

# **Hyperthermia with Mild Electrical Stimulation Protects Pancreatic $\beta$ -Cells From Cell Stresses and Apoptosis**

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Short running title: Heat with electric current prevents  $\beta$ -cell apoptosis.

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## Abstract

Induction of heat shock protein (HSP) 72 improves metabolic profiles in diabetic model mice. However, its impact on pancreatic  $\beta$ -cells is not known. The present study investigated whether HSP72 induction can reduce  $\beta$ -cell stress signaling and apoptosis, and preserve  $\beta$ -cell mass.

MIN6 cells and db/db mice were sham-treated or treated with heat shock (HS) + mild electrical stimulation (MES) to induce HSP72. Several cellular markers, metabolic parameters and  $\beta$ -cell mass were evaluated.

HS+MES treatment or HSP72 overexpression increased the HSP72 protein levels and decreased TNF- $\alpha$ -induced JNK phosphorylation, ER stress and pro-apoptotic signal in MIN6 cells. In db/db mice, HS+MES treatment for 12 weeks significantly improved the insulin sensitivity and glucose homeostasis. Upon glucose challenge, a significant increase in insulin secretion was observed *in vivo*. Compared with sham treatment, the HSP72, insulin, PDX-1, GLUT2 and IRS-2 levels were upregulated in the pancreatic islets of HS+MES-treated mice, whereas JNK phosphorylation, nuclear translocation of FOXO1 and NF- $\kappa$ B p65 were reduced. Apoptotic signals, ER stress and oxidative stress markers were attenuated.

Thus, HSP72 induction by HS+MES treatment protects  $\beta$ -cells from apoptosis by attenuating JNK activation and cell stresses. HS+MES combination therapy may preserve pancreatic  $\beta$ -cell volume to ameliorate glucose homeostasis in diabetes.

## INTRODUCTION

Type 2 diabetes (T2D) is one of the major causes of morbidity and mortality around the world (1). Both insulin resistance and pancreatic  $\beta$ -cell dysfunction are the main pathophysiological features of T2D. Current lines of evidence suggest that visceral fat accumulation is strongly associated with the pathogenesis of metabolic syndrome (2), and also with T2D and insulin resistance (3).

Cellular stresses such as oxidative stress and endoplasmic reticulum (ER) stress have been considered to be critical factors that cause or worsen insulin resistance as well as  $\beta$ -cell dysfunction in T2D (4-7). These stress pathways can be activated by metabolic alterations in diabetes and metabolic syndrome, resulting in augmentations to further deteriorate the metabolic abnormalities. Recent studies have shown that obesity compromising ER function results in insulin resistance and T2D that are partially dependent on JNK activation (8).

HSP72 is a major inducible molecular chaperone and plays central roles in protein synthesis, folding, re-folding and transport (9). Constitutive overexpression of HSP72 blocks the apoptotic cell death initiated by cellular stresses such as heat shock (HS), ceramide, ethanol, ionizing irradiation, tumor necrosis factor (TNF)- $\alpha$  and ischemia (10). Whole-body hyperthermia causes HSP72 overexpression in the heart, resulting in phosphatidylinositol 3-kinase (PI-3K)-dependent activation of Akt in association with protection against cardiac ischemia-reperfusion injury (11). Hyperthermia is also known to activate Akt in both PI-3K-dependent and -independent manners (12).

Regarding diabetes, decreased expression of HSP72 in the skeletal muscle of T2D patients has been reported, and this reduction is correlated with the degree of insulin resistance (13-15). In fact, induction of HSP72 by any means, such as whole-body hyperthermia, transgenic overexpression of HSP72 in muscle or administration of an HSP72 co-inducer, is beneficial for treating

hyperglycemia in diabetic humans and animal models (15-20). Indeed, it has been postulated that HSP72 attenuates the activation of the JNK pathway, which is involved in the pathogenesis of both insulin resistance and  $\beta$ -cell failure (15; 21). Moreover, we previously reported that the combination of HS+MES ameliorates insulin resistance in high-fat-fed diabetic mice (22; 23). In addition to hyperthermia, MES itself directly activates Akt in muscle cells (24). This combined therapy of HS+MES significantly reduces visceral fat accumulation, and ameliorates glucose homeostasis in high-fat-fed mice with restoration of insulin signaling (22).

