Mechanisms of hypoglycemic seizures in mice

Thesis for the degree of Doctor of Philosophy (PhD)

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Dedicated to my family, teachers and friends
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ABBREVIATIONS

ATP, Adenosine triphosphate;

BGL, Blood Glucose Level;

BAD, Bcl-2 associated death promoter;

BHB, Beta-hydroxybutyrate;

CNS, Central Nervous System;

CST, Cell signaling technology;

cyclic AMP, cyclic adenosine monophosphate;

DCCT, Diabetic control and complication trail study;

EDIC, Epidemiology of diabetic interventions and complications;

EGTA, Ethylene glycol tetraacetic acid;

F, Fasting;

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase;

H, Hour;

HAAF, Hypoglycemia associated autonomic failure;

HCL, Hydrochloride;

HFD, High fat diet;

HRP, Horseradish-peroxidase;

\textit{i.p}, intraperitoneally;
IIHS, Insulin induced hypoglycemic seizures;

INS, Insulin;

Na$_2$EDTA, Disodium ethylenediaminetetraacetic acid;

Na$_3$VO$_4$, sodium orthovanadate;

NaCl, Sodium chloride;

NIK, NF-kappaB inducing kinase;

NIRKO, Neuronal specific insulin receptor knockout;

NMDA, N methyl D aspartate;

PGC, Peroxisome proliferator-activated receptor-gamma coactivator

PEPCK, phosphoenolpyruvate carboxykinase;

PFC, Prefrontal cortex;

PKA, Protein kinase A;

PMSF, Phenylmethylsulfonyl fluoride

PVN, Paraventricular Nucleus

U, Unit;

USP, United States Pharmacopeia;
ABSTRACT

Intensive insulin therapy increased the life expectancy by reducing the micro and macro vascular complications in diabetic patients but it is associated with major complication, the hypoglycemia associated with coma or seizures. In order to understand the physiological mechanism in hypoglycemia, I investigated the 1) segmental brain glucose sensing and functional protein expression by analyzing the Bcl-2 associated death promoter (BAD) and AKT (protein kinase B) during fasting hypoglycemia, 2) glucagon signaling associated glucose production in brain and liver during fasting hypoglycemia and 3) β-hydroxybutyrate during fasting hypoglycemia associated insulin induced hypoglycemic seizures in male C57BL6/J mice.

BAD-deficient mice and Neuronal specific insulin receptor knockout (NIRKO) mice display impaired counter-regulatory hormonal responses during hypoglycemia. In my study, I investigated the fasting mediated expression of p-BAD<sup>ser155</sup> and p-AKT<sup>ser473</sup> in different regions of brain (prefrontal cortex, hippocampus, midbrain and hypothalamus). Overall in support with previous findings, fasting mediated hypoglycemia activates prefrontal cortex insulin signaling which influences the hypothalamic paraventricular nucleus mediated activation of sympathoadrenal hormonal responses.

The sustained glucagon infusion increases the hepatic glucose production, but this
effect is transient due to hypothalamic glucagon signaling. In hypoglycemia, glucagon acts as a major defense to sustain the blood glucose level and this raises the question regarding glucagon signaling associated glucose production in prolonged fasting hypoglycemia. In my study, I investigated the glucagon signaling in hypothalamus by analyzing the glucagon receptor expression and liver glucose production by analyzing p- protein kinase A (PKA) \textsuperscript{Ser/Thr} substrate and phosphoenolpyruvate carboxykinase (PEPCK) expression at different fasting time (4, 8, 12, 18, 24, 30, 36 and 42 h) using western blotting technique. Overall, the hypothalamic glucagon signaling possibly attenuates the liver p-PKA \textsuperscript{Ser/Thr} substrate and it may not affect the liver glucose production due to sustained elevation in PEPCK expression.

Severe hypoglycemia induced by insulin is associated with coma or seizures.

Generally fasting reduces seizures in animal models but fasting predisposes insulin induced hypoglycemic seizures (IIHS). So I suspect the duration of fasting may possibly affect the onset of seizures and I investigated the IIHS by administering 8 Units (U) insulin /k.g., intraperitoneally to 6 and or h fasted male C57BL6/J mice.

Overall, the quick seizure onset in 6 h fasted mice was possibly due to autonomic failure associated impaired glucose production and the late seizure onset in 24 h fasted mice was possibly due to progressive elevation of ketone bodies which may support brain metabolism.
INTRODUCTION

History of Diabetes Mellitus

- Diabetes mellitus, the rare condition was first recognized by ancient Egyptians (1500 B.C.E) in which a person urinate excess and lost weight [1].

- The Greek physician Aretaeus, used the term diabetes mellitus (80-138 C.E), by observing the urine had a sweet taste [1].

- Matthew Dobson (1776) measured the glucose concentration of patient urine and found it was increased in diabetes mellitus patients [1].

- Diabetes mellitus (19th century) was recognized in clinic and considered as fatal within weeks to months after diagnosis and there was no treatment available at that time [1].

Pathogenesis of Diabetes Mellitus

Diabetes is a complex, heterogeneous disorder.

- Type 1 diabetes mostly occur in young people and is due to selective autoimmune destruction of pancreatic beta cell, which leads to insulin deficiency [2].

- Type 2 diabetes mostly occurs in older adults, but the age of onset decreasing now. The increase in body weight, high fat diet and lack of physical activity are the major factors considered for the prevalence of diabetes [2].
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Insulin discovery

- Joseph von Mering and Oskar Minkowski (1889) removed the pancreas from dogs and they found that removing pancreas leads to diabetes, which gives the clue that pancreas and regulation of glucose levels [1].

- Edward Albert Sharpey-Schafer (1910) hypothesized that, diabetes was due to deficiency by a chemical compound in pancreas, and he called insulin based on the Latin word insula, meaning island [1].

- Frederick Banting and Charles Best (1921) discovered insulin and together with James Collip and John Macleod, they purified the hormone insulin and were the first to treat patient with diabetes [1].

Insulin therapy and its complications

Insulin therapy increased the life expectancy in diabetic patients but it is associated with macro vascular complications (coronary artery disease, peripheral artery disease and stroke) and micro vascular complications (diabetic nephropathy, neuropathy and retinopathy). Later it was suspected that uncontrolled hyperglycemia were behind these complications [3].

Diabetic control and complication trail study

Diabetic control and complication trail study (DCCT) (1983 - 1993) was designed to test the hypothesis that elevated plasma glucose concentration is responsible for the
complication in diabetes. The study was divided into two patient groups.

- **Conventional (goal: clinically well-being, called the standard treatment group)**
- **Intensive (goal: normalization of blood glucose level, called intensive treatment group)**

In their study results intensive therapy reduces the risk of eye disease (76%), kidney disease (50%) and nerve disease (60%) [4].

**Epidemiology of diabetic interventions and complications study**

The DCCT trail was ended in 1993, and the follow study Epidemiology of diabetic interventions and complications (EDIC) was continued using the same 90% participants from DCCT to study the cardio vascular risk parameters.

In their study results, intensive therapy reduces the risk of cardiovascular risk event (42%), non-fatal heart attack, stroke or death from cardiovascular causes (57%) [4].

**Risk of intensive insulin therapy**

One of the major problems associated with intensive insulin therapy is hypoglycemia.

According to DCCT study, there were 3,788 episodes of severe hypoglycemia (requiring assistance), 1,027 episodes were associated with coma or seizures. Overall the rate of severe hypoglycemia is 61.2% per 100 patient-years and the risk of hypoglycemia was persisted throughout the DCCT study period [5].
**Physiology of hypoglycemic counter-regulation in normal subjects**

When the blood glucose drops, the key physiological changes occur in the body to maintain the blood glucose level are

- Decrease in pancreatic $\beta$-cell insulin secretion.
- Increase in pancreatic $\alpha$-cell glucagon secretion.
- Increase in hormones such as adreno-medullary epinephrine, growth hormone and corticosteroids.

