseih et al. (Hsieh et al., 2000) then went on to examine whether elimination of the hepatic A-P venous glucose gradient within the liver would alter the changes in net hepatic and peripheral glucose uptake induced by portal glucose delivery in the generation of the portal glucose signal. The study was again carried out under hyperglycemic and hyperinsulinemic conditions with basal glucagon levels. In two experimental groups, glucose was infused into the hepatic portal vein at ~4 mg/kg/min and a peripheral vein at a variable rate to double the hepatic glucose load, while glucose was exclusively infused into a peripheral vein in a control group. In one of the portal glucose infusion groups, saline was infused into 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 induced by portal glucose delivery. NHGU was 2.3±0.4 mg/kg/min in the control group, and it increased to 4.3±0.6 mg/kg/min in response to the portal glucose delivery, while it was 2.5±0.8 mg/kg/min when the hepatic A-P glucose gradient was eliminated. The authors concluded that the

glucose level at the hepatic artery plays an important role in generation of the effect of portal glucose delivery on glucose uptake by liver.

As a corollary to these data, one could speculate that ligation of the hepatic artery would result in augmentation of NHGU during peripheral glucose delivery, since the hepatic arterial glucose level would always be lower than that in the portal vein. On the other hand, hepatic artery ligation would not lead to enhancement in NHGU in response to the portal glucose delivery, in comparison with peripheral glucose delivery, because the maximal effects of the negative A-P gradient should be present at all times. Moore et al. (Moore et al., 2003) performed a study in which the hepatic artery was chronically ligated in conscious dogs. They demonstrated that NHGU in response to hyperinsulinemia and glucose delivered via a peripheral vein was not significantly different in dogs that had undergone the hepatic artery ligation than in sham dogs. Furthermore, portal glucose delivery still resulted in a significant increment in NHGU in dogs that had undergone the ligation. These data suggest that there may be more than one reference site for comparison to portal vein glucose levels, and that redundancy exists such that when the primary sensing site is lost, control reverts to other sites.

Another piece of evidence to support the notion of neural involvement in generation of portal glucose signal comes from the findings that when the liver was denervated, the hepatic response to the portal glucose delivery was blunted. Adkins-Marshall et al. (Adkins-Marshall et al., 1992) determined in conscious dogs that portal glucose delivery had no effect on NHGU, in the presence of a

hyperglycemic-hyperinsulinemic clamp, following a total hepatic denervation; thus, hepatic denervation blunted the ability of the liver to discriminate between portal and peripheral glucose delivery.

Since it has been suggested that the portal signal may be neurally mediated, the parasympathetic and sympathetic nerves that innervate the liver need to be considered. Autonomic nerves innervate the liver along three routes: the portal vein, the hepatic artery, and the bile ducts, and the autonomic nerves from the hypothalamus could control glycogen metabolism (Shimazu, 1967; Shimazu *et al.*, 1966). Tiniakos et al. (Tiniakos *et al.*, 1996) showed that both parasympathetic and sympathetic efferent innervation is involved in hepatic hemodynamics, bile flow regulation, and control of carbohydrate and lipid metabolism. Shimazu et al. showed that stimulation of the sympathetic efferent resulted in an increase in hepatic glucose output by stimulating glycogen phosphorylase (Shimazu, 1981; Shimazu, 1987; Shimazu, 1983) and PEPCK activity. In contrast, stimulation of the parasympathetic efferents led to activation of glycogen synthase and inactivation of PEPCK in the liver (Shimazu, 1981; Shimazu, 1987; Shimazu, 1983). Furthermore, the hepatic vagus nerve, in which ~90% of the fibers are afferent (Stumpel *et al.*, 1997) has been shown to convey information regarding plasma glucose and other nutrients to the brain (Sakaguchi et al., 1982).

DiCostanzo et al. (Dicostanzo et al., 2006) examined the importance of the hepatic sympathetic innervation and its role in mediating the effects of the portal glucose delivery in 42-h-fasted conscious dogs. They 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. One group of dogs underwent selective sympathetic denervation. This was achieved by cutting the nerves at the celiac nerve bundle near the common hepatic artery. Control dogs underwent a sham procedure. The study was carried out under hyperglycemic conditions with basal arterial insulin and glucagon levels in both groups. In the first 90 min, glucose was infused peripherally to double the hepatic glucose load, then glucose was infused intraportally (~4 mg/kg/min), and the peripheral glucose infusion was reduced so as to maintain the hepatic glucose load. This was followed by a 90 min period during which glucose was not infused portally and peripheral glucose infusion was increased to again maintain the hepatic glucose load. NHGU in the sham dogs averaged 1.4±0.4, 2.9±0.3, and 2.0±0.3 mg/kg/min while NHGU averaged 2.5±0.4, 3.2±0.2, 3.3±0.5 mg/kg/min in denervated dogs, in three periods, respectively. These data suggested that the sympathetic nerves do indeed exert an inhibitory tone on hepatic glucose uptake and the removal of this inhibition by hepatic sympathetic denervation enhanced NHGU during hyperglycemia in the absence of portal signal. Furthermore, the increment of NHGU in response to portal glucose delivery was significantly blunted. Thus, the portal signal may lead to the removal of an inhibitory tone to the liver, which in turn allows NHGU to increase.

It should be noted that intrahepatic nerves containing nitric oxide (NO), which takes part of the nonadrenergic noncholinergic (NANC) neurotransmission (Rand *et al.*, 1995), have been identified in a variety of mammalian species

traveling with the structures of the hepatic hilus, including rats, guinea pigs, and cats (Esteban *et al.*, 2001; Esteban *et al.*, 1998; Esteban *et al.*, 1997; Peinado *et al.*, 2002). Varicose nerve fibers immunoreactive for neuronal nitric oxide synthase (nNOS) form a dense plexus around the larger portal veins, hepatic arteries, and bile ducts (Esteban *et al.*, 2001; Peinado *et al.*, 2002). Therefore, it is conceivable that the observation by DiCostanzo et al. was influenced by the surgical intervention of NANC nerves, complicating the interpretation of their data.

One study examined the role of the hepatic vagus nerve in hepatic glucose metabolism in rats which were subjected to hepatic vagotomy or sham operation (Matsuhisa et al., 2000). They found that hepatic glucose uptake was markedly reduced in rats with hepatic vagotomy compared with sham rats in response to the portal glucose delivery in the presence of a euglycemic-hyperinsulinemic clamp. It appears that innervation of the hepatic vagus nerve is important for the regulation of hepatic glucose uptake.

Cardin et al. (Cardin *et al.*, 2004) then showed that in the presence of hyperinsulinemia and hyperglycemia brought about by portal glucose infusion, halting electrical transmission in the vagus nerves using the vagal cooling technique had no effect on NHGU. It appears that this finding does not support the concept that the vagus nerves play a role in the transmission of the portal glucose signal. However, it is possible that afferent signaling via the vagus nerve had been maximally suppressed by portal glucose delivery, and thus vagal cooling would have resulted in no further reduction in vagal afferent firing nor effect on efferent sympathetic nerve activity to the liver. To further explore the

possibility, a recent study by DiCostanzo et al. (DiCostanzo et al., 2007) examined the role of the vagus nerve in mediating the portal signal in the absence of portal glucose infusion. They hypothesized that in the absence of the portal glucose signal, vagal blockade would simulate a rise in portal glucose causing decreased afferent vagal firing, which in turn enhance net hepatic glucose uptake. The study was performed under hyperglycemic and hyperinsulinemic conditions in the presence of basal glucagon, and the vagus nerves were cooled or sham-cooled. The result showed that net hepatic glucose uptake did not differ in the two groups before (2.2±0.5 vs. 2.9±0.8 mg/kg/min, respectively) or during the coil perfusion period (3.0±0.5 vs. 3.4±0.6 mg/kg/min, respectively). These data indicated that interruption of vagal signaling in the presence of hyperinsulinemia and hyperglycemia, without portal glucose delivery, had no effect on NHGU, thus it is unlikely that vagus nerve is a determinant of the afferent limb of NHGU. However, the role of the efferent vagus nerve in response to the portal glucose delivery cannot be ruled out since Shiota et al. (Shiota et al., 2000) showed that intraportal infusion of acetylcholine at 3 ug/kg/min enhanced net hepatic glucose uptake by ~65% in the presence of hyperinsulinemia and hyperglycemia in the absence of portal glucose delivery.

More evidence supporting neural regulation of glucose uptake by the liver in response to portal signal comes from studies on the effects of neural mediators on hepatic glucose uptake. Norepinephrine (NE), the major sympathetic neurotransmitter, affects glucose metabolism in hepatocytes via α1-adrenergic receptors (Garceau *et al.*, 1984). Intraportal NE infusion (50 ng/kg/min,

which elevated the hepatic sinusoidal NE concentration to 4,100 pg/ml) markedly increased hepatic glucose production by selectively stimulating hepatic glycogenolysis (Chu *et al.*, 1998). Besides NE, another neuro-transmitter, neuropeptide Y (NPY), which is released during sympathetic signaling to the liver (Taborsky *et al.*, 1994), has been recently shown to stimulate net hepatic glucose uptake in conscious dogs during the intraportal NPY infusion (Nishizawa *et al.*, 2008).

Another piece of evidence supporting neural control of the liver comes from the time course of the effects of the portal glucose signal on the liver. If the hepatic response to the portal signal involves a neural system, both the transmission of the signal and the hepatic response should be rapid. Pagliassotti et al. (Pagliassotti *et al.*, 1996b) examined the time course of the effects of the portal glucose signal on the liver and showed that the speed of the response is consistent with neural mediation.

In that study, when both glucagon and insulin levels were kept at basal, the hepatic glucose load was doubled by peripheral glucose infusion in all groups, they showed that in the absence of the portal signal, the liver took up a very small amount of glucose (~0.4 mg/kg/min). When the portal signal was generated under the same experimental conditions, there was a rapid increase in net hepatic glucose uptake, which reached a maximum (~2.7 mg/kg/min) within 15 min and was sustained. Thus, the portal glucose signal activates the liver much more quickly than would otherwise be the case. It is worth noting that the dissipation of the effect of the portal signal on NHGU is also very rapid (~15 min),

indicating that uncoupling is also very efficient (Hsieh *et al.*, 1998; Hsieh *et al.*, 1999b).

It is also important to understand the intracellular mechanism by which hepatocytes are activated in response to the portal glucose signal and the fate of the extracted glucose. When levels of insulin and glucagon were maintained at basal values, hyperglycemia in the absence of portal glucose was associated with little accumulation of glycogen. In the presence of the portal glucose signal, there was a significant increase in glycogen deposition (Pagliassotti *et al.*, 1996b). The portal signal caused rapid glucose uptake by the liver, and a significant accumulation in hepatic glycogen deposition, which resulted from the activation of liver glycogen synthase. Thus, hyperglycemia alone was unable to activate glycogen synthase, but glycogen synthase was activated in a time-dependent manner in response to portal glucose delivery. It seems that an increase in glucose-6-phosphate levels and a reduction in phosphorylation of glycogen synthase within hepatocytes may be responsible for the metabolic responses induced by portal glucose delivery.

Net hepatic glucose flux is the balance between the rate of glucose entry and exit. These rates are dependent on the rate of glucose phosphorylation catalyzed by glucokinase (GK) and the rate of dephosphorylation of glucose-6-phosphate catalyzed by glucose-6-phosphatase. Another potential intracellular regulatory point is the translocation of GK within the hepatocyte. It has been shown that in the unstimulated state, GK is sequestered in the hepatocyte nucleus, where it is bound to its regulatory protein (GKRP), and it is allosterically

inhibited due to a decreased affinity for glucose (Agius *et al.*, 1997; van Schaftingen *et al.*, 1997). A rise in intracellular fructose-1-phosphate due to fructose uptake by the liver (Davies *et al.*, 1990) or treatment with high concentration of glucose (Agius *et al.*, 2000) in isolated hepatocytes led to dissociation of GK from GKRP, allowing GK to enter the cytosol, where it catalyzes the phosphorylation of glucose, thus promoting glucose entry into hepatocytes. Chu et al. (Chu *et al.*, 2004) determined that in response to intraduodenal glucose delivery in conscious rats, GK was exported from the nucleus within 10 min as determined evidenced by the ratio of the nuclear to the cytoplasmic immunofluorescence of GK, whereas GKRP, the binding protein that tethers GK in the nucleus did not change its location. The results suggested that GK but not GKRP translocates rapidly in a manner that corresponds with enhancement of NHGU in response to glucose ingestion. Furthermore, the resulting rise in glucose-6-phosphate following GK translocation would then result in the activation of glycogen synthase, and these two changes would drive glucose uptake and glycogen storage by the liver.

It has been established that portal signal plays a key role in the coordination of the metabolic response to glucose ingestion, however, more work still needs to be carried out to understand the mechanism of this signaling system.

Other Mediators of Net Hepatic Glucose Uptake

To date, it remains to be elucidated how the coordinated responses of

muscle and liver to portal glucose delivery come about. It has been shown that other regulators of NHGU may exist, such as GLP-1, nitric oxide, and serotonin etc.

GLP-1

Glucagon-like peptide-1 amide (GLP-1), is released by the L cells of the intestine in the postprandial period. It improves glucose tolerance by both stimulating insulin and decreasing glucagon secretion and slowing gastric emptying (Drucker, 2006). It has been reported that treatment with GLP-1 increases glycogen sythase activity, decreases glycogen phosphorylase, and promotes glycogen storage in a dose-dependent manner in isolated rat hepatocytes (D'Alessio *et al.*, 2004). It is interesting to note that, at the whole body level, not like commonly used oral hypoglycemic agents, GLP-1 exerts its incretin effect only when hyperglycemia is present. In addition to its incretin effects, evidence from animal models indicates that both high physiological and pharmacological doses of GLP-1 can directly stimulate NHGU, independent of its well-recognized effects on insulin and glucagon secretion (Dardevet et al., 2004; Nishizawa et al., 2003). In line with these findings, a recent study in dogs showed that intraportal infusion of Vildagliptin (DPP-4 inhibitor) augmented hepatic glucose disposal by means beyond the effects of GLP-1 on insulin and glucagon (Edgerton *et al.*, 2009). On the other hand, the increase in glucose clearance in response to intraportal glucose delivery was ablated, when the GLP-1 receptor antagonist exendin-(9-39) was infused intraportally in mice (Burcelin *et al.*, 2001). Conflicting data are available regarding the non-incretin effects of GLP-1 in

humans, probably due to the methodological differences, with most not showing a significant direct effect (Meneilly *et al.*, 2001; Vella *et al.*, 2001) or a small effect (Prigeon *et al.*, 2003)of GLP-1 on hepatic glucose metabolism. Thus, the potential direct role of GLP-1 on hepatic glucose metabolism in the humans remains unclear.

Portal infusion of GLP-1 and glucose, but not systemic glucose, can produce decreased peripheral glucose levels independent of insulin, indicating a functional relationship between portal glucose and GLP-1 actions (Ionut *et al.*, 2005). Consistent with this observation, Johnson et al. (Johnson et al., 2008) determined that a physiological elevation of GLP-1 in the hepatic portal vein does not increase NHGU and whole body glucose uptake when hyperglycemia is induced by peripheral glucose infusion, suggesting that a physiological increase in GLP-1 augments glucose utilization only when GLP-1 and a negative arterial-portal glucose gradient co-exist. These data clearly showed the GLP-1 has the ability to regulate NHGU. Given that there are no data supporting that GLP-1 secretion could be changed by portal glucose delivery, and portal glucose delivery can promote NHGU when the secretion of endogenous GLP-1 was inhibited by somatostatin, it is unlikely that GLP-1 is mechanistically responsible for metabolic response induced by portal signal. It is possible, however, that GLP-1 and portal signal have interactive effects on hepatic glucose metabolism in the postprandial state, and that the activation of the portal glucose signal and GLP-1 actions might be a fundamental requirement for hepatic glucose uptake in response to a carbohydrate meal.

Nitric Oxide

Nitric oxide (NO) is a potent biological mediator produced in a variety of tissues during the catabolism of L-arginine to L-citrulline catalized by nitric oxide synthase (NOS). The latter exists in three isoforms including endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) forms. All three isoforms of NOS have been reported to be expressed in liver (McNaughton *et al.*, 2002). Intrahepatic nerves containing the neuroeffector nitric oxide (NO), which comprises part of the nonadrenergic noncholinergic (NANC) nerve system (Rand *et al.*, 1995), have been identified in a variety of mammalian species traveling within the structures of the hepatic hilus, including rat, guinea pig, and cat (Esteban *et al.*, 2001; Esteban *et al.*, 1998; Esteban *et al.*, 1997). Varicose nerve fibers immunoreactive for neuronal nitric oxide synthase (nNOS), the enzyme that catalyzes the formation of NO, form a dense plexus around the larger portal veins, hepatic arteries, and bile ducts. These fibers diminish in numbers in the distal branches of these structures. In the guinea pig, nNOS colocalized with both NPY and CGRP, suggesting both an afferent and an efferent function (Esteban *et al.*, 2001). NO intralobular fibers have been found. In the guinea pig, but not in the other species studied, nitrergic innervation of the hepatic veins was also identified (Esteban *et al.*, 2001; Peinado *et al.*, 2002).

Physiological levels of NO can stimulate glucose uptake and oxidation in skeletal muscle and adipocytes (Roy *et al.*, 1998). Moreover, chronic administration of L-NAME, a NOS inhibitor in drinking water, decreased glucose tolerance in rats (Balon *et al.*, 1999). eNOS knockout mice have

hyperinsulinemia and impaired insulin-stimulated glucose uptake relative to control mice (Duplain *et al.*, 2001). These findings suggest that NO could play an important regulatory role in glucose metabolism.

In vitro studies have shown that NO can have direct effects on isolated hepatocytes, which result in an inhibition of gluconeogenesis, a reduction in glycogen synthesis and an increase in glucose output (Horton *et al.*, 1994; Sprangers *et al.*, 1998). Lautt and others (Lautt, 2005) have investigated the role of hepatic NO in regulating whole body glucose metabolism. They hypothesized the existence of a novel neurohumoral mechanism by which hepatic parasympathetic nerves, through permissive release of a putative insulin sensitizing substance (HISS) from the liver, regulate peripheral glucose disposal. According to their theory, following a meal, insulin results in the release of HISS, which requires a parasympathetic permissive reflex that signals the presence of feeding. The parasympathetic nerves work through activation of cholinergic muscarinic receptors in the liver and subsequent production of NO, which then increases the release of HISS from the liver. This, in turn, stimulates peripheral glucose uptake, however, nothing is known about the direct effect of NO on hepatic glucose uptake *in vivo*.

Our preliminary data showed that intraportal infusion of a nitric oxide donor, SIN-1(3-morpholynosydnonimine), in the presence of portal glucose delivery, was associated with hypotension and decreased NHGU during a hyperglycemic (2X basal) hyperinsulinemic (4X basal) clamp in 42-h-fasted conscious dogs. This inhibitory effect of intraportal SIN-1 infusion on NHGU was

rapid and significant, suggesting that a decrease in NO may be involved in mediation of portal glucose signal. The question then arises as to how intraportal SIN-1 infusion leads to decreased NHGU. Supported by our observation of increased heart rate and lipolysis in this study, one possibility is that an indirect mechanism could be involved, such that an increase in sympathetic drive to the liver resulting from hypotension led to a drop of NHGU. Furthermore, our recent finding that hepatic sympathectomy increased NHGU in response to peripheral glucose infusion suggests that the sympathetic nerves exert a tonic inhibition on NHGU (Dicostanzo *et al.*, 2006). Another possibility is that NO had a direct effect on the hepatocytes since in vitro studies have clearly shown that endogenous and exogenous NO can alter signaling in the liver (Billiar *et al.*, 1989).

It is generally agreed that the most prominent NO signaling pathway involves activation of soluble guanylate cyclase (sGC). sGC converts guanosine triphosphate (GTP) to cGMP, which promotes diverse responses through several potential pathways, including protein kinase G (PKG), cGMP phosphodiesterase (PDE), and AMP-activated protein kinase (AMPK) (Lira *et al.*, 2007; Zhang *et al.*, 2008). Alternatively, cGMP-independent NO reactions may occur as well, including interaction of NO with superoxide anion to generate peroxynitrite, or direct nitrosylation of cysteine thiol residues of target proteins like glutathione (Rockey *et al.*, 2004). Studies showed that NO produced by hepatocytes and neighboring Kupffer cells can in turn stimulate sGC and increase cGMP levels in the liver (Billiar *et al.*, 1989; Curran *et al.*, 1989). Thus, these intracellular pathways could be responsible for the change of glucose uptake by the liver

seen in the preliminary study. Thus, specific aim I is to assess effects of the change of nitric oxide on net hepatic glucose uptake in conscious dogs, and the specific aims III and IV are to understand the intracellular mechanism of the effects of NO on net hepatic glucose uptake.

5-HT

Neuropeptides involved in the innervation of the liver include neuropeptide Y, substance P, vasoactive intestinal peptide, glucagon-like peptides, somatostatin, serotonin, etc, (Akiyoshi *et al.*, 1998; el-Salhy *et al.*, 1993; Stoyanova *et al.*, 1998). Serotonin is localized within neurons in the enteric nervous system (Ormsbee *et al.*, 1985), and serotonergic nerves are found associated with the branches of the portal vein, hepatic artery, bile duct in the connective tissue of portal tracts (McCuskey, 2004).

There is growing evidence showing that selective serotonin reuptake inhibitors (SSRI), which prolong the action of endogenous serotonin, may facilitate glycemic control in patients with type 2 diabetes (Breum *et al.*, 1995; Maheux *et al.*, 1997; Potter van Loon *et al.*, 1992). These findings suggest that serotonin may play a regulatory role in controlling glucose metabolism. Park and Choi (Park *et al.*, 2002) demonstrated that oral treatment with the SSRI fluoxetine for 8 weeks increased glucose disposal during a hyperinsulinemic euglycemic clamp in both sham-operated and 90%-pancreatectomized rats without changing food intake or body weight. Serotonin (5-hydroxytryptamine; 5-HT) has been shown to dose dependently decrease serum glucose levels in mice (Sugimoto *et al.*, 1990; Yamada *et al.*, 1989). Interestingly, type 2 diabetes is associated with

reduced levels of serotonin in blood (Martin et al., 1995). Furthermore, there is growing body of work showing that selective serotonin reuptake inhibitors (SSRI), which increase the level of endogenous serotonin, may impact glycemic control in people with type 2 diabetes (Breum *et al.*, 1995; Maheux *et al.*, 1997; Potter van Loon *et al.*, 1992). Taken together, these findings suggest that serotonin may play a regulatory role in controlling glucose metabolism.

Splanchnic glucose uptake, which is impaired in type 2 diabetic individuals (Basu *et al.*, 2001; Ludvik *et al.*, 1997), accounts for the disposal of ~1/3 of a moderately sized oral glucose load, and the liver accounts for the majority of this disposal (Bajaj *et al.*, 2002; Cherrington, 1999). Data support the possibility that hepatic serotonin levels might have an impact on net splanchnic/hepatic glucose uptake. For example, intraportal infusion of 5-HT (Moore et al., 2004b) or 5-hydroxytryptophan (5-HTP) (Moore *et al.*, 2005b), the immediate precursor of 5-HT, enhanced net hepatic glucose uptake (NHGU) in conscious dogs during a hyperinsulinemic hyperglycemic clamp. Further, it has been demonstrated that intraportal delivery of the SSRI fluvoxamine to the liver increased NHGU with concomitant stimulation of hepatic glycogen storage (Moore et al., 2004a). However, due to the limitations of drug specificity, whether these effects were caused by an inhibition of serotonin reuptake, or whether some other mechanism was involved, remains unclear.

Escitalopram (S-enantiomer of citalopram), another commonly prescribed antidepressant, has the highest selectivity for the serotonin transporter (Klein et al., 2006). Compared with fluvoxamine, it has little or no affinity for noradrenergic

or dopaminergic transporters (Owens et al., 2001). Compared to other antidepressants, this drug has relatively modest effects on appetite and weight as well as lower potential for drug interactions (Culpepper, 2002; Hyttel, 1994). Because of these characteristics, it serves as a useful tool for determining the effects of serotonin on hepatic glucose metabolism in the absence of the previously described confounding variables. Therefore, the purpose of specific aim II is to further assess the role of inhibition of serotonin level in the liver on NHGU by determining the effect of escitalopram on NHGU in conscious dogs.

Specific Aims

Specific Aim I: To Examine the Effect of an Intraportal Nitric Oxide (NO) Donor (SIN-1) on Net Hepatic Glucose Metabolism in the Presence of the Portal Glucose Signal

Specific Aim 1 assessed effects of the increase in nitric oxide in the liver on NHGU in the 42-h-fasted conscious dog under hyperinsulinemic hyperglycemic conditions. Portal infusion of the NO donor SIN-1, in the presence of the portal glucose signal, was used to increase NO levels within the liver and hepatic substrate balance was calculated in the presence or absence of SIN-1, while in another group, SIN-1 was given intraportally at the same rate into a leg vein. Consequently, Aim I increased the power of the preliminary data and addressed the question of whether the effect of SIN-1 on NHGU in the liver was

secondary to hypotension induced by SIN-1 infusion or a direct effect of NO on the liver.

Specific Aim II: To Assess Effects of Intraportal Infusion of the SSRI Escitalopram (Lexapro) on NHGU

Specific aim 2 assessed the role of inhibition of serotonin level in the liver on NHGU by determining the effect of intraportal infusion of a SSRI escitalopram on NHGU in the 42-h-fasted conscious dog under hyperinsulinemic hyperglycemic conditions. The hypothesis is that inhibition of serotonin level in the liver by intraportal infusion of a SSRI escitalopram enhances NHGU.

