Acute Euglycemia Restores the Direct Hepatic Effect of Insulin on Glucose Production (GP) in Depancreatized Diabetic Dogs

by

Neehar Gupta

A thesis submitted in conformity with the requirements for the degree of Master of Science, Graduate Department of Physiology
University of Toronto

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ABSTRACT

Acute Euglycemia Restores the Direct Effect of Insulin on Glucose Production in Depancreatized Diabetic Dogs

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Degree of Master of Science, 1999
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In our previous studies in normal dogs and humans, insulin had both a peripheral extrahepatic effect and a direct effect in suppressing glucose production (GP) whereas the direct effect was undetectable in moderately hyperglycemic diabetic dogs. The purpose of this thesis was to investigate whether euglycemia restores the direct effect of insulin on GP in diabetic dogs. Insulin was infused portally (POR) or peripherally at half the rate (1/2PER) to match the peripheral insulin levels and therefore obtain a selective POR vs. 1/2PER difference in hepatic insulin levels. A greater suppression of GP was seen with POR than 1/2PER during euglycemia. The effect of portal (POR REPL) vs. peripheral basal replacement doses (PER REPL) was also analyzed. To obtain the same basal euglycemia, a greater degree of peripheral insulinemia was required with PER REPL than POR REPL which is also consistent with a direct effect of insulin in suppressing GP. Therefore, acute insulin-induced restoration of euglycemia in diabetic dogs restores the direct hepatic effect of insulin on GP. Our studies show that the physiological route of insulin delivery results in better regulation of GP and in less peripheral hyperinsulinemia than the peripheral route.
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<td>GP</td>
<td>Glucose Production</td>
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<td>CHD</td>
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<td>G6Pase</td>
<td>Glucose-6-Phosphatase</td>
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<td>IP</td>
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<td>STZ</td>
<td>Streptozotocin</td>
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1. **GENERAL INTRODUCTION**

1.1 History

The concept of the ability to change the composition of blood by various organs in the body has been studied ever since Claude Bernard in the mid 1800s. Bernard introduced the idea of internal secretion based on experiments showing that an organ modifies the chemical composition of the blood that passes through it. He showed that the liver can release sugar directly into the blood (1). Ever since the recognition of diabetes mellitus as a disease entity, considerations of interrelations of the pancreas with liver metabolism have occupied a prominent role. In Minkowski’s untreated depancreatized dogs (2), it was shown that in the liver, the glycogen content rapidly became low, the fat content slowly increased and ketone production increased. It was therefore recognized that deranged liver metabolism was associated with symptoms of hyperglycemia. The role of the liver in diabetes was first demonstrated by Kausch (3) in 1893. Kausch demonstrated that hepatectomy led to hypoglycemia both in normal birds and in previously depancreatized birds. This technique was perfected and confirmed by Mann (4,5) in the hepatectomized dog. It became apparent from these studies that the liver is necessary for the most prominent sign of diabetes, namely hyperglycemia. Furthermore, Soskin (6) found that various agents which normally elicit hyperglycemia in the intact dog failed to do so in hepatectomized animals. These studies led to the conclusion that the bulk of blood sugar was manufactured in the liver.

After the discovery of insulin, it was anticipated that insulin would be the central hormone in correcting the abnormalities of the liver associated with diabetes. This was borne out by the experiments which demonstrated that administration of insulin to a
depancreatized animal having a fatty liver, low glycogen levels, and intense ketosis corrected these impairments. Initially, the administration of insulin did not lead to an increase in hepatic glycogen, but in fact led in most instances to hepatic glycogenolysis (7). It was thought that the reason for this was that many commercial brands of insulin contained glucagon, and that the hepatic glycogenolysis following injection of insulin is a result of glucagon and not insulin. This hypothesis was confirmed by Abel who crystallized pure insulin and showed that his preparation did not have any hyperglycemic effect after injection (8).

Once a role for the liver in diabetes was determined, the next question was the interrelation between the liver and insulin. There were some experiments in the early 1930s that showed that insulin could directly inhibit glycogenolysis in the liver. For example, the experiments of Molitor and Pollack (9) on perfused livers, and Seckel (10) on liver slices indicated an inhibition of glycogenolysis. In spite of these reports which indicated a direct inhibitory effect of insulin on net glucose release by the liver, this was still a very controversial topic. It was known at this point that the liver was primarily responsible for glucose production, and therefore it made sense that insulin would be necessary to adequately suppress glucose production following a meal. However, Lundsgaard's laboratory clearly demonstrated that perfused livers of cats and rabbits were non-responsive to insulin added to the perfusion medium (11). Renold et al. (12) performed the same experiments in vitro and found no effect of insulin upon glucose metabolism in liver slices. Contrary to common belief, the author concluded that the effect of insulin on the uptake of sugar by the liver was indirect, but that the insulin action on the periphery was a direct one. In the experiments cited up to the mid 1950s, it appeared that neither insulin nor glucose
could directly affect the metabolism of the isolated liver. In contrast, several investigators reported an action of insulin or glucose administration on the liver metabolism in vivo \( (13,14) \). Holt et al. \((15)\) using \(^{14}\)C- labelled glucose, showed that insulin injected into the tail vein of mice increased the glycogen content of peripheral muscles and decreased the glycogen in liver cells (because of hypoglycemia), whereas infusion of insulin into the portal vein caused a tenfold increase in the liver glycogen content without any change at the periphery. In summary, the bulk of the available evidence at the time pointed towards an indirect effect of insulin on glucose production \((12,16-18)\). Considering the normal physiology of how and where insulin is secreted, the above conclusions needed further exploration.
1.2 The Portal-Peripheral Insulin Gradient

Physiologically, insulin is released from the β cells of the islets of Langerhans of the pancreas, and enters the portal circulation on a direct course to the liver. In the liver, approximately 50% of the insulin is extracted (19), where the remaining 50% exits the liver and enters the peripheral circulation. Upon exiting the liver, this insulin is diluted because of the peripheral circulation’s greater volume than the portal volume (20,21). Therefore, because of hepatic insulin extraction and peripheral dilution of insulin, insulin levels are greater in the portal circulation than in the peripheral circulation, that is, there is a portal-peripheral insulin gradient. There is also a hepatic-peripheral insulin gradient, since approximately 72% of the hepatic vascularization is through the portal vein, and 28% by the hepatic artery (22,23). It is known that the liver is primarily responsible for glucose production, and it was thought that it was required for the liver to be exposed to such high levels of insulin so that hepatic glucose production can be adequately suppressed.

Individuals with type 1 diabetes, who have no endogenous insulin secretion, are treated with subcutaneous injections of insulin, which result in peripheral absorption of insulin. Therefore, with peripheral delivery of insulin, the hepatic insulin levels cannot be greater than the peripheral levels. It is thought that in the absence of a portal-peripheral gradient, peripheral hyperinsulinemia is needed to adequately insulinize the liver, in order to suppress glucose production. However, the liver must be under-insulinized because the doses of insulin which would be required to mimic the normal intraportal levels would induce hypoglycemia because of their peripheral effects (21). There is increasing evidence that peripheral hyperinsulinemia is a risk factor for atherosclerosis (24), and arterial hypertension (25).
1.3 Pathogenesis of atherosclerosis in diabetes and hypertension

The prevalence of atherosclerotic vascular disease is markedly increased among individuals with diabetes mellitus and hypertension. Its major clinical manifestations are consequences of atherosclerosis of coronary arteries, cerebral arteries and large arteries of lower extremities. Thus, atherosclerotic vascular disease is the major cause of mortality and significant morbidity in diabetic and hypertensive people (26). Autopsy studies have repeatedly demonstrated that atherosclerosis in diabetic subjects is more extensive and accelerated. In the Framingham study (27), among diabetic subjects the 20 year incidence of new Coronary Heart Disease (CHD) events was 1.7 times higher in men and 2.7 times higher in women than in non-diabetic people.

Chronic hyperinsulinemia has been associated with the development of cardiovascular disease (CVD) (28-30). Since hyperinsulinemia usually co-exists with insulin resistance (31), it is difficult to distinguish the effect of hyperinsulinemia on the development of CVD from that of insulin resistance. Insulin resistance itself may be associated with CVD. For example, it was determined that the degree of insulin resistance was independently correlated with carotid intima thickness, which is an independent risk factor for CVD (32). Another example is the association between insulin resistance and increased plasma levels of plasminogen activator inhibitor-1 (PAI-1) (33). PAI-1 is a risk factor for CVD because of its ability to enhance blood coagulation by suppressing fibrinolysis. PAI-1 levels are also increased by hyperinsulinemia (34).

There may be a direct relationship between hyperinsulinemia and atherosclerosis (35). The pathogenesis of atherosclerosis consists of a number of stages that begin with the appearance of fatty streaks on the sub-endothelial layer of a blood vessel and end with severe
encroachment of the lumen. Cytokines and growth factors that are produced from injured endothelial cells, platelets and foam cells cause migration of smooth muscle cells from the medial layer of the vessel into the intima (36) thus contributing to the formation of the atherosclerotic plaque and to a reduction in the diameter of the lumen. The reduction in vessel diameter due to plaque formation causes local blood flow to become more turbulent, thus leading to a greater deposition of platelets (36). Additionally, the plaque itself is rather unstable, and if ruptured or site of thrombus formation, might shut off blood flow downstream completely, leading to tissue death.

It has been shown that insulin may independently promote plaque formation. For example, in response to chronic insulin treatment, increased lipid synthesis has been observed in arterial tissue (37). Insulin has also been known to stimulate the development of fatty streaks (38) and to cause thickening of the arterial wall in experimental animals (39). Vascular smooth muscles have receptors for insulin (40). When insulin binds to smooth muscle cells, it causes them to proliferate (35). In addition, receptors for insulin have been found on endothelial cells cultured from bovine pulmonary vessels, aorta and fat capillaries, and on endothelial cells of human artery endothelium and umbilical vein (41). However, the effect of insulin on endothelial function may be protective against atherosclerosis by being counter-atherogenic (42).

Apart from atherosclerosis, it has been suggested that hyperinsulinemia and insulin resistance are closely linked with essential hypertension (28,43). More direct support for a causative link between insulin and hypertension is provided by the observation that a sustained insulin infusion for weeks induced hypertension (44,45) and that interruption of the insulin infusion reversed the elevated blood pressure to normal level in rats.
Insulin has been shown to increase renal sodium retention and stimulate sympathetic nervous system activity which leads to constriction of blood vessels and increased cardiac output (46). These factors all contribute to an increase in blood pressure which is also a risk factor of CVD (41). However, insulin also has vasodilatory properties, and therefore, it is the combination of hyperinsulinemia and insulin resistance which may the most harmful in hypertension as it is in CVD.
1.4 Recent Studies

The direct effect of insulin has long been a controversial issue (16). The direct hepatic action of insulin had been disclaimed by most investigators since a number of studies in vitro had failed to demonstrate clearly a consistent and reproducible effect of insulin upon hepatic glucose metabolism (12,47,48). After 1960, many studies examined whether the initial passage of insulin through the liver altered its subsequent metabolic activity qualitatively or quantitatively by comparing the effect of an equal dose of insulin administered via the portal vein or via a peripheral vein on the magnitude of the hypoglycemia and the magnitude of the change in peripheral glucose utilization.

In some of these studies, a direct effect of insulin was found (49-52). In one study (50), insulin was infused at two different rates in diabetic dogs, either intraportally or peripherally. At the high rate (0.05 U.kg\(^{-1}\).h\(^{-1}\)), GP was reduced to normal range by both the peripheral and portal route. However, at the low insulin infusion rate (0.006 U.kg\(^{-1}\).h\(^{-1}\)), intraportal insulin infusion suppressed GP to 4 mg.kg\(^{-1}\).min\(^{-1}\) (basal = 6.2 mg.kg\(^{-1}\).min\(^{-1}\)), whereas peripheral infusion at this rate had no significant effect. Brown et al. (51) demonstrated the complete reversal of diabetes when the venous drainage from fetal pancreatic transplants was diverted to the portal circulation, but not when the venous drainage was into the systemic circulation. Goriya et al. (52,53), using open-loop insulin infusion device in pancreatectomized dogs, observed that less insulin was necessary to maintain a normal fasting blood glucose when it was infused into the portal system compared with a peripheral vein.

There have also been studies that have found no practical advantage of portal venous over peripheral venous administration of insulin, which is consistent with an absent or
minimal direct effect of insulin on hepatic glucose metabolism (54). Rizza et al. (55) hypothesized that a lack of a differential effect between portal and peripherally administered insulin on hepatic glucose metabolism may be due to maximal insulin levels. In their study, they wished to determine whether the disposal of a mixed meal differed when insulin was given via the portal venous as compared with a peripheral venous route by a closed-loop infusion system (artificial pancreas). Insulin was administered portally until euglycemia was achieved and maintained for 2 hours. At this point, a standard liquid meal was given. They showed that glucose production and glucose utilization were the same with portal or peripheral insulin infusion. However, the use of the artificial pancreas at that time was associated with over-insulinization.

