# β-cell-protective effect of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) as a glutamate dehydrogenase (GDH) activator in db/db mice

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# β-cell-protective effect of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) as a glutamate dehydrogenase (GDH) activator in db/db mice

Directed by Professor Hyun Chul Lee

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Seung Jin Han

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This certifies that the Doctoral Dissertation of Seung Jin Han is approved.

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The Graduate School
Yonsei University

December 2011

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I would like to dedicate this research to God who has endowed me with wisdom and the ability to accomplish everything I have done.

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ABSTRACT

# β-cell-protective effect of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) as a glutamate dehydrogenase (GDH) activator in db/ db mice

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(Directed by Professor Hyun Chul Lee)

2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) is an activator of glutamate dehydrogenase (GDH), which is a mitochondrial enzyme with an important role in insulin secretion. We investigated the effect of BCH on the high glucose (HG)-induced reduction in glucose-stimulated insulin secretion (GSIS), the high glucose/palmitate (HG/PA)-induced reduction in insulin gene expression, and HG/PA-induced β-cell death. We also studied whether long-term treatment with BCH lowers blood glucose and improves β-cell integrity in db/db mice.

We evaluated GSIS, insulin gene expression, and DNA fragmentation in INS-1 cells exposed to HG or HG/PA in the presence or absence of BCH. An in vivo study was performed, in which seven-week-old diabetic db/db mice were treated with BCH (0.7g/kg, n = 10) and with placebo (n = 10) every other day

for 6 weeks. After treatment, an intraperitoneal glucose tolerance test and immunohistologic examinations were performed.

Treatment with BCH blocked HG-induced GSIS inhibition and the HG/PA-induced reduction in insulin gene expression in INS-1 cells. In addition, BCH significantly reduced HG/PA-induced INS-1 cell death and phospho-JNK expression. BCH treatment improved glucose tolerance and insulin secretion in db/db mice. BCH treatment also increased the ratio of insulin-positive β-cells to total islet area ($p < 0.05$) and reduced the percentage of β-cells expressing cleaved caspase 3 ($p < 0.05$). In conclusion, the GDH activator BCH improved glycemic control in db/db mice. This anti-diabetic effect may be associated with improved insulin secretion, preserved islet architecture, and reduced β-cell apoptosis.

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Key words : 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), glucolipotoxicity, beta-cell, glutamate dehydrogenase

# β-cell-protective effect of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) as a glutamate dehydrogenase (GDH) activator in db/ db mice

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## I. INTRODUCTION

Pancreatic β-cells produce the hormone insulin, whose action on target tissues maintains glucose homeostasis. Mitochondria play a pivotal role by generating signals that couple glucose sensing to insulin secretion[1, 2]. The mitochondrial matrix enzyme glutamate dehydrogenase (GDH), which catalyzes the interconversion of glutamate and the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate, is an important enzyme in the regulation of insulin secretion in pancreatic β-cells[3].The function of GDH as the regulator of insulin secretion was established through studies of the insulin-releasing capacity of

2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH)[4, 5]. These studies showed that BCH increased insulin secretion by activating GDH and that the insulinotropic action of BCH correlated with its ability to increase the oxidative deamination of glutamate. The discovery that gain-of-function mutations in the gene encoding GDH cause hyperinsulinism/hyperammonemia syndrome underlined the importance of GDH in glucose homeostasis in humans [6-8].

The activation of GDH by allosteric activators such as leucine and BCH as well as the overexpression of constitutively activated GDH induce insulin secretion through enhanced glutamate oxidation [5, 9-12]. It is therefore generally accepted that the activation of GDH enhances oxidative deamination of glutamate and increases ATP production by supplying mitochondria with α-ketoglutarate, thereby stimulating insulin secretion [4, 5, 11, 13].

Based on the results of previous studies, glucose concentration appears to be altered in GDH-overexpressing islets in a way that increases glucose-stimulated insulin secretion (GSIS). [11, 12]. One study reported that at low glucose concentration, GDH overexpression in islets potentiated insulin secretion [11]. However, another study showed that this was potentiated at high glucose concentration [12]. First- and second-phase insulin secretion was impaired in β-cell specific GDH deletion mice, indicating that GHD-dependent amplification of the secretory response may affect both phases [14]. It was noted in a recent study that by ADP-ribosylating GDH, SIRT4 repressed the activity

of GDH, thereby downregulating insulin secretion in pancreatic β-cells [15].

Recently, we demonstrated that metabolic impairment in mitochondria is associated with β-cell glucolipotoxicity[16]. According to these results, long-term treatment of INS-1 cells with high glucose/palmitate (HG/PA) impaired energy-producing metabolism and caused depletion of TCA cycle intermediates. Treatment with BCH not only maintained the TCA cycle intermediate pool but also had a strong protective effect against HG/PA-induced β-cell death. Therefore, we initially examined whether BCH improved β-cell glucolipotoxicity, as measured by HG-induced reduction in glucose-stimulated insulin secretion (GSIS), HG/PA-induced reduction in insulin gene expression, and HG/PA-induced β-cell death. Next, we investigated which death-related signal is affected by BCH. We also studied the effects of BCH on glycemic control and pancreatic β-cell integrity in db/db mice.

## II. MATERIALS AND METHODS

### 1. Cells

INS-1 rat insulinoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 g/mL streptomycin, and 10 mM HEPES, at 37℃ in a humidified atmosphere of 95% air and 5% $CO_2$.

### 2. Preparation of palmitate

Palmitate/BSA conjugates were prepared as described previously[16]. Briefly, a 20 mM solution of palmitate in 0.01 M NaOH was incubated at 70 ℃ for 30 min and the fatty acid soaps were then complexed with 5% fatty acid-free BSA in phosphate-buffered saline (PBS) in 1:3 volume ratio. The complexed fatty acids consisted of 5 mM palmitate and 3.75% BSA. The palmitate/BSA conjugates were diluted in 10% FBS culture medium (approximately 0.4% BSA) and administered to cultured cells.

### 3. Measurement of insulin secretion and content

INS-1 cells ($2 \times 10^5$) growing in 24-well plates were washed twice with KRB buffer (24 mM $NaHCO_2$, 1.2 mM $MgCl_2$, 1 mM HEPES, 129 mM NaCl, 4.8 mM KCl, 1.2 mM $KH_2PO_4$, 2.5 mM $CaCl_2$, 0.2% BSA, and 0.2 mM glucose, pH 7.4) and then incubated in the same buffer for 1 h. Insulin secretion was

stimulated by treatment with 0.2 mM or 16.7 mM glucose for 2 h. At the end of the incubation period, the amount of insulin released into the supernatant was quantified using a rat insulin radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO).

To determine insulin content, the cells were washed twice with KRB buffer and incubated overnight in an ethanol/HCl buffer at 4 ℃. At the end of the incubation period, the supernatant was collected and subjected to rat insulin RIA.