Specific Aim III: To Determine the role of sGC/cGMP Pathway in the Regulation of Net Hepatic Glucose Uptake, and Whether the Regulation of NHGU by NO is sGC/cGMP Dependent

The possibility raised by specific aim 1 is that hepatic NO, or some downstream signal that it generates, has an inhibitory effect on glucose uptake by the liver. Thus, the intracellular mechanism by which the effects of NO on NHGU are brought about need to be assessed. Therefore, specific aim 3 assessed the role of sGC/cGMP in the regulation of NHGU, and whether the regulation of NHGU by NO is sGC/cGMP-dependent in the 42-h-fasted conscious dogs under hyperinsulinemic hyperglycemic conditions. In one experimental group, a potent and specific sGC inhibitor ODQ was infused intraportally in the 42-h-fasted conscious dog under hyperinsulinemic hyperglycemic conditions, and NHGU was quantified. In another experimental group, ODQ was given intraportally to inhibit sGC/cGMP pathway, and then NO

donor SIN-1 was given intraportally later. The hypothesis is that a sGC-dependent mechanism has the ability to regulate NHGU, and the inhibition of sGC/cGMP pathway would blunt the effect of NO on NHGU.

Specific Aim IV: To Explore the Effect of an Intraportal Infusion of a cGMP Analog, 8-Bromo-cGMP, on Net Hepatic Glucose Metabolism

In Aim4, a membrane-permeable cGMP analog, 8-Bromo-cGMP, was infused intraportally in the 42-h-fasted conscious dog under hyperinsulinemic hyperglycemic conditions to determine the effect of an increase in hepatic cGMP on NHGU. We hypothesized that the increase in hepatic cGMP levels enhances NHGU.

CHAPTER II

MATERIALS AND METHODS

Animals and Surgical Procedures

Animal Care

Experiments were conducted on healthy 42h fasted conscious mongrel dogs (20-26 kg) of either sex that had been fed once daily a diet of meat (Kal Kan, Vernon, CA) and chow (Purina Lab Canine Diet No. 5006; Purina Mills, St. Louis, MO) comprised of 34% protein, 14.5% fat, 46% carbohydrate, and 5.5% fiber (~ 1500 kcal/d) based on dry weight. Water was available *ad libitum.* A fast of this duration was chosen because it produces a metabolic state resembling that in the overnight-fasted human and results in liver glycogen levels in the dog that are at a stable minimum (Adkins-Marshall *et al.*, 1990). Each dog was only used for one experiment. The animals were housed in a facility that met American Association for Accreditation of Laboratory Animal Care guidelines, and the protocols were approved by the Vanderbilt University Medical Center Animal Care and Use Committee.

Surgical Procedures

Approximately 16 days prior to the study, each dog underwent a laparotomy under general anesthesia (0.01 mg/kg buprenorphine HCl and 5

mg/kg proppofol 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 silicone rubber 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 silicone rubber 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 silicone rubber 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 previously 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.

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 the ends of the catheters were secured 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 the flow probe leads were exteriorized 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 (Deseret Medical, Becton Dickinson, Sandy, UT) were inserted percutaneously into the left and right cephalic veins and into a saphenous vein for the infusion of dye, glucose and

drug 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 stand quietly in a Pavlov harness throughout the experiments. 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 (Goresky *et al.*, 1975). 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 syringe 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). 1 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 6 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 3% perchloric acid (PCA; Fisher Scientific, Fair Lawn, New Jersey). The tube was vortexed, centrifuged, and the supernatant was stored in a separate tube at -70 ºC for later analysis of metabolite levels. A portion of this sample was used for the measurement of lactate and glycerol. In specific aim II, 1 ml of whole blood was aliquoted and immediately transferred to -70 ºC for the future assay of 5-HT concentrations. 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 in 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). One ml plasma for glucagon measurement 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 on 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 and calculation of hepatic plasma flow, as will be described later, and then frozen at -70 ºC until insulin was measured. All solutions were placed at -70 ºC 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 Pharmaceutical Inc., Dearborn, MI) at 1 ml/5 kg, and the abdomen was opened so that liver samples could be immediately freeze clamped *in situ* with precooled Wallenburger tongs and stored at -70 ºC. Liver samples were later pulverized under liquid nitrogen and then stored at -70 ºC for future use. The positions of the catheter tips in their respective vessels were verified at the time of the necropsy.

Sample Analysis

Plasma Glucose

Plasma glucose levels were determined during the experiment using the glucose oxidase method (Kadish *et al.*, 1969) with a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). The reaction sequence was as follows:

$$\alpha\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid and } H_2O_2 \quad (1)$$

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + H_20 \quad (2)$$

$$H_2O_2 + 2H^+ + 2I^- \xrightarrow{\text{molybdate}} I_2 + 2\,H_20 \quad (3)$$

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 37 ºC. The specific reactions were as follows:

Acyl-CoA synthetase

NEFA + ATP + CoA ----------→ Acyl-CoA + AMP + Ppi (4)

Acyl-CoA oxidase

Acyl-CoA + O_2 ----------→ 2,3-trans-enoyl-CoA + H_2O_2 (5)

2 H_2O_2 + 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline + 4- aminoantipyrine

Peroxidase

----------→ Purple color adduct

(6)

Whole Blood Metabolites: Lactate and Glycerol

Whole blood concentrations of lactate and glycerol were measured according to the method of Llyod et al (Lloyd *et al.*, 1978) 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:

Lactate deyhdrogenase

Lactate + NAD^+ ----------→ Pyruvate + NADH + H^+ (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 assay involved the following reaction:

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

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

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 (Wide, 1966). 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 (Morgan, 1963). Insulin antibodies and ^{125}I tracers were obtained from Linco Research Inc (St. Charles, MO). A 100 µl aliquot of plasma was incubated for 18 h at 4 ºC 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 (2nd antibody) and 100 µl IgG carrier for 30 min at 4 ºC. 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/free 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.

Glucagon

Immunoreactive plasma glucagon was also measured using a double antibody RIA (Linco) (Ensick, 1983). The protocol was modified by utilizing primary and secondary antibodies specific for glucagon (glucagon antibodies and ^{125}I tracers from Linco). A 100 µl aliquot of plasma was incubated for 24 h at 4 ºC 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 4 ºC. The samples was then incubated with 100 µl goat anti-guinea pig IgG (2nd antibody) and 100 µl IgG carrier for 2 h at 4 ºC. 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 oxyntomodulin (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 (Foster *et al.*, 1974) 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 31 ºC 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

A high-performance liquid chromatography (HPLC) method was used to determine epinephrine and norepinephrine levels as previously described by Goldstein et al (Goldstein *et al.*, 1981). 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 0.1 M perchloric acid (PCA) according to Anton and Sayre (Anton *et al.*, 1962).

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% and 4-6% for epinephrine and norepinephrine respectively 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 -70 ºC. 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.

Serotonin (5-HT)

A high-performance liquid chromatography (HPLC) method utilizing an Antec Decade (oxidation: 0.7) electrochemical detector was used to determine serotonin (5-HT) levels from liver tissue and whole blood samples. Liver tissue (5-10 mg) was homogenized in 500 µl of 0.1M TCA, which contained 0.01 M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8), using a tissue dismembrator (Fisher Scientific). Homogenates were spun at 10,000 g for 20 minutes. The supernatant was removed and then analyzed for serotonin concentrations. The pellet was saved for protein analysis. 125 µl of the whole blood sample was mixed with 125 µl of the extraction solvent (0.8 M perchloric acid, 0.1 M ascorbic acid and 10 mM EDTA), vortexed for 15 sec and spun at 10,000 g for 10 min, and the supernatant was then collected for the 5-HT assay.

Samples (20 µl) were injected using a Waters 717+ autosampler onto a Phenomenex Nucleosil (5u, 100A) C18 HPLC column (150 x 4.60 mm). 5-HT eluted with a mobile phase consisting of 89.5% 0.1M TCA, 0.01 M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Solvent was delivered at 0.7 ml/min using a Waters 515 HPLC pump. HPLC control and data acquisition were managed by Millennium 32 software. Daily calibration curves were generated by supplementing sample with stock 5-HT solutions to yield final concentrations of: 1, 3, 6, 12, 25, 50, 75, 100 ng/ml, using dihydroxybenzylamine

(DHBA) as internal standard. 5-HT was determined from the whole blood/liver extract by comparison with the standard curve. Additionally, recovery of 5-HT was estimated using equal volumes of 5-HT standards for comparison with sample standards. The interassay CVs was 3-11% and 4-6% for 5-HT for the entire range of the dose response curve.

Hepatic Glycogen Content

Liver glycogen content was determined using a modification of the method of Keppler and Decker (Moore *et al.*, 1991). Frozen liver tissue was ground to powder using mortars and pestles, and weighed (~200 mg) while kept chilled with liquid nitrogen. The sample weight (mg) was multiplied by 5 to determine the volume (ml) of 0.6 N PCA to be used. The tissue was combined with PCA then thoroughly homogenized using an Ultra-Turrax homogenizer (TP18/10, Janke & Kunkel, IKA Werk, GmbH & Co., Staufen, Germany). A 200 µl aliquot was then combined with 100 µl $KHCO_3$ to neutralize the sample. 500 µl of amyloglucosidase (2 mg/ml in 0.4 M sodium acetate buffer) was incubated in a shaker bath at 40 ºC for 2 hours. Glycogen concentration was then calculated by subtracting the glucose concentration in duplicate samples not treated with the enzyme from samples incubated with amyloglucosidase.

Hepatic cGMP

Liver cGMP levels were determined using cGMP EIA Kit (Cayman Chemical, Ann Arbor, MI). Frozen liver tissue was pulverized with mortars and

pestles, while kept chilled with liquid nitrogen. 300 mg of ground tissue were then placed into a regular 10 ml clean plastic tube with 1.5 ml 5% trichloroacetic acid (TCA). The sample was then kept on ice and homogenized using an Ultra-Turrax homogenizer (TP18/10, Janke & Kunkel, IKA Werk, GmbH & Co., Staufen, Germany). The precipitate was removed by centrifugation at 1,500 g for 10 minutes, and 1 ml of supernatant was transferred to a 15 mL clear polypropylene centrifuge tubes (Corning Inc., Corning, NY). TCA was extracted from the supernatant sample using water-saturated ether, which was made by adding Ultrapure water (Cayman Chemical, Ann Arbor, MI) to ether (Sigma-Aldrich, St. Louis, MO) until layers formed, of which the top layer was used. 4-5 ml of ether was added to 1 ml of supernatant. They were mixed, and the organic and aqueous phases were separated. The top layer (ether) was carefully removed and the extraction was repeated three times. The residual ether was further removed from the aqueous layer by heating the sample to 70℃ for ~15 min. 50 µl of sample or cGMP standard was added to an EIA plate with 50 µl cGMP AChE Tracer and 50 µl cGMP Antiserum. The plate was then covered with plastic film and incubated 18 hours at room temperature. The next day, the plate was emptied and rinsed carefully 4-5 times using Wash Buffer (Cayman Chemical, Ann Arbor, MI), and 200 µl of Ellman's Reagent was added to each well. The plate was then covered with plastic film and aluminum film, and incubated on an orbital shaker (Stovall Life Science Inc., Greensboro, NC) at room temperature for 120 min. Light absorbance of the sample was measure by reading the plate on a Packard Multi Probe Robotic Liquid Handling System

(Perkin Elmer; Shelton, CT) using a filter with a wavelength of 425 nm and bandwidth of 35 nm. The log of the amount of cGMP in the samples was inversely proportional to the log of (sample bound/maximum bound). The concentration of cGMP in the liver sample (pmol/ml) was determined by comparison to the standard curve. The sample detection range was 0.2-20 pmol/ml.

Hepatic Phosphodiesterase 3

Phosphodiesterase 3 (PDE3) has a low Km, and is a membrane-associated enzyme which mainly hydrolyzes cAMP (Maurice *et al.*, 2003; Muller *et al.*, 1992). Liver PDE3 activity was determined using a modification of the phosphodiesterase assay described by Loten et al. (Loten *et al.*, 1980). To prepare liver particulate and soluble phosphodiesterases, frozen liver tissue was ground to powder, and weighed (~200 mg) while kept chilled with liquid nitrogen. The sample weight (mg) was multiplied by 10 to determine the volume (ml) of isotonic sucrose (250 mM sucrose, 10 mM Tris-HC1, 1 mM EDTA, pH 7.5) to be used. The tissue was combined with isotonic sucrose then thoroughly homogenized using an Ultra-Turrax homogenizer (TP18/10, Janke & Kunkel, IKA Werk, GmbH & Co., Staufen, Germany). The homogenate was filtered through Dacron wool and centrifuged in 15-ml polycarbonate tubes (Beckman Coulter, Inc., Fullerton, CA) at 20,000 rpm for 45 min. The supernatant, which contains most of the soluble high Km phosphodiesterases, was discarded. The tube was carefully rinsed with buffered isotonic sucrose, without disturbing the pellet, and

allowed to drain. The pellet, which contains most of the low Km phosphodiesterase, was taken up in 4 ml of dilute Tris buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5) and rehomogenized with 4 strokes of the A pestle in a 7-ml with buffered isotonic sucrose. PDE3 measurements were performed in a final volume of 40 µl containing 50 mM Tris/HCl, pH 7.5, 25 mM MgCl, 0.33 mg/ml of bovine serum albumin, cyclic ^{3}H AMP (37.7 Ci/mmol; about 160,000 dpm) and sufficient unlabeled cyclic AMP to produce the desired substrate concentration. Tubes were incubated for 5 min at 30 ºC and the reaction was terminated by stop mix. 5 µl of snake venom was added to each tube (Crotalus atrox, 0.5 mg/ml), and incubated for 45 min at 30 ºC. 1 ml of water was added to each tube and mixed, and 1 ml of reaction mixture was loaded onto a column (0.7 x 2.5 cm) of quaternary aminoethyl (QAE)-Sephadex (A-25) in the formate form to separate adenosine from unreacted cyclic AMP. The column was then eluted with 3 ml of 20 mM ammonium formate, pH 7.4, and 4 ml of effluent was collected and counted in a Triton-based scintillation mixture. The phosphodiesterase assay was performed at a substrate concentration of 0.1 µmol/l, because at this concentration of cyclic AMP mainly low Km phosphodiesterase is measured. PDE3 activity (pmol ^{3}H cAMP hydrolyzed) was calculated by radioactivity counts (cpm) times 0.0000326 pmol/cpm, and normalized by protein dry weight (mg).

Western blotting

Frozen liver tissue (~50 mg) was ground to powder using mortars and pestles, while kept chilled with liquid nitrogen. The tissue was then combined with

1 ml of homogenizing buffer (50 mM Tris-HCl pH 7.0, 100 mM sucrose, 10% v:v glycerol, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 10 µL/mL buffer of phosphatase inhibitor cocktail 1 and 2, and protease inhibitor cocktail [Sigma; St. Louis, MO]) and thoroughly homogenized using an Ultra-Turrax homogenizer (TP18/10, Janke & Kunkel,. IKA Werk, GmbH & Co., Staufen, Germany). Homogenates were centrifuged at 2,500 g for 30 min, supernatants were removed and soluble protein concentration was determined using the Biorad protein assay (BioRad; Hercules, CA). Aliquots of supernatant were mixed 1:1 v:v with freshly prepared 2X SDS-PAGE loading buffer (100 mM Tris-HCl, pH. 6.8, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-mercaptoethanol), and boiled for 5 min. Samples were subjected to SDS-PAGE (12% resolving gel) followed by transfer to nitrocellulose membranes. Blocking was performed with 5% (wt/v) bovine serum albumin in Tris-buffered saline containing Tween-20 (TBST: 10 mM Tris-base, pH 7.0, 150 mM NaCl, 0.2% v:v Tween 20) for 1h at room temperature, and then incubated overnight with the appropriate primary antibody at 4 ºC. After 3x5 min washes with TBST, membranes were incubated with HRP-conjugated secondary antibody (Promega, Madison, WI) for 1h at room temperature, followed by 3 x 5 min washes in TBST. Proteins were visualized using ECL Plus Western detection reagents (GE Healthcare, Piscataway, NJ) and the ECL signals were detected after brief (5-60s) exposure to X-ray film. Bands were quantified using ImageJ software (http://rsb.info.nih.gov/ij/) and the intensity of the protein signal was normalized to total protein signal. β-actin was used as a loading control.

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 (Leevy *et al.*, 1962). 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 conclusions would be drawn if 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 (ΔT_{up} and ΔT_{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 (Hendrick, 1986).

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 (Greenway *et al.*, 1971).

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 when necessary 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 be quantitively recovered in the portal and hepatic veins if good mixing occurs. 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 (Brun, 1951). 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 40% of the sample time points during intraportal glucose infusion period.

Blood Pressure and Heart Rate

For specific aims I, III and IV, systolic and diastolic blood pressure and heart rate were determined throughout the experiments at each sampling time 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:

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

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 concentrations at 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. (Hsieh *et al.*, 1998; Pagliassotti *et al.*, 1996a). 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:

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

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 was determined using the indirect method because it is less subject to mixing problems. Nevertheless, there was no statistical difference between data determined 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:

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

where NHSU is net hepatic substrate uptake and could represent NHGU when glucose is the substrate.

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 (Greenway *et al.*, 1971) as explained earlier.

Net Hepatic Carbon Retention

Net hepatic carbon retention was calculated as the sum of the balances of glucose and lactate, once the latter was converted to glucose equivalents. The calculation of net hepatic carbon retention is an established approach to estimate hepatic glycogen accretion and has been described and validated previously (An *et al.*, 2008). It has the advantage of not being dependent upon an estimate of the prestudy glycogen content, which must be obtained from a separate set of 42-h-fasted dogs. The calculation omits the contribution of gluconeogenic substrates other than lactate and fails to account for the glucose oxidized by the liver. However, these two rates are relatively small, quantitatively similar, and offsetting (Satake *et al.*, 2002).

Nonhepatic Glucose Uptake and Clearance

The average nonhepatic glucose uptake (nonHGU) between two time points (T1 and T2) was calculated by the following overall equation:

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

where T1 and T2 represent the two measurement time points. Note that the (($NHGU_{T1}$ + $NHGU_{T2}$)/2) term will be negative when the liver is taking up glucose. 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 (Altszuler *et al.*, 1956), and 0.65 represents the pool fraction (Cowan *et al.*, 1971).

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:

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

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

All data are presented as means ± S.E.M.. Time course data were analyzed with Two Way Repeated Measures ANOVA, and One Way ANOVA was used for any comparisons of other mean data. Post-hoc analysis was carried out using the Student-Newman-Keuls method. Statistical significance was accepted at $P<0.05$. The specific statistical tests used in the studies are described in the experimental design sections of chapters III-VI.

CHAPTER III

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

Aim

The purposes of specific aim 1 were two-fold. The first was to increase the power of the SIN-1 study initiated by DiCostanzo et al. The second was to determine whether the effect of SIN-1 on NHGU in the liver was secondary to hypotension induced by SIN-1 infusion or a direct effect of NO on the liver.

Experimental design

Each experiment consisted of a 90-min equilibration period (-120 to –30 min), a 30-min basal period (-30 to 0 min), and a 240-min experimental period (0 to 240 min), which was divided into a 90 minute period (P1) followed by a 150 minute period (P2). In all experiments a constant infusion of indocyanine green dye (0.076 mg/min; Sigma Immunochemicals, St. Louis, MO) was initiated at -120 min via the left cephalic vein and p-aminohippuric acid (PAH; 0.4 mg/kg/min; Sigma) was infused via the left saphenous vein from -120 to 0 min. At 0 min, a constant infusion of somatostatin (0.8 μg/kg/min; Bachem, Torrance, CA) was begun via the left saphenous vein to suppress endogenous insulin and glucagon secretion. At the same time, basal glucagon (0.57 ng/kg/min; Glucagen, Novo Nordisk, Bagsvaerd, Denmark) and 4-fold basal insulin (1.2 mU/kg/min; Eli Lilly &

Co., Indianapolis, IN) infusions were started through the splenic and jejunal catheters. Glucose (20% dextrose) was delivered intraportally at 4mg/kg/min, and PAH was mixed with it at a concentration allowing a delivery rate of 0.4 mg/kg/min. In addition, a primed continuous infusion of 50% dextrose was begun via the right cephalic vein at time 0, so that the blood glucose could quickly be clamped at the desired hyperglycemic level (170-175 mg/dL). In P2, saline (SAL, n=8) was infused intraportally, or 3-morpholinosydnonimine (SIN-1; 4 µg/kg/min; Cayman Chemical, Ann Arbor, MI) was delivered intraportally (PoSIN-1, n=12) or peripherally via the right saphenous vein (PeSIN-1, n=10). The peripheral glucose infusion rate was adjusted in P2 to maintain a similar hepatic glucose load (HGL) to that seen in P1.

Results

Hemodynamic response

Systolic blood pressure did not change significantly over the course of the experiment in response to saline infusion. The systolic blood pressure in the PeSIN-1 and PoSIN-1 groups were similar to those seen in SAL in the basal period and P1 but dropped significantly during P2 (Table 3.1). Diastolic blood pressure also fell significantly during P2 in the PeSIN-1 and PoSIN-1 groups (Table 3.1). The average heart rate did not change over time in SAL, but it increased ($P<0.05$) during P2 in both the PeSIN-1 and PoSIN-1 groups (Table 3.1).

Hormone concentrations

The arterial and hepatic sinusoidal insulin levels increased three to four-fold and remained stable during P1 and P2 in all groups (Table 3.2). Arterial and hepatic sinusoidal plasma glucagon concentrations remained basal in all groups (Table 3.2).

Hepatic blood flow, blood glucose concentrations, and hepatic glucose load

Portal vein blood flow decreased ~20% during P1 in all groups in response to somatostatin infusion (Table 3.3). There was a concomitant small increase in hepatic arterial flow, and as a consequence, total hepatic blood flow did not change significantly during the first experimental period. There were small increases in portal vein and hepatic arterial blood flow in all groups during P2, such that total flow was completely restored to baseline values in each group, and there were no significant differences among groups.

Arterial blood glucose levels increased in all groups from a basal value of 82±1 to 174±3 mg/dL during P1 and P2 (Fig. 3.2). The hepatic glucose loads increased proportionally and did not differ significantly among groups at any time (Fig. 3.2).

Net hepatic glucose balance and change of net hepatic fractional extraction of glucose from baseline

All groups exhibited a similar rate of net hepatic glucose output during the basal period. Coincident with the start of the experimental period (4X basal insulin, basal glucagon and hyperglycemia), all groups switched from net

production to net uptake of glucose, with the rates (4.8±0.4, 4.8±0.4 and 5.1±0.4 mg/kg/min in SAL, PoSIN-1 and PeSIN-1, respectively) not being significantly different among groups during the last 30 minutes of P1 (Fig. 3.3). Subsequently, the rate of NHGU remained relatively stable in SAL and PeSIN-1 (5.5±0.4 and 5.4±0.4 mg/kg/min), whereas in PoSIN-1 it was reduced to 4.2±0.3 mg/kg/min during P2 ($P<0.05$ vs. SAL and PeSIN-1). The change of hepatic fractional extraction of glucose from baseline followed a similar pattern, falling significantly in response to intraportal SIN-1 infusion (Fig. 3.3).

Glucose infusion rates, nonhepatic glucose uptake

The glucose infusion rate in all three groups increased over time. There was a tendency for the total glucose infusion rate to be lower in PoSIN-1, in line with the reduction in NHGU seen in response to portal SIN-1 infusion (Fig. 3.4). Nonhepatic glucose uptake increased modestly over time in all groups, but did not differ among groups (Fig. 3.4).

Lactate metabolism and net hepatic carbon retention

The arterial blood lactate concentrations rose in all groups during P1 and P2 relative to the basal period. After the experimental period began, net hepatic lactate balance changed from uptake to output, and output remained evident in all groups during P2 (Table 3.4). Net hepatic carbon retention did not differ between PeSIN-1 and SAL at any time but was reduced in PoSIN-1 during P2 (3.8±0.2 in PoSIN-1 vs. 4.9±0.3 in SAL and 4.7±0.2 mg glucose

equivalents/kg/min in PeSIN-1, respectively, P<0.05).

Glycerol and nonesterified fatty acid metabolism

Arterial blood glycerol concentrations and net hepatic glycerol uptake were reduced 65–75% by hyperglycemia and hyperinsulinemia and remained suppressed in all groups throughout P1 and P2. The suppression of glycerol was, however, partially reversed during P2 in the PeSIN-1 and PoSIN-1 but not the SAL group (29±5, 48±5 and 45±5 mg/kg/min in SAL, PoSIN-1 and PeSIN-1, respectively) (Table 3.4). Arterial nonesterified fatty acid (NEFA) concentrations and net hepatic NEFA uptake changed in a pattern similar to glycerol, decreasing 80–90% during P1 in all groups. There was, however, no rebound during P2 in the PeSIN-1 or PoSIN-1 groups (Table 3.4).

Discussion

The rate of hepatic glucose uptake *in vivo* depends on a complex set of variables, including neural, hormonal and substrate signals (Pagliassotti *et al.*, 1992a). In the present study, we took great care to control these inputs so as to allow precise assessment of the effect of SIN-1 delivery on NHGU. To our knowledge, this study is the first *in vivo* investigation to explore the effects of the NO donor SIN-1 on net hepatic glucose uptake in the presence of portal glucose delivery, as well as elevated plasma insulin and glucose levels. Under these conditions, portal infusion of the NO donor SIN-1 caused a ~24% decrease in net hepatic glucose uptake and hepatic glycogen accumulation. We were unable to

detect any effect of portal SIN-1 on glucose uptake by nonhepatic tissues or whole body glucose utilization. Peripheral SIN-1 infusion was without effect on either hepatic or nonhepatic glucose utilization.