These hormones act in the liver and to increase the liver glucose production to sustain the blood glucose level. The hormonal release were controlled by the brain, which has several glucose sensors and it also directly modulate liver glucose via vagus nerve [6].

**Pathophysiology of hypoglycemic counter-regulation in diabetic subjects**

In intensive insulin therapy diabetic patients, when the blood glucose level drops, there was no increase in glucagon and attenuated epinephrine which leads to failure in liver glucose production to sustain blood glucose level. This hormonal failure in hypoglycemia is called as hypoglycemia associated autonomic failure (HAAF).

In order to understand the HAAF mechanisms in diabetes, the researchers started to investigate the brain glucose sensing regions and the function of proteins that involved in controlling glucose production [7].

**Brain glucose sensing and control**
So far, there are different regions of brain were activated during hypoglycemia such as hypothalamus, prefrontal cortex, thalamus and nucleus solitary tract. Specifically the ventral medial hypothalamic region control the hormonal release of glucagon and epinephrine and there are several proteins like K –ATP channel, AMPK, were involved in sensing glucose detection.[8,9].
SCOPE OF THE STUDY

1. To investigate the glucose sensing region and functional protein expression involved in glucose sensing associated with hormonal regulation during fasting hypoglycemia, I investigate the protein expression of p-BAD<sup>ser155</sup> and p-AKT<sup>ser473</sup> in different regions of brain (prefrontal cortex, hippocampus, midbrain and hypothalamus).

2. In hypoglycemia, glucagon acts as a major defense to sustain the blood glucose level and this raises the question regarding glucagon signaling in prolonged fasting hypoglycemia. To understand the glucagon signaling in fasting hypoglycemia, I investigate the glucagon signaling in hypothalamus and liver glucose production at different fasting time (4, 8, 12, 18, 24, 30, 36 and 42 h).

3. Severe hypoglycemia is associated with coma or seizures. The previous reports suggesting that fasting (48 h) in mice causes resistance to insulin induced hypoglycemic seizures (IIHS) but in rats fasting (14-16 h) predisposes IIHS. So I suspect the duration of fasting may possibly affect the onset of seizures and in this study I investigated the IIHS by administering 8 Units (U) insulin /k.g., intraperitoneally to 8 weeks old male C57BL6/J mice.
CHAPTER I

Fasting mediated increase in p-BAD\textsuperscript{ser155} and p-AKT\textsuperscript{ser473} in the prefrontal cortex of mice
Introduction

BAD (BCL2 associated death promoter) is a member of BCL2 family of cell death and survival proteins. Phosphorylation of BAD inhibits apoptosis. Apart from the apoptotic function, BAD role is well recognized in metabolism. BAD\textsuperscript{ser155} and glucokinase reside in a mitochondrial complex and dephosphorylation of BAD\textsuperscript{ser155} diminishes glucokinase activation that blunts mitochondrial respiration in response to glucose [10].

BAD\textsuperscript{−/−} mice and whole brain RNA interference mediated knockdown of BAD in wild type C57BL/6J mice display impaired counter regulatory hormonal responses after 2-Deoxy-D-Glucose induced glucoprivation and insulin induced hypoglycemia [11]. In addition to that primary BAD\textsuperscript{−/−} and BAD\textsuperscript{ser155A} neurons and astrocytes decrease glucose and increase the uptake of beta-hydroxybutyrate (BHB) for mitochondrial oxidation and BAD\textsuperscript{−/−} and BAD\textsuperscript{ser155A} mice are seizure resistant in several animal models [12].

Starvation or fasting, leads to decrease in blood glucose level (BGL), the hypoglycemia. The physiological defenses against hypoglycemia are regulated by central and peripheral mechanisms. In the periphery pancreatic alpha cells increase glucagon and beta cells decrease insulin. The brain influences the endocrine and sympathoadrenal mediated activation of counter regulatory hormones and neurogenic
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symptoms. This leads to increase in glucose production in liver, carbohydrate
ingestion and shifts to the production of alternative fuel [13,14].

Glucose is the primary fuel to the brain under the normal physiological condition. The
brain utilizes ketone bodies such as BHB and acetoacetate in fasting, starvation and
chronic feeding of ketogenic diet. Ketosis decreases glucose metabolism and
increases ketone body metabolism in brain [14–17]. In addition to that fasting reduces
seizure severity in animal models except in insulin induced hypoglycemic seizures
[18–21].

From the above reports both fasting and BAD-deficient mice have several common
physiological roles such as altering counter-regulatory hormones, increasing BHB
utilization, decreasing glucose utilization and reduced seizures severity in animal
models.

Insulin role is critical in response to hypoglycemia. Insulin increase, decrease and not
alter counter-regulatory hormonal responses. Also neuronal specific insulin receptor
knockout mice (NIRKO) have attenuated sympathoadrenal response during
hypoglycemia [22]. In the periphery, especially in liver insulin phosphorylates its
downstream kinase Protein kinase B or AKT and inhibits gluconeogenesis and
glycogenolysis [23]. To understand the insulin signaling and BAD regulation, I
investigated the fasting mediated expression of p-BADser155 in different regions of the
brain and p-AKT\textsuperscript{ser473} in different regions of the brain and liver.

Materials and methods

Experimental design

Group 1 of non-fasted mice (n=6) had free access to food and water ad libitum. Group 2 of fasted mice (n=6) had free access to water but not food for 18 h. Group 3 of non-fasted mice (n=4) had free access to food and water ad libitum. Group 4 of fasted mice (n=5) had free access to water but not food for 24 h. Mice were sacrificed after the blood glucose measurement between (11.00 A.M. – 3.00 P.M.). I collected different parts of the brain such as prefrontal cortex, hippocampus, hypothalamus and midbrain [Fig.1], and liver. The reason for the collection of different parts of the brain is 1) hypothalamus acts a glucose sensor [24] 2) hypoglycemia activates medial prefrontal cortex [25] 3) hippocampus is highly vulnerable to seizures and (Avoli 2007) 4) fasting predisposes insulin induced hypoglycemic seizures by altering substantia nigra K(\text{ATP}) channel [21]. Substania nigra located in the midbrain and I took the entire midbrain. The collected samples were snap-frozen with liquid nitrogen and immediately stored at -80ºC.
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**Fig. 1.** The different regions (arrow pointed) of the brain separated for western blotting analysis

**Blood collection for glucose measurement**

Blood samples were collected from tail bleeds and BGL was checked using FreeStyle Freedom glucometer
Homogenization of brain and liver tissues

Briefly, the tissue was homogenized in lysis buffer 20 mM Tris-HCL (pH-7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, and 1 mM Na₃VO₄. 0.1 mM PMSF, 1 µg/ml aprotonin, 1 µg/ml leupeptin and phosphatase arrest III (1:1000) using a tissue homogenizer for 20 s and centrifuged at 800 g for 15 min. The supernatant was stored at –80°C. The protein concentrations of the resulting solutions were determined by the bicinchoninic acid method.

Protein analysis by Western blotting

Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween (TBS-T) and incubated using the following antibodies: Primary antibodies used were as follows p-BADser155 were normalized to GAPDH and β-Actin and p-AKTser473 were normalized to total AKT. All the antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and used at a dilution of 1:1000. After washing for three times with TBS-T, the membranes were incubated with appropriate horseradish-peroxidase (HRP) conjugated secondary antibodies for 1 h at room temperature. Further, the membranes were washed three times with TBS-T and then developed using a chemiluminescence detection system (Amersham Biosciences,
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Buckinghamshire, UK). The blots were scanned and the signals were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). I observed p-AKT\textsuperscript{ser473} and total AKT expressed in 60 (kDa) molecular weight. I observed p-BAD\textsuperscript{ser155} expression in between 35-45 (kDa) molecular weight.