In this study, we used MIN6 cells and db/db mice to assess whether HSP72 induction by HS+MES treatment can improve  $\beta$ -cell function *in vitro* and *in vivo*. Our results showed that the combination of HS+MES significantly increased HSP72 protein levels in MIN6 cells and reduced JNK activation, ER stress and pro-apoptotic signal induced by TNF- $\alpha$ . Furthermore, HS+MES treatment increased the insulin contents and reduced apoptotic signals as well as cellular stress markers in  $\beta$ -cells of db/db mice. Thus, induction of HSP72 by HS+MES treatment may protect pancreatic  $\beta$ -cells against apoptosis through inhibition of JNK and ameliorate glucose homeostasis in diabetes.

## RESEARCH DESIGN AND METHODS

**Animals.** Six-week-old male db/db mice (BKS.Cg-m<sup>+/+</sup>Leprdb/J: Leprdb/Leprdb mice) or wild type littermates were obtained from Charles River Laboratories Inc. (Kanagawa, Japan) and housed in a vivarium, in accordance with the guidelines of the Animal Facility Center of Kumamoto University. The mice were maintained on a standard chow diet and water *ad libitum*.

All procedures were approved by the Animal Care and Use Committee of Kumamoto University.

**Heat shock (HS) + mild electrical stimulation (MES) treatment.** The treatment of HS+MES was performed as described in elsewhere (22). Briefly, mice were treated with or sham-treated with HS+MES twice a week for 12 weeks. HS (42°C)+MES (0.6V/cm, 55 pulses/sec, 0.1 millisecond duration) were delivered to the mice through rubber pads with a generator, Biometronome™ (Tsuchiya Gum Co., Ltd.).

**Glucose tolerance test** Glucose tolerance test was performed as described in elsewhere (22).

**Immunohistochemistry.** Antibodies summarized Supplementary Data 1. were used to investigate individual protein expression by immunohistochemistry of frozen pancreatic sections. Practical method of immunohistochemical analysis was described elsewhere (22). The insulin-positive areas and islet sizes were evaluated using a BZ-II Analyzer (Keyence). At least 20 fields from three independent animals were randomly examined.

**Laser capture microdissection (LCM) and total RNA isolation.** Pancreatic sections were prepared at 10 μm thickness using a cryostat (Leica, Wetzlar, Germany) and mounted on RNase-free treated glass slides. After tissue staining by HistoGene LCM Frozen Section Staining Kit (Arcturus, Mountain View, CA, USA), the islets were irradiated with a laser using the PixCell system (Arcturus, Mountain View, CA, USA). The peripheral area was first removed and then the β-cell-rich core area was collected. For each specimen, at least 500 hits were used to obtain

enough RNA for amplification.

**Quantitative real time RT-PCR.** Total RNA was extracted from LCM dissected islets using NucleoSpin miRNA (MACHEREY-NAGEL, Düren, Germany). The first-strand cDNA synthesis from 1 µg of total RNA was primed with oligo (dT) (Takara, Tokyo, Japan). A LightCycler System (Roche Diagnostics, Meylan, France) was used to quantify the transcripts. The quantitative results for these mRNA levels were normalized by the β-actin mRNA levels. The sequence of primers used were indicated in Supplementary data 2.

**Insulin content.** To assess the insulin content, the pancreas was rapidly removed, homogenized and extracted in acid ethanol overnight at 4°C. The insulin contents were measured by ELISA using a kit from Linco Research Inc. (St. Charles, MO).

**Culture of MIN6 cells.** MIN6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mmol/L glucose, 15% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 5 µL/L, β-mercaptoethanol at 37°C and 5% CO<sub>2</sub>. After reaching 60–80% confluence, the cells were treated with MES (0.6 V/cm; 55 pulses/s; peak duration, 0.1 ms) and HS (42°C) for 10 min (22; 23). At 10 h after the treatment, whole cell lysates were extracted.

**TNF-α treatment and HSP72 overexpression in MIN6 cells.** MIN6 cells were treated with MES and/or HS for 10 min. At 10 h after the treatment, the cells were incubated with or without recombinant mouse TNF-α (25 ng/mL) for 6 h, and then lysed. The lysates were extracted for Western blotting. The relative intensities of the protein expression levels were analyzed using the Image J software (NIH, Bethesda, MD). For HSP72 overexpression, an Hsp72 cDNA-containing plasmid (22) was transfected using TransIT<sup>®</sup>-LT1 (Takara, Tokyo, Japan).