Although there are several NO donors available (e.g., SNAP, V-PYRRO/NO, DEA/NO), we specifically chose to use SIN-1 in light of its metabolic fate. *In vivo*, SIN-1 decomposes non-enzymatically in a two step reaction with the second step yielding NO and superoxide (O2.-), which can readily form peroxynitrite (ONOO-) (Padmaja *et al.*, 1993). NO or peroxynitrite can then nitrosylate glutathione to generate S-nitroso-glutathione (GSNO) (Ji *et al.*, 1999). According to Lautt's HISS hypothesis, GSNO can act as an endogenous NO reservoir and serve as an intermediate between NO synthesis and guanylate cyclase activation in the HISS pathway (Guarino *et al.*, 2004). In addition, we found SIN-1, in contrast to the other NO donors, has little effect on total hepatic blood flow, so that total hepatic blood flow did not differ among groups in the current study.

In contrast to the liver, where blood flow was minimally altered, there was a marked overall hemodynamic response to SIN-1 infusion whether it was given into a peripheral vein or the hepatic portal vein. This was undoubtedly due to the well known vasodilatory effects of NO. Systolic and diastolic blood pressure both decreased significantly when the NO donor was infused, regardless of the route of infusion, while the heart rate rose secondary to hypotension. The drop in blood pressure and the rise in heart rate tended to be modestly greater when SIN-1 was given peripherally. Although markers of NO levels were not measured in the

present study, this drop may have been associated with higher levels of SIN-1 in peripheral blood when the compound was infused through a peripheral vein as opposed to the hepatic portal vein.

There are several possible ways that SIN-1 could affect the liver. First, it may act directly on hepatocytes to reduce NHGU. *In vivo*, hepatocytes are exposed to autogenously derived NO as well as NO derived from non-parenchymal cells (Taylor *et al.*, 1998). Both iNOS and eNOS are expressed in hepatocytes; additionally, iNOS has been found in hepatic Kupffer and Ito cells in various species, while nNOS has been localized in some nerve fibers in the rat liver (Alexander, 1998; Feelisch *et al.*, 1999; Wei *et al.*, 2002). Horton et al. (Horton *et al.*, 1994) demonstrated that the NO donors S-nitroso-N-acetylpenicillamine (SNAP) and SIN-1 inhibit gluconeogenesis from isolated rat hepatocytes in a time- and dose-dependent manner. They suggested that the mechanism by which this occurs involves a decrease in amount of phosphoenolpyruvate carboxykinase (PEPCK) protein. On the other hand, Sprangers et al. (Sprangers *et al.*, 1998) showed that glycogen synthesis from glucose in rat hepatocytes was inhibited by the NO donor SNAP due to decreased glycogen synthase activity (less conversion of glycogen synthase *b* into *a* by synthase phosphatase). They also found that glycogen synthesis is more sensitive to inhibition by NO than is gluconeogenesis (Sprangers *et al.*, 1998). Borgs et al. (Borgs *et al.*, 1996) observed that NO infusion (34 µmol/L) increased the rate of glucose output in the perfused rat liver ~3 fold due to a stimulation of glycogenolysis which occurred as a result of activation of glycogen

phosphorylase. Similarly, an *in vivo* study carried out by Ming et al. (Ming *et al.*, 2000) showed that portal SIN-1 infusion potentiates norepinephrine-induced glucose output from the liver in cats, and this potentiation is blocked by inhibition of guanylate cyclase, a key signaling molecule downstream of NO. Although the underlying mechanisms still remain to be elucidated, it appears that the regulation of hepatic metabolism by NO may involve downstream signals such as protein nitrosylation and/or multiple cGMP-dependent pathways.

It is also possible, however, that SIN-1 reduces NHGU through an indirect effect on the liver. Such an indirect effect could have come about as a result of an increase in sympathetic drive to the liver secondary to the hypotension resulting from intraportal SIN-1 infusion. The fact that an increase in sympathetic drive occurred during SIN-1 infusion is supported by our finding that the heart rate increased in response to the drug and by our observation that lipolysis increased in response to SIN-1, as indicated by the increase in the arterial blood glycerol level. Increased sympathetic input to the liver would have been expected to reduce NHGU (Dicostanzo *et al.*, 2006). In our earlier study, eliminating sympathetic input to the liver by selective sympathetic denervation augmented the NHGU seen in response to elevated glucose and insulin levels (Dicostanzo *et al.*, 2006). The PeSIN-1 group was therefore included to assess the possibility that SIN-1 could have an indirect effect on the liver. In the PeSIN-1 group, SIN-1 was given via a leg vein in order to bring about a similar or greater hemodynamic response to that seen with portal vein SIN-1 infusion. In this way, sympathetic input to the liver was increased by an amount equal to or greater than when SIN-

1 was given intraportally. In the PeSIN-1 group, NHGU averaged ~5.5 mg/kg/min during P1 and P2 and did not differ from the rate evident during saline infusion, indicating that increased sympathetic input to the liver, if it occurred, did not reduce NHGU. This then indicates that the decrease in NHGU seen when SIN-1 was infused portally was not secondary to hypotension. The increases in heart rate and lipolysis seen in P2 in response to SIN-1 infusion actually tended to be slightly greater when SIN-1 was given into a peripheral vein than when it was given into the portal vein. Despite this, there was no decrease in NHGU or hepatic glycogen synthesis, suggesting that NO regulates hepatic glucose metabolism through a direct action on the liver. Further studies need to be performed to determine the mechanism by which this regulation comes about.

Major extrahepatic glucose consumers include skeletal muscle, adipose tissue, brain, etc. In our previous studies in which insulin and glucose were elevated in conscious dogs, we found that muscle glucose uptake (measured using a hindlimb balance technique) accounted for a significant portion of the increase in nonhepatic glucose uptake (Galassetti *et al.*, 1998). In the present experiments, nonhepatic glucose uptake in response to intraportal SIN-1 infusion was not greater than when saline was given or SIN-1 was infused via a leg vein.

In 1997, Lautt (Lautt, 2005) proposed that the liver produces HISS and that the latter enhances muscle glucose uptake and muscle insulin sensitivity. HISS generation is reported to involve the hepatic parasympathetic nervous system, the production of NO and the interaction of NO with glutathione in the liver (Guarino *et al.*, 2003; Guarino *et al.*, 2004). In a study carried out by Sadri et

al. (Sadri *et al.*, 1997), intraportal, but not intravenous, administration of L-NAME, a nonselective nitric oxide synthase antagonist, significantly reduced whole body insulin sensitivity as measured under euglycemic conditions using the rapid insulin sensitivity test (RIST) in rats. This suggests that NOS inhibition in the liver resulted in a drop in hepatic NO, which resulted in peripheral insulin resistance. This insulin resistance was reversed by SIN-1 infusion into the portal vein, but not into a peripheral vein (Sadri *et al.*, 1999). Thus, the insulin resistance that was induced by the inhibition of NOS in the liver was reversed by providing NO directly to the liver. However, our present data do not support the concept that hepatic NO action promotes glucose uptake in peripheral tissues under hyperglycemic conditions. There are several possible explanations as to why this was the case. First, HISS was thought to be active only in the fed state in rodents (Seredycz *et al.*, 2006). Thus, failure to observe an effect of portal SIN-1 infusion on nonhepatic glucose uptake in 42-h fasted dogs could be due to the fast duration or a species difference. In addition, most HISS experiments that have been conducted under euglycemic conditions (Guarino *et al.*, 2003; Guarino *et al.*, 2004; Lautt, 2005; Moore *et al.*, 2002; Sadri *et al.*, 1999; Sadri *et al.*, 1997), while in the present study, a hyperinsulinemic hyperglycemic clamp was used, and as a result, muscle glucose uptake may have reached a rate that would make a response to a hepatic NO signal hard to detect. The current study sheds no light on the mechanism by which HISS works or the nature of the putative signaling substance “HISS”.

In line with our current finding, that intraportal infusion of the NO donor SIN-1 reduced NHGU in the presence of hyperinsulinemia and hyperglycemia, normal dogs that received an intraportal infusion of the NOS inhibitor L-NAME (L-NG-nitroarginine methyl ester) exhibited enhanced NHGU under hyperinsulinemic and hyperglycemic conditions (Moore *et al.*, 2005a).

In conclusion, we demonstrate for the first time that intraportal infusion of an NO donor, SIN-1, reduced NHGU and net hepatic carbon retention under hyperglycemic and hyperinsulinemic conditions in conscious 42-h-fasted dogs. The lack of changes in NHGU and glycogen storage when the same hemodynamic response was seen in response to peripheral SIN-1 administration suggests that the effects of portal SIN-1 probably came about as a result of a direct effect of NO on hepatocytes. The molecular mechanism by which NO brings about its hepatic effect needs to be elucidated. The effects on nonhepatic glucose uptake in response to intraportal infusion of SIN-1 were not detected, so that total body glucose disposal tended to be reduced by hepatic NO elevation. These findings indicate that hepatic NO may play a role in directing the disposition of glucose reaching the liver via the portal vein, and, by extension, an oral glucose load, into liver.

Table 3.1: Heart rate, systolic and diastolic blood pressure during the basal and experimental periods in conscious 42-h-fasted dogs given saline or SIN-1 infused into the portal vein or into a peripheral vein

Group	Basal Period	Experimental Period	
		Period 1	Period 2
Systolic Blood Pressure, mmHg			
SAL	178±5	173±5	163±8
PoSIN-1	164±3	167±4	117±5*†
PeSIN-1	175±6	178±6	120±5*†
Diastolic Blood Pressure, mmHg			
SAL	96±4	94±4	95±5
PoSIN-1	87±2	89±3	78±3*†
PeSIN-1	95±2	93±2	80±2*†
Heart Rate, beats/min			
SAL	94±5	100±4	101±7
PoSIN-1	88±6	86±4	107±5*
PeSIN-1	96±5	88±5	120±10*

Data are means±S.E.M.; n=7 in the saline (SAL) group, n=12 in the group that received 3-morpholinosydnonimine (SIN-1) in the portal vein (PoSIN-1), and n=10 in the group that received SIN-1 in the peripheral vein (PeSIN-1)
**= Significant statistical difference (P<0.05) from basal period within the group*
†= Significant statistical difference (P<0.05) from SAL group

Table 3.2: Hormone concentrations during the basal and experimental periods in conscious 42-h-fasted dogs given saline or SIN-1 infused into the portal vein or into a peripheral vein

Group	Basal Period	Experimental Period	
		Period 1	Period 2
Arterial Plasma Insulin, µU/ml			
SAL	9±2	26±3*	26±2*
PoSIN-1	7±1	21±2*	22±2*
PeSIN-1	7±1	21±3*	25±3*
Hepatic Sinusoidal Insulin, µU/ml			
SAL	19±3	74±6*	81±4*
PoSIN-1	19±3	89±6*	78±6*
PeSIN-1	21±5	94±9*	95±12*
Arterial Plasma Glucagon, pg/ml			
SAL	45±7	51±4	44±6
PoSIN-1	36±4	42±3	37±3
PeSIN-1	37±3	43±5	39±5
Hepatic Sinusoidal Glucagon, pg/ml			
SAL	59±7	69±6	61±6
PoSIN-1	45±4	59±4*	51±4
PeSIN-1	42±3	64±6*	61±4*

Data are means±S.E.M; n=8 in SAL goup, n=12 in PoSIN-1 group and n=10 in PeSIN-1 group
**= Significant statistical difference (P<0.05) from basal period within the group*

Table 3.3: Average hepatic arterial, portal, and total hepatic blood flow during the basal and experimental periods in conscious 42-h-fasted dogs given saline or SIN-1 infused into the portal vein or into a peripheral vein

Group	Basal Period	Experimental Period	
		Period 1	Period 2
Average Hepatic Arterial Blood Flow, ml/kg/min			
SAL	6.0±0.5	9.4±0.8*	10.1±1.1*
PoSIN-1	4.8±0.4	5.8±0.5†	6.9±0.5*†
PeSIN-1	5.8±0.6	7.0±0.8†	7.4±0.7†
Average Hepatic Portal Blood Flow, ml/kg/min			
SAL	22.2±2.0	18.2±1.5*	19.3±1.6
PoSIN-1	22.2±1.3	17.5±1.2*	20.0±1.2
PeSIN-1	22.2±1.8	17.3±1.3*	18.5±1.2
Average Total Hepatic Blood Flow, ml/kg/min			
SAL	28.2±2.5	27.6±2.3	29.4±2.7
PoSIN-1	27.0±1.7	23.3±1.7	26.9±1.7
PeSIN-1	28.0±2.4	24.3±2.1	25.9±1.9

Data are means±S.E.M.; n=8 in SAL goup, n=12 in PoSIN-1 group and n=10 in PeSIN-1 group
** = Significant statistical difference (P<0.05) from basal period within the group*
† = Significant statistical difference (P<0.05) from SAL group

Table 3.4: Average lactate, glycerol and NEFA concentration and net hepatic balance during the basal and experimental periods in conscious 42-h-fasted dogs given saline or SIN-1 infused into the portal vein or into a peripheral vein

Group	Basal Period	Experimental Period	
		Period 1	Period 2
Arterial Blood Lactate, µmol/L			
SAL	412±50	1390±134	1174±116
PoSIN-1	327±17	1049±45*	966±45*
PeSIN-1	467±41	1033±91	1010±73
Net Hepatic Lactate Balance, µmol/kg/min			
SAL	-6.1±0.9	10.0±2.1	7.8±1.8
PoSIN-1	-5.3±0.3	6.8±1.0	4.5±0.9*
PeSIN-1	-4.5±0.7	11.7±2.3	7.6±2.0
Arterial Blood Glycerol, µmol/L			
SAL	99±14	35±6	29±5
PoSIN-1	88±6	36±4	48±5*
PeSIN-1	101±5	38±3	45±5*
Net Hepatic Glycerol Uptake, µmol/kg/min			
SAL	1.9±0.3	0.7±0.1	0.6±0.1
PoSIN-1	1.7±0.2	0.6±0.1	1.0±0.1*
PeSIN-1	2.0±0.2	0.7±0.1	0.9±0.2
Arterial Plasma NEFA, µmol/L			
SAL	1034±81	183±23	140±16
PoSIN-1	976±41	136±18	143±20
PeSIN-1	853±53	129±20	110±19
Net Hepatic NEFA Uptake, µmol/kg/min			
SAL	3.8±1.2	0.2±0.1	0.3±0.1
PoSIN-1	3.4±0.3	0.3±0.1	0.4±0.1
PeSIN-1	1.9±0.6	0.2±0.1	0.2±0.1

Data are means±S.E.M.; n=8 in SAL goup, n=12 in PoSIN-1 group and n=10 in PeSIN-1 group
*All values in each group during the experimental period are significantly different (P<0.05) from the basal period. * = Significant statistical difference (P<0.05) from SAL group*
Negative values for balance data indicate net hepatic uptake

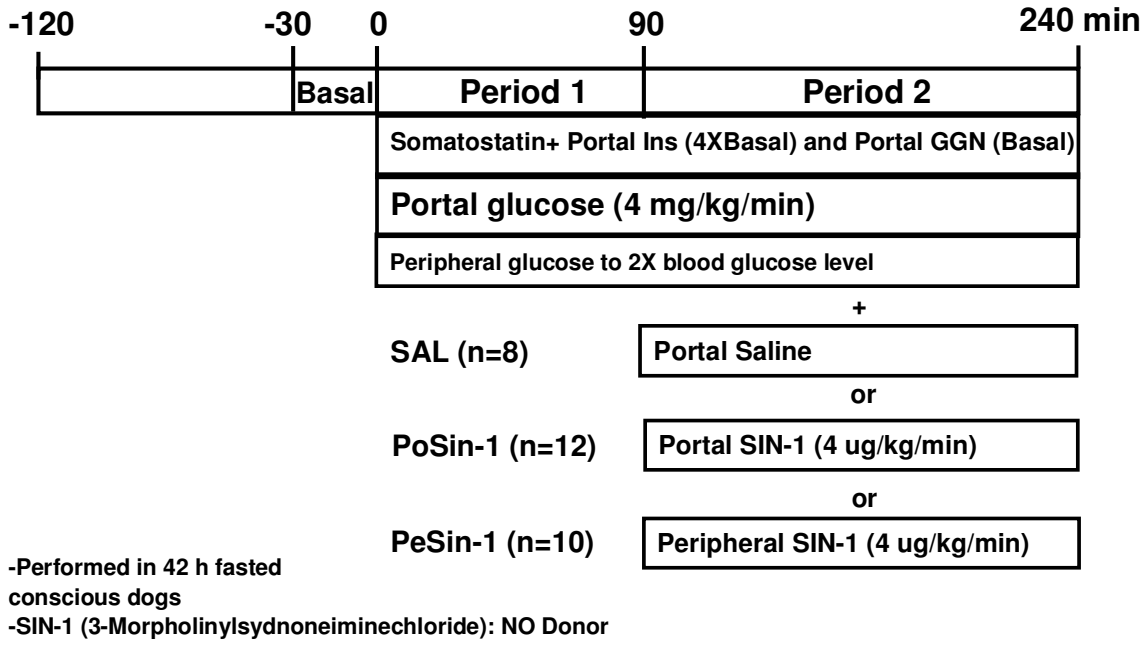

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Figure 3.1: Schematic representation of the study. The protocol comprises the basal (-30-0 min) and experimental periods (period 1, 0-90 min; period 2, 90-240 min). Somatostatin was infused peripherally and insulin (4-fold basal) and glucagon (basal) were given intraportally, whereas glucose was delivered intraportally (4 mg/kg/min) and peripherally at a variable rate to increase the hepatic glucose load 2-fold basal during period 1 and period 2. The saline (SAL) group (n = 8) received intraportal saline during period 2; PoSIN-1 group (n = 12), received intraportal 3-morpholinosydnonimine (SIN-1, 4 µg/kg/min) during period 2. PeSIN-1 group (n = 10) received peripheral SIN-1 (4 µg/kg/min) during period 2.

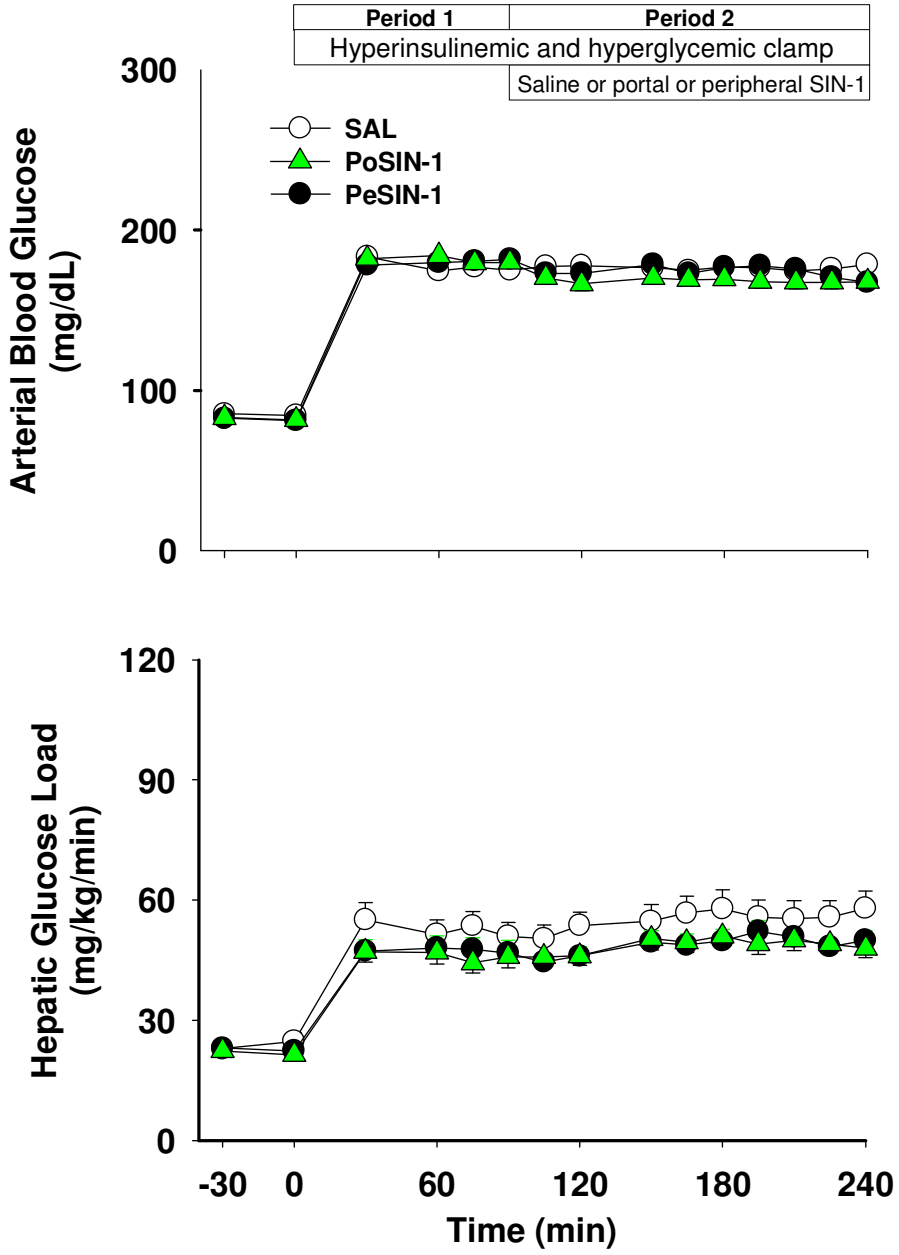

Figure 3.2: Arterial blood glucose and hepatic glucose loads in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 3.1 for description of study conditions. Data are means±S.E.M.

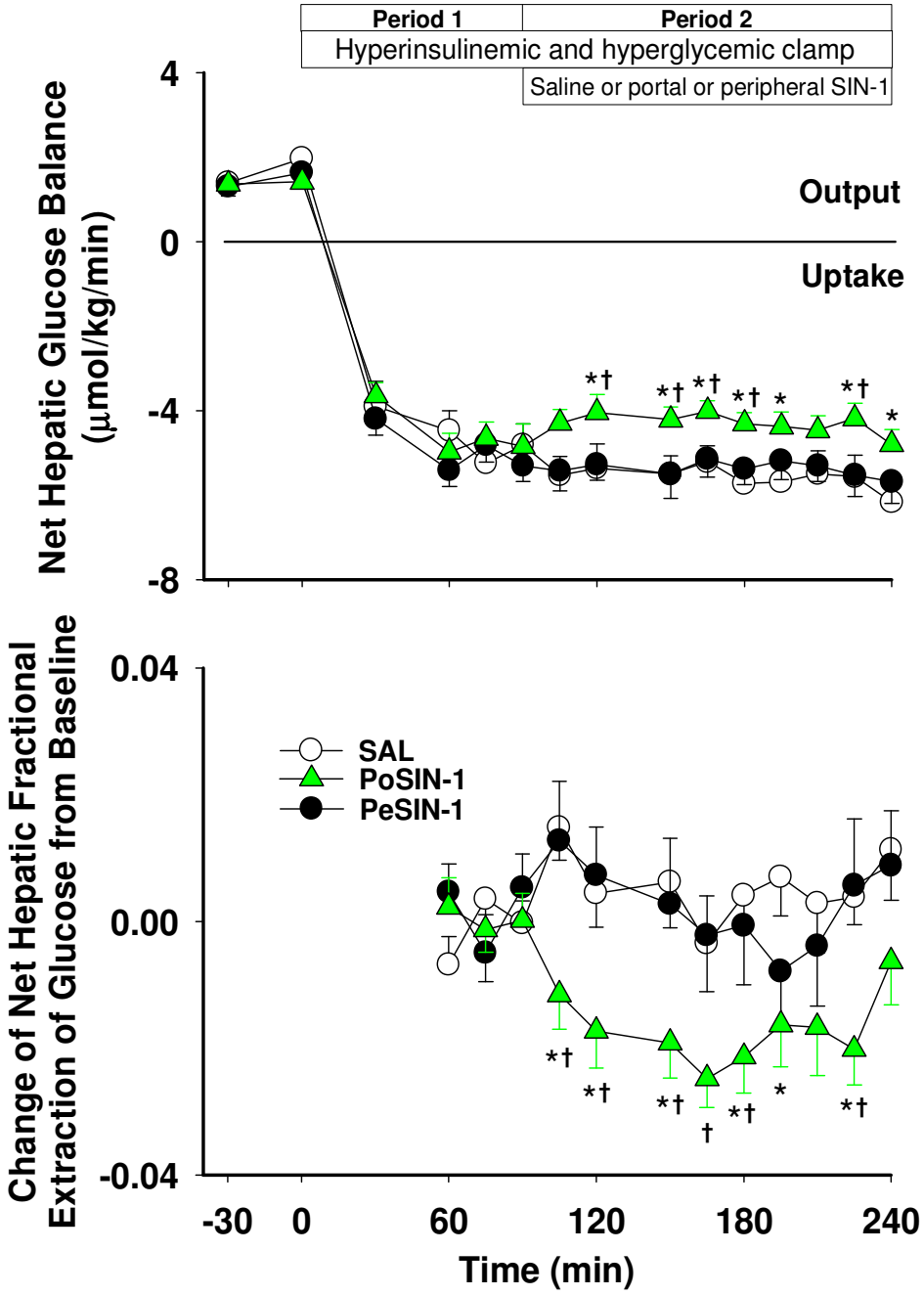

Figure 3.3: Net hepatic glucose uptake and change of net hepatic fractional extraction of glucose from baseline in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 3.1 for description of study conditions. Data are means±S.E.M. *P<0.05 compared with SAL group. †P<0.05 compared with the PeSIN-1 group.