4. Apoptosis ELISA assay

Cells seeded in 96-well plates were grown in RPMI 1640 containing 11.1 mM glucose until they reached 70% confluence. They were then treated with 25 mM glucose (HG) and 0.4 mM palmitate (PA) in the presence or absence of BCH for 24 h. The cells were lysed, and cytosolic levels of oligonucleosomes, produced as a result of apoptosis-related DNA degradation, were quantified using a Cell Death Detection ELISA Plus kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

5. Quantitative real- time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA

synthesis was performed using 1 μg of total RNA and an avian myeloblastosis virus reverse transcription system. PCR was performed with a SYBR Green PCR Master Mix (PE Applied Biosystems, www.appliedbiosciences.com) according to the manufacturer's instructions. The following primers were used: insulin1, CTGGTGGAGGCTCTGTACCT (forward) and GTGCAGCACT GATCCACAAT (reverse); and GAPDH, ATGATTCTACCCACGGCAAG (forward) and CTGGAAGATGGTGATGGGTT (reverse). All amplification was performed using the following thermal cycling conditions: 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s. The real-time PCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System (PE Applied Biosystems). Expression data were normalized to the values for the control gene GAPDH to yield the relative abundance.

6. Immunoblotting

Whole proteins were extracted by differential centrifugation (10,000x g, 10 min) and protein concentrations in lysates were determined using protein assay kits (Bio-Rad, Hercules, CA). An equal volume of 2 x SDS sample buffer [125 mM Tris.Cl, (pH 6.8), 4% SDS, 4% 2-mercaptoethanol, 20% glycerol] was added to cell lysates, and equivalent amounts of protein (30 μg) were loaded onto 10–15% polyacrylamide gels, electrophoresed, and then

electrophoretically transferred onto Polyvinylidene Fluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking the membranes with 5% skimmed milk for 30 min, target antigens were reacted with anti-phospho-JNK (Thr183/Tyr185) (Cell Signaling, Beverly, MA) or anti-total JNK or anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as primary antibody and then with the appropriate secondary antibody: horseradish peroxidase-conjugated anti-rabbit immunoglobulin G for anti-total JNK and anti-phospho-JNK antibodies, or anti-goat immunoglobulin G for anti-β-actin antibody. Immunoreactive bands were then developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Arlington Height, IL). Band intensity was determined by densitometric analysis using a one-dimensional Quantity One® 1D image analysis system.

7. Animals and treatments

Five-week-old male diabetic db/db (C57BLKS/J-leprdb/leprdb) mice and male non-diabetic db/+ (C57BLKS/J-leprdb/+) mice were purchased from Japan SLC (Shizuoka, Japan). The animals were housed three per cage in a temperature-controlled room (22℃) under a 12-h/12-h light/dark cycle with lights on at 6 a.m. The animals were given free access to a standard chow diet and water. Following 2 weeks of adaptation, the mice were randomly divided into three groups ($n$ = 10/group): saline-injected non-diabetic db/+ mice,

saline-injected diabetic db/db mice (db/db control mice), and BCH-injected diabetic db/db mice. Prior to use, BCH (Sigma, St. Louis, MO) was dissolved in saline to yield a 10 mM stock solution. BCH (0.7 g/kg) was injected intraperitoneally every other day for 6 weeks. All institutional guidelines for the care and use of animals were followed.

8. Intraperitoneal Glucose Tolerance Test (IPGTT)

After 6 weeks of treatment, the mice were fasted for 16 h prior to the IPGTT, to allow complete drug washout. Glucose (2 g/kg) was administered via injection into the peritoneal cavity**.** Blood samples were collected from tails using heparinized calibrated micropipettes at 0, 60, and 180 min after glucose loading. Blood was immediately centrifuged at 1,000 rpm for 10 min at 4℃, and the plasma was collected and stored at -80℃ until assayed. Plasma glucose levels were measured using the glucose oxidase method, and plasma insulin levels were determined using an RIA kit (Linco Research).

9. Histology, immunohistochemistry, and immunofluorescence

At 14 weeks of age, the mice were killed. The pancreas of each mouse was removed, fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned to a thickness of 3 μm. Pancreatic sections were stained first with hematoxylin and eosin (H&E), and then for insulin (with a guinea pig

anti-insulin antibody; Dako Corp., Carpinteria, CA, USA) and glucagon (rabbit anti-glucagon antibody; Cell Signaling Technology). Additional sections were co-stained with fluorescently labeled antibodies specific for insulin (Dako Corp.) and cleaved caspase 3 (Cell Signaling Technology). Fluorescence signals were visualized using a confocal microscope (Carl Zeiss MicroImaging GmbH), with excitation at 448 nm and emission at 515 nm. Overall numbers of cleaved caspase 3-positive β-cells in each stained section were counted at ×400 magnification. The frequency of cleaved caspase 3-positive β-cells was determined as a percentage of the total number of β-cells in each tissue section. Histological images were analyzed using ImageJ software (NIH Image, USA).

10. Statistical analyses

All data are expressed as means ± standard error (SE). Statistically significant differences were identified using Student's *t*-test (for assays with two sample sets) and one-way analysis of variance (ANOVA) in conjunction with the Tukey correction (for multiple experimental groups). Statistical analyses were performed using SPSS software ver. 13.0 (SPSS Inc., Chicago, IL, USA). A $p$ value of $<0.05$ was deemed to indicate statistical significance.

## III. RESULTS

1. Potentiating effect of BCH on insulin secretion in INS-1 cells

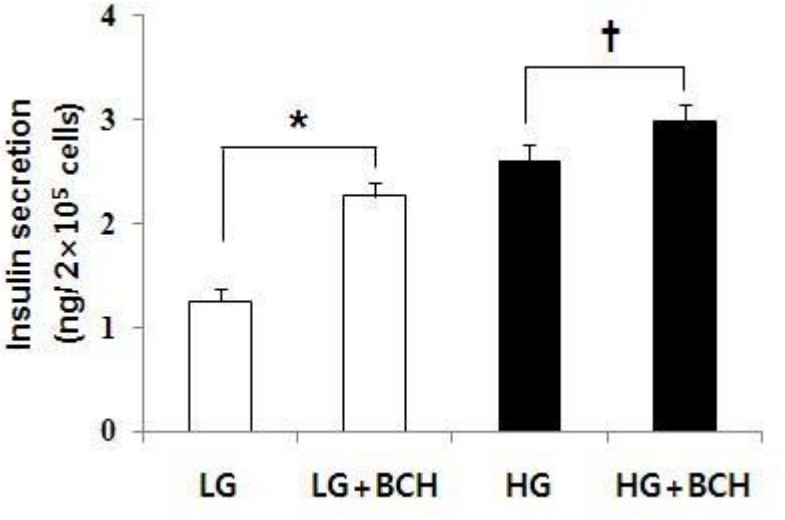
Insulin secretion by INS-1 cells was stimulated by treatment with 0.2 mM glucose (LG) or 16.7 mM glucose (HG) for 2 h, in conjunction with 10 mM BCH. As shown in Fig. 1A, HG increased insulin secretion about 2.0-fold compared with LG. BCH augmented LG- and HG-stimulated insulin secretion approximately 1.8- and 1.2-fold, respectively. Thus, BCH potentiated insulin secretion stimulated by LG and HG in INS-1 cells.

