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Constitutively active heat shock factor 1 enhances glucose-driven insulin secretion

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Abstract

Weak pancreatic β-cell function is a cause of type 2 diabetes mellitus. Glucokinase regulates insulin secretion via phosphorylation of glucose. The present study focused on a system for the self-protection of pancreatic cell by expressing heat shock factor (HSF) and heat shock protein (HSP) to improve insulin secretion without inducing hypoglycemia. We previously generated a constitutively active form of human HSF1 (CA-hHSF1). An adenovirus expressing CA-hHSF1 using the cytomegalovirus promoter was generated to infect mouse insulinoma cells (MIN6 cells). An adenovirus expressing CA-hHSF1 using a human insulin promoter (Ins-CA-hHSF1) was also generated to infect rats. We investigated whether CA-hHSF1 induces insulin secretion in MIN6 cells and whether Ins-CA-hHSF1 can improve blood glucose and serum insulin levels in healthy Wister rats and type 2 diabetes mellitus model rats. CA-hHSF1 expression increased insulin secretion 1.27-fold compared with the overexpression of wild-type hHSF1 in MIN6 cells via induction of HSP90 expression and subsequent activation of glucokinase. This mechanism is associated with activation of both glucokinase and neuronal nitric oxide synthase. Ins-CA-hHSF1 improved blood glucose levels in neonatal streptozotocin-induced diabetic rats. Furthermore, Ins-CA-hHSF1 reduced oral glucose tolerance testing results in healthy Wister rats because of an insulin spike at 15 minutes; however, it did not induce hypoglycemia. CA-hHSF1 induced insulin secretion both in vitro and in vivo. These findings suggest that gene therapy with Ins-CA-hHSF1 will be able to be used to treat patients with type 2 diabetes mellitus and impaired glucose tolerance without causing hypoglycemia at fasting.

1. Introduction

Studies on the pathophysiology of type 2 diabetes mellitus have revealed 2 causative defects: insulin resistance and impaired β-cell function. Furthermore, reduced insulin secretion by β-cells is common in type 2 diabetes mellitus patients, particularly in Asians [1,2]. Most type 2 diabetes mellitus patients show a net decrease in β-cell mass [3]. Sulfonylureas are thus prescribed for most type 2 diabetes mellitus patients. However, high blood glucose levels inhibit insulin secretion and eventually cause β-cell apoptosis [4], leading to "secondary sulfonylurea failure."

Our aim was to examine a potential method for improving insulin secretory function by pancreatic β-cells through the enhancement of a system that acts as a cytoprotectant using the overexpression of heat shock proteins (HSPs). Heat shock proteins are induced because of cellular stress [5]. Stress can be any sudden change in the cellular environment to which the cell is not prepared to respond, such as heat shock, oxidative stress, and hypoxia. Almost all types of cellular stresses induce HSPs. Owing to the generality of this

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phenomenon, HSPs are often called *stress proteins*. The rationale behind this phenomenon is that, after stress, the need for the chaperone function of HSPs is increased, thus triggering their induction. This need is caused by the increased amount of damaged proteins, by the inhibition of their elimination via the proteasome, and by the damage of the chaperones themselves [6].

Diabetes mellitus is an age-related disease, and reducing heat shock factor (HSF) 1 activity accelerates tissue aging and shortens life span [7]. Heat shock proteins and HSF1 might support insulin secretory actions and prevent β-cell apoptosis due to hyperglycemia or glucotoxicity. Heat shock at 42°C induces insulin secretion in vitro [8], although the mechanisms remain unclear.

Heat shock proteins have been classified into families according to their approximate molecular weight and homology, that is, HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs. Heat shock proteins have been reported to exert a protective effect on the pancreas. HSP27 is a member of the small HSPs family that has antioxidant properties and inhibits the mitochondrial apoptotic pathway; constitutive HSP27 overexpression protects islets from cytokine-induced injury [9]. HSP32, also known as *heme oxygenase 1*, generates carbon monoxide and heme metabolites. The heme metabolites produced, including bilirubin and biliverdin, display significant antioxidant properties [10]. Furthermore, carbon monoxide has been shown to stimulate insulin release by β-cells [11]. HSP70 also protects the pancreas against stress and prevents intracellular activation of trypsinogen in pancreatic acinar cells [12]. Furthermore, heat shock, which is involved with HSP70 expression, inhibits cytokine-induced inducible nitric oxide synthase (iNOS) expression; iNOS is an isoform of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) [13]. However, iNOS induces nitric oxide (NO) production more strongly than other NOSs. Excessive NO is a toxic effector molecule in β-cells [14]. We previously studied the pleiotropic effects of statins in vascular endothelial cells and found that statins improve vascular endothelial cell function via induction of HSPs [15]. In addition, we generated an adenovirus encoding the constitutively active form of HSF1 (CA-hHSF1) to induce vigorous expression of HSPs [16]. CA-hHSF1 is constitutively active through a deletion in the second of 3 leucine zipper domains, preventing the enzyme from forming the monomeric, inactivated state [17]. We found that CA-hHSF1 markedly improved vascular endothelial function as compared with wild-type hHSF1. Moreover, this improvement of vascular endothelial function by CA-hHSF1 and wild-type hHSF1 is observed under routine conditions without cell stress agents. Given our promising findings for vascular endothelial cells and that HSPs protect β-cells from stress, we examined the ability of CA-hHSF1 to improve insulin secretion under hyperglycemic conditions, identified which HSP contributes to this improvement, and determined whether CA-hHSF1 induces insulin secretion under hyperglycemic conditions without β-cell dysfunction.

Regarding insulin secretion, glucokinase (GCK), also called *hexokinase IV*, is an important glucose-sensitive enzyme for insulin secretion. However, exposure to chronically elevated glucose reduces GCK expression [18]. Neuronal nitric oxide synthase is an isoform of eNOS that is expressed mainly in the brain, but is also expressed in pancreatic β-cells. In HEK293 cells, HSP90 activates nNOS via calmodulin-dependent protein kinase II (CaMKII) [19,20]; and CaMKII also induces insulin secretion in MIN6 cells [21]. On the basis of this combined evidence, we examined the effects of nNOS and GCK on insulin secretion in MIN6 cells under conditions of CA-hHSF1, wild-type hHSF1, and HSP overexpression. We also examined the effects of CA-hHSF1 on blood glucose and serum insulin levels in type 2 diabetes mellitus model rats showing impaired insulin secretion and in healthy Wister rats.

