Brain adaptations of insulin signaling kinases, GLUT 3, p-BADser155 and nitrotyrosine expression in various hypoglycemic models of mice

Vigneshwaran Pitchaimani, Somasundaram Arumugam, Rajarajan Amirthalingam Thandavarayan, Vengadeshprabhu Karuppagounder, Mst Rejina Afrina, Remya Sreedhar, Meilei Harimaa, Masahiko Nakamura, Kenichi Watanabe, Satoru Kodama, Kazuya Fujihara, Hirohito Sone

Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, 956-8603, Japan
Department of Cardiovascular Sciences, Houston Methodist Research Institute, Houston, TX, 77030, USA
Department of Cardiology, Yamanashi Prefectural Central Hospital, Kofu, Yamanashi, 400-8506, Japan
Department of Pharmacology & Toxicology, National Institute of Pharmaceutical Education and Research (NIPER) - Kolkata, Kolkata-700054, India
Department of Pharmacology, Faculty of Pharmacy, University of Dhaka, Dhaka-1212, Bangladesh
Department of Laboratory Medicine and Clinical Epidemiology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori Cho-Aku, Niigata, 951-8510, Japan
Department of Hematology, Endocrinology and Metabolism, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

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ABSTRACT
Aim and objective: Insulin-induced moderate or severe hypoglycemia (MH or SH) impairs cognition and SH causes neuronal death. On the contrary, alternate day fasting (ADF) protects the brain during excitotoxic stress and improves cognitive function. Unlike the scenario in the periphery, insulin and its relationship towards brain glucose uptake and metabolism are considered to be less significant. Yet, the hypoglycemia associated brain metabolism is not clearly understood. The authors broadly investigated the brain metabolism in various hypoglycemic models such as insulin-induced MH, SH, SH with glucose reperfusion, 24 h fasting and ADF in the cortex or hippocampus of C57BL6/J mice. The authors analyzed the protein expression of insulin signaling kinases (plays a key role in neuronal survival and memory), Bcl-2 associated death promoter (p-BADser155) (dephosphorylation inhibits glucokinase activity and reduces glucose or increases ketone body metabolism in the brain), neuronal-specific glucose transporter 3 (GLUT 3) and nitrotyrosine (marker of nitric oxide which is involved in neuronal glucose uptake via GLUT 3) using western blotting analysis.

Results: Insulin-induced MH or SH differentially regulated the brain insulin signaling kinases. The expression of p-BADser155 decreased in all hypoglycemic models except the insulin-induced MH in hippocampus. The trended higher GLUT 3 and increased nitrotyrosine expression of insulin-induced SH were restored after glucose reperfusion. The trended higher or increased GLUT 3 and nitrotyrosine expression of ADF were positively correlated with serum beta-hydroxybutyrate levels.

Conclusion: During hypoglycemia, it can be suggested that the brain might decrease glucose metabolism via glycolysis or prefer ketone body metabolism (except the insulin-induced MH in hippocampus) by modifying the p-BADser155 expression. In addition to the ketone body metabolism, the brain might adapt to uptake glucose in insulin-induced SH or ADF by modifying the GLUT 3 or nitrotyrosine expression.

Abbreviations: ADF, Alternate day fasting; BAD, Bcl-2 associated death promoter; BGL, Blood glucose level; CST, Cell signaling technology; EGTA, Ethylene glycol tetraacetic acid; GLUT, Glucose transporter; HCL, Hydrochloride; HMP shunt, Hexose monophosphate shunt; HRP, Horseradish-peroxidase; i.m, intramuscular; i.p, intraperitoneally; i.v, intravenous; Na2EDTA, Disodium ethylenediaminetetraacetic acid; Na3VO4, Sodium orthovanadate; NaCl, Sodium chloride; NADPH, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; MCT, Monocarboxylate transporters; NMDA, N-methyl-D-aspartate; OGD, Oxygen-glucose deprivation; PFK 1, Phosphofructokinase 1; p70S6K, Ribosomal protein S6 kinase; PMSF, Phenylmethylsulfonyl fluoride; TCA cycle, Tricarboxylic acid cycle; U, Unit; USP, United States Pharmacopoeia

Corresponding author. Department of Laboratory Medicine and Clinical Epidemiology for Prevention of Noncommunicable Diseases, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori Cho-Aku, Niigata, 951-8510, Japan.
E-mail addresses: wataken@med.niigata-u.ac.jp (K. Watanabe), ybbkodama@gmail.com (S. Kodama), kafujihara-dm@umin.ac.jp (K. Fujihara), sone@med.niigata-u.ac.jp (H. Sone).