Statistical analysis

Statistical analysis was performed using two tailed Students \textit{t}-test.

Results and discussion

Blood glucose level

In group 1 the BGL of non-fasted mice was 143.5 ± 5.68 mg/dl. In the group 2 the basal BGL was 148.3 ± 6.94 mg/dl and after 18 h fasting the BGL was significantly reduced into 78 ± 5.4 mg/dl. In the group 3 the BGL of non-fasted mice was 137.0 ± 2.94 mg/dl. In the group 4 the basal BGL was 131.8 ± 3 mg/dl and after 24 h fasting the BGL was significantly reduced into 87.8 ± 4.87 mg/dl.

p-BAD\textsuperscript{ser155} expression in different regions of the brain.

In the 18 h fasted mice I observed a significant increase in p-BAD\textsuperscript{ser155} expression in prefrontal cortex, but significant decrease in p-BAD\textsuperscript{ser155} in hippocampus, midbrain and decrease but not significant in hypothalamus (Fig.2, 3).

To confirm again, I checked in 24 h fasted mice and I observed a significant increase in p-Bad\textsuperscript{ser155} in the prefrontal cortex and a significant decrease in the hypothalamus.
My results suggest that except prefrontal cortex all the other regions may utilize BHB as an alternative fuel by decreasing p-BAD$_{\text{ser155}}$ during hypoglycemia. In the brain, neuronal activity leads to increase in cerebral blood flow and hypoglycemia increases the regional cerebral blood flow to medial prefrontal cortex, in particular anterior cingulate cortex [25]. Stress impairs prefrontal cortex [27]. Fasting mediated hypoglycemia increases stress hormones and one study reported that increase in glucagon, epinephrine and corticosterone without hypoglycemia not increase neuronal activity in the cingulate cortex of rats and they suggest that increase in neuronal activity is only by hypoglycemia [28–31]. In my results p-BAD$_{\text{ser155}}$ specifically increases in prefrontal cortex and decreases in hypothalamus, the glucose sensing area of brain, suggesting that prefrontal cortex p-BAD$_{\text{ser155}}$ may get involved in counter regulatory hormonal response activation during hypoglycemia and it raises the question that prefrontal cortex sense only glucose and not uptake the alternative fuel BHB. Fasting decreases p-BAD$_{\text{ser155}}$ in hippocampus, the area which is considered highly vulnerable to seizures (Avoli, 2007) suggesting that fasting may increase the uptake of BHB by decreasing p-BAD$_{\text{ser155}}$ or it may open K( ATP) channel to reduce seizure severity.
Fasting predisposes insulin induced hypoglycemic seizures and it is believed that K(ATP) channel in substantia nigra plays a significant role in fasting mediated predisposition and severity of seizures [21]. In my study, fasting significantly decreases p-BAD$^{\text{ser155}}$ in midbrain and my study doesn’t explain the midbrain role in fasting mediated predisposition of seizures.

**p-AKT$^{\text{ser473}}$ expression in different region of brain and liver**

S. J. Fisher’s group reported that neuronal insulin receptor knockout mice has impaired counter regulatory hormonal response during hypoglycemia [32]. Hypothalamus acts as a glucose sensor and they injected insulin into the mediobasal hypothalamus and intracerebroventricular (2.8 mm posterior to bregma, on the suture line, to a targeted depth 10.1 mm) the place which may not exactly targeting the prefrontal cortex and they found no change in the counter regulatory hormonal levels [24]. This suggests that hypothalamus is not the area where insulin signaling mediate counter regulatory hormonal response.
Fig. 2. Bar graph obtained by densitometry analysis Western blot data of different regions of the brain. **p < 0.01, *p < 0.05 considered as significant. (Group1 - Fed vs Group2 - 18 h fasted). Results are mean ± S.E.M represents the ratio of (p-BAD$^{ser155}$/GAPDH) and (p-AKT$^{ser473}$/total AKT). Data were analyzed using two tailed Unpaired t test.
Fig. 3. Representative western blot data for Group 1 (Fed) vs Group 2 (18 h fasted).
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Fig. 4. Bar graph obtained by densitometry analysis Western blot data of prefrontal cortex and hypothalamus. *p < 0.05 considered as significant. (Group3 - Fed vs Group4 - 24 h fasted). Results are mean ± S.E.M represents the ratio of (p-BADser155/β-Actin) and (p-AKTser473/total AKT). Data were analyzed using two tailed Unpaired t test and Mann-Whitney t test.

Insulin signaling phosphorylates its downstream kinase AKT and AKT phosphorylation is believed to phosphorylate BADser155. There is also another view that phosphorylation of BAD is essential for the glucose induced insulin release in islets [33,34]. I believed that BAD and AKT functions are interconnected and I checked the AKT expression in different regions of the brain.
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In the 18 h fasted mice I observed an increase \( \text{p-AKT}^{\text{ser473}} \) in prefrontal cortex but not significant. In the hippocampus \( \text{p-AKT}^{\text{ser473}} \) was significantly decreased. In the midbrain \( \text{p-AKT}^{\text{ser473}} \) was decreased but not significant and no change was observed in hypothalamus (Fig.2, 3).

To confirm again, I checked in the 24 h fasted mice and I found a significant increase in \( \text{p-AKT}^{\text{ser473}} \) in the prefrontal cortex and significant decrease in the hypothalamus.

**Fig. 5.** Representative western blot data for Group 3 (Fed) vs Group 4 (24 h fasted).
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(Fig.4, 5).

My results suggest that prefrontal cortex is the area where insulin signaling may get involved in counter regulatory hormonal activation.

There has been a long debate regarding insulin synthesis in CNS. The central insulin levels and insulin receptor activation are not correlated with the peripheral insulin levels [35,36]. Preproinsulin mRNA I and II are detected in the fetal nervous system [37]. This led me to the assumption that CNS insulin synthesis is independent and not it depends on the peripheral insulin. Recent studies indicating that CNS insulin synthesis is little and the brain depends on the pancreatic insulin. Insulin crosses the blood brain barrier and partially gets saturated at euglycemic levels and it transiently phosphorylates AKT in the cerebral cortex and hippocampus [38–40]. Fasting decreases insulin transport to cerebrospinal fluid and it decreases AKT phosphorylation in the hippocampus and cortex [38,41].

In my results 18 h decreases p-AKT\textsuperscript{ser473} but not significant and 24 h fasted mice significantly decreases p-AKT\textsuperscript{ser473} (Fig.6, 7) in the liver suggesting that fasting decreases insulin signaling, thereby it increases the gluconeogenesis and glycogenolysis in liver. Overall fasting decreases the p-AKT\textsuperscript{ser473} in the periphery (liver) and different parts of the brain (hippocampus, midbrain and hypothalamus)
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Fig. 6. Bar graph obtained by densitometry analysis Western blot data of liver. *p < 0.05 considered as significant. (Group 1 - Fed vs Group 2 - 18 h fasted) and (Group 3 - Fed vs Group 4 - 24 h fasted). Results are mean ± S.E.M represents the ratio of (p-AKT_{ser473}/total AKT). Data were analyzed using two tailed Unpaired t test.

Fig. 7. Representative western blot data for Group 1 (Fed) vs Group 2 (18 h fasted) and Group 3 (Fed) vs Group 4 (24 h fasted)

except prefrontal cortex. My study suggests that CNS insulin synthesis is independent in prefrontal cortex and other areas of the brain may depend on pancreatic insulin.