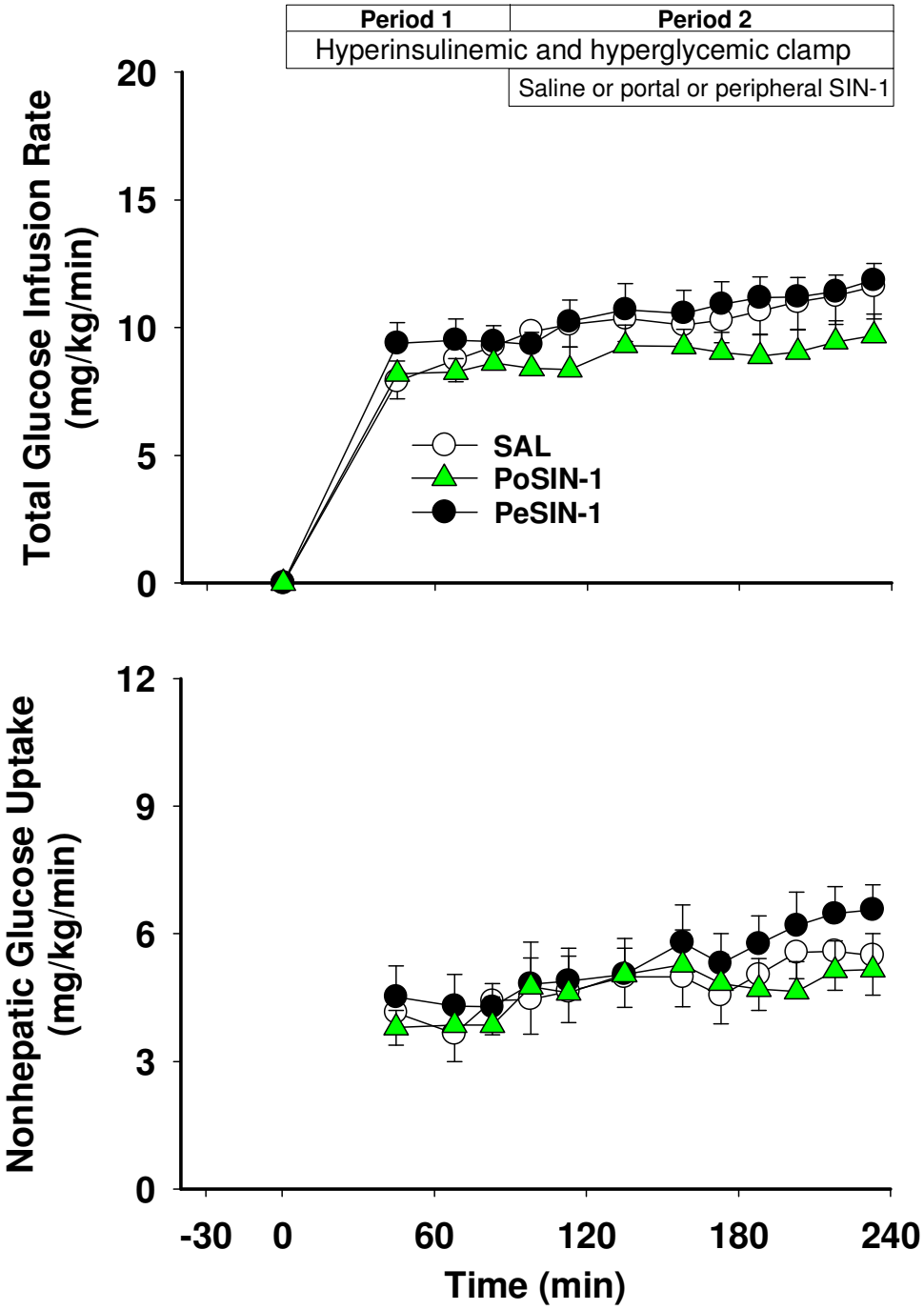

Figure 3.4: Glucose infusion rate and nonhepatic glucose uptake in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 3.1 for description of study conditions. Data are means±S.E.M.

CHAPTER IV

EFFECTS OF INTRAPORTAL INFUSION OF THE SSRI ESCITALOPRAM (LEXAPRO) ON NET HEPATIC GLUCOSE UPTAKE

Aim

To examine whether escitalopram enhances net hepatic glucose uptake in conscious dogs under fixed hyperinsulinemic and hyperglycemic conditions, when the role of the liver in glucose disposition is very important to glucose homeostasis.

Experimental Design

Animals were divided into three experimental groups in a randomized study setting. Each experiment consisted of a 90-min equilibration period (-120 to -30 min), a 30-min basal period (-30 to 0 min), and a 270-min experimental period (0 to 270 min), which was divided into a 90 minute period (P1) followed by a 180 minute period (P2). In all experiments a constant infusion of indocyanine green dye (0.076 mg/min; Sigma Immunochemicals, St. Louis, MO) was initiated at –120 min via the left cephalic vein. At 0 min, a constant infusion of somatostatin (0.8 μg/kg/min; Bachem, Torrance, CA) was begun via the left saphenous vein to suppress endogenous insulin and glucagon secretion. Basal glucagon (0.57 ng/kg/min; Glucagen, Novo Nordisk, Bagsvaerd, Denmark) and 4-fold basal insulin (1.2 mU/kg/min; Eli Lilly & Co., Indianapolis, IN) infusions

were then started through the splenic and jejunal catheters and maintained for the duration of the study. In addition, at time 0, a primed continuous infusion of 50% dextrose was started via the right cephalic vein, so that the blood glucose could be quickly be clamped at the desired hyperglycemic level (~165 mg/dL). In P2, saline was infused intraportally in the control group (SAL, n=11), while escitalopram oxalate (Forest Research Institute, Jersey City, NJ) was infused intraportally at 2 µg/kg/min (L-ESC, n=6) or 8 µg/kg/min (H-ESC, n=7). Intraportal infusion of the drug facilitated liver targeting and reflected the route of the drug entry seen clinically. The peripheral glucose infusion rate was adjusted in P2 to maintain a similar hepatic glucose load (HGL) to that seen in P1.

Results

5-HT levels

The 5-HT levels in the liver were significantly increased in response to intraportal escitalopram infusion at 8 µg/kg/min (H-ESC vs. SAL, $P<0.05$) (Table 4.1). Arterial, portal and hepatic vein blood 5-HT concentrations did not change significantly in any group throughout the study (Table 4.1). There was a consistent (with the exception of L-ESC group during P2) albeit small net hepatic production of serotonin in each group (Table 4.1).

Hormone concentrations

The arterial and hepatic sinusoidal insulin concentrations increased three- to fourfold and remained stable during P1 and P2 in all groups (Table 4.2). Arterial and hepatic sinusoidal plasma glucagon concentrations, on the other hand, remained near basal (Table 4.2). The mean plasma cortisol concentrations also remained statistically unchanged from basal in each group (Table 4.2).

Hepatic blood flow, blood glucose concentrations, and hepatic glucose load

Portal vein blood flow decreased ~20% in all groups during P1 in response to somatostatin infusion (Table 4.3). There tended to be a concomitant and somewhat offsetting increase in hepatic arterial flow. Nevertheless, total hepatic blood flow tended to be slightly reduced during P1 and P2 in all groups.

Arterial blood glucose concentrations increased in all groups from a basal value of 82±2 to 164±3 mg/dL during P1 and P2 (Fig. 4.2). The hepatic glucose loads increased proportionally and did not differ significantly among groups at any time (Fig. 4.2).

Net hepatic glucose balance and net hepatic fractional glucose extraction

All groups exhibited a similar rate of net hepatic glucose output during the basal period. Coincident with the start of the experimental period (4X basal insulin, basal glucagon and hyperglycemia), all groups switched from net output to net uptake of glucose, with the rates not being significantly different among groups during P1 (Fig. 4.3). Subsequently, the rate of net hepatic glucose uptake

remained relatively stable in SAL and L-ESC (2.4±0.3 and 2.4±0.2 mg/kg/min), whereas it increased in H-ESC and averaged 4.1±0.6 mg/kg/min during the last hour of P2 ($P<0.05$ vs. SAL). The net hepatic fractional glucose extraction followed a similar pattern, increasing significantly in response to intraportal escitalopram infusion at 8 µg/kg/min (Fig. 4.3).

Glucose infusion rates, nonhepatic glucose uptake

The glucose infusion and nonhepatic glucose uptake rates increased over time in all groups (Fig. 4.4). In response to intraportal escitalopram infusion at 8 µg/kg/min, the area under the curve (AUC) for the change from baseline in glucose infusion rate was significantly greater than in the other two groups (128±27 in H-ESC vs. 49±10 in SAL and 66±15 mg/kg/min X180 min in L-ESC group, $P<0.05$) during P2 (90–270 min). Nonhepatic glucose uptake did not differ significantly among groups at any time.

Lactate metabolism, net hepatic carbon retention and hepatic glycogen content

The arterial blood lactate concentrations rose in all groups during P1 and P2. After the experimental period began, net hepatic lactate balance changed from uptake to output, and the output rates remained elevated in all groups during P2 (Table 4.4). Net hepatic carbon retention (mg glucose equivalents/kg/min) did not differ between SAL, L-ESC and H-ESC at any time during P1 but it increased in H-ESC relative to the other two groups during P2 (3.8±0.5 in H-ESC vs. 2.8±0.2 in SAL and 2.7±0.1 L-ESC group, respectively,

P<0.05). In agreement with this, the hepatic glycogen content at the end of study was greater in H-ESC than in L-ESC and SAL (5.6 ± 0.4 vs. 3.3± 0.2 and 4.0 ± 0.4 g /100 g liver, respectively, P<0.05).

Glycerol and nonesterified fatty acid metabolism

Arterial blood glycerol concentrations and net hepatic glycerol uptakes were reduced by 40–65% in response to hyperglycemia and hyperinsulinemia and remained suppressed in all groups throughout P1 and P2 (Table 4.4). Arterial plasma nonesterified fatty acid concentrations and net hepatic nonesterified fatty acid uptakes changed in a pattern similar to glycerol, decreasing 85–95% during P1 and P2 in all groups (Table 4.4).

Discussion

To our knowledge, this study is the first *in vivo* investigation to explore the effects of the SSRI escitalopram on the ability of the liver to take up and store glucose. In the presence of fourfold basal insulin and basal glucagon, hyperglycemia caused net hepatic glucose uptake of ~2.0 mg/kg/min during P1 in all three groups. Portal infusion of the SSRI escitalopram at 2 µg/kg/min (L-ESC) did not significantly enhance net hepatic glucose uptake during P2 compared with the control group (SAL). However, when the infusion rate was increased to 8 µg/kg/min (H-ESC), net hepatic glucose uptake was ~60% greater than the corresponding rate in SAL. Similar to net hepatic glucose uptake, net hepatic carbon retention did not differ among groups during P1. The enhancement of net

hepatic glucose uptake during P2 in H-ESC was accompanied by a ~60% increase in net hepatic carbon retention, and the hepatic glycogen content at the end of the study was greater in H-ESC than in SAL and L-ESC. At the same time, glucose uptake by nonhepatic tissues was not significantly altered by escitalopram infusion. There was a tendency (not statistically significant) for arterial and sinusoidal insulin levels to be higher in the L-ESC and H-ESC groups than in SAL group in period 2 when escitalopram was given. Since somatostatin inhibited insulin secretion, as confirmed by reduced arterial c-peptide levels (data not shown), this could have resulted from a drug induced decrease in insulin clearance or more than likely a random difference in insulin clearance in the L-ESC and H-ESC groups. In an earlier study, it was demonstrated that SSRI Sertraline increases plasma insulin levels in rats without changing peripheral insulin sensitivity (Gomez *et al.*, 2001). Nevertheless, it seems unlikely that the tendency of insulin to be higher in the H-ESC group was responsible for the enhancement of net hepatic glucose uptake seen in that group since it was a small change and the same tendency for insulin to be higher in the L-ESC group did not result in increased net hepatic glucose uptake.

Evidence shows that serotonin has a range of biological functions including acting as a neurotransmitter and as a regulator of vascular tone and glucose metabolism. Treatment with serotonin or its precursor tryptophan induces hypoglycemia in rodents (Smith *et al.*, 1977; Yamada *et al.*, 1989), whereas 5-HT receptor antagonists cause hyperglycemia (Wozniak *et al.*, 1991). In addition, SSRIs improve glucose tolerance and insulin sensitivity in obese and

diabetic rats (Gomez *et al.*, 2001; Picarel-Blanchot *et al.*, 1994) and humans (Breum et al., 1995; Maheux et al., 1997; Potter van Loon et al., 1992). However, the mechanisms that account for hypoglycemia or improved glucose tolerance in individuals with diabetes after treatment with SSRIs are not fully understood.

In our previous study, infusion of the SSRI fluvoxamine into the liver through the hepatic portal vein increased net hepatic glucose uptake and hepatic carbon storage under hyperglycemic hyperinsulinemic conditions in conscious dogs (Moore et al., 2004a). Fluvoxamine inhibits reuptake of serotonin into the presynaptic nerve terminals, but whether this is the mechanism by which it brings about an increase in net hepatic glucose uptake remains unknown. In the present study, escitalopram was chosen because it is the most selective SSRI available, having a very low affinity for serotonergic (5-HT_{1-7}), alpha- and beta-adrenergic, dopamine (D_{1-5}), histamine (H_{1-3}), muscarinic (M_{1-5}), and benzodiazepine receptors (Waugh *et al.*, 2003). Since some of these receptors could influence glucose metabolism, using escitalopram allows us to more definitively study the effect of serotonin on hepatic glucose metabolism. The patterns of enhancement in net hepatic glucose uptake and net hepatic carbon retention in response to intraportal escitalopram infusion at 8 µg/kg/min were similar to those seen during intraportal infusion of fluvoxamine, confirming the role of hepatic serotonin in the regulation of hepatic glucose uptake. Fluvoxamine may also have inhibited peripheral glucose uptake since it was shown to significantly reduced nonhepatic glucose uptake albeit at a single time point at the end of our earlier study (Moore et al., 2004a). There was no such effect of escitolapram on nonhepatic glucose

metabolism in the present study. It should be noted, however, that there was a tendency for an elevation in the plasma epinephrine level to occur when fluvoxamine was infused but not when escitolapram was given (data not shown), thereby potentially explaining the reduction in nonhepatic glucose uptake.

5-HT receptor subtypes have been grouped into seven families, 5-HT receptors 1 to 7, based on the second messenger system they employ as well as their sequence homologies. Each family comprises several variant subtypes. With the exception of the 5-HT_3 receptor, 5-HT receptors are G-protein-coupled receptors. 5-HT_1, 5-HT_2 and 5-HT_4 receptors are found in relative abundance in the liver (genecards.org). 5-HT_4 receptor is coupled to Gs, which activates adenylyl cyclase (AC), and increases synthesis of cAMP. cAMP in turn activates protein kinase A (PKA), and PKA phosphorylates downstream protein kinases. PKA also causes activation of glycogen phosphorylase in hepatocytes thereby triggering the degradation of hepatic glycogen. 5-HT_2 receptor is coupled to Gq, which activates phospholipase C (PLC), yielding diacylglycerol (DAG). DAG activates protein kinase C (PKC), which then activates downstream protein kinases. By contrast, 5-HT_1 receptor is coupled to Gi, which inhibits AC and PKA. It is possible that the activation of hepatic 5-HT_1 receptor will cause inactivation of PKA, and in turn result in an increase in net hepatic glucose uptake and hepatic glycogen deposition in response to escitalopram. The latter possibility is supported by a recent study which showed that a 5-HT_1 receptor agonist could enhance glycogen synthesis in cultured hepatocytes (Hampson et al., 2007).

Twenty mg escitalopram by mouth OQD is commonly prescribed to treat depression. It has been shown in human subjects that i.v. infusion of escitalopram at a rate of 10 mg/h (~2 µg/kg/min) brings about a similar pharmacokinetic profile to 20 mg of the drug p.o. (Sogaard et al., 2005). In the present study, when escitalopram was infused intraportally at 2 µg/kg/min, a dose which was believed to mimic the pharmacokinetic profile in humans taking 20 mg escitalopram daily, no change of net hepatic glucose uptake was observed. However, net hepatic glucose uptake was significantly enhanced in response to the portal infusion of escitalopram at 8 µg/kg/min. Little is known about how the pharmacokinetics of escitalopram in dogs and humans compare, but an early citalopram (escitalopram racemate) study showed that its half life is much shorter and its systemic plasma clearance rate is much higher in dogs than in humans (Fredricson Overo, 1982). This suggests that in order to mimic the pharmacokinetic profile in humans, higher doses of escitalopram are needed in the dog model. Therefore, the enhancement of net hepatic glucose uptake seen in the present study with a high dose of escitalopram may reflect that seen in humans with a typical oral dose of the drug. The enhancement of net hepatic glucose uptake was accompanied by an increase in net hepatic carbon retention, which reflects hepatic glycogen deposition. In a net sense, ~85% of the glucose taken up by the liver in response to the drug was deposited in liver glycogen, while most of the remaining glucose left the liver as lactate. It appears that portal escitalopram infusion had no effects on nonhepatic glucose uptake, although it is hard to conclusively rule out such a possibility because of the variability

associated with the measurement of this parameter. This means that the increase in whole body glucose disposal seen in response to intraportal escitalopram infusion at 8 µg/kg/min was most likely the result of augmented net hepatic glucose uptake.

As noted above, the augmentation of net hepatic glucose uptake and hepatic glucose storage could have resulted from a direct interaction of endogenous serotonin with its receptor on hepatocytes. Recently, it was reported that physiological concentrations of serotonin or a 5-HT_1 receptor agonist have direct stimulatory effects on glycogen synthesis in primary cultures of rat hepatocytes (Hampson et al., 2007). It is also possible, however, that the increase in net hepatic glucose uptake seen in the present study could have been brought about by a neural signal initiated in the liver. A rise in hepatic serotonin levels resulting from intraportal serotonin injection can decrease the afferent firing rate in the hepatic branch of the vagus nerve, similar to the effect seen in response to intraportal glucose injection (Niijima, 1981) (Niijima, 1996). The vagus nerve communicates with the hypothalamus via the hypothalamic projections to the medulla oblongata and the liver could receive autonomic input as a result of hypothalamic nuclei receiving serotonergic input (Buijs et al., 2003; Hosoya, 1985). Furthermore, stimulation of hypothalamic autonomic efferent can enhance hepatic glycogen synthase activity (Shimazu, 1967). These investigations suggest a mechanism by which a change of serotonin levels in the liver could elicit a neural signal which may be coordinated by the hypothalamus to enhance net hepatic glucose uptake. In line with this theory, we observed an

increase in 5-HT levels in the liver in response to intraportal escitalopram infusion at 8 µg/kg/min. Consistent with our previous observations with fluvoxamine, however, we did not detect a significant increase in 5-HT levels in the blood. Such measurements may not be very sensitive readouts for alterations in neurotransmitter levels in the synapse (Ahren et al., 1988), given the variance of the analytical method and the limited power to detect a change of 5-HT levels (the power calculation was set based on detecting differences in net hepatic glucose balance). We also cannot rule out the involvement of central serotonergic sensing since the infused SSRI escitalopram can penetrate the blood-brain barrier and may thus activate the brain serotonergic system directly. The central serotonergic system has recently been shown to regulate peripheral glucose metabolism without changing energy expenditure, locomotor activity or body weight (Zhou et al., 2007). These authors showed that 5-HT_{2C} receptor agonists significantly improved glucose tolerance and reduced the plasma insulin level in mouse models of obesity and type 2 diabetes. Furthermore, they showed that the mechanism required downstream activation of melanocortin-4 receptors in the CNS. The precise workings of the serotonin receptors/pathways regulating hepatic glucose metabolism require further elucidation. Highly selective receptor agonists/antagonists will undoubtedly provide valuable mechanistic insights into the role of serotonin receptors in modulating glucose homeostasis.

In conclusion, our data indicate that intraportal infusion of a SSRI, escitalopram, enhanced net hepatic glucose uptake and hepatic glycogen deposition under hyperglycemic and hyperinsulinemic conditions in conscious

42-h-fasted dogs. These findings raise the possibility that serotonin may play a role in directing the disposition of an oral glucose load. Hepatic-targeted SSRIs might therefore help to control postprandial hyperglycemia in individuals with diabetes.

Table 4.1: Serotonin levels and net hepatic balance in conscious 42-h-fasted dogs given saline, or a low or high dose escitalopram infusion into the hepatic portal vein

		Experimental Period	
Group	**Basal Period**	**Period 1**	**Period 2**
5-HT Levels in Liver, ng/mg protein			
SAL	N/A	N/A	543±141
L-ESC	N/A	N/A	799±208
H-ESC	N/A	N/A	908±72*
Arterial Blood 5-HT, µg/L			
SAL	1140±283	1054±238	968±250
L-ESC	835±176	835±165	702±126
H-ESC	805±203	766±217	728±172
Portal Blood 5-HT, µg/L			
SAL	1235±322	1139±243	933±200
L-ESC	873±149	780±126	733±123
H-ESC	901±240	834±202	730±173
Hepatic Blood 5-HT, µg/L			
SAL	1268±299	1150±220	1041±226
L-ESC	889±131	861±138	694±113
H-ESC	945±230	861±175	769±197
Net Hepatic 5-HT Balance, µg/kg/min			
SAL	1.5±2.0	1.0±0.6	2.6±1.5
L-ESC	0.7±1.8	1.8±1.1	-0.7±1.4
H-ESC	2.0±1.8	0.7±1.3	0.9±1.3

N/A=liver sample is not available due to the fact that biopsy was only taken after completion of experiments
Data are means±S.E.M.. n=6 dogs in each group
SAL=the group that received saline; L-ESC=the group that received escitalopram in the portal vein at 2 µg/kg/min; H-ESC=the group that received escitalopram in the portal vein at 8 µg/kg/min
Negative values for balance data indicate net hepatic uptake
** = Significant statistical difference (P<0.05) from the saline group*

Table 4.2: Hormone concentrations during the basal and experimental periods in conscious 42-h-fasted dogs given saline, or a low or high dose escitalopram infusion into the hepatic portal vein

Group	Basal Period	Experimental Period	
		Period 1	Period 2
Arterial Plasma Insulin, µU/ml			
SAL	8±1	19±1*	22±2*
L-ESC	8±1	22±2*	26±2*
H-ESC	8±1	23±2*	26±3*
Hepatic Sinusoidal Insulin, µU/ml			
SAL	22±3	81±13*	78±10*
L-ESC	24±8	94±8*	98±6*
H-ESC	25±4	95±7*	98±8*
Arterial Plasma Glucagon, pg/ml			
SAL	43±4	41±3	40±3
L-ESC	40±6	45±9	44±8
H-ESC	35±4	43±3	37±3
Hepatic Sinusoidal Glucagon, pg/ml			
SAL	50±4	56±4	53±4
L-ESC	47±5	61±7	57±8
H-ESC	40±4	63±4*	51±3
Arterial Cortisol, µg/dL			
SAL	4±1	5±1	3±1
L-ESC	3±1	3±1	3±1
H-ESC	4±1	5±1	3±1

Data are means±S.E.M.; n=11 dogs in the saline group (SAL), n=6 in the group that received escitalopram at 2 µg/kg/min (L-ESC) and n=7 in the group that received escitalopram at 8 µg/kg/min (H-ESC)
**= Significant statistical difference (P<0.05) from basal period within the group*

Table 4.3: Average hepatic arterial, portal, and total hepatic blood flow during the basal and experimental periods in conscious 42-h-fasted dogs given saline, or a low or high dose escitalopram infusion into the hepatic portal vein

		Experimental Period	
Group	**Basal Period**	**Period 1**	**Period 2**
Average Hepatic Arterial Blood Flow, ml/kg/min			
SAL	5.1±0.4	5.9±0.5	6.8±0.9
L-ESC	5.2±0.4	5.9±0.8	6.9±1.1
H-ESC	5.0±0.2	5.5±0.3	6.0±0.6
Average Hepatic Portal Blood Flow, ml/kg/min			
SAL	22.6±1.4	17.9±1.2*	17.9±1.2*
L-ESC	25.6±0.7	18.3±0.8*	17.3±0.6*
H-ESC	21.4±1.7	16.3±1.2*	17.4±1.1
Average Total Hepatic Blood Flow, ml/kg/min			
SAL	27.7±1.4	23.8±1.4	24.7±1.4
L-ESC	30.8±0.9	24.2±1.4*	24.8±1.5
H-ESC	26.4±1.7	21.8±1.4	23.5±1.2

Data are means±S.E.M.; n=11 dogs in the saline group (SAL), n=6 in the group that received escitalopram at 2 µg/kg/min (L-ESC) and n=7 in the group that received escitalopram at 8 µg/kg/min (H-ESC)
**= Significant statistical difference (P<0.05) from basal period within the group*

Table 4.4: Average lactate, glycerol and NEFA concentrations and net hepatic balances during the basal and experimental periods in conscious 42-h-fasted dogs given saline, or a low or high dose escitalopram infusion into the hepatic portal vein