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1. Introduction

In an average of 6.5-year follow-up of the Diabetic Control and Complication Trial, the insulin-dependent diabetes mellitus patients in both the intensive (n = 711) and conventional (n = 730) treatment group experienced 3788 episodes of severe hypoglycemia (requiring assistance) and 1027 episodes of coma or seizures (The Diabetes Control and Complications Trial Research Group, 1997). Moderate hypoglycemia (MH) (with consciousness) was managed with oral administration of carbohydrate while severe hypoglycemia (SH) (without consciousness and experiencing coma or seizures) was treated by administration of dextrose and glucagon, until the blood glucose level (BGL) was stabilized to restore consciousness (Ford et al., 2013). However, the reperfusion of glucose after SH increased the neuronal damage in animal models (Suh et al., 2007). Besides, acute insulin-induced hypoglycemia impaired executive cognitive functions in humans (Graveling et al., 2013). Despite hypoglycemia being a barrier in diabetes, there is no comprehensive understanding of insulin-induced hypoglycemia associated brain metabolism.

Fasting hypoglycemia or fasting-mediated increase in ketone bodies spares the glucose metabolism in the brain (Redies et al., 1989; Zhang et al., 2013). Alternate day fasting (ADF) protects the brain during excitotoxic stress (Anson et al., 2003), improves the cognitive function and decreases the oxidative stress in mice (Li et al., 2013). There is no comprehensive understanding of fasting or ADF hypoglycemia associated brain metabolism.

After the carbohydrate ingestion, the pancreas releases high levels of insulin and regulates the systemic metabolism such as glucose uptake or glycolysis in the peripheral tissues. Insulin promotes phosphorylation of several downstream kinases such as protein kinase B (AKT), ribosomal protein S6 kinase (p70S6K) and extracellular signal-regulated kinase (ERK) to exert its physiological functions (Kubota et al., 2012). Insulin signaling plays a key role in neuronal survival, improves learning and memory, synaptic maintenance, neuronal circuitry formation and dendritic arbor development (Banks et al., 2012). Insulin crosses the blood-brain barrier in a saturable transport mechanism, but its role in brain glucose uptake and metabolism is considered to be less significant. There is no comprehensive understanding of insulin-induced MH or SH associated brain metabolism in this regard.

Study Overview

| Study 1 | Group 1 - Non Fasted (NF). Group 2 - 24 hour Fasted (24hF). Group 3 or 4 - 2 U insulin induced moderate hypoglycemic mice sacrificed at 10 min (INS10) or 30 min (INS30). Group 5 - 2 U insulin induced severe hypoglycemic mice sacrificed at 90 min (INS90). |
| Study 2 | Group 1 - Received normal saline (Sham). Group 2 - 8 U insulin induced severe hypoglycemic mice sacrificed at 180 min (INS G-). Group 3 - 8 U insulin induced severe hypoglycemic mice received 3.5 mg/kg/p.o. dextrose at 120 min and sacrificed at 180 min (INS G+). |
| Study 3 | Group 1 - Non Fasted (NF). Group 2 - 24 hour Fasted (24hF). Group 3 - Alternate Day Fasted (ADF). |

Fig. 1. Study overview. The figure represents an overview of the entire study design. The authors performed three studies and investigated the various hypoglycemic models such as insulin-induced moderate hypoglycemia (nearly 50 mg/dl), severe hypoglycemia (˂ 20 mg/dl) and severe hypoglycemia with glucose reperfusion, fasting and alternate day fasting hypoglycemia.
2. Material and methods

2.1. Experimental conditions

The mice were housed in 12:12 h light:dark cycle schedule with a controlled temperature of 23 ± 2 °C and humidity 55 ± 15%. 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.

2.2. Study 1

The authors used three months old male C57BL6/J mice. The mice used in the study were divided into five groups: Group 1 (n = 4), Non-fasted (NF), fed ad libitum; Group 2 (n = 5), 24 h Fasted (24 hF), had free access to water but not food for 24 h, Group 3 (INS10) or Group 4 (INS30) (n = 5 each), food was removed before an hour of 2 U insulin was administered to induce MH (nearby 50 mg/dl) and the mice were immediately sacrificed at 10 or 30 min and Group 5 (INS90) (n = 5), food was removed before an hour of 2 U insulin was administered to induce SH (“~20 mg/dl) and the mice were immediately sacrificed at 90 min. The cervical dislocation was performed to sacrifice the mice after the BGL measurement. The cerebral cortex (the region above the hippocampus) and hippocampus tissue were collected for the western blotting analysis. The authors specifically chose the cerebral cortex and hippocampus since the earlier studies suggested that the above-mentioned regions were quite vulnerable to hypoglycemic insults (Suh et al., 2007) (Fig. 1).

2.3. 2 U insulin dose calculation and administration

The authors used pancreatic insulin sigma I0516 (10 mg/ml) derived from bovine (Clodfelder-Miller et al., 2005). It contained not less than 27 USP U/Mg. Based on the manufacturer’s instructions pertaining to some activity being lost during the manufacturing process, the dose was calculated to be 24 U/mg. Based on the blood glucose level of 2 U/kg insulin dissolved in normal saline and administered intraperitoneally (i.p) to groups 3, 4 and 5.

2.4. Study 2

In study 2, two months old male C57BL6/J mice were chosen and divided into three groups. The mice were fasted for 6 h in all the groups. The administration of saline or 8 U insulin was considered as 0 min. Group 1 (Sham, n = 4) received normal saline (NS) at 0 min. Group 2 (INS G-, n = 5) received 8 U insulin (i.p) at 0 min and 3.5 mg/kg bwt dextrose (i.p) to groups 3, 4 and 5.