It is well established that the connection between medial prefrontal cortex and hypothalamic pituitary adrenal axis. Paraventricular nucleus (PVN) of hypothalamic
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cells involved in endocrine and sympathoadrenal mediated activation of hormones [42]. Hypoglycemia in NIRKO mice decreases c-fos, as a marker of neuronal activity in PVN and fasting increase c-fos in PVN [22,43].

In summary, I suggest a possible mechanism, that fasting induced hypoglycemia triggers the insulin mediated signaling by phosphorylating AKT and BAD expression in the prefrontal cortex. The prefrontal cortex influences the neuronal activity of PVN of hypothalamus; thereby it activates the endocrine and sympathoadrenal mediated hormonal activation (Fig. 8).

My study is the preliminary study and I believe it help us to understand the glucose sensing mechanism of brain. In my study I didn’t determine the lactate, glycogen utilization and BHB tissue levels of prefrontal cortex. Also I didn’t check the other regions like nucleus solitary tract and thalamus for their involvement in hypoglycemia mediated activation, which has to be further investigated [13,44]
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**Fig. 8.** Hypothetical model, in which the hypoglycemia is detected in prefrontal cortex (PFC) and it may modulate sympathoadrenal hormonal response by activating the hypothalamic paraventricular nucleus (PVN).

I reported the results of this chapter in the topic and journal of

**Fasting mediated increase in p-BAD\textsuperscript{ser155} and p-AKT\textsuperscript{ser473} in the prefrontal cortex of mice. Neurosci Lett. 2014;579:134-39.**
CHAPTER II

Continuous monophasic glucagon signaling after fasting hypoglycemia in mice
Introduction

The alpha cells from the pancreatic islet of Langerhans release glucagon [45]. Glucagon activates glucagon receptors and it is distributed in several tissues. Specifically, the glucose production controlling tissue such as the hypothalamus and the glucose-producing tissue such as liver, kidney and small intestine have glucagon receptors [46–49]. The glucagon receptor activation increases the cyclic adenosine monophosphate (AMP) – Protein Kinase A (PKA) axis pathway to increase the hepatic glucose production [50,51]. However, even in the case of sustained arterial glucagon, the increase in net splanchnic glucose production is transient [52]. Three decades after, the mechanism behind the transient glucose production after glucagon infusion were identified due to hypothalamic glucagon signaling which inhibits the hepatic glucose production via vagus nerve [53].

In iatrogenic or fasting hypoglycemia, the glucagon acts as a major defense to increase the hepatic glucose production [13,14,54]. If the hypothalamic glucagon signaling inhibits the glucose production in the liver, then it raises the question regarding glucagon signaling and glucose production in prolonged fasting hypoglycemia. So in this study, I investigated the fasting hypoglycemia at different time interval glucagon signaling of hypothalamus by determining the protein expression of glucagon receptor and its downstream protein, p-PKA$^{Ser/Thr}$ substrate.
and peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC-1). In the liver, I investigated glucagon signaling and glucose production by determining the protein expression of glucagon receptor, p-PKA \( \text{Ser/Thr} \) substrate and phosphoenolpyruvate carboxykinase (PEPCK) using western blotting analysis.

**Materials and Methods**

**Animals**

I used 8 - 9 weeks old male C57BL6/J mice. The mice were housed in 12 : 12 h light: dark cycle schedule with a controlled temperature 23 ± 2°C and humidity 55 ± 15%. The non-fasted (NF) group had free access to food and water (n=4). The fasted group at different time interval (4, 8, 12, 18, 24, 30, 36 and 42 h), or (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF) had free access to water but not food throughout the fasting period (n=5 each group). The mice are nocturnal and I started fasting in all the groups in a specific time between 4.30 - 5.30 P.M. and I sacrificed by cervical dislocation on their respective hours. The brain were separated and the tissues such as hypothalamus and liver were collected and snap frozen in liquid nitrogen and immediately stored in -80°C. All experiments with animals were performed in accordance with the national guidelines and approved by the animal care committee of Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan.

**Blood collection for glucose measurement**
Blood samples were collected from tail bleed and blood glucose level (BGL) was checked using FreeStyle Freedom glucometer.

**Homogenization of hypothalamus and liver tissue**

Briefly, the tissue was homogenized in lysis buffer 20 mM Tris-HCL (pH-7.5), 150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, and 1 mM Na$_3$VO$_4$, 0.1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and phosphatase arrest III (1:1000) using a tissue homogenizer for 20 s and centrifuged at 800 g for 15 min. The supernatant was stored at -80°C. The protein concentrations of the resulting solutions were determined by the bicinchoninic acid method.

**Protein analysis by western blotting**

Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween (TBS-T) and incubated overnight using the following antibodies: Primary antibodies were used as follows Glucagon Receptor (sc-66912), p- PKA (Ser/Thr) substrate (CST- 9621), PEPCK (sc-74825), Glyceraldehyde-3-phosphate dehydrogenase or GAPDH (CST 14C10) and β-actin (CST-4970) used at a dilution of 1:1000 or 8000 in TBS-T buffer. After washing three times with TBS-T, the membranes were incubated with appropriate horseradish peroxidase (HRP)
conjugated secondary antibodies for 1 h at room temperature. Further, the membranes were washed three times with TBS-T and then developed using a chemiluminescence detection system (Pierce ECL western blotting substrate plus - NC132132JP and Immunostar - LD 290-69904). The blots were scanned with C-Digit blot scanner (LI - COR) and the signals were quantified with Image Studio Lite (LI - COR) software.

Statistical analysis

Statistical analysis was performed using two-tailed Unpaired t test and two-tailed, one way ANOVA followed by Tukey's test.

Results

Blood glucose level

There was no significant difference in BGL at the basal level of fasting groups (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF) vs NF group. After fasting, the BGL was significantly decreased in the entire fasting groups vs NF group (Table 1).
Table 1. Blood glucose level (BGL). This table represents the BGL of basal and after fasting in all the groups. There was a significant difference (**p < 0.001) in BGL in the entire fasted groups vs non-fasted group. Results are mean ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey’s test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose level (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Non Fasted</td>
<td>157.2 ± 12.56</td>
</tr>
<tr>
<td>4 h Fasted</td>
<td>137.6 ± 5.46</td>
</tr>
<tr>
<td>8 h Fasted</td>
<td>151.2 ± 8.51</td>
</tr>
<tr>
<td>12 h Fasted</td>
<td>152.4 ± 9.83</td>
</tr>
<tr>
<td>18 h Fasted</td>
<td>150.6 ± 4.11</td>
</tr>
<tr>
<td>24 h Fasted</td>
<td>144.8 ± 5.64</td>
</tr>
<tr>
<td>30 h Fasted</td>
<td>157.6 ± 2.78</td>
</tr>
<tr>
<td>36 h Fasted</td>
<td>133.8 ± 3.20</td>
</tr>
<tr>
<td>42 h Fasted</td>
<td>127.0 ± 7.76</td>
</tr>
</tbody>
</table>

