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Hypothalamic glucagon signaling in fasting hypoglycemia

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ABSTRACT

Aims: Sustained glucagon infusion increases 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 this study, we investigated the proteins associated with hypothalamic glucagon signaling and liver gluconeogenesis during fasting hypoglycemia.

Main methods: 8–9 week old, male C57BL6/J mice were fasted for 4, 8, 12, 18, 24, 30, 36 or 42 h. In the hypothalamus, we investigated glucagon signaling by analyzing the glucagon receptor and its downstream protein, peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC-1) expression. In the liver, we investigated gluconeogenesis by analyzing p-protein kinase A (PKA)$^{Ser/Thr}$ substrate and phosphoenolpyruvate carboxykinase — cytosolic (PEPCK-C) expression using the western blotting technique.

Key findings: The elevated or trended higher hypothalamic glucagon receptor and PGC-1 expressions at 18 and 42 h were correlated with the attenuated liver p-PKA$^{Ser/Thr}$ substrate expression. The attenuated hypothalamic glucagon receptor and PGC-1 expressions at 12, 24, 30 and 36 h were correlated with the elevated or trended higher liver p-PKA$^{Ser/Thr}$ substrate expression.

Significance: The hypothalamic glucagon signaling during fasting hypoglycemia might have been modulated by circadian rhythm and this possibly attenuates the liver p-PKA$^{Ser/Thr}$ substrate to modify the gluconeogenesis pathway. This mechanism will help to understand the hyperglucagonemia associated complications in diabetes.

1. Introduction

The alpha cells of the pancreatic islet of Langerhans release glucagon [1]. Glucagon activates glucagon receptors and it is distributed in several tissues. Specifically, the glucose production controlling tissue such as the hypothalamus and glucose-producing tissues such as the liver, kidney and small intestine have glucagon receptors [2–5]. The glucagon receptor activation increases the cyclic adenosine monophosphate (AMP)–protein kinase A (PKA) axis pathway to increase hepatic glucose production [6,7]. However, even in the case of sustained arterial glucagon, the increase in net splanchnic glucose production is transient [8]. Three decades after, the mechanism behind the transient glucose production after glucagon infusion was identified due to hypothalamic glucagon signaling which inhibits the hepatic glucose production via the vagus nerve [9].

In iatrogenic or fasting hypoglycemia, the glucagon acts as a major defense to increase the hepatic glucose production [10–12]. If hypothalamic glucagon signaling inhibits glucose production in the liver, then it raises the question regarding glucagon signaling and glucose production in prolonged fasting hypoglycemia. In this study, we investigated the prolonged fasting hypoglycemia glucagon signaling of the hypothalamus by determining the protein expression of the glucagon receptor and its downstream protein, peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC-1). Also, we investigated the proteins involved in liver gluconeogenesis by determining the expression

Abbreviations: BGL, blood glucose level; CST, cell signaling technology; Cyclic AMP, cyclic adenosine monophosphate; EGTA, ethylene glycol tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCL, hydrochloride; HRP, horseradish-peroxidase; JST, Japan standard time; Na_2EDTA, disodium ethylenediaminetetraacetic acid; Na_3VO_4, sodium orthovanadate; NaCl, sodium chloride; NIK, NF-kappaB inducing kinase; PGC-1, peroxisome proliferator-activated receptor-gamma coactivator 1; PEPCK-C, phosphoenolpyruvate carboxykinase — cytosolic; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride.

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Table 1
Blood glucose level.