2.5. 8 U insulin dose calculation and administration

We have chosen 0.33 mg/kg bwt insulin, which is equivalent to 8 U/kg insulin. Insulin was dissolved in normal saline and administered intraperitoneally (i.p) to group 2 and 3.

2.6. Study 3

The authors used two months old male C57BL6/J mice - Group 1 (n = 4), Non-fasted (NF), fed ad libitum; Group 2 (n = 5), 24 h Fasted (24 hF), had free access to water but not food for 24 h and Group 3 (n = 5), alternate day fasted (ADF), had free access to water but not food for 24 h. The procedure mentioned above was repeated as a total of refeeding thrice and fasting four times. The mice were anesthetized with ether briefly (1–2 min) and sacrificed by cervical dislocation after the blood collection or BGL measurement (Fig. 1).

2.7. Blood collection

Blood samples were collected from tail bleeds and the BGL was checked using Freestyle Freedom glucometer. For the beta-hydroxybutyrate (BHB) assay, the mice were anesthetized with ether 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.

2.8. Beta-hydroxybutyrate assay

In study 3, the serum samples (n = 4) were analyzed using Biovision BHB colorimetric assay kit (K632-100).

2.9. Homogenization of hippocampus and cortex tissue

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 aprotinin, 1 μg/ml leupeptin. It was followed by phosphatase arrest III (I: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.

2.10. 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: AKT (CST - 9272), p-AKTser473 (CST-9271), p70S6K (CST-9252), p-ERK1/2 (CST - 9102), p-p70S6kThr389 (CST - 9205), ERK1/2 (CST - 9102), b-actin (CST - 8457), p-AKTser473 (CST-9271), p-AKTser473 (CST-9271), p-AKTser473 (CST-9271). The membranes were probed with secondary antibodies and detected by enhanced chemiluminescence (ECL). The bands were quantified using Image J software.

Table 1

| Group | Blood glucose level (mg/dl) |
|-------|-----------------------------|
|       | 0 h or min | 24 hF | 10 min | 30 min | 90 min |
| NF    | 120 ± 6.7  |       |       |       |       |
| 24 hF | 127.6 ± 4.3| 58.2 ± 5***|       |       |       |
| INS10 | 141.2 ± 13.5|       | 67.8 ± 6***|       |       |
| INS30 | 125 ± 6.9  |       |       | 54.8 ± 1.7***|       |
| INS90 | 119 ± 8.5  |       |       |       | ~ 20***|

The blood glucose level was significantly decreased in 24 hF, INS10, INS30 or INS90 vs NF. ***p < 0.001 considered as significant vs NF. Results are mean ± S.E.M. and the data were analyzed using one-way ANOVA followed by Tukey’s test. Abbreviation: Non-Fasted (NF); 24 h fasted (24 hF); 2 U insulin-induced hypoglycemia at 10, 30 or 90 min (INS10, INS30 or INS90).
9297), GLUT3 (sc-74399), Nitrotyrosine (sc-65385), and β actin (CST-4970) were used at a dilution of 1:1000 in TBS-T buffer. After washing thrice 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 thrice 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.

2.11. Statistical analysis

Statistical analysis was performed using two-tailed, one-way ANOVA followed by Tukey's test.

3. Results

3.1. Study 1

The BGL in 24 hF, INS10, INS30 and INS90 significantly (**p < 0.001) decreased compared to NF (Table 1).

Firstly, the authors analyzed the expression of insulin signaling kinases in 24 hF. A trended lower or significantly (**p < 0.01 and ***p < 0.001) decreased expression of insulin signaling kinases such as p-AKTser473 (Fig. 2A–C), p-pS6KThr389 (Fig. 3A–C) or p-ERK1/2 (Fig. 3E–G) in 24 hF of hippocampus or cortex was observed.

Next, the expression of insulin signaling kinases in 2 U insulin-induced MH or SH was analyzed. In INS10, p-AKTser473 was significantly (**p < 0.01) decreased, but there was no change observed in INS30 or INS90 in the hippocampus or cortex (Fig. 2A–D and Fig. 5A). However, the authors observed a trended higher p-pS6KThr389 or p-ERK1/2 in INS10 of hippocampus (Fig. 3A–E and Fig 5A) but were significantly (p < 0.05, **p < 0.01 and ***p < 0.001) decreased in INS30 or INS90 of hippocampus (Fig. 3B–F and Fig 5A). Unlike INS10 of hippocampus, p-pS6KThr389 or p-ERK1/2 were significantly (**p < 0.01) decreased in INS10 of cortex (Fig. 3C–G and Fig 5A). Besides, p-pS6KThr389 was significantly (**p < 0.05) decreased in INS30 or INS90 of cortex (Figs. 3D and 5A) and there was no change observed in p-ERK1/2 in INS30 or INS90 of cortex (Figs. 3H and 5A).

p-BADser155 expression was significantly (**p < 0.01) decreased in INS10 of hippocampus or cortex (Fig. 4A and C and Fig. 5A), but it was restored in INS30 of hippocampus (Figs. 4B and 5A). In the INS30 of cortex, there was a trend to restore. Yet, the authors observed a significant (*)p < 0.05, (**p < 0.01 and ***p < 0.001) decrease in p-BADser155 (Figs. 4D and 5A). Like INS10, a significant (**p < 0.01) decrease in p-BADser155 in INS90 of hippocampus or cortex was observed (Fig. 4B, D and Fig 5A).

