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The Double-edged Effect of Insulin on the Neuronal Cell Death Associated with Hypoglycemia on the Hippocampal Slice Culture

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It is well known that the central nervous system (CNS) is vulnerable to hypoglycemia and hyperglycemia. Insulin is indispensable for serum glucose control and diabetes patients are on the relative or absolute deficient state of insulin. The role of insulin on the CNS, however, has not been fully elucidated, yet. To reveal the role of insulin on the neuronal survival, we have used in vitro system of an organotypic hippocampal slice culture from rat, and examine the neuronal cell death at the various glucose concentrations in the presence or absence of insulin. When glucose concentrations is varied to 0, 1, 3, 5 and 30mM in the incubation medium, the neuronal cell death was minimum at 5mM, and no neuronal survival was observed under 1mM on the CA1. On the dentate gyrus granule cells (DG), on the other hand, the significant neuronal survival was observed even as low as 1mM. In the presence of 1nM concentration of insulin, the neuronal cell death curve showed the U-shape, and the minimum death point was 3-5mM glucose concentrations at the CA1. At the DG, insulin did not show the protective effect up to 48 hours culture regardless of glucose concentration. In the absence of glucose, insulin accelerated the neuronal cell death both in the CA1 and DG. We concluded that insulin has a double-edged effect on the neuronal cell death dependent on glucose concentration, and that the CA1 and the DG have a different sensitivity to insulin in terms of cell survival.

It has been well known that the central nervous system is vulnerable to hypoglycemia. Insulin regulates a blood glucose level and its deficiency causes diabetes. An action of insulin on the central nervous system has not been enough elucidated, yet. Recent reports have suggested that the type 2 diabetes is one of the risk factors for the decay of cognitive function and the blockade of insulin signal cascade may be involved for its pathology (17, 21, 22). And after ischemic events, insulin acts directly on the brain to reduce ischemic brain necrosis independent of hypoglycemia (26). Hypoglycemia causes lethal consequences during insulin treatment. Clinically, it is mandatory to avoid nocturnal hypoglycemia especially in case of treating elderly and the type1 diabetic patients, and it is known that 2 to 4% of the type1 diabetic patients die by an excess of insulin administration (15). The harm effects of hypoglycemia, therefore, are well known and the protection method is mainly to keep blood glucose in adequate levels. The insulin action per se during hypoglycemia against the CNS, however, is merely understood.

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In this paper, we examined the direct action of insulin on the neuronal cell death at a variety of glucose concentrations by using cultured hippocampal slices. Our experimental results suggested that the CNS damage during hypoglycemia would exaggerate by insulin therapy itself and the caution may be necessary not only the glucose levels but also the insulin therapy itself during hypoglycemia.

MATERIALS AND METHODS

The experiments were conducted according to the guidelines for animal experimentation at the Kobe University School of Medicine, and conform to the relevant National Institution of Health guidelines.

Preparation of organotypic hippocampal slices

Hippocampal slices were made from the septal half of the hippocampus using a standard method (20). Briefly, 9- to 11-days Wistar rats (Hartley, SLC, Japan) were anesthetized with 98% diethyl I ether and decapitated. The hippocampus were rapidly dissected at 4-6 °C and cut into 450μm slices using a McIllwain Tissue Chopper (Mickle Laboratory Engineering Co., Ltd, UK). Slices were then transferred onto membranes (pore diameter: 30μm, Millicell-CM, Millipore, Bedford, MA, USA), and placed into a six-well microplate (Costar Corning Inc, NY, USA) with 1mL of slice culture medium per well. The culture medium contained 50% Eagle's minimal essential medium (MEM) (Gibco, CA, USA), 25% Hanks' Balanced Salt Solution (HBSS)(Gibco, CA, USA), 25% heat-inactivated horse serum (Gibco, CA, USA) containing 1% penicillin/streptomycin. The medium was changed every 3 days. Slices were kept in culture for 14 days before study and the six-well microplates were stored at 37 °C in a 5% CO2 incubator under a 95% humidity atmosphere (Sanyo, Tokyo, Japan).

