

MECHANISMS OF HYPOTHALAMIC AND SMALL INTESTINAL NUTRIENT SENSING

by

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A thesis submitted in conformity with the requirements for the degree of

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“Mechanisms of hypothalamic and small intestinal nutrient sensing”

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General Abstract

Nutrient sensing pathways in both the brain and gut decrease hepatic glucose production. Hypothalamic activation of lactate metabolism decreases glucose production, but it is unknown whether the hypothalamus detects circulating lactate to maintain glucose homeostasis. In the gut, lipids decrease glucose production via a neuronal network but the downstream signaling mechanisms are unknown. We tested whether circulating lactate activates central lactate metabolism to decrease glucose production *and* postulated that duodenal protein kinase C (PKC) acts downstream of lipids to decrease glucose production through a neuronal network. We report that central lactate metabolism is required for the maintenance of glucose homeostasis in the presence of circulating lactate *and* that activation of duodenal PKC is required for lipids to decrease glucose production. This shows the importance of the brain and gut in the regulation of glucose production, and could pave the way for restoration of glucose homeostasis in disease.

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Be the change you wish to see in this world -Mahatma Gandhi

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List of Abbreviations

ACC	Acetyl-Coenzyme A carboxylase
ACS	Acyl-Coenzyme A synthetase
ACSF	Artificial cerebrospinal fluid
AMPK	Adenosine-mono-phosphate-activated protein kinase
BIM	Bisindolymaleimide
BBB	Blood-brain-barrier
C75	Cerulein
CNS	Central nervous system
CPT-1	Carnitine palmitoyltransferase-1
CCK	Cholecystokinin
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
FA	Fatty acid(s)
FA-CoA	Fatty-acyl Coenzyme A
FAS	Fatty acid synthase
FFA	Free fatty acids
G3P	Glycerol-3-phosphate
GLI	Glycenclamide
GPR	G-protein coupled receptor
HFD	High-fat diet
Icv	Third cerebral ventricle
IH	Intra-hypothalamic
IP	Intraperitoneal
IP3	Inositol 1,4,5-triphosphate
IRS	Insulin receptor substrate
IV	Intravenous
K _{ATP}	Adenosine-tri-phosphate-sensitive potassium (channel)
LCFA	Long-chain fatty acid(s)
LCFA-CoA	long-chain fatty acyl Coenzyme A
LDH	Lactate dehydrogenase
MBH	Mediobasal hypothalamus
MCD	Malonyl-coenzyme A decarboxylase
MCT	Monocarboxylate transporter

NMDA	N-methyl-D-aspartate
NTS	Nucleus of the solitary tract
OAG	1-oleoyl-2-acetyl-sn-glycerol
OXA	Oxamate
PDH	Pyruvate dehydrogenase
PI	Phosphatidylinositol
PKC	Protein kinase c
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
ROT	Rottlerin
SAL	Saline
SRIF	Somatostatin
TLR	Toll-like receptor
TG	Triglyceride(s)
UDP	Uridinediphosphoglucose
VEH	Vehicle

Publications that Contributed to this Thesis

Kokorovic A, Cheung GW, Rossetti L and Lam TK. Hypothalamic sensing of circulating lactate regulates glucose production. *Journal of Cellular and molecular Medicine*: 13(11-12), 4403-8 (2009).

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Introduction

1.1 OBESITY AND TYPE 2 DIABETES

Obesity and its related co-morbidities, particularly type 2 diabetes mellitus, are becoming an increasing concern in our society. With more than 1 billion people overweight [1], it is evident that the disease has reached epidemic proportions. The cause of this increased incidence is largely the result of consumption of high caloric diets, combined with an overall decrease in physical activity [2].

Obesity is tightly associated with insulin resistance, in which the body is unable to effectively promote glucose disposal in response to insulin [3]. In skeletal muscle and adipose tissue, this manifests itself as the inability of insulin to elicit glucose uptake, whereas in the liver it is the inability of insulin to decrease hepatic glucose production. These defects, combined with impaired insulin secretion, result in hyperglycemia [3].

The key characteristic of type 2 diabetes is fasting hyperglycemia, which arises partly due to an inappropriate elevation of hepatic glucose production [4]. If left untreated, diabetes and the associated hyperglycemia cause serious complications, including microvascular [5]and

cardiovascular disease [6], as well as some cancers [7], which dramatically affect the quality of life of the individual. Hence, great interest lies in understanding the biological and physiological mechanisms governing glucose homeostasis, and current research is focused on developing better therapies to treat this debilitating condition.

The mechanisms linking obesity with insulin resistance and type 2 diabetes are not yet fully understood. However, research has shown that some of the nutrient sensing mechanisms designed to maintain glucose homeostasis are impaired in diet-induced insulin resistance, partially contributing to the increased glucose production, and resulting hyperglycemia [8]. It is the focus of this thesis to examine the nutrient sensing mechanisms in the brain and the small intestine which act to regulate glucose homeostasis.

1.2 NUTRIENT SENSING IN THE CENTRAL NERVOUS SYSTEM

It has long been recognized that the central nervous system (CNS) is a critical regulator in the body's ability to maintain whole body homeostasis. Homeostatic mechanisms assure that a stable internal environment is maintained, regardless of exterior changes. The hypothalamus has been implicated in maintaining a number of these parameters, including body temperature [9], blood pressure [10], energy homeostasis [11], as well as glucose homeostasis [8]. In particular, it has been found that the hypothalamus can sense nutrients, such as lipids and glucose [12], to regulate the latter two parameters. Since these discoveries, the notion of hypothalamic *nutrient sensing* (an acute accumulation of nutrients) mechanisms has been forwarded.

In this section I will discuss the central nervous system of energy and glucose homeostasis by lipid and glucose/lactate sensing.

1.2.1 Glucose/Lactate Sensing

Glucose is critical to the brain, since it serves as the primary source of fuel for this vital organ. However, the discovery of glucose sensing neurons in the brain alluded to the notion that central glucose may also serve a physiological purpose [12]. Accordingly, research has demonstrated that in addition to being a fuel substrate, central glucose metabolism regulates energy intake [13-15] and as has recently been demonstrated, hepatic glucose production [16].

Glucose metabolism in the brain occurs via its metabolite lactate, in a proposed mechanism that is termed the *astrocyte-neuron lactate shuttle* [17], which couples neuronal activity to glucose utilization [18]. According to this hypothesis, the neurotransmitter glutamate enters astrocytes through a Na⁺ powered concentration gradient, whereby intracellular Na⁺ levels are increased, and glutamate is converted to glutamine. This results in the activation of the ATPase pump, which promotes astrocytic glycolysis. The end substrate of glycolysis, pyruvate, is then converted to L-lactate by the muscle form of lactate dehydrogenase, (LDH)-A. Accumulation of L-lactate in the astrocyte results in its efflux into the extracellular fluid, via the monocarboxylate transporter (MCT)-1. L-lactate is subsequently taken up by neurons via the MCT-2 transporter, and following its entry into neurons is reconverted into pyruvate by the heart form of LDH-B. Lastly, pyruvate is

converted to acetyl-CoA by pyruvate dehydrogenase (PDH), which activates ATP-sensitive potassium (K_{ATP}) channels.

A recent discovery has highlighted the importance of central glucose in the regulation of peripheral glucose levels [16]. An acute infusion of glucose into the third cerebral ventricle (icv) of rodents rapidly decreased hepatic glucose production, as assessed by a basal insulin pancreatic clamp, by inhibiting both gluconeogenesis and glycogenolysis [16]. This effect was recapitulated with administration of icv L-lactate, as predicted by the *astrocyte-neuron lactate shuttle* [17] hypothesis. To further delineate the biochemical steps required for this effect, icv oxamate (OXA, a competitive inhibitor of LDH) was co-infused with L-lactate or glucose. In both cases, OXA blunted the observed decrease in hepatic glucose production, demonstrating that conversion to pyruvate is necessary. This was further supported by intra-hypothalamic (IH) infusion of dichloroacetate (an activator of PDH via its inhibition of PDH kinase), which resulted in a suppression of glucose production. In an independent study, it was shown that K_{ATP} channel activation decreases hepatic glucose production [19]. In parallel with the notion that glucose metabolism ultimately results in K_{ATP} channel activation, icv co-infusion of the K_{ATP} channel blocker glibenclamide (GLI) with glucose or L-lactate also blunted their ability to decrease glucose production [16]. Hence, these elaborate studies

demonstrate that hypothalamic glucose metabolism regulates peripheral glucose levels via the activation of K_{ATP} channels.

In the presence of elevated circulating glucose levels, hepatic glucose production is markedly suppressed [20]. Since glucose readily crosses the blood-brain-barrier (BBB) via facilitated transport [21], it is plausible that the effect of circulating glucose on glucose production is at least in part due to the central action of glucose. This was indeed found to be the case. When hyperglycemia was induced in rodents receiving IH OXA infusion, the expected decrease in glucose production was blunted by 40%, indicating that hypothalamic glucose metabolism is indeed required for the regulation of glucose fluxes [16]. Interestingly, the ability of glucose to regulate its own production is blunted in type 2 diabetes, since hyperglycemia is unable to decrease hepatic glucose production [22]. It remains to be determined whether this defect lies in an inability of central glucose to decrease glucose production. If this is the case, restoration of hypothalamic glucose sensing could lead to therapeutics for the restoration of glucose levels in type 2 diabetic individuals.

The relative importance of central glucose and lactate sensing mechanisms in the maintenance of peripheral glucose levels is further supported by the fact that direct infusion of lactate or glucose into the ventromedial hypothalamus of rodents severely blunts the hypoglycemic

counterregulatory response [23]. Specifically, under hyperinsulinemic hypoglycemic conditions, glucagon and epinephrine release are severely diminished with the stimulation of central glucose/lactate metabolism. Hence, *central* sensing of circulating glucose levels plays a pivotal role in restoring plasma glucose levels during the hypoglycemic counterregulatory response.

Contrary to the effects of circulating glucose on glucose production, intravenous lactate infusions actually increase hepatic gluconeogenesis [24]. Since glucose production is the net sum of gluconeogenesis and glycolysis, it would be expected that the result would be an increase in glucose production. However, this is not observed due to a compensatory decrease in glycogenolysis [24]. This phenomenon is termed *hepatic autoregulation*, and is observed for lactate as well as free fatty acids (FFA) [24-26]. *It is yet unknown whether hypothalamic lactate sensing mechanisms are designed to counteract the direct stimulatory effect of circulating lactate on hepatic gluconeogenesis to maintain glucose homeostasis. This will be addressed in the first Aim of this thesis.*

1.2.2 Free fatty acid sensing

In addition to its ability to sense glucose and lactate, the CNS is also able to detect changes in levels of FFA. While FFA are not used as a primary energy source centrally, experimental evidence has demonstrated that they serve as important indicators of energy status, allowing the brain to regulate energy and glucose homeostasis accordingly.

It is still debated as to how FFA cross the BBB to gain access to neurons, as there is support for both a passive diffusion model [27-29], as well as evidence to indicate that transport molecules are required [30]. Whichever the proposed mechanism, the biochemical pathway of fatty acid (FA) metabolism following its entry into the brain cells is well known [12]. FA are immediately esterified into fatty-acyl CoA (FA-CoA) by the enzyme acyl-CoA synthetase (ACS). FA-CoA subsequently enter the mitochondria through the acyltransferase carnitine palmitoyltransferase-1 (CPT-1) transporter (located on the outer mitochondrial membrane), where they undergo β -oxidation to generate acetyl-CoA, the entry molecule for the *citric acid cycle*. Following its conversion into citrate and exit from the mitochondria into the cytoplasm, acetyl-CoA can be regenerated from citrate. Here, it is converted into malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC), which can be inhibited by the AMP-activated protein kinase (AMPK). Malonyl-CoA potently suppresses CPT-1 activity,

thereby regulating β -oxidation, and is converted into FA by the enzyme fatty acid synthase (FAS). Alternatively, FA-CoA can be used to generate diacylglycerol (DAG) and triglycerides (TG) via incorporation into the glycolytic end-product glycerol-3-phosphate (G3P). DAG is an activator of the signaling molecule protein kinase C (PKC), which in turn activates K_{ATP} channels. As noted earlier, hypothalamic glucose and lactate metabolism also results in the activation of K_{ATP} channels to lower hepatic glucose production. Hence, the biochemical pathways of lipid and glucose/lactate may converge to collectively regulate glucose homeostasis.

The notion that lipids may be sensed by the CNS to act as a monitor of energy status came from a study in which mice treated centrally with FAS inhibitors demonstrated decreased food intake and body weight [31]. This anorectic effect of central lipid metabolism was followed by the discovery that direct infusion of the long-chain fatty acid (LCFA) oleic acid into the third cerebral ventricle of rats potently diminishes not only food intake, but also hepatic glucose production [32]. Interestingly, the short-chain FA octanoic acid did not elicit the same metabolic effect, demonstrating the requirement of LCFA specifically to induce decreased glucose production and energy intake [32]. In the same study, it was found that the LCFA effect was dependent on the activation of K_{ATP} channels, since the co-administration of oleic acid with the K_{ATP} channel blocker glibenclamide blunted the effect on hepatic glucose production.

In parallel with these data, numerous studies have manipulated various components of the FFA biochemical pathway to test their effects on energy and glucose homeostasis. At the center of these manipulations lies CPT-1 modulation by malonyl-CoA, since its regulation, both directly and indirectly, can control the cellular levels of available FA. The following studies would support the proposed hypothesis that modulation of malonyl-CoA/CPT-1 changes the availability of intracellular LCFA-CoA, which in turn affects energy and glucose homeostasis.

As previously mentioned, the enzyme ACC, which is inhibited by AMPK, catalyzes the formation of malonyl-CoA, which in turn potently inhibits CPT1. Central activation of AMPK increases food intake, while its inhibition decreases both food intake and body weight [33-36]. Since AMPK activation would ultimately result in decreased levels of cellular LCFA-CoA (via decreased malonyl-CoA mediated CPT-1 inhibition), it is likely that its effects on energy homeostasis are acting through LCFA-CoA.

Likewise, changes in central ACC activity have been shown to modulate energy and glucose homeostasis. Leptin, a well-known anorectic hormone, is known to activate ACC. Inhibition of central ACC activity in the presence of circulating leptin negates leptin's anorectic effect [37], which

may be due to a lack of LCFA-CoA accumulation as a result of decreased malonyl-CoA mediated CPT1 inhibition. Furthermore, increases in central citrate (an effector of ACC activity) decrease food intake and body weight [38], as well as lowering blood glucose levels [39].

Levels of malonyl-CoA can also be directly decreased with malonyl-coenzyme A decarboxylase (MCD), and indeed over-expression of this enzyme in the hypothalamus of rats increased food intake and body weight, as well as impaired suppression of glucose production [40]. Conversely, malonyl-CoA levels can be increased by inhibition of FAS, the enzyme converting malonyl-CoA into FA. Indeed, treatment of mice with the FAS inhibitor cerulenin (C75) results in decreased food intake and body weight [31]. This finding was further supported with brain and beta cell-specific FAS knockout mice, which show a similar phenotype [41].

From the previous studies, it is evident that hypothalamic fatty acid sensing in part maintains glucose homeostasis. These studies, however, administered LCFA directly into the hypothalamus, thereby raising the concern of whether the effects are of physiological relevance. Hence, studies were conducted to test the effects of hypothalamic FA sensing on glucose homeostasis in the presence of *circulating* FA.