2. Methods

2.1. Materials

Dulbecco modified Eagle medium (DMEM), fetal calf serum (FCS), streptomycin, and penicillin were purchased from GibcoBRL (Grand Island, NY). Antibodies to HSF1 and HSPs were purchased from Stressgen (Victoria, British Columbia, Canada). Other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Adenovirus for overexpression was generated using the Adeasy system (Stratagene, La Jolla, CA), whereas 17-(allylamino)-17-demethoxygelanamycin (17-AAG) was purchased from Calbiochem (San Diego, C A). N^G-nitro-arginine methyl esterhydrochloride (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP), *S*-nitroso-L-glutathione (GSNO), 3-bromo-7-nitroindazole (3-Br-7-Ni), N^{ω}-propyl-L-arginine (NPLA), and KN-93 were purchased from Cayman Chemical (Ann Arbor, MI).

Glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide hydrate (NADH), and adenosine triphosphate (ATP) were purchased from Roche Diagnostics (Penzberg, Germany). A mouse insulin radioimmunoassay kit was purchased from Linco Research (St Charles, MO), and a rat insulin enzyme-linked immunoassay kit was purchased from Mercodia (Uppsala, Sweden).

2.2. Cell culture

MIN6 cells were kindly provided by Dr Yoshitomo Oka (Tohoku University, Sendai, Miyagi, Japan) [22]. MIN6 cells were cultured at 37°C in DMEM supplemented with 20% FCS. MIN6 cells were passaged every 3 to 6 days. Cells from passages 35 to 40 were used for experiments.

2.3. Measurement of insulin secretion in vitro

MIN6 cells were grown 3.5 cm in 6-well plates at 1.5×10^6 cells per well and in triplicate. The following day, adenovirus was added; and the cells were incubated for 48

hours. Cells were washed twice with 3 mmol/L glucose in HEPES-buffered Krebs-Ringer bicarbonate (KRB) buffer. After a 30-minute preincubation, the medium was removed; and cells were washed twice with KRB buffer containing 25 mmol/L glucose. After 1 hour, the medium was collected. The supernatant was stored at −80°C. After the medium was harvested, cells were removed from the plate with trypsin and counted. For long (24 hours) incubations, cells were washed twice with DMEM containing 25 mmol/L glucose and 20% FCS. After 24 hours, the medium was collected and centrifuged. The supernatant was stored at −80°C. After the medium was harvested, cells were removed from the plate with trypsin and counted. Insulin was measured using a radioimmunoassay kit (Linco Research) according to the protocol supplied by the manufacturer. All measurements of insulin secretion were normalized to cell counts.

2.4. Small interfering RNA transfections

Mouse nNOS small interfering RNA (siRNA) siRNA and nontargeting siRNA were purchased from Dharmacon (Lafayette, CO). The si-nNOS and nontargeting siRNA were transfected into MIN6 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and transfection was performed according to the instructions from the manufacturer.

2.5. Protein extraction and Western blot analysis

Cells were treated with adenovirus encoding wild-type hHSF1 or CA-hHSF1. After 2 days, total cellular protein was extracted, using Laemmli buffer, as described previously [15].

2.6. Protein extract in rat tissue

After oral glucose tolerance test (OGTT), rats were anesthetized. We removed the pancreas from the rats. Tissue was washed twice with ice-cold Tris buffer saline (pH 7.4); and 0.3 g of pancreas tissue was put in 2 mL of ice-cold lysate buffer containing 50 mmol/L Tris/HCl pH 7.4, 50 mmol/L NaCl, 4 mmol/L EDTA, 10% glycerin, 2 mmol/L dithiothreitol (DTT), and protease inhibitor. The tissue was gently homogenized in a glass-Teflon homogenizer on ice and centrifuged at 10 000*g*, and the supernatant was stored at −80°C until use. Before electrophoresis on sodium dodecyl sulphate polyacrylamide gels, we added 2× Laemmli buffer to the tissue extract.

2.7. GCK assay

Cells (4.0×10^6 cells per well) were grown in 6-well plates. The following day, cells were infected with adenovirus. After 48 hours, cells were harvested in 20 mmol/L KH_2PO_4 (pH 7.0) containing 100 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.1% bovine serum albumin, 1 mmol/L EDTA, 100 mmol/L DTT, and 5% glycerol. Cells were gently homogenized in a glass-Teflon homogenizer and centrifuged at 10 000*g* [23], and the supernatant was stored at −80°C. Glucose phosphorylating activity was determined by adding the cell extract to plastic microtubes containing 50 mmol/L HEPES-NaOH (pH 7.4), 8 mmol/L KCl, 8 mmol/L $MgCl_2$, 0.5 mmol/L NAD, 5 mmol/L ATP, and 1 U/mL glucose 6-phosphate dehydrogenase. The rate of NADH formation was monitored by measuring changes in absorbance at 340 nm. Hexokinase and GCK activities were determined at 0.5 and 50 mmol/L glucose, respectively. Glucokinase activity (activity at 50 mmol/L glucose-hexokinase activity) represented 75% of all glucose phosphorylating activity in insulinoma cell extracts [24]. Glucokinase activity was normalized to the concentration of protein in the insulinoma cell extract as measured using the Bio-Rad (Hercules, CA) protein assay.

2.8. Adenoviruses for expressing CA-hHSF1; hHSF1; human HSP32, HSP70, and HSP90; and LacZ

CA-hHSF1 is hHSF1 with the deletion of amino acids 186 to 202 and was generated by polymerase chain reaction (PCR) as previously described [16]. Adenoviruses for expressing human HSP32, HSP70, and HSP90 were generated as previously described [16].

2.9. Immunoprecipitation

Immunoprecipitation was performed largely according to the manufacturer's procedure. Cells were washed 4 times with ice-cold 1× buffer (50 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 7.5). Next, 1× buffer with a protein inhibitor cocktail, phosphatase inhibitor cocktail, and 0.5 mmol/L DTT was added. Cells were harvested by scraping and were homogenized by 10 strokes using a Dounce homogenizer. The cell homogenate was centrifuged at 12 000*g* for 10 minutes, after which the supernatant was centrifuged at 100 000*g* for 45 minutes. Before addition of antibody, we took a separate, identical amount of sample to determine the control expression of GCK in the cytosol. The solubilized sample was incubated with 2 μg/mL of mouse monoclonal anti-nNOS antibody for 3 hours at 4°C with gentle rotation and was then incubated with 50 μL protein G–agarose overnight. The pellet was washed 4 times with 1× buffer containing a protease inhibitor cocktail and then washed with 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5 mmol/L DTT, and a protease inhibitor cocktail. After washing, the pellet was eluted with 75 μL of 2× sodium dodecyl sulphate buffer and stored at −80°C until use. Protein was detected using a 1:1000 dilution of rabbit anti-nNOS or anti-GCK antibody.