Treatment of hippocampal slices

Slices in the six-well microplates at day 14 were washed, and the basic medium was replaced with various agents for the treatment. The basic medium contained 90mM NaCl, 4mM KCl, 0.1mM MgCl2, 0.1mM KH2PO4, 0.5mM MgSO4, 0.1mM Na2HPO4, 0.5mM NaH2PO4, 14mM NaHCO3, 1.2mM CaCl2, 10mM glucose, about 2mM essential and non-essential amino acids, and 0.02mM vitamins. In order to investigate the changes in neuronal toxicity due to the glucose concentration, various glucose concentrations (0mM, 1mM, 3mM, 5mM, and 30mM) were added to the medium that was used to treat the slices. Moreover, the change in the neuronal death rate was investigated both with and without insulin loading at a concentration of 1nM. (insulin: Humulin® R, Eli Lilly, Indiana, USA)

Assessment of cell death in hippocampal slices

The propidium iodide (PI) method was used in the assessment of neuronal death in hippocampal slices at 24h, 48h, and 72h after each treatment in the CA1 and the dentate gyrus granule cells (DG) of the hippocampus. To label the nuclei of dead neurons, 4.6μg/mL of PI (Sigma, St. Louis, Mo, USA) was added to the wells of the culture microplates for 15min. PI is a polar compound that only enters cells with damaged cell membranes, where it binds to nucleic acids within the cells and develops a bright red fluorescence. The dye is basically non-toxic to neurons, and is used as an indicator of neuronal integrity and cell viability (11). Thus the intensity of fluorescence correlates with the cell death fraction. After 15 min, digital images of the PI fluorescence were obtained with an inverted fluorescence microscope (4×objective) equipped with a digital camera (Olympus IX70, Tokyo, Japan). After the final image, all the neurons were killed by adding 10 μ M N-methyl-D-aspartic acid（NMDA）and the final PI fluorescence intensity was

calibrated as 100% cell death. The mean intensity (green values) of the PI fluorescence were measured using an image program MacScope (Ver 2.6.1, Mitani Inc, Osaka, Japan).

Statistical analysis

Values were expressed as mean ± standard error of the mean (SEM) from three independent experiments. The statistical significance was established by ANOVA followed by a post-hoc test, and then the non-paired t-test was employed using StatView software (v.5.0.1.0; SAS Institute Inc., Cary, NC, USA). $p<0.05$ was considered to be statistically significant.

RESULTS

CA1 neuronal cell death in the presence or absence of serum

Serum is widely used for maintenance of cultured neuronal cell viability. To know the extent of nerve protection effects of serum in our experimental settings, the neuronal cell death was evaluated in the presence or absence of the heat inactivated horse serum (Gibco, CA, USA) in the culture medium. The glucose concentration in the medium was kept at 30mM, the concentration that is usually commercially available. After 72 hour culture, the neuronal cell survival was better in the presence of serum (n=30) in comparison with the absence of serum (n=11) and the cell death rate was 22.7 ± 6.3% and 40.8 ± 6.2%, respectively (non-paired t-test; $p<0.05$) (Fig.1).