In 1987, the general concept that the insulin level within the hepatic sinusoids was responsible for the hormone's inhibitory effect of the liver was re-examined. It was noted that in obese non-diabetic humans (56), suppression of glucose production could occur in response to insulin infusion even when the estimated portal insulin concentrations did not rise. Insulin was infused into a peripheral vein and euglycemia was maintained with a glucose infusion. Endogenous insulin secretion, estimated from C-peptide concentrations, decreased by about 50% over the course of the experiment. Based on the fall in endogenous insulin release and the prevailing peripheral insulin concentration, the authors estimated that the portal vein insulin concentration probably remained unchanged. Nevertheless glucose production was suppressed by about 80% at the end of their study. This study was of importance because it was the first recent study to focus attention on the indirect effect of insulin to inhibit glucose production by the liver.
However, there is a problem in the interpretation of previous comparisons of intraportal vs. systemic insulin administration. In earlier studies, insulin was infused at an equal rate via either route \((21,49,50,54,57,58)\). Because about half the insulin infused intraportally is extracted during its first pass through the liver, with intraportal infusion systemic insulin levels were much lower, and portal insulin levels were likely much higher than with equidose systemic infusion. The failure to match either intraportal or systemic concentrations clouds direct comparison between intraportal vs. matched systemic hormone infusion. A study was performed by Ader and Bergman \((59)\) to clarify the importance of the direct vs. the indirect effects of insulin vis-à-vis suppression of hepatic glucose production in normal dogs. Insulin was infused intraportally or peripherally. However, in this case, insulin was infused intraportally at rates exactly double those given peripherally. Because about half of the insulin is extracted by the liver, this design was able to match systemic insulin levels despite varying portal-peripheral insulin gradients. This was the first study to match peripheral insulin levels with both portal and peripheral insulin infusions. In their experiments, Ader and Bergman infused somatostatin to suppress endogenous insulin and glucagon secretion and infused fixed doses of glucagon. Euglycemia was maintained by glucose infusion. In this model GP was found to be suppressed proportionally to peripheral but not hepatic insulin levels.

To examine whether insulin's peripheral effect is independent of the prevailing glucagon levels \((60)\), euglycemic glucose clamps with somatostatin were performed in conscious normal dogs where insulin was infused portally or peripherally at half the portal infusion rate, and glucagon was not replaced. It was found that the effect of insulin to suppress hepatic glucose production under fasting conditions was determined predominantly
by systemic rather than portal insulin concentrations as seen in Ader and Bergman’s study (59).

Our laboratory conducted a similar protocol on healthy normal dogs at both basal and suprabasal insulin levels (61). Insulin was infused through the portal intravenous route (POR). The same insulin dose as infused portally was infused in the periperial circulation (PER). The calculated hepatic insulin levels were greater with the POR treatment than the PER treatment as with the PER treatment insulin is diluted in the systemic circulation before reaching the liver. The peripheral insulin levels however were much higher with the PER treatment than the POR treatment as a large portion of insulin in the POR treatment is extracted by the liver before reaching the peripheral circulation (19). Unfortunately, with the above two protocols, it is not possible to separately investigate either the hepatic or the peripheral effect of insulin on GP, since, as described above, with the same dose of insulin given peripherally or portally neither the peripheral nor the portal insulin levels are equal. To match the peripheral insulin levels, a third treatment was performed. Insulin was infused peripherally at a dose equal to half the dose infused portally, ie. insulin was infused at a dose equal to the amount of insulin exiting the liver during the POR treatment, due to 50% hepatic insulin extraction. Therefore, the dose of the third treatment (1/2PER) was equal to 50% of the POR (and PER, because the doses of POR and PER are the same) treatment. The 1/2PER treatment resulted in equal peripheral insulin levels with those of the POR treatment, but the calculated hepatic insulin levels were much lower when compared to the POR treatment. With different hepatic insulin levels, any differences in glucose production can be attributed to this difference in insulin. The insulin infusion rates to obtain basal insulin levels were 0.125mU/kg.min portally (POR) or peripherally (PER), or 0.0625 mU/kg.min
peripherally (1/2PER). The insulin infusion rates to obtain suprabasal insulin levels were 0.45 mU/kg.min portally or 0.225 mU/kg.min peripherally.

In the normal dogs at both basal and suprabasal insulin levels, PER suppressed GP more than POR or 1/2PER. Since peripheral levels of insulin were greater in PER despite lower hepatic insulin levels, this is consistent with an indirect effect of insulin in suppressing GP. However, in the normal dogs at both basal and suprabasal insulin levels, POR did suppress GP more than the 1/2PER group. Since calculated hepatic insulin levels were greater with POR while peripheral levels were matched, this is consistent with a direct effect of insulin on the liver in suppressing GP. The direct effect of insulin appeared to be greater at low insulin levels.

This is consistent with a collaborative study of our laboratory with Dr. Lewis (62) in which a similar protocol was conducted in normal humans. Portal hyperinsulinemia (portal study) was produced using a programmed intravenous tolbutamide infusion algorithm (63,64). The reason for this is that portal cannulation is too invasive to be performed in humans. Tolbutamide is a substance that can be delivered peripherally and stimulate the pancreas to secrete insulin into the portal vein. This way, insulin can be secreted at a constant rate, and this rate can be matched with a full-dose peripheral insulin dose. In a second treatment, full-dose exogenous insulin was delivered through a peripheral vein. Lastly, a third treatment was set up in order to match peripheral insulin levels by delivering insulin peripherally at half the rate of the portal study. It was found that GP was suppressed to the greatest extent with full dose peripheral insulin infusion (PER). Tolbutamide stimulated insulin delivery (POR) was the second most effective treatment in suppressing GP.
and half-peripheral insulin delivery (1/2PER) was the least effective. These are the same results as found in normal dogs (61).

Further evidence of a direct effect of insulin on hepatic glucose production was found by Maheux et al. (65) in normal humans. Their data showed that portal insulin can act directly on the liver to inhibit GP independent of an increase in peripheral plasma insulin concentration.

In all of the above studies, whether insulin was given via a peripheral vein or the portal vein, both the systemic and liver sinusoid insulin concentrations rose concurrently. Cherrington’s group was interested in directly assessing the effect of a selective rise in systemic insulin (brought about in the absence of a change in portal insulin) or portal insulin (brought about in the absence of a change in systemic insulin) on GP in the conscious overnight fasted normal dog (66). The selective rise in systemic insulin was accomplished by administering insulin portally for a 40 min. control period and then stopping the portal infusion and giving insulin into a leg vein at twice the portal vein infusion rate. With this technique, the arterial (peripheral) insulin concentration increased dramatically but the portal vein insulin concentrations were kept constant. Their data confirmed the ability of arterial insulin to inhibit GP. The selective rise in portal insulin was achieved by increasing the portal vein insulin concentration in the absence of any change in the arterial plasma insulin level. Unlike in the previous protocol, a combination of peripheral and portal insulin was administered. After a 40-minute control period, the peripheral insulin infusion was turned off and the portal insulin infusion rate was increased. It was shown that there was no decrease in hepatic gluconeogenesis despite a suppression of GP. From these observations, it was concluded that the change in GP reflected a decrease in glycogenolysis.
Another study was performed by the same group where the hepatic effects of a selective increase in hepatic sinusoidal insulin brought about by insulin infusion into the hepatic artery was compared with those resulting from insulin infusion into the portal vein (67). It was found that GP decreases in response to a selective increase in hepatic sinusoidal insulin, regardless of whether it comes about because of administration of insulin in the hepatic artery or portal vein.

A recent study done by our lab and Dr. Vranic’s laboratory (68) addressed the question whether in diabetic dogs, the inhibition of GP is primarily dependent upon hepatic or peripheral insulin levels, and therefore is due to insulin’s direct or indirect effect on glucose production.

It is important to study a diabetic model if one wants to address the therapeutic question of the effects of subcutaneous insulin treatment of diabetes. Diabetic people lack endogenous insulin secretion and therefore the normal portal-peripheral insulin gradient is abolished. Insulin must be administered subcutaneously and it is absorbed into the peripheral circulation. However, with peripheral absorption, the levels of insulin in the portal vein can never be greater than those in the peripheral circulation and therefore a portal-peripheral insulin gradient cannot be set up as is in normal physiology. To investigate whether this non-physiological route of insulin delivery has dramatic effects on insulin action, the diabetic model must be studied. In this study, the depancreatized dog was used as a model of insulin-dependent diabetes mellitus. This is a good model for type 1 diabetes because lacking a pancreas, the dog is not able to secrete any insulin. With respect to glucagon, despite the fact that there is no pancreas, and therefore no pancreatic α cells, the gastric mucosa is able to secrete glucagon (69). High physiological doses of insulin
(0.9mU/kg.min), comparable to those secreted during the post-prandial period were infused via the portal intravenous route (POR). To compare the effects of the portal route of insulin administration to that of the peripheral insulin delivery as in the treatment of diabetes, the same insulin dose as infused portally was infused in the peripheral circulation (PER) since insulin is absorbed peripherally after subcutaneous injection. A third treatment was set up with insulin being delivered at half the portal rate, peripherally (1/2PER) to match peripheral insulin levels.

It was found that GP was suppressed more with PER than equidose portal (POR) insulin infusion. 1/2PER insulin infusions resulting in the same peripheral insulin levels as POR suppressed GP equally, despite the difference in hepatic insulin levels. Thus, it was found that the suppression of GP was proportional not to hepatic but peripheral insulin levels. These observations refer to depancreatized diabetic dogs under conditions of moderate hyperglycemia. In these dogs, the results of Giacca et al. were similar to those obtained by Ader and Bergman in normal dogs.

The greater suppression of GP by the PER treatment was accompanied by a larger decrease in the gluconeogenic substrates alanine and glycerol, as well as free fatty acids (FFA). Consistent with the higher peripheral insulin levels obtained with the PER treatment than the POR and 1/2PER treatments, peripheral glucagon levels declined more with peripheral insulin infusion than portal or half-dose peripheral infusions.

In the previous study by Giacca and Vranic the insulin levels were in the high physiological range. It is possible that in all three treatments the direct effect of insulin on GP was maximal and so further suppression of GP could only be achieved by insulin’s indirect effect. By lowering the insulin level, a direct effect of insulin could be unmasked.
There are contrasting reports in the literature regarding this point. At basal insulin levels and unclamped (declining) glucose levels, a direct effect of insulin on GP could be detectable in one study in diabetic dogs, with rapid saturation at increasing insulin doses (50). However, in Ader and Bergman's study, also carried out at basal insulin levels but in normal dogs, no direct action of insulin was detected during a euglycemic clamp. In this latter study with low insulinization, the confounding effects of somatostatin, used to inhibit insulin secretion in normal dogs, might have been predominant (59).

Therefore our laboratory performed a study under conditions of low insulinization (0.125 mU/kg.min) in diabetic depancreatized dogs (70). The hypothesis was that now because the direct effect of insulin on hepatic glucose production is not maximal, this direct effect proportional to the hepatic insulin levels will add to the indirect effect, proportional to the peripheral insulin levels. The experiment was designed to measure the insulin-induced suppression of GP by comparing portal and peripheral insulin infusions resulting in levels of peripheral venous insulin found in the fasting state (0.125 mU/kg.min PER and POR and 0.0625 mU/kg.min 1/2PER).

In this study in the diabetic depancreatized dogs under conditions of moderate hyperglycemia and basal insulin levels, it was found that the suppression of GP was proportional to peripheral insulin levels, not to hepatic insulin levels. GP was suppressed to a greater extent with PER insulin infusion than equidose POR infusion. The POR and 1/2PER infusions were equally potent in suppressing GP. Therefore, despite the insulin levels being lowered to the post-absorptive range, this did not unmask the direct hepatic effect of insulin on glucose production.
To determine whether the diabetic state reduces the direct effect of insulin in humans, nine patients with diet treated type 2 diabetes and moderate hyperglycemia were studied in collaboration with Dr. Lewis (71). In contrast with our studies in non-diabetic individuals, glucose production was not more suppressed at steady state in POR versus 1/2PER despite much higher hepatic insulin levels in POR. Therefore, this was the first study to show hepatic resistance to the acute direct suppressive effect on insulin of hepatic glucose production in patients with type 2 diabetes.

Therefore, in our previous studies in normal dogs and humans (61,62), insulin had both a peripheral indirect and a direct effect in suppressing glucose production. The direct effect was undetectable in our studies in moderately hyperglycemic (~10mM) depancreatized dogs (68,70) and type 2 diabetes patients (71). We hypothesized that this is due to hepatic insulin resistance and/or hyperglycemia.
1.5 Mechanisms of the direct and indirect effects of insulin on GP

With knowledge of a direct and indirect effect of insulin on glucose production, the next question was the mechanism whereby glucose production is suppressed.

Insulin can inhibit hepatic glycogenolysis (72,73) and gluconeogenesis, and promote glycogen formation by directly controlling some key regulatory enzymes in the liver. For this reason, it is thought that suppression of hepatic glucose production can be achieved through a direct hepatic effect (74). Once insulin binds to its hepatic insulin receptor, through a series of phosphorylations, it activates protein phosphatase-1, which then dephosphorylates glycogen phosphorylase, inactivating it, and glycogen synthase, activating it. Furthermore, protein kinase B becomes activated, inactivating glycogen synthase kinase 3, which prevents glycogen synthase from being turned off. These series of phosphorylations and dephosphorylations allows glucose to be stored as glycogen, through the action of glycogen synthase and by inactivating glycogen phosphorylase, prevents glucose from being formed, thereby suppressing glucose production.