2.10. Animals

Institutional guidelines for the use and care of laboratory animals were followed. Rats were purchased from Charles River Laboratory Japan (Yokohama, Japan). All experimental procedures were performed in accordance with the guidelines of animal care and experimentation committee at Gunma University.

2.11. OGTT in rat

The OGTT was performed after an 8-hour fast by administering 1 g/kg glucose in a volume of 10 mL/kg. Blood samples were obtained before and 15, 30, 60, and 120 minutes after glucose challenge. Blood glucose levels were determined using a Glutest E (Sanwa Medical, Nagoya, Japan) [25].

2.12. Generation of type 2 diabetes mellitus model rats

To create a model of type 2 diabetes mellitus, neonatal streptozotocin (nSTZ)-diabetic rats were generated by intraperitoneal injection with 100 mg/kg of streptozotocin on the day of birth. At 5 to 6 weeks old (130-150 g), when nonfasting blood glucose level was 6.67 to 8.33 mmol/L, rats were anesthetized; and the pancreas was injected directly with 1.0×10^8 plaque-forming units (pfu) of Ins-CA-hHSF1– or LacZ-encoding adenovirus via an abdominal incision. Nonfasting blood glucose and body weight were measured every other day for 1 week before and 1 week after infection. An OGTT was also performed the day before and 5 days after infection.

Healthy Wister, nondiabetic male rats at 8 weeks old were anesthetized; and the pancreas was injected directly with 1.0×10^8 pfu of Ins-CA-hHSF1– or LacZ-encoding adenovirus via an abdominal incision. Five days after the infection, an OGTT was performed; and serum insulin level was measured.

2.13. Data analysis

Data are presented as means ± standard deviation of at least 3 separate experiments. The significance of intergroup differences was determined by analysis of variance. Values of $P < .05$ were considered to indicate a significant difference.

3. Results

3.1. Effects of overexpression of hHSF1, CA-hHSF1, HSP32, HSP70, and HSP90 on insulin secretion in MIN6 cells

We examined whether overexpression of wild-type hHSF1 or CA-hHSF1 induces insulin secretion in MIN6 cells. Insulin secretion was induced by hHSF1 in MIN6 cells (45.5 ± 9.85 fmol/10^4 cells per hour, n = 6, $P < .05$) (Fig. 1A). Moreover, CA-hHSF1 induced insulin secretion more strongly than wild-type hHSF1 in MIN6 cells (1.27-fold, n = 6, $P < .05$ at a multiplicity of infection of 10). We next investigated which HSPs contribute to insulin secretion and proinsulin expression by examining the effects of overexpressing CA-hHSF1, HSP32, HSP70, and HSP90. In MIN6 cells overexpressing HSP90, 60-minute treatment with 25 mmol/L glucose enhanced insulin secretion to 97.1 ± 12.4 fmol/10^4 cells per hour (n = 9, $P < .05$) (Fig. 1B).

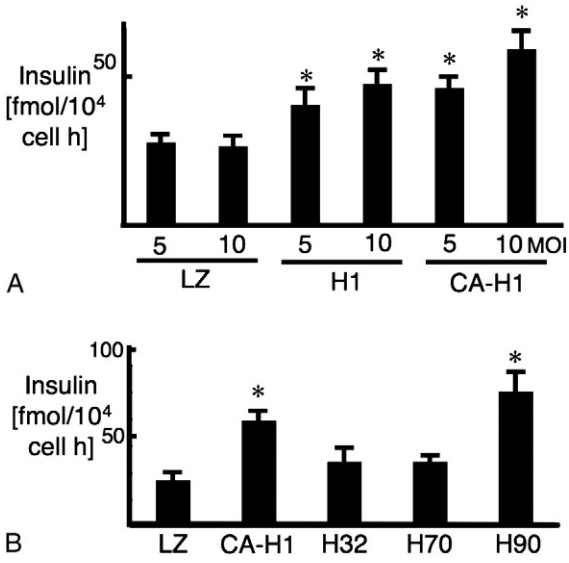


Fig. 1. Overexpression of CA-hHSF1 and HSPs induces insulin secretion in MIN6 cells. MIN6 cells were transfected with adenovirus containing the gene for CA-hHSF1 (CA-H1), hHSF1 (H1), HSP32 (H32), HSP70 (H70), HSP90 (H90), or LacZ (LZ; control). A and B, After 2 days, medium was replaced with KRB buffer; and insulin levels were assessed by a radioimmunoassay. Each column and bar represent the mean ± SD for 3 separate experiments performed in duplicate. *$P < .01$ vs LacZ-infected cells (n = 6).

3.2. Effect of overexpressing human HSP32, HSP70, and HSP90 on insulin secretion under other conditions in MIN6 cells

To further examine which HSPs contribute to insulin secretion in other circumstances, we examined long incubation with either high or low glucose levels. In the presence of 25 mmol/L glucose for 24 hours, a higher level of insulin secretion was seen with overexpression of HSP32 (912 ± 140 fmol/10^4 cells, n = 9, $P < .05$ vs LacZ-infected cells) than with overexpression of HSP90 (614 ± 44.7 fmol/10^4 cells, n = 9, $P < .05$ vs LacZ-infected cells) (Fig. 2A). In the presence of 3 mmol/L glucose, overexpression of CA-hHSF1, HSP32, or HSP90 for 24 hours did not promote insulin secretion (Fig. 2B).