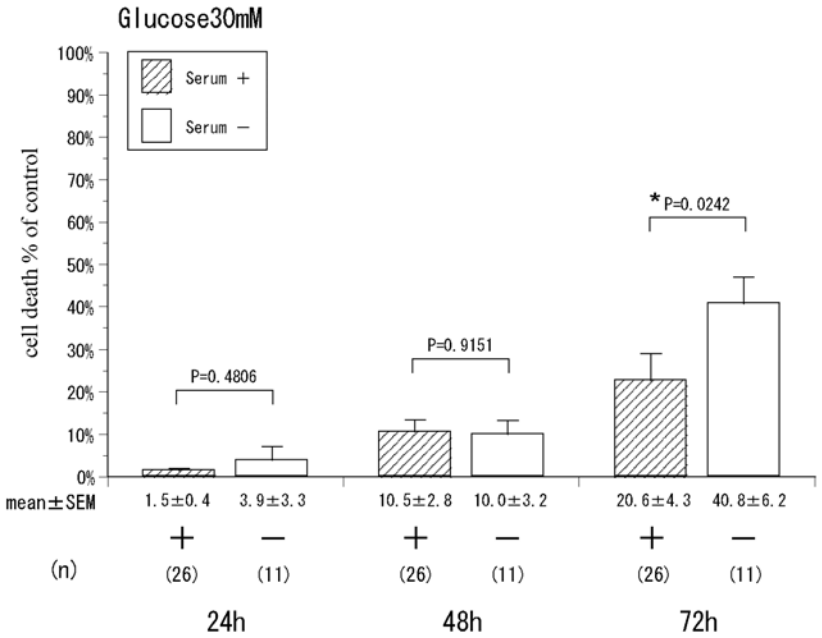

Fig. 1. CA1 neuronal cell death in the presence or absence of serum.
The glucose concentration in the medium was kept at 30mM, the concentration that is usually commercially available. After 72-hour culture, the neuronal cell survival was better in the presence of serum (n=30) in comparison with the absence of serum (n=11) and the cell death rate was 22.7±6.3% and 40.8±6.2%, respectively (*: non-paired t-test; $p<0.05$.)

The CA1 neuronal cell death during low glucose

The difference in cell death rates was examined in various glucose concentrations (0mM, 1mM, 3mM, 5mM, and 30mM) under the environment lacking serum on the CA1 pyramidal cell. Glucose 0mM (n=9) and 1mM (n=14) resulted prominently high cell death rates after 48 hour culture and the cell death rates were 57.0±6.5% and 53.7±7.4%, respectively. After 72 hour, the rate further increased and the each rate was 83.6± 4.9% and 92.9±1.2%, respectively. The cell death rates showed the U-shaped curve against the glucose concentrations and neuronal cell death was minimum at 5mM glucose (n=9) (Fig. 2)(One-way ANOVA; $p<0.0001$, Scheffe's F test; $p<0.01$).