In INS10, INS30 or INS90, there was no significant difference in GLUT3 expression. However, a trended lower GLUT3 in INS30 and no change in GLUT3 in INS90 of hippocampus or cortex was observed (Fig. 4E–H and Fig 5A).

3.2. Study 2

At 0 min, there was no significant difference in the BGL in all the groups (Sham, INS G- or INS G) (Fig. 6A). After the 8 U insulin administration, the BGL drastically decreased in INSG- or INS G+ from 30 min. The mice experienced seizures at 51.50 ± 4.4 (INS G-) min and 45.82 ± 1.8 (INS G+) min and there was no significant difference in the seizure onset time (Fig. 6B). At 120 min, the BGL in INSG- and INS G+ were less than 20 mg/dl and the INS G+ received dextrose (3.5 mg/kg/p.o.). At 180 min, the BGL rose in INS G+ (76.20 ± 7.81 mg/dl) and there was a significant difference (###p < 0.001) in the BGL between INS G- (< 20 mg/dl) versus INS G+ (76.20 ± 7.81 mg/dl).

Fig. 2. p-AKTser473 expression in the hippocampus or cortex. Bar graphs depict the fold change derived from densitometric analysis of Western blot data. **p < 0.01 considered as significant versus NF. Results expressed as mean ± S.E.M represent the ratio of p-AKTser473/t-AKT. Data were analyzed using one-way ANOVA followed by Tukey’s test. Abbreviation: NF - non-fasted; 24 hF - 24 h fasted; INS10 or INS30 - 2 U insulin-induced moderate hypoglycemia (nearly 50 mg/dl). The mice were sacrificed at 10 min or 30 min; INS90 - 2 U insulin-induced severe hypoglycemia (˂ 20 mg/dl, experienced seizure-like behavior at 68.75 ± 3.7 min) and the mice were sacrificed at 90 min.
There was a significant (***p < 0.001) decrease in p-BADser155 expression observed in INSG- or INSG+ of hippocampus or cortex. The trended higher expression of GLUT 3 in INSG- was restored after the glucosereperfusion in INSG+ of hippocampus or cortex. Like GLUT 3, the nitrotyrosine expression was significantly (*p < 0.05 and **p < 0.01) increased in INSG- and it was restored after the glucose reperfusion in INSG+ of hippocampus or cortex (Fig. 6 C). Based on
the manufacturer instructions, the authors calculated the nitrotyrosine expression nearly to the molecular weight of 55 kDa (Supplementary Fig. 1 and 2). Moreover, the authors analyzed the GLUT 3 and nitrotyrosine expression in a separate gel.

3.3. Study 3

On day 8, a significant (**p < 0.001) difference in BGL of 24 hF or ADF compared to NF was observed (Table 2). There was no change in p-BADser155 in 24 hF of hippocampus but significantly (*)p < 0.05)
Fig. 5. (A). Expression level of insulin signaling kinases. The figure represents the overall expression level of insulin signaling kinases, p-BADser155 and GLUT 3. The gray shade indicates the moderate hypoglycemia (nearly 50 mg/dl). *p < 0.05, **p < 0.01 and ***p < 0.001 considered as significant vs NF. Results are mean ± S.E.M represent the ratio of p-AKTser473/t-AKT, p-pS6KThr389/t-pS6K, p-ERK1/2/t-ERK1/2, p-BADser155/β-actin and GLUT 3/β-actin. Data were analyzed using one-way ANOVA followed by Tukey’s test. (B). Proposed model for the differential regulation of insulin signaling kinases. In the present study, a decrease in insulin signaling kinases expression was observed at 10 or 30 min. The finding could be attributed to the sudden moderate hypoglycemia experienced by the brain or some changes in the insulin transporter in the blood brain barrier which restricted the insulin entry. However, the proposed mechanism must be investigated further.
Fig. 6. (A, B). Blood glucose level and seizure onset. After 6 h fasting, at 0 min, the blood glucose level (BGL) (Sham, 122.8 ± 14.46; INS G-, 123.8 ± 11.12; or ING +, 120.7 ± 4.97 mg/dl) in all the three groups were not significantly different. At 0 min, 8 U insulin administration drastically decreased the BGL in INS G- and ING + from 30 min and the mice experienced seizures at 51.50 ± 4.4 (INSG-) or 45.82 ± 1.8 (INS G+) min, which were not significantly different. At 120 min, the BGL in INS G- and ING + were less than 20 mg/dl and the authors administered dextrose (3.5 mg/kg/p.o.) in INS G+. At 180 min, the BGL rose in INS G+ (76.20 ± 7.81 mg/dl) and there was a significant difference in BGL between INS G+ versus INS G- (˂ 20 mg/dl).