2. Preventive Effect of BCH on HG-induced GSIS inhibition and HG-induced reduction of insulin content in INS-1 cells

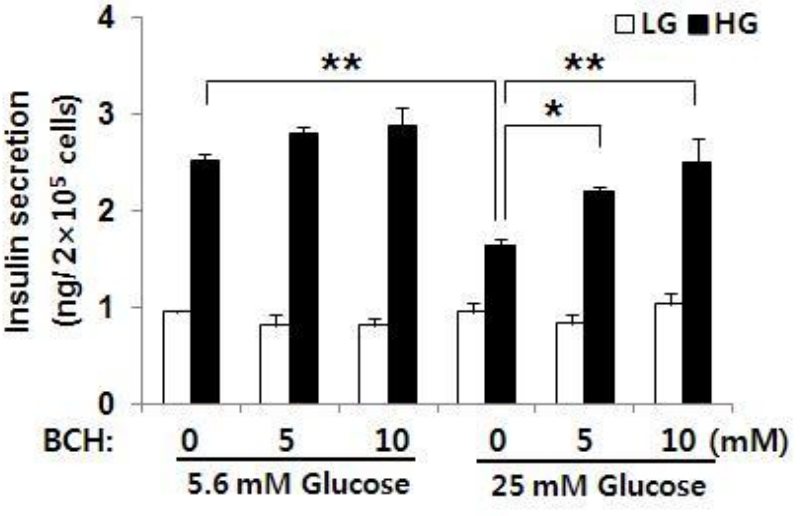
To determine whether treatment with BCH can prevent HG-induced GSIS inhibition in β-cells, INS-1 cells were incubated with BCH in medium containing 5.6 mM or 25 mM glucose for 24 h, and the amount of insulin secreted in response to glucose stimulation (LG, 0.2 mM or HG, 16.7 mM) for 2 h was measured by RIA. As shown in Fig. 1B, GSIS in INS-1 cells was impaired by long-term exposure to HG, whereas treatment with BCH significantly prevented HG-induced GSIS inhibition. Treatment with BCH did not affect LG-induced insulin secretion. In addition, while long-term incubation with HG significantly reduced insulin content, treatment with BCH significantly

restored HG-induced reduction of insulin content in a concentration-dependent manner (Fig. 1C)

A.




B.



C.

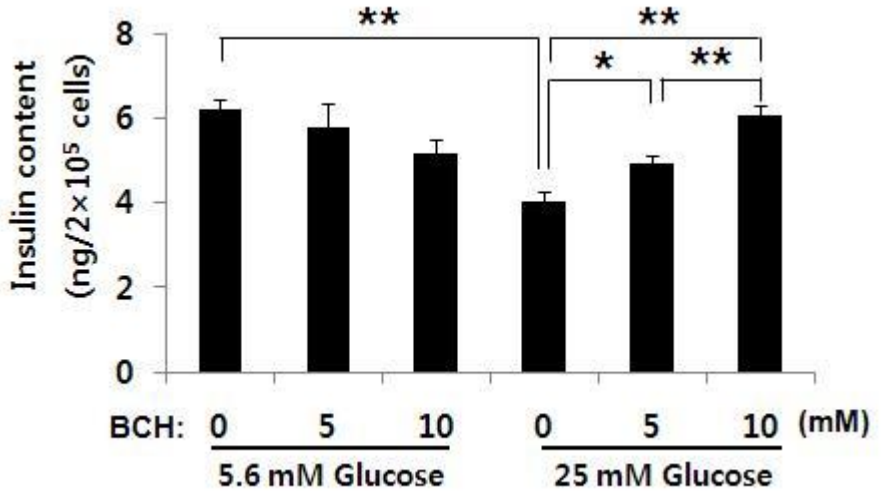



**Figure 1.** Protective effects of BCH on HG-induced GSIS inhibition and HG-induced reduction of insulin content in INS-1 cells.

(A) Potentiation of GSIS by BCH in INS-1 cells.

Insulin secretion by INS-1 cells was stimulated by treatment with 10 mM BCH for 2 h in the presence of 0.2 mM glucose (low glucose; LG) or 16.7 mM glucose (high glucose; HG). The insulin released into the medium was quantified by insulin RIA. Data are expressed as means ± SE of three independent experiments. *$p < 0.05$ vs. LG; †$p < 0.05$ vs. HG.

(B) Preventive effect of BCH on HG-induced GSIS inhibition in INS-1 cells.

INS-1 cells were treated with 5mM or 25 mM glucose for 24 h in the absence or presence of 5 mM or 10 mM BCH. The cells were incubated in KRB buffer for

1 h and then stimulated with 0.2 mM glucose (LG) or 16.7 mM glucose (HG) for 2 h. The insulin released into the medium was quantified by insulin RIA. Data are expressed as means ± SE of three independent experiments. *$p < 0.05$, **$p < 0.01$.

(C) Preventive effect of BCH on HG-induced insulin content decrease in INS-1 cells.

INS-1 cells were treated with 5mM or 25 mM glucose for 24 h in the absence or presence of 5 mM or 10 mM BCH. The cells were washed twice with KRB buffer and incubated overnight in an ethanol/HCl buffer at 4 ℃. At the end of the incubation period, the supernatant was collected and subjected to rat insulin RIA. Data are expressed as means ± SE of three independent experiments. *$p < 0.05$, **$p < 0.01$.

3. Protective effect of BCH on the HG/PA-induced reduction in insulin gene expression in INS-1 cells

The exposure of INS-1 cells to 25 mM glucose and 0.4 mM palmitate (HG/PA) for 12 h reduced insulin gene expression by 60% compared with BSA control treatment (Fig. 2). Co-treatment with BCH blocked the inhibitory effect of HG/PA on insulin gene expression in a concentration-dependent manner.