3.3. Effect of overexpressing human HSP90, hHSF1, and CA-hHSF1 on insulin secretion in MIN6 cells

To confirm that overexpression of CA-hHSF1 induces insulin secretion via HSP90, we used 17-AAG, an ansamycin antibiotic that binds to the ATP binding site of HSP90 and disrupts association with client proteins [26]. In addition, we examined whether overexpression of wild-type hHSF1 or CA-hHSF1 induced HSP90 expression in MIN6 cells. Insulin secretion induced by overexpression of wild-type hHSF1, CA-hHSF1, or HSP90 was inhibited by 17-AAG (Fig. 3A). Furthermore, both CA-hHSF1 and hHSF1 induced HSP90 expression in MIN6 cells (Fig. 3B). These results suggest that both

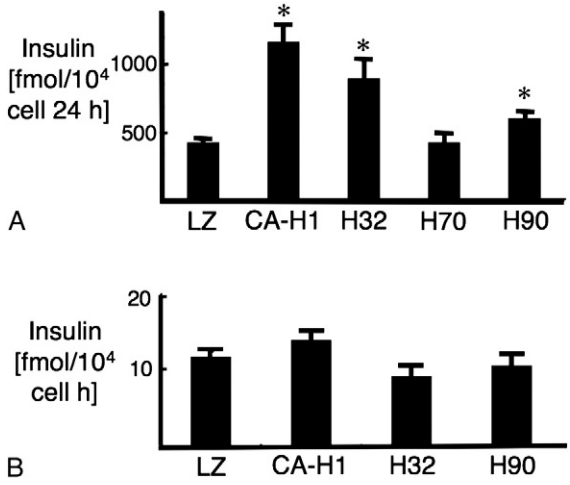


Fig. 2. Overexpression of HSP90 induces insulin secretion at high glucose in MIN6 cells. MIN6 cells were transfected with adenovirus containing the gene for CA-hHSF1 (CA-H1), hHSF1 (H1), HSP32 (H32), HSP70 (H70), HSP90 (H90), or LacZ (LZ; control). A, After 1 day, medium was replaced with DMEM containing 25 mmol/L glucose for 24 hours for the insulin assay. B, After 2 days, medium was replaced with KRB buffer; and insulin release was assayed with 3 mmol/L glucose. Columns and bars represent mean ± SD for 3 separate experiments performed in duplicate. *$P < .01$ vs vehicle-treated cells (n = 9).

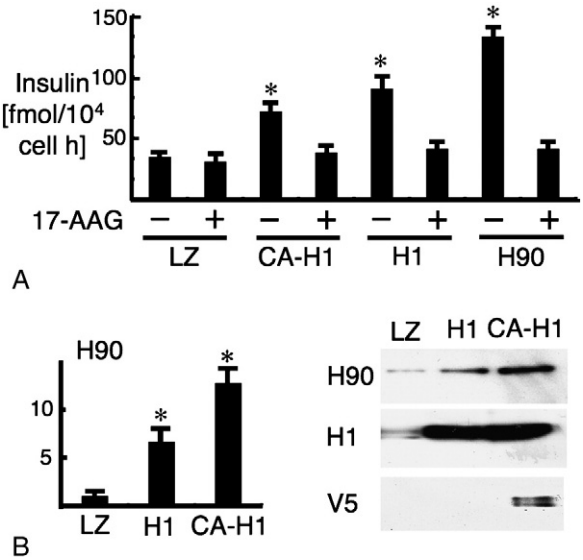


Fig. 3. CA-hHSF1 and HSF1 induce insulin secretion via HSP90 in MIN6 cells. A, MIN6 cells were transfected with adenovirus containing the gene for CA-hHSF1 (CA-H1), HSF1 (H1), HSP90 (H90), or LacZ (LZ; control). The following day, cells were treated with or without 500 nmol/L 17-AAG (an inhibitor of HSP90); and after 24 hours, the medium was replaced with KRB buffer. Insulin secretion was then assayed with 25 mmol/L glucose. Columns and bars represent mean ± SD for 3 separate experiments performed in duplicate. *$P < .01$ vs vehicle-treated cells (n = 9). B, MIN6 cells were transfected with adenovirus containing the gene for CA-hHSF1 (CA-H1), hHSF1 (H1), or LacZ (LZ; control). After 2 days, total cellular protein was extracted; and levels of HSP90 (H90), HSF1 (H1), and V5 (tag protein of CA-hHSF1) were assessed by Western blotting. Columns and bars represent mean ± SD for 3 separate experiments performed in duplicate. *$P < .01$ vs vehicle-treated cells (n = 3).

wild-type hHSF1 and CA-hHSF1 induce insulin secretion via HSP90.

3.4. Effects of HSP90 on CaMKII and nNOS in MIN6 cells

In HEK293 cells, HSP90 activates nNOS via CaMKII [19,20]. We examined whether CA-hHSF1, HSF1, or HSP90 overexpression activated nNOS via CaMKII in MIN6 cells. Overexpression of HSP90 and CA-hHSF1 significantly enhanced the phosphorylation of CaMKII in MIN6 cells (HSP90: 1.78- ± 0.20-fold [n = 6] vs LacZ-infected cells [$P < .05$], CA-hHSF1: 1.66- ± 0.18-fold [n = 6] vs LacZ-infected cells [$P < .05$]) (Fig. 4A). In addition, phosphorylation of nNOS was inhibited by the CaMKII-selective inhibitor KN-93 and nNOS (2.34- ± 0.120-fold [n = 6] vs LacZ-infected cells [$P < .05$]) (Fig. 4B). These results suggest that HSP90 activates nNOS by enhancing phosphorylation by CaMKII.