(C). p-BADser155, GLUT 3 and nitrotyrosine expression in the hippocampus or cortex. Bar graphs depict the fold change derived from densitometric analysis of Western blot data. *p < 0.05, **p < 0.01 and ***p < 0.001 considered as significant vs Sham. ###p < 0.001 considered as significant vs INS G+. Results expressed as mean ± S.E.M represented the BGL, seizure onset time and the ratio of p-BADser155/β-actin, GLUT 3/β-actin and nitrotyrosine/β-actin. Data were analyzed using one-way ANOVA followed by Tukey’s test. Abbreviation: Sham – received normal saline; INS G - received 8 U insulin, experienced severe hypoglycemia and seizures; INS G+ - received 8 U insulin, experienced severe hypoglycemia with seizures with glucose reperfusion.
Table 2

| Blood glucose level (mg/dl) | Group | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 ( euthanized) |
|----------------------------|-------|-------|-------|-------|-------|-------|-------|-------|---------------------|
| NF                         | –     | –     | –     | –     | –     | –     | –     | –     | 138.9 ± 6.26        |
| 24 hF                      | –     | –     | –     | –     | –     | –     | –     | –     | 133.4 ± 5.80        |
| ADF                        | 143.4 ± 12.14 | 60.8 ± 3 | 145.6 ± 5.6 | 78.4 ± 3.99 | 30 ± 3.19 | 66.2 ± 2.08 | 123.2 ± 8.52 | 57.80±2.05 | ***                  |

In day 8, the blood glucose level significantly decreased in 24 hF or ADF vs NF. **p < 0.01 considered as significant vs NF. Results are mean ± S.E.M. and the data were analyzed using one-way ANOVA followed by Tukey's test. Abbreviation: Non-fasted (NF), fed ad libitum; 24 h Fasted (24 hF), had free access to water but not food for 24 h; Alternate day fasted (ADF), had free access to water but not food for 24 h. The procedure was repeated as a total of refeeding thrice and fasting four times.

decreased in 24 hF of cortex. Further, a significant (\*p < 0.05 and \**p < 0.01) decrease in p-BADser155 in ADF of hippocampus or cortex was noticed (Fig. 7 A).

A higher trend or significantly (\*p < 0.05 and \**p < 0.01) increased GLUT 3 or nitrotyrosine expression in 24 hF of hippocampus or cortex was observed. However, there was no significant difference in GLUT 3 or nitrotyrosine expression in 24 hF of hippocampus or cortex. The authors observed a trended higher nitrotyrosine expression in ADF of hippocampus or cortex was noticed (Fig. 7A).

The serum BHB levels were significantly (**p < 0.001) increased in 24 hF or ADF compared to NF. Furthermore, a significant (Øp < 0.05) increase in BHB levels in ADF compared to 24 hF was noticed (Fig. 7B).

4. Discussion

The extensive literature revealed that so far, there were only few studies that investigated the brain insulin signaling kinases expression after the peripheral insulin administration. In one study, the anesthetized mice were administered with 1 mU insulin and the BGL maintained nearly 110 mg/dl between 5 and 20 min, transiently increased the cerebral p-AKTser473 or p-ERK1/2 and the expression of the kinases reached its maximum at 10 min and returned to baseline at 20 min (Freude et al., 2005). In another study, the awake mice under went 4 mU hyperinsulinemic-euglycemic clamp settings and after the duration of 1–1.5 h, there was no change in hippocampal or hypothalamus p-AKTser473 (Stanley et al., 2016). In addition to the studies mentioned above, overnight fasted mice received 5 U insulin and it drastically decreased the BGL nearly 20 mg/dl at 10 min. The expression of p-AKTser473 in the hippocampus or cortex increased at 5 min and it returned to baseline at 10 min (Clodfelder-Miller et al., 2005).

To the author’s knowledge, the present study is the first of its kind to analyze the insulin signaling kinases expression during MH (nearly 50 mg/dl). Contrary to the earlier studies, a decrease in p-AKTser473 was observed in INS10 (Fig. 2A, C and 5A) and restored in INS30 (Fig. 2B, D and 5A) of hippocampus or cortex.

In the hepatoma cells, the insulin signaling promotes phosphorylation of AKT but not pS6K at 5 min. Phosphorylation of pS6K began at 15 min (Kubota et al., 2012). The above observation led the authors to investigate the other insulin signaling kinases such as p-pS6KThr389 or p-ERK1/2. As expected, a trended higher expression of p-pS6KThr389 or p-ERK1/2 was observed in INS10 of hippocampus (Fig. 3A, E and 5A), thereby suggesting that the insulin was likely to promote the phosphorylation of its downstream kinases before or at 10 min. Further, a decrease in p-pS6KThr389 or p-ERK1/2 was observed in INS30 of hippocampus and INS10 of cortex (Fig. 3B, C, 3F, 3G and 5A). Also, the authors observed a decrease in p-pS6KThr389 in INS30 of cortex (Fig. 3D and 5A).