Insulin has additional short term effects to those mentioned above. The effect of insulin on hepatic glucose production has been studied in anesthetized rats in the post-absorptive state (75). It has been shown that insulin increases the level of fructose 2,6-bisphosphate, via an increase in the Vmax of 6-phosphofructo-2-kinase and concomitantly decreased activity of fructose-2,6-bisphosphatase, resulting in a 5-fold increase in the ratio of kinase/phosphatase. Fructose 2,6-bisphosphate when formed from elevated levels of fructose 6-phosphate, activates phosphofructokinase in the liver and drives glycolysis while suppressing fructose 1-6-bisphosphatase and gluconeogenesis (76).
Insulin also has long term effects on the gene expression of a number of hepatic enzymes involved in glucose metabolism (77). The role of insulin in the regulation of glucokinase (GK) activity is largely at the transcriptional level. Specifically, insulin causes a prompt increase in the rate of GK gene transcription in vivo (78,79) and in vitro (80). The mechanism underlying the promotion of GK gene transcription by insulin appears to be via the phosphorylation or dephosphorylation of one or more transcription factors that alters either their function or DNA binding (77). Barzilai and Rossetti (81) examined the effect of chronic insulin deficiency followed by acute insulin replacement on hepatic GK mRNA and Vmax. Vmax (the Michaelis-Menten constant) is defined as the concentration of substrate when the reaction rate is 1/2 of the maximum reaction rate Vmax. The maximum reaction rate is achieved by saturating the enzyme with its substrate. Chronic insulin deficiency (6-weeks) following 90% pancreatectomy in rats resulted in a 2-fold decrease in hepatic GK mRNA and Vmax and the acute administration of insulin resulted in a restoration of both parameters. These data suggest that insulin has the ability to up-regulate glucokinase activity by increasing GK gene expression, however further studies are required to evaluate whether insulin also has short term effects of GK activity, independent of GK gene expression.

Glucose-6-phosphatase (G6Pase) represents another target for the direct regulation of hepatic glucose production by insulin as shown in a report by Gardner et al (82). Specifically, these authors demonstrated that increases in insulin under euglycemic conditions in vivo inhibit flux through G6Pase coupled with a modest decline in cytosolic glucose-6-phosphate (G6P) concentration. This implies that the suppressed flux through G6Pase is not a result of a decrease in the amount of substrate, but rather that insulin does exert a direct inhibitory effect on G6Pase. The mRNA level of the G6Pase catalytic subunit
has been shown to be increased in insulin deficiency (83,84) thereby indicating that insulin regulates G6Pase gene transcription. The mechanism by which insulin suppresses G6Pase gene transcription was addressed recently by Streeper et al (85). Using mouse G6Pase-chloramphenicol acetyltransferase (CAT) fusion genes transiently expressed in hepatoma cells, these authors discovered that deletion of the G6Pase promoter sequence between 271 and 159 completely abolished the suppressive action of insulin on G6Pase gene transcription. They went on to describe the presence of a multicomponent insulin response sequence in this region. Similar to GK, it is unclear whether insulin also has short term effects on G6Pase.

Apart from G6Pase, another important gluconeogenic enzyme is phosphoenolpyruvate carboxykinase (PEPCK). PEPCK is a key enzyme in the synthesis of glucose in the liver and kidney. It has been shown that insulin also inhibits the gene expression of PEPCK by reducing mRNA levels (86).

The effects of insulin on the gene expression of the enzymes involved in glycogen metabolism (glycogen synthase and glycogen phosphorylase) are currently unclear (87,88).

In addition to the direct hepatic action of insulin, insulin has a number of extrahepatic effects that may also influence hepatic glucose production. Insulin's antilipolytic action controls the supply of FFA and glycerol from peripheral tissues to the liver, and insulin reduces the output of gluconeogenic substrate amino acids from muscle. Insulin is thought to be the most powerful inhibitor of FFA release (89,90). In vitro studies of isolated human adipocytes (91) and studies in human volunteers (92) found that suppression of lipolysis is more sensitive to insulin than is stimulation of glucose utilization. Furthermore, in normal humans, suppression of lipolysis was found to be more sensitive to insulin than suppression of hepatic glucose production (93). This supply of precursors (alanine, glycerol, lactate) and
energy substrates (free fatty acids) for gluconeogenesis is an important determinant of hepatic glucose production. In addition, insulin inhibits glucagon secretion, providing another possible indirect mechanism for suppression of hepatic glucose production. The mechanisms of the direct and indirect effect of insulin on glucose production have been addressed in a number of in vivo studies. In these studies, mainly the mechanism of the acute suppressive effect of insulin on GP has been addressed.

In Dr. Cherrington's paper (66), in one protocol the insulin level in the systemic circulation was increased in the absence of changes in portal insulin. In another protocol, the insulin level in the portal vein was increased in the absence of any change in the arterial (peripheral) plasma insulin level. This way, they were able to study the direct effect of insulin. With a decrease in both hepatic glucose output and glucose production there was no decrease in hepatic gluconeogenesis. From these results, it is evident that the change in glucose production reflected a decrease in glycogenolysis. This is consistent with the notion that the short term direct effects on insulin in hepatic glucose metabolism are mainly mediated by activation/deactivation of enzymes involved in glycogen metabolism.

With respect to the indirect effects of insulin, the fall in glucose caused by the rise in systemic insulin appeared to result from two major actions of the hormone. First, the rise in arterial insulin caused a slight reduction in the availability of gluconeogenic precursors to the liver, and as a result net hepatic gluconeogenic precursor uptake fell. As well, net hepatic lactate production increased in response to the selective rise in arterial insulin. The source of this lactate was glucose-6-phosphate which underwent glycolysis within the liver rather than being exported as glucose. Therefore it was found that the indirect effect of insulin on the liver appeared to alter the gluconeogenic rate and the fate of glucose within the hepatocyte.
The increase in net hepatic lactate production was particularly intriguing as it correlated in
time almost perfectly with a decline in plasma FFA and the fall in net hepatic FFA uptake. It
was thought that it was the fall in lipolysis that explained the increased net hepatic lactate
output.

Three studies were performed in which insulin suppression of GP was studied under
conditions in which FFA reductions with insulin were prevented. In one study (94),
hyperinsulinemic-euglycemic clamps were performed in normal dogs in which FFA were
either allowed to decline or were prevented from declining with Liposyn (a triglyceride
emulsion) and heparin. Results indicated that preventing the fall in FFA during insulin
blocks the suppression of GP. These data provided direct support for the concept that insulin
suppression of FFA is a necessary link in the overall effect of insulin to suppress GP. In a
second study, a selective rise in arterial plasma insulin was brought about in two groups (95)
of normal dogs. In one, the fall in plasma FFA and glycerol was prevented by infusing
Intralipid (also a triglyceride emulsion) and heparin (+FFA) while the other was a control (-
FFA). In the -FFA group the results were as in the earlier study. Prevention of the fall in
the plasma FFA during the selective increase in arterial plasma insulin on the other hand
reduced the fall in GP. In the FFA clamped group, there was no increase in net hepatic
lactate output in response to the rise in arterial insulin, indicating that it was indeed the fall in
net hepatic FFA uptake which triggered the re-direction of G6P to lactate rather than
glucose. Over the last ten years, a number of other studies have shown independently that
FFA concentrations can influence GP (96,97).

A third study which we performed in collaboration with Dr. Lewis, examined the role
of FFA in mediating the extrahepatic effects of insulin in humans (98). Like previously, two
groups were studied, one with tolbutamide infusion (POR group), the other with equidose peripheral infusion (PER group), however a dose of heparin and Intralipid was administered to prevent the insulin-induced suppression of FFA. A euglycemic clamp was performed. It was shown that the previously shown greater suppression of GP with equimolar peripheral versus portal insulin was eliminated or reversed if FFA were prevented from decreasing suggesting an important role of FFA in mediating the extrahepatic effects of insulin on GP.

Apart from insulin’s action on fat and muscle, there are two other ways in which a selective rise in extrahepatic insulin is able to suppress glucose production.

α-Insulin can act on the α cell to inhibit glucagon secretion. By glucagon being suppressed, GP is also suppressed. Therefore insulin can mediate the extrahepatic effect by preventing the release of glucagon from the α cells. However, in normal physiology, glucagon is suppressed directly in the α cell by insulin in the pancreas. The pancreas is upstream to the portal vein and therefore when an experimental approach is set up where insulin is infused in the portal vein to mimic normal physiology, it is not entirely accurate since glucagon cannot be suppressed by intra-islet insulin. Conversely, in diabetes, the α cell is controlled by peripheral insulin, as there is no endogenous insulin secretion. A study was conducted by our laboratory to assess the role of glucagon with respect to insulin’s direct and indirect effects in suppressing GP in depancreatized dogs (99). Insulin was infused portally, peripherally and ½ dose peripherally as seen in the previous study (68). However, in this study, glucagon was clamped at high levels, levels which are seen in poorly controlled diabetes. Where previously full dose peripheral insulin infusion suppressed GP more than portal, in this study, it was shown that equidose portal and peripheral insulin infusions suppressed GP to the same extent suggesting that glucagon may mediate part of the
indirect effect of insulin. As well, portal insulin infusion was more potent than ½ dose peripheral in suppressing GP, suggesting that glucagon can also unmask the direct effect of hepatic insulin levels on GP. This was also shown in our collaborative study with Dr. Lewis in humans (100), and was confirmed in a study in normal dogs (101).

Additionally, physiologic increments in peripheral insulin concentration in conscious dogs can decrease renal glucose production (102). The role of the kidney in glucose metabolism is thought to be minor under most circumstances. Cahill et al. (103) demonstrated that net renal glucose output was negligible in the post-absorptive state, but it increased substantially with 4-6 weeks of fasting. More recently, animal studies have documented the ability of the kidney to significantly increase glucose output in conditions of insulin deficiency (104). Although these observations underscore the potential role of the kidney in glucose regulation in specific circumstances, they have been interpreted to indicate that the kidney is unimportant under most conditions. In vitro studies have indicated that the kidney is capable of both glucose production and utilization. Krebs and co-workers (105,106) demonstrated that the biochemical machinery is in place of the proximal convoluted tubule to efficiently convert 3-carbon precursors to glucose, particularly glycerol and glutamine. Cersosimo et al. (102) set out to investigate under in vivo conditions whether simultaneous renal glucose production and utilization occur in conscious dogs. It was shown that tracer-determined renal glucose production and utilization accounted for ~30% of glucose turnover in post-absorptive dogs. Physiological hyperinsulinemia suppressed renal glucose production and stimulated renal glucose uptake by ~75%. However, the contribution of the kidney to tracer-determined GP in the post-absorptive state is still controversial since
Ekberg et al (107) showed that GP by the human kidney in the post-absorptive state makes at most only a minor contribution (~5%) to tracer-determined GP.
1.6 Routes of Insulin Treatment and Chronic Effects of Insulin on GP

Goriya et al. (53) studied the effect of long term portal insulin therapy in unrestrained, and conscious pancreatectomized dogs who carried a programmable insulin pump for 163-224 days. When compared to peripheral infusions, 20% less exogenous insulin was required and peripheral fasting insulin levels were 30% lower. In the post-absorptive state, blood glucose was normal as was plasma insulin. It was therefore shown that portal insulin replacement in pancreatectomized dogs could normalize both blood glucose and insulin in the fasting state.

In a similar study (108), insulin was delivered portally in one group of dogs for 164-224 days and into the peripheral circulation in another group of dogs for 123-365 days. It was shown that metabolic regulation achieved with portal insulin replacement was closer to normal than that achieved with peripheral infusion. It is possible that the chronic effects of insulin may be different than the acute effects (the direct effect on GP may be more important because of induction of hepatic enzymes by insulin).

In humans however, portal insulin therapy is not feasible. SC infusion of insulin is the conventional method of insulin treatment because it is relatively non-invasive. However, with peripheral absorption of insulin, the portal-peripheral insulin gradient is abolished. Many studies have examined alternative routes of insulin treatment that restore this gradient. It has been shown that insulin that is delivered to the peritoneal cavity is absorbed almost entirely by the portal circulation (109) and thereafter is delivered to the liver, thus mimicking physiological pancreatic insulin secretion. As well, because intraperitoneal (IP) insulin therapy leads to a reduction in the peripheral levels of insulin and may therefore reduce the risk of atherosclerosis and hypertension, much attention has been paid to this form of treatment. It has been shown that subjects with type 1 diabetes that were given insulin
intraperitoneally had lower systemic insulin levels in the presence of the same glucose levels as observed during subcutaneous (SC) insulin infusion (110). Intraperitoneal (IP) insulin delivery results in a large first-pass hepatic insulin clearance of 40-70% (109,111) and therefore, a portal-peripheral insulin gradient. The greater hepatic insulinization with IP than SC delivery inhibits GP which leads to a reduction in peripheral insulin levels necessary to achieve normoglycemia (112,113). Implantable IP insulin pumps have been manufactured and tested in patients with type 1 diabetes (114,115). Long term studies are needed to determine whether the reduction in insulin levels obtained with IP treatment is associated with a reduction in diabetic complications. Furthermore, the use of implantable pumps to administer insulin is still considered to be a rather invasive method.