		Experimental Period	
Group	**Basal Period**	**Period 1**	**Period 2**
Arterial Blood Lactate, µmol/L			
SAL	327±58	673±84	783±81
L-ESC	395±32	934±98	855±73
H-ESC	551±90	1025±192	986±103
Net Hepatic Lactate Balance, µmol/kg/min			
SAL	-7.3±1.1	3.5±4.3	3.0±2.8
L-ESC	-6.6±0.9	6.8±1.6	3.6±1.4
H-ESC	-5.2±1.5	4.1±1.5	3.5±1.8
Arterial Blood Glycerol, µmol/L			
SAL	82±14	43±11	34±15
L-ESC	97±9	34±5	24±4
H-ESC	98±7	46±10	31±7
Net Hepatic Glycerol Uptake, µmol/kg/min			
SAL	1.8±0.4	1.0±0.4	0.6±0.3
L-ESC	2.1±0.3	0.5±0.1	0.5±0.1
H-ESC	1.4±0.2	0.7±0.1	0.6±0.2
Arterial Plasma NEFA, µmol/L			
SAL	803±114	108±11	90±18
L-ESC	909±75	133±17	96±14
H-ESC	1027±57	138±20	100±14
Net Hepatic NEFA Uptake, µmol/kg/min			
SAL	4.4±1.5	0.2±0.1	0.1±0.1
L-ESC	3.2±0.4	0.4±0.1	0.2±0.1
H-ESC	3.0±0.5	0.4±0.1	0.2±0.1

Data are means±S.E.M.; n=11 dogs in the saline group (SAL), n=6 in the group that received escitalopram at 2 µg/kg/min (L-ESC) and n=7 in the group that received escitalopram at 8 µg/kg/min (H-ESC) All values in each group during the experimental period were significantly different (P<0.05) from the basal period. Negative values for balance data indicate net hepatic uptake

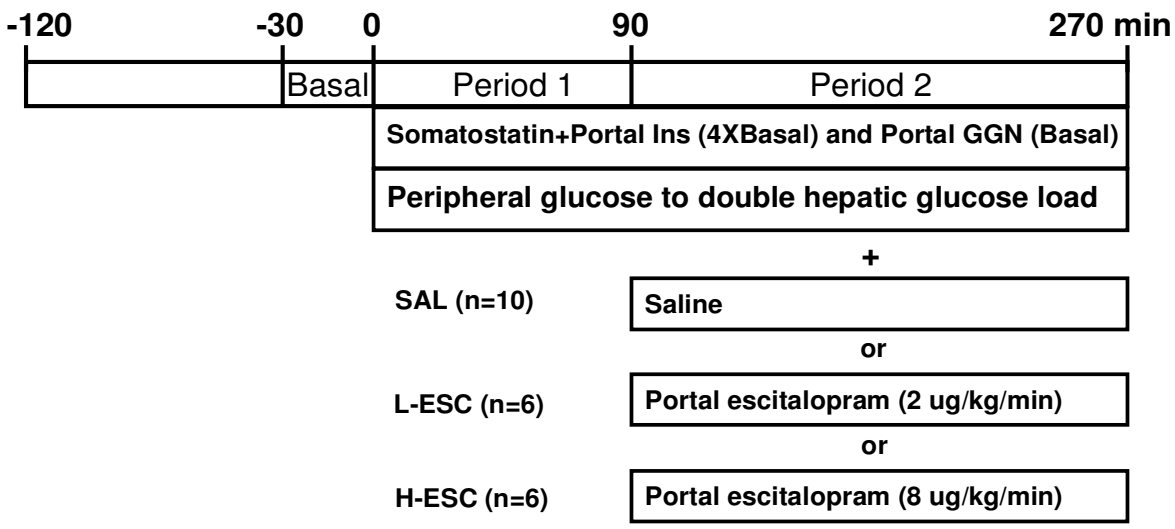

Figure 4.1: Schematic representation of the study. The protocol comprises the basal (-30-0 min) and experimental periods (period 1, 0-90 min; period 2, 90-270 min). Somatostatin was infused peripherally and insulin (4-fold basal) and glucagon (basal) were given intraportally, while glucose was delivered peripherally at a variable rate to increase the hepatic glucose load 2-fold basal during period 1 and period 2. The saline (SAL) group (n=11) received intraportal saline during period 2. L-ESC group (n=6), received intraportal escitalopram (2 µg/kg/min) during period 2. H-ESC group (n=7), received intraportal escitalopram (8 µg/kg/min) during period 2

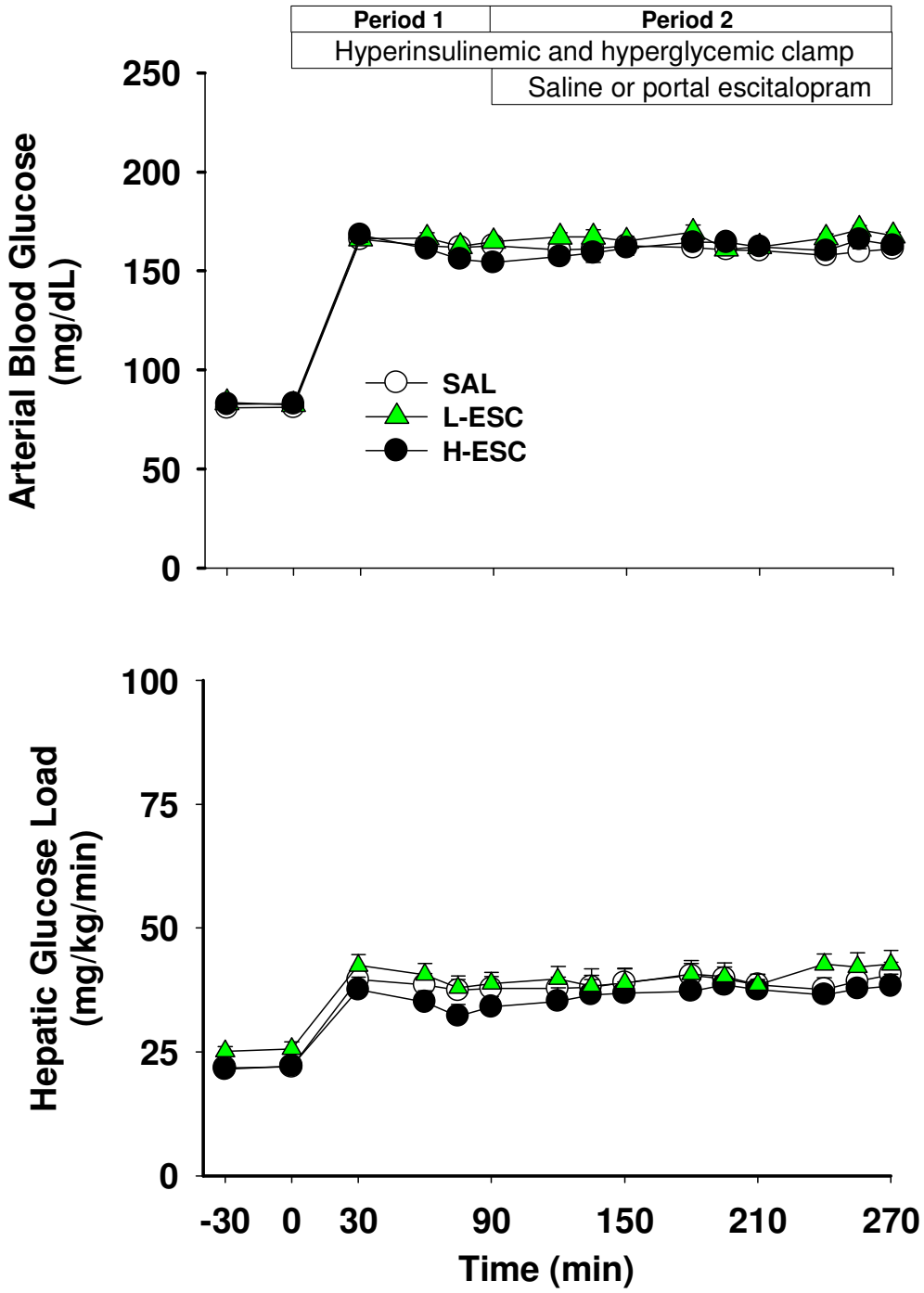

Figure 4.2: Arterial blood glucose and hepatic glucose loads in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 4.1 for description of study conditions. Data are means ± S.E.M.

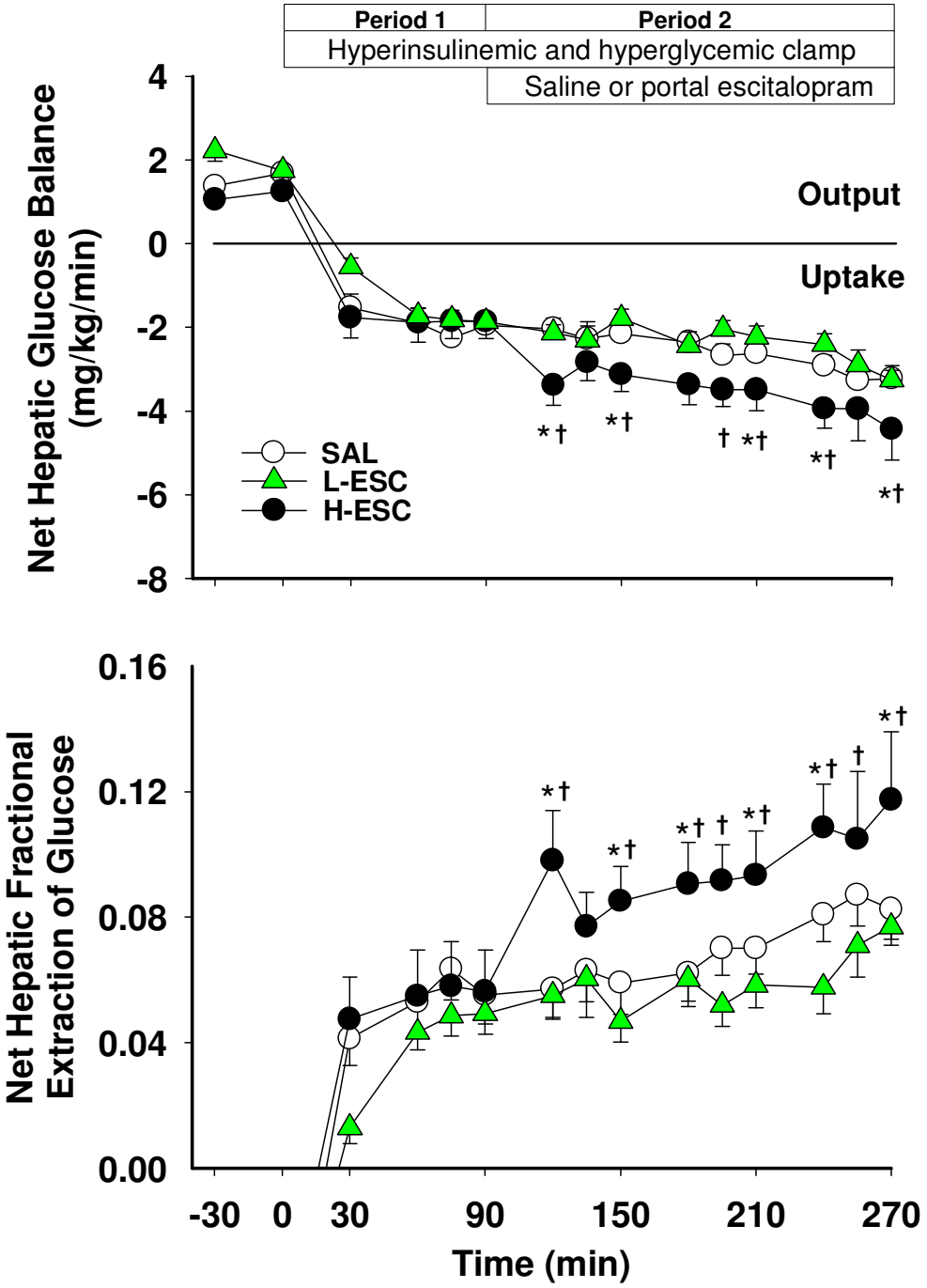

Figure 4.3: Net hepatic glucose uptake and net hepatic fractional extraction of glucose in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 4.1 for description of study conditions. Data are means±S.E.M; *P<0.05 compared with SAL group. †P<0.05 compared with L-ESC group.

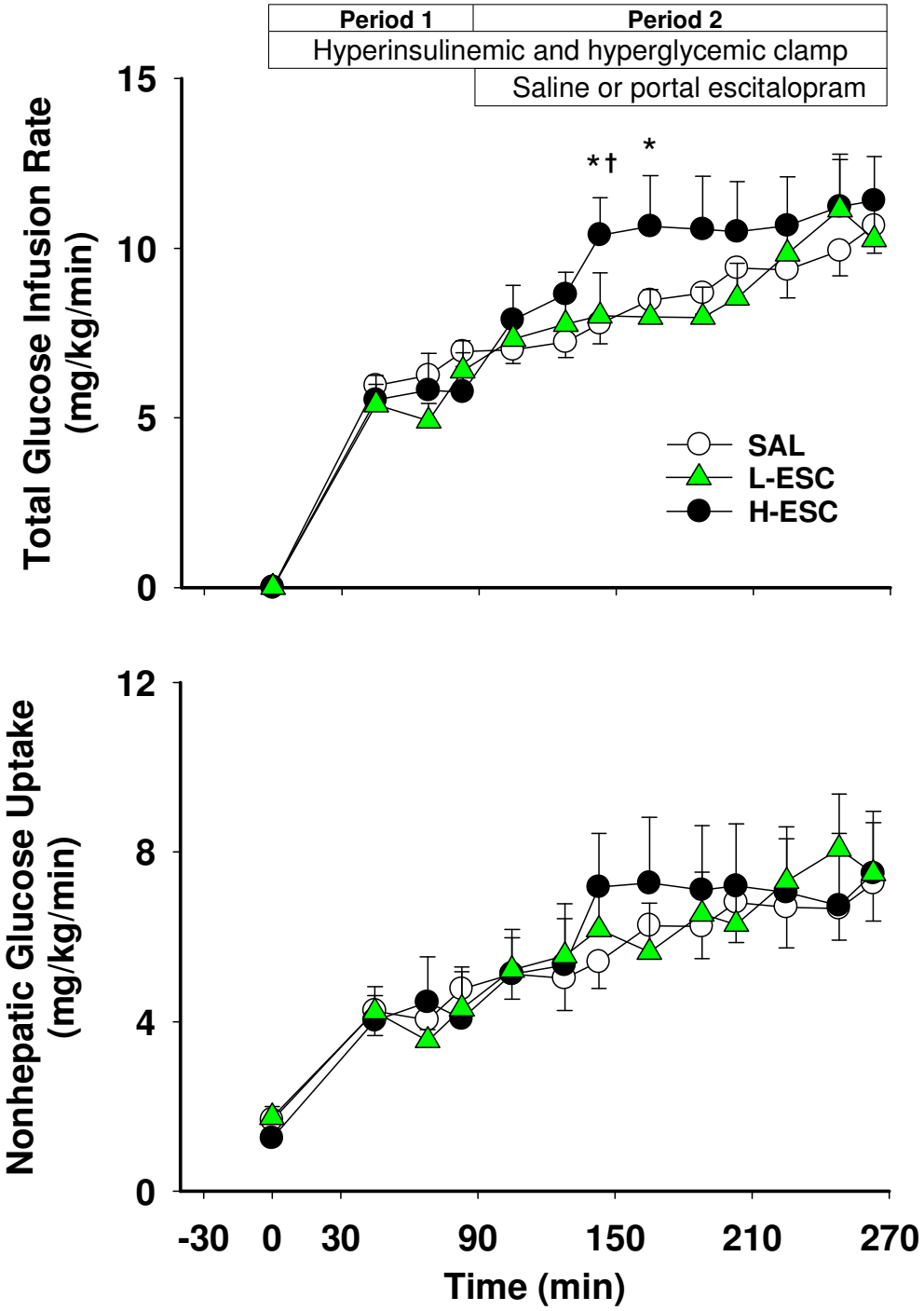

Figure 4.4: Glucose infusion rate and nonhepatic glucose uptake in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 4.1 for description of study conditions. Data are means±S.E.M; *P<0.05 compared with SAL group. †P<0.05 compared with L-ESC group.

CHAPTER V

THE ROLE OF sGC/cGMP PATHWAY IN THE REGULATION OF NET HEPATIC GLUCOSE UPTAKE

Aim

We previously showed in the conscious dog that increased hepatic NO resulting from portal SIN-1 infusion inhibits NHGU. Because NO can activate soluble guanylate cyclase (sGC), the aim of the present study was to determine whether a sGC/cGMP dependent mechanism is responsible for the regulation of NHGU by hepatic NO.

Experimental Design

As described in Fig. 5.1, each experiment consisted of a 90-min equilibration period (-120 to -30 min), a 30-min basal period (-30 to 0 min), and a 270-min experimental period (0 to 270 min), which was divided into a 90 minute period (P1) followed by a 180 minute period (P2). In all experiments a constant infusion of indocyanine green dye (0.076 mg/min; Sigma Immunochemicals, St. Louis, MO) was initiated at –120 min via the left cephalic vein. At 0 min, a constant infusion of somatostatin (0.8 μg/kg/min; Bachem, Torrance, CA) was begun via the left saphenous vein to suppress endogenous insulin and glucagon secretion. At the same time, basal glucagon (0.57 ng/kg/min; Glucagen, Novo

Nordisk, Bagsvaerd, Denmark) and 4-fold basal insulin (1.2 mU/kg/min; Eli Lilly & Co., Indianapolis, IN) infusions were started through the splenic and jejunal catheters and maintained for the duration of the study. In addition, at time 0, a primed continuous infusion of 50% dextrose was started via the right cephalic vein so that the blood glucose could be quickly be clamped at the desired hyperglycemic level (~160 mg/dL). In P2, saline was infused intraportally in the control group (SAL, n=10), and ODQ (Cayman Chemical Company, Ann Arbor, MI) was infused intraportally at 0.8 µg/kg/min in the ODQ (n=10) and ODSN (n=6) groups, while SIN-1 (Cayman Chemical Company, Ann Arbor, MI) was infused intraportally at 4 µg/kg/min in the ODSN group for the last 90 min of P2. The peripheral glucose infusion rate was adjusted as needed to maintain a similar hepatic glucose load (HGL) to that seen in P1.

Results

Hormone concentrations

The arterial and hepatic sinusoidal insulin levels increased three to four-fold and remained stable during P1 and P2 in all groups (Table 5.1). Arterial and hepatic sinusoidal plasma glucagon concentrations, on the other hand, remained basal throughout the study in all groups (Table 5.1). The mean plasma cortisol concentrations also remained basal and unchanged in each group (Table 5.1).

Hepatic blood flow, blood glucose concentrations, and hepatic glucose load

Portal vein blood flow decreased ~20% in all groups during P1 in response to somatostatin infusion (Table 5.2). There was a concomitant and offsetting (~20%) increase in hepatic arterial flow. Consequently, total hepatic blood flow tended to be slightly reduced during both P1 and P2 in all groups.

Arterial blood glucose levels increased in the all groups from a basal value of 80±2 to 159±2 mg/dL during P1 and P2 (Fig. 5.2). The hepatic glucose loads increased proportionally in all groups and did not differ significantly among groups at any time (Fig. 5.2).

Net hepatic glucose balance and net hepatic fractional glucose extraction

All groups exhibited a similar rate of net hepatic glucose output during the basal period. Coincident with the start of the experimental period (4X basal insulin, basal glucagon and hyperglycemia), all groups switched from net output to net uptake of glucose, with the rates (2.2±0.3, 2.2±0.3, and 2.6±0.4 mg/kg/min in SAL, ODQ and ODSN groups, respectively) not being significantly different among groups (Fig. 5.3). Subsequently, the rate rose to 3.1±0.3 mg/kg/min in SAL, whereas it increased to 4.7±0.5 and 4.5±0.6 mg/kg/min in the ODQ and ODSN groups, respectively, during the last hour of the experiment ($P<0.05$ vs. SAL). The net hepatic fractional extraction of glucose followed a similar pattern, increasing significantly and similarly in response to intraportal ODQ infusion in the absence or presence of SIN-1 (Fig. 5.3).

Glucose infusion rates, nonhepatic glucose uptake

The glucose infusion and nonhepatic glucose uptake rates increased over time in all groups (Fig. 5.4). In the ODQ and ODSN groups, the glucose infusion rate was significantly increased over that required in the saline group. Nonhepatic glucose uptake did not differ significantly between groups at any time, although there was a tendency for it to be increased in the presence of ODQ.

Lactate metabolism, net hepatic carbon retention and hepatic glycogen content

The arterial blood lactate concentrations rose in all groups during P1 and remained elevated in P2. After the experimental period began, net hepatic lactate balance changed from uptake to output, and the output rates remained increased in all groups during P2 (Table 5.3). Net hepatic carbon retention (mg glucose equivalents/kg/min) did not differ among groups at any time during P1 but was increased relative to the control group during P2 in response to ODQ, regardless of the presence or absence of SIN-1, (3.8±0.2 in ODQ and 3.8±0.4 in ODSN vs. 2.4±0.2 in the SAL group, $P<0.05$).

AMP-activated protein kinase signaling in the liver

Compared with the saline group (n=4), intraportal infusion of ODQ (n=5) and intraportal infusion of ODQ and SIN-1 (n=5) were associated with a decrease of ~30% in the phosphorylation of Thr-172 in AMP-activated protein kinase (AMPK) as well as a decrease in the phosphorylation of Ser-79 in acetyl-CoA carboxylase (ACC) in the liver (Fig. 5.5).

Glycerol and nonesterified fatty acid metabolism

Arterial blood glycerol concentrations and net hepatic glycerol uptake were reduced by 55–70 % in response to hyperglycemia and hyperinsulinemia and remained suppressed in all groups throughout P1 and P2 (Table 5.3). Arterial plasma nonesterified fatty acid (NEFA) concentrations and net hepatic NEFA uptake changed in a pattern similar to glycerol, decreasing ~85% during P1 and remaining suppressed in P2 in all groups (Table 5.3).

Discussion

To our knowledge, this report is the first *in vivo* work to explore effects of the sGC dependent pathway on net hepatic glucose uptake under hyperglycemic hyperinsulinemic conditions. Under these conditions, portal infusion of the sGC inhibitor ODQ caused a ~45% increase in net hepatic glucose uptake and a ~55% increase in hepatic glycogen accumulation. Hepatic cGMP levels were reduced by ~25% ($P<0.05$, data not shown) in response to ODQ, confirming the inhibition of sGC in the liver by ODQ. Intraportal infusion of SIN-1 did not attenuate the enhancement of NHGU induced by ODQ. At the same time, the rate of glucose uptake by nonhepatic tissues tended to increase, although we were unable to detect any significance due the variability of the signal. As a result, whole body glucose utilization increased ~30% in response to intraportal ODQ infusion.

Our previous study showed that portal but not peripheral infusion of the nitric oxide donor SIN-1 inhibited NHGU, suggesting that the effect of NO was

the result of a direct effect within the liver (An *et al.*, 2008). This concept is supported by the expression of all three isoforms of NOS in the liver. Both iNOS and eNOS are expressed in hepatocytes; additionally, iNOS has been found in hepatic Kupffer and Ito cells in various species, while nNOS has been identified in some nerve fibers in the rat liver (Alexander, 1998; Feelisch *et al.*, 1999; Wei *et al.*, 2002). There are also other experiments supporting the concept that NO regulates hepatic glucose metabolism. Borgs et al. (Borgs *et al.*, 1996) observed that NO infusion (~0.15 μmol/g liver/min) increased the rate of glucose output in the perfused rat liver as a result of activation of glycogen phosphorylase. Horton et al. (Horton *et al.*, 1994) demonstrated that the NO donors S-nitroso-N-acetylpenicillamine (SNAP) and SIN-1 inhibited gluconeogenesis in isolated rat hepatocytes and they suggested that this may be associated with a decrease in phosphoenolpyruvate carboxykinase (PEPCK) activity. Sprangers et al. (Sprangers *et al.*, 1998) showed that glycogen synthesis from glucose in rat hepatocytes was inhibited by the NO donor SNAP due to decreased glycogen synthase activity (less conversion of glycogen synthase *b* into *a*).

Activation of sGC and production of cGMP is one of the main signaling pathways associated with the biological action of NO. It has been shown that glucose transport in cardiomyocytes was inhibited by different membrane-permeant cGMP analogues whereas inhibition of endogenous cGMP production with ODQ increased glucose transport (Bergemann *et al.*, 2001). To date, however, no one has shown whether this downstream target is responsible for the changes in glucose metabolism seen in response to NO/NO donor treatment.

ODQ is a potent and specific inhibitor of sGC, and as a result is an important pharmacological tool used in differentiating cGMP dependent and -independent effects (Hwang *et al.*, 1995). By infusing this drug intraportally to inhibit sGC in the liver of the conscious dog, the present study was clearly able to show that inhibition of sGC augmented net hepatic glucose uptake under conditions of elevated insulin and glucose. Furthermore, intraportal infusion of SIN-1 concomitantly with SIN-1 did not attenuate this effect. These findings suggest the sGC/cGMP dependent pathway has the ability to regulate net hepatic glucose uptake in the whole animal. Further, it appears that the sGC/cGMP pathway is responsible for the inhibition of NHGU resulting from SIN-1 infusion seen in our previous experiments (An *et al.*, 2008). Major extrahepatic glucose consumers include skeletal muscle, adipose tissue, brain, etc. In the present experiments, nonhepatic glucose uptake tended to increase when ODQ was infused. This finding is not consistent with previous observations by others that inhibition of hepatic guanylyl cyclase in response to the intraportal administration of the GC inhibitors Methylene Blue or ODQ significantly reduced glucose uptake at peripheral tissues in rats (Guarino *et al.*, 2004). This inconsistency could be related to the use of different experimental designs, different duration of fast, the use of different species or that fact that our data regarding nonhepatic glucose uptake do not indicate a real difference.