Insulin crosses the blood-brain barrier (BBB) in a saturable transport mechanism (Banks et al., 2012). Previous studies suggested that the peripheral insulin administration in different conditions such as euglycemia, overnight fasting with SH increased the insulin signaling kinases expression before or at 10 min and it returned to baseline at 20 or 30 min (Clodfelder-Miller et al., 2005; Freude et al., 2005). The finding as mentioned above, suggested that insulin entry into the brain might reach saturation after 10 min. Contrary to that, in the present study, the authors observed a decrease in insulin signaling kinases expression at 10 or 30 min (Fig. 5A). The finding might be attributed to the sudden MH experienced by the brain or some changes in the insulin transporter in the BBB that restricted the insulin entry.

Insulin-induced SH increases neuronal death (Suh et al., 2007). Hypoglycemic coma for 30 min, phosphorylated the AKT in the cortex and their group suggested that the phosphorylation of AKT may play a protective role (Ouyang et al., 2000). In the present study, there was no change in AKT expression; but the expression of ERK and P6S6K decreased or no change was observed in INS90 of hippocampus or cortex (Fig. 5A). Overall, the differential expression of insulin signaling kinases during SH might be regulated by non-canonical pathways.

Oxygen-glucose deprivation (OGD) (in vitro model of ischemia) decreased the neuronal cell viability. The addition of metformin (insulin sensitizer) moderately increased the neuronal cell viability whereas the insulin receptor tyrosine kinase inhibitor tyrphostin A47 decreased the neuronal cell viability, thereby suggesting that the insulin signaling protects the neurons during OGD (Mielke et al., 2006). In contrast, the addition of insulin to the hippocampal neuronal culture decreased the cell viability under glucose deprivation (Tanaka et al., 2008). In the present study, the decrease in insulin signaling during MH or SH may be either beneficial or detrimental to the brain. However, the proposed mechanism must be investigated further. Overall, the authors proposed a hypothetical model for insulin-induced hypoglycemia associated kinases expression in the brain (Fig. 5B).

Proteomic analysis revealed that phosphofructokinase 1 (PFK 1), an important regulatory enzyme in glycolysis, is a BAD associated protein. BAD phosphorylation is required for the activation of PFK 1 (Deng et al., 2008). In addition, the proapoptotic protein BAD and glucokinase reside in a complex. Glucokinase also known as hexokinase IV, facilitates the phosphorylation of glucose to glucose-6-phosphate in the glycolysis pathway. Unlike other hexokinases, glucokinase has a weak affinity for glucose and it is not inhibited by glucose-6-phosphate. Glucokinase activity varies based on glucose concentrations; thereby, it acts as a glucose sensor (de Backer et al., 2016; Lenzen, 2014). Glucose deprivation results in the dephosphorylation of BAD which inhibits glucokinase activity (Danial et al., 2003). In order to understand the BAD role in glycolysis, oxygen consumption rate (OCR) was investigated using different carbon substrates (Danial et al., 2003). The addition of glucose in BAD +/- hepatocytes increased the OCR, whereas in BAD -/- hepatocytes decreased the OCR. The fructose (bypasses the two main regulatory steps in glycolysis) and pyruvate (the end product of glycolysis) increased the OCR in BAD -/- hepatocytes (Danial et al., 2003). Moreover, the addition of glucose in BAD -/- astrocytes and neurons decreased the OCR whereas the addition of BHB increased the OCR. Overall, this suggesting that BAD deficiency might involve in glycolysis inhibition and prefer BHB oxidation in brain cells (Giménez-Cassina et al., 2012) (Supplementary Fig. 3).
in INS30 of hippocampus and there was a trend to restore in INS30 of cortex (Fig. 4A, B, 4C, 4D and 5A); thereby suggesting that the insulin-induced MH in INS10 might decrease glucose metabolism via glycolysis whereas in INS30, the brain might try to restore glucose metabolism. Even though insulin crosses the BBB in a saturable transport mechanism, prolonged and sustained insulin-induced MH in humans, does not affect the brain glucose metabolism via glycolysis or tricarboxylic acid (TCA) cycle (van de Ven et al., 2011). Further, the authors only achieved the MH in INS10 or INS30 (Table 1) and additional studies are required to investigate the p-BADser155 expression in prolonged MH.

Monocarboxylate transporters (MCT) are plasma membrane transporters that carry the lactate or ketone bodies. Insulin-induced MH does not alter or decreases the MCT2 in different regions of the brain (Vavaiya et al., 2007). In the present study, the authors did not analyze

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**Fig. 7.** (A). p-BADser155, GLUT 3 and nitrotyrosine expression in the hippocampus or cortex. Bar graphs depict the fold change derived from densitometric analysis of Western blot data. (B). Serum beta-hydroxybutyrate (BHB) levels. The BHB levels in 24 hF and ADF increased when compared to NF. Also, the BHB level in ADF increased when compared to 24hF. *p < 0.05, **p < 0.01 and ***p < 0.001 were considered as significant vs NF. #p < 0.05 considered as significant vs 24 hF. Results expressed as mean ± S.E.M represented the serum BHB levels and the ratio of p-BADser155/β-actin, GLUT 3/β-actin and nitrotyrosine/β-actin. Data were analyzed using one-way ANOVA followed by Tukey's test. Abbreviation: NF - non-fasted; 24 hF - 24 h fasted; ADF – Alternate day fasted.
the serum BHB in MH or SH. The peripheral BHB administration prevents hypoglycemic neuronal death (Julo-Amilpas et al., 2015) and 24 h fasting associated elevation of BHB increased the seizure onset time in insulin-induced hypoglycemic seizures in mice (Pitchaimani et al., 2016), which indirectly suggests that the brain may uptake ketone bodies during SH.