**Statistical analyses.** Quantitative data are presented as the means ± SD of at least three independent experiments. Statistical analyses were based on Student's *t*-test for paired or unpaired

data as appropriate. For comparison with multiple groups, ANOVA was carried out. Values of  $p < 0.05$  were considered to indicate statistical significance.

## RESULTS

### **HS+MES treatment increases HSP72 protein and decreases phospho-JNK, ER stress and pro-apoptotic signal in MIN6 cells.**

To examine the effects of HS+MES on pancreatic  $\beta$ -cells, we used MIN6 cells, and determined the HSP72 protein levels at 10 h after a 10-min treatment with HS (42°C) and/or MES (0.6 V/cm; 0.1-ms duration; 55 pulses/sec). HSP72 protein expression was dramatically induced by HS+MES (10.5-fold increase compared with control cells,  $p < 0.000001$ ), while HS alone caused a mild increase in HSP72 (3.3-fold increase,  $p < 0.0001$ ) and MES alone did not (Fig. 1A). To examine whether the induced HSP72 could suppress JNK activation, the effects of HS+MES treatment on the activation of JNK induced by TNF- $\alpha$  (25 ng/mL) were investigated. TNF- $\alpha$  stimulation caused significant increases in the phospho-JNK levels, and these increases were completely suppressed by HS+MES treatment to comparable levels to TNF- $\alpha$ -unstimulated cells (Fig. 1B and E; 61.3% reduction in phospho-JNK1; 43.1% reduction in phospho-JNK2/3). To confirm whether these effects were mediated by HSP72, an HSP72 expression vector (22) was used to overexpress HSP72 protein in MIN6 cells. After HSP72 overexpression by plasmid transfection, TNF- $\alpha$ -induced JNK phosphorylation was similarly attenuated to the levels as in the HS+MES treatment (Fig. 1C; 55.8% reduction in phospho-JNK1; 28.4% reduction in phospho-JNK2/3), suggesting that HS+MES attenuates JNK activation upon TNF- $\alpha$  stimulation mainly through HSP72 induction. To further confirm whether the inhibition of HSP72 cause opposite phenomenon, Hsp72 siRNA treatment was superimposed on HS+MES. HS+MES with scrambled si-RNA (s-si: Fig. 1D) increased HSP72 levels and reduced p-JNK as observed in Fig. 1B. HS+MES with Hsp72 si-RNA treatment partially decreased HSP72 expression and restored p-JNK activation (Fig. 1D and F). Moreover, ER stress marker, BiP and pro-apoptotic signal, cleaved caspase-3 levels were



determined in the same experimental system. The alterations of these markers were quite similar as observed in p-JNK changes (Fig. 1B, C and D).

These data indicate that HSP72 induced by HS+MES and JNK activation, ER stress, pro-apoptotic signal were inversely correlated.

As HSP72 and PI-3K/Akt signal suppress the JNK signaling (11), Akt phosphorylation was investigated. When MIN6 cells were stimulated with insulin, increased phospho-Akt was observed in HS+MES or overexpression of HSP72 compared to the untreated. HS+MES or HSP72 induction partially but significantly restored phospho-Akt levels under TNF- $\alpha$  stimulation (Fig. 1B and C). This effect was cancelled by Hsp72 siRNA treatment (Fig. 1D).

### **HS+MES treatment ameliorates glucose homeostasis and increases insulin secretion in**

#### **db/db mice.**

We previously showed that HS+MES treatment ameliorates insulin resistance and glucose homeostasis in high-fat-fed mice (22). To examine whether HS+MES treatment exerts similar effects in insulin-deficient db/db mice (25). From 6 weeks of age, db/db mice were subjected to the treatment with MES (0.6 V/cm; 0.1-ms duration; 55 pulses/sec) and HS (42°C) for 10 min twice a week for 12 weeks (22). Untreated db/db mice were sham-treated without HS or MES.

Until week 12 of the treatment, there were no detectable differences in the body weights and food intakes between the two groups (Fig. 2A and B). However, after 8 weeks of treatment, the fasting blood glucose level was significantly lower in the HS+MES-treated mice than in the control mice (527.0 $\pm$ 43.2 vs. 403.8 $\pm$ 18.6 mg/dL,  $p=0.03$ ; Fig. 2C). The fasting serum insulin level was also significantly decreased (1.75 $\pm$ 0.23 vs. 0.98 $\pm$ 0.25 ng/mL,  $p=0.03$ ; Fig. 2C), whereas the serum adiponectin and leptin levels were indistinguishable between the two groups (Fig. 2C). Both the fasting and random fed blood glucose levels were significantly lower as early as week 4

of the treatment, and these effects were sustained until week 8 of the treatment (Fig. 2D). On a glucose tolerance test at week 8 of the treatment, significant suppression of the blood glucose excursion was observed at 30 min (Fig. 2E). The serum insulin levels during the glucose tolerance test showed significant increases at 30 and 60 min (Fig. 2F).