An alternative to the delivery of insulin via the IP route is pancreatic islet or whole pancreas transplantation, when a kidney transplant and therefore immunosuppression is also required. It has been shown that patients who have undergone pancreas transplantation have good glycemic control and are not dependent on exogenous insulin, even in the long term (>1 year) (116). Two types of pancreas transplantation exist, one with systemic pancreatic venous drainage of insulin and one with portal pancreatic venous drainage. The question of chronic systemic or portal infusion of insulin on insulin sensitivity and glucose tolerance has been addressed. It has been shown that diversion of pancreatic venous drainage to the systemic circulation increases the peripheral insulinemia, decreases insulin sensitivity and decreases the suppression of glucose production (117-119). It was concluded that physiological insulin delivery and therefore portal drainage during transplantation may be relevant for the complete normalization of glucoregulation in diabetes.
A simpler procedure compared to whole pancreas transplantation is the implantation of insulin-secreting pancreatic islet cells. This procedure also establishes good glycemic control and temporary insulin-independence (120). The metabolic effects of insulin derived from renal subscapular islet grafts either with systemic delivery of insulin through renal venous drainage or with portal delivery of insulin from islet graft in the same site was studied in streptozotocin (STZ) diabetic rats (121). It was shown that systemic delivery of insulin was associated with hyperinsulinemia, and insulin resistance. These abnormalities were prevented with portal delivery of insulin. In an older study, no differential effects of the two routes of delivery were seen, except for higher glycogen levels with portal insulin delivery (122). In these islet replacement studies, results are clouded by different islet viability in different sites of implantation. Since islet transplantation is a considerably less invasive procedure compared to pancreas transplantation, further studies designed to prevent graft failure and prolong insulin-independence are warranted.
1.7 Rationale for Thesis

Our previous studies in normal dogs and humans (61,62) showed that insulin had both a peripheral indirect and a direct effect in suppressing glucose production. However, in our studies in moderately hyperglycemic (~10mM) depancreatized dogs (68,70) and type 2 diabetic patients (71), the direct effect was undetectable. We hypothesized that this is due to hepatic insulin resistance and/or hyperglycemia.

The mechanism of GP inhibition by insulin in normal physiology might differ from the mechanism of GP suppression by insulin in diabetes for a number of reasons. First, normal dogs are euglycemic and have no insulin resistance whereas diabetic dogs are usually hyperglycemic and insulin resistant. Hepatic insulin resistance in the diabetic dog model may decrease or eliminate a direct effect of insulin on GP. Secondly, hyperglycemia can inhibit glycogenolysis, thereby shifting GP to gluconeogenesis (123,124). It is known that insulin acts directly on the liver by suppressing glycogenolysis (72,73), and possibly because of suppression by hyperglycemia, further suppression by insulin is not possible. Reduced glycogenolysis could limit the direct effect of insulin in suppressing GP, whereas the ratio of gluconeogenesis to glycogenolysis under diabetic conditions (104,125) might account for a greater indirect effect of insulin on GP than in non-diabetic dogs through a decrease in gluconeogenic substrates. Possibly, by clamping the blood glucose level at euglycemia, the suppression of glycogenolysis by hyperglycemia is abolished, and insulin is allowed to maximally suppress glycogen breakdown in the liver.

One of the aims of the present thesis was to determine whether in depancreatized diabetic dogs, under conditions of euglycemia, a direct effect could be restored, as seen in the normal dogs of the previous study. It is possible that the lack of a direct effect of insulin on
GP in the diabetic dogs was due to moderate hyperglycemia. Therefore, a similar protocol was conducted where instead of moderate hyperglycemia, these dogs were clamped at euglycemia. Two treatments were conducted, a high physiological dose of insulin (0.9mU/kg.min) was infused in the portal vein (POR), and an insulin dose was given peripherally to equal the amount of insulin exiting the liver during the portal treatment (PER) and thus match the peripheral insulin levels. The hypothesis was that at euglycemia, we would be able to observe a restoration of the direct effect of insulin and therefore GP would be suppressed to a greater extent with POR insulin treatment as compared to PER insulin treatment, at matched peripheral insulin levels.

One possible criticism to the above discussed experimental protocol is that the insulin levels needed to achieve euglycemia are higher than those needed to achieve moderate hyperglycemia. It is possible that the postulated restoration of the direct effect of insulin on hepatic glucose production by insulin induced euglycemia is not due to euglycemia but to higher insulin levels. In our previous studies, an insulin dose was administered until moderate hyperglycemia was achieved and then kept constant for the remainder of the experiment. To achieve euglycemia, more insulin is required, and this extra insulin could possibly induce a direct effect. Therefore we set up a second protocol where insulin was administered until we reached moderate hyperglycemia, as performed previously. However, to examine the effect of euglycemia, the glucose level was allowed to fall until euglycemia, where it was then clamped for the remainder of the experiment. This way, the insulin levels are the same in both cases and therefore any difference in hepatic glucose production can be attributed to euglycemia.
Furthermore, with this protocol we also wished to study if a direct effect is indeed restored by euglycemia, how acute this restoration is. Where the direct effect would not be expected to be present during the hyperglycemic part of the experiment, during the euglycemic period, this direct effect should start to become evident. We wished to study how long it takes for euglycemia to restore the direct hepatic effect of insulin on GP (ie. is it chronic or acute euglycemia that is needed for a direct effect to take place).

The last aim of this thesis was to look at the effect of the direct and indirect effect of insulin on GP from the perspective of treatment of a type 1 diabetic patient. Two treatments were set up, one in which insulin was administered through the peripheral circulation in a replacement dose to achieve euglycemia (PER REPL), as in the traditional treatment of a type 1 diabetic subject. In the second treatment, insulin was administered into the portal vein, on a direct route to the liver (POR REPL). We wished to compare the effects of treating a diabetic with insulin peripherally, as in the standard treatment, with infusing insulin into the portal vein, as seen in normal physiology. Insulin was delivered by either route until euglycemia was achieved. At the same glucose levels, the question was, how much insulin would be needed. This was measured by assaying the amount of insulin in the peripheral circulation in both treatments. If less insulin was present in the peripheral circulation with the POR REPL as compared to PER REPL, then a direct effect of insulin in suppressing GP was present because otherwise glucose levels would be higher with POR REPL, due to less peripheral insulin. It is well known that the peripheral insulin levels required to achieve normal glycemia in type 1 diabetes patients are higher than normal insulin levels. Peripheral hyperinsulinemia in insulin treated diabetic patients can be due to insulin resistance and/or to the route of non-physiological insulin administration. We wished
to study the latter reason. The rationale was, if less insulin was present in the peripheral circulation with one treatment as opposed to the other, with the same levels of glycemia, then that treatment was a safer treatment because peripheral hyperinsulinemia is a risk factor for atherosclerosis (24), and arterial hypertension (25).
1.8 Hypotheses

1. Euglycemia restores the direct effect of insulin on GP in depancreatized diabetic dogs during a glucose clamp.

2. Only a brief period of euglycemia is required to restore the direct effect of insulin on GP.

3. The level of peripheral insulin necessary to achieve normoglycemia with portal insulin replacement (POR REPL) is lower than with peripheral insulin replacement (PER REPL).
2. METHODS

2.1 Experimental animals and surgical procedures

This study was performed on seven post-absorptive depancreatized male dogs. Depancreatized dogs were chosen as they are a model of type 1 diabetes mellitus. Due to the volume of blood sampled during each experiment, mongrel dogs weighing 25-35 kg were selected. The dogs were put through a 3 week conditioning period during which time they were immunized for distemper, hepatitis, parvo virus, and rabies. They were given antibiotics orally twice daily for eight days, starting three days before surgery and intra-muscularly on the day of surgery (500 mg, Cephalexin, Novopharm, Toronto, Ontario, Canada). The dogs were fasted for 24 hours before surgery to allow for their stomach contents to be emptied. The animals were given an intramuscular (IM) injection of atropine (0.05 mg/ml Atropine Sulphate, M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada) to stop throat secretions, an analgesic (0.3 mg Buprenorphine HCl; Rickett & Colman Pharmaceuticals, Hull, Great Britain) and a neuroleptic (0.5 mg/kg Atravet, Ayerst Laboratories, Montreal, Quebec, Canada) to sedate the animal. Thereafter, an intravenous dose (0.02 mg/kg) of an ultra short acting barbiturate, sodium thiopental (Abbot Laboratories, Montreal, Quebec, Canada) was administered at which time an intubation tube was positioned in the dog’s trachea. At this point, the dogs’ necks and abdomen were shaved. Once shaved, the dogs necks and abdomen were disinfected with alcohol and Betadine (Purdue Frederick Inc. Pickering, Ontario, Canada). Anaesthesia was maintained through 0.5% halothane (Halocarbon Laboratories, New Jersey, USA) in carrier gas consisting of
60% nitric oxide (Canox, Toronto, Ontario, Canada) and 40% oxygen (Canox, Toronto, Ontario, Canada).

The surgeries were performed in the Department of Comparative Medicine of the University of Toronto under sterile conditions. The abdominal cavity was opened with a midline laparotomy and visceral fat was removed as necessary to allow easy access to the spleen and pancreas. At this time, the pancreas was completely removed while care was taken to preserve duodenal vascularization through the pancreato-duodenal vessels.

In all dogs, a silastic cannula (0.05” internal diameter, Baxter Healthcare Corporation, McGaw Park, IL) was inserted into the portal vein through a branch of the splenic vein and advanced until the tip was approximately 1.0 cm beyond the point of confluence of the splenic vein with the portal vein, i.e. approximately 5 cm from the branching point of the portal vein into its left and right bifurcations to the liver. Three silastic cannulae (one 0.05” internal diameter and two 0.03” internal diameter) were inserted into the jugular vein and advanced into the superior vena cava. In addition, a silastic cannula (0.05” internal diameter) was inserted into the carotid artery and advanced into the aortic arch. The carotid cannula served for arterial sampling and the jugular and portal cannulae served for infusions. The thicker jugular line was used for the viscous dextrose infusate. The cannulae were tunnelled subcutaneously and exteriorized at the back of the neck. They were filled with heparin (1000U/ml, Hepalean, Organon Teknika, Ontario, Canada) to prevent the blood from clotting in the cannulae. The lines were then bandaged around the dog’s neck. An analgesic was administered halfway through the surgery intravenously, and intramuscularly post-surgically to prevent post-surgery pain. In addition, a small dose of
NPH insulin (Iletin II NPH insulin, Eli Lilly, Co. Indianapolis, USA.) was injected subcutaneously to regulate glycemia.
2.2 Post-Surgical Animal Care

After surgery, the amount of food given to the dogs was slowly increased until they received enough food to maintain a constant body weight. This food consisted of a mixture of dry chow (minimum 25% protein, minimum 9% fat, maximum 4% fibre, maximum 12% moisture, maximum 10% ash, maximum 2% added minerals, Purina Mills, St. Louis, MO) mixed with canned meat (10% protein, 5% fat, 78% moisture, Derby Pet Food, Brampton, Ontario, Canada) once a day. The dog food was supplemented with folic acid (5mg, Novopharm Ltd, Toronto, Ontario, Canada) and iron (70mg ferrous gluconate, Novopharm Ltd, Toronto, Ontario, Canada) to assist erythrocyte production and thus prevent anemia due to blood sampling during the experiments. In addition, pancreatic enzymes (Cotazym, Organon Canada, Toronto, Ontario, Canada) were given orally to replace the lost exocrine function of the pancreas. Regular porcine (Iletin II regular insulin, Eli Lilly Co., Indianapolis, USA) and NPH porcine insulin in an approximate ratio of 1:2 was injected subcutaneously. Glycemia was measured using a glucometer (Bayer, Etobicoke, Ontario, Canada). The insulin dose was adjusted accordingly to keep fasting glycemia at 7-9mmol/l.

The cannulae were regularly flushed (every 3-4 days) with saline and filled with heparin to maintain patency and avoid clotting and the neck incision was cleaned with peroxide and Betadine. Body weight, body temperature, haematocrit, stools and food intake were monitored regularly. All procedures were in accordance with the Canadian Council on Animal Care standards and were approved by the Animal Care Committee of the University of Toronto.

Only dogs who were healthy, with a haematocrit above 35% and under relatively well controlled diabetes (blood glucose 7-9 mmol/l) were allowed to undergo experiments. The
dogs received the normal amount of food the day before the experiment and a lower amount of insulin. The regular insulin dose was usually unaffected while the NPH insulin was reduced to \( \frac{1}{2} \) or \( \frac{1}{3} \) of the previous day's dose. This was performed to obtain early morning hyperglycemia and to ensure that all NPH insulin was eliminated before the experiment. As well, 50 ml of blood was taken and spun down, and the plasma was used for making up the solutions the day of the experiment and later for tracer assays. After the experiment, food was given and a reduced insulin dose was administered as appropriate, to avoid overlap with the regular daily dose. The experiments were performed after at least an 18 hour overnight fast and were repeated at least 10 days after the previous experiment to allow for restoration of the haematocrit level.
2.3 Experimental Design

2.3.1 Basal Euglycemia / Euglycemic Clamp (Study 1)

At the start of the experiment, the depancreatized dogs were hyperglycemic (>400 mg/dl) because they had received a reduced NPH insulin dose, the day before. Once all the solutions were made and the experiment was ready to begin in these dogs, regular porcine insulin was initially infused intraportally, starting at a high dose. The dose was then gradually reduced to basal levels in the goal of obtaining constant euglycemia (90-120 mg/dl). The dose was kept fixed for at least 30 minutes before sampling. When glucose levels decreased below 250 mg/dl, a bolus of tracer (42.5 μCi of 3-³H tritiated glucose, New England Nuclear, Boston, MA) was given and a continuous tracer infusion (0.24 μCi/ml) was started at time = -210 minutes to enable the measurements of GP and GU. The bolus was designed to decrease the equilibration period (126).