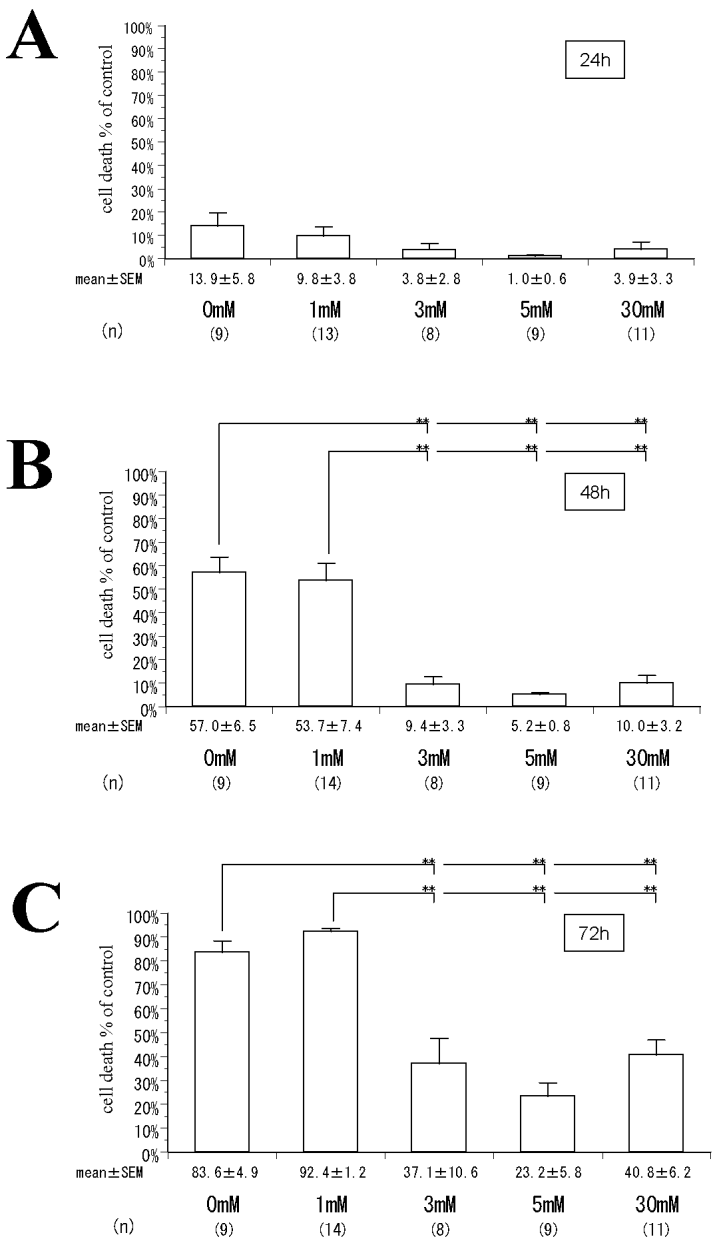

Fig. 2. The CA1 neuronal cell death during low glucose.

Various glucose concentrations (0mM, 1mM, 3mM, 5mM, and 30mM) under the environment lacking serum on the CA1 pyramidal cell. Glucose 0mM (n=9) and 1mM (n=14) resulted prominently high cell death rates after 48 hour culture and the cell death rates were 57.0±6.5% and 53.7±7.4%, respectively. After 72 hour, the rate further increased and the each rate was 83.6± 4.9% and 92.9±1.2%, respectively. The cell death rates showed the U-shaped curve against the glucose concentrations and neuronal cell death was minimum at 5mM glucose (n=9) (Fig. 2) (*:One-way ANOVA; P=0.0008, Scheffe's F test; $p<0.05$) (**:One-way ANOVA; P<0.0001, Scheffe's F test; $p<0.01$).

The DG granule cell death during low glucose

The difference in cell death rates was examined in various glucose concentrateions (0mM, 1mM, 3mM, 5mM, and 30mM) under

the environment lacking serum on the DG granule cell. The cell death rates increased maximally at 0mM glucose and the cell death rate after 48 hours was 59.2±5.3%. The DG granule cell was kept relatively well alive even in 1mM glucose condition and the death rate after 48 hours was only 14.5 ± 5.0%. The cell death rates did not show the U-shaped curve against the glucose concentrations and neuronal cell death was most inhibited at 3mM glucose (one-way ANOVA; $p<0.0001$, Scheffe's F test; $p<0.01$) (Fig. 3).