Body weight

There was no significant difference in body weight at the basal level of fasting groups (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF) vs NF group. After fasting, the body weight was significantly decreased relative to their basal body weight and the percentage decrease in body weight was exponentially decreased with the fasting time (Table 2).
**Table 2. Body weight.** This table represents the body weight of basal and after fasting in all the groups. There was a significant difference (*p < 0.05, **p < 0.01 and ***p < 0.001) in body weight in all the fasted groups relative to their basal level and the percentage decrease in body weight exponentially decreased with fasting time. Results are mean ± S.E.M. Data were analyzed using Unpaired t test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal (gm)</th>
<th>Fasted (gm)</th>
<th>% decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Fasted</td>
<td>21.76 ± 1.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 h Fasted</td>
<td>23.24 ± 0.16</td>
<td>21.34 ± 0.16***</td>
<td>8.17 ± 0.26</td>
</tr>
<tr>
<td>8 h Fasted</td>
<td>23.24 ± 0.73</td>
<td>20.74 ± 0.63*</td>
<td>10.73 ± 0.48</td>
</tr>
<tr>
<td>12 h Fasted</td>
<td>23.64 ± 0.67</td>
<td>20.46 ± 0.59**</td>
<td>13.45 ± 0.62</td>
</tr>
<tr>
<td>18 h Fasted</td>
<td>22.88 ± 0.48</td>
<td>19.50 ± 0.48**</td>
<td>14.79 ± 0.90</td>
</tr>
<tr>
<td>24 h Fasted</td>
<td>21.88 ± 0.62</td>
<td>17.64 ± 0.34***</td>
<td>19.28 ± 0.9</td>
</tr>
<tr>
<td>30 h Fasted</td>
<td>22.08 ± 0.52</td>
<td>17.64 ± 0.47***</td>
<td>20.13 ± 0.48</td>
</tr>
<tr>
<td>36 h Fasted</td>
<td>24.22 ± 0.8</td>
<td>19.34 ± 0.66**</td>
<td>20.14 ± 0.30</td>
</tr>
<tr>
<td>42 h Fasted</td>
<td>24.28 ± 0.51</td>
<td>18.74 ± 0.36***</td>
<td>22.78 ± 1.09</td>
</tr>
</tbody>
</table>

**Liver and brain weight ratio**

In all the fasting groups (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF), the liver weight to the body weight ratio was significantly decreased vs NF group and there was no change in the brain (hindbrain and cerebellum removed) weight to the body weight ratio in all the fasting groups vs NF group (Table 3).
Mechanisms of hypoglycemic seizures in mice

Table 3. The ratio of liver and brain weight. This table represents the ratio of liver and brain weight (hindbrain and cerebellum removed) to the body weight. There was a significant difference (*p < 0.05 and ***p < 0.001) in the ratio of liver weight to the body weight in the entire fasted group vs non-fasted group. There was no significant difference in the ratio of brain weight to the body weight in the entire fasted group vs non-fasted group. Results are mean ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey’s test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio : Liver weight / Body weight</th>
<th>Ratio : Brain weight / Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Fasted</td>
<td>0.050 ± 0.00</td>
<td>0.0152 ± 0.00</td>
</tr>
<tr>
<td>4 h Fasted</td>
<td>0.043 ± 0.00*</td>
<td>0.0151 ± 0.00</td>
</tr>
<tr>
<td>8 h Fasted</td>
<td>0.041 ± 0.00***</td>
<td>0.0152 ± 0.00</td>
</tr>
<tr>
<td>12 h Fasted</td>
<td>0.041 ± 0.00***</td>
<td>0.0137 ± 0.00</td>
</tr>
<tr>
<td>18 h Fasted</td>
<td>0.041 ± 0.00***</td>
<td>0.0143 ± 0.00</td>
</tr>
<tr>
<td>24 h Fasted</td>
<td>0.039 ± 0.00***</td>
<td>0.0145 ± 0.00</td>
</tr>
<tr>
<td>30 h Fasted</td>
<td>0.038 ± 0.00***</td>
<td>0.0152 ± 0.00</td>
</tr>
<tr>
<td>36 h Fasted</td>
<td>0.037 ± 0.00***</td>
<td>0.0145 ± 0.00</td>
</tr>
<tr>
<td>42 h Fasted</td>
<td>0.031 ± 0.00***</td>
<td>0.0139 ± 0.00</td>
</tr>
</tbody>
</table>

Glucagon receptor expression in hypothalamus

In the 18hF and 42hF groups, the glucagon receptor expression was significantly (**p < 0.01) increased compared to NF group. However, there were no observed changes in the glucagon receptor expression of 4hF, 12hF, 24hF, 30hF and 36hF groups compared to NF group. The glucagon receptor expression trended higher in 8hF group compared to NF group, but the difference did not reach significance (Fig. 9, 15A).
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Fig. 9. Glucagon receptor expression in the hypothalamus. Bar graph obtained by fold change densitometry analysis of western blot data. **p < 0.01 considered as significant vs NF. Results are mean ± S.E.M represents the ratio of (Glucagon receptor / β-actin). Data were analyzed using one-way ANOVA followed by Tukey’s test. Non-Fasted (NF), Fasted at different time intervals (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF). In 36hF, one sample gets damaged.

p-PKA Ser/Thr substrate expression in hypothalamus

There was a significant decrease in p-PKA Ser/Thr substrate expression in 4hF and 8hF and there was no changes were observed in other groups. (Fig. 10, 15B).
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Fig. 10. p-PKA \textsuperscript{Ser/Thr} substrate expression in the hypothalamus. Bar graph obtained by fold change densitometry analysis of western blot data. \textbf{***}p < 0.001 considered as significant vs NF. Results are mean ± S.E.M represents the ratio of (p-PKA \textsuperscript{Ser/Thr} substrate / β-actin). Data were analyzed using one-way ANOVA followed by Tukey’s test. Non-Fasted (NF), Fasted at different time intervals (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF). In 36hF, one sample gets damaged.

PGC-1 expression in hypothalamus

In the 42hF group, the PGC-1 expression was significantly (*p < 0.05) increased compared to NF group. However, there were no observed changes in the PGC-1 expression of 4hF, 8hF, 24hF, 30hF and 36hF groups compared to NF group. The
PGC-1 expression trended higher in 12hF group compared to NF group, but the
difference did not reach significance (Fig. 11, 15C).

**Fig. 11. PGC-1 expression in the hypothalamus.** Bar graph obtained by fold change
densitometry analysis of western blot data. We observed two bands and we calculated
the lower band for PGC-1 expression nearly the molecular weight 55 kDa. *p < 0.05
considered as significant vs NF. Results are mean ± S.E.M represents the ratio of
(PGC-1 / β-actin). Data were analyzed using one-way ANOVA followed by Tukey’s
test. Non-Fasted (NF), Fasted at different time intervals (4hF, 8hF, 12hF, 18hF, 24hF,
30hF, 36hF and 42hF). In 36hF, one sample got damaged.

**Glucagon receptor expression in liver**

There were no observed changes in glucagon receptor in all the groups (4hF, 8hF,
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12hF, 18hF, 24hF, 30hF, 36hF and 42hF) of liver (Fig. 12, 15D).

Fig. 12. Glucagon receptor expression in liver. Bar graph obtained by fold change densitometry analysis of western blot data. Results are mean ± S.E.M represents the ratio of (Glucagon receptor/ β-actin). Data were analyzed using one-way ANOVA followed by Tukey’s test. Non-Fasted (NF), Fasted at different time intervals (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF

**p-PKA**<sub>Ser/Thr</sub> substrate expression in liver

In the 8hF, 12hF, 30hF and 36hF groups, the p-PKA<sub>Ser/Thr</sub> substrate expression was significantly (*p < 0.05 and **p < 0.001) increased compared to NF group. However, there were no observed changes in the p-PKA<sub>Ser/Thr</sub> substrate expression of 18hF and 42hF groups compared to NF group. The p-PKA<sub>Ser/Thr</sub> substrate expression trended
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higher in 4hF and 24hF groups compared to NF group, but the difference did not reach significance (Fig.13, 15E).