### **HS+MES treatment increases HSP72 and insulin expression levels in pancreatic $\beta$ -cells of**

#### **db/db islets.**

We previously reported that the combination of HS+MES effectively induces HSP72 expression in various tissues *in vivo* (22). In the present study, we consistently observed that cytoplasmic HSP72 protein expression was increased in the islets of treated db/db mice (Fig. 3D) compared with the islets of sham-treated db/db mice (Fig. 3A) at the 12<sup>th</sup> weeks of treatment. Western blotting analyses revealed that the HSP72 protein levels were increased 5.7-fold ( $p=0.0029$ ) in the HS+MES group (Fig. 3G: left) compared with sham-treated mice. Hsp72 mRNA levels were also confirmed to be increased 5.4-fold compared with sham-treated db/db islets (Fig. 3G: right.  $p=0.023$ ), assessed by quantitative real-time RT-PCR (qRT-PCR) using laser capture microdissection (LCM). In parallel with the decreased insulin secretion, sham-treated db/db mice showed reduced numbers of insulin-positive cells. The insulin protein expression in each cell was also decreased (Fig. 3B). However, when the mice were treated with HS+MES, the number of insulin-positive cells and the intensity of the insulin immunoreactivity in each cell were greatly increased in the treated islets (Fig. 3E). Most insulin positive cells co-expressed HSP72 in the treated islets (Fig. 3F). The insulin-2 mRNA levels from islets dissected by LCM and insulin contents in the islets of the treated mice were also significantly increased compared with those in sham-treated db/db mice (Fig. 3H and I). The insulin-positive area determined by fluorescent microscope in each islet of HS+MES treated db/db mice was increased by almost 4-fold compared to sham-treated db/db islet (wt:  $2969.4 \pm 226.4 \mu\text{m}^2$ , wt HS+MES:  $3097.2 \pm 431.8$

$\mu\text{m}^2$ , sham-treated db/db:  $561.0 \pm 42.4 \mu\text{m}^2$  ( $p=0.0021$ , v.s wt), db/db HS+MES:  $2284.9 \pm 272.1 \mu\text{m}^2$  ( $p=0.00004$ , v.s. sham-treated db/db),  $n=4$  for each group), while the islet sizes were comparable between the two groups (sham-treated db/db:  $3028.1 \pm 153.6 \mu\text{m}^2$  vs. db/db HS+MES:  $2987 \pm 220.4 \mu\text{m}^2$ ,  $p=0.83$ ).

Thus, HS+MES treatment preserved  $\beta$ -cell mass and insulin secretion *in vivo*. However, *in vitro* experiments in MIN6 cells showed different effects. Insulin secretion in MIN6 cells both at 6 and 10 hr after HS+MES treatment was decreased by  $\sim 30\%$  compared to control (Supplementary Figure 1A). This reduction in insulin secretion inversely paralleled with AMP-kinase (AMPK) activation (Supplementary Figure 1B). When MIN6 cells were treated with HS+MES, p-AMPK levels were significantly upregulated at 1hr after treatment, and diminished thereafter. Overexpression of dominant negative-AMPK completely cancelled HS+MES induced reduction of insulin secretion (data not shown).

**JNK phosphorylation and FOXO1 nucleo-cytoplasmic translocation are reduced in HS+MES-treated db/db islets.**

It has been reported that the JNK pathway, which is known to being activated by variety of stress signals, is abnormally accelerated in various tissues under diabetic conditions (26; 27). When JNK is activated, dimerized JNK translocates into the nucleus, and activates downstream targets such as c-jun. To analyze the subcellular localization of JNK, we performed immunohistochemical staining with an anti-phospho-JNK antibody. The results revealed that phospho-JNK was mainly localized in the nuclei in sham-treated db/db islets (Fig. 4A: a, c and e), whereas the HS+MES-treated db/db islets showed drastic suppression of the nuclear accumulation of phospho-JNK (Fig. 4A: b, d and f). Phospho-JNK was also confirmed to being decreased by Western blotting (phospho-JNK1:  $37 \pm 7\%$  reduction vs. sham-treated db/db mice ( $p < 0.0001$ ); phospho-JNK2/3:  $48 \pm 6\%$  reduction vs. sham-treated db/db mice ( $p=0.003$ )),

while the JNK protein expression levels remained unaltered (Fig. 3C). These observations suggest that the JNK activation and cellular stress milieu were attenuated by the HS+MES treatment.