As previously mentioned, increases in levels of circulating FFA (or lactate) causes an increase in gluconeogenesis. However, in nondiabetic humans and dogs, an increase in glucose production is not observed due to a concomitant decrease in glycogenolysis [24-26]. This metabolic adaptation is referred to as hepatic autoregulation, and in the case for FFA is impaired in type 2 diabetes, since the expected decrease in glycogenolysis in the presence of circulating LCFA does not occur to maintain euglycemia [42]. In a recent study, it was found that lipid-induced hepatic autoregulation requires the effect of central LCFA-CoA to decrease hepatic glucose production [43]. In the presence of a physiological rise in the levels of circulating lipids, inhibition of hypothalamic fatty acid esterification to generate LCFA-CoA with triacsin C administration **or** hepatic vagotomy negates the inhibitory effects of hypothalamic lipids on glucose production [43]. This leads to a rise in glucose production and a disruption in glucose homeostasis in the presence of an elevation in circulating lipids. Furthermore, this effect requires the activation of hypothalamic K_{ATP} channels. Hence, hypothalamic sensing of circulating lipids is required to counteract the direct lipid-induced stimulation on hepatic gluconeogenesis to maintain euglycemia. Although it has been determined for lipids, *it has not been determined whether the hypothalamic sensing of circulating lactate to decrease glucose production is required to offset the lactate-induced stimulation of gluconeogenesis, thereby mediating hepatic autoregulation. This will be addressed in the first Aim of this thesis.*

1.2.3 Protein Kinase C

It is evident from the aforementioned literature that changes in the availability of central LCFA-CoA regulate both glucose and energy homeostasis. Although this has been well established, the associated molecular/signaling mechanisms need further elucidation. Emerging research has demonstrated that a potential key molecule mediating the lipid effect may be protein kinase C, and will be discussed in this section.

Activation of Protein Kinase C

The various protein kinase C (PKC) isoforms are involved in an immense array of biological functions. The PKC superfamily is a family of serine/threonine kinases and consists of at least 10 distinct isoforms, divided into three subgroups based on their mode of activation and sequence homology (for review, see [44]). The classical PKC isoforms consist of α , β , and γ subtypes, and require both Ca^{2+} and DAG for activation. The novel PKC isoforms (δ , ϵ , η , and θ) also require DAG for activation, but are calcium independent. Hence, the classical and novel PKCs can be activated by the stimulation of G protein coupled receptors as well as receptor tyrosine kinases. Both of these signal transduction pathways involve the activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate into the second messengers DAG and inositol 1,4,5-

triphosphate (IP3). IP3 subsequently binds to its receptor on the endoplasmic reticulum, resulting in the release of Ca^{2+} . In addition to this acute elevation of DAG, DAG levels can also be increased chronically during periods of increased TG availability, as is the case with obesity [45]. In these circumstances, DAG is synthesized de novo in the endoplasmic reticulum via the G3P/G3P acyl transferase pathway. Interestingly, DAG can also be synthesized de novo from glucose [46] and acutely from insulin receptor activation [47], suggesting that hyperglycemia and hyperinsulinemia both contribute to increased PKC activation.

Lastly, the atypical PKC isoforms (ζ and λ) are both calcium and DAG independent, but rather are activated by phosphatidylinositol (PI)- 3-dependent kinase in response to insulin receptor substrate (IRS) and activation of the PI 3-kinase pathway [44].

Protein Kinase C Acts Downstream of LCFA-CoA to Regulate Glucose Homeostasis

It has long been known that this insulin resistant state is associated with lipid oversupply. The conversion of FA into long-chain fatty acyl CoA (LCFA-CoA) results in the generation of intracellular signaling molecules, which stimulate their respective signaling pathways to induce insulin resistance (reviewed in [48]). As previously discussed, DAG is one of the intracellular signaling molecules generated from FA [45], and therefore FFA metabolism is able to activate the classical

and novel forms of PKC. It is presumable, then, that increased availability of FFA, as is the case in diet-induced obesity, would be associated with PKC activation.

Indeed, a number of studies have shown various PKC isoforms, predominantly of the novel class, act downstream of lipid to induce insulin resistance in skeletal muscle [49-53], liver [54-57] and brain [58]. In the skeletal muscle and liver, PKC is thought to exert its effects via altered phosphorylation of IRS-1 [59-62], a key regulator of the insulin signaling cascade, as well as phosphorylation of the insulin receptor itself [63]. In parallel with these findings, palmitic acid exposure in the CNS by either direct infusion or oral gavage in rodents impairs hypothalamic insulin signaling and is accompanied by increased PKC θ activation (as measured by membrane translocation) [58]. Furthermore, knockdown of PKC θ specifically in the arcuate nucleus was found to attenuate the obesity phenotype induced by high-fat feeding and improve insulin signaling [58]. This study suggests that the obesity and insulin resistance associated with high-fat feeding are the result of hypothalamic PKC θ activation and its suppression of the insulin signaling pathway.

In contrast to these detrimental effects of central PKC activation in response to lipid, Ross et al [64] found a beneficial role of hypothalamic PKC δ on glucose homeostasis. The study found that PKC δ is sufficient and necessary for CNS lipid-sensing mechanisms to lower glucose production [64].The

general PKC activator 1-oleoyl-2-acetyl-sn-glycerol (OAG) was infused into the hypothalamus of rats *in vivo*, and the effects on glucose production were assessed under a basal insulin clamp setting. It was found that OAG infusion decreases hepatic glucose production without increased glucose uptake. When OAG was co-infused with PKC inhibitors, bisindolymaleimide (BIM) or Rottlerin (ROT), this effect was abolished. Lastly, central administration of the same PKC inhibitors into rats receiving intravenous lipid infusion caused an elevation of hepatic glucose production, indicating that hypothalamic PKC δ activation is required for circulating lipids to decrease hepatic glucose production.

With the finding that various isoforms of PKC act downstream of lipids in skeletal muscle, liver as well as the brain, it would be a logical presumption that PKC may also act downstream of lipid in other organs to regulate glucose homeostasis. Interestingly, the small intestine has recently emerged as a key regulator of glucose homeostasis, paving the way for the possibility that PKC may exert effects in the small intestine.

1.3 NUTRIENT SENSING IN THE SMALL INTESTINE

From the aforementioned discussion, it is evident that nutrient sensing mechanisms, via the metabolism of glucose/lactate or lipids, in the hypothalamus are critical to regulating both energy and glucose homeostasis. However, the brain is not the only organ implicated in employing nutrient sensing mechanisms to regulate these two critical homeostatic parameters.

The gut has been receiving increasing attention due to its newly emerging role as the first line of metabolic defense against energy and nutrient imbalance. Once nutrients enter the upper portion of the small intestine, the duodenum, nutrient sensing mechanisms are activated so that the body can facilitate appropriate physiological responses to adapt to the increased nutrient flux. The focus of the research has been on upper intestinal lipid sensing, which acts to regulate both energy and glucose homeostasis via a *gut-brain* and *gut-brain-liver* axis, respectively.

1.3.1 Gut-brain Axis : Energy Homeostasis (Figure 1)

Although seemingly straight forward, food intake in humans is a complex biological function, consisting of signal integration at multiple levels, with processing occurring in the hypothalamus.

The hypothalamus receives inputs from multiple sources, which can be triggered by not only emotional, social and behavioural cues [65], but also from hormones (in the upper intestine, cholecystokinin (CCK)) [66-69] and nutrient signals, namely lipids [70-74]. These nutrient signals stem from food ingestion at the level of the gut that, in the preabsorptive state, sends signals to the hypothalamus to change feeding behaviour in an effort to maintain overall energy balance [75].

In line with this notion, accumulating evidence in rodents and humans has established that accumulation of lipid in the duodenum suppresses food intake through a neuronal network [76-80]. This effect requires the lipase mediated hydrolysis of TG into FA, since inhibition of lipase reduces the satiation effect [76;77]. In line with these findings, studies have shown that administration of intraduodenal FA can suppress energy intake in both rats and humans [81-85]. Interestingly, as has been shown with central nutrient sensing mechanisms, the length of the FA acyl chain is important in promoting this satiation effect. The satiety is elicited only by LCFA with 12 or more carbons, whereas short and medium chain fatty acids with 11 or less carbons do not induce the same effect

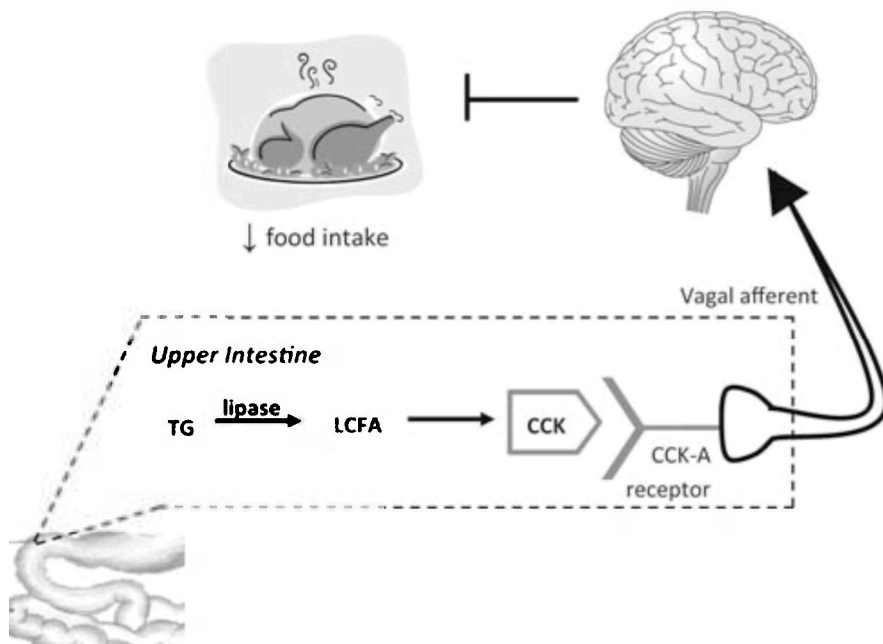
[81-85]. Hence, lipid ingestion acts as a signal of energy surfeit and triggers a rapid homeostatic response to decrease subsequent energy intake. The mechanism whereby lipid acts to decrease food intake is via the release of the gut-peptide hormone CCK [66-69]. CCK is released in response to lipids, and has been shown to mimic the effects of lipids on food intake in both rodents and humans [66-69]. This leads to the interesting possibility that perhaps downstream CCK signaling in obesity and diabetes is disrupted, offering a potential target for therapeutics.

The satiation effect of upper intestinal lipids requires neuronal activation to send signals to the hypothalamus. Intraduodenal co-infusion of lipids with the general anesthetic tetracaine prevented the lipid induced decrease in energy intake [86]. Hence, neuronal activation at the duodenum is required for the suppression of feeding behaviour. Further evidence of this comes from studies in which the subdiaphragmatic vagal afferents innervating the duodenum are denervated. These subdiaphragmatic vagotomies abolish the ability of upper intestinal lipids to suppress feeding in rodents [87]. By activating the subdiaphragmatic vagal nerve, lipid signals arrive at the nucleus of the solitary tract (NTS) to activate neurons in this hindbrain region [88]. The NTS has been implicated as an important integration and relay center for numerous mammalian biological functions [89;90] ; hence, it is presumable that NTS efferents emanate to the hypothalamus, which then sends outputs to change feeding behaviour. Although the mechanisms of the lipid-induced gut-

brain axis are not fully understood, recent findings have shed some insight. In response to feeding, small intestinal mucosal cells produce the lipid messenger oleoylethanolamide which is known to decrease meal frequency via its activation of the peroxisome proliferator-activated receptors-alpha [70;71;91;92]. This oleoylethanolamide induced effect on satiety requires the uptake of dietary oleic acid, which activates oleoylethanolamide mobilization [72]. Furthermore, it has been shown that the plasma lipid N-acylphosphatidylethanoalamine is secreted into the circulation from the small intestine in response to dietary lipid, resulting in decreased food intake [73]. N-acylphosphatidylethanoalamine infusion directly into the hypothalamus reproduces this satiety effect, demonstrating the involvement of the CNS [73]. More effort is required to fully dissect the downstream signaling molecules involved in the lipid-induced satiation effect; this is of utmost importance in order to understand the dysregulation that occurs in the disease state.

The aforementioned studies have established the existence of a lipid-induced gut brain axis to regulate energy homeostasis. Hence, lipid sensing mechanisms in the small intestine act as an indicator of nutrient surfeit, sending signals to the CNS to decrease energy intake. Although the role of lipid derived molecules on the small intestinal lipid induced satiety effects has been explored, the downstream signaling cascades remain to be determined.

Figure 1



Adapted from Kokorovic A, Cheung C, Lam TK. Upper intestinal lipids regulate energy and glucose homeostasis. *Cell Mol Life Sci*66: 3023-3027, 2009. [co-first author]

Lipids trigger a gut-brain axis to decrease food intake. As lipids enter the duodenum in the form of TG, they are esterified to LCFA by the enzyme lipase. This results in the activation of the gut peptide hormone CCK, which activates duodenal CCK-A receptors in the duodenum to trigger vagal afferent signals. The vagal afferents terminate in the NTS, resulting in a decrease in food intake and body weight. TG=triglyceride, LCFA=long chain fatty acid, CCK=cholecystokinin

1.3.2 Gut-brain-liver Axis : Glucose Homeostasis (Figure 2)

The gut, specifically the upper small intestine, plays an important role in the sustenance of energy homeostasis by regulating satiety behaviour in response to lipid ingestion [76-80]. In addition to this important metabolic effect, it has recently been discovered that lipid sensing mechanisms in the duodenum activate a *gut-brain-liver* axis to regulate hepatic glucose production [74]. Hence, nutrient sensing in the small intestine is critical in the maintenance of two parameters of utmost importance in diabetes and type 2 obesity, namely energy and glucose homeostasis.

Lipid administration directly into the duodenum of rats resulted in a decrease in hepatic glucose production during a euglycemic pancreatic clamp, in the presence of near basal insulin levels and no changes in other glucoregulatory hormones [74]. When lipid was co-administered with the triascin C, which prevents FA esterification to form LCFA-CoA, *or* tetracaine the effect on glucose production was blunted, demonstrating the requirement of LCFA-CoA specifically and neuronal transmission. Furthermore, vagal deafferentation experiments in rats receiving intraduodenal lipid infusion also reversed the effect, demonstrating that the vagus nerve is necessary. Since vagal afferents from the duodenum target the NTS, it was tested whether activation of N-Methyl-D-Aspartate (NMDA) receptors in this important brain region is required for the lipid effect on

glucose production. Indeed, infusion of the NMDA receptor inhibitor MK801 directly into the NTS of rodents receiving intraduodenal lipid infusion reversed the glucose production suppression effect. Lastly, it was tested whether signal transmission through the efferent branch of the vagal nerve to the liver is required. Indeed, in rats in which the hepatic branch of the vagus nerve was transected (hepatic vagotomy), intraduodenal lipid infusion did not decrease hepatic glucose production. Hence, duodenal lipid signals are relayed from the NTS to the liver via vagal hepatic innervation.