There are several possible ways that NO/sGC/cGMP could affect NHGU. Many biological effects of NO and cGMP in the cell are mediated by PKG (Vaandrager *et al.*, 1996), so it is possible that the PKG-dependent pathway is

the downstream signal responsible for the regulation of NHGU by NO. Evidence supports a role for PKG in glucose metabolism. PKG was found to phosphorylate GSK-3β, a key regulator of glycogen synthase, on Ser 9, thereby inhibiting its enzyme activity both *in vitro* and *in vivo* (Zhao *et al.*, 2005). Young et al. showed that activation of PKG by cGMP-stimulated glucose oxidation in skeletal muscle, likely through the phosphorylation and activation of UCP-3 (Young *et al.*, 1998). On the other hand, MacKenzie et al. showed that cGMP can modulate glucose transport in cultured vascular smooth muscle cells, but effects of cGMP are unlikely to be mediated through the action of PKG since the PKG inhibitor KT-5823, at doses up to 1μM, did not block the stimulation of glucose transport by an cGMP analog, 8-bromo-cGMP (MacKenzie *et al.*, 2001). In addition, the expression of PKG levels are low in the liver (MacKenzie *et al.*, 2001; Walter, 1981), and very few metabolic enzymes in the liver have been identified as physiological targets regulated by PKG. Further studies will be necessary to explore the role of PKG relative to the sGC/cGMP pathway in the regulation of hepatic glucose uptake. It has been well established that cAMP leads to activation of cAMP-dependent protein kinase (PKA), which in turn phosphorylates many intracellular enzymes involved in the regulation of glycogen metabolism, gluconeogenesis, and glycolysis (Exton, 1987). Since cGMP-inhibits the cAMP-specific phosphodiesterase (PDE3), which could hydrolyze cAMP, it is possible that ODQ could lower hepatic cGMP thereby removing the inhibition on PDE3, which could, in turn, reduce hepatic cAMP levels and potentially alter glucose metabolism. However, the hepatic PDE3 activity (pmol/min/mg dry

weight) in the present study was not different between groups ($3.6x10^{-3}\pm0.3x10^{-3}$ in ODQ vs. $3.2x10^{-3}\pm0.3x10^{-3}$ in SAL group). This indicates that PDE3 does not appear to be the mechanism responsible for the metabolic response seen in the present study.

Last, it is possible that NO/sGC/cGMP may regulate NHGU through an AMPK dependent pathway. It has recently been reported that NO can activate AMPK in endothelium and muscle via a sGC/cGMP-dependent pathway (Lira et al., 2007; Zhang et al., 2008). In line with this concept, we demonstrated that intraportal infusion of ODQ was associated with a decrease (~30%) in the phosphorylation of Thr-172 in AMPK without changing it protein level. This observation is confirmed by a decrease (~45%) in the phosphorylation of Ser-79 in ACC, a marker of AMPK activity, in the liver. It remains unclear whether the AMPK pathway is required for the metabolic response seen in the present study.

AMPK has been proposed to act as a 'metabolic master switch' mediating the adaptation to systemic and cellular energy status. In the past few years, AMPK has been implicated in the control of hepatic glucose homeostasis (Viollet *et al.*, 2006), although data are still controversial. Bergeron et al. (Bergeron *et al.*, 2001) showed that systemic infusion of AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), an AMPK activator, suppressed hepatic glucose output in overnight-fasted normal and obese rats. This finding was supported by an *in vitro* experiment which showed that treatment with an adenovirus expressing a constitutively active form of AMPK alpha2 reduced glucose output in cultured hepatocytes (Foretz *et al.*, 2005), and an *in vivo*

observation that liver-specific AMPK alpha2 KO mice had increased fasting hepatic glucose production (Andreelli *et al.*, 2006). These studies were in contrast to those of Iglesias et al., who demonstrated that a intraperitoneal injection of AICAR caused a marked increment in net hepatic glycogen breakdown in 5-h-fasted rats (Iglesias *et al.*, 2002). In agreement with Iglesias et al, Pencek and colleagues showed that intraportal AICAR infusion in 18-h-fasted conscious dogs led to an increase in hepatic glucose output in the presence of basal intraportal glucagon, high physiological insulin, and hyperglycemia, euglycemia, or hypoglycemia (Camacho *et al.*, 2005b; Pencek *et al.*, 2005). They were also able to show that the increase in hepatic glucose output in response to AICAR infusion was due to a stimulation of net hepatic glycogenolysis. The latter observation is supported by the finding that AMPK can inactivate glycogen synthase and activate phosphorylase (Polekhina *et al.*, 2003). The differences between these studies and the results by Bergeron et al. can be attributed to differences in hepatic glycogen content. Consistent with differences in hepatic glycogen levels being responsible for these differences is the observation that the stimulation of NHGO in response to an intraportal AICAR infusion was lost when given to a 42-h-fasted dog in which liver glycogen was markedly reduced (Pencek et al., unpublished data). Camacho et al. (Camacho *et al.*, 2005a) speculated that the stimulation of hepatic glucose output by AICAR in 18-h-fasted dogs was due to an off-target drug effect since the increase in glucose output induced by AICAR remained even in the presence of high physiological insulin levels (~15 fold), when AMPK activation was presumably inhibited, as evidenced

by ACC (Ser79) phosphorylation not being different between AICAR treated and saline treated animals. It needs to be noted that the AMPK activity in that study was not measured, and ACC, the index of AMPK activity could be regulated by insulin via an AMPK independent mechanism and that may compromise the conclusion that AMPK was inhibited by elevated insulin (Brownsey *et al.*, 2006). Nevertheless, AMPK plays an important role in the regulation of hepatic glucose production, glucose homeostasis, and development of AMPK activators could become a therapy for the metabolic abnormalities associated with type 2 diabetes (Viollet *et al.*, 2006; Zhang *et al.*, 2009). The current study showed that a reduction in AMPK phosphorylation was associated with an enhancement of NHGU induced by intraportal infusion of ODQ during conditions that mimic the postprandial state. The speculation that inactivation of AMPK in the liver could promote NHGU is supported by a recent study relating to downstream effect of AMPK activation by Mukhtar et al. (Mukhtar *et al.*, 2008). They found that AMPK activation by AICAR inhibited glucose phosphorylation and glucokinase translocation in hepatocytes, which could potentially inhibit glucose uptake. The inhibition of glucose-induced GK translocation by AICAR was associated with phosphorylation of both GKRP and the cytoplasmic glucokinase binding protein, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) (Mukhtar *et al.*, 2008). Whether the inactivation of AMPK is involved in the regulation of NHGU by hepatic NO is at present unclear and needs to be further investigated.

We previously indicated that portal infusion of the NO donor SIN-1 reduced NHGU in the presence of the portal glucose delivery, hyperglycemia and

hyperinsulinemia in conscious dog (An *et al.*, 2008). This result is consistent with the observation that intraportal infusion of NOS inhibitor L-NAME enhanced NHGU under hyperinsulinemic and hyperglycemic conditions, and the augmentation was partially reversed by giving SIN-1 intraportally (Nishizawa *et al.*, 2008). The present results combined with these earlier findings raise the possibility that the hepatic NO/sGC/cGMP action may be responsible for the effect of oral/ portal glucose delivery on NHGU. One may postulate that a pathway involved in the alteration of the NO action in the liver may comprise part of the feeding signal that triggers an increase in hepatic glucose uptake, although the pattern of hepatic NO changes in response to feeding and fasting remain to be established.

In conclusion, we demonstrate for the first time that intraportal infusion of ODQ to inhibit sGC in the liver, increased NHGU and net hepatic carbon retention under hyperglycemic and hyperinsulinemic conditions in conscious 42-h-fasted dogs. It appears that AMPK pathway is involved, although the exact molecular mechanism by which NO/sGC/cGMP brings about its hepatic effect needs to be elucidated. These findings indicate that hepatic NO/sGC/cGMP may play a role in directing the partition of glucose entry the body via the portal vein, and, by extension, an oral glucose load, between the liver and muscle.

Table 5.1: Hormone concentrations during the basal and experimental periods in conscious 42-h-fasted dogs given saline, ODQ or ODQ+SIN-1 infused into the portal vein

Group	Basal Period	Experimental Period	
		Period 1	Period 2
Arterial Plasma Insulin, µU/ml			
SAL	8±1	20±2*	23±2*
ODQ	7±1	20±2*	22±2*
ODSN	6±1	18±2*	21±1*
Hepatic Sinusoidal Insulin, µU/ml			
SAL	23±3	85±12*	83±8*
ODQ	24±5	80±8*	85±10*
ODSN	19±3	89±8*	83±6*
Arterial Plasma Glucagon, pg/ml			
SAL	39±4	39±3	36±4
ODQ	41±4	40±4	38±3
ODSN	33±4	35±4	35±3
Hepatic Sinusoidal Glucagon, pg/ml			
SAL	44±6	52±4	49±5
ODQ	47±7	53±6	54±5
ODSN	40±6	50±3	47±3
Arterial Cortisol, µg/dL			
SAL	4±1	4±1	3±1
ODQ	4±1	4±1	2±1
ODSN	4±1	3±1	3±1

Data are means±S.E.M.; n=10 in SAL and ODQ groups. N=6 in ODSN group.
**= Significant statistical difference (P<0.05) from basal period within the group*
Basal period:-30-0 min and experimental period 1: 0-90 min; period 2: 90-270 min

Table 5.2: Average hepatic arterial, portal, and total hepatic blood flow during the basal and experimental periods in conscious 42-h-fasted dogs given saline, ODQ or ODQ+SIN-1 into the portal vein

Group	Basal Period	Experimental Period	
		Period 1	Period 2
Average Hepatic Arterial Blood Flow, ml/kg/min			
SAL	5.4±0.4	6.4±0.5	7.3±0.8*
ODQ	6.1±0.8	7.8±1.1	8.6±1.0*
ODSN	5.0±0.4	5.0±0.4*	5.7±0.4
Average Hepatic Portal Blood Flow, ml/kg/min			
SAL	24.1±2.0	19.4±1.9*	18.4±1.8*
ODQ	26.4±2.4	20.0±2.2*	20.1±2.0*
ODSN	23.3±3.2	18.3±2.2	19.5±2.3
Average Total Hepatic Blood Flow, ml/kg/min			
SAL	29.5±2.1	25.8±2.1	25.7±2.1
ODQ	32.5±2.8	27.7±3.0	28.7±2.6
ODSN	28.3±3.5	23.3±2.4	25.2±2.5

Data are means±S.E.M.; n=10 in SAL and ODQ groups. N=6 in ODSN group.
** = Significant statistical difference (P<0.05) from basal period within the group*

Table 5.3: Average lactate, glycerol and NEFA concentration and net hepatic balance during the basal and experimental periods in conscious 42-h-fasted dogs given saline, ODQ or ODQ+SIN-1 into the portal vein

		Experimental Period	
Group	**Basal Period**	**Period 1**	**Period 2**
Arterial Blood Lactate, µmol/L			
SAL	506±119	966±72	852±62
ODQ	461±52	1033±144	909±99
ODSN	438±61	848±122	866±124
Net Hepatic Lactate Balance, µmol/kg/min			
SAL	-6.0±1.1	4.8±1.0	3.5±1.1
ODQ	-5.4±1.4	5.3±1.8	3.0±1.2
ODSN	-4.9±0.9	8.0±2.6	6.3±1.9
Arterial Blood Glycerol, µmol/L			
SAL	84±8	38±7	35±6
ODQ	88±9	37±7	29±6
ODSN	74±14	29±7	31±8
Net Hepatic Glycerol Uptake, µmol/kg/min			
SAL	1.2±0.2	0.4±0.1	0.5±0.1
ODQ	1.8±0.2	0.5±0.2	0.6±0.2
ODSN	1.4±0.2	0.5±0.1	0.6±0.2
Arterial Plasma NEFA, µmol/L			
SAL	949±106	154±36	127±29
ODQ	955±96	140±27	146±39
ODSN	883±161	101±21	121±33
Net Hepatic NEFA Uptake, µmol/kg/min			
SAL	2.1±0.4	0.3±0.1	0.3±0.2
ODQ	2.7±0.4	0.3±0.1	0.4±0.2
ODSN	2.1±0.4	0.3±0.1	0.4±0.2

Data are means±S.E.M.; n=10 in SAL and ODQ groups. N=6 in ODSN group.
All values in each group during the experimental period are significantly different (P<0.05) from the basal period. Negative values for balance data indicate net hepatic uptake

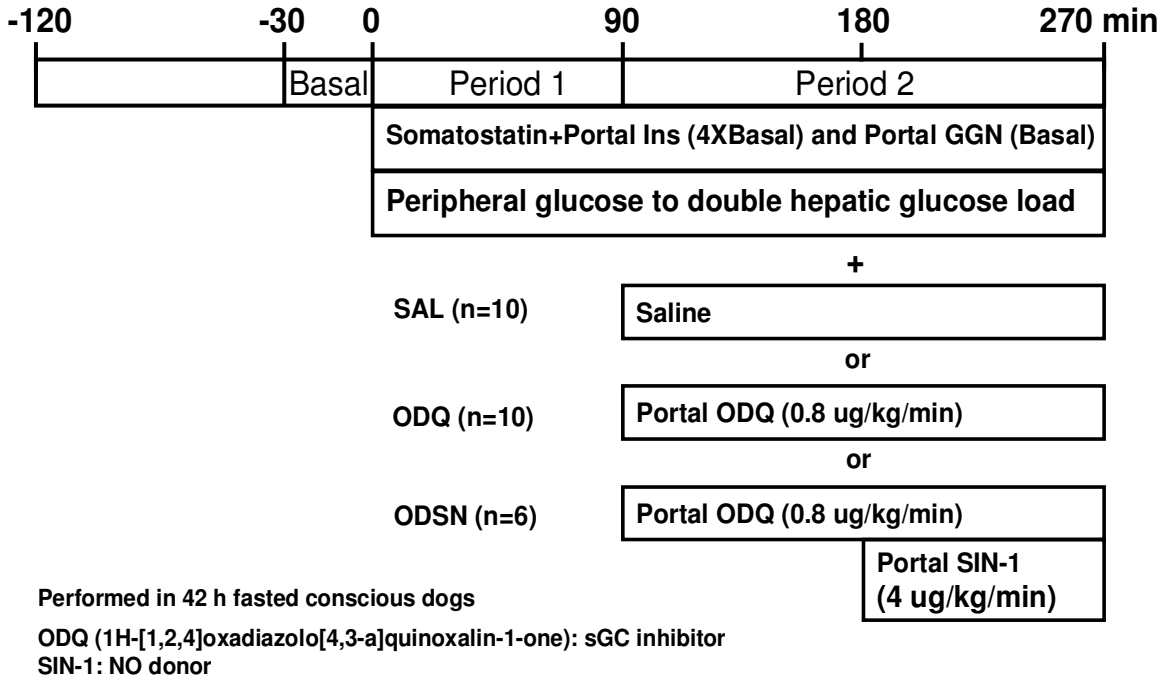

Figure 5.1: Schematic representation of the study. The protocol comprises the basal (-30-0 min) and experimental periods (period 1, 0-90 min; period 2, 90-270 min). Somatostatin was infused peripherally and insulin (4-fold basal) and glucagon (basal) were given intraportally, while glucose was delivered peripherally at a variable rate to increase the hepatic glucose load 2-fold basal during period 1 and period 2. The saline (SAL) group (n=10) received intraportal saline during period 2. ODQ group (n=10), received intraportal ODQ. ODSN group (n=6), received intraportal ODQ during period 2 and SIN-1 in the last 90 min of period 2.

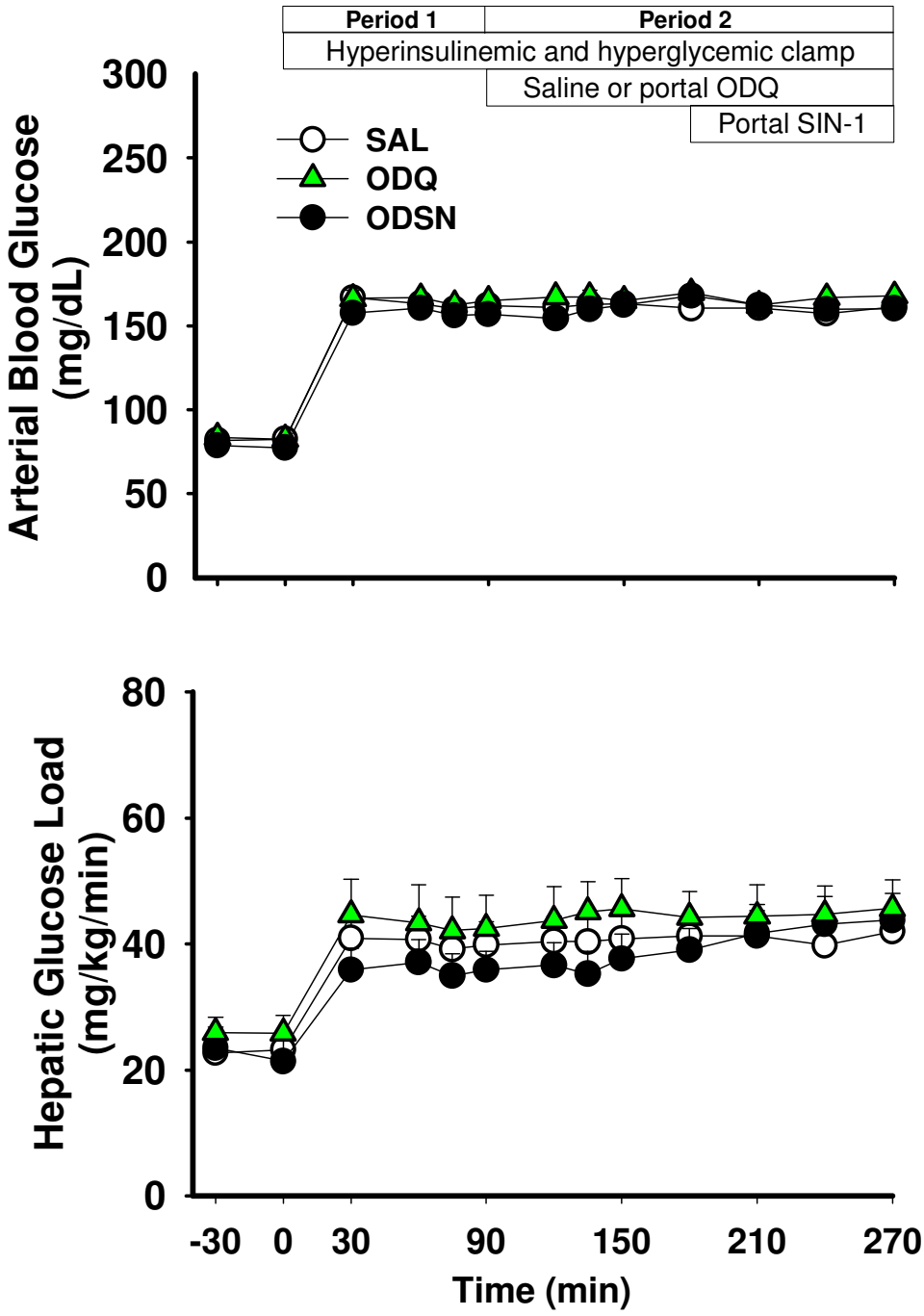

Figure 5.2: Arterial blood glucose and hepatic glucose loads in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 5.1 for description of study conditions. Data are means ± S.E.M.

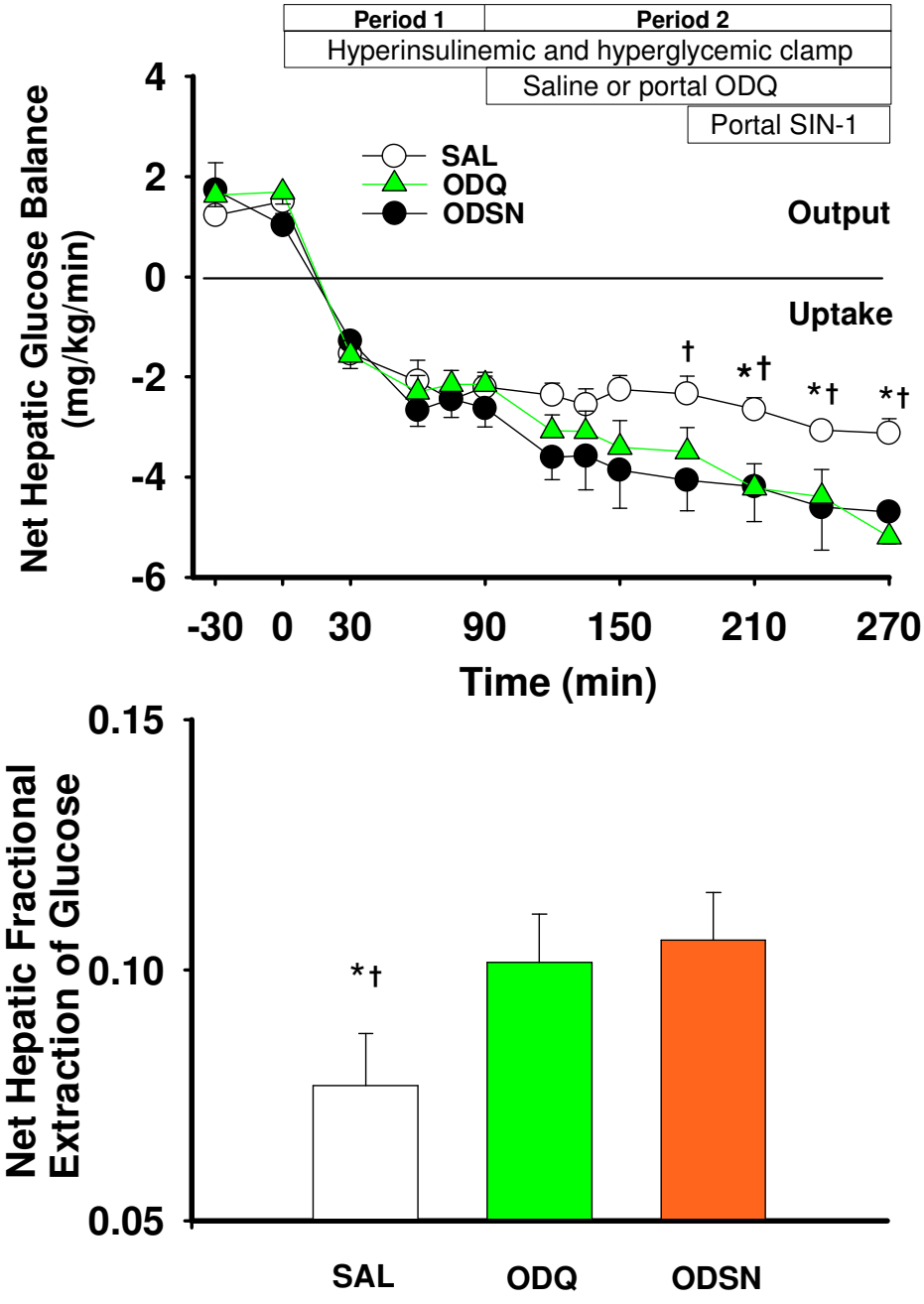

Figure 5.3: Net hepatic glucose uptake and net hepatic fractional extraction of glucose in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 5.1 for description of study conditions. Net hepatic fractional extraction of glucose data represent the averaged values for the last hour in each group. Data are means±S.E.M.; *P<0.05 compared with the ODQ group. †P<0.05 compared with the ODSN group.

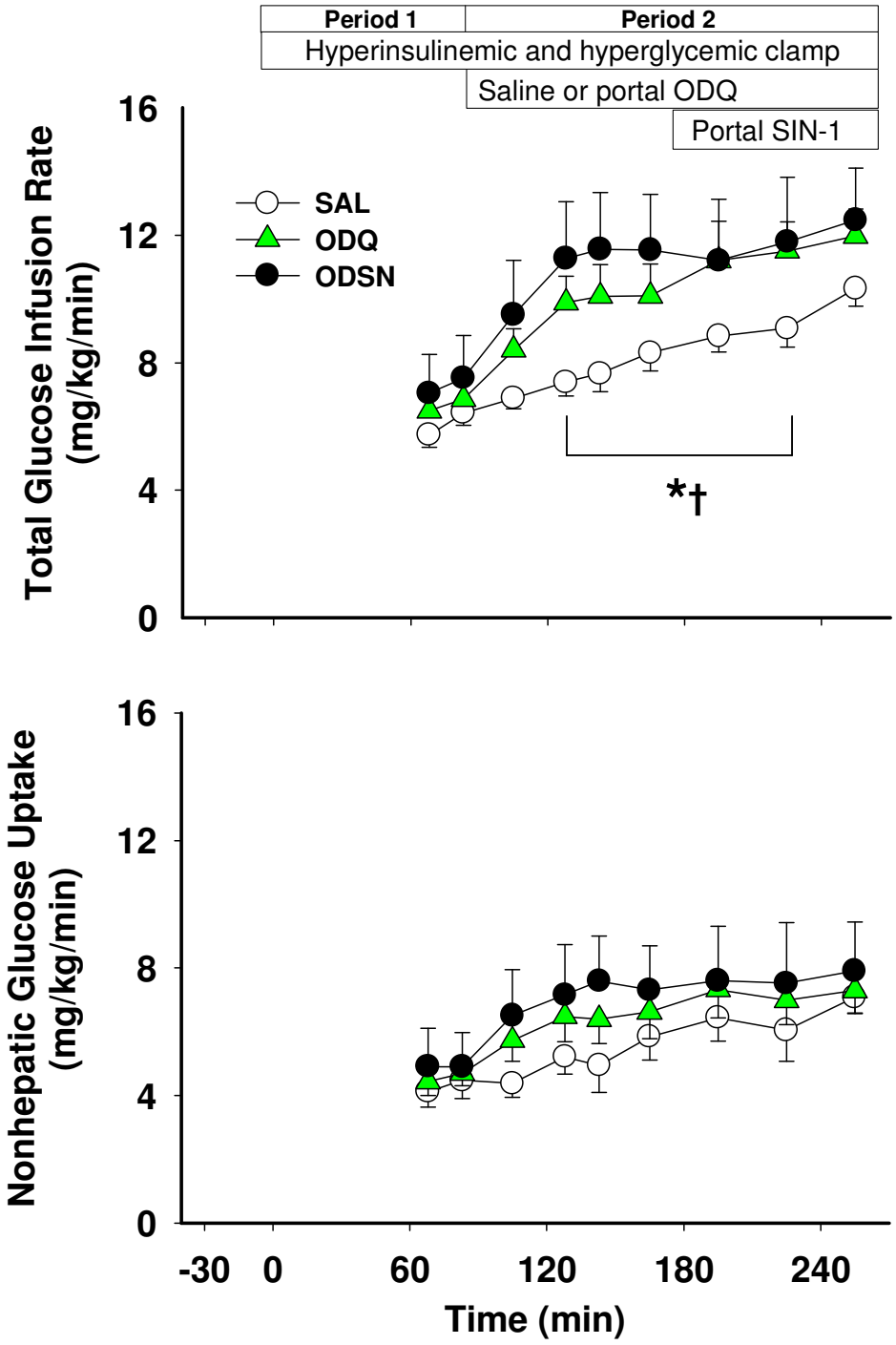

Figure 5.4: Glucose infusion rate and nonhepatic glucose uptake in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 5.1 for description of study conditions. Data are means±S.E.M. *P<0.05 compared with the ODQ group. †P<0.05 compared with the ODSN group.