The forkhead transcription factor FOXO1 is an important transcription factor that plays key roles in apoptosis, cellular proliferation, differentiation and glucose metabolism (28). To investigate the expression and intracellular localization of FOXO1, we carried out immunostaining with an anti-FOXO1 antibody. In sham-treated db/db islets, nuclear accumulation of FOXO1 was observed (Fig. 4B: a, c and e). FOXO1 changed its intracellular localization from the nucleus to the cytoplasm upon HS+MES treatment (Fig. 4B: b, d and f).

#### **PDX-1 nuclear localization and ER stress markers in HS+MES-treated db/db islets.**

JNK activation was reported to induce the nucleo-cytoplasmic translocation of the important transcription factor pancreatic duodenal homeobox (PDX)-1 (29). Although PDX-1 was expressed at lower levels in nuclei in sham-treated db/db islets (Fig. 5A: a, c and e), the number of signals and intensity of PDX-1 expression were dramatically increased in the HS+MES-treated group (Fig. 5A: b, d and f). PDX-1 protein expression was decreased in sham-treated db/db mice compared to wt mice with or without HS+MES treatment. Upon HS+MES treatment, PDX-1 expression was partially recovered (Fig. 5A, g: 3.6-fold increase.  $p=0.0082$ ). PDX-1 mRNA expression in isolated islets by LCM showed significant increase in HS+MES-treated group (Fig 5A, g: 3.1-fold increase.  $p=0.016$ ).

ER stress is one of the key mediators of  $\beta$ -cell dysfunction (30; 31). The expression of an early ER stress marker, BiP, was suppressed in the islets of HS+MES treated db/db mice, in contrast to its upregulation in the sham-treated db/db islets (Fig. 5B: a, b and e). Since excessive ER stress signals cause  $\beta$ -cell apoptosis *in vivo*, we investigated the apoptotic signals. The pro-apoptotic transcription factor C/EBP homolog protein (CHOP) is activated under severe ER stress

conditions to induce cell elimination by apoptosis (30; 31). Nuclear induction of CHOP was considerably suppressed in the HS+MES treated db/db mice islets (Fig. 5B: c and d). CHOP mRNA expression was increased by 4.9-fold in the islets of sham-treated db/db mice compared to wt with or without HS+MES. CHOP mRNA in the islets of HS+MES-treated db/db mice was significantly decreased by  $56\pm 7\%$  (Fig. 5B, f,  $p=0.0006$ ). ER stress milieu was also investigated by alternative splicing of X-box binding protein (XBP)-1. Spliced form of XBP-1 (XBP-1s) protein was determined by Western blotting. XBP-1s level was increased by 3.4-fold ( $p=0.0021$ ) in sham-treated db/db mice compared to wt, and was significantly decreased by  $52.2\%$  ( $p=0.015$ ) upon HS+MES treatment (Fig. 5C).

**Another molecular markers in  $\beta$ -cells treated with HS+MES.** It was recently reported that calcineurin/nuclear factor of activated T-cells (NFAT) signaling regulates  $\beta$ -cell growth and appropriate  $\beta$ -cell functions (32). To assess the effects of HS+MES treatment on the calcineurin/NFAT pathway, NFAT and one of its downstream targets, glucose transporter (GLUT) 2, were visualized by immunohistochemistry. In sham-treated db/db islets, NFAT was predominantly located in the cytoplasm of cells with lower expression of insulin (Supplementary Fig. 2a). Upon HS+MES treatment, nuclear translocation of NFAT was observed in cells with higher insulin expression (Supplementary Fig. 2b). GLUT2, which participates in glucose sensing in  $\beta$ -cells, was increased in the membrane fraction of treated db/db islets compared with sham-treated db/db islets (Supplementary Fig. 2c and d). GLUT2 protein expression was decreased by  $83\pm 12\%$  in sham-treated db/db mice compared to wt. Upon HS+MES treatment in db/db mice, GLUT2 expression was almost restored ( $78\pm 9\%$  of wt: Supplementary Fig. 2g). Insulin receptor substrate (IRS)-2, an important insulin signaling molecule for  $\beta$ -cell growth (33), showed lower expression in sham-treated db/db islets, and was greatly increased by HS+MES

treatment (Supplementary Fig. 2e, f and h). Cleaved-caspase-3 was also downregulated in the HS+MES islets (Supplementary Fig. 3A: a and b). Another early apoptotic marker, annexin V displayed similar trend (Supplementary Fig. 3A: c and d).