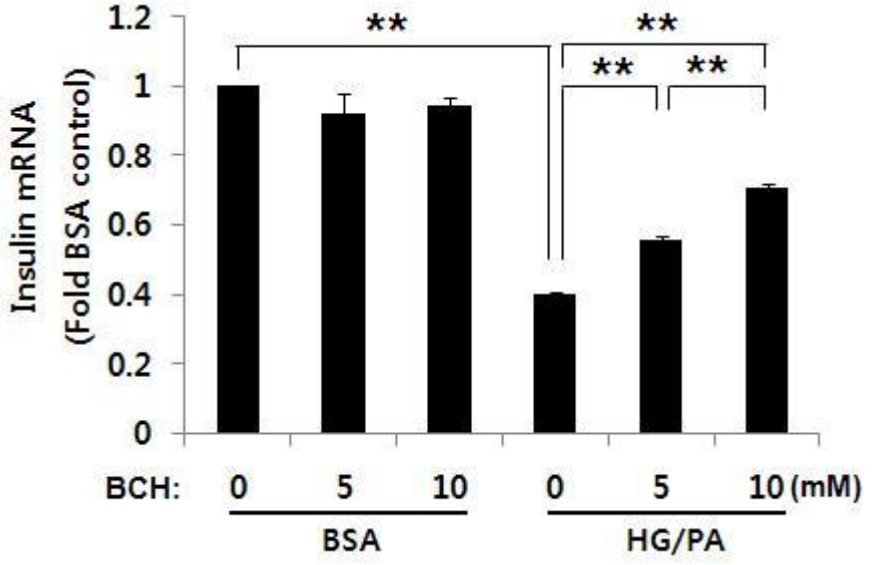



**Figure 2.** Protective effects of BCH against HG/PA-induced downregulation of insulin gene expression in INS-1 cells.

INS-1 cells were treated with 25 mM glucose and 0.4 mM palmitate (HG/PA) in the presence or absence of BCH for 12 h. Insulin mRNA levels were quantified

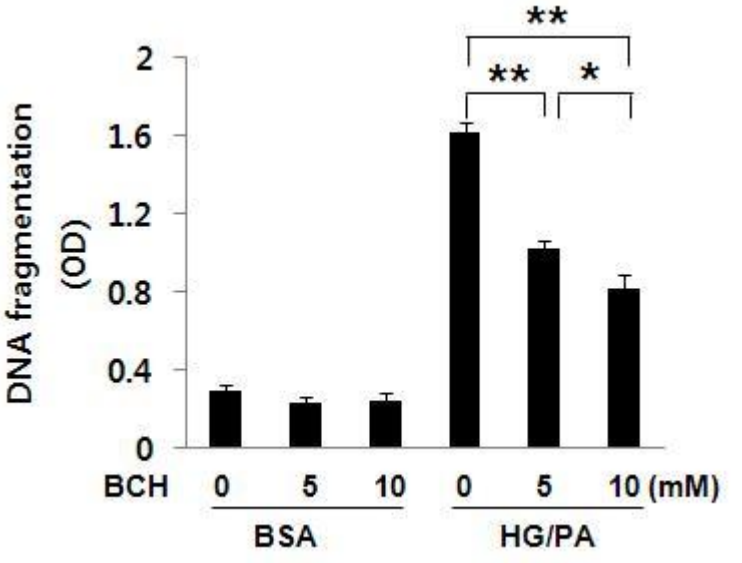
by real-time RT-PCR and normalized to the GAPDH expression level. Relative quantification was used to calculate the change in insulin mRNA expression, expressed as fold change relative to BSA control-treated INS-1 cells.

Data are expressed as means ± SE of three independent experiments. $^{**}p < 0.01$.

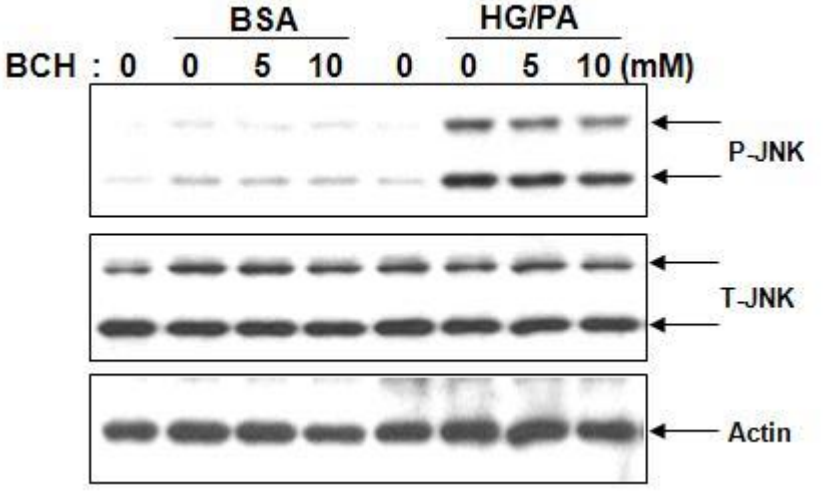
4. Protective effect of BCH against HG/PA-induced INS-1 cell death

A cell death detection assay revealed fragmented DNA in INS-1 cells exposed to HG/PA for 24 h (Fig. 3A). HG/PA increased cytosolic DNA fragmentation from 0.3 to 1.6, indicating that it induced apoptotic cell death in INS-1 cells. BCH protected against HG/PA-induced DNA fragmentation in a concentration-dependent manner. As JNK is known to be a critical mediator in HG/PA-induced β-cell apoptosis, we investigated whether JNK activation was associated with the protective effect of BCH. Treatment with BCH attenuated HG/PA-induced phospho-JNK expression in a concentration-dependent manner (Fig 3B, C).

**A.**


DNA fragmentation
(OD)
2
1.6
1.2
0.8
0.4
0
**
**
*
BCH 0 5 10 0 5 10 (mM)
BSA
HG/PA

**B.**


BSA
HG/PA
BCH : 0 0 5 10 0 0 5 10 (mM)
P-JNK
T-JNK
Actin

**C.**

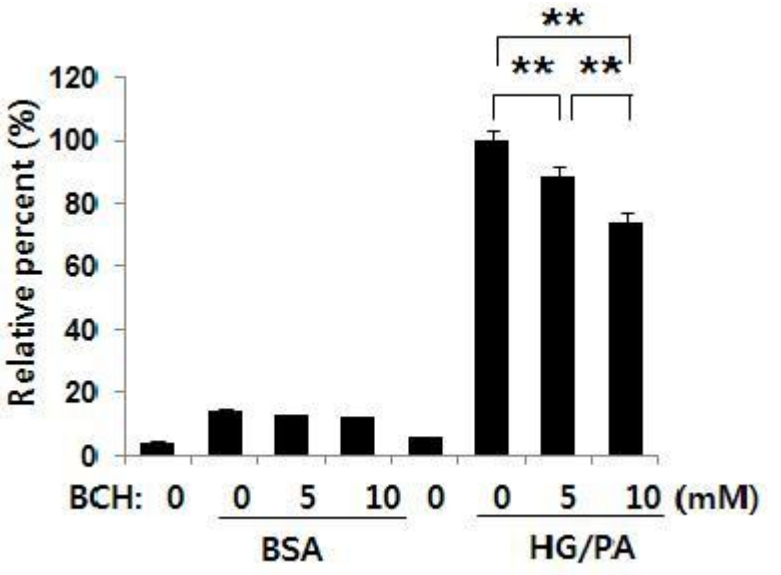



**Figure 3.** Protective effect of BCH against HG/PA-induced INS-1 cell apoptosis.

(A) Protective effect of BCH against HG/PA-induced DNA fragmentation. INS-1 cells were treated with 25 mM glucose and 0.4 mM palmitate (HG/PA) in the presence or absence of BCH for 24 h. The level of DNA fragmentation was measured using a Cell Death Detection ELISA. Data are expressed as means ± SE of four independent experiments. *$p < 0.05$, **$p < 0.01$ vs. HG/PA-treated INS-1 cells.