Insulin-induced SH (INS90, INS G-) or SH with glucose reperfusion (INS G+) decreased the p-BADser155 (Fig. 4B, D, 5A and 6C), suggesting that the brain might adapt to decrease glucose metabolism via glycolysis or prefer ketone body metabolism. Further, it could be inferred that the brain was likely to inhibit glycolysis even after the glucose reperfusion.

Activation of the NMDA receptor increased the surface expression of GLUT 3, whereas the NMDA receptor antagonist prevented it. The addition of glutamate in cortical neurons phosphorylated the nNOS and the NMDA receptor-induced surface expression of GLUT 3 was blocked by the neuronal nitric oxide synthase (nNOS) inhibitor. Overall, NMDA receptor activation by glutamate induces the expression of GLUT 3 via the nNOS pathway, thereby increases the intracellular glucose (Ferreira et al., 2011).

The authors observed a trended lower GLUT 3 expression in INS30 of hippocampus or cortex (Fig. 4F, H and 5A). In humans, acute hypoglycemia decreased brain glutamate (Terpstra et al., 2014). So, the trended lower GLUT 3 in INS30 of hippocampus or cortex was likely to be mediated by the glutamate-NMDA receptor axis pathway. Prolonged insulin-induced MH does not affect brain glucose metabolism (van de Ven et al., 2011) but impairs cognition (Graveling et al., 2013). Further, the insulin role in brain glucose uptake is considered to be less significant (Banks et al., 2012), thereby raising the question if the prolonged insulin-induced MH affected the glutamate levels and if the glutamate interfered with glucose uptake via GLUT 3. The queries raised have to be investigated further.

Moreover, there was no change in GLUT 3 expression in INS90 (experienced seizures) of hippocampus or cortex (Fig. 4F, H and 5A). Insulin-induced SH increases the glutamate and NMDA receptor activity (McGowan et al., 1995) and the GLUT 3 expression is regulated by glutamate/NMDA receptor/nitric oxide pathway (Ferreira et al., 2011). The authors speculated if an extension of SH with seizure duration would elevate the GLUT 3 expression. Earlier, the authors have reported that the mice fasted for 6 h duration and the administration of 8 U insulin predisposed the seizure onset time (Pitchaimani et al., 2016). The same procedure was followed in study 2 and the trended higher GLUT 3 was observed in INS G-. Moreover, GLUT 3 expression was correlated with increased nitrotyrosine (marker of nitric oxide) expression (Fig. 6 C), thereby suggesting that SH with seizures in INS G-modified the GLUT 3 expression, possibly via glutamate/NMDA receptor/nitric oxide/GLUT 3 axis pathway. After the glucose reperfusion, the mice regained their consciousness and the expression of GLUT 3 or nitrotyrosine were restored in INS G+. The above finding suggested that the glucose reperfusion might decrease the SH with seizure-induced excess glutamate signaling.

Moreover, glutamate excitotoxicity leads to hypoglycemic neuronal death. The production of superoxide is mainly from mitochondrial electron transport chain, dehydrogenase complex of mitochondrial TCA cycle and the regeneration of NADPH by hexose monophosphate (HMP) shunt. SH with glucose reperfusion (possibly enter into the neuron via GLUT 3) increases the neuronal damage only via HMP shunt-dependent NADPH oxidase-associated superoxide production (Suh et al., 2007).

Overall, during glucose reperfusion in SH, the glucose might enter into the neuron via GLUT 3 and the neuron is likely to shift the metabolism from glycolysis to HMP shunt, thereby SH with glucose reperfusion resulting in NADPH oxidase-dependent superoxide production.

Studies by others report 24 h fasting or ADF decreased the insulin signaling kinases expression in the brain (Clodfelder-Miller et al., 2005; Lu et al., 2011). In the present study, the authors observed a trended lower or decreased insulin signaling kinases (p-AKTser473 p- pS6KThr389 or p-ERK1/2) expression in 24 hF (Fig. 2A, C, 3A, C, E and 3G), while the ADF insulin signaling kinases expression were not analyzed. Insulin-induced MH impaired the executive cognitive functions in humans (Graveling et al., 2013). On the contrary, ADF improved the cognitive functions in mice (Li et al., 2013). The defective insulin signaling has been linked to cognitive impairment (Bloemer et al., 2014). So, the decrease in insulin signaling kinases expression in insulin-induced MH or ADF and its functional role in cognitive impairment have to be investigated further.