**Fig. 13.** p-PKA$^{\text{Ser/Thr}}$ substrate expression in liver. Bar graph obtained by fold change densitometry analysis of western blot data. *p < 0.05 and ***p < 0.001 considered as significant vs NF. Results are mean ± S.E.M represents the ratio of (p-PKA$^{\text{Ser/Thr}}$ substrate / GAPDH). Data were analyzed using one-way ANOVA followed by Tukey’s test. Non-Fasted (NF), Fasted at different time intervals (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF).
PEPCK expression in liver

We investigated the PEPCK expression to understand the gluconeogenesis. In the 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF groups, the PEPCK expression was significantly (*p < 0.05, **p < 0.01 and ***p < 0.001) increased compared to NF group. However, there was no observed change in the PEPCK expression of 4hF group compared to NF group (Fig. 14, 15F).

![Fig. 14. PEPCK expression in the liver. Bar graph obtained by fold change densitometry analysis of western blot data. *p < 0.05, **p < 0.01 and ***p < 0.001 considered as significant vs NF. Results are mean ± S.E.M represents the ratio of (PEPCK / GAPDH). Data were analyzed using one-way ANOVA followed by Tukey’s test. Non-Fasted (NF), Fasted at different time intervals (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF).]
Discussion

The elevated glucagon receptor and PGC-1 expressions of the hypothalamus in the 18hF and 42hF were correlated with the attenuated liver p-PKA $^{\text{Ser}/\text{Thr}}$ substrate expression of the 18hF and 42hF (Fig. 9, 11, 13, 15A, 15C and 15E). Also, the attenuated glucagon receptor and PGC-1 expressions of the hypothalamus in the 12hF, 24hF, 30hF or 36hF were correlated with the elevated liver p-PKA $^{\text{Ser}/\text{Thr}}$ substrate expression of the 12hF, 24hF, 30hF or 36hF (Fig. 9, 11, 13, 15A, 15C and 15E). This suggesting that the elevated hypothalamic glucagon signaling possibly attenuates the liver p-PKA $^{\text{Ser}/\text{Thr}}$ substrate to modify the gluconeogenesis pathway, but the another gluconeogenic protein, the PEPCK were not correlated with the liver p-PKA $^{\text{Ser}/\text{Thr}}$ substrate expression (Fig. 13, 14, 15E and 5F). This may be due to the following reasons. Apart from the cyclic AMP-PKA axis induced PEPCK expression, the fasting-induced hormones such as the growth hormone and glucocorticoid also modify the PEPCK-C expression [51], fasting also increase the growth hormone and glucocorticoid levels [14,56,57]. This may induce liver glucose production by increasing PEPCK expression via another axis [51]. So, the sustained and exponential increase in PEPCK expression was possibly due to the growth hormone or glucocorticoid in fasting hypoglycemia.
Mechanisms of hypoglycemic seizures in mice

A
Fold change Glucagon Receptor \( \beta \)-adin
Hypothalamus

B
Fold change p-PK(α-substrate) \( \beta \)-adin
Hypothalamus

C
Fold change PGC-1/ \( \beta \)-adin
Hypothalamus

D
Fold change Glucagon Receptor \( \beta \)-adin
Liver

E
Fold change p-RO(a-substrate) GAPDH
Liver

F
Fold change PEPCK GAPDH
Liver
**Fig. 15 (A, B, C, D, E and F).** This figure represents the fold change protein expression of hypothalamic glucagon receptor, p-PKA Ser/Thr substrate or PGC-1 expression and liver glucagon receptor, p-PKA Ser/Thr substrate and PEPCK expression. *p < 0.05, **p < 0.01 and ***p < 0.001 considered as significant vs NF. Results are mean ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey’s test. Non-Fasted (NF), Fasted at different time intervals (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF).

Also, the hypothalamic p-PKA Ser/Thr substrate in all the groups were not correlated with the hypothalamic glucagon receptor and PGC 1 (Fig. 9, 10, 11, 15A, 15B, 15C). This may be due to the following reasons. The hypothalamus has different nuclei and still it is unclear which nucleus have glucagon receptor. So, the unchanged and decreased expression of the hypothalamic p-PKA Ser/Thr substrate was possibly due to other nuclei expression in hypothalamus.

Hyperglucagonemia induced hyperglycemia is well established in type 1 and type 2 diabetic animal models and the inhibition of glucagon action considered as a major therapeutic strategy towards diabetes [58–60]. P.I. Mighiu et al., clearly mentioned that, glucagon infusion in medial basal hypothalamus of three days high fat diet (HFD) fed rats not alters the hypothalamic glucagon signaling or liver glucose production and the intravenous glucagon extremely increase the BGL in HFD-fed rats and they suggest, the hypothalamic glucagon resistance is responsible for the extremely high uncontrolled BGL in diabetes [53].

In addition to the hypothalamic glucagon resistance, I suggest that there is a slight
possibility to consider the other mechanisms and continuous monophasic glucagon signaling in diabetes, because of the following reasons. 1) HFD increases inflammation in the liver and specifically, NF-kappaB inducing kinase (NIK) promotes glucagon response. Depletion of NIK in the liver decreases glucagon-induced hepatic glucose production \[61\]. This report suggests that, in addition to the hypothalamic glucagon resistance, the liver inflammation also increase glucagon-induced hepatic glucose production. 2) In both the diabetic and normal subjects, the sustained and elevated arterial glucagon increases the glucose production, but transient and it further increases the glucose production again after a time period \[52,62\]. This report suggests that even in diabetes the glucose production is transient and increased later which support the continuous monophasic glucagon signaling. So, further studies are required to confirm the other possible mechanisms in diabetic models.

Before interpreting my results, it is important to consider that, I did not check serum glucagon in fasting hypoglycemia, but several chronic fasting studies in humans significantly increases the glucagon levels \[14,54,63\] and also I did not perform hepatic vagotomy to confirm the hypothalamic glucagon receptor signaling attenuate the liver PKA. So further studies are required to confirm the mechanism involved in continuous monophasic glucagon signaling in hypothalamus and liver.
Conclusion

Overall my data suggest that, glucagon signaling in fasting hypoglycemia is continuous and monophasic. The hypothalamic glucagon signaling may inhibit liver p-PKA or modify glucose production and this mechanism will help to understand hyperglucagonemia associated glucose production in diabetes (Fig. 13).

**Fig. 16.** Glucagon signaling induced hepatic glucose production is transient and later it was found due to the hypothalamic glucagon signaling which inhibits hepatic glucose production. In fasting hypoglycemia glucagon acts as major defense and in my results I observed a continuous monophasic glucagon signaling in hypothalamus and liver.
CHAPTER III

Fasting time duration modulate the onset of insulin induced hypoglycemic seizures in mice
**Introduction**

Hypoglycemia is one of the frequent complaints in the emergency department [64]. In a 6.5 years follow in Diabetic Control and Complication Trail, there were 1,027 episodes of coma or seizures [5]. Fasting decreases the blood glucose level (BGL) and it increases the alternate fuel ketone bodies such as beta-hydroxybutyrate [14]. The increased level of beta-hydroxybutyrate in fasting may play an important role in seizure protection [65]. Specifically in insulin induced hypoglycemic seizures (IIHS), 48 h fasted mice were resistant to IIHS and 14 - 16 h fasted rats predisposes IIHS [21,66]. This raises a question that, why fasting increases (anticonvulsant) and decreases (proconvulsant) seizure onset time after insulin induced severe hypoglycemia in mice and rat respectively? I suspect that the time duration in fasting may play a role in the seizure onset. So in this study I investigated the effect of 8 U IIHS in nonfasted group and two fasted group (6 h and 24 h) in mice.

**Materials and Methods**

**Animals**

I used 8 weeks old male C57BL6/J mice. The mice were housed in 12 : 12 h light: dark cycle schedule with a controlled temperature 23 ± 2°C and humidity 55 ± 15%. I divided them into three groups. Group 1 mice (non- fasted) had free access to food
and water (n=9), group 2 mice (6 h fasted) had free access to water but not food for 6 h (n=10) and group 3 mice (24 h fasted) had free access to water but not food for 24 h (n=8). All experiments with animals were performed in accordance with the national guidelines and approved by the animal care committee of Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan.