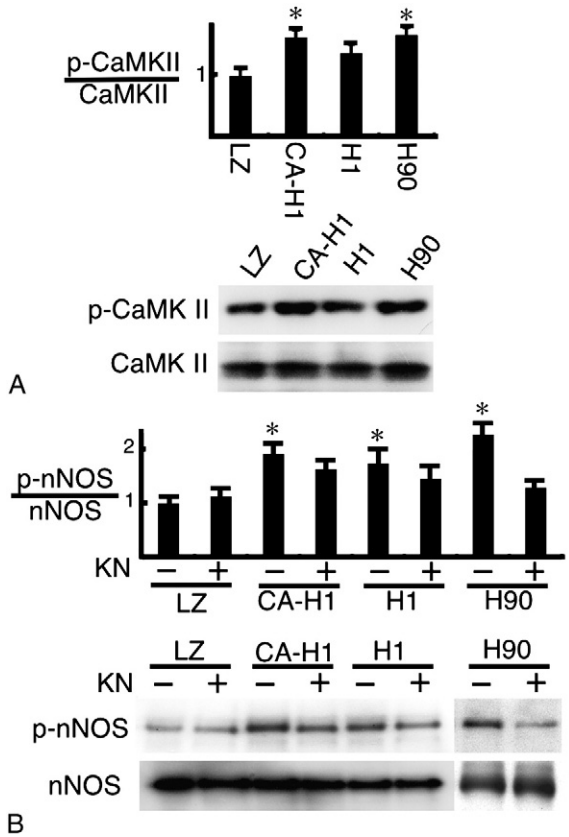


Fig. 4. Overexpression of HSP90 induces nNOS activity via CaMKII activation. MIN6 cells were transfected with adenovirus containing the gene for CA-hHSF1 (CA-H1), hHSF1 (H1), HSP90 (H90), or LacZ (LZ; control). A, After 2 days, total cellular protein was extracted; and levels of phosphorylated CaMKII (p-CaMKII) and total CaMKII protein (control) were assessed by Western blotting. B, After 2 days, cells were treated for 30 minutes with 10 μmol/L KN-93 (a CaMKII inhibitor: KN) or dimethylformamide (vehicle control); and then total cellular protein was examined for levels of phosphorylated nNOS (p-nNOS) and total nNOS protein by Western blotting. Columns and bars represent mean ± SD for 3 separate experiments performed in duplicate. *$P < .01$ vs vehicle-treated cells (n = 6).

3.5. Effects of HSP90 on GCK activity by CaMKII and nNOS in MIN6 cells

We further examined whether overexpression of HSP90 enhances GCK activity via activation of nNOS and CaMKII. KN-93 (a CaMKII-selective inhibitor), 3-Br-7Ni (an inhibitor of nNOS), NPLA (a selective inhibitor of nNOS, substrate of L-arginine), and siRNA targeting nNOS suppressed the enhancement of GCK activity by HSP90 in MIN6 cells (Fig. 5A, B). Furthermore, HSF1 and CA-hHSF1 induced GCK activity via activation of CaMKII. These results suggest that HSP90 expression leads to GCK activation by first activating CaMKII, which then phosphorylates and activates nNOS, finally stimulating GCK.

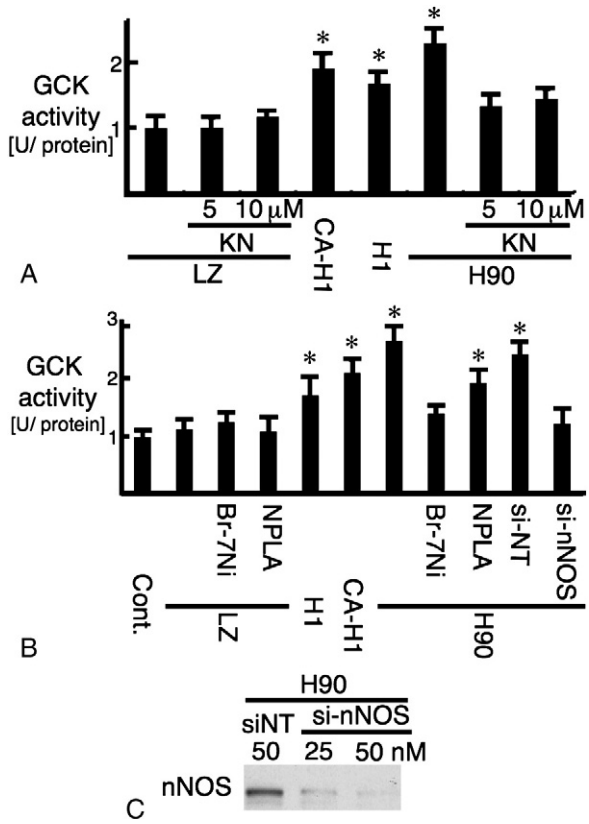


Fig. 5. Overexpression of HSP90 induces GCK activation via nNOS activation. MIN6 cells were transfected with adenovirus containing the gene for CA-hHSF1 (CA-H1), hHSF1 (H1), HSP90 (H90), or LacZ (LZ; control). A, After 2 days, cells were treated for 30 minutes with 5 or 10 μmol/L of KN-93 (a CaMKII inhibitor: KN) or dimethylformamide (vehicle control); and glucokinase (GCK) activity was assayed. B, After 6 hours, cells were transfected for 36 hours with 50 nmol/L of mouse nNOS siRNA (si-nNOS) or nontargeting siRNA (siNT) or for 1 hour with 10 nmol/L 3-Br-7-Ni (Br-7Ni) (an inhibitor of nNOS), 15 nmol/L NPLA (an inhibitor of nNOS), or dimethyl sulfoxide (DMSO) (vehicle control); and GCK activity was assayed. C, Transfection with 25 or 50 nmol/L of mouse nNOS siRNA (si-nNOS) or nontargeting siRNA (siNT) was carried out 6 hours after transfection with HSP90 adenovirus. After 36 hours, total protein was extracted; and the level of nNOS protein was assessed by Western blotting to confirm the knockdown of nNOS expression by mouse nNOS siRNA. Columns and bars represent mean ± SD for 3 separate experiments performed in duplicate. *$P < .01$ vs vehicle-treated cells (n = 9).

3.6. Effect of HSP90 on GCK and nNOS in MIN6 cells

To determine whether activation of GCK by nNOS is direct or mediated by NO production, we examined the effect of 2 general NOS inhibitors: L-NAME and L-NMMA. Neither general NOS inhibitor prevented activation of GCK by HSP90 overexpression (Fig. 6A). We also found that the NO donors SNAP and GSNO inhibited GCK activity in MIN6 cells. Excess NO is toxic to β-cells. Therefore, these NO donors did decrease GCK activity. In addition, we found that L-arginine–like compounds of NOS inhibitors such as L-NAME, L-NMMA, and NPLA did not inhibit GCK activation via HSP90 overexpression. However, 7Ni-Br significantly inhibited GCK activation by HSP90. L-Arginine–like compounds are targets of substrate binding on the catalytic heme domain. However, 7Ni-Br inhibits nNOS activity by means of occupying the nNOS cofactor site [27]. These results suggest that GCK activation via nNOS activation is important for nNOS association with the cofactor site. In addition, these findings suggest that nNOS directly interacts with GCK. Therefore, we examined immunoprecipitated nNOS and identified GCK in complex with nNOS. HSP90 induced binding between nNOS and GCK in the cytosol of MIN6 cells (Fig. 6B-1). This binding was inhibited by 7Ni-Br treatment, suggesting that the nNOS cofactor site is important for GCK binding and activity.