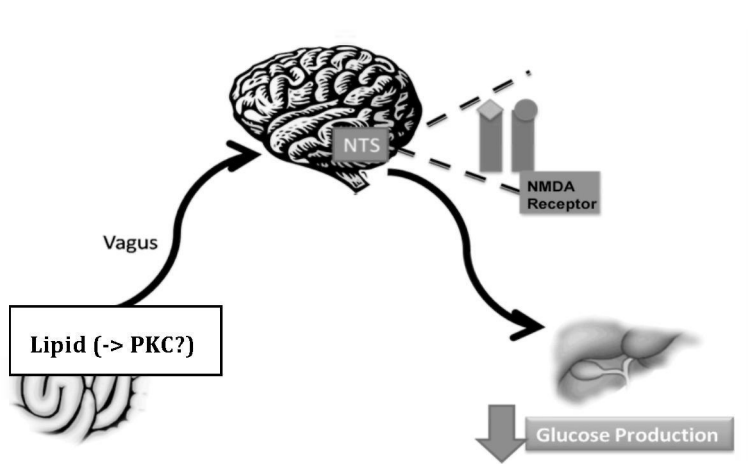
These experiments demonstrate the existence of an upper intestinal lipid-induced gut-brain-liver neuronal axis, which represents one of the first lines of metabolic defenses against nutrient excess to provide metabolic balance by downregulating glucose production on nutrient exposure. Various mechanisms remain to be elucidated to gain a complete understanding of how upper intestinal lipids regulate glucose homeostasis through the intestine-brain-liver neuronal network. *It will be the focus of the second Aim of this thesis to dissect the molecular mechanisms lying downstream of LCFA-CoA in order to gain understanding of the signaling cascade involved in mediating the lipid-induced gut-brain-liver axis.* Nonetheless, in combination with the previously mentioned gut-brain neuronal axis in the regulation of energy homeostasis, lipid-induced activations of the gut-brain and gut-brain-liver neuronal networks allow transient control of energy and glucose homeostasis upon the ingestion of lipids.

It is interesting to note that some of the mediators of lipid metabolism discussed above are, in addition to being expressed in the hypothalamus, also found in the small intestine. In line with the finding that LCFA-CoA in the small intestine of rodents triggers *gut-brain-liver* axis to lower hepatic glucose production [73], it remains a possibility that convergent mechanisms may be shared between the brain and intestine in the regulation of peripheral glucose levels. For example, PKC acts downstream of LCFA-CoA in the hypothalamus to decrease hepatic glucose production [64]. PKC is also expressed in the small intestine [93-95], and therefore it would be plausible that PKC activation downstream of LCFA-CoA in the small intestine may mediate the ability of lipids to lower hepatic glucose production. *This possibility will be addressed in the second Aim of this thesis.*

Furthermore, it has been found that levels of CPT-1 are increased in the intestine of mice fed on a HFD [96]; this would decrease the availability of LCFA-CoA, thereby potentially contributing to the observed glucose dysregulation in this model. Moreover, ACC is also expressed in the small intestine [97]; combined with the fact that leptin receptors are also present [98], there is a possibility that leptin-mediated regulation of ACC in the small intestine could contribute to the regulation of glucose homeostasis through accumulation of LCFA-CoA. Evidently, these areas need further exploration, and should be the focus of future research efforts.

In addition to the aforementioned *gut-brain-liver* axis to effectively decrease hepatic glucose production in response to intraduodenal lipid infusion, the existence of a portal vein-brain-liver axis has also recently been revealed [99]. Following gastric bypass surgery in rodents fed on a high-fat diet (HFD), there is a restoration of insulin sensitivity and decrease in glucose production, which requires portal vein innervation. Furthermore, it was found that an intragastric infusion of glucose triggers a portal-brain-muscle axis to effectively increase insulin stimulated glucose uptake [100]. Hence, the gut is able to regulate glucose homeostasis by various nutrient sensing mechanisms.

Figure 2



Lipids trigger a gut-brain-liver axis to decrease glucose production. As lipids enter the duodenum, they trigger vagal afferents, which terminate in the NTS. This results in the activation of NMDA receptors, leading to a decrease in hepatic glucose production. We postulate that PKC acts downstream of lipids to trigger this effect.

1.4 NUTRIENT SENSING IN DISEASE MODELS

Under normal conditions, increased nutrient flux activates a series of biochemical and physiological responses aimed at maintaining energy and glucose homeostasis. Hence, nutrient sensing mechanisms ensure that stable body weight and blood glucose levels are maintained despite acute changes in nutrient consumption. Unfortunately, this tightly coupled system seems to be dysregulated under conditions of high-fat feeding [8].

With respect to nutrient sensing in the CNS, high fat feeding in rodents has shown to disrupt the lipid sensing mechanisms designed to decrease hepatic glucose production. Namely, the administration of oleic acid icv into rodents fed on a HFD for merely 3 days blunts the ability of the LCFA to decrease glucose production [101]. This deficiency at least in part due to an increase in CPT-1 expression in response to high-fat feeding [102]. As previously discussed, CPT-1 regulates the transport of LCFA-CoA from the cytosol to the mitochondria. Hence, regulation of CPT-1 activity controls the levels of available LCFA-CoA. Increased CPT-1, then, would decrease the extracellular accumulation of LCFA-CoA in hypothalamic cells, thereby impairing the LCFA-CoA mediated control of glucose homeostasis. In parallel with this, restoration of LCFA-CoA in the hypothalamus of rodents fed on HFD (via inhibition of CPT-1) restores glucose homeostasis [102]. In fact, another

key mediator of lipid metabolism, specifically malonyl CoA (a key inhibitor of CPT-1 activity) has been shown to be decreased in HFD fed rodents [102]. These findings hold exciting implications for the treatment of the glucose defects associated with obesity and diabetes, since restoration of hypothalamic lipid metabolism via CPT-1 modulation (ie. through pharmacological agents) could potentially restore defects in glucose homeostasis.

Although lipids are unable to reduce glucose production in response to high-fat feeding, studies have shown that lactate administered into the hypothalamus of rodents fed on a HFD can still decrease hepatic glucose production [103]. This is an exciting discovery, since it implies that lactate can surpass the defects in hypothalamic nutrient sensing in disease models. Hence, activation of lactate metabolism centrally could be the key to restoring glucose homeostasis.

As discussed, upper intestinal lipids in the form of LCFA can induce satiation via the gut-brain axis in normal rats and humans [81-85]. However, this effect is not reproducible in rats fed on a HFD for 3 weeks [104]. Moreover, the degree to which the satiety effect is blunted is highly dependent on the composition of the diet, with higher fat content being associated with more impairment [104]. These findings demonstrate that lipid overconsumption leads to a disruption in the gut-brain axis, suggesting that individuals adapted to high-fat feeding may be unresponsive to the LCFA induced

satiety effect. Since excessive energy intake, particularly of foods rich in saturated fats, is attributable to obesity, this disrupted satiety signal holds important implications. Namely, restoration of this defect could potentially lead to decreasing food intake in obese individuals, contributing to a potential cure for this debilitating disease.

In addition to blunting the satiety effect, high-fat feeding in rats for merely 3 days negates the ability of lipids to induce a gut-brain-liver axis to lower glucose production [74]. To date, the mechanisms lying downstream of LCFA-CoA to mediate the *gut-brain-liver* axis are unknown, and therefore it is not yet possible to determine where the defect is occurring. It remains a possibility, then, that elucidation of the downstream signaling cascade may reveal precisely where the defect lies, allowing for restoration of nutrient sensing mechanisms and glucose homeostasis during high-fat feeding.

Evidently, the feedback mechanisms designed to tightly maintain appropriate levels of nutrients in the body are disrupted in an environment with excessive nutrient influx. This paves the way for a detrimental and self-sustaining cycle, whereby excessive nutrient intake results in impaired nutrient sensing mechanisms, causing further intake of nutrients (inability to induce satiety), and increases in the amount of circulating nutrients (inability to decrease glucose production). With

the advent of an obesity/type 2 diabetes epidemic, it is vital to focus research on restoring these defects in nutrient sensing mechanisms in an effort to treat these debilitating conditions.

General Hypothesis and Aims

Nutrient sensing mechanisms in the brain and the small intestine play pivotal roles in the regulation of energy and glucose homeostasis, two parameters which are disrupted in obesity and its associated co-morbidity, type 2 diabetes. Since obesity is a great concern in our society, it is of utmost importance to decipher the mechanisms by which nutrient sensing mechanisms maintain whole body energy and glucose homeostasis. The unveiling of these mechanisms could potentially lead to novel treatment for this debilitating disease.

The overall purpose of this thesis is to elucidate the nutrient sensing mechanisms in the brain (**Aim 1**) and small intestine (**Aim 2**) that regulate glucose production. Although the brain and intestine are two distinct anatomical areas with unique functions, their nutrient sensing mechanisms act redundantly to decrease hepatic glucose production and food intake.

AIM 1

Previous reports have demonstrated that direct delivery of lactate into the hypothalamus lowers glucose production in normal and early-onset diabetic and obese rodents [16;103]. Since circulating lactate directly increases liver gluconeogenesis without altering glucose production [24], we postulate that *hypothalamic lactate sensing is designed to counteract the direct stimulatory effect of circulating lactate on hepatic gluconeogenesis to maintain glucose*

homeostasis. This will be addressed in **AIM 1** of this thesis. Briefly, we will inhibit hypothalamic lactate metabolism in the presence of circulating lactate, and assess the effect this will have on glucose production, using the pancreatic clamp technique and tracer dilution methodology.

AIM 2

Direct delivery of lipids into the duodenum of the small intestine decreases hepatic glucose production [74]. In peripheral tissues, protein kinase C (PKC) acts downstream of lipids to decrease insulin sensitivity [49-57] . PKC activation in the hypothalamus has also been implicated in decreasing glucose production. Based on these findings, we hypothesize that ***PKC activation in the duodenum of the small intestine is sufficient and necessary for lipids to trigger a gut-brain-liver axis to decrease hepatic glucose production.*** This will be assessed in **AIM 2** of this thesis. We will activate PKC in the duodenum to test whether it can lower glucose production, and confirm it is through a gut-brain-liver axis. We will also inhibit PKC activation in the presence of duodenal lipids to see whether the lipid-induced decrease in hepatic glucose production is blunted. The pancreatic clamp technique in conjunction with tracer dilution methodology will be used.

General Materials and Methods

All study protocols described were reviewed and approved by the Institutional Animal Care and Use Committee of the University Health Network.

Animals

8-9 week old male Sprague-Dawley rats, weighing between 250-300g (obtained from Charles River Laboratories, Montreal QC) were used for our studies. Rats were housed in individual cages, and were maintained on a standard light-dark (12h-12-h) cycle with access to regular rat chow (Harland Teklad 6% Mouse/ Rat Diet; composition 52% carbohydrate, 31% protein, 17% fat; total calories provided by digestible nutrients 3.83kca/g), with access to water *ad libitum*. The rats were allowed 5 days to acclimatize upon arrival, before surgeries were performed.

Surgical Procedures

Stereotaxic Surgery

In accordance with the atlas of the rat brain, animals were implanted stereotaxically with indwelling cannula (Plastics One Inc., Roanoke, VA) as previously described. Briefly, rats were anesthetized with intraperitoneal ketamine (Ketalean; Bimeda-MTC, Cambridge, Ontario) in combination with xylazine (Rompun; Bayer), and then fixed onto a stereotaxic machine (David Kopf

Instruments, Tujunga, CA) with ear bars and a nose piece which was set to +5.0mm. For implantations into the third cerebral ventricle, 22-gauge stainless steel single guide cannulae were used; for the mediobasal hypothalamic OR nucleus of the solitary tract implantations 26-gauge stainless steel double guide cannulae were used.

Implantations were performed according to the following coordinates:

Third cerebral ventricle (icv): 2.5 mm posterior of bregma, 0.0 mm lateral from midline, 8.0 mm below skull surface

Mediobasal hypothalamus (MBH): 3.1 mm posterior of bregma, 0.4 mm lateral from midline, 9.6 mm below skull surface

Nucleus of the solitary tract (NTS): 0.00 mm on occipital crest, 0.4 mm lateral to midline, 7.9 mm below skull surface

Rats were given a one week period of recovery time following stereotaxic surgery, housed in individual cages and maintained on a standard light-dark (12h-12-h) cycle with access to regular

rat chow and water *ad libitum*. If rats were deemed to be fully recovered, based on daily food intake and body weight, subsequent surgical procedures were performed.

Vascular Catheterizations

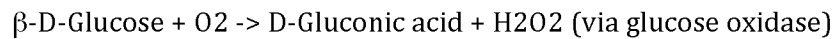
Rats which were to undergo pancreatic (basal insulin) – euglycemic clamps received indwelling catheters into the right internal jugular vein and left carotid artery for infusions and blood sampling purposes. Rats were first anesthetized with IP ketamine (Ketalean; Bimeda-MTC, Cambridge, Ontario) in combination with xylazine (Rompun; Bayer). The catheters were made of polyethylene tubing (PE 50; Clay Adams), with a 15 mm cuff-extension of Silastic tubing (Corning). Following insertion of the catheters into the vein and artery, they were tunneled subcutaneously and exteriorized. A bolus of approximately 0.2 mL of 10% heparinized saline was given into both cannulae to maintain potency. Cannulae were air sealed using metal pins.

Biochemical Analyses

Plasma glucose

Plasma glucose levels were measured with a glucose analyzer (Glucose Analyzer GM9, Analox Instruments, Lunenburg, MA). Plasma was obtained from a 10 μ L sample of blood, following centrifugation at 6000rpm for approximately 10 seconds. 10 μ L of plasma was used for the

determination of plasma glucose levels. The glucose analyzer measures the rate of oxygen consumption between the glucose in the obtained plasma sample and glucose oxidase as follows:



Under the assay conditions, the rate of this reaction (and oxygen consumption) is directly proportional to the plasma glucose concentration. The rate of oxygen consumption is determined with a polarographic oxygen sensor. Clark-type amperometric oxygen electrodes, bearing a voltage between them able to reduce the dissolved oxygen, are placed into the plasma sample which was injected into the analyzer. Since the partial pressure of oxygen in the plasma is proportional to the limiting current, one may determine its partial pressure.

Plasma glucose tracer specific activity

During the pancreatic clamp procedure, blood samples were obtained from the rats at 10 minute intervals. 50 μ L of plasma was obtained via centrifugation at 6000rpm for 30 seconds. To determine the radioactivity of [3-3H] in plasma, 100 μ L Ba(OH)₂ and 100 μ L ZnSO₄ were added to the plasma aliquot to deproteinize it. The mix was briefly vortexed, then centrifuged for 7 minutes at 6000 rpm at 4°C. The supernatant (protein-free) was obtained. Since tritium on the C-3 position of glucose is

lost to water during glycolysis, the supernatant was evaporated overnight in order to remove the tritiated water. Hence, the radioactivity determined by liquid scintillation counting represents the radioactivity of the [3-3H] glucose in the plasma only.

Plasma Insulin, glucagon and adiponectin

Plasma insulin, glucagon and adiponectin levels were determined by radioimmunoassay (RIA) using kits from Linco Research (St. Charles, MO), and was followed according to manufacturer's instructions. The principle of RIA remains the same for all hormones. In brief, a fixed concentration of a labeled tracer antigen (125I-labeled insulin, glucagon or adiponectin) is incubated with a constant dilution of antiserum (guinea pig anti-rat insulin/glucagon serum; rabbit anti-rat adiponectin serum), assuring that there is unbound antigen remaining. Therefore, when the plasma sample containing the unlabeled antigen (insulin/glucagon/adiponectin) is added to the mixture, there is competition for antibody binding between the labeled and unlabeled antigen. Based on the principle of substrate binding, the percent of labeled vs unlabeled antigen binding is directly proportional to the relative concentration. One may then separate the labeled antibody-bound antigen from the unbound labeled antigen, and by counting the radioactivity of the fractions, determine the concentration of the antigen (insulin/glucagon/adiponectin) from a predetermined standard curve.