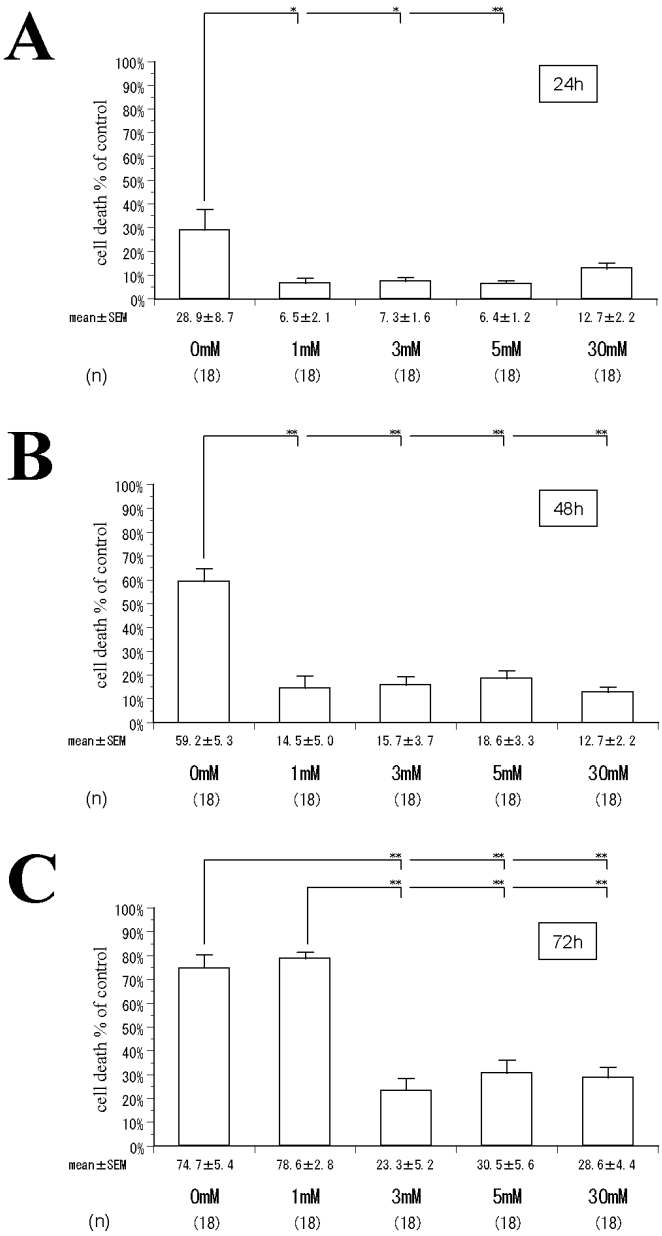

Fig. 3. The DG granule cell death during low glucose.

Various glucose concentrations (0mM, 1mM, 3mM, 5mM, and 30mM) under the environment lacking serum on the DG granule cell. The cell death rates increased maximally at 0mM glucose and the cell death rate after 48 hours was 59.2±5.3%. The DG granule cell was kept relatively well alive even in 1mM glucose condition and the death rate after 48 hours was only 14.5 ± 5.0%. The cell death rates did not show the U-shaped curve against the glucose concentrati-onns and neuronal cell death was most inhibited at 3mM glucose (one-way ANOVA; $p<0.0001$, Scheffe's F test; $p<0.01$) (Fig. 3).

CA1 neuronal cell death in the presence or absence of insulin

The difference in the cell death rates on the CA1 pyramidal neuron was examined in the presence or absence of 1nM insulin during treatment with a variety of glucose concentrations (0mM, 3mM, and 30mM). In the presence or absence of insulin at 3mM glucose, the observed cell death rates after 48 hours were 10.3±1.2% and 38.1±9.1%, respectively, and after 72 hours were 22.4±3.8% and 54.5±8.2%, respectively ($p<0.05$) (Fig.4B). Thus, the presence of insulin significantly improved the cell survival at 3mM glucose concentrations up to 72hours. In the case of 0mM glucose, the insulin addition surprisingly deteriorated the cell survival and the cell death rates of the presence or absence of insulin were 67.0 ± 10.5% and 37.3 ± 8.9%, respectively ($p<0.05$) (Fig. 4A). At 30mM glucose condition, the insulin addition did not give any significant effects on the cell survival ($p=0.789$) (Fig. 4C).