3.7. Effects of CA-hHSF1 overexpression in healthy Wister rats

We further examined the effects of CA-HSF1 on male rats that had not been treated with streptozotocin. Overexpression of CA-hHSF1 reduced blood glucose levels at 15 minutes in the OGTT (Fig. 7A). CA-hHSF1 was seen to increase serum insulin level at 15 minutes (304.2 ± 48.06 pmol/L for CA-hHSF1 vs 210 ± 43.34 pmol/L for LacZ [n = 8, $P < .05$]) (Fig. 7B). However, no significant differences in fasting or nonfasting blood glucose levels were seen between CA-hHSF1 expression and LZ expression rats (Fig. 7C).

3.8. Effects of CA-hHSF1 overexpression in type 2 diabetes mellitus model rats

We examined the effects of CA-hHSF1 in nSTZ male diabetic rats, as a model of type 2 diabetes mellitus. Overexpression of CA-hHSF1 reduced the blood glucose level at 60 and 120 minutes in the OGTT (Fig. 8A).

3.9. Effects of CA-hHSF1 overexpression in the pancreases of rats

Despite the low efficiency of transfection of Ins-CA-hHSF1 in healthy Wister rats, Ins-CA-hHSF1 increased insulin levels in OGTT. We examined the expression of HSF1, HSP90, HSP32, CaMKII, nNOS, and GCK expression in rat pancreases. As shown in Fig. 9, Ins-CA-hHSF1 induced HSF1, HSP90, HSP32, and nNOS expression in the pancreases of rats; however, as shown in Fig. 4B, CA-HSF1,

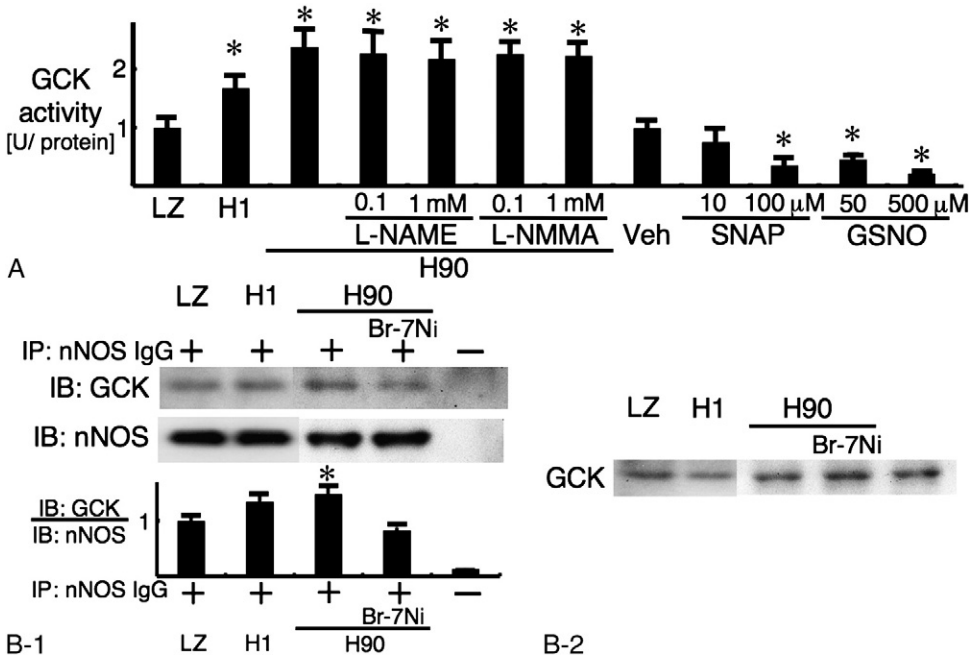


Fig. 6. Overexpression of HSP90 directly activates GCK via nNOS. MIN6 cells were transfected with adenovirus containing the gene for hHSF1 (H1), HSP90 (H90), or LacZ (LZ; control). A, After 1 day, cells were treated with 2 general NOS inhibitors (L-NAME, L-NMMA), 2 nitric oxide donors (SNAP, GSNO), or DMSO (vehicle control) for 24 hours; and GCK activity was assayed. B, After 2 days, cells were treated with 10 nmol/L 3-Br-7-Ni (Br-7Ni) (an inhibitor of nNOS) or DMSO (vehicle control) for 1 hour. Cells were then immunoprecipitated with mouse monoclonal nNOS antibody. B-1, IP: nNOS indicates immunoprecipitation with mouse monoclonal nNOS antibody. IB: nNOS indicates immunoblot with rabbit monoclonal nNOS antibody. The GCK activities were normalized to the concentration of cellular protein. B-2, At immunoprecipitation, before addition of mouse monoclonal nNOS antibody, we took the same cell lysates for examination of GCK expression in cytosol. In all experiments, the value for the control cell was set at 1.0. Columns and bars represent the mean and SD, respectively, for 3 separate experiments performed in duplicate. *$P < .01$ vs vehicle-treated cells (n = 6).

HSF1, and HSP90 failed to induce nNOS expression in MIN6 cells.

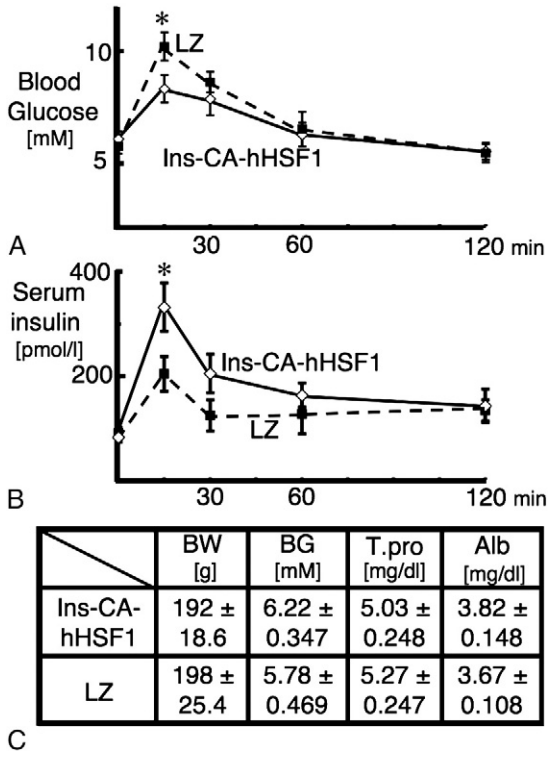
4. Discussion

We examined the role of the following mechanism for CA-hHSF1–induced insulin secretion both in vitro and in vivo. In vitro, CA-hHSF1 induces HSP90 expression, which activates CaMKII, resulting in the phosphorylation of nNOS [19,28]. Activated nNOS then directly activates GCK and finally induces insulin secretion by insulinoma cells. However, with 3 mmol/L glucose, CA-hHSF1 did not induce insulin secretion. Likewise, CA-hHSF1 in vivo did not reduce fasting glucose levels in healthy Wister rats.