The protocols provided by the supplier were used. A predetermined standard curve was generated using provided hormone standards. ¹²⁵I-labeled hormone (50μL) and rat antibody specific for the hormone (50μL) were added to the standards, as well as to the plasma samples obtained from the experiment (50μL), followed by vortexing. The samples were incubated overnight at 4°C, and 1 mL of precipitating reagent was added to the mixture followed by vortexing, and a 20 min incubation at 4°C. The samples were then centrifuged, with the pellet containing the antibody-bound hormone. The radioactivity of the pellet was counted with a gamma counter (Perkin Elmer 1470). The counts (B) for the samples and the standards were expressed as a percentage of the mean counts of the total binding reference tubes (Bo):

$$\% \text{ total binding} = \% B/Bo = \text{sample/standard}/Bo \times 100\%$$

Using the standard curve generated, one can determine the hormone concentration of the experimental sample.

*note: Insulin and adiponectin RIA kits use a 2 day protocol; glucagon uses a 3 day protocol, due to an additional overnight incubation of the standards and samples at 4°C with the unbound glucagon antibody.

Calculations

The pancreatic clamp experiments in combination with tracer-dilution methodology allow for the determination of glucose production and uptake in the samples. Throughout the experiment, [3-3H] glucose tracer was infused at a constant rate to allow tracer equilibration. With the use of a steady state formula [105], the rate of glucose appearance, R_a , which is equivalent to the rate of glucose disappearance, R_d , can be determined:

$$R_a=R_d=\text{constant tracer infusion rate } (\mu\text{Ci}/\text{min})/\text{specific activity } (\mu\text{Ci}/\text{mg})$$

Under basal steady state conditions, the rate of glucose appearance is equal to the rate of its disappearance, which is also equal to the rate of glucose production. Under the setting of a pancreatic clamp, exogenous glucose is infused to maintain euglycemia; therefore the rate of glucose production is the difference between the R_d and the rate of glucose infusion during the experiment.

Statistical Analysis

Data are presented as means \pm S.E. The statistical differences between the treatment groups were determined using two-way analysis of variance, which was followed by the Turkey post hoc test with a probability of $p < 0.05$ taken as significant.

Aim 1: Mechanisms of Hypothalamic Nutrient Sensing

The findings in this Aim were published as:

Kokorovic A, Cheung GW, Rosseti L, Lam TK. Hypothalamic sensing of circulating lactate regulates glucose production. *Journal of Cellular and Molecular Medicine*. 2009; Nov-Dec;13(11-12):4403-8.

4.1 Abstract

Recent evidence has implicated the hypothalamus as being a critical regulator of whole body glucose homeostasis via its ability to sense nutrients. In particular, infusions of the glucose metabolite lactate directly into the hypothalamus of rodents decreases hepatic glucose production. However, it is currently unknown whether these hypothalamic lactate sensing mechanisms are responsive to circulating lactate to regulate glucose production and maintain glucose homeostasis *in vivo*. It is the purpose of this study to determine whether hypothalamic sensing of circulating lactate is required for the maintenance of glucose homeostasis. Intravenous lactate was infused into rodents with or without inhibition of hypothalamic lactate-sensing mechanisms. Lactate metabolism was inhibited centrally using three independent approaches. To determine the effect of the treatments on glucose metabolism *in vivo*, tracer-dilution methodology in combination with the pancreatic clamp technique were employed. It was found that in the presence of a physiologically significant rise in circulating lactate levels, pharmacological inhibition of hypothalamic lactate

metabolism, using the lactate dehydrogenase inhibitor oxamate OR the ATP-sensitive potassium channel blocker glibenclamide, resulted in increased glucose production. In order to pinpoint the hypothalamic region responsible for this effect, oxamate was also infused directly into the mediobasal hypothalamus, resulting in an increase in glucose production in the presence of similar levels of plasma lactate. In conclusion, these data demonstrate that hypothalamic sensing of circulating lactate is required to maintain glucose homeostasis in normal rodents.

4.2 Materials and Methods

Animal Preparation

Following 7 days of recovery from stereotaxic *icv* or MBH surgery (see General Materials and Methods), rats whose daily food intake and body weight had recovered to pre-surgical values underwent the vascular catheterization surgery (see General Materials and Methods). Recovery from surgery was once again monitored by measuring daily food intake and body weight, and 4-5 post-catheterization the rats underwent the pancreatic clamp studies.

Chemicals used for Infusions

- 1) Saline (SAL) control (intravenous (iv) infusion)
- 2) L-lactate (Sigma; 100 μ mol/kg•min, pH 7.0; iv infusion)
- 3) *icv* OXA (lactate dehydrogenase inhibitor; dissolved in artificial cerebrospinal fluid (ACSF); Sigma; 3 μ L bolus; 50mM at 5 μ L/hr)
- 4) MBH OXA (lactate dehydrogenase inhibitor; dissolved in ACSF; Sigma, 0.33 μ L bolus, 50mM at 0.33 μ L/hr)
- 4) GLI (K_{ATP} channel blocker; dissolved in 5% DMSO; Sigma; 100 μ M at 5 μ L/hr; *icv* infusion)
- 5) Vehicle (VEH) control (5% DMSO for glibenclamide control studies; ACSF for oxamate control studies)

The concentrations of OXA and GLI were chosen based on previous literature demonstrating that at these concentrations, the chemicals alone do not affect glucose kinetics, but are able to blunt the glucose production lowering effect of direct central lactate infusion [16].

Clamp Procedure

The animals were restricted to approximately 58kcal of food the night before the surgeries to maintain the same nutritional status. The experiment lasted a total of 360 min. A subgroup of conscious, unrestrained rats received an infusion of *icv* VEH or OXA or GLI (t=0 min) for the duration of the experiment; in a separate set of experiments, a different subgroup of rats received an infusion of MBH VEH or OXA. Following a 120 minute *icv* pre-infusion period, intravenous (*iv*) sodium L-lactate or SAL with a primed-continuous infusion of [3-3H]-glucose (Perkin Elmer; 40 μ Ci bolus; 0.4 μ Ci/min) were initiated (t=120 min) and maintained throughout the experiment, allowing for the determination of glucose production (mg/kg•min) and uptake with tracer-dilution methodology. After 60 minutes of tracer infusion, plasma samples for assessment of radioactive [3-3H]-glucose specific activity and glucose levels were taken at 10 minute intervals for the remainder of the experiment. The steady state basal period glucose production was taken to be from t=180-240 min. At t=240 min the pancreatic clamp was initiated with a continuous infusion of insulin (1.5

mU/kg•min) and somatostatin (SRIF, 3µg/kg•min), and 25% glucose was administered as needed to maintain euglycemia (plasma glucose concentration of approx 8mM). Plasma samples for the determination of lactate, insulin, adiponectin and glucagon levels were taken at 10 minute intervals from t=330-360.

Biochemical Analyses

*For analysis of plasma insulin, glucagon, adiponectin, glucose; and tracer specific activity please refer to General Materials and Methods

Plasma Lactate

Plasma lactate concentrations were determined using a kit in accordance with manufacturer's instructions (Sigma Diagnostics, Stl Louis, MO). The principle is as follows: Lactate oxidase converts lactic acid to pyruvate and hydrogen peroxide. In the presence of the hydrogen peroxide, peroxidase produces a coloured dye with a specific absorption maximum (540nm) (through the oxidative condensation of chromogen precursors). Therefore, one can measure the increase in absorbance at 540nm to determine the plasma lactate concentrations in the sample.

4.3 Results

Sodium L-lactate was infused iv into conscious, unrestrained Sprague Dawley male rats (100 $\mu\text{mol}/\text{kg}\cdot\text{min}$, pH=7) to elevate plasma lactate concentrations by ~ 2 -2.5 fold (Figure 3a). *Iv* lactate infusion did not increase glucose production during the pancreatic clamps when plasma glucose and gluco-regulatory hormones were maintained at basal levels. In the final two hours of lactate elevation, pancreatic-insulin clamps in combination with tracer dilution methodology were employed to evaluate the effects of treatment on glucose kinetics (Figure 3a). Circulating hormone levels (insulin, adiponectin, glucagon) as well as plasma glucose were comparable in all groups during the clamps (Figure 3 e,f).

Central icv OXA administration (50mM at 5 $\mu\text{L}/\text{hr}$) decreased the exogenous glucose infusion rate required to maintain euglycemia during the clamp procedure to 1 mg/kg $\cdot\text{min}$ (icv OXA) compared to 6 mg/kg $\cdot\text{min}$ (icv vehicle), in the presence of a similar elevation of plasma lactate (Figure 4a). Glucose production was increased with OXA treatment to 10 mg/kg $\cdot\text{min}$ compared to 6 mg/kg $\cdot\text{min}$ (icv vehicle) (Figure 4b). Consistent with this finding, central GLI administration also decreased the exogenous glucose infusion rate required to maintain euglycemia to 1.5 mg/kg $\cdot\text{min}$ compared to 6 mg/kg $\cdot\text{min}$ (icv vehicle) (Figure 4a). This was the result of an increase in glucose production, which

was 10 mg/kg•min compared to 6 mg/kg•min (icv vehicle) (Figure 4b). Glucose uptake remained unchanged in both OXA and GLI treatments (Figure 4c)

To determine the central neuroanatomical localization of the lactate sensing mechanisms, we ablated lactate sensing specifically within the mediobasal hypothalamus. This was accomplished by combining *iv* lactate infusions (100 μmol/kg•min) with bilateral administration of OXA (50mM at 0.33μL/hr) into the MBH (Figure 5a). It was confirmed that vehicle infusions did not affect normal glucose kinetics in the presence of *iv* lactate. MBH OXA infusion caused a decrease in the glucose infusion rate required to maintain euglycemia in the same degree of plasma lactate elevation (Figure 5b). This was the result of an increase in the rate of glucose production (Figure 5c), without a change in the rate of glucose uptake (Figure 5d).

We have successfully demonstrated, for the first time, that hypothalamic sensing of circulating lactate is required for the maintenance of glucose homeostasis in normal rodents.

4.4: Aim 1 Figures

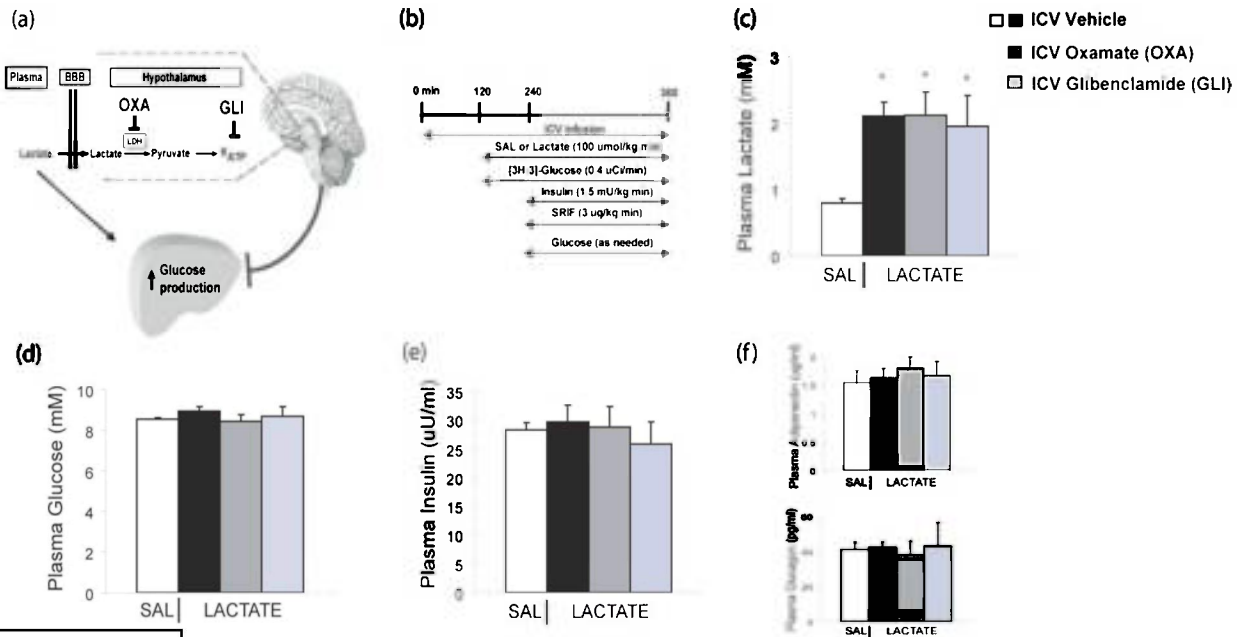


Figure 3

(A) Working hypothesis. The hypothalamic metabolism of lactate to pyruvate and the subsequent activation of the KATP channels are required to maintain glucose homeostasis in response to systemic lactate elevations. Lactate dehydrogenase inhibitor oxamate. KATP channel blocker glibenclamide. **(B)** Experimental design. Somatostatin (SRIF). During the final 30 min. of the clamps, **(C)** intravenous lactate infusion elevated plasma lactate levels compared to control (saline, SAL) by ~2- to 2.5-fold. * $P < 0.001$ versus SAL. **(D)** Plasma glucose, **(E)** plasma insulin, **(F)** plasma adiponectin and glucagon levels were comparable in all groups during the clamps.

- ICV Vehicle
- ICV Oxamate (OXA)
- ICV Glibenclamide (GLI)

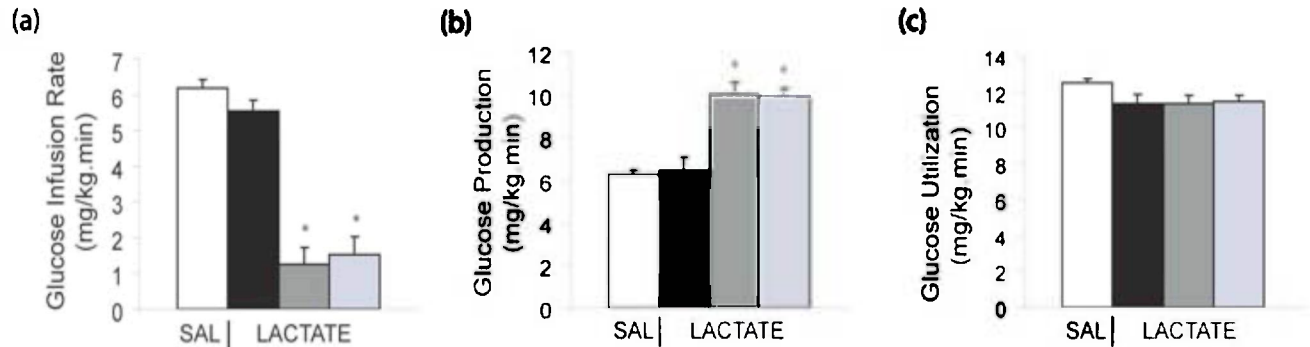


Figure 4

Central sensing mechanisms of circulating lactate regulate glucose production. In the presence of systemic elevation of lactate, direct inhibition of central lactate metabolism via i.c.v. administration of lactate dehydrogenase inhibitor oxamate (OXA) (compared to control) led to a marked **(A)** decrease in exogenous glucose infusion rate and **(B)** increase in GP during the final 30 min. of the clamps. Similarly, direct inhibition of central KATP channels via i.c.v. administration of blocker glibenclamide **(A)** decreased exogenous glucose infusion rate and **(B)** increased GP in response to systemic lactate infusions. **(C)** Glucose utilization/uptake was comparable in all groups. *P < 0.001 versus SAL with i.c.v. vehicle or lactate with i.c.v. vehicle.