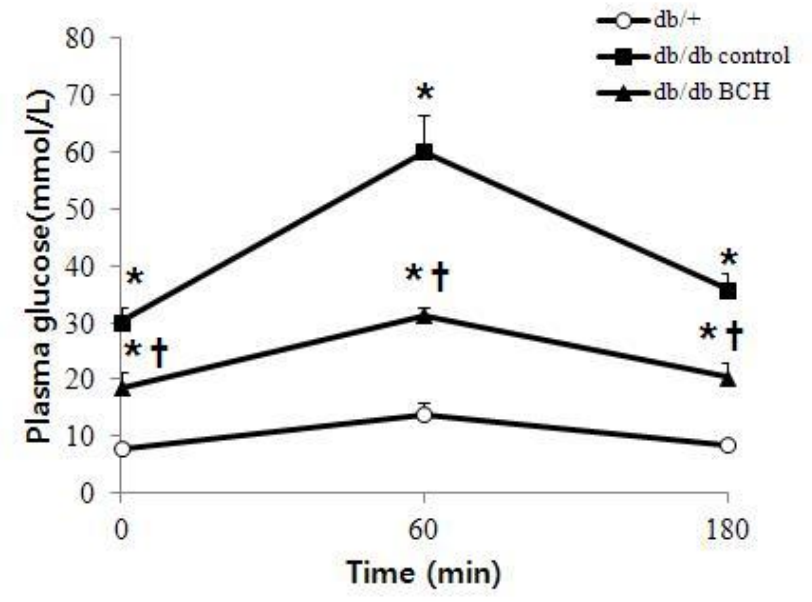
(B) Preventive effect of BCH on JNK activation during HG/PA-induced INS-1 cell apoptosis. INS-1 cells were treated with 25 mM glucose and 0.4 mM

palmitate (HG/PA) in the presence or absence of BCH for 12 h. Phospho-JNK levels were analyzed by immunoblotting using an anti-phospho-JNK antibody. (C) Quantitative analysis of phospho-JNK expression was performed using a densitometer. The histogram shows the means ± SE for densitometric scans of protein bands from four independent experiments, normalized by comparison with actin and expressed as a percentage of HG/PA control treated INS-1 cells (set to 100%). $^{**}p < 0.01$.

5. Effect of BCH treatment on body weight, plasma glucose level, and plasma insulin level during IPGTT

After 6 weeks of treatment, body weight was significantly higher in db/db control mice (43.6 ± 2.5 g) than in non-diabetic db/+ mice (37.2 ± 0.8 g). However, there was no difference in body weight between db/db control mice and db/db mice treated with BCH (44.5 ± 2.2 g). As shown in Fig. 4A, db/db control mice exhibited marked glucose intolerance, as confirmed by the results of an IPGTT. Interestingly, BCH treatment significantly improved glucose tolerance and insulin secretion (Fig. 4A, B).

**A.**


db/+
db/db control
db/db BCH
Plasma glucose(mmol/L)
80
70
60
50
40
30
20
10
0
0
60
180
Time (min)
*
*†

**B.**

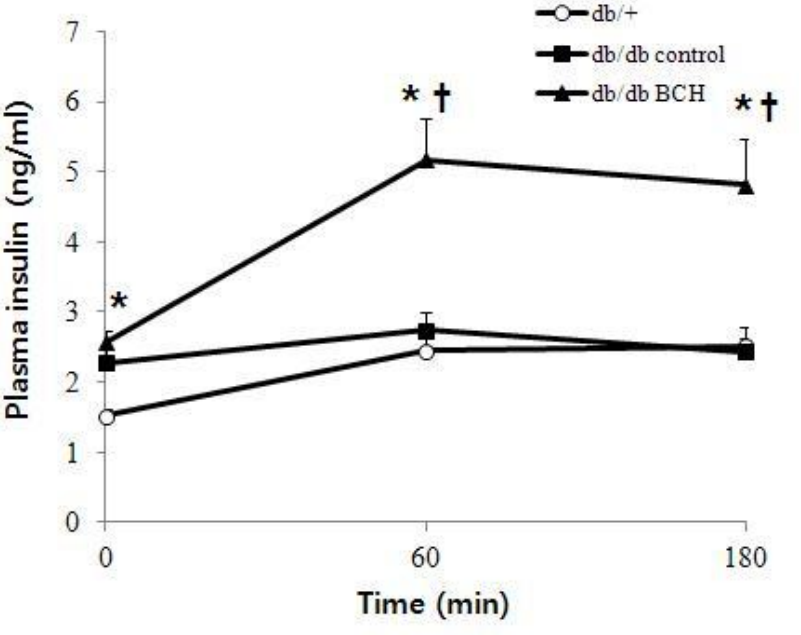

db/+
db/db control
db/db BCH
Plasma insulin (ng/ml)
7
6
5
4
3
2
1
0
0
60
180
Time (min)
*
*†