The equilibration period was at least 40 minutes long. Both the low portal insulin infusion and the tracer infusion were continued throughout the experiment. At time = -40, basal sampling started every 10 minutes for 40 minutes. At time = 0, insulin was infused either portally (POR) or at approximately half the dose peripherally (1/2PER) to match the peripheral insulin levels obtained with the POR treatment (due to insulin extraction by the liver) and also to obtain a large difference in the estimated hepatic insulin levels. The dose that matched the peripheral insulin levels in our dogs were slightly less than 50% of the previous dose (see results). All insulin infusions were prepared in saline containing approximately 4% (w/v) of the dog’s own plasma. The plasma was added to prevent insulin from sticking to the glass beaker used for preparation of the infusate.
Plasma glucose was clamped at the initial pre-clamp glycemic level for 3 hours with a variable exogenous dextrose infusion (50% dextrose, Abbott Laboratories, Montreal, Quebec, Canada) which was adjusted accordingly to plasma glucose concentrations determined every 5 minutes. Glucose was clamped to isolate the effects of insulin per se from the effect of lowering glycemia and to avoid any release of counter-regulatory hormones. The euglycemic clamp required the dextrose infusion to be spiked with 3-2H glucose tracer (hot glucose) according to Finegood et al. (127, 128) to prevent the decline in the glucose specific activity during the glucose clamp, and thus, minimize errors which are associated with the use of a one-compartment, fixed-pool volume model method for calculations of GP (129,130).

Arterial samples were taken every 10 minutes for 40 minutes in the basal period and then every 5 minutes for the next 3 hours of the hyperinsulinemic clamp. The blood samples were taken for insulin, glucagon, free fatty acids, lactate, alanine, and for 3-2H glucose. The blood samples for 3-2H glucose were collected in tubes containing 2.5 mg of sodium fluoride (Fisher), to prevent glucose degradation, and dried heparin (50 U.S.P. units per 1.0 ml sample) as an anticoagulant. The samples for glucagon, insulin and free fatty acid analysis were collected in tubes containing 0.125 ml of 24 mg/dl ethylenediamine tetraacetic acid (EDTA; Sigma) to prevent clotting and 0.125 ml of Trasylol (2000 kallikrein IU; FBA, NY) per 2.5 ml of blood. Trasylol is a broad spectrum proteolytic inhibitor that protects against loss of glucagon immunoreactivity. Blood samples for alanine, lactate and catecholamines were collected in tubes containing an equal volume of 10% perchloric acid (BDH Inc. Toronto, Ont.) to deproteinize the sample by precipitation thereby clearing the sample. The assays for the above metabolites and catecholamines have not been done yet. The sample
must be clear to reduce errors in the fluorometric assay methods. All tubes were centrifuged at 1500 rpm at 4°C for 15 minutes. Once centrifuged, the plasma from the tubes for tracer, insulin, glucagon and free fatty acids were transferred to new storage tubes. The supernatants of tubes for alanine, glycerol, and lactate were transferred, re-spun and then transferred again to new tubes. All tubes were then stored at -20°C.
EXPERIMENTAL PROTOCOL 1

Dog Model: Depancreatized dog with carotid, jugular and portal cannulations

Portal insulin replacement to achieve and maintain **euglycemia**

3-$^3$H glucose tracer

Insulin
0.9 mU/kg.min POR or
$\sim0.45$ mU/kg.min 1/2PER

Variable hot glucose infusion to maintain a euglycemic clamp

-160 -40 0 Sampling period 180

Time (min)
2.3.2 Basal Hyperglycemia / Euglycemic Clamp

A second protocol was conducted which was similar to the first one. At the start of the experiment, the dogs were hyperglycemic and a portal insulin dose was administered. However, instead of starting the basal period when the glucose level had reached euglycemia, as in the first protocol, it was started at moderate hyperglycemia (160 – 200 mg/dl) as in the previous study (68,70). After the 40 minute basal period, an additional insulin dose was administered either portally or peripherally. Unlike the first protocol where the glucose level was clamped at the basal level, in this protocol, the glucose levels were allowed to fall until euglycemia was achieved, and then clamped for the remainder of the 3 hours at euglycemia with a variable exogenous dextrose infusion. Whereas in the first study a “hot glucose” infusion was required to maintain the plasma glucose specific activity, in this study, since a very low rate of exogenous dextrose was needed to maintain the clamp, cold dextrose could be used as the infusate without affecting the specific activity. As in study 1, blood samples were taken throughout the clamp for the same determinations as described above.
EXPERIMENTAL PROTOCOL 2

Dog Model: Depancreatized dog with carotid, jugular and portal cannulations

Portal insulin replacement to achieve moderate hyperglycemia

3-³H glucose tracer

Insulin
0.9 mU/kg.min POR or
~0.45 mU/kg.min 1/2PER

Variable hot glucose infusion once euglycemia is achieved, to maintain a euglycemic clamp

-160 -40 0 Sampling period 180
Time (min)
2.3.3 Basal Euglycemia / Insulin Replacement Studies (Protocol 3)

A third study was conducted in which insulin was infused peripherally instead of portally until euglycemia was achieved. At this point, blood samples were taken only for a 40 minute basal period. Samples were taken for the same determinations as described above. The purpose of this above experiment was to compare the insulin levels needed to achieve the same level of glycemia during portal replacement (POR REPL) as in the basal period of study 1 (for each dog, the results of the two experiments of study 1 in the basal period were averaged), or peripheral insulin replacement (PER REPL).
**EXPERIMENTAL PROTOCOL 3**

**Dog Model:** Depancreatized dog with carotid, jugular and portal cannulations

- Portal or Peripheral insulin replacement
to achieve and maintain euglycemia

- $3^{-3}H$ glucose tracer

<table>
<thead>
<tr>
<th>Sampling period</th>
<th>-160</th>
<th>-40</th>
<th>0</th>
<th>180</th>
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<tbody>
<tr>
<td>Time (min)</td>
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2.4 Laboratory methods

2.4.1 Plasma Glucose Assay

Plasma glucose concentrations were measured by the glucose oxidase method (131) on a glucose analyzer (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). A 10 ul sample of plasma containing D-glucose is injected into a solution containing oxygen and glucose oxidase. The glucose reacts with the oxygen in the following reaction which is catalyzed by glucose oxidase:

\[
\text{D}-\text{glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{Glucose Oxidase}} \text{gluconic acid} + \text{H}_2\text{O}_2
\]

In this reaction, oxygen is used at the same rate of glucose to form gluconic acid. A polarographic oxygen sensor is used to detect oxygen consumption which is directly proportional to the glucose concentration in the sample. Results are obtainable within thirty seconds after sample addition. The data are accurate to 3mg/dl. The analyzer was calibrated before use and frequently during the experiment with the 150 mg/dl Beckman Certified Glucose Standard Solution.

2.4.2 Plasma Tracer Assay

For determination of 3-[\text{\textsuperscript{3}H}]-glucose radioactivity, the plasma samples were thawed in cold water. A sample of tracer infusate used was diluted to 1/1000 its concentration and treated as samples. Once all the samples had thawed, they were spun at 2500 rpm for ten minutes, and then 0.2 ml were transferred to new tubes. At this point, the plasma was deproteinized
in equal volumes of 5% (w/v) zinc sulphate and 0.3 N barium hydroxide (BDH) (Sigma Diagnostics, St. Louis, Minnesota, U.S.A.). 1 ml of double distilled water was also added. The samples were deproteinized to reduce quenching of the emitted energy from the tritium label by the protein when being counted. After ten minutes, the samples were spun down, and the supernatant was transferred to a set of new tubes to be spun down once again. 1 ml of each solution was then transferred to a set of scintillation vials and the vials were placed in an oven overnight to allow for evaporation to eliminate tritiated water. Once this was completed, 1 ml of double distilled water and 10 ml of liquid scintillation solution (Ready Safe, Beckman) was added, the tubes were vortexed, and radioactivity from 3-[³H]-glucose was measured in a beta-scintillation counter (Camberra Packard).

2.4.3 Plasma Insulin Assay

Plasma insulin levels were determined using the kit from Pharmacia AB, Uppsala, Sweden. Pharmacia Insulin RIA is a double antibody radioimmunoassay. Insulin in the sample competes with a fixed amount of ¹²⁵I-labelled insulin for the binding sites on the specific antibodies. Bound and free insulin are separated by the addition of a second antibody immuno-adsorbent followed by centrifugation and decanting. The radioactivity in the pellet is then measured. The radioactivity is inversely proportional to the quantity of insulin in the sample. Each package of Pharmacia Insulin RIA contains reagents for 100 tubes, sufficient for 44 samples and one standard curve in duplicate.

A standard curve is first constructed using insulin standards with known concentrations (0, 3, 10, 30, 100 and 240 uU/ml) in duplicate. We used dog insulin standard. Dog insulin has the same primary structure as pork insulin. Samples were also assayed in
duplicate. 100 µl of sample were pipetted into the appropriate vials. Then $^{125}$I-insulin was added (50µl to all tubes) and then the antibody was added (50 µl to all tubes). At this point, the contents of the tubes should all be green. The rack was then shaken to ensure mixing and was incubated for 2 hours at room temperature. Following this, the suspension was decanted and incubated for ½ hour at room temperature. Then the supernatant was centrifuged for 10 minutes at 1500g. The tubes were decanted and then left to stand ½ minute upside down on absorbent paper. For the most optimal results, the tube rack should be moved (upside down) onto a dry absorbent paper and tapped a couple of times against the absorbent paper to get rid of residual drops. The precipitate is firmly packed and none of it is lost with vigorous tapping. The tubes were then counted to determine the radioactivity.

The calculation of results is as follows:

1. The counts (B) for each of the standards and unknowns are expressed as a percentage of the mean counts of the "0=standard" (Bo).

$$\% \text{ activity bound} = \frac{B(\text{standard or unknown}) \times 100\%}{Bo}$$

2. The percentage values obtained for the insulin standards are plotted against the concentration on a lin-log paper and a standard curve is constructed.

3. The concentration of the unknown samples is read from the standard curve.

The coefficient of interassay variation as determined on reference plasma was less than 7%. 
2.4.4 Plasma Glucagon Assay

The plasma glucagon assay was performed using the kit from Diagnostic Products Corporation (DPC, Los Angeles, California). Antibody glucagon is an $^{125}$I radioimmunoassay designed for the quantitative measurement of glucagon in EDTA plasma. The DPC's Double Antibody Glucagon Procedure is a sequential radioimmunoassay. After preincubation of the blood sample with anti-glucagon antibody, $^{125}$I labelled glucagon competes with labelled glucagon in the sample for antibody sites. After incubation for a fixed time, separation of bound from free glucagon is achieved by the PEG-accelerated double-antibody method, followed by centrifugation. The precipitate containing the antibody-bound fraction is then counted, and the glucagon concentrations are read from a calibration curve. The whole assay involves two overnight incubations. Glass tubes rather than plastic tubes should be employed for the assay, due to the tendency of glucagon to adsorb to plastic surfaces. Just prior to the assay, the 2000 pg/ml Glucagon (‘Master) Calibrator is diluted in the Glucagon Zero Calibrator. Each dilution is vortexed thoroughly before proceeding to the next dilution. 200 µl of the zero calibrator A is pipetted into NSB tubes (non-specific binding), and into the maximum binding tubes, and 200 µl of each of the remaining calibrators B through F (287, 144, 29, 14, 7 pmol/l respectively) into appropriate tubes (this was done in duplicate). Then 200ul of each sample and each control is pipetted into appropriate tubes (this was also done in duplicate). 100 ul of Glucagon Antiserum (BLUE) is then added to all tubes except the NSB and T tubes, and they are vortexed. The rack is then covered and incubated for 24 hours at 2-8°C. The racks are covered to avoid evaporation. Then, 100 ul of $^{125}$I-glucagon (clear) is added to all tubes, which are all subsequently vortexed. The rack is then covered again and incubated for another 24 hours at
2-8°C. The T-tubes (total counts) are counted as they do not need further processing. Then 1.0 ml of cold Precipitating Solution (red) is added to all tubes, and vortexed. The tubes are then centrifuged for 15 minutes at 1500g. The supernatant is aspirated and the precipitate is counted for 1 minute.