Nuclear factor kappa B (NF- $\kappa$ B) signaling is crucial for transmitting cellular stresses (34). Nuclear localization of NF- $\kappa$ B p65 was predominant in sham-treated db/db islets, but decreased in treated islets (Supplementary Fig. 3B: a and b). Oxidative stress marker, 8-Hydroxy-2'-deoxyguanosine (8-OHdG) was increased in sham-treated db/db islets, but were attenuated in HS+MES treated db/db islets (Supplementary Fig. 3B: c and d).

## DISCUSSION

Cellular stress signaling pathways, such as ER stress and oxidative stress have recently been recognized as important factors for the development and progression of T2D. Cell protection is one of the key ideas for preserving or even restoring pancreatic  $\beta$ -cell function under diabetic conditions.  $\beta$ -cells are considered to be relatively sensitive to these stresses, because the cells are continuously stimulated to produce insulin to overcome the demand for insulin in the state of insulin resistance (31). In the present study, we focused on the effects of HS+MES on  $\beta$ -cell protection.

HSP72 is one of the major inducible HSPs and plays significant roles in protein synthesis, folding and cell survival (9). In response to stresses, which lead to denaturation of proteins, HSP72 prevents protein aggregation and induces refolding of damaged proteins into their native states (35). Importantly, it has been revealed that HSP72 can modulate the functions of several molecules, including JNK (36). HSP72 interacts physically with JNK, thereby suppressing both JNK activation and JNK-mediated cell death (37). The JNK pathway is known to be activated under diabetic conditions and to be passively involved in the progression of insulin resistance and suppression of insulin biosynthesis (27). JNK-inhibitory peptide treatment was reported to improve insulin resistance and ameliorate glucose tolerance in diabetic mice (21).

We previously reported that HSP72 was greatly induced by HS+MES in various tissues (22). Using this combined treatment, we found that HSP72 was considerably increased in db/db mice islets and MIN6 cells in the present study. Upregulation of HSP72 appears to be very effective for suppressing JNK pathway. Recently, HSP72 induction by various methods, such as long-term hyperthermia, muscle-specific transgenic of HSP72 and HSP72 co-inducer BGP-15 treatment with hyperthermia, were also reported to protect against visceral adiposity and insulin resistance

(15; 17). We and others also found that geranylgeranylacetone, another HSP72-inducer, displays similar metabolic advantages to HS+MES treatment (19; 20), suggesting that induction of HSP72 may be beneficial for improving diabetic pathophysiology.

To further investigate the mechanisms of the preservation of  $\beta$ -cells through HSP72 induction by HS+MES, we focused on the JNK pathway. PI-3K/Akt suppresses the JNK in islets, and JNK inhibition increases the viability of human islets (38). The activation of JNK suppresses insulin biosynthesis and also interferes with the actions of insulin. PDX-1 is a homeodomain-containing transcription factor that plays pivotal roles in the development and differentiation of  $\beta$ -cells (39). Oxidative stress or activation of JNK decreases the activity of Akt in  $\beta$ -cell line HIT-T15 cells, leading to decreased phosphorylation and increased nuclear localization of FOXO1, which is followed by enhanced cytoplasmic translocation of PDX-1 and finally results in suppression of insulin biosynthesis (40). PDX-1 has also been suggested to be involved in glucokinase and GLUT2 gene expression. In the present study, we have revealed that HS+MES-treated db/db islets show inhibition of phosphorylated JNK, nucleo-cytoplasmic translocation of FOXO1 and increased PDX-1 intensity in their nuclei, indicating that this combined treatment ameliorates  $\beta$ -cell integrity in diabetic animals, at least in part through inhibition of JNK. Indeed, whole body hyperthermia in rats attenuates the inhibition of glucose induced insulin release by LPS stimulation (41). It is also reported that induction of HSP72 by glutamine supplementation decreases ischemic damage in rat islets (42).