We examined nNOS by HSP90 induction of insulin secretion in MIN6 cells. However, nNOS also generates NO; and a large amount of NO induces β-cell apoptosis [14]. Three separate genes encode the 3 isoforms of NOS. Whereas eNOS and nNOS are constitutively expressed, iNOS is usually only expressed during inflammation. Furthermore, iNOS produces much more NO than the other isoforms. Some reports have shown that inhibitors of NOS enhance insulin secretion [29], although nonselective or excessively high concentrations of NOS inhibitors can cause different results through partial inhibition of various isoforms. In β-cells, nNOS is found on membranes of insulin granules [30]. Some investigators have reported that nNOS inhibits insulin secretion via NO production [31,32]. However, we found L-arginine–like compounds of NOS inhibitors, including nNOS selective inhibitors, did not inhibit GCK activation via HSP90 overexpression. We therefore examined the direct effects of nNOS, rather than those via NO production, on insulin secretion. In terms of insulin secretion, GCK plays a key role in whole-body glucose homeostasis by catalyzing the phosphorylation in glucose-sensing cells such as pancreatic β-cells, hepatocytes, and anterior pituitary cells [33,34].

Glucokinase phosphorylates glucose to form glucose-6-phosphate and has a low K_m for glucose, and is thus considered to be a glucose sensor. CA-hHSF1 did not induce hypoglycemia in healthy Wister rats or insulin secretion at glucose concentrations less than 3 mmol/L. This is most likely because glucose is a substrate of GCK. Hypoglycemia is the worst adverse effect of diabetes mellitus therapy. Thus, the failure to induce hypoglycemia is a very advantageous characteristic of CA-hHSF1 for the treatment of diabetes.

The inactivating GCK mutation is known as a *maturity-onset diabetes of the young* subtype. However, activating mutations of GCK are even more infrequent than maturity-onset diabetes of the young. Some reports have described familial activating GCK mutations as inducing hypoglycemia



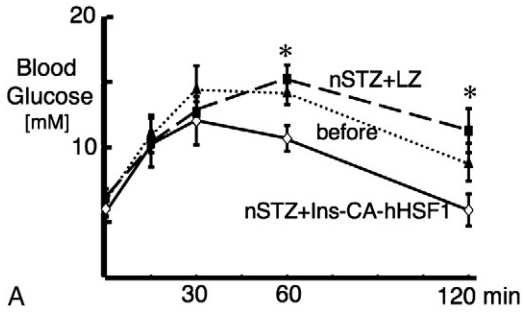
	BW [g]	BG [mM]	T.pro [mg/dl]	Alb [mg/dl]
Ins-CA-hHSF1	192 ± 18.6	6.22 ± 0.347	5.03 ± 0.248	3.82 ± 0.148
LZ	198 ± 25.4	5.78 ± 0.469	5.27 ± 0.247	3.67 ± 0.108

C

Fig. 7. CA-hHSF1 decreases blood glucose levels and increases serum insulin levels in healthy rats. A to C, CA-hHSF1 reduces blood glucose levels in the OGTT in healthy Wister male rats. Five-week-old healthy Wister male rats were infected with adenovirus for expressing Ins-CA-hHSF1 (Ins-CA-hHSF1; ◊) or LacZ (LZ; ■). A, After 5 days, an OGTT was performed. B, Serum insulin levels during the OGTT. Results are represented as mean ± SD (n = 8). C, Profile for Ins-CA-hHSF1–infected and LZ-infected Wister control male rats 1 day after the OGTT at postmeal. *$P < .01$ vs LacZ-infected Wister rats (n = 8). BW indicates body weight; BG, blood glucose; Tpro, total protein; Alb, albumin.

[35], and a K_{ATP} channel mutation is also known to cause congenital hyperinsulinism and hypoglycemia. The K_{ATP} channel mutation induces inappropriate insulin release. However, activating GCK mutations leads to the resetting of the glucose-stimulated insulin release threshold [35]. We thus speculated that activation of GCK is desirable for diabetes mellitus therapy without hypoglycemia.

Based on our studies of insulinoma cells, we expected that overexpression of HSF1 would help protect stressed β-cells, and predicted that CA-hHSF1 could improve insulin secretion by both weakened β-cells and healthy β-cells in vivo. We therefore examined the effects of expressing CA-hHSF1 in rat models of type 2 diabetes mellitus and in healthy Wister male rats under high-glucose conditions. We generated CA-hHSF1–expressing adenovirus, under the control of a human insulin promoter specifically expressed in β-cells, to transfect pancreatic β-cells intravenously from the splenic artery. However, the rats were too small for intravenous injection; therefore, we injected the adenovirus directly into the pancreas through an abdominal incision. This procedure was technically quite simple; however, there were 2 problems. First, the abdominal incision and direct injection into the pancreas induced acute pancreatitis; and second, the adenovirus had a low transfection efficiency into β-cells when directly injected in the pancreas. Therefore, we transfected rats that were not wild type for hHSF1 with CA-hHSF1. β-Cell deficits exist in type 2 diabetes mellitus. However, lean type 2 diabetes mellitus cases have no less than a 41% deficit in relative β-cell volume [36]. This figure suggests that a small amount of β-cells in islets contributes to insulin secretion. Moreover, CA-hHSF1, HSF1, or HSP90 did not induce nNOS expression in MIN6 cells. However, in vitro CA-hHSF1 remarkably induced nNOS expression in pancreas. Therefore, despite the low efficiency of transfection of Ins-CA-hHSF1 in healthy Wister rats, Ins-CA-hHSF1 increased serum insulin level on OGTT in healthy Wister rats. However, nSTZ-diabetic rats already have reduced β-cell numbers. Thus, the β-cell deficit ratio is very important for improvement by Ins-CA-hHSF1 because of low efficiency of transfection. In nSTZ-diabetic rats, STZ was administered on the day of birth. At 4 weeks of age, insulin secretion was exhausted; and the blood glucose level gradually increased [37]. During this period, hyperglycemia leads to a vicious cycle of further losses in β-cell function and free radical production, a process referred to as *glucotoxicity* [38]. We chose nSTZ-*moderate*-diabetic rats, with nonfasting blood glucose levels between 6.67 and 8.33