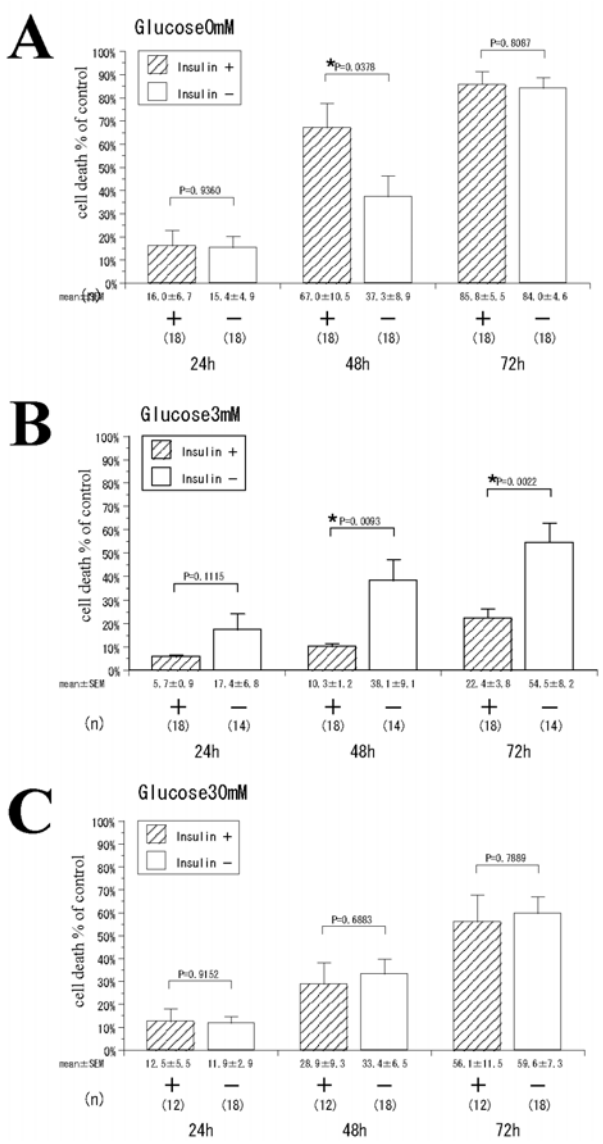

Fig. 4. CA1 neuronal cell death in the presence or absence of insulin.

The presence or absence of 1nM insulin during treatment with a variety of glucose concentrations (0mM, 3mM, and 30mM). In the presence or absence of insulin at 3mM glucose, the observed cell death rates after 48 hours were 10.3±1.2% and 38.1±9.1%, respectively, and after 72 hours were 22.4±3.8% and 54.5±8.2%, respectively (*: non-paired t-test; $p<0.05$) (Fig.4B). Thus, the presence of insulin significantly improved the cell survival at 3mM glucose concentrations up to 72hours. In the case of 0mM glucose, the insulin addition surprisingly deteriorated the cell survival and the cell death rates of the presence or absence of insulin were 67.0 ± 10.5% and 37.3 ± 8.9%, respectively (*: non-paired t-test; $p<0.05$) (Fig. 4A). At 30mM glucose condition, the insulin addition did not give any significant effects on the cell survival (non-paired t-test; $p=0.789$) (Fig. 4C).

The DG granule cell death in the presence or absence of insulin

The difference in cell death rates on the DG granule cell was examined in the presence or absence of 1nM insulin. The presence and absence of insulin at 3mM glucose showed no significant difference after 48 hours (22.1± 3.2 % and 25.5±3.6%, respectively) (p=0.4963). After 72 hours, the insulin showed minor protective effect against the cell death (31.8 ± 4.2% and 46.6 ± 4.1%, respectively) (p=0.0161). At 0mM glucose, the insulin addition deteriorated the cell survival and the cell death rates after 48 hours in the presence or absence of insulin were 47.2 ± 9.2% and 23.9 ± 3.7%, respectively (p<0.05)（Fig. 5A）. At 30mM glucose condition, no significant difference was observed between the insulin and non-insulin groups (p=0.2074) (Fig. 5C).