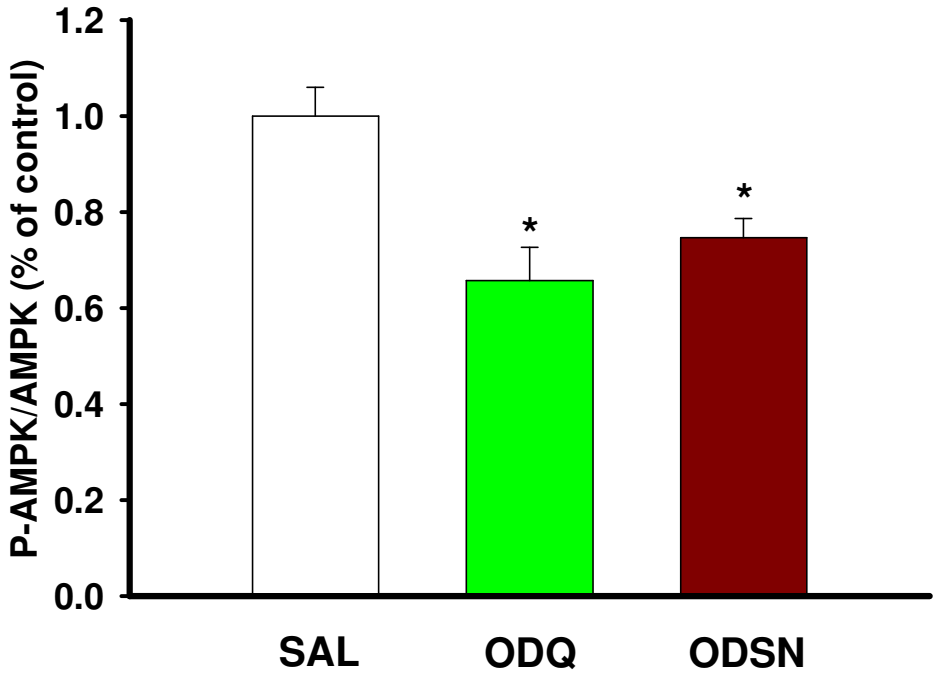

Figure 5.5: ODQ decreases phosphorylation of AMPK at Thr172 and ACC at Ser79 in the liver (biophy taken at the end of the experiments). See Fig. 5.1 for description of study conditions. Data are means±S.E.M.; *P<0.05 compared with SAL group. The blots shown are representative of at least four blots obtained from independent assays.

CHAPTER VI

THE EFFECT OF AN INTRAPORTAL INFUSION OF A CGMP ANALOG 8-BROMO-CGMP ON NET HEPATIC GLUCOSE METABOLISM

Aim

We previously showed that increased hepatic NO resulting from portal SIN-1 infusion inhibits NHGU, and the inhibition of hepatic sGC/cGMP resulting from portal ODQ infusion enhances NHGU. Thus, the aim of the present study was to determine whether increased hepatic cGMP would inhibit NHGU.

Experimental Design

As shown in Fig. 6.1, each experiment consisted of a 90-min equilibration period (-120 to -30 min), a 30-min basal period (-30 to 0 min), and a 270-min experimental period (period 1, 0-90 min; period 2, 90-180 min; period 3, 180-270 min). In all experiments a constant infusion of indocyanine green dye (0.076 mg/min; Sigma Immunochemicals, St. Louis, MO) was initiated at –120 min via the left cephalic vein. At 0 min, a constant infusion of somatostatin (0.8 µg/kg/min; Bachem, Torrance, CA) was begun via the left saphenous vein to suppress endogenous insulin and glucagon secretion. At the same time, basal glucagon (0.57 ng/kg/min; Glucagen, Novo Nordisk, Bagsvaerd, Denmark) and 4-fold basal insulin (1.2 mU/kg/min; Eli Lilly & Co., Indianapolis, IN) infusions were started through the splenic and jejunal catheters and maintained for the duration of the

study. Glucose (20% dextrose) was delivered intraportally at 4mg/kg/min, and PAH was mixed with it to allow a delivery rate of 0.4 mg/kg/min. In addition, a primed continuous infusion of 50% dextrose was begun via the right cephalic vein at time 0 so that the blood glucose could quickly be clamped at the desired hyperglycemic level (~170 mg/dL). In P2, saline was infused intraportally in the control group (SAL, n=10), and a membrane permeable cGMP analog, 8-Bromo-cGMP (Tocris Bioscience, Ellisville, MO) was infused intraportally at 50 µg/kg/min in the CGMP (n=6) groups. In P3, in three dogs from the cGMP group, the peripheral glucose infusion rate was adjusted to maintain a similar arterial glucose to that seen in P1, and in the other three dogs, arterial blood glucose was lowered to compensate for the increase in hepatic blood flow thus allowing the hepatic glucose load (2X basal) to remain matched to that in P1.

Results

Hemodynamic response

Blood pressure did not change significantly over the course of the experiment in response to saline infusion (Table 6.1). The systolic blood pressure in the CGMP/L and CGMP/H groups were similar to those seen in SAL in the basal period and P1 but tended to decrease in P2, and dropped significantly during P3 (Table 6.1). Diastolic blood pressure also fell significantly during P3 in response to intraportal 8-Br-cGMP infusion (Table 6.1). The average heart rate

did not change over time in SAL but it tended to increase during P2 and P3 in the CGMP/L and CGMP/H groups (Table 6.1).

Hepatic blood flow, blood glucose concentrations, and hepatic glucose load

Portal vein blood flow decreased by ~20% in all groups during P1 in response to somatostatin infusion (Table 6.2). There was a concomitant and offsetting (~20%) increase in hepatic arterial flow. As a result, total hepatic blood flow tended to be slightly reduced during P1 in all groups. Hepatic portal blood flow increased by ~30% in response to 8-Br-cGMP during P2 and P3. Consequently, total hepatic blood flow remained near basal during P2 and P3 in the SAL group, while it increased by 27% and 16% in CGMP/L, and by 32% and 31% in CGMP/H during P2 and P3, respectively.

The arterial blood glucose level increased in the all groups from a basal value of 85±2 to 172±2 mg/dL during P1 and P2. It was sustained at that level during P3 in the SAL and CGMP/H groups, while it was allowed to drop to 127±2 mg/dL during P3 in the CGMP/L group (Fig. 6.2). The hepatic glucose loads increased to 2X basal during P1, P2 and P3 in the SAL group, and during P1 in CGMP/L and CGMP/H groups. It increased by ~30% in the CGMP/L group and ~25% in the CGMP/H group, respectively, during P2 because of the increase in portal vein blood flow. The hepatic glucose loads remained elevated during P3 in the CGMP/H group, but returned to the 2X basal in the CGMP/L group during P3 (Fig. 6.2).

Hormone concentrations

The arterial and hepatic sinusoidal insulin levels increased three- to fourfold during P1 and P2 in all groups (Table 6.3). Compared with P1, the elevated sinusoidal insulin levels declined by ~25% in response to 8-Br-cGMP in P2 and P3 (Table 6.2). Arterial plasma glucagon concentrations remained near basal throughout the study, but hepatic sinusoidal glucagon concentrations fell by ~20% in response to 8-Br-cGMP (Table 6.2). The mean plasma cortisol concentrations also remained statistically unchanged from basal in each group (Table 6.2). Norepinephrine (nmol/L) rose to 238±48 and 203±47 ($p<0.05$ v.s. 84±8 in P1, and epinephrine (nmol/L) rose to 161±47 and 216±100 (NS v.s. 136±34 in P1) in response to the hypotention induced by 8-Br-cGMP during P2 and P3, respectively.

Net hepatic glucose balance and net hepatic fractional glucose extraction

All groups exhibited a similar rate of net hepatic glucose output during the basal period (Fig. 6.3). Coincident with the start of the experimental period (4X basal insulin, basal glucagon and hyperglycemia), all groups switched from net output to net uptake of glucose, with the rates (4.8±0.4, 4.3±0.9 and 4.9±0.5 mg/kg/min in SAL, CGMP/L and CGMP/H groups during P1, and 5.4±0.5, 4.7±0.9 and 5.4±0.7 mg/kg/min in SAL, CGMP/L and CGMP/H groups during P2, respectively) not being significantly different between groups (Fig. 6.3). Subsequently, NHGU rose to 5.8±0.5 mg/kg/min in SAL during P3, whereas it decreased to 2.6±1.0 mg/kg/min ($P<0.05$ vs. SAL) in the CGMP/L group, and to

4.7±0.4 mg/kg/min in the CGMP/H group. In response to 8-Br-cGMP during P3, the average rate of NHGU was reduced by ~35% (P<0.05 vs. SAL) (Fig. 6.3). The net hepatic fractional extraction of glucose was 0.09±0.01, 0.10±0.01 and 0.11±0.01 in the SAL group. In response to 8-Br-cGMP, it deceased from 0.08±0.03 to 0.06±0.02 in P2, and 0.05±0.02 in P3 in the CGMP/L group, and from 0.10±0.02 to 0.09±0.01 in P2, and 0.08±0.02 in P3 in the CGMP/H group, respectively (Fig. 6.3). During P3, the change of net hepatic fractional extraction of glucose from baseline was ~0.01 in the SAL group, but was~ -0.03 (P<0.05 v.s. SAL) in response to intraportal 8-Br-cGMP infusion (Fig. 6.3).

Glucose infusion rates, nonhepatic glucose uptake

The glucose infusion rate increased over time in the SAL group but it changed less in the CGMP/H group (Fig. 6.4). In the CGMP/L group, the glucose infusion rate increased over time during P1 and P2, but was significantly decreased over that required in the saline group during period 3. Nevertheless, nonhepatic glucose uptake did not differ significantly among groups at any time (Fig. 6.4).

Lactate metabolism, net hepatic carbon retention and hepatic glycogen content

The arterial blood lactate concentrations rose in all groups during P1 and remained elevated in P2. After the experimental period began, net hepatic lactate balance changed from uptake to output, and the output rates remained elevated in all groups during P2 and P3 (Table 6.4). Net hepatic carbon retention (mg

glucose equivalents/kg/min) did not differ among groups at any time during P1 but decreased in response to 8-Br-cGMP relative to the control group during P3 (2.1±0.8 in CGMP/L [P<0.05 vs. SAL], 4.0±0.2 in CGMP/H, and 5.2±0.3 in SAL group).

Glycogen synthase and phosphorylase in the liver

Compared with the saline group (n=4), intraportal infusion of 8-Br-cGMP (n=6) was associated with a decrease in glycogen synthase activity (GS ratio L/H Glc6P 0.03±0.03 in CGMP v.s.0.11±0.12 in SAL, P<0.05), and no change of glycogen phosphorylase activity (GPh ratio -/+ AMP 0.13±0.15 in CGMP v.s. 0.11±0.14 in SAL) in the liver was detected.

Glycerol and nonesterified fatty acid metabolism

Arterial blood glycerol concentrations and net hepatic glycerol uptake were reduced by 30–70% in response to hyperglycemia and hyperinsulinemia and remained suppressed in all groups throughout P1 and P2 (Table 6.4). The suppression of glycerol was, however, partially reversed during P2 and P3 in response to 8-Br-cGMP but not saline (28±5, 31±6 in SAL and 54±10, 60±13 µmol in response to 8-Br-cGMP [average value of CGMP/L and CGMP/H groups] during P2 and P3, respectively). Arterial plasma nonesterified fatty acid (NEFA) concentrations and net hepatic NEFA uptake changed in a pattern similar to glycerol, decreasing 60-90% during P1 and remaining suppressed in P2 and P3 in all groups (Table 6.4). There was, however, no apparent rebound during P2

and P3 in response to 8-Br-cGMP (Table 6.4).

Discussion

Our earlier experiments showed that an increase in hepatic NO resulting from intraportal infusion of the NO donor SIN-1 inhibited NHGU (An *et al.*, 2008). In addition, the inhibition of hepatic cGMP production by intraportal infusion of the sGC inhibitor ODQ enhanced NHGU and hepatic glycogen accumulation. In the present study, we showed that intraportal infusion of a cGMP analog, 8-Br-cGMP reduced NHGU in the presence of hyperinsulinemia, hyperglycemia, basal glucagon and portal glucose delivery in 42-h-fasted dogs. This finding confirmed our previous data that the cGMP pathway in the liver has the ability to regulate NHGU. Furthermore, this finding and the observation that NHGU was inhibited when SIN-1 was infused intraportally suggest that cGMP dependent pathway is the likely mechanism by which NO regulates NHGU.

In the present study, intraportal infusion of cGMP analog, 8-Br-cGMP, reduced the ability of the liver to take up glucose. Systolic and diastolic blood pressure both decreased ~25% when the 8-Br-cGMP was infused, and this was undoubtedly due to the vasodilatory effects of the compound. The fact that there was an increase in sympathetic drive during 8-Br-cGMP infusion is supported by our findings that the heart rate rose secondary to hypotension, norepinephrine and epinephrine levels increased, and by our observation that lipolysis increased in response to the drug, as indicated by the increase in the arterial blood glycerol level. Increased sympathetic input to the liver would have been expected to

reduce NHGU (Dicostanzo *et al.*, 2006). However, in our previous experiments (An *et al.*, 2008), when SIN-1 was given via a leg vein to bring about a similar hemodynamic response to that seen in the present study, and sympathetic input to the liver was increased by a similar amount, there was no decrease in NHGU or hepatic glycogen synthesis , suggesting that increased sympathetic input to the liver in the present study was not the mechanism responsible for the reduction of NHGU.

At the same time, 8-Br-cGMP significantly increased hepatic portal blood flow by ~30% during P2, while the hepatic arterial flow was not significantly altered. As the liver is a low-resistance organ, hepatic arterial flow is mainly determined by the blood pressure and the resistance at the hepatic artery, whereas hepatic portal flow is mainly determined by resistance at gastrointestinal vessels. The fact that hepatic arterial blood flow remained unchanged in the present study was probably due to the offsetting effects of the decrease in blood pressure and the decrease in hepatic arterial resistance in response to 8-Br-cGMP infusion. Portal blood flow, on the other hand, increased by ~30% probably because the effects of the reduction of the resistance of the gastrointestinal vessels were greater than the drop of the blood pressure. As a result of these vascular effects, the total hepatic blood flow increased by ~25%, the total hepatic glucose load increased, and the hepatic sinusoidal insulin and glucagon levels declined in accord with the change in blood flow, while NHGU was not significantly different among groups during P2. It remains unknown how much difference there is between the effects of increasing the hepatic glucose

load by increasing hepatic blood flow with the glucose level constant and the effects of increasing the glucose level with hepatic blood flow constant (Shiota *et al.*, 2000). In order to minimize the potential metabolic effect of the increase in hepatic glucose load associated with the change of blood flow, in the CGMP/L group, we lowered the arterial glucose levels from ~170 to ~125 mg/dL during P3 to match the level of hepatic glucose load in P1. As a result, the total hepatic glucose load returned to 2X basal, while NHGU was significantly reduced by 55% ($P<0.05$) in the CGMP/L group infusion during P3. NHGU tended to decrease by ~20% in CGMP/H group during P3, when the arterial glucose levels remained at ~170 mg/dL, and the hepatic glucose load was increased due to the change of the blood flow. When the data from two CGMP groups were analyzed together, we found that the NHGU was reduced by 35% ($P<0.05$) in response to 8-Br-cGMP during P3 versus the SAL group, when the total hepatic glucose load was virtually the same. It should be noticed that in period 3, there was a ~25% and ~20% reduction of insulin and glucagon levels in the liver, respectively, in response to 8-Br-cGMP. One may postulate that this reduction of sinusoidal insulin could be partially responsible for the reduction of NHGU. However, the changes in the sinusoidal hormone concentrations were small and the consequences of the decreases in the glucagon and insulin would have been offsetting. It seems unlikely, therefore, that changes in insulin and glucagon mediated the decrease in NHGU caused by 8-Br-cGMP. Thus despite the imperfect of the study, the data support the concept that cGMP decreases NHGU in vivo.

It has been shown that there is crosstalk between cAMP and cGMP signaling pathways (Algara-Suarez *et al.*, 2004). Thus, it is conceivable that 8-Br-cGMP infusion in the present study also activated PKA in the liver, which could have been responsible for the change of NHGU. However, the assay of hepatic glycogen phosphorylase, one of the markers of PKA, was not changed in response to 8-Br-cGMP, suggesting that was not the case.

It has been demonstrated that NO can activate AMPK in endothelium and muscle via a sGC/cGMP dependent pathway (Lira et al., 2007; Zhang et al., 2008), a process that may involve CaM kinase (Zhang *et al.*, 2008). We recently showed that lowering of hepatic cGMP in dogs was associated with a reduction in the AMPK and ACC phosphorylation in the liver. These results suggest that elevated hepatic NO or cGMP may limit NHGU through the activation of AMPK in the liver. This hypothesis is supported by the finding that AMPK can inactivate glycogen synthase and activate phosphorylase (Polekhina *et al.*, 2003), and a recent study which showed that AMPK activation by AICAR inhibits glucokinase translocation and glucose phosphorylation in hepatocytes, as a result of phosphorylation of both GKRP and PFK2 (Mukhtar *et al.*, 2008). These data fit into our current observation that the activity of hepatic glycogen synthase was significantly reduced in response to 8-Br-cGMP.

Evidence has suggested that AMPK plays a role in hepatic glucose metabolism, and could be a therapeutic target for people with type 2 diabetes (Viollet *et al.*, 2006; Zhang *et al.*, 2009). This notion has been supported by a number of studies. Bergeron et al. (Bergeron *et al.*, 2001) showed that systemic

infusion of AICAR, an AMPK activator, suppressed hepatic glucose output in overnight-fasted rats. This finding was supported by an *in vitro* experiment which showed that treatment with an adenovirus expressing a constitutively active form of AMPK alpha2 reduced glucose output in cultured hepatocytes (Foretz *et al.*, 2005), and an *in vivo* observation that liver-specific AMPK alpha2 KO mice had increased fasting hepatic glucose production (Andreelli *et al.*, 2006). It has been shown that activation of AMPK in mouse liver can inhibit hepatic gluconeogenesis and decrease glycogen content (Foretz *et al.*, 2005). It should be noted that the reduction of hepatic glucose output induced by activation of AMPK in above studies was mainly due to the suppression of gluconeogenesis, since the glycogen content of the livers was virtually zero (Bergeron *et al.*, 2001; Foretz *et al.*, 2005; Viollet *et al.*, 2006). On the other hand, when there was glycogen in the liver, Iglesias et al. demonstrated that an intraperitoneal injection of AICAR resulted in a marked increment in net hepatic glycogen breakdown in rats (Iglesias *et al.*, 2002), likewise Pencek et al. showed that intraportal AICAR infusion in 18-h-fasted conscious dogs caused an increase in glycogenolysis and hepatic glucose output (Camacho *et al.*, 2005b; Pencek *et al.*, 2005). Thus, it appears that the different outcomes regarding hepatic glucose output seen in response to the activation of hepatic AMPK in the aforementioned studies can be explained by differences in hepatic glycogen levels, in particular, the balance between the increase in glycogenolysis, if any, and the suppression of hepatic gluconeogenesis. Therefore, it is not surprising to find an association between inactivation of AMPK and increased hepatic glucose uptake and storage in the

present work.

In conclusion, we demonstrated that intraportal infusion of 8-Br-cGMP decreased NHGU under hyperglycemic and hyperinsulinemic conditions in conscious 42-h-fasted dogs. The exact molecular mechanism by which NO/sGC/cGMP brings about its hepatic effect remains to be established. Whether this pathway is involved in the metabolic response to feeding also needs to be determined.

Table 6.1: Heart rate, systolic and diastolic blood pressure during the basal (-30-0 min) and experimental periods (period 1, 0-90 min; period 2, 90-180 min; period 3, 180-240 min) in conscious 42-h-fasted dogs given saline, or 8-Br-cGMP infused into the portal vein

		Experimental Period		
Group	**Basal Period**	**Period 1**	**Period 2**	**Period 3**
Systolic Blood Pressure, mmHg				
SAL	178±5	173±5	168±8	163±10
CGMP/L	175±8	183±10	164±1	127±14*†
CGMP/H	180±1	173±11	170±7	150±10*
Diastolic Blood Pressure, mmHg				
SAL	96±4	94±4	95±6	102±10
CGMP/L	79±3	83±6	73±12	54±5*†
CGMP/H	90±2	84±8	80±7	67±12*†
Heart Rate, beats/min				
SAL	94±5	100±4	94±10	106±9
CGMP/L	91±25	85±23	109±22	115±21
CGMP/H	100±15	86±14	125±17	136±31

Data are means±S.E.M.; n=8 in the saline (SAL) group, n=3 in the group that received 8-Br-cGMP in the portal vein and and arterial glucose was maintained at ~125 mg/dL in period 3(CGMP/L), and n=3 in the group that received 8-Br-cGMP in the portal vein and and arterial glucose was maintained at ~170 mg/dL in period 3 (CGMP/H)

**= Significant statistical difference (P<0.05) from basal period within the group*

†= Significant statistical difference (P<0.05) from SAL group

Table 6.2: Average hepatic arterial, portal, and total hepatic blood flow during the basal and experimental periods in conscious 42-h-fasted dogs given saline, or 8-Br-cGMP infused into the portal vein

		Experimental Period		
Group	**Basal Period**	**Period 1**	**Period 2**	**Period 3**
Average Hepatic Arterial Blood Flow, ml/kg/min				
SAL	6.2±0.6	9.5±0.9*	10.1±1.1*	10.8±1.4*
CGMP/L	4.3±0.2	5.2±0.9†	5.4±0.6†	5.7±0.6
CGMP/H	5.2±1.2	6.4±1.3	5.7±1.2	5.7±1.0
Average Hepatic Portal Blood Flow, ml/kg/min				
SAL	22.2±2.0	18.4±1.5	19.0±1.6	19.2±1.8
CGMP/L	29.7±3.1	22.5±3.2	37.8±4.6†	33.7±5.4†
CGMP/H	21.6±4.5	16.9±4.2	29.7±6.6†	29.4±8.5
Average Total Hepatic Blood Flow, ml/kg/min				
SAL	28.3±2.2	27.9±2.1	29.1±2.4	30.0±2.7
CGMP/L	34.1±4.1	27.7±3.4	43.3±4.9†	39.5±6.0
CGMP/H	26.8±4.7	23.3±4.6	35.4±6.5	35.1±8.4

Data are means±S.E.M.; n=10 in the saline (SAL) group, n=3 in the group that received 8-Br-cGMP in the portal vein and and arterial glucose was maintained at ~125 mg/dL in period 3(CGMP/L), and n=3 in the group that received 8-Br-cGMP in the portal vein and arterial glucose was maintained at ~170 mg/dL in period 3 (CGMP/H)
** = Significant statistical difference (P<0.05) from basal period within the group*
†= Significant statistical difference (P<0.05) from SAL group

Table 6.3: Hormone concentrations during the basal and experimental periods in conscious 42-h-fasted dogs given saline, or 8-Br-cGMP infused into the portal vein

		Experimental Period		
Group	**Basal Period**	**Period 1**	**Period 2**	**Period 3**
Arterial Plasma Insulin, µU/ml				
SAL	8±1	25±3*	25±2*	25±2*
CGMP/L	9±1	22±2*	25±3*	24±4*
CGMP/H	8±2	19±2*	25±2*	25±3*
Hepatic Sinusoidal Insulin, µU/ml				
SAL	21±4	79±8*	85±10*	80±8*
CGMP/L	18±5	89±9*	78±4*	77±4*
CGMP/H	20±7	91±18*	60±11*	60±10*
Arterial Plasma Glucagon, pg/ml				
SAL	45±7	50±4	46±6	46±6
CGMP/L	35±8	37±4	36±4	35±3
CGMP/H	33±5	38±5	39±4	33±5
Hepatic Sinusoidal Glucagon, pg/ml				
SAL	58±7	69±6	65±6	62±7
CGMP/L	43±11	53±7	44±7	39±6
CGMP/H	44±4	55±5	47±5	43±4
Arterial Cortisol, µg/dL				
SAL	3±1	5±1	3±1	4±1
CGMP/L	3±1	3±1	5±1	5±2
CGMP/H	2±1	3±1	4±1	4±1