To obtain results in terms of concentration from the logit-log representation of the calibration curve, first for each pair of tubes the average NSB-corrected counts per minute are calculated:

\[
\text{Net Counts} = \text{Average CPM} - \text{Average NSB CPM}
\]

Then the binding of each pair of tubes as a percent of maximum binding (MB) is determined, with NSB-corrected counts of the A tubes taken as 100%:

\[
\text{Percent Bound} = \left( \frac{\text{Net Counts}}{\text{Net MB Counts}} \right) \times 100\%
\]

Using the logit-log graph paper provided with the kit, the Percent Bound is plotted along the vertical axis against the Concentration along the horizontal axis for each of the nonzero calibrators, and the straight line is drawn to approximate the path of these points. Results for the unknowns may then be read from the line by interpolation. The coefficient of interassay variation as determined on reference plasma was less than 16%.
2.4.5 *Plasma Free Fatty Acid Assay*

The FFA concentrations were determined with the fluorometric method of Miles et al. This procedure uses the reactions described below and a fluorometer (Sequoia-Turner, Model 450 Fluorometer) to produce NAD\(^+\) and allow the determination of plasma FFA concentrations:

\[
\text{FFA} + \text{CoA} + \text{ATP} \xrightarrow{\text{Acyl CoA}} \text{acyl CoA} + \text{AMP} + \text{Ppi} \\
\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2 \text{ADP} \\
2 \text{ADP} + 2 \text{phosphoenolpyruvate} \xrightarrow{\text{Pyruvate kinase}} 2 \text{ATP} + 2 \text{pyruvate} \\
2 \text{pyruvate} + 2 \text{NADH} \xrightarrow{\text{Lactate dehydrogenase}} 2 \text{lactate} + 2 \text{NAD}^+ 
\]

A standard curve (1-7 nM) is made by diluting oleate (Sigma) in potassium phosphate buffer. The standards, samples and control plasma were pipetted into glass tubes. Tris assay buffer (NADH, ATP, PEP, myokinase, pyruvate kinase, lactic dehydrogenase, acyl-CoA synthetase (all Sigma)) was added to the tubes which were then mixed and allowed to sit for 10 minutes at room temperature. The tubes were then pre-read in the fluorometer. CoA was then added to allow the reactions to take place. The tubes were mixed and incubated for 75-90 minutes before being post-read in the fluorometer. The post-reading was subtracted from the pre-reading to determine the decrease in fluorescence due to NADH oxidation. A standard curve was constructed and the sample readings were interpolated off the curve. The interassay variation, as determined by reference plasma measurements, was 9%.
2.5 Calculations

GP was calculated as the endogenous rate of appearance measured with 3-[3-H]-glucose. A modified one-compartment model of Steele was used to account for the endogenously infused mixture of labelled and unlabelled glucose (128). Data were smoothed with the optimal segments routine using the optimal error algorithm (132).

The amount of tracer in the dextrose infusate was based on estimates of suppression of glucose production and dextrose requirements. The following equation by Finegood et al. (128), modified as in Giacca et al. (68) to account for partial suppression of glucose production, was used to calculate the specific activity of the dextrose infusate:

\[
SA_{ginf} = \frac{I \times ((GINF_{ss} / Ra(b)) - F) \times 1000}{GINF_{ss} \times BW}
\]

where:
- \(SA_{ginf}\): specific activity of the dextrose infusate (uCi/g)
- \(I\): constant tracer infusion rate (uCi/min)
- \(GINF_{ss}\): steady state glucose infusion rate (mg/kg.min)
- \(Ra(b)\): basal glucose production (mg/kg.min)
- \(F\): steady state suppression of glucose production, \(Ra(b) - Ra_{ss} / Ra(b)\)
- \(Ra_{ss}\): steady state glucose production (mg/kg.min)
- \(BW\): weight of the dog (kg)

The initial estimates of \(GINF_{ss}\), \(Ra(b)\) and \(F\) were those used in (68).
The following equation was used to calculate the amount of tracer to be added to cold glucose to obtain the SA_{inf} required:

\[
T_{INF\text{vol}} = \frac{S_{a\text{inf}} \times G \times V}{T_{INF} + (S_{a\text{inf}} \times (G-C))}
\]

where:
- \(T_{INF\text{vol}}\) = ml of tracer infusate to be added to dextrose infusate
- \(S_{a\text{inf}}\) = specific activity of the dextrose infusate (uCi/g)
- \(V\) = final volume of the dextrose infusate (ml)
- \(T_{INF}\) = radioactive concentration of tracer infusate (uCi/ml)
- \(G\) = cold glucose concentration in the dextrose infusate (g/ml)
- \(C\) = concentration of carrier glucose in the tracer infusate (g/ml)

The estimated portal insulin levels were calculated using the Fick principle of dye dilution modified by Ader and Bergman (59).

\[
I_{PO} = \frac{INF_{1} + I_{PE}}{PPF}
\]

\(I_{PO}\), and \(I_{PE}\) are the portal, and systemic insulin levels. \(INF_{1}\) and \(PPF\) are the portal insulin infusion rates, and portal blood flow, respectively. The portal plasma flow was assumed to be 500 ml/min (59). To calculate the estimated hepatic insulin levels, we assumed that 72% of the blood flow to the liver was from the portal circulation and that the remaining 28% was from the hepatic artery (22).
Statistics

The data were expressed as ± mean standard error of the mean (SEM). The calculations were done on the last 90 minutes when a new steady state was obtained. Two way analysis of variance (ANOVA) was carried out for differences between experimental groups as all experiments were paired. Data were also analyzed within each group for differences between the experimental periods. Calculations were performed with SAS software (SAS Statistical Analysis Systems, Cary, NC).
3. RESULTS

3.1 Basal Euglycemia / Euglycemic Clamp (Study 1)

The following results are based on an n=6 for POR and 1/2PER. The plasma glucose levels were maintained constant at euglycemia (POR 111.6±2.8; 1/2PER 109.6±2.1 mg/dl, figure #4). Plasma glucose specific activity increased minimally from the basal values of 2.7±0.2 (POR) and 2.6±0.2 uCi/g (1/2PER) to 2.9±0.1 (POR) and 2.7±0.2 uCi/g (1/2PER) during the clamp period (figure #5). The increase was ~8% and ~5% in the POR and 1/2PER groups respectively. The portal dose to achieve and maintain euglycemia was 0.68±0.16 (POR) and 0.70±0.19 mU/kg.min (1/2PER). The basal peripheral insulin levels were 10.2±1.2 and 9.4±0.5 uU/ml in the POR and 1/2PER groups respectively (figure #6). The POR treatment raised the peripheral insulin levels to 24.7±2.2 uU/ml. The 1/2PER treatment raised insulin levels to 26.2±1.4 uU/ml. There was no difference in the increase in peripheral insulin levels during the clamp between POR and 1/2PER.

The estimated basal portal insulin levels were 51.4±6.4 and 56.6±2.6 uU/ml in the POR and 1/2PER treatments. During the clamp, POR and 1/2PER raised portal insulin levels to 121.1±9.1 and 74.0±1.9 uU/ml. The portal insulin levels with POR were significantly greater than 1/2PER (p=0.0001). The estimated basal hepatic insulin levels were 39.6±4.9 and 43.4±1.9 uU/ml in the POR and 1/2PER treatments respectively (figure #7). During the clamp, hepatic insulin levels in POR were significantly higher than in 1/2PER (POR 94.1±6.2; 1/2PER 60.9±1.4 uU/ml; p=0.0001; figure #7).

The hepatic extraction of insulin was calculated to be 55.7%. This is slightly higher than is conventionally known to be (19). Furthermore, this is higher than what was found
previously in our laboratory. For this reason, the peripheral insulin dose administered was not exactly half that given portally, but slightly lower (0.39 mg/kg.min; 44.3%) in order to match peripheral insulin levels.

The GU in the basal periods was 3.2±0.3 and 3.4±0.3 mg/kg.min, (n.s.) in the POR and 1/2PER treatments respectively. The GU during the clamp was 4.9±0.3 and 5.2±0.5 mg/kg.min, (n.s.) in the POR and 1/2PER treatments respectively (figure #8). Metabolic Clearance Rate (MCR) in the basal period was 2.9±0.2 and 3.1±0.3 ml/kg.min, (n.s.) and in the clamp period was 4.5±0.3 and 4.8±0.4, (n.s.) in the POR and 1/2PER groups respectively (figure #9). The glucose infusion rates (GINF) necessary to maintain glycemia constant, are shown in figure #10. The GINF was significantly greater in the POR (p=0.02) than 1/2PER (POR 2.8±0.3 vs. 1/2PER 2.4±0.3 mg/kg.min).

Basal endogenous GP was 2.9±0.3 and 3.2±0.4 mg/kg.min, (n.s.) in the POR and 1/2PER treatments respectively. When the additional insulin was given, GP was suppressed to a significantly greater extent with POR (p=0.0001) than 1/2PER (POR 2.2±0.3 vs. 1/2PER 2.8±0.4 mg/kg.min) (figure #11).

The basal glucagon levels were 68.6±12.1 and 58.9±5.9 pg/ml, (n.s.) in the POR and 1/2PER groups. During the clamp, the glucagon levels were similar in both treatments (POR 59.0±4.9; PER 52.2±4.4 pg/ml) (figure #12).

The basal FFA were 890.4±120.9 and 913.7±132.2 uEq/l in the POR and 1/2PER groups. During the clamp, the FFA levels were similar in both treatments (POR 514.9±100.2; 1/2PER 539.6±109.3 uEq/l) (figure #13).
Figure #4
Plasma glucose levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Euglycemia / Euglycemic Clamp
Glucose Levels

-○- 1/2 PER
-●- POR

GLUCOSE (uU/ml)

-50 0 50 100 150 200

TIME (min)
n=6
Figure #5
Plasma glucose specific activity in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Euglycemia / Euglycemic Clamp
Plasma Glucose Specific Activity

\[ \text{SPECIFIC ACTIVITY (uCi/g)} \]

\[ \text{TIME (min)} \]

- - 1/2 PER
- - POR

n=6
Figure #6
Peripheral insulin concentrations in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Euglycemia / Euglycemic Clamp
Peripheral Insulin Levels

---

PERIPHERAL INSULIN (uU/ml)

TIME (min)

- ○ 1/2 PER
- ● POR

n=6
Figure #7
Estimated Hepatic Insulin levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; p=.0001.

Basal Euglycemia / Euglycemic Clamp
Estimated Hepatic Insulin Levels

![Graph showing estimated hepatic insulin levels](image-url)
Figure #8
Glucose utilization in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).
Figure #9
Metabolic clearance rate in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Euglycemia / Euglycemic Clamp
Metabolic Clearance Rate

![Graph showing metabolic clearance rate over time for POR and 1/2PER conditions.]
Figure #10
Glucose infusion rates in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (p=0.02).

Basal Euglycemia / Euglycemic Clamp
Glucose Infusion Rates

[Graph showing glucose infusion rates over time for POR and 1/2PER conditions, with n=6 indicated.]
Glucose production in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; p=.0001.
Figure #12
Glucagon levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

**Basal Euglycemia / Euglycemic Clamp**

**Glucagon Levels**

![Graph showing glucagon levels during basal euglycemia and during a euglycemic clamp. The graph compares glucagon levels in portally (POR) and peripherally (1/2PER) infused groups. The x-axis represents time in minutes, and the y-axis represents glucagon levels in pg/ml. The graph indicates a decrease in glucagon levels over time in both groups, with POR showing slightly higher levels compared to 1/2PER. The error bars indicate variability.]
Figure #13
Free fatty acid levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=6) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=6) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Euglycemia / Euglycemic Clamp
Free Fatty Acid Levels

![Graph showing free fatty acid levels over time for POR and 1/2PER conditions.](image-url)
3.2 Basal Hyperglycemia / Euglycemic Clamp (Study 2)

The following results are based on an n=5 for POR and 1/2PER. The basal plasma glucose levels were maintained constant at moderate hyperglycemia (POR 189.5±5.3; 1/2PER 181.1±9.3 mg/dl). Throughout the course of the clamp, the glucose levels declined until euglycemia was reached and maintained for the remainder of the clamp (Figure #14). Plasma glucose specific activity increased slightly from the basal values of 2.1±0.1 (POR) and 2.3±0.2 (1/2PER) to 2.5±0.1 (POR) and 2.6±0.2 (1/2PER) during the clamp period (figure #15). The increase was ~20% and ~13% in the POR and 1/2PER groups respectively. The portal dose to achieve moderate hyperglycemia during the basal period was 0.3±0.0 (POR) and 0.2±0.0 mU/kg.min, (n.s.) (1/2PER). The basal peripheral insulin levels were 6.7±0.8 and 5.4±0.3 uU/ml in the POR and 1/2PER groups respectively (figure #16). The POR treatment raised the peripheral insulin levels to 23.2±2.9 uU/ml. The 1/2PER treatment raised insulin levels to 23.8±2.4 uU/ml. There was no difference in the increase in peripheral insulin levels during the clamp between POR and PER.

The estimated basal portal insulin levels were 25.3±2.8 and 20.3±2.6 uU/ml in the POR and 1/2PER treatments. During the clamp, POR and 1/2PER raised portal insulin levels to 98.2±4.4 and 38.6±3.4 uU/ml. The portal insulin levels with POR were significantly greater than 1/2PER (p=0.0001). Insulin extraction by the liver was calculated to be 55%. The 1/2PER insulin dose administered after basal was 0.39 mg/kg.min. The estimated basal hepatic insulin levels were 20.1±2.2 and 16.2±1.9 uU/ml in the POR and 1/2PER treatments respectively (figure #17). During the clamp, hepatic insulin levels were
significantly higher (p=0.0001) in POR than 1/2PER (POR 77.2±3.5; 1/2PER 34.4±2.9 uU/ml; figure#17).