Recent evidence has indicated that the calcineurin/NFAT pathway regulates multiple factors that control  $\beta$ -cell growth and appropriate endocrine functions (32). In the HS+MES-treated mice, NFAT was translocated into the nucleus, and GLUT2, a downstream target of NFAT, was upregulated, suggesting that NFAT pathway are involved in the improved  $\beta$ -cell function as well



as JNK pathway.

Pancreatic  $\beta$ -cell apoptosis upon ER stress is mainly regulated by the inductions of CHOP and caspase-3 (31), which were significantly decreased by HS+MES treatment. As ER stress markers, BiP and XBP-1s expression were both downregulated by the HS+MES treatment, suggesting that the induction of HSP72 by HS+MES may attenuate the ER stress milieu as well as oxidative stress signaling confirmed by 8-OHdG reduction. These stress signals can also be activated by chronic inflammatory signals, such as NF- $\kappa$ B. Nuclear translocation of NF- $\kappa$ B increases TNF- $\alpha$  mRNA expression. Although we haven't examined the local expression of TNF- $\alpha$ , NF- $\kappa$ B p65 nuclear translocation was attenuated by this treatment.

We also found that IRS-2, which is an important molecule for insulin signaling and  $\beta$ -cell proliferation (43), was upregulated in the treated islets. This effect may also contribute to the protection of the  $\beta$ -cells against apoptosis, however, the mechanisms of IRS-2 upregulation need to be studied.

The preventive effect of HS+MES on  $\beta$ -cell degradation in db/db mice may be mainly caused by the suppression of apoptosis through JNK inactivation. The glucose-lowering effect of HS+MES seems to be due primarily to preserving insulin production rather than preventing insulin resistance, because significant upregulation of insulin secretion in HS+MES treated db/db mice showed a relatively small improvement of glucose excursion on i.p.GTT (Fig. 2 E and F). However, it remains uncertain whether the improved  $\beta$ -cell function observed *in vivo* is directly caused by HS+MES or through a secondary effect of reduced systemic glucose toxicity and insulin resistance. Since HS+MES may modulate certain numbers of molecules, collective analyses of gene and/or protein expressions need to be carried out for further elucidation.

Thus, HS+MES certainly prevents  $\beta$ -cell apoptosis and preserves  $\beta$ -cell mass *in vivo*, however, *in vitro* experiments using MIN6 cells repeatedly shows decreased insulin secretion (~30%) by HS+MES. This phenomenon may be explained by transient activation of AMPK by HS+MES, because AMPK activation reduces insulin secretion (44). HS+MES activate AMPK at 1 hr after the treatment, and was diminished thereafter. We believe that preserved  $\beta$ -cell mass (4 times greater than that of sham-treated db/db islets) can respond to glucose challenge even the each cell's insulin secretion capability was attenuated. Alternatively, transient activation of AMPK may decrease insulin secretion, but does not last long *in vivo*. It may also be possible that activation of AMPK contribute to protect  $\beta$ -cells (45).

In conclusion, we have revealed the induction of HSP72 in db/db islets by HS+MES, which results in inactivation of JNK and enhancement of PDX-1 nuclear translocation, thereby leading to increased insulin biosynthesis, accompanied by a reduced ER stress and oxidative stress milieu. Taken together, our findings indicate that HS+MES treatment not only improves insulin resistance in insulin-sensitive tissues, but also contributes to the survival of  $\beta$ -cells in diabetes. This treatment may hold promise as a novel therapeutic approach to human diabetes and metabolic syndrome.

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T.K. researched data, contributed to discussion and wrote manuscript. K.S and R.M. researched data. S.K. researched data and contributed to discussion. H.A. researched data. MA. S. reviewed/edited manuscript. J.K. H.M. and N.F. contributed to discussion. H.K and E.A. reviewed/edited manuscript.

Dr. Tatsuya Kondo is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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## Figure legends

Fig. 1. HS+MES treatment induces HSP72 and suppresses phospho-JNK in MIN6 cells.