Data are means±S.E.M.; n=10 in the saline (SAL) group, n=3 in the group that received 8-Br-cGMP in the portal vein and and arterial glucose was maintained at ~125 mg/dL in period 3(CGMP/L), and n=3 in the group that received 8-Br-cGMP in the portal vein and arterial glucose was maintained at ~170 mg/dL in period 3 (CGMP/H)
**= Significant statistical difference (P<0.05) from basal period within the group*

Table 6.4: Average lactate, glycerol and NEFA concentration and net hepatic balance during the basal and experimental periods in conscious 42-h-fasted dogs given saline, or 8-Br-cGMP infused into the portal vein

	Experimental Period			
Group	**Basal Period**	**Period 1**	**Period 2**	**Period 3**
Arterial Blood Lactate, µmol/L				
SAL	412±50	1390±134*	1202±122*	1155±141*
CGMP/L	425±159	1065±183*	1027±175*	1036±184*
CGMP/H	535±179	1135±157*	1030±163*	1151±71*
Net Hepatic Lactate Balance, µmol/kg/min				
SAL	-6.1±0.9	10.0±2.1*	8.4±2.1*	6.6±1.7*
CGMP/L	-5.8±0.3	10.5±3.4*	7.6±2.4*	4.7±1.5*
CGMP/H	-3.2±2.8	10.6±3.8*	6.5±1.4*	8.5±2.1*
Arterial Blood Glycerol, µmol/L				
SAL	99±14	35±5*	28±5*	31±6*
CGMP/L	111±8	41±14*	50±19*	48±15*
CGMP/H	81±12	40±3*	58±2*	73±13
Net Hepatic Glycerol Uptake, µmol/kg/min				
SAL	1.9±0.3	0.7±0.1*	0.6±0.1*	0.7±0.1*
CGMP/L	2.3±0.3	0.7±0.3*	1.5±0.5	1.5±0.5
CGMP/H	1.4±0.2	0.7±0.2*	1.7±0.4	2.0±0.7
Arterial Plasma NEFA, µmol/L				
SAL	1006±78	221±23*	146±17*	131±16*
CGMP/L	1185±65	237±26*	171±42*	145±27*
CGMP/H	950±173	289±74*	324±81*	299±92*
Net Hepatic NEFA Uptake, µmol/kg/min				
SAL	3.8±1.3	0.3±0.1*	0.3±0.1*	0.4±0.1*
CGMP/L	3.6±0.2	0.4±0.1*	0.3±0.3*	0.4±0.4*
CGMP/H	2.7±0.7	0.8±0.4*	1.1±0.3*	0.5±0.9*

Data are means±S.E.M.; n=10 in the saline (SAL) group, n=3 in the group that received 8-Br-cGMP in the portal vein and and arterial glucose was maintained at ~125 mg/dL in period 3(CGMP/L), and n=3 in the group that received 8-Br-cGMP in the portal vein and arterial glucose was maintained at ~170 mg/dL in period 3 (CGMP/H)
** = Significant statistical difference (P<0.05) from basal period within the group*
Negative values for balance data indicate net hepatic uptake

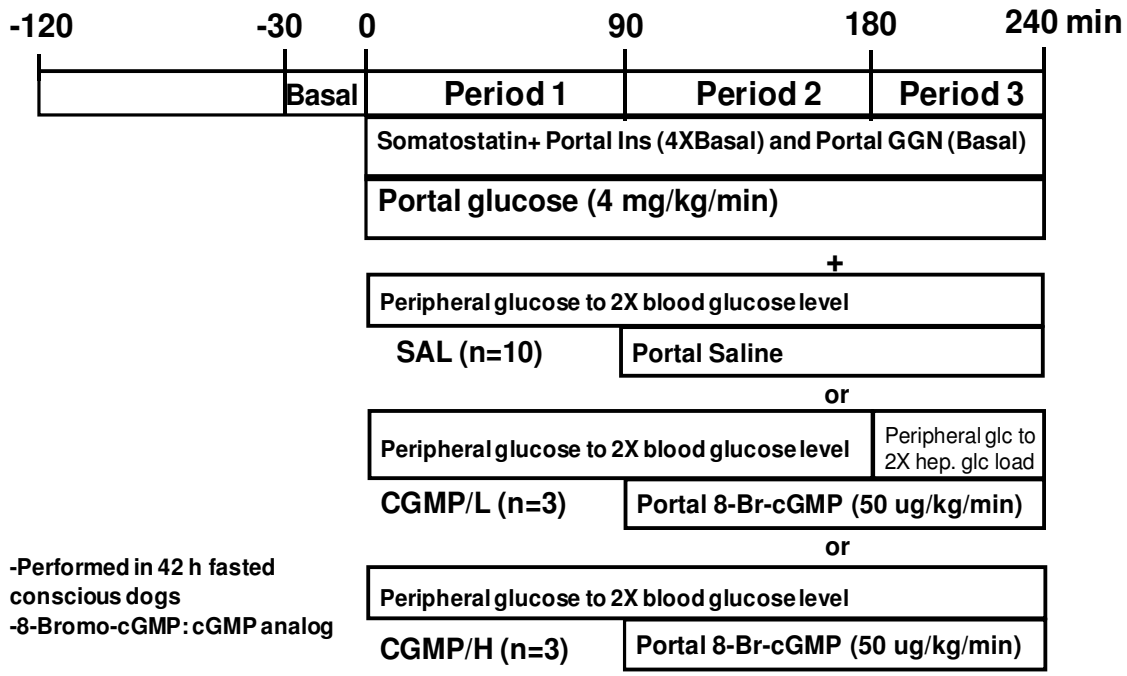

Figure 6.1: Schematic representation of the study. The protocol comprises the basal (-30-0 min) and experimental periods (period 1, 0-90 min; period 2, 90-180 min; period 3, 180-240 min). Somatostatin was infused peripherally and insulin (4-fold basal) and glucagon (basal) were given intraportally, whereas glucose was delivered intraportally (4 mg/kg/min) and peripherally at a variable rate to increase the hepatic glucose load 2-fold basal or arterial blood glucose 2-fold basal during period 1, 2 and 3. The saline (SAL) group (n=10) received intraportal saline during period 2 and 3. Out of six subjects received intraportal 8-Bromo-cGMP at 50 µg/kg/min during period 2 and 3, three had lowered arterial glucose to match the hepatic glucose load to the in period 1(CGMP/L, n=3), and three of them had arterial glucose maintained to that in period 1 (CGMP/H, n=3).

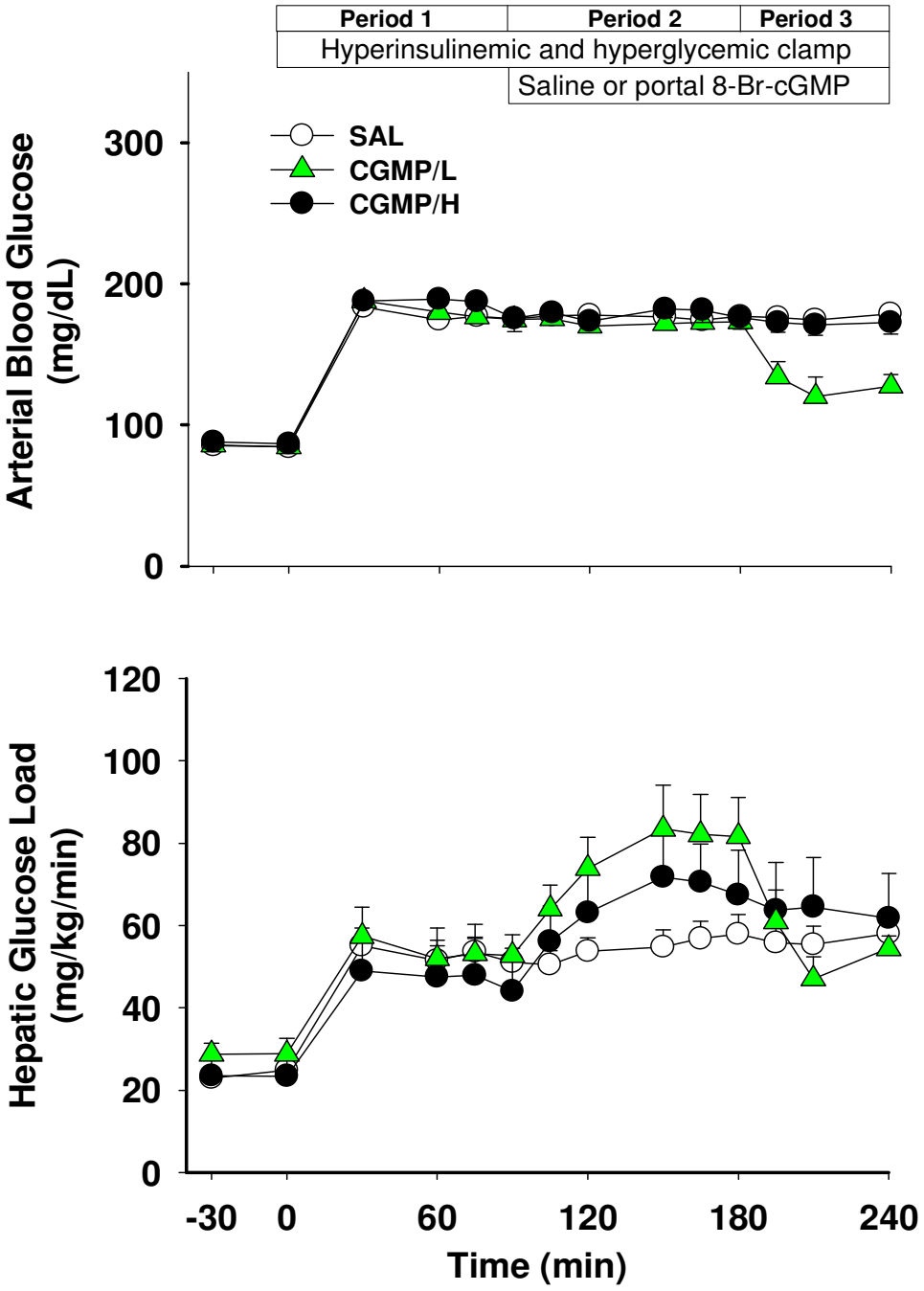

Figure 6.2: Arterial blood glucose and hepatic glucose loads in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 6.1 for description of study conditions. Data are means ± S.E.M.

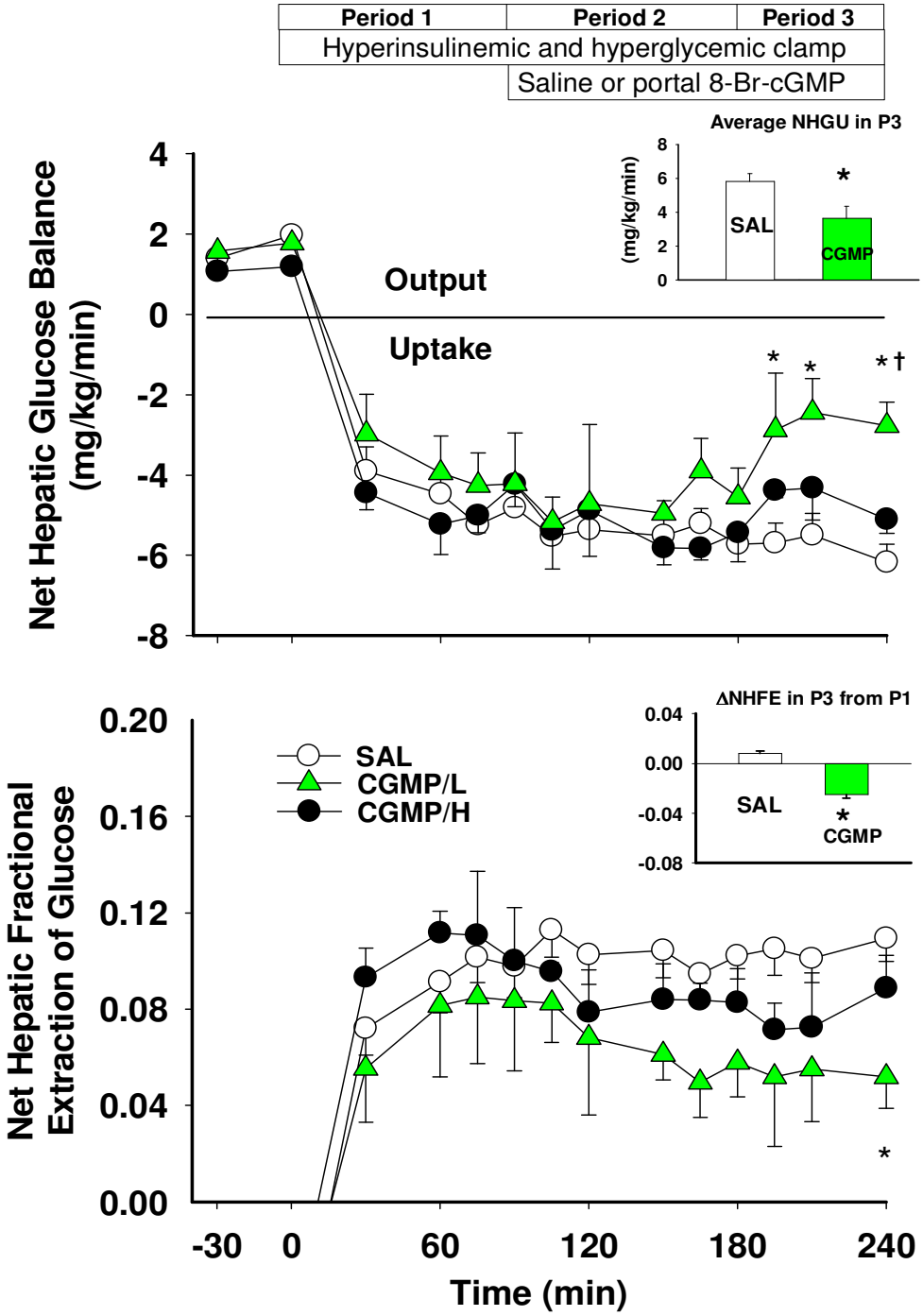

Figure 6.3: Net hepatic glucose uptake and net hepatic fractional extraction of glucose in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 6.1 for description of study conditions. Averaged values during period 3 in the saline group (n=10) and the 8-Br-cGMP treated group (n=6) are expressed as histograms. Data are means±S.E.M.; *P<0.05 compared with SAL group. †P<0.05 compared with CGMP/H group

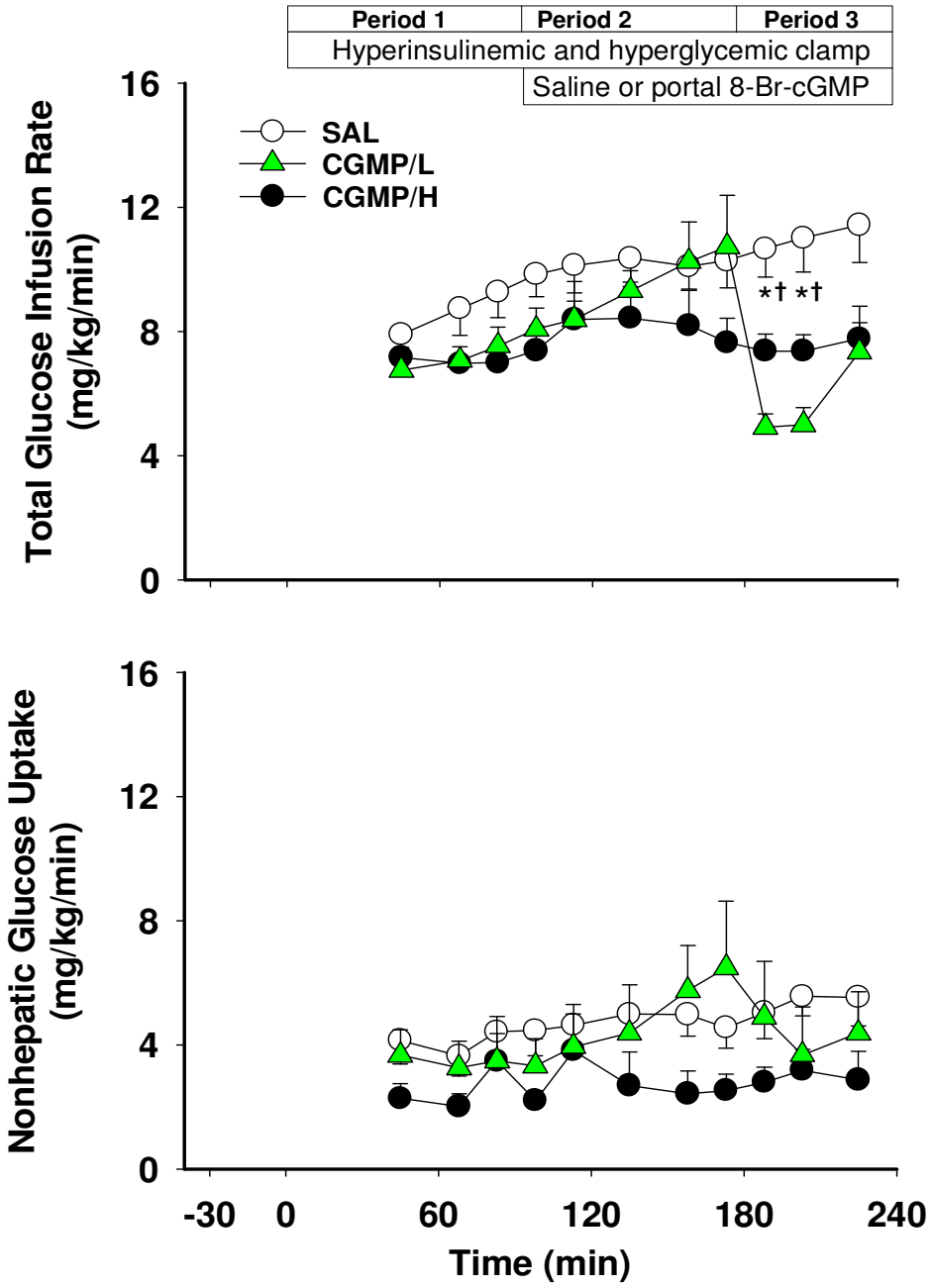

Figure 6.4: Glucose infusion rate and nonhepatic glucose uptake in 42-h-fasted conscious dogs during the basal and experimental periods. See Fig. 6.1 for description of study conditions. Data are means±S.E.M.; *P<0.05 compared with CGMP/L group. †P<0.05 compared with CGMP/H group

CHAPTER VII

SUMMARY AND CONCLUSIONS

The liver plays a critical role in the uptake and storage of glucose after a carbohydrate meal. It has been shown in conscious dogs, that during a continuous intraduodenal glucose infusion, in a net sense, ~53% of the absorbed glucose is taken up by the liver, with the majority being stored as glycogen (Moore *et al.*, 1991). A time course study in humans showed that in response to mixed meal ingestion (three meals during the day, 5 h apart), hepatic glycogen concentration increased continuously throughout the day without any detectable decrease after breakfast or lunch, and peaked at ~4 h after dinner (Hwang *et al.*, 1995). Thus, the postprandial uptake of glucose by the liver, which occurs over 14-16 h of the day, plays an important part in the body's ability to maintain glucose homeostasis.

It has been suggested that hepatic glucose uptake is impaired in individuals with type 2 diabetes (Basu *et al.*, 2000). Likewise, postprandial hepatic glycogen storage is defective in individuals with type 1 (Hwang *et al.*, 1995) and type 2 diabetes (Krssak *et al.*, 2004). Impairments in net hepatic glucose uptake and hepatic glycogen synthesis exacerbate postprandial hyperglycemia, which in turn contributes to kidney and nerve damage, as well as increased risk for the development of cardiovascular disease (Hays *et al.*, 2008). Given the substantial increase in the prevalence of diabetes in recent years

(Spanakis *et al.*, 2009), it is critical to understand how hepatic glucose uptake is normally regulated as well as the cause of dysregulation in this process in insulin resistant individuals.

It is well established that hepatic insulin/glucagon levels, the hepatic glucose concentration/load, and the route of glucose delivery are three major determinants of NHGU (Cherrington, 1999). The role of the latter in the mediation of NHGU has been an area of research for two decades, but the mechanism by which it exerts its effects is still not completely understood.

Our attention turned towards biological mediators, such as NO, and 5-HT, since *in vitro* data suggested that these factors may play a role in the regulation of NHGU and, further, could be involved in the portal glucose signal (Lautt, 2004; Moore *et al.*, 2004b). Although a link between hepatic NO production and the portal glucose signal is still not established, it has been shown that the expression of iNOS in the liver is markedly increased in diabetic rats (~70%) (Madar *et al.*, 2005) and in diabetic mice (~150%) (Fujimoto *et al.*, 2005). These data, along with the observation that the response of the liver to the portal glucose signal is impaired in insulin resistant animals (Coate et al., unpublished data), suggest that increased hepatic NO may be involved in the impaired response to the portal glucose signal in diabetes.

In the first part of my dissertation, intraportal infusion of an NO donor, SIN-1, was shown to reduce NHGU and net hepatic carbon retention under hyperglycemic and hyperinsulinemic conditions in conscious 42-h-fasted dogs. Further, the lack of changes in NHGU and glycogen storage when the same

hemodynamic response was seen in response to peripheral SIN-1 administration suggested that the effects of portal SIN-1 came about as a result of a direct effect of NO on hepatocytes. These findings raise the possibility that hepatic NO plays a physiologic role in the regulation of NHGU.

Our conclusions from specific aim I led us to further investigate the effect of hepatic NO in regulating NHGU. cGMP-independent NO reactions include interaction of NO with superoxide anion to generate peroxynitrite, nitrosylation of cysteine thiol residues and nitration of tyrosine on target proteins. It is well known, however, that NO can activate sGC and thereby increase the production of cGMP. Thus, in the second part of the dissertation, the role of sGC/cGMP in the mediation of NHGU was determined. Intraportal infusion of ODQ to inhibit sGC and the production of cGMP in the liver, resulted in increased NHGU and net hepatic carbon retention under hyperglycemic and hyperinsulinemic conditions in conscious 42-h-fasted dogs. Further, we showed that intraportal infusion of SIN-1 did not attenuate the enhancement of NHGU. At a mechanistic level, we showed that the increase in NHGU was associated with a reduction in phosphorylation of AMPK and ACC. We next showed that intraportal infusion of 8-Br-cGMP, a cGMP analog, decreased NHGU and net hepatic carbon retention under hyperglycemic and hyperinsulinemic conditions in conscious 42-h-fasted dogs. These data suggest that NO can regulate NHGU as a result of the alteration of the sGC/cGMP pathway in the liver.

The findings from the fourth part of the dissertation showed that that intraportal infusion of a SSRI, escitalopram, enhanced net hepatic glucose

uptake and hepatic glycogen deposition under hyperglycemic and hyperinsulinemic conditions in conscious 42-h-fasted dogs. These data suggest that serotonin may play a role in the regulation of NHGU and the portal glucose signal, and raised the possibility that hepatic-targeted SSRIs might help to control postprandial hyperglycemia in individuals with diabetes.

The work presented in this dissertation demonstrates that NHGU can be suppressed by the increase in hepatic NO or cGMP, which resulted from the intraportal infusion of an NO donor SIN-1 or a cGMP analog 8-Br-cGMP, respectively. Conversely, NHGU was enhanced by the decrease in hepatic cGMP resulting from the intraportal infusion of a sGC inhibitor ODQ. It is now established that the expression of nNOS, eNOS and iNOS can be regulated at the transcriptional level under various conditions (Kroncke *et al.*, 2000). For instance, high glucose was shown to decrease iNOS expression and nitric oxide production in cultured smooth muscle cells (Nishio *et al.*, 1996). It has also been shown that NOS activity can be regulated within one minute through phosphorylation under various physiological and pathological conditions such as stimulation of cytokines, hormones, etc. (Schulz *et al.*, 2005). Thus, it is conceivable that under basal condition, there is an inhibitory tone of hepatic NO/cGMP that restrains NHGU, and that this inhibitory signal is rapidly removed in response to portal glucose delivery thereby enhancing NHGU. In addition, we showed that NHGU was enhanced by an increase in hepatic 5-HT which resulted from the intraportal infusion of the SSRI escitalopram. Taken together, these above data give rise to the possibility that portal glucose delivery results in

removal of an inhibitory signal (NO) and the simultaneous augmentation of a stimulatory signal (5-HT), thereby leading to an increase in NHGU. This hypothesis needs to be further investigated. Thus, future experiments are required to determine whether the level of hepatic NO/cGMP changes in response to portal glucose delivery. If so, which isoforms of NOS in the liver are involved should be determined. On the mechanistic level, one direction is to determine the role of PKG in the mediation of NHGU by using introportal delivery of the inhibitor of PKG (cGMP-dependent protein kinase) delivered intraportally. Another direction is to investigate whether there is a cGMP independent pathway that contributes to the metabolic effects caused by hepatic NO. Studies are currently underway to examine the effect of PKG in the regulation of NHGU.

The studies described in this dissertation have a number of important implications. First, the fact that NO/cGMP and 5-HT are natural constituents of hepatocytes, along with the data from this work, suggest that they may comprise part of an endogenous pathway involved in regulation of hepatic glucose uptake *in vivo*. Thus, these data suggest a possible mechanism by which the portal glucose signal may work. Second, since hepatic NOS is upregulated, and portal glucose signal/hepatic glucose uptake is impaired in insulin resistance, understanding the regulation of NHGU by NO may provide insight into mechanisms underlying this disease in the liver. Third, mechanisms that decrease hepatic NO/cGMP may be effective in countering impaired hepatic glucose uptake, and a compound with similar hepatic actions might therefore help to correct postprandial hyperglycemia in individuals with diabetes.

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