Group	Blood glucose level (mg/dl)	
	Basal	Fasted
Non-fasted	157.2 ± 12.56	–
4 h fasted	137.6 ± 5.46	87.60 ± 1.69***
8 h fasted	151.2 ± 8.51	89.00 ± 2.09***
12 h fasted	152.4 ± 9.83	84.60 ± 3.48***
18 h fasted	150.6 ± 4.11	84.60 ± 6.33***
24 h fasted	144.8 ± 5.64	70.40 ± 2.01***
30 h fasted	157.6 ± 2.78	69.80 ± 9.56***
36 h fasted	133.8 ± 3.20	51.80 ± 1.74***
42 h fasted	127.0 ± 7.76	88.20 ± 10.17***,#

This table represents the BGL of basal level and after fasting in all the groups. Results are mean ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey's test.
*** $p < 0.001$ vs non-fasted.
# $p < 0.05$ vs 36 h fasted.

of the p-PKA$^{Ser/Thr}$ substrate and phosphoenolpyruvate carboxykinase — cytosolic (PEPCK-C) using western blotting analysis.

2. Materials and methods

2.1. Animals

We used 8–9 week old male C57BL6/J mice. The mice were housed in a 12:12 h light:dark cycle schedule with a controlled temperature of 23 ± 2 °C and humidity of 55 ± 15%. The non-fasted (NF) group had free access to food and water (n = 4). The groups fasted at different time intervals (4, 8, 12, 18, 24, 30, 36 or 42 h or 4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF or 42hF) had free access to water but not food throughout the fasting period (n = 5 each group). The mice are nocturnal and we started fasting in all the groups at a specific time between 16:30 and 17:30 Japan standard time (JST). We sacrificed them by cervical dislocation at their respective fasting duration. The brain was separated and the tissues such as the 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 national guidelines and approved by the animal care committee of Niigata University of Pharmacy and Applied Life Sciences (Approval No. H2706-02), Niigata, Japan.

2.2. Blood collection for glucose measurement

Blood samples were collected by tail bleed and blood glucose level (BGL) was checked using a FreeStyle Freedom glucometer.

Table 2
Body weight.

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

This table represents the body weight of basal level and after fasting in all the groups. There was a significant difference in body weight in all the fasted groups relative to their basal level and the percentage decrease in body weight decreased with fasting time. Results are mean ± S.E.M. Data were analyzed using unpaired *t* test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

2.3. 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_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, and 1 mM Na_3VO_4, 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1:1000 phosphatase arrest III) 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.

2.4. 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 primary antibodies: glucagon receptor (sc-66912), p-PKA$^{Ser/Thr}$ substrate (CST-9621), PGC-1 (sc-13067), PEPCK-C (sc-74825), glyceraldehyde-3-phosphate dehydrogenase or GAPDH (CST-14C10) and β-actin (CST-4970) used at a dilution of 1:1000–8000 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 or Immunostar — LD 290-69904). The blots were scanned with a C-Digit blot scanner (*LI-COR*) and the signals were quantified with Image Studio Lite (*LI-COR*) software.

2.5. Statistical analysis

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

3. Results

3.1. Blood glucose level

There was no significant difference in BGL at the basal level of entire fasting groups (4hF, 8hF, 12hF, 18hF, 24hF, 30hF, 36hF and 42hF) compared to the NF group. After fasting, the BGL was significantly decreased (***$p < 0.001$) in the entire fasting groups compared to the NF group. Specifically, in the 42hF group, the BGL was significantly increased (#$p < 0.05$) compared to 36hF (Table 1).

Table 3
The ratio of liver and brain weight.

Group	Ratio: liver weight/body weight	Ratio: brain weight/body weight
Non-fasted	0.050 ± 0.00	0.0152 ± 0.00
4 h fasted	0.043 ± 0.00*	0.0151 ± 0.00
8 h fasted	0.041 ± 0.00***	0.0152 ± 0.00
12 h fasted	0.041 ± 0.00***	0.0137 ± 0.00
18 h fasted	0.041 ± 0.00***	0.0143 ± 0.00
24 h fasted	0.039 ± 0.00***	0.0145 ± 0.00
30 h fasted	0.038 ± 0.00***	0.0152 ± 0.00
36 h fasted	0.037 ± 0.00***	0.0145 ± 0.00
42 h fasted	0.031 ± 0.00***	0.0139 ± 0.00

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