**Insulin dose and administration**

I used pancreatic insulin (sigma I0516 10 mg/ml) derived from bovine source [38]. It containing not less than 27 USP U/mg and based on the manufacture instructions that some activity may lost during manufacturing process, I calculated the dose for 24 U/mg. So I have chosen 0.33 mg/kg insulin which is equivalent to 8 U/kg insulin dissolved in normal saline and administered intraperitoneally (i.p). The administration of insulin injection was considered as 0 min and I observed the behavioral seizures symptoms for upto 4 h duration.

**Blood collection for glucose measurement**

Blood samples were collected from tail bleeds and BGL was checked using Freestyle Freedom glucometer.

**Beta-hydroxybutyrate assay**

I used separate mice for group 1(non-fasted, n=4), group 2 (6 h fasted, n=5) and group 3 (24 h fasted, n=5) for the beta-hydroxybutyrate assay. The mice were anesthetized
with pentobarbital 50mg /kg (i.p) and the blood was collected using retro-orbital puncture. The serum was separated by centrifugation (3000 g) for 15 min at 4°C and stored at -80°C. The serum samples were analyzed using Bio vision beta-hydroxybutyrate colorimetric assay kit (K632-100).

**Statistical analysis**

Statistical analysis was performed using two tailed Unpaired t test and one way ANOVA followed by Tukey’s test.

**Results**

**Body weight**

The initial body weight between group 1 (23.77 ± 0.8 g) vs group 2 (22.89 ± 0.3 g) vs group 3 (22.69 ± 0.4 g) were not significantly different. The group 2 mice were fasted for 6 h and the body weight was significantly decreased from 22.89 ± 0.3 to 21.79 ± 0.3 g. The group 3 mice were fasted for 24 h and the body weight was significantly decreased from 22.69 ± 0.4 to 18.86 ± 0.56 g (Table 4).
**Table 4.** This table represents the body weight of before and after in fasted mice. 6 h fasting significantly decreases (*p < 0.05) the body weight in group 2 and the 24 h fasting significantly decreases (***p < 0.001) the body weight in group 3. Results are mean ± S.E.M. Data were analyzed using Unpaired *t* test.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Non fasted</th>
<th>6 h fasted</th>
<th>24 h fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td>23.77 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td>22.89 ± 0.3</td>
<td>21.79 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td></td>
<td>22.69 ± 0.4</td>
<td></td>
<td>18.86 ± 0.56***</td>
</tr>
</tbody>
</table>

**Blood glucose level**

The initial BGL between group 1 (161.4 ± 6.58 mg/dl) vs group 2 (163.6 ± 4.2 mg/dl) vs group 3 (164.8 ± 4.1 mg/dl) mice were not significantly different. The group 2 mice were fasted for 6 h and the BGL was significantly decreased from 163.6 ± 4.2 to 121.1 ± 5.9 mg/dl. The group 3 mice were fasted for 24 h and the BGL was significantly decreased from 164.8 ± 4.1 to 74.63 ± 4.5 mg/dl (Table 5).
**Table 5.** This table represents the BGL of before and after in fasted mice. 6 h fasting significantly decreases (*p < 0.05) the BGL in group 2 and the 24 h fasting significantly decreases (***p < 0.001) the BGL in group 3. Results are mean ± S.E.M. Data were analyzed using Unpaired t test.

<table>
<thead>
<tr>
<th>Blood glucose level (mg/dl)</th>
<th>Non fasted</th>
<th>6 h fasted</th>
<th>24 h fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>161.4 ± 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>163.6 ± 4.2</td>
<td>121.1 ± 5.9*</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>164.8 ± 4.1</td>
<td></td>
<td>74.63 ± 4.5***</td>
</tr>
</tbody>
</table>

**Blood glucose level after insulin induced hypoglycemia**

Before the insulin administration at 0 min, there was a significant difference in basal BGL between group 1 (161.4 ± 6.58 mg/dl) vs group 2 (121.1 ± 5.9 mg/dl) vs group 3 (74.63 ± 4.5 mg/dl). After the insulin administration at 30 min, the BGL was significantly decreased between group 1 (51.56 ± 2.27 mg/dl), vs group 2 (26.10 ± 3.3 mg/dl) and group 3 (22.63 ± 1.25 mg/dl), and not significantly different between group 2 vs group 3. At 60 min, the BGL was significantly decreased between group 1 (39.22 ± 2.11 mg/dl), vs group 2 (< 20 mg/dl) and group 3 (< 20 mg/dl). At 90 min, the BGL was significantly decreased (p < 0.05) between group 1 (23.33 ± 1.6 mg/dl), vs group 2 (< 20 mg/dl) but not vs group 3. At 120 and 180 min, in all the three groups, the BGL was below 20 mg/dl. At 240 min, the BGL was increased slightly in group 1 (25.60 ± 2.8 mg/dl) and there was a significant difference between group 1 vs
Mechanisms of hypoglycemic seizures in mice

group 2 and group 3 (Table 6).

**Table 6.** This table represents the BGL at different time interval after insulin administration in all the groups. (*p < 0.05) and (***p < 0.001) considered as significant between group 1 vs group 2 and group 3. (**p < 0.001) considered as significant between group 2 vs group 3. Results are mean ± S.E.M. Data were analyzed using one way ANOVA followed by Tukey’s test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose level (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Group 1</td>
<td>161.4 ±</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td>Group 2</td>
<td>121.1 ±</td>
</tr>
<tr>
<td></td>
<td>5.9 *</td>
</tr>
<tr>
<td>Group 3</td>
<td>74.63 ±</td>
</tr>
<tr>
<td></td>
<td>4.5 ***</td>
</tr>
</tbody>
</table>

###

**Behavioral observation and seizure onset**

After the insulin administration, I observed the several behavioral changes like, tail flicking or rotation, all legs extended and the body touches ground, shivering, running, hind leg pushing down and body extend upward, head bend backward, tonic stiffness, jump, clonus, trying barrel rotation and barrel rotations. Not all mice experienced all the behavior. Previous study suggesting that jump, clonus and barrel rotations were considered as syndrome of hypoglycemic seizures and verified by EEG [21]. So in my
study I observed the first symptoms of jump, clonus or barrel rotation considered as seizure onset. The seizure onset time in group 1 was 109.7 ± 4.39 min. The seizure onset time in group 2 was 46.50 ± 3.95 min, which was significantly decreased when compared to group 1. The seizure onset time in group 3 was 165.4 ± 13.26 min, which was significantly higher when compared to group 1 and group 2 (Fig. 17).

**Fig. 17.** After the insulin administration, the onset of seizures considered as first symptom of jump or clonus or barrel rotations. The seizure onset time was significantly decreased (***p < 0.001) in group 2 when compared to group 1. The seizure onset time was significantly higher in group 3 when compared to (***p < 0.001) group 1 and (###p < 0.001) group 2. Results are mean ± S.E.M. Data were analysed using one way ANOVA followed by Tukey’s test.

**Seizure induced mortality**

The mortality rate in group 1 was 44%, group 2 was 20% and in group 3 was 25%.

Before the mice dying, in all the groups, I observed a specific clonus behavior and
this might responsible for the death of animals.

**Serum beta-hydroxybutyrate levels**

There was no significant difference in beta-hydroxybutyrate levels between group 1 vs group 2 and there was a significant increase in group 3 when compared to group 1 and group 2 (Table 7).

**Table 7.** This table represents the serum beta-hydroxybutyrate levels of all the groups. There was significant increase (**p < 0.001**) in beta-hydroxybutyrate levels in group 3 vs group 1 and group 2. Results are mean ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey’s test.