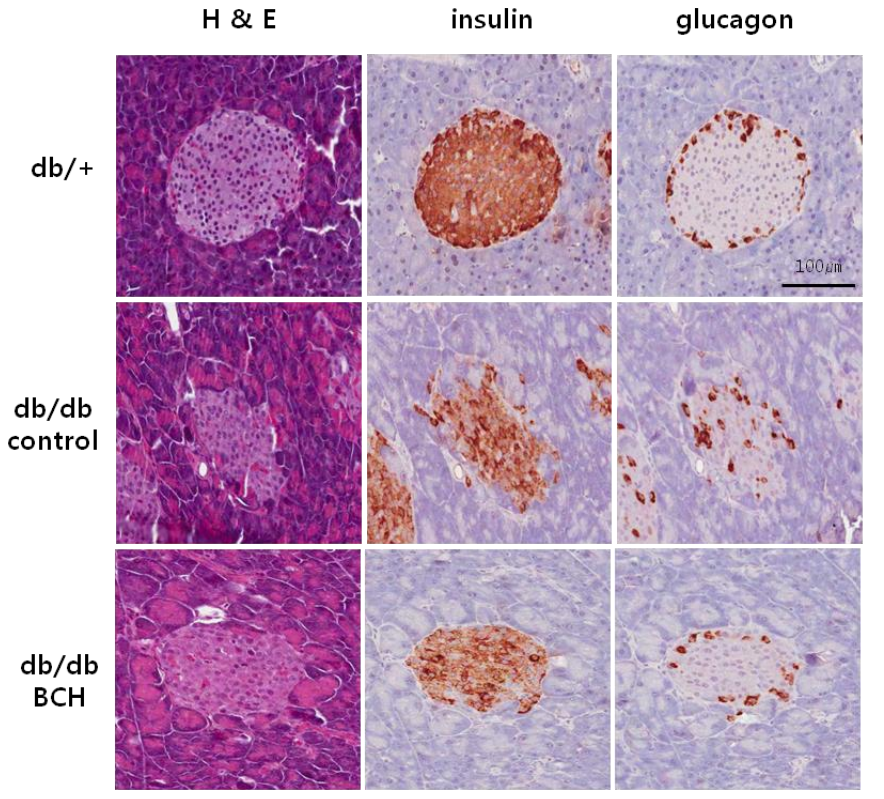
**Figure 4.** Effect of BCH on plasma levels of glucose (A) and insulin (B) during an intraperitoneal glucose tolerance test (IPGTT). Data are presented as means ± SE ($n$ = 10 per group). *$p$ < 0.05 vs. db/+ mice; †$p$ < 0.05 vs. db/db control mice.

6. Effect of BCH treatment on islet morphology

Pancreatic tissue from db/db control mice showed islet destruction and decreased β-cell insulin staining compared with db/+ mice (Fig. 5A). In addition, islets from db/db control mice contained glucagon-positive α-cells, which had infiltrated the entire islet. Tissue from BCH-treated db/db mice displayed relatively normal islet morphology, significantly reduced numbers of α-cells in the islet core, and a more normal distribution of β-cells and α-cells. The ratios of insulin-positive β-cells to total islet area and glucagon-positive α-cells to total islet area were calculated from acquired digital images. As shown in Fig. 5B, the insulin-positive β-cells accounted for 79.2 ± 1.2% of the total islet area in nondiabetic db/+ mice, but only 42.2 ± 1.7% in db/db control mice ($p$ < 0.05). Treatment with BCH significantly increased the insulin-positive β-cell-to-total islet area ratio (56.0 ± 1.2%, $p$ < 0.05). On the other hand, the glucagon-positive α-cell-to-total islet area ratio was higher in db/db control mice than in nondiabetic db/+ mice (16.6 ± 2.2% vs. 10.3 ± 0.8%, $p$ < 0.05; Fig. 5C). Treatment with BCH significantly reduced the glucagon-positive α-cell-to-total

islet area ratio (to 11.2 ± 0.5%).

**A.**



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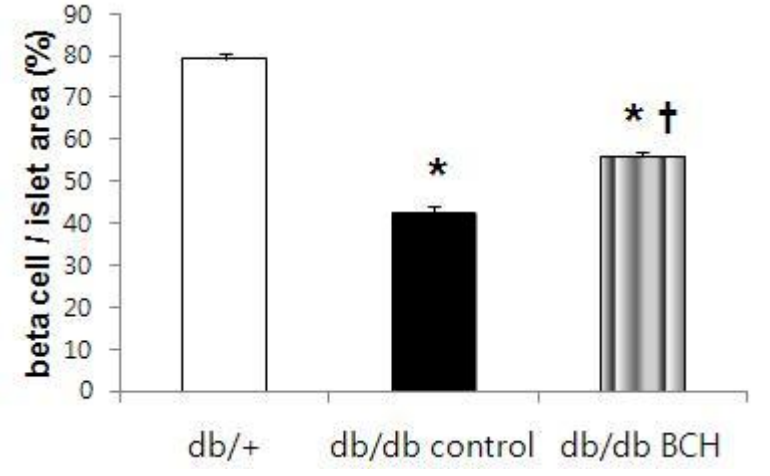



**C.**

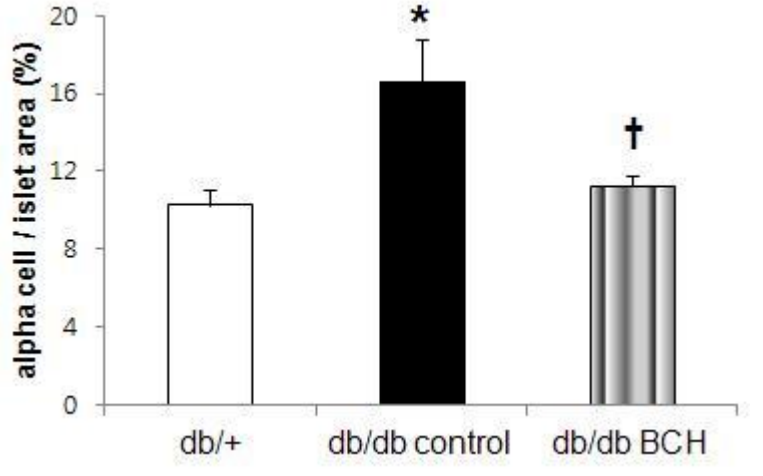



**Figure 5.** Immunohistochemical analysis of islets

(A) Typical islets from db/+ mice treated with saline, db/db mice treated with saline (db/db control), and db/db mice treated with BCH (db/db BCH) were

stained with hematoxylin and eosin (H&E), and with anti-insulin antibody and anti-glucagon antibodies.

(B) Ratio of insulin-positive β-cell area to total islet area. Digital images of immunostained pancreas sections (such as those shown in A) were used to calculate the insulin-positive β-cell area as a percentage of the total islet area. *$p < 0.05$ vs. db/+ mice; †$p < 0.05$ vs. db/db control mice.