Fasting increased the plasma and brain BHB in human subjects (Pan et al., 2000). Also, the increase in plasma BHB reduced the cerebral metabolic rate of glucose in rats (LaManna et al., 2009; Zhang et al., 2013). In the present study, 24 hF or ADF increased the serum BHB levels (Fig. 7B). Moreover, the p-BADser155 expression decreased in 24 hF or ADF (Fig. 4A, C and 7A), suggesting that the brain might prefer ketone body metabolism or decreases glucose metabolism.

In an earlier study, 72 h fasting increased the GLUT 3 expression in the brain (Nagamatsu et al., 1994). In the present study, there was a trended higher nitrotyrosine but not GLUT 3 expression in 24 hF (Fig. 7A). The serum BHB levels in ADF were significantly higher when compared to 24 hF and there was a correlation between ADF serum BHB levels and GLUT 3 expression (Fig. 7B). In humans, plasma BHB gradually increased over the days and reached a plateau (Cahill, 2006). So, further studies are required to confirm the relationship between the prolonged fasting associated serum BHB levels and GLUT 3 expression.

Like ADF, recurrent episodes of insulin-induced hypoglycemia without seizures increased the GLUT 3 expression (Lee et al., 2000; Uehara et al., 1997). Also, recurrent episodes of insulin-induced MH elevated the brain glutamate levels compared to a single episode of MH in type 1 diabetic subjects (Terpstra et al., 2014). The above findings suggested that the brain might adapt to modify the GLUT 3 expression in response to multiple episodes of hypoglycemia.

Table 3

| S. No | Various hypoglycemic models                                                                 | GLUT 3                                                                 | p-BADser155                                                                 |
|-------|------------------------------------------------------------------------------------------|----------------------------------------------------------------------|--------------------------------------------------------------------------------|
| 1     | Fasting hypoglycemia (24 hF)                                                             | no change                                                           | decreased                                                                     |
| 2     | Alternate day fasting hypoglycemia (ADF)                                                 | no change or increased                                              | decreased                                                                     |
| 3     | Insulin induced moderate hypoglycemia at 10 or 30 min (INS10 or INS30)                    | no change at 10 min but trended lower                                | decreased at 10 min but trended to restore or restored                        |
| 4     | Insulin induced severe hypoglycemia, experienced seizures (INS G-)                      | at 30 min                                                          | decreased at 30 min                                                          |
| 5     | Insulin induced severe hypoglycemia, experienced seizures with glucose reperfusion (INS G+) | trended higher                                                     | decreased                                                                     |

Hypoglycemia alters the brain glutamate signaling which may increases the intracellular glucose via GLUT 3 expression. Dephosphorylation of BADser155 inhibit glycolysis, reduces glucose or increases ketone body metabolism in the brain. Here, we summarized the overall GLUT 3 and pBADser155 (hippocampus and cortex) expression in various hypoglycemic models.
5. Summary

Prior to the interpretation of results of the present study, it is important to consider that the authors did not examine the hypoglycemia associated protein expression in different brain cells such as astrocytes or neurons. From a broader perspective, the report has been summarized in the following order. First, insulin-induced MH (INS10 or INS30) and SH (INS90) differentially regulated the insulin signaling kinases expression. Second, the expression of p-BADser155 was decreased in all hypoglycemic models (except insulin-induced MH of hippocampus INS30) suggesting that the brain might decrease glucose metabolism via glycolysis or prefer ketone body metabolism. Third, the trended higher GLUT 3 and increased nitrotyrosine expression of SH with seizures in INS G-was restored after the glucose reperfusion in INS G+. The finding suggested that during SH with seizures, the brain might adapt to uptake glucose in addition to ketone body metabolism. Fourth, the trended higher or increased GLUT 3 and nitrotyrosine expression in ADF were positively correlated with ADF serum BHB levels, thereby indicating that ADF brain might adapt to uptake glucose in addition to the ketone body metabolism (Table 3).

CRediT authorship contribution statement

Vigneshwaran Pitchaimani: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. Somasundaram Arumugam: Formal analysis, Investigation. Rajarajan Amirthalingam Thandavarayan: Writing - review & editing. Vengadeshprabhu Karuppagounder: Investigation. Mst Rejina Afrin: Investigation. Remya Sreedhar: Investigation. Meilei Harima: Writing - review & editing. Masaaki Nakamura: Writing - review & editing. Kenichi Watanabe: Supervision, Project administration, Funding acquisition, Writing - review & editing. Satoru Kodama: Writing - review & editing. Kazuya Fujihara: Writing - review & editing. Hirohito Sone: Writing - review & editing.

Declaration of competing interest

The authors declare no competing financial interests.

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Hirohito Sone: Investigation. Kazuya Fujihara: Writing - review & editing. Meilei Harima: Writing - review & editing. Vengadeshprabhu Karuppagounder: Investigation. Mst Rejina Afrin: Investigation. Remya Sreedhar: Investigation. Meilei Harima: Writing - review & editing. Masaaki Nakamura: Writing - review & editing. Kenichi Watanabe: Supervision, Project administration, Funding acquisition, Writing - review & editing. Satoru Kodama: Writing - review & editing. Kazuya Fujihara: Writing - review & editing. Hirohito Sone: Writing - review & editing.

Declaration of competing interest

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Appendix A. Supplementary data

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