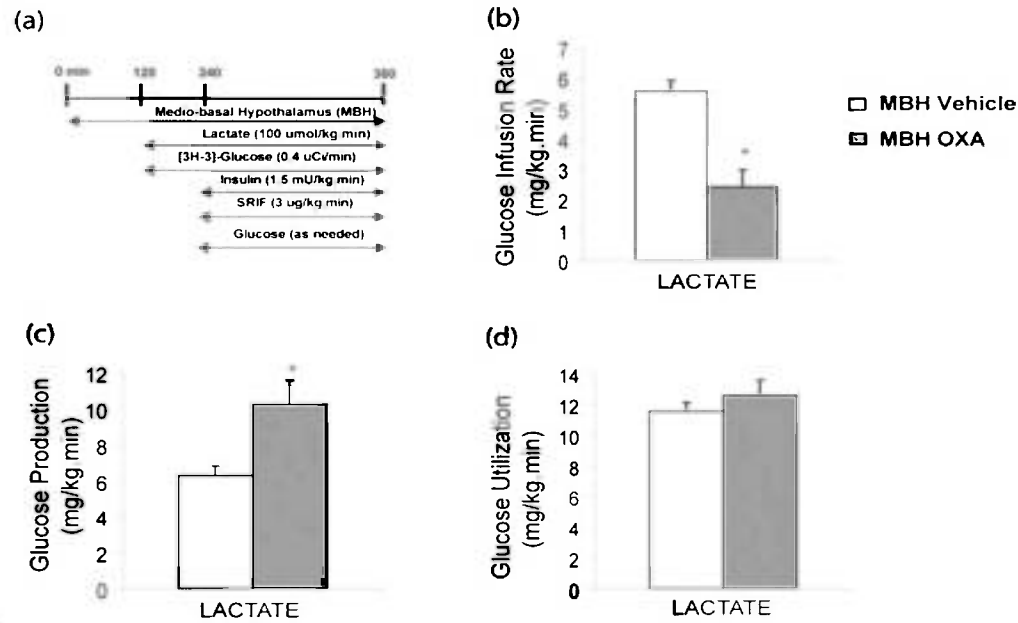


Figure 5

Hypothalamic sensing of circulating lactate regulates glucose production (GP). (A)

Experimental design. Somatostatin. In the presence of systemic lactate elevation, direct inhibition of lactate metabolism within the mediobasal hypothalamus (MBH) via MBH administration of oxamate (compared to control) (B) decreased exogenous glucose infusion rate and (C) increased GP during the final 30 min. of clamps. (D) Glucose utilization/uptake was comparable in all groups. *P < 0.001 versus lactate with MBH vehicle.

4.5 Discussion & Limitations

The hypothalamus has long been known to be a critical regulator of whole body homeostatic parameters. In particular, it has been shown that this important brain area can integrate signals to regulate vital bodily functions, such as temperature [9], blood pressure [10] and as was recently shown glucose levels [12]. Specifically, it has been shown that an acute increase in lipids [32;102] or glucose/lactate [16;103] specifically in the hypothalamus can decrease hepatic glucose production. These studies were performed in rodents, in which the nutrients were infused directly into the third cerebral ventricle under a pancreatic clamp setting, in which insulin and glucoregulatory hormones are maintained at near basal levels, and glucose is infused systemically as needed to maintain euglycemia. This is in direct opposition with the effects of lipid and lactate in the periphery, whereby they act on the liver to increase gluconeogenesis [24-26]. However, there is no concomitant increase in glucose production, due to a compensatory decrease in glycogenolysis [24-26]. To prove the relevance of these findings in a physiological setting, central lipid metabolism was inhibited in the presence of circulating lipids and pancreatic clamps were performed [43]. It was found that the hypothalamus can sense lipids and decrease glucose production to oppose the direct stimulatory effects of lipids on hepatic gluconeogenesis [43]. Hence, even in a physiological setting central nutrient sensing mechanisms play roles of utmost importance in maintaining glucose homeostasis.

Until our study, the effects of central lactate metabolism in the presence of circulating lactate were unknown. In order to elucidate the mechanisms of lactate sensing that are responsible for the maintenance of euglycemia despite a direct stimulatory effect of lactate on the liver, we tested the ability of the hypothalamus to sense an elevation of plasma lactate by 2-2.5 fold to restrain glucose production and maintain glucose homeostasis in normal rodents. To do this, we performed euglycemic pancreatic clamps in rodents, in which insulin and gluco-regulatory hormones were maintained at near basal levels. Consistent with previous reports, we found that an elevation of circulating lactate did not have an effect of glucose production [24]. In line with the fact that hypothalamic lipid sensing mechanisms are responsible for the lipid induced metabolic restraining effect on glucose production [43], we propose that hypothalamic lactate sensing employs a similar mechanism. In other words, we postulate that activation of central lactate metabolism is required to maintain euglycemia by offsetting the increase in hepatic gluconeogenesis in the presence of circulating lactate. Therefore, direct inhibition of hypothalamic lactate sensing should lead to a disruption in glycemic control mechanisms, and result in an increase in glucose production in the presence of circulating lactate. In order to test this hypothesis, we inhibited hypothalamic lactate sensing mechanisms by two different approaches and performed pancreatic clamps to assess their effect on glucose production. We found that the inhibition of hypothalamic lactate metabolism

increased glucose production in the presence of systemic lactate infusion. *Therefore, hypothalamic lactate metabolism is responsible for restraining glucose production in the presence of elevated levels of circulating lactate, thereby maintaining glucose homeostasis.*

We have shown that central lactate metabolism specifically in the MBH is required for the maintenance of glucose homeostasis in the presence of systemic lactate infusions. This region of the brain contains the arcuate nucleus, which is densely populated with neuronal subtypes implicated in regulating the aforementioned parameters [106]. The neurons most commonly implicated in mediating these effects are (a) those which express the end products of pro-opiomelanocortin (POMC), namely α -melanocyte stimulating hormone (α -MSH) and (b) those which express neuropeptide Y (NPY) and agouti-related peptide (AgRP). Activation of POMC neurons results in decreased food intake [106;107]; on the contrary, activation of NPY/AgRP neurons results in an increase in food intake [106;108;109]. In addition to their effects on energy balance, these neurons have also been implicated in regulating glucose homeostasis. For example, central infusions of melanocortin agonists improves glucose homeostasis [110;111], whereas infusion of NPY causes insulin resistance [112-114]. Given the relevance of these neurons in the regulation of glucose homeostasis, it would be of interest to pinpoint their importance in mediating lactate metabolism. Particularly, whether inhibition of lactate metabolism specifically in these neurons (ie. A POMC or

AgRP/NPY specific knockout of LDH-B) can blunt the ability of central lactate metabolism to regulate glucose homeostasis. This hypothesis remains to be tested.

Although the MBH has been implicated in being an important area of energy and glucose homeostasis, other brain regions have emerged as important regulators of these parameters as well. As previously mentioned, infusion of lactate or glucose directly into the ventromedial hypothalamus blunts the hypoglycemic counterregulatory response, demonstrating the importance of this brain area in regulating glucose homeostasis [23]. Furthermore, it has been shown that the NTS (brainstem region) is important in mediating the ability of duodenal lipid to decrease both energy intake and glucose production [89;90]. Further evidence for brainstem regulation of glucose homeostasis comes from a study in which activation of NMDA receptors directly in the dorsal vagal complex of rodents is sufficient to lower hepatic glucose production [115]. Hence, other brain regions, in addition to the MBH, regulate glucose homeostasis. It would be of interest to determine whether activation of lactate metabolism in these aforementioned areas would result in a decrease in glucose production. Further studies need to be performed to test this possibility.

Our study was performed in conscious, unrestrained rodents receiving an acute lactate infusion for a duration of 4 hours, which increased plasma lactate levels to a degree similar to what is seen

during exercise [116]. Interestingly, hyperlactinemia has been shown to induce insulin resistance by decreasing insulin stimulated glucose uptake in skeletal muscle [117]. These studies were performed in anesthetized rodents undergoing a hyperinsulinemic clamp. In our study, we did not observe the expected change in glucose uptake parameters as determined by tracer dilution methodology. This discrepancy may be due to the fact that (a) we performed a basal insulin clamp (the lactate effect is insulin stimulated) (b) Our levels of lactate following the acute infusion were lower by 2-fold, and therefore it may not have been at a high enough level to induce the effect (c) Our rats were conscious and unrestrained, without influence of anaesthetics.

An additional limitation to the study stems from the fact that, although we found changes in overall glucose production in response to our treatments, we are not in a position to attribute these to changes in gluconeogenesis or glycogenolysis, since these parameters were not measured in our hands. Although previous studies have shown that circulating lactate increases hepatic gluconeogenesis with a concomitant decrease in glycogenolysis [24], we are unable to claim this to be true in our study. The latter can be measured with the use of ^{14}C -labeled hepatic uridinediphosphoglucose [UDP]-glucose, ^{14}C -labeled lactate and tritiated glucose [118]. Essentially, gluconeogenesis can be estimated from the specific activities of the ^{14}C -labeled [UDP]-glucose, and hepatic phosphoenolpyruvate after the infusion of ^{14}C -labeled lactate and tritiated glucose.

Glycogenolysis is then the difference between hepatic glucose production and gluconeogenesis.

Although we were unable to determine these factors, previous literature has confirmed the finding that circulating lactate increases gluconeogenesis, with a decrease in glycogenolysis [24], and therefore we may attribute the increase in glucose production observed in the presence of our hypothalamic treatments as a result of increased glycogenolysis.

4.6 Future Directions

This is the first finding that highlights the ability of the hypothalamus to detect elevations in circulating lactate to restrain glucose production and maintain glucose homeostasis in normal rodents. This is in parallel with the finding that central lipid metabolism restrains glucose production to offset the effects of circulating lipids [43]. In type 2 diabetic patients, circulating lipids increase hepatic glucose production [42]. This is likely due to defective hypothalamic lipid sensing mechanisms, since direct infusion of lipid into the hypothalamus of rats on a high fat diet does not decrease glucose production [32;102]. In contrast to these findings, circulating lactate does not increase glucose production even in patients with metabolic stress conditions [119]. This is consistent with the fact that central lactate metabolism is intact in early onset of diabetic and obese rodents [103]. It has not yet been tested whether hypothalamic lactate sensing mechanisms are intact in diabetic and obese human subjects. However, based on the aforementioned findings in rodents, we postulate that lactate sensing mechanisms would indeed be intact in humans with type 2 diabetes and obesity. Hence, it becomes an intriguing possibility that activation of central lactate metabolism in diabetic and obese individuals may in fact be able to offset the effects of circulating lipids to partially restore glucose levels. Although this remains to be tested, there lies the possibility of employing therapeutic strategies aimed at enhancing central lactate metabolism to treat the hyperglycemia associated with type 2 diabetes and obesity.

In order to begin addressing this possibility, future studies need to test whether hypothalamic sensing of circulating lactate is intact in obese/diabetic rodents. It has been demonstrated that direct administration of lactate centrally in rodents with uncontrolled diabetes and diet-induced insulin resistance is able to lower glucose production [103], however whether this central sensing is functional in the presence of circulating lactate remains to be explored. If this is indeed the case, it would be an appropriate platform to begin deciphering whether these mechanisms are indeed intact in obese and diabetic human subjects.

In order to employ central lactate sensing as a therapeutic agent for treating hyperglycemia, one would need to activate central lactate metabolism. This feat may be achieved by manipulating the known biochemical pathway. For example, activation of the enzyme LDH-B in neurons would lead to the downstream activation of K_{ATP} channels, ultimately resulting in decreased hepatic glucose production. Hence, by manipulating components of the lactate biochemical pathway one may be able to develop pharmacological agents to treat hyperglycemia. Hence, activation of central lactate metabolism holds potential for restoring glucose homeostasis, however further studies are required.

Aim 2: Mechanisms of Small Intestinal Nutrient Sensing

5.1 Abstract

It has been shown that an acute lipid infusion into the duodenum of rodents triggers a gut-brain-liver axis to lower hepatic glucose production. However, the molecular/signaling mechanisms lying downstream of lipids to induce this effect remain largely unknown. In parallel, protein kinase c (PKC)- δ in the hypothalamus of rodents is required for hypothalamic lipids to lower glucose production. Furthermore, PKC- δ acts downstream of lipids in the peripheral tissues liver and muscle to regulate insulin sensitivity. Based on these observations, the hypothesis that duodenal PKC acts downstream of lipids to induce the gut-brain-liver axis to effectively lower hepatic glucose production was tested. The pancreatic clamp technique in combination with tracer-dilution methodology were used to assess the effect of duodenal treatments on glucose metabolism *in vivo*. The general PKC activator 1-oleoyl-2-acetyl-sn-glycerol (OAG) was infused into the duodenum of conscious, unrestrained rats, and it was found that PKC activation decreases hepatic glucose production. When duodenal OAG was co-infused with the general PKC inhibitor bisindolylmaleimide (BIM) or the PKC- δ specific inhibitor rottlerin, this effect of glucose production was abolished. Furthermore, when neuronal transmission at the level of the duodenum was ablated with the co-infusion of the general anesthetic tetracaine, the lowering of glucose production was blunted; this suggests that a neuronal network is involved. Next, the N-methyl-D-aspartate receptor

inhibitor, MK801, was infused into the nucleus of the solitary tract concomitantly with duodenal OAG. Again, glucose production was not lowered, suggesting that neuronal signals initiated at the duodenum in response to PKC activation require NMDA receptors in the NTS to regulate glucose production. Furthermore, hepatic vagotomy (transection of the hepatic branch of the vagus nerve) prevented duodenal OAG from lowering glucose production. To demonstrate that PKC activation is acting downstream of lipid, we co-infused BIM or ROT with lipids into the duodenum, and found that glucose production was not decreased. We tested the physiological relevance of our studies with a fasting-refeeding protocol and found that duodenal PKC activation is required to lower glucose levels and maintain glucose homeostasis in response to refeeding. Lastly, we found that high-fat feeding for 3 days blunted OAG's ability to decrease hepatic glucose production. Together, these data demonstrate that duodenal PKC activation acts downstream of lipids to induce the gut-brain-liver axis to effectively decrease hepatic glucose production.

5.2 Materials and Methods

Animal Preparation

A subgroup of rats undergoing the pancreatic clamp received duodenal cannulations in conjunction with vascular catheterization and NTS stereotaxic surgery or selective hepatic branch vagotomy when required (see General Materials and Methods for vascular catheterization and stereotaxic surgery). A different subgroup of rats undergoing fasting-refeeding experiments received duodenal cannulations only. Animals were to recover fully following surgery, as measured by daily food intake and body weight before the experiments were performed (pancreatic clamp subgroup 5-7d; fasting-refeeding subgroup 4d).

Surgical Procedures

Duodenal Cannulation

Rats were anesthetized with intraperitoneal (IP) ketamine (Ketalean; Bimeda-MTC, Cambridge, Ontario) mixed with xylazine (Rompun; Bayer). Duodenal cannulation surgeries were performed as described [74]. A laparotomy incision was made on the ventral midline and abdominal muscle wall, allowing for access to the gastrointestinal tract in the peritoneum. The upper portion of the duodenum was identified as being 1.5cm proximal of the pyloric sphincter. Once the duodenum was isolated, a 25-gauge needle was used to puncture the ventral aspect of the duodenum, in a region

where the vascular arcade was minimal in order to prevent bleeding. A saline-filled 18 cm catheter made of silicone tubing (0.04 in. ID, 0.085 in. OD; Sil-Tec, Technical Products, USA) with a 0.5 cm extension of smaller silicone tubing (0.025 in. ID, 0.037 in. OD; Sil-Tec, Technical Products, USA) surrounded by Marlex mesh was inserted into the duodenal lumen approximately 2 cm downstream of the pyloric sphincter. The cannula was secured onto the exterior outer serosal surface duodenum with tissue adhesive (Vetbond) and 6-0 silk suture. The proximal portion of the cannula exited the abdominal cavity through the site where the laparotomic incision was made, and the abdominal muscle layer was sutured with 4-0 silk sutures. A small (approximately 2 cm) midline incision was made through the skin on the back of the neck, immediately rostral to the interscapular area. Through this incision, the proximal portion of the tubing was tunneled subcutaneously from the abdominal cavity to the back of the neck. The abdominal and neck incisions were closed off with 4-0 silk sutures. The duodenal catheter was flushed before being air sealed with a metal pin.