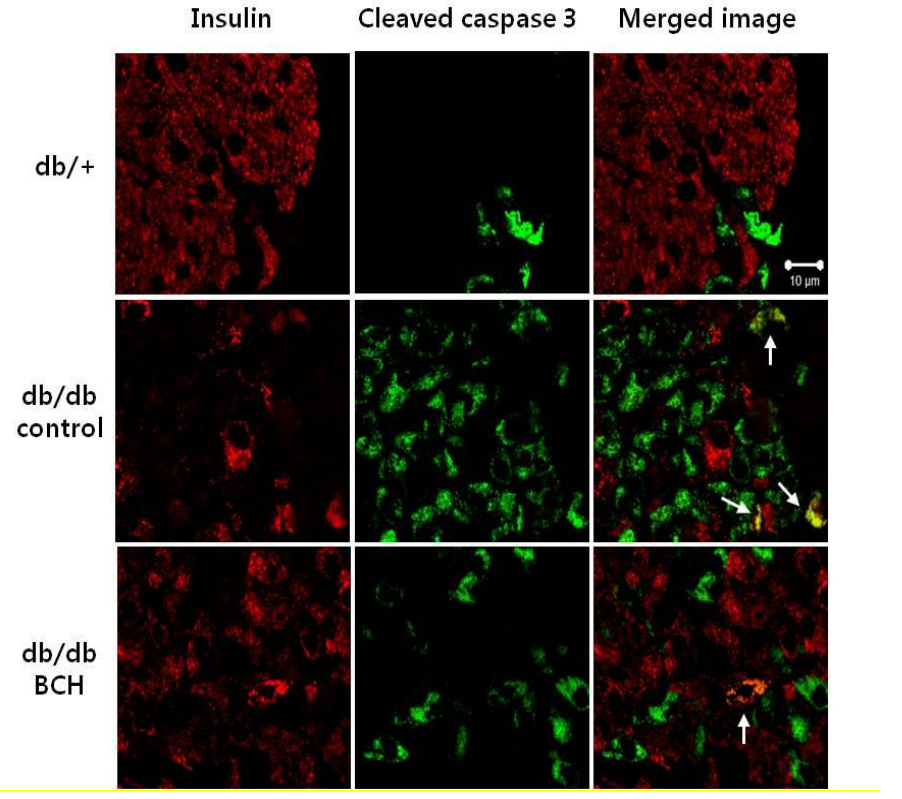
(C) Glucagon-positive α-cell area as a percentage of the total islet area.

*$p < 0.05$ vs. db/+ mice; †$p < 0.05$ vs. db/db control mice.

7. Effect of BCH treatment on cleaved capase 3 expression in β-cells

We performed immunofluorescence analyses to determine whether the expression of cleaved caspase 3 in β-cells was altered in animals treated with BCH. The expression of cleaved caspase-3 was significantly lower in BCH-treated db/db mice compared with db/db control mice (5.4 ± 0.9 vs. 15.6 ± 2.8, $p < 0.05$; Fig. 6A, B)

**A.**



**B.**

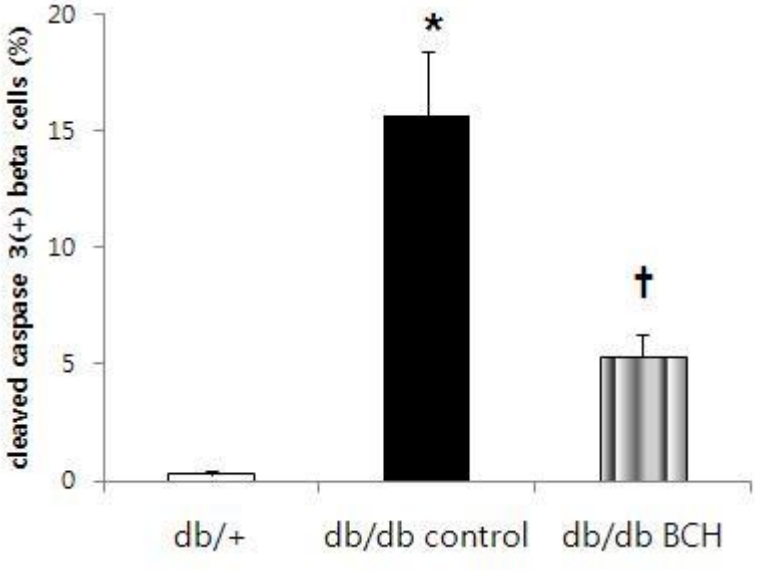



**Figure 6.** Effect of BCH on β-cell expression of cleaved caspase 3.

(A) Effect of BCH on β-cell expression of cleaved caspase 3, as assessed by immunofluorescence. Representative images of islets stained for insulin (red) and cleaved caspase 3 (green) are shown.

(B) Cleaved caspase 3-positive β-cell frequencies. Tissue sections from saline-treated db/+ mice and db/db mice treated with saline (db/db control) or BCH (db/db BCH) were analyzed. Data are expressed as means ± SE ($n$ = 6 per group). *$p < 0.05$ vs. db/+ mice; †$p < 0.05$ vs. db/db control mice.

## IV. DISCUSSION

We have known for a while that BCH induces hypoglycemia and stimulates insulin release[17-19]. Later, it was shown that the sole mechanism of action of BCH is the activation of GDH[4, 5]. Until now, most studies on BCH as a GDH activator have focused on its function in insulin secretion, and not any other functions in β-cells.

In the present study, we found that BCH reversed HG-induced GSIS inhibition and protected against HG/PA-induced reduction of insulin gene expression as well as HG/PA-induced β-cell cytotoxicity *in vitro*. Furthermore, in accord with its protective effect against HG/PA-induced glucolipotoxicity, BCH preserved β-cell mass and improved glycemic control in db/db mice. To our knowledge, this is the first report demonstrating the protective effects of BCH on β-cell function and islet architecture in a type 2 diabetes animal model.

Chronic exposure to elevated levels of glucose and fatty acids is thought to contribute to the progression of type 2 diabetes by impairing β-cell function and inducing β-cell apoptosis, effects that are collectively referred to as β-cell glucolipotoxicity[20-22]. Mitochondria mediate the response of β-cells to extracellular glucose by generating ATP and initiating a cascade of events culminating in the release of insulin. However, β-cells chronically exposed to high levels glucose and free fatty acids showed reductions in mitochondrial membrane potential and ATP production[21]. Furthermore, decreased levels of

various mitochondrial enzymes in islet cells under hyperglycemic and hyperlipidemic conditions support the theory that mitochondrial dysfunction contributes to β-cell glucolipotoxicity [23, 24].

We hypothesized that the anti-diabetic effect of BCH results from improved mitochondrial function owing to the activation of GDH. The first contributor to this effect of BCH is the stimulation of insulin secretion. Islets acutely exposed to high glucose levels showed an increase in insulin release, whereas a decrease in insulin release was observed in islets exposed chronically to high glucose[25, 26]. Our results showed that acute treatment with BCH potentiated insulin secretion and that long-term treatment blocked HG-induced GSIS inhibition in INS-1 cells, and increased insulin secretion in db/db mice. These results are in accord with previous studies of the effects of BCH on insulin secretion [4, 5, 9]. Liu et al. reported that increased anaplerotic flux caused by BCH is an important metabolic process underlying second-phase insulin secretion[27]. On the other hand, prolonged exposure to fatty acids impairs insulin gene expression in the presence of high glucose[28, 29]. BCH also restored suppressed insulin gene expression under glucolipotoxic conditions in INS-1 cells. Based on these results, we concluded that BCH treatment may improve β-cell function.

The second mechanism of the anti-diabetic effect of BCH is protection against HG/PA-induced β-cell apoptosis. In our previous study, when the HG/PA-induced reduction in viability began to be detected, TCA cycle

intermediate levels in HG/PA-treated INS-1 cells were very low[16]. In addition, the rates of glucose and palmitate oxidation and the level of ATP were reduced in HG/PA-treated cells. Treatment with BCH increased the levels of TCA cycle intermediates, restored ATP levels, enhanced the oxidation rate, and protected against HG/PA-induced INS-1 cell apoptosis.