The GU in the basal periods was 3.6±0.1 and 3.5±0.4 mg/kg.min, (n.s.) in the POR and 1/2PER treatments respectively. GU remained relatively constant during the clamp except in both groups except in the last 50 minutes, where GU was significantly higher in 1/2PER (p<0.05). The values at the last time point (180 minutes) were 3.9±0.3 and 5.0±0.6 mg/kg.min in the POR and 1/2PER treatments respectively (figure #18). MCR in the basal period was 1.9±0.1 and 2.0±0.3 ml/kg.min, (n.s.) and in the clamp period was 3.3±0.3 and 3.6±0.3 in the POR and 1/2PER groups respectively (figure #19). The dextrose infusion rates necessary to maintain glycemia constant are shown in figure #20. Both MCR and GINF were higher in 1/2PER than POR in the last 50 minutes (p<0.05).

Basal endogenous GP was 3.2±0.3 and 3.3±0.3 mg/kg.min, (n.s.) in the POR and 1/2PER treatments respectively. When the additional insulin was given, GP was suppressed to the same extent for most of the clamp. During the last 1/2 hour, the POR treatment suppressed GP to a greater than the 1/2PER treatment, p<0.036 (figure #21).

The basal glucagon levels were 59.2±8.4 and 61.3±6.2 pg/ml, (n.s.) in the POR and 1/2PER groups. During the clamp, the glucagon levels were similar in both treatments (POR 54.8±7.1; 1/2PER 48.8±6.5) (figure #22).

The basal FFA were 981±85.8 and 1062.4±72.6 uEq/l, (n.s.) in the POR and 1/2PER groups. During the clamp, the FFA levels were similar in both treatments (POR 524.2±63.3; PER 441.6±57.4) (figure #23).
Figure #14
Plasma glucose levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Hyperglycemia / Euglycemic Clamp
Glucose Levels
Figure #15
Plasma glucose specific activity in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Hyperglycemia / Euglycemic Clamp
Plasma Glucose Specific Activity

![Graph showing plasma glucose specific activity over time for POR and 1/2PER conditions.](image-url)
Figure #16
Peripheral insulin concentrations in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).
Figure #17
Estimated hepatic insulin levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; p=.0001.

Basal Hyperglycemia / Euglycemic Clamp
Estimated Hepatic Insulin Levels

CALCULATED HEPATIC INSULIN (uU/ml)

1/2 PER
POR

TIME (min)

n=5
Figure #18
Glucose utilization in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; p<0.05 in the last 50 minutes.

Basal Hyperglycemia / Euglycemic Clamp
Glucose Uptake
Figure #19
Metabolic clearance rate in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; p<0.05 in the last 50 minutes.

Basal Hyperglycemia / Euglycemic Clamp
Metabolic Clearance Rate

TIME (min)

METABOLIC CLEARANCE RATE (ml/kg.min)

n=5
Figure #20
Glucose infusion rates in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; p<0.05 in the last 50 minutes.

Basal Hyperglycemia / Euglycemic Clamp
Glucose Infusion Rates
Figure #21
Glucose production in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; p<0.036 in the last 30 minutes.

Basal Hyperglycemia / Euglycemic Clamp
Glucose Production
Figure #22
Glucagon levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp. POR vs. 1/2PER; (n.s.).

Basal Hyperglycemia / Euglycemic Clamp
Glucagon Levels
Free fatty acid levels in response to full dose insulin infusion (0.9 mU/kg.min) portally (POR; n=5) or ~45% (0.39 mU/kg.min) peripherally (1/2PER; n=5) during a euglycemic hyperinsulinemic clamp.

Basal Hyperglycemia / Euglycemic Clamp
Free Fatty Acid Levels

![Graph showing free fatty acid levels](image-url)
3.3 Basal Euglycemia / Insulin Replacement Studies (Study 3)

The following results are based on an n=6 for POR REPL and PER REPL. The plasma glucose levels were constant at euglycemia (POR REPL 110.4±2.5; PER REPL 113.4±3.6 mg/dl, (n.s.); figure #24). The insulin dose to achieve euglycemia was 0.69±0.08 (POR REPL) and 0.34±0.04 mU/kg.min (PER REPL).

Endogenous GP (GP=GU) in basal steady state conditions like those of this protocol was 2.9±0.2 and 2.6±0.1 mg/kg.min, (n.s.) in the POR REPL and PER REPL treatments respectively (figure #25).

The peripheral insulin levels were significantly higher in the PER REPL (p=0.0005) than in POR REPL (PER REPL 17.0±2.1; POR REPL 11.9±1.1 uU/ml; figure #26). The estimated portal insulin levels were 55.2±5.7 and 17.0±2.1 in the POR REPL and PER REPL treatments. The estimated hepatic insulin levels were 43.1±4.3 and 17.0±2.1 uU/ml in the POR REPL and PER REPL treatments respectively (figure #27). Both the estimated hepatic and portal insulin levels were significantly greater in the POR REPL than the PER REPL treatment (p=0.0001). Insulin extraction by the liver, calculated from the rates of the systemic insulin delivery rates was 58.3%.

The basal glucagon levels were similar in both treatments (POR REPL 61.3±5.0; PER REPL 59.9±3.7) (figure #28).

The FFA were 858.6±98.0 and 878.8±112.5 umol/l, (n.s.) in the POR REPL and PER REPL groups (figure #29).
Basal Euglycemia / Insulin Replacement Studies
Glucose Levels

Figure #24
Plasma glucose levels in response to portal (0.69 mU/kg.min) insulin replacement (POR REPL; n=6) or peripheral (0.34 mU/kg.min) insulin replacement (PER REPL; n=6) to achieve and maintain euglycemia.
Figure #25
Glucose production in response to portal (0.69 mU/kg.min) insulin replacement (POR REPL; n=6) or peripheral (0.34 mU/kg.min) insulin replacement (PER REPL; n=6) to achieve and maintain euglycemia.

Basal Euglycemia / Insulin Replacement Studies
Glucose Production

![Graph showing glucose production with bars for POR REPL and PER REPL]
Peripheral insulin levels in response to portal (0.69 mU/kg.min) insulin replacement (POR REPL; n=6) or peripheral (0.34 mU/kg.min) insulin replacement (PER REPL; n=6) to achieve and maintain euglycemia.

Basal Euglycemia / Insulin Replacement Studies
Peripheral Insulin Levels

![Bar chart showing peripheral insulin levels for POR REPL and PER REPL treatments, with a significant difference indicated by a star (*) and p = 0.0005.](chart.png)

n=6
Calculated Hepatic insulin levels in response to portal (0.69 mU/kg.min) insulin replacement (POR REPL; n=6) or peripheral (0.34 mU/kg.min) insulin replacement (PER REPL; n=6) to achieve and maintain euglycemia.

Basal Euglycemia / Insulin Replacement Studies

Calculated Hepatic Insulin Levels

![Bar graph showing calculated hepatic insulin levels for portal (POR REPL) and peripheral (PER REPL) insulin replacement.](image)

- **POR REPL**
- **PER REPL**

**CALCULATED HEPATIC INSULIN (uU/ml)**

- n=6
- p = 0.0001
Figure #28
Glucagon levels in response to portal (0.69 mU/kg.min) insulin replacement (POR REPL; n=6) or peripheral (0.34 mU/kg.min) insulin replacement (PER REPL; n=6) to achieve and maintain euglycemia.

Basal Euglycemia / Insulin Replacement Studies
Glucagon Levels

![Glucagon Levels Graph]

- **POR REPL**
- **PER REPL**

n=6
Figure #29
Free fatty acid levels in response to portal (0.69 mU/kg.min) insulin replacement (POR REPL; n=6) or peripheral (0.34 mU/kg.min) insulin replacement (PER REPL; n=6) to achieve and maintain euglycemia.

Basal Euglycemia / Insulin Replacement Studies
Free Fatty Acid Levels
4. DISCUSSION

4.1. Basal Euglycemia / Euglycemic Clamp (Study 1)

In this study in the diabetic depancreatized dogs, at matched peripheral insulin levels, POR insulin infusion was more effective than 1/2PER insulin infusion in suppressing glucose production during a euglycemic clamp. This is in contrast to our previous studies (68,70) which have shown, under conditions of moderate hyperglycemia that both POR and 1/2PER insulin infusions suppressed GP to the same extent.

Once the dogs were clamped at euglycemia with an initial portal insulin dose, an additional insulin dose was administered either portally (POR) or peripherally (1/2PER) to match the peripheral insulin levels. Previously, the peripheral dose was chosen to be half of the portal dose (1/2PER) due to the assumption of 50% insulin extraction by the liver (19). However, in our dogs insulin extraction by the liver was found to be greater than 50% and therefore a dose slightly lower than half the portal dose was required to match peripheral insulin levels. The 1/2PER treatment resulted in equal peripheral insulin levels to those of the POR treatment, but the calculated portal and hepatic insulin levels were much lower when compared to the POR treatment. With a selective difference in hepatic insulin levels, any differences in glucose production can be attributed to this difference in insulin.

The glucose clamp technique was used in this study as well as in the previous study. This allowed us to investigate the effect of insulin without the confounding effects of counter-regulatory hormones. A radio-labelled glucose infusate was used to maintain the plasma glucose specific activity constant throughout the clamp, therefore minimizing any errors caused by a change in specific activity when calculating glucose production with a
fixed pool-mono compartmental model (127,128). In this study, we wished to investigate whether the lack of a direct effect of insulin on GP in the previous study was due to the high glucose level. The hypothesis was that at euglycemia, this direct effect would be restored.

The peripheral insulin levels were the same in both treatments (POR and 1/2PER). This was because the peripheral insulin dose was ~45% that of the portal dose, due to ~55% hepatic extraction.

GU was matched in both cases. This was expected since the peripheral insulin levels were matched. GU increased in both treatments from basal. This occurred due to the additional dose of insulin administered at time 0. These results were the same as seen previously in our studies in depancreatized dogs at the same insulin dose (68). The GINF was significantly greater in the POR treatment, compared to the 1/2PER treatment. This was interesting because GINF is dependent on both GU and GP, and with matched GU, this observation indicated that GP was suppressed to a greater extent with POR than 1/2PER.

Indeed, unlike the previous study, the suppression of GP in the diabetic dogs was greater with POR than with 1/2PER insulin infusion. The fact that a greater suppression of GP was found with POR in our study leads to the conclusion that euglycemia acutely restores the direct hepatic effect of insulin on GP. This is consistent with our findings in normal dogs (61) and humans (62).

Glucagon and FFA levels decreased to the same extent as expected because peripheral insulin levels were the same in both treatments. With respect to glucagon, this is in accordance with the suppressive effect of peripheral insulin on glucagon secretion, as the glucagon secretory cells in the gastric mucosa of the depancreatized dog are exposed to
peripheral insulin levels with both portal and peripheral infusions because the site of portal infusion is downstream from the pancreas.

It can therefore be concluded that in diabetic depancreatized dogs, at matched peripheral insulin levels, POR insulin infusion was more effective than 1/2PER insulin infusion in suppressing glucose production during a euglycemic clamp. In our previous studies (68,70) it was shown under conditions of moderate hyperglycemia that both POR and 1/2PER insulin infusions suppressed GP to the same extent, however at euglycemia as has been shown here, the suppression of GP with POR insulin infusion was significantly greater than that of 1/2PER insulin infusion. This indicates that euglycemia acutely restores the direct hepatic effect of insulin of glucose production in depancreatized diabetic dogs.
4.2. Basal Hyperglycemia / Euglycemic Clamp (Study 2)

Since the insulin levels needed to achieve euglycemia are higher than those needed to achieve moderate hyperglycemia, it is possible that the restoration of the direct effect of insulin on GP in study 1 was not due to euglycemia but to higher insulin levels. Compared to our previous studies where moderate hyperglycemia was achieved through insulin infusion and then kept constant for the remainder of the experiment, in this study, euglycemia was achieved and therefore more insulin was required. It is possible that this extra insulin could induce a direct effect. Therefore in this protocol insulin was administered until we reached moderate hyperglycemia, as performed previously. To examine the effect of euglycemia, the glucose level was allowed to fall until euglycemia was reached. Euglycemia was then maintained for the remainder of the experiment by the glucose clamp technique. In this protocol, the basal insulin levels were the same as in the previous study (70) and therefore any difference in hepatic glucose production between POR and 1/2PER can be attributed to euglycemia. It was found that near the end of the clamp, when euglycemia was reached, there was a greater suppression of glucose production with POR as compared to 1/2PER. However because the duration of our clamp was only 3 hours, the direct effect was only present for \(~30\) minutes. It would have been interesting to extend our clamp for an additional hour to see if the direct effect persists as long as euglycemia is maintained.

Another aspect that we wished to study with this protocol was the acuteness of the effect of euglycemia in restoring the direct effect. In other words, we wished to observe once euglycemia was achieved, how long it took before the direct effect becomes manifest.

In depancreatized dogs under conditions of basal hyperglycemia at matched peripheral insulin levels, POR insulin infusion appeared to be more effective than PER
insulin infusion in suppressing GP once euglycemia was achieved. It was shown previously that when the glucose levels were maintained as moderate hyperglycemia, POR and 1/2PER insulin infusion suppressed GP to the same extent.