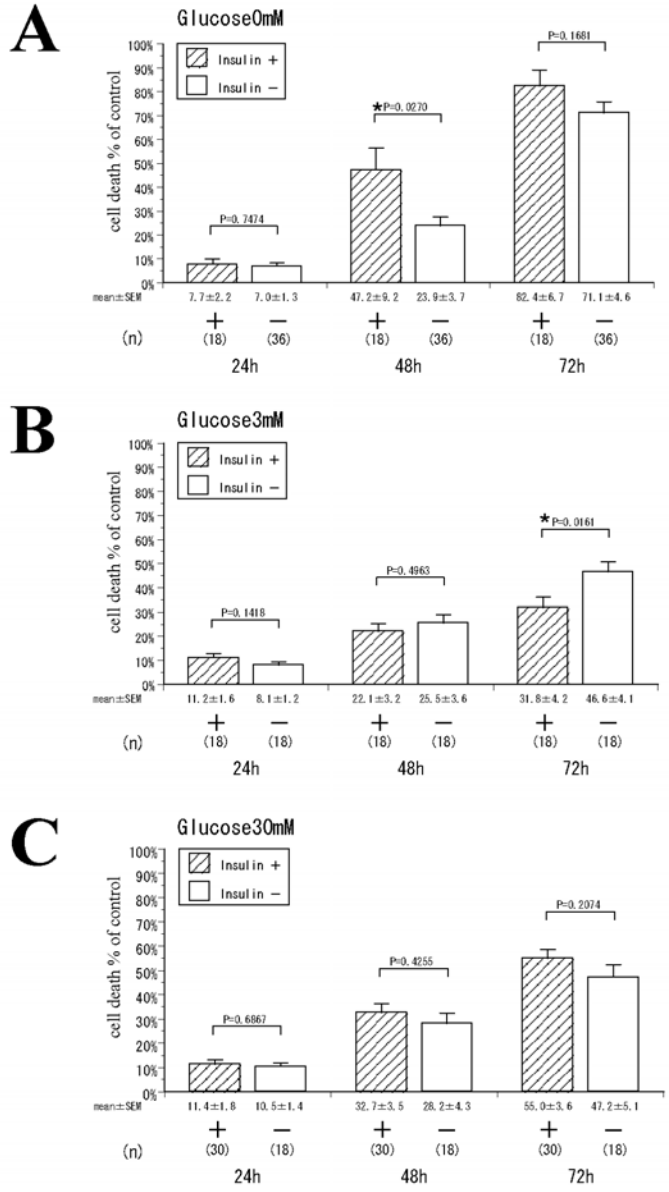

Fig. 5. The DG granule cell death in the presence or absence of insulin.

The presence or absence of 1nM insulin. The presence and absence of insulin at 3mM glucose showed no significant difference after 48 hours (22.1± 3.2 % and 25.5±3.6%, respectively) (non-paired t-test; p=0.4963). After 72 hours, the insulin showed minor protective effect against the cell death (31.8 ± 4.2% and 46.6 ± 4.1%, respectively) (*: non-paired t-test; p=0.0161). At 0mM glucose, the insulin addition deteriorated the cell survival and the cell death rates after 48 hours in the presence or absence of insulin were 47.2 ± 9.2% and 23.9 ± 3.7%, respectively (*: non-paired t-test; p<0.05)（Fig. 5A）. At 30mM glucose condition, no significant difference was observed between the insulin and non-insulin groups (non-paired t-test; p=0.2074) (Fig. 5C).

DISCUSSION

In the present experiment, we examined the insulin effects on the neuronal cell death during low glucose condition, and found that the insulin protected neuronal cell with low glucose, but increased neuronal cell death in case of glucose free condition (Fig. 4A, Fig. 5A). Moreover, while the DG had more tolerant against low glucose, the neuroprotective effect of insulin during low glucose had more prominent on the CA1 than the DG.

Neurotrophic effects of serum

When serum is contained, a low neuronal cell death rate was observed in comparison with addition of insulin only (1nM), suggesting that a variety of factors contained in serum work for neuronal cell protection in addition to insulin (Fig. 1). Neurotrophic factors such as NGF (nerve growth factor) or BDNF (brain derived neurotrophic factor), and vitamin B family in serum are supposed to restrain apoptosis and promote neuronal survival (18, 27). Our results were coincident to these previous results.