The JNK pathway is known to be a critical mediator of fatty acid-induced endoplasmic reticulum (ER) stress[30]. Bachar et al. showed that glucose increased JNK phosphorylation in response to palmitate-induced ER stress and that JNK inhibition reduced β-cell apoptosis, underlining the importance of this pathway in mediating glucolipotoxicity-induced β-cell apoptosis[31]. In the present study, BCH suppressed HG/PA-induced JNK activation in INS-1 cells. We also confirmed the protective effects of BCH against β-cell apoptosis in db/db mice. BCH protected against loss of β-cell mass and preserved the architecture of pancreatic islets. Another mechanism may be the reduction in β-cell overwork due to insulin resistance, as BCH treatment improved insulin sensitivity in insulin tolerance tests in db/db mice (data not shown). We cannot therefore exclude the possibility that the anti-diabetic effect of BCH was attributable to enhanced glycemic control and that BCH thus indirectly reduced glucolipotoxicity.

Finally, we suggest that the activation of GDH by BCH may enhance mitochondrial energy metabolism by increasing anaplerotic flux, thereby

improving β-cell function and preventing β-cell apoptosis.

In a recent study, transgenic mice with β-cell-specific deletion of GDH exhibited a 60% reduction in GDH activity, an increased percentage of disorganized islets, and a partial reduction in glucose-stimulated insulin secretion[14]. These results are consistent with our findings that GDH is important in insulin secretion and islet integrity. However, when they were fed a normal-calorie diet, these transgenic mice did not demonstrate a difference in IPGTT glucose excursion compared with control mice. Because of this, the authors suggested that although insulin secretion was strongly limited, maximal GDH capacity is not required to maintain glucose homeostasis under normal-calorie conditions. Thus, we believe that if these transgenic mice with β-cell-specific deletion of GDH were fed other high-nutrient diets such as high-glucose or high-free fatty acid diets, they would develop glucose intolerance.

Treatment with BCH was not lethal and did not affect body weight, weights of major organs, food intake, or physical appearance (Supplementary Table 1). We did not detect any abnormalities upon autopsy of BCH-treated db/db mice. Moreover, treatment with BCH did not cause liver toxicity, as assessed by serum AST and ALT levels (Supplementary Table 1). These findings suggest that the dose of BCH used did not cause any toxicity in db/db mice.

The dose of BCH used in the present study and other study may be too high

for therapeutic usefulness in the treatment of type 2 diabetes[17]. A new chemical that can effectively mimic BCH at lower dosages (i.e., μmol amounts) is needed. In addition, the effect of BCH can be influenced by the supply of glutamate.

## V. CONCLUSION

In summary, we demonstrated that treatment with the GDH activator BCH improved glucose tolerance in db/db mice, a type 2 diabetes animal model. Improved glycemic control was associated with improved insulin secretion, increased insulin-positive β-cell area as a percentage of total islet area, restoration of normal islet architecture, and reduced β-cell apoptosis. The results suggest that pharmacological activation of GDH may represent a novel therapy for type 2 diabetes.

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## APPENDICES

Supplementary Table 1.

Effect of BCH on food intake, weights of major organs, and serum AST and ALT levels in db/db mice

| Parameter | db/db control | db/db BCH |
|---|---|---|
| Food intake (g/mouse/day) | 5.8 ± 0.5 | 5.0 ± 0.5 |
| Organs weight (g) | | |
| Liver | 2.5 ± 0.3 | 2.4 ± 0.2 |
| Heart | 0.13 ± 0.02 | 0.16 ± 0.02 |
| Kidney | 0.22 ± 0.01 | 0.23 ± 0.02 |
| Serum AST (U/L) | 259 ± 48 | 137 ± 15 |
| Serum ALT (U/L) | 48 ± 5 | 37 ± 6 |

Data are presented as means ± SE.

ABSTRACT(IN KOREAN)

# db/ db 마우스에서 glutamate dehydrogenase (GDH) 활성제인 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH)의 베타세포 보호효과

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한 승 진

2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH)는 인슐린 분비능 조절에 중요한 미토콘드리아 효소인 glutamate dehydrogenase (GDH)의 활성제이다. 본 연구에서는 BCH가 고혈당에 의한 포도당 자극 인슐린 분비능의 저하에 미치는 효과와 고혈당과 지방산에 의해 인슐린 유전자 발현 억제 및 베타세포 사멸에 미치는 효과를 살펴보고자 하였다. 또한 제 2형 당뇨병 모델인 db/db 마우스에 BCH 장기투여시 혈당 강하 효과와 베타세포의 integrity에 미치는 효과를 분석하고자 하였다.

고혈당에 의한 포도당 자극 인슐린 분비능과 고혈당과 지방산에 의한 인슐린 유전자 발현과 DNA fragmentation에 BCH 투여효과를 확인하기 위해 INS-1 세포주를 사용하였다. 생체 내 실험을 위해서는 7주령의 db/db 마우스 10마리씩 두 군으로 나누어서 한 군에는 0.7g/kg의 BCH를, 다른 군에는 위약을 격일로 6주간 투여하였다. 투여를 마친 후 복강내 당부하 검사와 췌장 조직의 면역염색을 시행하였다.

BCH 투여는 혈당에 의한 포도당 자극 인슐린 분비능의 저하와

고혈당과 지방산에 의한 인슐린 유전자 발현 감소를 막았다. 또한 BCH는 고혈당과 지방산으로 유도된 INS-1 세포의 사멸과 JNK 인산화를 감소시키는 효과를 나타내었다.

BCH를 db/db 마우스에서 투여하였을 때 당부하 검사상 혈당이 감소하였고 인슐린 분비가 증가되었다. 췌장의 면역조직염색 결과를 분석한 결과 BCH 투여시 췌도 내 인슐린 염색되는 베타세포의 상대적 면적이 증가된 반면 글루카곤이 염색되는 알파세포의 상대적 면적은 감소되었다. 또한 베타세포 사멸을 나타내는 cleaved caspase 3 발현되는 베타세포의 백분율을 감소되었다.

결론적으로 GDH 활성제인 BCH는 db/db 마우스에서 항당뇨 효과를 나타내었다. 이러한 BCH의 항당뇨 효과는 인슐린 분비능 증가와 베타세포 사멸 감소, 및 췌도의 조직학적 형태를 보존하는 효과를 통해서 나타내는 것으로 보인다. 따라서 GDH의 약물적 활성화는 제 2형 당뇨병 치료의 새로운 target 될 수 있을 것이다.

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핵심되는 말 : 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), 포도당지방독성, 베타세포, glutamate dehydrogenase