Selective Hepatic Branch Vagotomy

The subgroup of rats requiring hepatic branch vagotomy underwent the procedure at the same time as the duodenal and vascular catheterizations were performed. The hepatic vagotomy was performed as previously described [74]. A laparotomy incision was made on the ventral midline

and abdominal muscle wall, allowing for access to the gastrointestinal tract in the peritoneum. The stomach was isolated and retracted into saline-soaked cotton gauze in order to expose the descending esophagus and the subdiaphragmatic vagal trunk; a gastrohepatic ligament was isolated and severed using fine forceps. The hepatic branch of the ventral subdiaphragmatic vagal trunk was transected using microcautery, which effectively severs the hepatic vagus. Transection of the hepatic vagus nerve prevents neuronal communication between the liver and the brain, with minimal disruption of the hepato-duodenal sub-branch.

Chemicals for Intraduodenal and NTS Infusions

1) SAL

2) OAG – general PKC activator; Calbiochem; stock solution made in DMSO, working solution dissolved in saline; 250 μ M

3) BIM – general PKC inhibitor; Tocris Bioscience; stock solution made in DMSO, working solution dissolved in saline; 240 μ M

4) ROT – PKC- δ specific inhibitor; Calbiochem; stock solution made in DMSO, working solution dissolved in saline; 60 μ M

5) Tetracaine – general anesthetic; Sigma; stock solution made in DMSO, working solution dissolved in saline; 1mg/mL

6) MK801 - N-methyl-D-aspartate receptor inhibitor; Sigma; stock solution made in saline, working solution dissolved in saline; 5 μ g/mL at 0.03ng/min

7) 20% Intralipid (0.03kcal/min)

The intraduodenal infusions were given from t=150-200 at a rate of 0.01 mL/min (a bolus of 0.12 mL/min for 1 min was first administered) during the pancreatic clamp. At this rate, it has been found that there is no leakage into the circulation.

The dose of OAG used is based on a previous study in which it was infused directly into the hypothalamus of rodents; this concentration was found to decrease the hepatic glucose production during a pancreatic clamp.

The dose of ROT were used based on a previous study, in which it was found to block the effect of hypothalamic OAG on decreasing hepatic glucose production.

The dose of BIM was determined by a dose-response (60 μ M, 120 μ M, 240 μ M).

The doses of tetracaine and MK801 were chosen based on a previous study where co-administration with intraduodenal Intralipid blunted the lipid effect of decreased glucose production.

Clamp Procedure

Rats were restricted to approximately 58kcal the night before the experiments to ensure similar nutritional status. The pancreatic clamp experiment lasted a total of 200 minutes. At t=0 min, a primed continuous infusion of [3-3H]-glucose was initiated at a rate of 0.4 $\mu\text{Ci}/\text{min}$ (bolus of 40 μCi) and maintained throughout the experiment for the assessment of glucose kinetics using tracer-dilution methodology (see General Materials and Methods). Following 60 min after the onset of the infusion (t=60 min), which allows sufficient time for the tracer to equilibrate, blood samples were taken at 10 minute intervals (for collection of plasma to determine the specific activity of [3-3H]-glucose and for the analysis of glucose levels) for the remainder of the protocol. The basal steady state glucose production rate was taken to be from t=60 min to t=90 min. At t=90 min, the pancreatic clamp (basal insulin) was initiated; a continuous infusion of insulin (0.8 mU/kg/min) and somatostatin (3 $\mu\text{g}/\text{kg}/\text{min}$) was given to inhibit endogenous insulin and glucagon secretions. A variable infusion of 25% glucose was given to maintain euglycemia (euglycemic glucose levels were based on the levels determined during the basal steady state period), beginning at 30 minutes following the onset of the clamp (t=120 min). Intraduodenal infusions were initiated at t= 150 min and maintained until the end of the experiment. For experiments involving NTS treatment, MK801 was administered beginning at t=90 min and lasted until the end of the experiment. Additional blood samples were taken at t=90 min, 180 min and 200 min to determine plasma insulin levels.

Fasting-Refeeding Protocol

A subgroup of rats that had received duodenal catheters underwent the fasting-refeeding protocol to assess the physiological relevance of the pancreatic clamp findings. Rats were fasted at 4pm the day before the experiments were to be started. At 4pm the following day (ie. following a 24h fast), a 10 minute pre-infusion (at t= -10 min) of the general PKC inhibitor BIM (240 μ M) or saline (control) was administered at a rate of 0.01mL/min (bolus of 0.12mL; same dose and infusion rate as the clamp studies), and maintained for the remainder of the experiment which lasted a total of 20 minutes. Food (regular chow) was given back to the animals at t= 0 min. Food intake and glucose levels were measured every 10 minutes (ie. at t= -10, 0, 10, 20 min).

High-fat feeding model

Following duodenal and vascular catheterization surgery, a subgroup of rodents was placed on a lard-oil enriched high-fat diet for 3 days. The composition of the high-fat diet is: 45% carbohydrate, 22% protein, 33% fat; total calories provided per gram is 5.14. Daily food intake was monitored, and it was confirmed that the HFD-fed rodents ingested at minimum the same amount of calories as the rodents on regular chow. Once this was determined, the HFD-fed rodents underwent the pancreatic clamp procedure to test whether duodenal PKC activation is able to trigger the gut-brain-liver axis in this model.

5.3 Results

Duodenal PKC suppresses hepatic glucose production (Figure 6)

In order to test our hypothesis that PKC activation is able to trigger the gut-brain-liver axis to lower glucose production, we first had to demonstrate that duodenal PKC activation can lower glucose production in a clamped setting. Following infusion of the PKC activator, OAG, into the duodenum an increase in the glucose infusion rate required to maintain euglycemia, as compared to a saline infused control, during the clamp was observed (Figure 6a). This increase in glucose infusion rate was fully accounted for by a decrease in hepatic glucose production (Figure 6c). Hence, duodenal PKC activation can lower glucose production independent of changes in levels of circulating insulin and gluco-regulatory hormones. Co-infusion of OAG with both the general PKC inhibitor BIM and specific PKC delta inhibitor ROT into the small intestine abolished the observed decrease in glucose production during the clamp. The glucose infusion rate was comparable to saline control. Clamp studies were performed to confirm that BIM or ROT administered independently do not affect glucose kinetics. Glucose uptake levels did not change significantly across all treatment groups (Figure 6d). These data support the hypothesis that duodenal PKC- δ can decrease hepatic glucose production in normal rodents.

Duodenal PKC suppresses glucose production through a gut-brain-liver axis (Figure 7)

Once we confirmed that duodenal PKC activation can decrease hepatic glucose production in a clamped setting, we needed to confirm that this effect was due to its ability to trigger the *gut-brain-liver* axis. To test whether neuronal activation at the level of the duodenum is required, we infused tetracaine concomitantly with OAG. There was no decrease in hepatic glucose production during the pancreatic clamp (Figure 7c), with glucose infusion rate similar to saline infused controls (Figure 7a). Glucose uptake did not change across the treatment groups (Figure 7d). Tetracaine alone did not affect glucose kinetics. This confirms that neuronal signals at the level of the duodenum are required for a decrease in hepatic glucose production.

Next, we needed to test whether the duodenal innervation is sending signals to the CNS. In a subgroup of rodents, during the infusion of OAG into the duodenum, we infused vehicle control OR MK801 (the NMDA receptor inhibitor) into the NTS of the same animal. In the vehicle treated animals, there was an increase in the glucose infusion rate required to maintain euglycemia during the clamp (Figure 7a), which was accounted for by a decrease in hepatic glucose production (Figure 7c), as expected. When the MK801 was infused into the NTS concomitantly with duodenal OAG, the glucose infusion rate required to maintain euglycemia was comparable to duodenal saline infusion during the clamp (Figure 7a), and there was no decrease in hepatic glucose production (Figure 7c).

Glucose uptake did not change across all treatment groups (Figure 7d). MK801 alone did not affect glucose kinetics. Hence, NMDA receptor activation in the NTS is required for the duodenal OAG-induced decrease in hepatic glucose production.

Lastly, we needed to test whether signals from the NTS are sent to the liver via the hepatic vagal nerve to decrease glucose production. Therefore, we performed hepatic vagotomy surgeries and infused OAG into the duodenum of this subgroup of rodents. The glucose infusion rate required to maintain euglycemia was comparable to duodenal saline infusion during the clamp (Figure 7a), and there was no change in hepatic glucose production (Figure 7c). Glucose uptake levels did were comparable across all groups (Figure 7d). Hepatic vagotomy alone did not affect glucose kinetics. Hence, vagal efferent signals are required for PKC activation to decrease hepatic glucose production.

These data support the hypothesis that duodenal PKC (likely the δ isoform) transmits neuronal signals to the NTS thereby activating NMDA receptors, which subsequently relays signals to the liver via the hepatic branch of the vagus nerve to lower hepatic glucose production.

PKC activation is required for lipids to lower glucose production (Figure 8)

We have successfully demonstrated that PKC activation can independently trigger the *gut-brain-liver* axis to lower hepatic glucose production in normal rodents. Given that duodenal lipids also trigger this axis, and that hypothalamic PKC activation is required for lipids to decrease glucose production, we wanted to test whether PKC acts downstream of lipids in the duodenum. We infused lipid into the duodenum and confirmed previous findings that lipids increase the glucose infusion rate required to maintain euglycemia during the clamp (Figure 8a), and decrease hepatic glucose production (Figure 8c) without affecting glucose uptake (Figure 8d). Next, we co-infused BIM or ROT with lipid into the duodenum. During the clamp, the glucose infusion rate required to maintain euglycemia was comparable to that of saline controls (Figure 8a), and there was no decrease in hepatic glucose production (Figure 8c). These data indicate that duodenal PKC acts downstream of the lipid-induced gut-brain-liver axis to lower hepatic glucose production.

Pharmacological inhibition of PKC activation in the gut disrupts glucose homeostasis during refeeding (Figure 9)

It is evident from our clamp studies that duodenal PKC activation acting downstream of lipids can decrease hepatic glucose production. These findings can be strengthened with a confirmation that this mechanism is also employed in a physiological setting. Hence, we tested whether PKC

activation is required for the maintenance of glucose homeostasis in response to nutrient consumption.

Intraduodenal saline administration began at -10 minutes, and plasma glucose levels were at 106mg/dL. Refeeding began at time= 0 minutes, where plasma glucose levels rose slightly. Plasma glucose levels and cumulative food intake rose in 10 and 20 min of refeeding for the intraduodenal saline-infused rats. Strikingly, infusion of the intraduodenal PKC inhibitor BIM (also started at -10 minutes) significantly increased plasma glucose levels by approximately 20mg/dL versus saline after 20 minutes of refeeding in the presence of matching cumulative food intake. This data indicates that PKC activation in the duodenum physiologically regulates glucose homeostasis, independent of changes in food intake.

Duodenal PKC activation fails to lower glucose production in response to high-fat feeding

(Figure 10)

It has been shown that rats fed on a 3 day HFD are unable to lower glucose production in response to intraduodenal lipid infusions [74]. Hence, we wanted to test whether PKC activation can surpass this defect to lower glucose production in a high-fat setting. We infused intraduodenal OAG into rats fed on both regular chow and high fat diet, in conjunction with performing pancreatic clamp

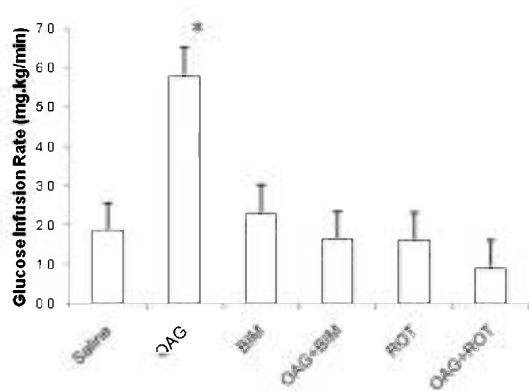
studies. OAG increased the glucose infusion rate required to maintain euglycemia (Figure 10a), and lowered glucose production in rats fed on a regular chow diet (Figure 10c), as expected. However, rats fed on a HFD for 3 days receiving duodenal OAG infusion did not show an increase in the glucose infusion rate required to maintain euglycemia (Figure 10a), and did not lower hepatic glucose production (Figure 10c). In fact, the OAG infused group on a HFD had results comparable to the saline infused animals fed on regular chow. Glucose uptake was comparable in all groups (Figure 10d).

Hence, high-fat feeding in rodents abolishes the ability of duodenal PKC to lower glucose production, a finding similar to those obtained for duodenal lipid studies. Evidently, high fat feeding results in a dysregulation of small intestinal sensing mechanisms regulating glucose homeostasis. Further studies need to be performed to address the possibility that these mechanisms are also dysregulated in obesity and diabetes, and to dissect the downstream signaling cascade regulating glucose production at the level of the duodenum, in an effort to rescue glucose homeostasis.

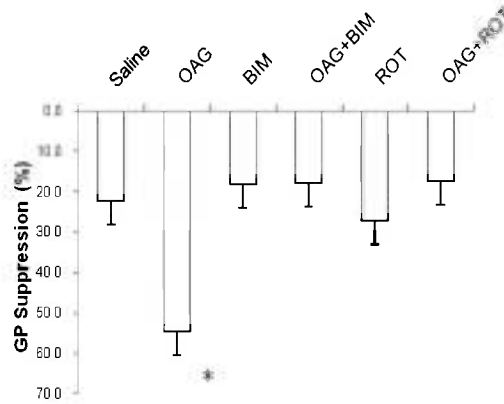
Together, these data show that duodenal PKC activation is required for lipids to trigger the gut-brain-liver axis to lower hepatic glucose production in normal rodents. We have also successfully shown that PKC activation is required for the regulation of blood glucose levels in response to feeding.