The peripheral insulin levels, as in the first protocol, were the same in both treatments. This was because the peripheral insulin dose was ~45% that of the portal dose, due to ~55% hepatic extraction. By matching peripheral insulin levels, any differences in glucose production between the two treatments could be attributed to the differences in hepatic insulin levels.

Unlike the first protocol, glucose uptake was not the same in both treatments. This was surprising considering the peripheral insulin levels were matched. It is quite possible that there were differences in insulin sensitivity between the two groups due to experimental variability despite the experiments being paired. This may be supported by the lower insulin infusion rates necessary to achieve euglycemia in the basal period in the 1/2PER group. GU did not change from basal in both experiments except for the last hour of the PER treatment where it increased. The lack of change of GU in the first part of the experiment was due to the glucose decline, as glucose clearance was increased by insulin as expected. The GINF was greater in the 1/2PER treatment, compared to the POR treatment, due to greater GU with 1/2PER compared to POR.

Up until about the 150 minute mark, GP in both treatments were suppressed to the same extent. After 150 minutes and for the remainder of the clamp however, the suppression of GP was greater with POR than 1/2PER. It could be argued that this observation is preliminary because the GU were not matched. However, despite less glucose utilization with the POR treatment, the GP suppression was greater than 1/2PER which would indicate
that the direct effect of insulin was likely underestimated in the more insulin resistant POR group. It is likely that at matched GU, the suppression of GP would be even greater than currently observed with POR compared to 1/2PER. Once euglycemia was achieved at ~100 minutes, it took ~50 minutes for the direct action of insulin to become manifest. This time course is consistent with an acute effect of correction of hyperglycemia on GP. Again, the 50 minute time may be overestimated due to the presence of some insulin resistance in POR vs. 1/2PER. Glucagon levels and FFA decreased to the same extent when measured in the peripheral circulation. This is due to the same reasons as in the previous protocol for the peripheral insulin levels were matched in both treatments.
4.3. Basal Euglycemia / Insulin Replacement Studies (Study 3)

In the third and final protocol, we wished to look more closely at the treatment of a type 1 diabetic patient. Due to lack of insulin secretion by the pancreas, the normal physiological portal-peripheral insulin gradient is abolished. This is important because type 1 diabetic patients are treated with subcutaneous injections of insulin, which result in peripheral absorption of insulin. For this reason, portal levels of insulin can never be greater than peripheral levels. It is thought that in the absence of a portal-peripheral gradient, peripheral hyperinsulinemia is needed to adequately insulinize the liver, in order to suppress glucose production. However, there is increasing evidence that peripheral hyperinsulinemia is a risk factor for atherosclerosis (24), and arterial hypertension (25).

Two treatments were set up, one in which insulin is administered through the peripheral circulation (PER REPL), as in the traditional treatment of a type 1 diabetic. In the second treatment, insulin was administered into the portal vein, on a direct route to the liver (POR REPL). We wished to compare the effects of treating a diabetic subjects with insulin peripherally, as in the standard treatment, with infusing insulin into the portal vein, as seen in normal physiology. Insulin was delivered by either route until euglycemia was achieved.

With similar glucose levels, the level of insulin necessary to achieve this desired level of glycemia was measured. When measured in the peripheral circulation, a significantly greater level of insulin was needed with PER REPL than POR REPL to achieve the same euglycemic level. Hepatic insulin extraction was calculated at ~60%. Furthermore, the insulin doses required to achieve euglycemia in both treatments were also measured. The POR REPL insulin dose was 0.69 and the PER REPL insulin dose was 0.34 mU/kg.min. For no direct effect to be present, the PER REPL dose would have been ~40% that of the POR
REPL dose due to 60% insulin extraction. However, the PER REPL dose was ~50% of the POR REPL dose indicating that a direct effect was present.

Therefore in our depancreatized dogs under conditions of fasting insulin induced euglycemia, at matched glucose levels and GP, less peripheral insulin was required with POR REPL than with PER REPL insulin infusion. This suggests that POR REPL achieved the same glucose levels and GP because of a direct effect of the greater hepatic insulinization. If the direct effect was not present, the glucose levels in the POR REPL would be higher than what was observed.
5. General Discussion

We have shown here for the first time in depancreatized diabetic dogs that correction of hyperglycemia acutely restores insulin's direct effect on glucose production. This is consistent with our previous findings in normal dogs (61) and humans (62). The difference in GP suppression between POR and 1/2PER was even greater than that seen in our previous studies in normal dogs (61). It might be argued that the studies in normal dogs were carried out with a lower insulin dose (both POR and 1/2PER) However, the direct effect of insulin was greater in the normal dogs at a low insulin dose compared to a high insulin dose. For this reason, it is safe to say that if the same protocol were conducted in our depancreatized dogs at a lower insulin dose, the difference between POR and 1/2PER would be even greater than already shown, indicating that the restoration of insulin's direct effect on GP by acute euglycemia was likely complete.

Soskin and Levine (133) first proposed that mammalian liver can rapidly change its glucose output in response to changes in the circulating glucose concentration independent of hormonal signals. It is now recognized that the plasma glucose concentration in part regulates GP (134,135). The general notion is that hyperglycemia suppresses GP. Recently, a lot of information has become available about the mechanism(s) by which hyperglycemia, independent of insulin, inhibits GP. Rossetti et al. (124) found that hyperglycemia caused a marked inhibition of hepatic glucose production mainly through the suppression of glycogenolysis and the increase in GK flux with no apparent changes in the fluxes through gluconeogenesis and G6Pase. With glycogenolysis already maximally suppressed, hepatic insulin which suppresses glycogenolysis may not further suppress GP. For this reason, the
direct effect of insulin on GP is lost. It is thought that at euglycemia, glycogenolysis is not suppressed and therefore insulin is able to directly suppress GP. It was known that inhibition of net hepatic glycogenolysis was a key component of glucose action to inhibit GP, however a mechanism needed to be derived. Recently, a study examined the mechanism by which glucose and insulin inhibit net hepatic glycogenolysis in humans (136). It was shown that hyperglycemia, per se, inhibits net hepatic glycogenolysis primarily through inhibition of glycogen phosphorylase flux (136), and that hyperinsulinemia inhibits net hepatic glycogenolysis primarily through stimulation of glycogen synthase flux (136). However, with hyperinsulinemia and hyperglycemia, inhibition of glycogen phosphorylase and activation of glycogen synthase are not necessarily coupled and coordinated. The masking of insulin’s direct effect by hyperglycemia would explain why at moderate hyperglycemia, during a hyperinsulinemic glucose clamp, a direct effect of insulin on hepatic glucose production was not found in our depancreatized diabetic dogs (68,70).

The role of glycogenolysis in the direct effect of insulin is also evident from the results in our studies evaluating the effect of glucagon on the direct effect of insulin. When glucagon levels were kept constant by a high-dose portal glucagon infusion in diabetic dogs, equimolar peripheral infusion was no longer more potent than portal insulin in suppressing GP and POR insulin infusion became more potent than 1/2PER infusion (100). This could result because in the presence of glucagon the direct effect of insulin in suppressing hepatic GP is potentiated. Mittelman et al. (101) recently showed that the direct portal effect of insulin in suppressing hepatic GP is enhanced when glucagon levels are elevated with a glucagon infusion in somatostatin-treated dogs. A more recent study examined the effect of elevated glucagon levels on insulin’s direct effect on hepatic GP in humans (100).
Tolbutamide was administered to allow for endogenous pancreatic insulin secretion (POR group). It was found that GP was suppressed to a greater extent during tolbutamide infusion than in the PER group (full dose) when glucagon was administered continuously throughout the basal and hyperinsulinemic periods. Therefore, it seems that glucagon sensitizes the liver to the direct suppressive effect of insulin. Previous *in vitro* studies have also shown that the effects of insulin in isolated hepatocytes or in the perfused liver are greatly accentuated in the presence of glucagon (137). It is known that with glucagon, glycogenolysis is accentuated, and it may be known that this facilitates insulin’s effect in directly suppressing GP by inhibiting glycogenolysis.

In the second study, we wished to study the acuteness of the effect of correction of hyperglycemia in restoring the direct effect. Once euglycemia was achieved, approximately 50 minutes later the direct effect started to become manifest. There was a tendency for the POR dose to suppress GP to a greater extent which became significant in the last 30 minutes. If the clamp were extended for an additional hour, more definite conclusions could be made with regards to the direct effect being restored.

In our study, we tested the effect of acute correction of hyperglycemia on insulin’s direct effect. An interesting question is whether there is an effect of *chronic* euglycemia in restoring insulin’s direct effect on GP. It is possible that the effect of chronic euglycemia on insulin’s direct effect may be by preventing the impairment of hepatic glucose metabolism due to insulin resistance and glucose toxicity. To study the effect of chronic euglycemia, tighter control of glycemia should have been achieved by continuously monitoring the dogs glycemic levels for a few days, and the experiments redone at hyperglycemia. However, it
has been shown here that acute euglycemia appears to completely restore the direct effect and for this reason there may not be much space for the effect of chronic euglycemia.

In the third study, it was noted that the levels of GP and GU were the same with both POR REPL and PER REPL. This is surprising since the levels of insulin are different. With higher insulin levels in the peripheral circulation and less in the portal circulation with PER REPL, it is expected that GP=GU would be higher. In addition, the levels of glucagon and FFA were similar in both protocols, presumably because the difference in the peripheral insulin levels was not great. It is possible that the greater peripheral insulin levels were enough to inhibit GP by a FFA and glucagon independent effect (effect on renal glucose production?) whereas the difference in insulin levels had no differential peripheral effects. This is not surprising since we have shown that the small higher peripheral insulin levels do not affect GU in these dogs (70). Another possibility is that 1/2PER group may have been more insulin resistant, however the slight hyperinsulinemia with PER REPL may not have been long enough to cause insulin resistance. It has been shown that at least 12 hours of hyperinsulinemia are needed to cause insulin resistance (138,139).

In our insulin replacement studies (study 3), it was shown that lower levels of insulin in the peripheral circulation were present with portal versus peripheral delivery to achieve euglycemia. This is important since the traditional route of insulin delivery in type 1 diabetic patients is subcutaneous. With subcutaneous injections, insulin is absorbed peripherally, and therefore the portal-peripheral insulin gradient is lost. We showed acutely that the same levels of glycemia can be achieved with less systemic insulin levels with POR REPL versus PER REPL, and therefore our results show that the peripheral route of insulin administration can induce peripheral hyperinsulinemia. We studied the acute effect of portal
versus peripheral insulin replacement. However, the chronic effect is more important in the
treatment of type 1 diabetes as in diabetes, insulin treatment is chronic. We have shown in
streptozotocin (STZ) diabetic rats that hepatic glucose production is suppressed to a greater
extent following chronic insulin treatment via the intraperitoneal (IP) cavity versus
subcutaneous (SC) route (112), despite a major difference in peripheral insulin levels,
suggesting that the direct effect of insulin is even more accentuated during chronic insulin
treatment. This is likely due to insulin’s effect on the gene expression and activity of hepatic
enzymes. In another study in STZ-diabetic rats, we have shown that the difference in hepatic
insulinization between chronic IP and SC insulin treatment can lead to a difference in hepatic
enzyme activities in vivo. It was shown (140) that G6Pase activity was elevated in untreated
diabetic rats but was restored to normal levels following chronic insulin treatment regardless
of route. GK activity was lower in untreated diabetes. SC insulin treatment only partially
restored GK levels to normal, however with IP treatment GK activity was increased to even
greater levels than normal. This suggests that the difference in hepatic insulinization
between the two routes of treatment results in differences in the activity of hepatic enzymes
and that GK is more sensitive than G-6-Pase to differences in hepatic insulin levels brought
about by chronic IP versus SC treatment in rats.

The question that was posed at the start of this study was that, if no direct effect of
insulin on GP is present in diabetes as seen previously at hyperglycemia, then SC insulin
treatment would be an adequate treatment modality as far as GP is concerned. However, our
studies have clearly shown that the direct effect is very apparent at euglycemia.
Furthermore, we have also shown that at basal euglycemia, lower levels of peripheral insulin
are required with portal insulin replacement therapy compared to peripheral insulin
replacement. Thus, the physiological route of insulin delivery has some advantage over the peripheral route and therefore further investigation or alternative routes of insulin treatment is justified.
6. **GENERAL SUMMARY AND CONCLUSIONS**

It in the current study, in depancreatized dogs under conditions of basal euglycemia, at matched peripheral insulin levels, POR insulin infusion was shown to be more effective than 1/2PER insulin infusion in suppressing GP during a euglycemic clamp. This clearly indicates that the greater hepatic insulinization with portal delivery has a direct effect of inhibiting GP. Furthermore, in moderately hyperglycemic depancreatized dogs, POR insulin infusion appeared to be more effective than PER insulin infusion in suppressing GP, once euglycemia was achieved.

Lastly, under conditions of euglycemia, less peripheral insulin was required to match glucose levels with POR REPL than with PER REPL insulin infusion which suggests that POR REPL achieved the same glucose levels because of a direct effect of the greater hepatic insulinization.

Since we have shown for the first time that the direct effect of insulin in suppressing GP is present with portal insulin treatment as compared to peripheral insulin treatment in depancreatized diabetic dogs under euglycemic conditions, further studies aimed at producing a safe and relatively non-invasive portal insulin delivery system are warranted.
7. REFERENCES