<table>
<thead>
<tr>
<th>Serum beta-hydroxybutyrate levels (mmol/l)</th>
<th>Non fasted</th>
<th>6 h fasted</th>
<th>24 h fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.30 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td>0.24 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td></td>
<td>2.64 ± 0.3 ***</td>
</tr>
</tbody>
</table>

**Discussion**

My results raise three major questions. 1) Does the decrease in BGL determine the seizure onset time? 2) Why the seizure onset time in 6 h fasted mice was quicker than non-fasted mice? 3) Why the seizure onset time in 24 h fasted mice was prolonged than nonfasted and 6 h fasted mice?

After the insulin administration the BGL was decreased in group 1 and it reaches below 20 mg/dl between the time periods of (90 – 120 min) which was correlated
with the seizure onset time of group 1 (109.7 ± 4.39 min) (Table 6) (Fig. 17). Also in
the group 2, after the insulin administration, the BGL was decreased and it reaches
below 20 mg/dl between the time periods of (30 – 60 min) which was correlated with
the seizure onset time of group 2 (46.50 ± 3.95 min) (Table 6) (Fig. 17). Contrary to
that, in group 3 the BGL was decreased below 20 mg/dl between the time periods of
(30 – 60 min) which was not correlated with the seizure onset time of group 3 (165.4
± 13.26 min) (Table 6) (Fig. 17). Overall this suggesting that, the decrease in BGL
was correlated with the seizure onset time in group 1 and group 2 but not correlated
with the group 3.

The above explanation does not explain why group 2 had quick decrease in BGL
correlated with seizure onset time. One can argue that, the basal BGL was
significantly higher before the insulin administration in group 1 vs group 2 and group
3 and this decreases the BGL quicker after insulin administration in group 2 and
group 3. But in Velísek L et al., study, they have clearly mentioned the difference in
basal BGL between fasted and non-fasted group not account for earlier onset of
seizure [21].

During hypoglycemia, the physiological defenses are to increase the release of
hormones such as glucagon or epinephrine which increases the liver glucose
production to sustain BGL [13]. In my study after the initial decrease in BGL, the
BGL of group 1 was slightly maintained at 60 or 90 min and decreased at 120 or 180 min and further increased again in 240 min, but in the group 2 or group 3, the BGL was not slightly maintained and decreased progressively from 60, 90, 120, 180 or 240 min (Table 6). This indirectly suggests that glucose production in the liver possibly impaired in group 2 and group 3. I also compared the Velísek L et al., study in which they took 40 mg/dl as a starting point in both non-fasted + insulin and fasted + insulin group [21]. The time taken for non-fasted + insulin group from the starting point 40 mg/dl to reach 18.3 ± 1.4 mg/dl was 106.0 ± 10.3 min (seizure onset time) but the time taken for fasted + insulin group from the starting point 40 mg/dl to reach 20 ± 0.5 mg/dl was 78.0 ± 3.8 min (seizure onset time) and there was a significant time difference between the groups (Fig. 18) [21].
Fig. 18. This figure represents the Velísek L et al., results [6]. In their study, there was a significant difference in basal BGL between non-fasted and fasted rats (not shown in figure). So after insulin administration they took 40 mg/dl as a starting point in both the groups. The time taken for non-fasted + insulin group from the starting point 40 mg/dl to reach 18.3 ± 1.4 mg/dl was 106.0 ± 10.3 min (seizure onset time) but the time taken for fasted + insulin group from the starting point 40 mg/dl to reach 20 ± 0.5 mg/dl was 78.0 ± 3.8 min (seizure onset time) and there was a significant time difference between the groups. This indirectly suggesting that fasted + insulin group had impaired glucose production and not sustain the BGL for longer time period like in non-fasted rats.

This also indirectly suggesting that the fasted + insulin group not able to sustain BGL like the non-fasted group possibly by impaired glucose production. Also in humans 72 h fasting along with insulin hypoglycemia impaired the hormonal defenses (glucagon, adrenaline and cortisol) against hypoglycemia [67]. So the quick decrease in BGL.
possibly by autonomic failure associated impaired glucose production. I suggest the impaired glucose production and the synthesis of ketone bodies was less in group 2 when compared to group 3 which might responsible for the quicker seizure onset in group 2 mice (Fig. 19).

In normal conditions, the basic property of neuron has to propagate action potential by releasing neurotransmitter like glutamate to communicate with other neurons. For that purpose it requires energy in the form of ATP derived mainly from glucose [68–70].

The disruption of ATP synthesis by mitochondrial toxins or by other compounds leads to seizure [71]. During severe hypoglycemia, the brain ATP and pyruvate levels fall and this activate NMDA receptor to induce seizures [72,73]. So the decreased BGL in group 1 and group 2 may decrease ATP or neuronal metabolism and this correlated with the seizure onset. During the progression of fasting hypoglycemia, the brain shifts the metabolic fuel from glucose to beta-hydroxybutyrate (ketone bodies) [74,75] and the increase in ketone bodies possibly maintain the neuronal metabolism which increases the seizure onset time in group 3.
**Mechanisms of hypoglycemic seizures in mice**

**Fig. 19.** This figure represents the hypothetical model for fasting time duration (0, 6 and 24 h) modulation on seizure onset time. The top arrow (↑ ketone bodies) indicates that the progressive increase in fasting time (0, 6 and 24 h) associated increase in ketone bodies such as beta-hydroxybutyrate. The bottom arrow (↑ impaired glucose production) indicates the autonomic failure after fasting + insulin hypoglycemia. The onset of seizure time was decreased in 6 h fasted mice was possibly due to impaired glucose production and less synthesis of ketone bodies and the onset of seizure time was increased in 24 h fasted mice was possibly due to increase in synthesis of alternate fuel (ketone bodies) which sustain brain metabolism.

Overall, the autonomic failure associated impaired glucose production may involve in quicker seizure onset in 6 h fasted mice and the time progressive production of ketone bodies, which sustain brain metabolism may involve in late seizure onset in 24 h fasted mice (Fig. 19)

<table>
<thead>
<tr>
<th>Fasted + 8 U insulin (i,p)</th>
<th>0 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seizure onset time (min)</td>
<td>109.7 ± 46.50 ±</td>
<td>165.4 ±</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>13.2</td>
<td>5</td>
</tr>
</tbody>
</table>
For the reproducibility of my results, I recommend to use 8 weeks old mice, because I suspect the age may affect the release of beta-hydroxybutyrate after fasting and in the rats, fasting for longer duration (48 h) may increase seizure onset time because the significant hormonal changes begin to occur after 24 h fasting hypoglycemia in rats [76].
Summary

- BAD and AKT phosphorylation in the prefrontal cortex may elicit counter-regulatory hormonal responses during hypoglycemia.
- Continuous monophasic glucagon signaling was observed in fasting hypoglycemia.
- Increased glucagon signaling in hypothalamus may attenuate the liver PKA or modify glucose production.
- Fasting duration 6 h and 24 h modulate insulin induced hypoglycemic seizure onset.
- 6 h fasting predispose seizure onset time possibly by impaired glucose production.
- 24 h fasting prolong the seizure onset time possibly by the increase in ketone bodies.
Mechanisms of hypoglycemic seizures in mice

Articles


Posters presented in conferences

1. Poster title - Brain metabolism in moderate and severe hypoglycemia of mice. American Diabetes Association 75th scientific sessions, June 5-9, 2015

   Boston, United States.


3. Poster title – Fasting mediated predisposition of insulin induced hypoglycemic seizures is independent of central hippocampal insulin signaling mechanism.
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(2nd prize) Colloquium on Drug Resistance Epilepsy, August 16-18, 2013, Bangalore, India.

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