5.4 Aim 2 Figures:

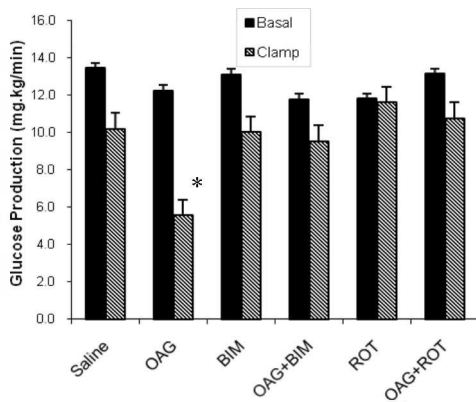
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B



C



D

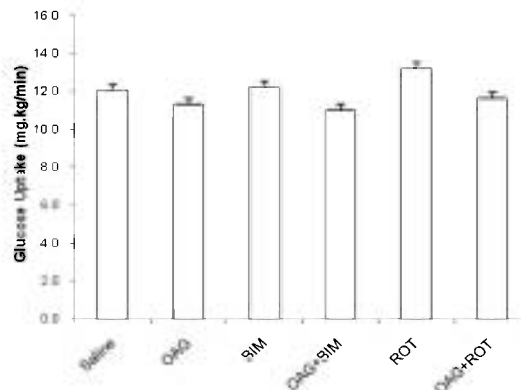


Figure 6: Duodenal PKC activation suppresses glucose production. During the pancreatic clamp, intraduodenal OAG infusion (**A**) increased GIR and (**B**) decreased GP compared to other groups. Co-administration of OAG with BIM *or* ROT abolished the OAG-induced (**A**) increase in GIR, and (**B**) decrease in GP. The inhibitors alone did not have an effect on GIR or GP. (**C**) Glucose uptake remained unchanged across treatment groups. OAG (n = 9), saline (n=10), BIM (n=5), OAG+BIM (n=5), ROT (n=5) and OAG+ROT (n=6). Values are shown as mean \pm s.e.m. *p<0.05

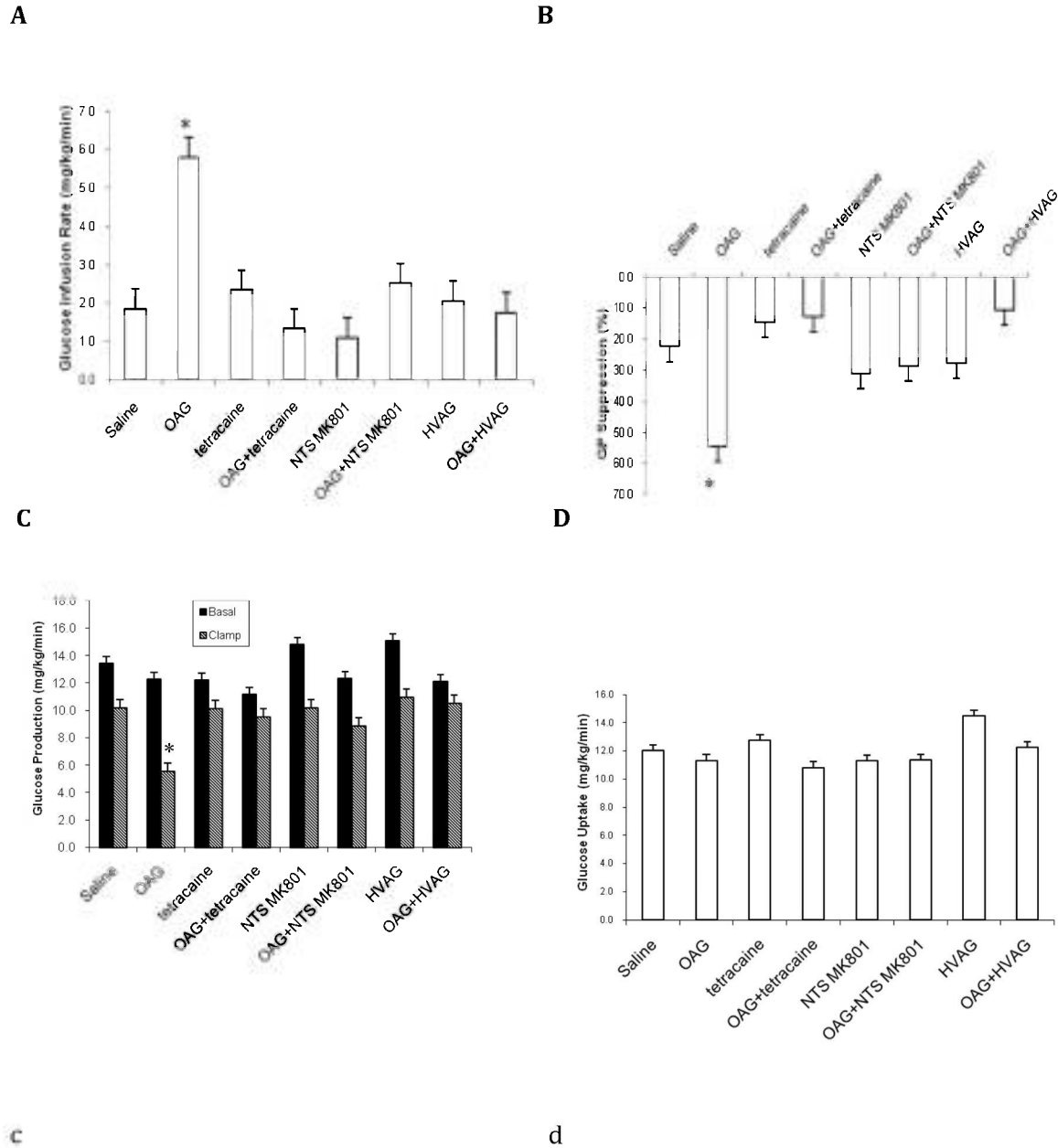


Figure 7: Duodenal PKC activation suppresses glucose production through a gut-brain-liver axis. During the pancreatic clamp, intraduodenal OAG infusion (**A**) increased GIR and (**B**) decreased GP. Co-infusion of intraduodenal OAG with tetracaine *or* NTS MK801 *or* in rodents with HVAG blunted the OAG-induced (**A**) increase in GIR and (**B**) decrease in GP. The inhibitors alone did not have an effect on GIR or GP. (**C**) Glucose uptake remained unchanged across all treatment groups. Saline (n=9), OAG (n=8), tetracaine (n=5), OAG+tetracaine (n=6), NTS MK801 (n=5), OAG+NTS MK801 (n=5), HVAG (n=5), OAG+HVAG (n=7). Values are shown as mean \pm s.e.m. *p<0.05

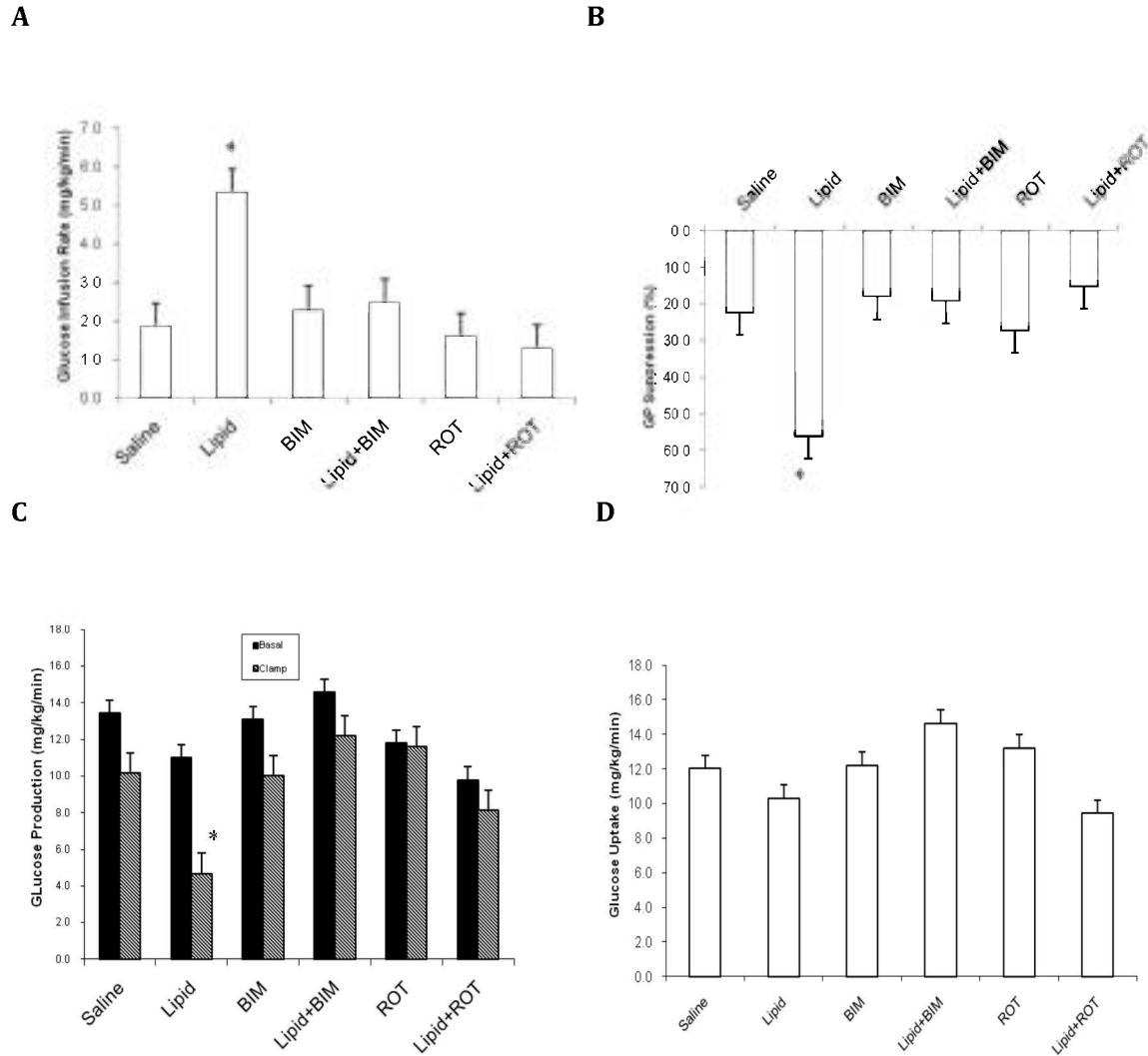
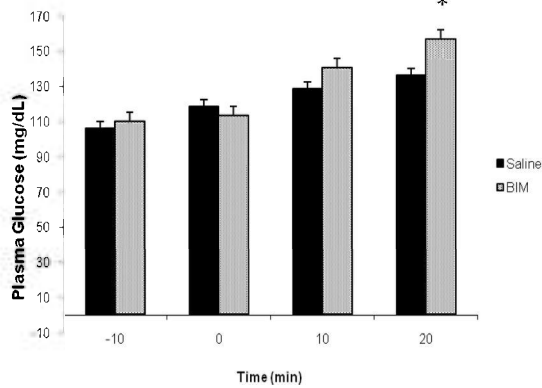
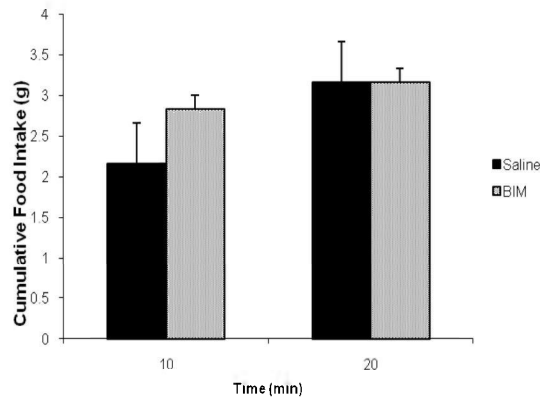


Figure 8: Duodenal PKC activation is required for lipids to suppress glucose production.

During the pancreatic clamp, intraduodenal Intralipid infusion (**A**) increased GIR and (**B**) decreased GP. Co-administration of Intralipid with BIM or ROT abolished the Intralipid-induced (**A**) increase in GIR and (**B**) decrease in GP with no effects of the inhibitors alone. (**C**) Glucose uptake remained unchanged across all treatment groups. Saline (n=8), lipid (n=9), BIM (n=5), lipid+BIM (n=5), ROT (n=5), lipid+ROT (n=6). Values are shown as mean ± s.e.m. *p<0.05

A**B****Figure 9: Pharmacological inhibition of PKC activation in the gut disrupts glucose**

homeostasis during refeeding. (A) At time -10 and 0 min, which represent the fasting state, rats receiving intraduodenal saline or BIM have similar plasma glucose levels. However, after 20 min of re-feeding, rats receiving intraduodenal BIM infusion have significantly higher plasma glucose levels compared to intraduodenal saline infused animals.

(B) Since cumulative food intake was comparable in all groups, the effects on glucose levels were independent of changes in food intake. Values are shown as mean \pm s.e.m. * $p < 0.05$

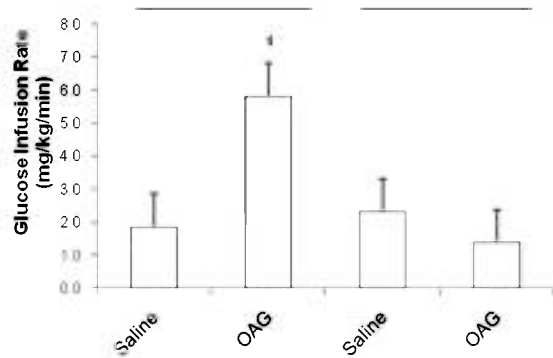
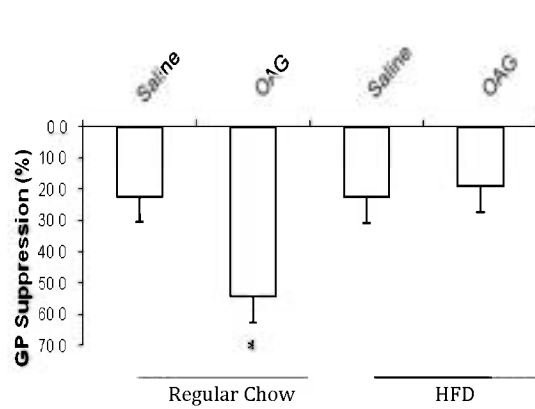
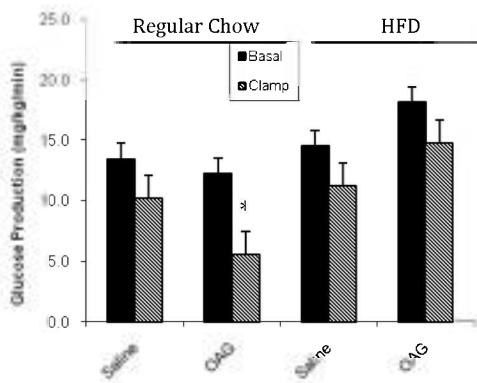
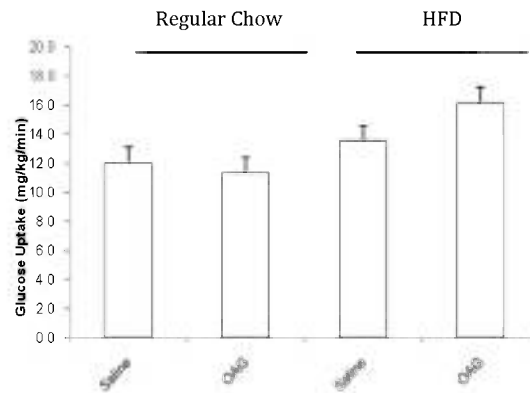
A**B****C****D**

Figure 10: Duodenal PKC activation fails to lower glucose production in response to high-fat feeding. During the pancreatic clamp, intraduodenal OAG infusion (**A**) increased GIR and (**B**) decreased GP in rodents fed on a regular chow diet. However, in rats fed on a HFD infusion of OAG failed to replicate the OAG-induced (**A**) increase in GIR and (**B**) decrease in GP. (**C**) Glucose uptake remained unchanged across all treatment groups. Saline regular chow (n=8), OAG regular chow (n=9), saline HFD (n=5), OAG HFD (n=5). Values are shown as mean \pm s.e.m. *p<0.05

5.5 Discussion and Limitations

The intestine is in a prime position to serve as a nutrient sensor, since it is one of the first sites of nutrient exposure following a meal. Hence, it is of no surprise that newly emerging research has implicated the intestine as being the first line of metabolic defense against both energy and nutrient excess.