The CA1 vulnerability to hypoglycemia

Selective vulnerability has been well known to date and particularly the CA1 is one of the most vulnerable sites in the CNS against a stress. It is commonly believed that glutamate excitotoxicity relates to selective vulnerability. Glutamate level in the hippocampus of mouse after ischemic stress was greater in the CA1 than that of the DG (6, 14, 25). Also it has been known that the extracellular glutamate level rises in a glucose-free condition (24). As for the functional selectivity, the field potential was reported to be well maintained with low glucose concentration on the DG compared with the CA1. Li et al explained these selectivity as the differential activity of phosphofructokinase (PFK), the key enzyme for glycolysis. And the DG, indeed, has a high PFK activity than that of the CA1 (10). We found that the DG showed more tolerance to hypoglycemia than the CA1 at 1mM glucose concentration (Fig. 2, Fig.3), indicating the lower dependency of the DG granule neurons on glucose for their survival. Albeit it is a well-known phenomenon, the precise mechanism of the difference of glucose sensitivity between the CA1 and the DG neurons will need more exploration.

The protective effect of insulin during low glucose

The culture medium was adjusted to prepare insulin at 1nM concentration. This concentration corresponds to a blood insulin level following a hypodermic injection of about 27 units of insulin as a conversion to a 50 kg human body (8). This amount is nearly equal to that used in a clinical treatment. There is a report on the experiment in that 4nM insulin successfully worked for the suppression of cell apoptosis in the CNS (23). Our results showed even the smaller dose of insulin could affect the neuronal cell death. In CA1, 3mM glucose with insulin treatment inhibited prominently the neuronal cell death (Fig. 4B). A question arose whether lower than 3mM glucose concentration with insulin might alter the cell death rate. We conducted the experiment at the condition of 1.5mM and 2mM glucose, and obtained an advantageous result for survival of the neuronal cell (data not show). In case of the DG, the insulin treatment did not inhibit the neuronal cell death (Fig. 5B, Fig. 5C). Insulin takes glucose actively in the cell through the GLUT4 translocation to convert ca. 50% of glucose to energy. Furthermore, insulin activates MAP kinase （mitogen-activated protein kinase） working as a cell propagation factor to support the neuronal cell (12). In addition, insulin induces the expression of BDNF (29). A cooperation of these factors takes probably an important role for survival of neurons. In the present study, the prominent inhibition of the neuronal cell death was found only in the CA1. The levels of mRNA of GLUT4 were found to express in higher degree in the CA1 than the DG (2). Therefore, the CA1 neurons will be affected more influence by insulin than the DG neurons, at least on

glucose transport. Moreover, it has been reported that the depression of the insulin signal in the CNS increases GSK activity (Glycogen Synthase Kinase-3), that may lead to induce the neuronal cell death (1). GSK-3 expresses more in the CA1 pyramidal neurons and these preference in the CA1 may a least partially explain the insulin-sensitive selective vulnerability of the CA1 (4).

The acceleration of cell death by insulin during glucose deprivation

It has long been alleged that a possible secondary action of insulin includes affecting an amino acid metabolism and a lipid metabolism to enhance protein synthesis and lipid synthesis resulting in inhibiting the use of a substrate other than glucose by the cell. Thus, the environment lacking an enough amount of glucose may allow insulin to work negatively for cell survival (7). AMPK (AMP-activated protein kinase) that enhances the glucose transportation in a hypoxia tissue, is reduced by insulin treatment in an ischemic heart muscle (3, 5, 9, 19, 28). It is possible that insulin may block the induction of AMPK during glucose deprivation, and thus result in increase of cell death. Interestingly, an in vivo experiment reported that the neuronal protection effect of insulin showed the U-shaped curve, having a maximum peak in 6 to 7mM of the glucose level, and insulin rather accelerated the neuronal cell death at 2 to 3mM or the lower concentration of glucose (30).

CONCLUSION

Insulin therapy is now a common strategy for diabetic treatment, and caution for its therapy has been paid mainly on the treatment related hypoglycemia. Our study indicated that in central nervous system, insulin indeed has double-edged effects, and while neuronal survival is promoted in the presence of the adequate concentrations of glucose, the hazard effect of hypoglycemia may be accelerated by the presence of insulin. The selective vulnerability did not exist in this hypoglycemia-related insulin neuronal toxicity. The further study for this mechanism especially on the molecular cascade may lead to the better clinical management for diabetic care particularly on the prevention of the CNS complication.

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