A: MIN6 cells were treated with HS and/or MES for 10 min. At 10 h after the treatment, the cells were harvested and the HSP72 protein levels were determined by Western blotting. B: After HS+MES treatment, MIN6 cells were incubated with or without TNF- $\alpha$  (25 ng/mL) for 6 h. The expression levels of HSP72, JNK, phospho-JNK, BiP and cleaved caspase-3 were determined by Western blotting. Upon insulin stimulation, phospho-Akt levels were also investigated. C: HSP72 protein was overexpressed by transfection of an Hsp72 cDNA-containing plasmid. TNF- $\alpha$  stimulation was carried out and the expression levels of HSP72, JNK, phospho-JNK, BiP and cleaved caspase-3 were determined as described for (B). D: Upon HS+MES with TNF- $\alpha$  treatment, scrambled si-RNA (s-si) or Hsp72 si-RNA was transfected. HSP72, JNK, p-JNK, BiP and cleaved caspase-3 were determined by Western blotting. E: Quantitative results of p-JNK levels in the data shown in (B). F: Quantitative results of p-JNK levels in the data shown in (D). \*\* $p < 0.01$ , N.S. (not significant) vs. indicated group.

Fig. 2. HS+MES treatment improves glucose tolerance and insulin secretion in db/db mice.

A and B: Weekly body weight increments (A) and food intakes (B) were measured during HS+MES treatment or sham-treatment in db/db mice. C: The fasting blood glucose, fasting insulin, adiponectin and leptin levels were measured after 8 weeks of treatment. D: The changes in the fasting and random fed glucose levels were plotted. E and F: The glucose tolerance and insulin secretion capability were evaluated by intraperitoneal injection of glucose (1 g/kg body weight) in HS+MES-treated and sham-treated db/db mice. Whole blood was collected from the

tail vein at the indicated time points for blood glucose and insulin measurements. Values are means  $\pm$  SEM ( $n=4-6$ ).  $*p<0.05$  vs. sham-treated db/db control mice.

Fig. 3. Increased expression of HSP72 and insulin in the pancreas of HS+MES-treated db/db mice. A to F: Sections of pancreas were immunostained for HSP72 and insulin with DAPI staining. Representative pancreatic sections from sham-treated db/db control and HS+MES-treated mice are shown. G: Left panel; Western blotting of HSP72 and actin using whole pancreatic tissues. Right panel; Hsp72 mRNA was measured by quantitative real-time RT-PCR (qRT-PCR). Islet samples were isolated using laser capture microdissection (LCM) as described in RESEARCH DESIGN AND METHODS. H and I: Insulin-2 mRNA by qRT-PCR (H) using LCM and the insulin content per nanogram of DNA (I) were measured in islets isolated from HS+MES-treated and sham-treated db/db mice. J: Insulin positive area was determined using fluorescent microscope BZ-8100. Values are means  $\pm$  SEM ( $n=50$ ) islets each from 3 mice of each genotype).  $*p<0.05$ ,  $**p<0.01$  vs. indicated group.

Fig. 4. HS+MES treatment suppresses JNK activation and FOXO1 nuclear expression in the pancreas of db/db mice. A and B: Sections of pancreas were immunostained for phospho-JNK (A: a~f) and FOXO1 (B: a~f). Representative pancreatic sections from sham-treated db/db and HS+MES-treated mice are shown. C: The levels of total JNK (46 kDa) and phospho-JNK (54 kDa) were determined by Western blotting in sham-treated and HS+MES-treated db/db mice. The expression levels of phospho-JNK were quantified as relative to the respective total JNK. Values are means  $\pm$  SEM ( $n=4-6$ ).  $++p<0.01$  vs. untreated-wt control mice.

$**p<0.01$  vs. sham-treated db/db mice.

Fig. 5. PDX-1 expression and ER stress signals in the pancreas.

A: Sections of pancreatic specimen were immunostained for PDX-1 (a~f). Representative pancreatic sections from sham-treated and HS+MES treated db/db mice are shown. PDX-1 protein expression assessed by Western blotting and PDX-1 mRNA expression measured by qRT-PCR were investigated (g). Relative PDX-1 protein and mRNA expression were quantified and corrected using respective internal controls ( $\alpha$ -tubulin for protein,  $\beta$ -actin for mRNA)(g).

B: Sections of pancreatic specimen were immunostained for BiP (a and b) and CHOP (c and d). Representative pancreatic sections from sham-treated and HS+MES treated db/db mice are shown. BiP mRNA (e) and CHOP mRNA (f) were quantified by qRT-PCR using LCM.

C: XBP-1s protein expression (identified as 54 kDa band) was determined by Western blotting and the expression levels were corrected using  $\alpha$ -tubulin. \* $p$ <0.05, \*\* $p$ <0.01 vs. indicated group.