	BW [g]	BG [mM]	T.pro [mg/dl]	Alb [mg/dl]
nSTZ+Ins-CA-hHSF1	145 ± 30.9	8.50 ± 2.03	4.36 ± 0.27	3.10 ± 0.29
nSTZ+LZ	151 ± 36.0	10.3 ± 2.24	4.65 ± 0.27	3.32 ± 0.21

B

Fig. 8. CA-hHSF1 decreases blood glucose level and increases serum insulin levels in neonatal streptozotocin-induced diabetes male rats. A and B, CA-hHSF1 improves blood glucose level of OGTT in neonatal streptozotocin-induced diabetes male rats. The OGTT was performed in 5-week-old neonatal streptozotocin-induced diabetic male rats before infection with adenovirus (before; ▲). The following day, rats were infected with adenovirus for expression of Ins-CA-hHSF1 (nSTZ + Ins-CA-hHSF1; ◊) or LacZ (nSTZ + LZ; ■). A, After 5 days, OGTT was performed. Blood glucose levels are shown for before and after infection with adenovirus. B, Profile for nSTZ + Ins-CA-hHSF1 and nSTZ + LZ diabetic male rats 1 day after the OGTT at postmeal. Results represent mean ± SD (n = 8). *$P < .01$ vs LacZ-infected neonatal streptozotocin-induced diabetic rats (nSTZ + LZ) (n = 8).

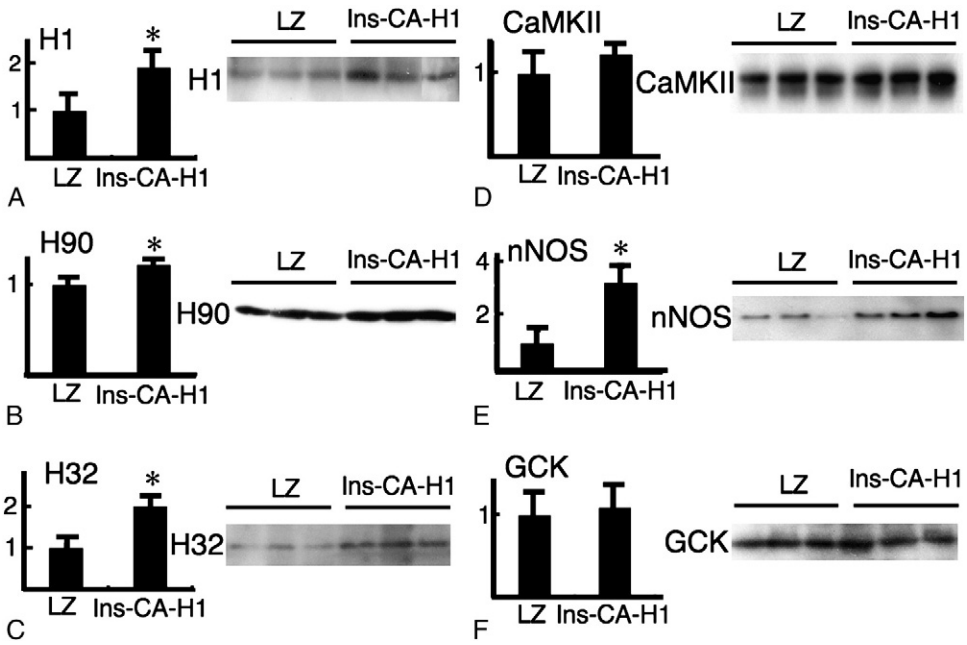


Fig. 9. CA-hHSF1 increased nNOS expression in the pancreases in healthy rats. A to E, Five-week-old healthy Wister male rats were infected with adenovirus for the expression of Ins-CA-hHSF1 (Ins-CA-H1) or LacZ (LZ). After the OGTT, protein from the pancreas tissue was extracted; and levels of HSF1 (H1), HSP90 (H90), HSP32 (H32), CaMKII, nNOS, and GCK protein were assessed by Western blotting. *$P < .01$ vs LacZ-infected Wister rats (n = 6).

mmol/L at 5 weeks of age, as a model of mild type 2 diabetes mellitus. We found that overexpression of CA-hHSF1 improved blood glucose levels on OGTT, especially after 60 minutes in 5-week-old nSTZ-diabetic rats. However, in nSTZ-diabetic rats with high glucose levels, which have very severe β-cell damage by STZ, Ins-CA-hHSF1 did not reduce blood glucose levels on OGTT because of the low efficiency of adenovirus transfer. HSP27 or HSP relieves islet apoptosis in response to cytotoxic agents or oxidative stress [9,39]. HSP32 also has antioxidant properties [10]. We found that CA-hHSF1 induced HSP32 in rat pancreas. We supposed that CA-hHSF1 improved the glucotoxicity of nSTZ-diabetic rats via the induction of HSP32 expression.

In healthy Wister rats, blood glucose was reduced at 15 minutes on OGTT as compared with controls. However, during the fasting and nonfasting periods, we observed no reductions in blood glucose levels in healthy rats. In addition, in MIN6 cells, CA-hHSF1 did not cause higher insulin secretion than in controls in the presence of 3 mmol/L glucose. These results show that CA-hHSF1 enhances glucose-induced insulin secretion and does not induce insulin secretion either in vitro or in vivo.

Heat shock proteins interact with a number of cellular systems and form efficient cytoprotective mechanisms for not only damaged normal cells but also malignant cells. Tumor cells require higher HSP90 activity than normal cells to maintain their malignancy [40]. An inhibitor of HSP90 is applied to anticancer drugs. Cancers of the pancreas mostly derive from pancreatic duct cells and exocrine cells, and endocrine cell cancer of the pancreas is rare. This is one of the reasons why CA-hHSF1 was combined with an insulin promoter that expresses only in β-cells.

In summary, the current results show that CA-hHSF1 induces insulin secretion more strongly than wild-type hHSF1 in MIN6 cells under high-glucose conditions caused by GCK activation. Overexpression of CA-hHSF1 in β-cells enhances glucose-driven insulin secretion not only in type 2 diabetes mellitus model rats but also in healthy Wister rats without causing hypoglycemia. CA-hHSF1 may thus be a useful and safe gene therapy for patients with diabetes mellitus and impaired glucose tolerance, especially for the reduction of postmeal hyperglycemia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.metabol.2010.07.029.

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