Here, we have revealed an important signaling cascade involving activation of the novel isoform of PKC- δ in the duodenum to trigger the *gut-brain-liver* axis to lower hepatic glucose production, without affecting glucose uptake. Importantly, we have found that PKC- δ acts downstream of intraduodenal lipids to activate this important neuronal network. The aforementioned findings were performed in rodents in a clamped setting, allowing the assessment of PKC activation in the regulation of glucose homeostasis without accompanying changes in insulin and gluco-regulatory hormones. In order to strengthen these findings and assess the role of PKC activation in a physiological setting, we also performed fasting-refeeding experiments. Consistent with our data obtained from the clamp setting, we found that PKC activation suppresses plasma glucose levels in response to feeding. Hence, we have successfully demonstrated using multiple approaches that duodenal PKC- δ activates a neuronal network to regulate glucose homeostasis in vivo. This is consistent with previous findings demonstrating that activation of hypothalamic PKC- δ decreases

hepatic glucose production in rodents [64]. Hence, we have strengthened the notion that activation of PKC- δ may play an important role in decreasing glucose levels. This finding may hold beneficial implications in the treatment of disease complications, namely type 2 diabetes where hyperglycemia due to increased glucose production contributes to a number of diabetic complications.

Our findings have therefore added to the knowledge of the duodenum as being an important regulator of whole body homeostatic parameters. The existence of a lipid-induced gut-brain axis to effectively decrease food intake and body weight has been established in both rodents and humans [76-80]. Furthering the notion of the intestine as being a regulator of vital homeostatic parameters was the finding of a lipid-induced *gut-brain-liver* axis to effectively decrease hepatic glucose production [74]. Incoming lipids are first esterified to LCFA-CoA by intestinal lipases [103;104]; LCFA-CoA subsequently activates the vagal afferent fibers innervating the duodenum [86], sending a signal to the NMDA receptors of the NTS [88]. NTS-initiated signals are then sent to the liver via the vagal efferents to effectively decrease hepatic glucose production. Our study is the first to show the signal transduction mechanisms which act downstream of LCFA-CoA in the intestine to trigger this important effect. We have demonstrated that the activation of PKC specifically in the duodenum sends a neuronal signal to the NTS through the vagus nerve, where NMDA receptors are

activated, resulting in activation of the hepatic vagal efferents to decrease hepatic glucose production. Interestingly, the activation of PKC in a model of early onset of insulin resistance is unable to trigger the gut-brain-liver axis to lower hepatic glucose production. This finding suggests that restoration of the mechanisms downstream of PKC signaling in obesity and type 2 diabetes may contribute to the normalization of glucose levels. Further studies aimed at dissecting these molecular mechanisms are required.

The literature regarding the role of PKC- δ on diabetic parameters has been examined with the use of genetic approaches. Generation of a PKC- δ whole-body knockout mouse demonstrated a phenotype in which knockout mice fed on regular chow exhibit glucose intolerance and impaired insulin secretion [120]. The mice were challenged with an intraperitoneal glucose test, and it was found that the mice were glucose intolerant with reduced glucose stimulated insulin secretion. An insulin tolerance test was also performed, however there was no observed decrease in insulin sensitivity. To delineate the mechanism involved in the glucose intolerance, the authors performed studies on isolated islets from the knockout mice. It was found that the mice exhibited decreased glucose stimulated insulin secretion. Furthermore, this was found to be the result of reduced PKC- δ mediated phosphorylation of the Munc18-1 protein, which is tightly involved in exocytosis events [121]. Hence, the PKC- δ knockout mice exhibit glucose intolerance as a result of decreased

exocytosis of insulin granules in islets, which is the result of decreased Munc18-1 phosphorylation.

In our study, the effects of insulin secretion were not determined. However, we postulate that the effects are not due to a change in insulin secretion, since insulin levels are maintained at near basal during the clamp procedure.

In contrast to the aforementioned study, a PKC- δ whole-body knockout generated by a different group revealed a phenotype in which knockout mice fed on a HFD exhibit improved glucose tolerance as a result of increased insulin sensitivity [122]. The knockout mice fed on a HFD were subjected to an intraperitoneal glucose tolerance test, and it was found that compared to the control littermates, the knockout mice were protected against HFD induced glucose intolerance. Interestingly, the authors also found that the knockout mice fed on regular chow demonstrated improved glucose tolerance, as a result of increased insulin sensitivity (as determined by decreased circulating insulin levels). The improved glucose tolerance was attributed to a decrease in lipid accumulation, which is correlated with reduced synthesis of hepatic sterol regulatory element binding transcription factor 1, FAS and ACC, which are molecules involved in lipogenesis.

These results hold interesting implications for our findings that activation of duodenal PKC- δ improves insulin sensitivity in rodents fed on a regular chow diet. Although the deleterious effect of the whole body knock-out on regular chow was attributed by the authors to a decrease in insulin

secretion, clamp studies were not performed such that a fair assessment of insulin sensitivity was not made. (Note: Clamp studies allow assessment of glucose kinetics in the presence of basal insulin levels; on the contrary, an insulin-stimulated glucose tolerance test requires a high dose of insulin. At such high insulin doses, the change in hepatic glucose production (and therefore hepatic insulin sensitivity) cannot be fairly assessed). A whole body knock out model would target PKC- δ in the intestine, thereby removing the beneficial effect of PKC- δ activation on insulin sensitivity. It remains to be determined whether restoration of PKC- δ signaling in the intestine would rescue the defects in the whole body knock-out mouse. Our findings, then, may also potentially explain why knockout mice fed on a HFD exhibit improved glucose tolerance. It is known that PKC- δ in the periphery is associated with insulin resistance in rodents fed a HFD [49-57]. Hence, knocking out PKC- δ in the periphery would have beneficial effects in rodents fed a HFD. Since we have shown that the PKC- δ 's ability to reduce glucose production is impaired in HFD-fed rodents, the intestinal loss of PKC- δ would not be deleterious. Overall, our findings allude to the notion that PKC- δ in the intestine specifically may play a pivotal role in maintaining whole body glucose homeostasis.

Since PKC- δ activation does not decrease glucose production in our model of early onset of insulin resistance, it is not a potential therapeutic agent for the treatment of hyperglycemia in diabetes and obesity. However, this finding may partly explain the remarkable improvement in glycemic control

witnessed following gastric bypass surgeries in type 2 diabetic individuals, even before weight loss occurs [123]. It has been observed that in addition to treating severe obesity, gastric bypass surgeries are followed by a remission of diabetes in many cases. The surgery involves minimizing stomach size, in addition to removing the duodenal segment of the small intestine and attaching the stomach to the jejunum. Hence, nutrient accumulation in the duodenum is bypassed. Since it has been shown that lipid sensing in the duodenum is impaired in models of early onset of insulin resistance [104], and we have now demonstrated that PKC- δ signaling is impaired in the same model, it is a possibility that bypassing the defects in the duodenum is restoring glucose homeostasis by activating nutrient sensing mechanisms in the jejunum and/or ileum. This hypothesis remains to be tested.

In conclusion, we have provided evidence that activation of the signaling molecule PKC- δ in the duodenum triggers a *gut-brain-liver* axis to decrease hepatic glucose production in normal rodents. This finding may contribute to the development of therapeutic strategies aimed at restoring glucose homeostasis in obesity and type 2 diabetes. However, our studies are not without limitations.

To activate PKC in the duodenum, we used the general PKC activator OAG, which is an analogue of the lipid metabolite DAG. Hence, this pharmacological activation is not specific to a particular PKC

isoform. To partly compensate for this, we used a PKC- δ specific inhibitor Rottlerin to test whether the glucose production lowering effect of OAG is reversed. However, some studies have suggested that this compound may in fact not be completely specific for PKC- δ [124]. To overcome these shortages due to unspecificity of pharmacological compounds, one would have to employ the use of molecular techniques such as adenovirus overexpression of PKC- δ specifically in the duodenum to confirm that the effect of decreased glucose production is due to activation of this specific isoform. Corollary, adenovirus overexpression of dominant negative PKC- δ , or shRNA targeted against PKC- δ selectively in the duodenum would blunt the effect of decreased glucose production, and confirm the specificity of this isoform in mediating the effect.

Although it has been shown from previous studies that a 50 minute duodenal infusion will not leak into the circulation [74], we are unsure whether leakage occurs into the other parts of the intestine (ie. the jejunum and ileum). Hence, there is a possibility that PKC activation in other gut segments may partly be contributing to the observed effect. Since PKC is expressed in other intestinal segments, we would need to exclude this possibility. This can be overcome by placing the intestinal infusion catheters into the jejunum or ileum, to see whether OAG infusion would indeed decrease hepatic glucose production. If such is the case, then we could not be in a position to claim that PKC in the duodenum is regulating glucose production, but rather the entire small intestine. If OAG did

not decrease hepatic glucose production in the jejunum or ileum, we would be in a position to claim that the duodenum is exerting the effect, since it would be impossible for leakage to occur from the jejunum or ileum to the upstream intestinal segments.

5.6 Future Directions

We have demonstrated a novel role of duodenal PKC- δ in regulating hepatic glucose production through a neuronal axis in normal rodents. We have shown that it acts downstream of LCFA-CoA to induce this effect, however the signaling/molecular mechanisms lying downstream of PKC- δ remain to be elucidated. A potential downstream mediator may be the gut peptide CCK. It has been shown that in the STC-1 cell line [125;126], PKC is able to stimulate the secretion of CCK. Furthermore, it has been shown that CCK acts downstream of lipids to trigger the *gut-brain-liver* axis to decrease glucose production via activation of CCK-A receptors in the duodenum. Hence, it is plausible that PKC- δ activation results in the stimulation of CCK release to act on duodenal CCK-A receptors. This link remains to be explored.

Although we are postulating that PKC, and subsequent CCK activation, mediate the lipid-induced decrease in hepatic glucose production, there remains a possibility that other mechanisms are involved. In particular, the gut expresses receptors which bind FA, such as TLR (toll-like receptor) [127] and G-protein coupled receptor (GPR)-40 [128], leading to the possibility that these receptors play a role in our described lipid effect. TLR receptors have a well-established role as mediating inflammatory pathways, however their role has recently been expanded to acting as regulators of insulin sensitivity in rodents. In particular, a study has found that TLR-4 knockout mice are

protected from decreased insulin signaling in muscle, and do not have reduced insulin sensitivity in response to systemic lipid infusion [129]. Another study demonstrated the importance of TLR-5 in mediating various metabolic parameters, with the finding that TLR-5 deficient mice have increased food intake and develop insulin resistance [130]. Hence, TLR are emerging as important mediators of energy and glucose homeostasis. GPR40 is also expressed in the gut, and mediates glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) secretion in response to FA stimulation [127]. Therefore, GPR40 activation also indirectly contributes to the regulation of glucose homeostasis, since the aforementioned incretins augment insulin action. Hence, there is a possibility that in response to our lipid infusion, binding of FA to TLR or GPR40 may contribute to the observed changes in glucose production. Although a possibility, it remains unlikely since previous studies have shown that the conversion of LCFA to LCFA-CoA is a required step in the lipid-induced gut-brain-liver axis to lower glucose production [75], and both these receptors bind FA directly. Hence, if there was a potential involvement, ablation of LCFA-CoA formation should not blunt the lipid effect. However, further studies need to be performed to test the involvement of these important gut receptors in the lipid-induced regulation of glucose homeostasis.

The focus of our gut nutrient studies was limited to the upper intestine, namely the duodenum. Although the duodenum is the main site of nutrient absorption, making it an ideal site for nutrient sensing mechanisms, nutrients have also been shown to reach the distal gut segments (jejunum and ileum). Hence, there remains the possibility that the jejunum and ileum may also be involved in intestinal nutrient sensing mechanisms. Indeed, studies have shown that lipid infusions into the jejunum or ileum decrease food intake [131]. However, the distal gut has not yet been implicated in regulating glucose homeostasis. Hence, it is plausible that PKC may act downstream of lipids in the distal gut to regulate glucose homeostasis.

General Discussion

The regulation of glucose homeostasis involves a complex interplay between various systems. The main regulator of plasma glucose levels in response to a glucose load is insulin, which is secreted by the pancreatic β cells. The role of insulin is to increase glucose uptake in the peripheral tissues (skeletal muscle and adipose) and to inhibit hepatic glucose production [132]. On the contrary, if glucose levels decrease, a counter-regulatory response is initiated, where glucagon stimulates glucose production [133]. In addition, the hormones epinephrine and norepinephrine, as well as cortisol and growth hormone can further augment plasma glucose levels during hypoglycemia [132]. Further regulation of glucose levels comes from incretin hormones, which augment insulin release and therefore indirectly lower glucose levels [132].

In addition to the aforementioned physiological responses to changes in glucose levels, nutrient sensing in the regulation of glucose and energy homeostasis has emerged as an exciting new avenue of research. Being such a new avenue, there are to date an unlimited number of possibilities that remain to be explored. These possibilities include deciphering the downstream molecular mechanisms of nutrient sensing which act in the hypothalamus and the gut.

In this thesis, we have begun to partially dissect these pathways. We have found that central lactate sensing in the presence of circulating lactate is required for the maintenance of homeostasis. Given that central lactate, and not FFA, is able to decrease hepatic glucose production in models of early onset of diet induced insulin resistance [32;103], this finding alludes to the notion that activation of lactate metabolism specifically may be applicable to treating glucose imbalance in obese and diabetic individuals. Furthermore, it has been found that glucose sensing in the portal vein triggers a neuronal network to regulate glucose uptake [99]. In line with the notion that the brain and the gut share similar biochemical and physiological pathways to regulate glucose homeostasis, it would be of interest to determine whether the glucose metabolite lactate could also trigger this effect.

We have also shown that PKC acts downstream of lipids in the duodenum to trigger the gut-brain-liver axis to decrease hepatic glucose production. This is the first attempt to elucidate the signaling mechanisms of lipids. Although PKC activation did not trigger this axis in a model of diet induced insulin resistance, our study has paved the way for other studies which further aim to dissect what signaling molecules lie downstream of PKC. Once the defect is pinpointed, pharmacological agents could be used to restore glucose homeostasis. Interestingly, hypothalamic PKC- δ activation in rodents fed on a HFD for 3 days is able to suppress glucose production [64]. Hence, it is of interest to pinpoint what are the differences in the downstream signaling events specific to PKC- δ in the

hypothalamus and the gut. This would pave way for restoring the defect in the gut, if the hypothalamic mechanisms are elucidated.

General Conclusion

Based on the findings of this thesis, and others, it seems evident that the gut and the brain share redundant physiological responses in their regulation of glucose production. In other words, nutrient sensing mechanisms both in the brain and the gut act in concert to decrease hepatic glucose production. Furthermore, the biochemical/molecular pathways also seem to converge. For example, LCFA-CoA in both the hypothalamus [64], and as we have shown, the duodenum results in a decrease in hepatic glucose production via activation of PKC. Hence, the brain and the gut present a united front in maintaining glucose homeostasis in the presence of nutrient excess. These findings may hold important implications for therapeutic strategies aimed at restoring glucose levels in obesity and type 2 diabetes. Namely, activation of a single biochemical pathway could provide dual benefits by triggering glucose restoration mechanisms in both the brain and gut. In order to achieve this feat, the molecular pathways governing glucose restoration need further elucidation.

Overall, the exciting new avenue of nutrient sensing mechanisms in the CNS and the gut has paved way for a milieu of possible interventions targeted to restore energy and glucose homeostasis in obese and diabetic individuals.

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