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Development of Akt-activated GSK3β inhibitory peptide

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ABSTRACT

Abnormal overexpression of GSK3β has been implicated in insulin resistance. Although many potent GSK3β inhibitors have been developed as drug candidates for anti-insulin resistance, the inhibitors are prone to show side effects because they interfere with normal GSK3β function without regulation. Recently, it was reported that the PPPSPxS motifs in the Wnt coreceptor LRP6 were able to directly inhibit GSK3β only when the motif was phosphorylated. Here, we generated a new GSK3β inhibitory peptide that can be activated by Akt by combining the PPPSPxS motif and an Akt target sequence. The peptide exhibited an inhibitory effect on GSK3β only when it was phosphorylated by Akt in a purified system and in cells when stimulated by insulin. Thus, our findings provide a novel concept for drugs against diseases that are involved in the abnormal GSK3β activity, including type 2 diabetes mellitus.

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1. Introduction

Glycogen synthase kinase 3β (GSK3β) is a serine-threonine kinase that was initially described as a key enzyme for phosphorylating and inhibiting glycogen synthase [1]. GSK3β plays fundamental roles in controlling various cellular functions, including cell division, proliferation, differentiation, and adhesion [2,3]. In response to insulin, GSK3β activity is suppressed by phosphorylation at Ser9 by Akt through an autoinhibitory mechanism in peripheral tissues [4–6].

Accumulating results suggest that hyperactivation of GSK3β in cells is associated with many human diseases, including type 2 diabetes [7,8]. Type 2 diabetes, which accounts for approximately 90% of all diabetes, characteristically begins with insulin resistance [9]. To date, many studies on this have been performed on the molecular mechanism for insulin resistance, although it still remains to be elucidated. As one of possible mechanisms, elevated GSK3β activity has been implicated in insulin resistance. GSK3β protein levels are significantly higher in muscle biopsies from patients with type 2 diabetes, and blood glucose levels are effectively decreased by inhibition of GSK3 in rodent models of type 2 diabetes [10–12]. This elevation of GSK3β inversely correlates with both glycogen synthase (GS) activity and insulin-stimulated glucose disposal [12]. The hyperactivation of GSK3β is also associated with the ribosomal protein S6 kinase isoform 1 (S6K1), which was implicated in the insulin resistance [13,14]. A recent study revealed that GSK3β positively regulates S6K1 [15], and thus use of drugs targeting GSK3β to treat type 2 diabetes was suggested. Even if insulin effectively activates Akt, the total GSK3β activity was not sufficiently suppressed by Akt in the muscle of the diabetic subject [16]. Thus these observations indicated that the efficient inhibition of GSK3β by Akt can be an important step to alleviate the insulin resistance.

GSK3β has been chosen as a potential therapeutic target in multiple diseases including type 2 diabetes mellitus [10,17]. LiCl was reported as the first GSK3β inhibitor in 1996 [18], and more than 30 small molecule GSK3 inhibitors have already been identified including SB216763 and SB415286 [10,19,20]. However, none of them has been approved for clinical use [21], probably because these inhibitors are prone to show side effects if they interfere with normal GSK3β functions. To resolve this unwanted side effect, we designed a GSK3β inhibitory peptide that can be activated by Akt in response to insulin stimulation. Our findings provide a new concept for drugs against diseases that are involved in the abnormal GSK3β activity.

2. Materials and methods

2.1. Plasmids and antibodies

DNA fragments encoding LRP6 motif C (residues 1565–1586 of human LRP6) were obtained by PCR from a human cDNA library, and inserted into the pGEX-TEV vector (a modified vector from

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pGEX-4T1) using *Eco*RI and *Xho*I sites to express the peptide in *Escherichia coli* as a GST-fusion protein with a TEV protease cleavage site. DNA fragments encoding EPVPPPPTPRSSRSRTTSFSES (called LRP-GSK) and EPVPPPPAPRSSRSRTTSFSES (called LRP-GSKmt) were obtained from synthesized genes by Bioneer (Daejeon, Korea) [31]. These DNA fragments were similarly inserted into the vector.

Constitutively active Akt was generated as described previously [32]. Briefly, a DNA fragment encoding the catalytic domain of mouse Akt (residues 126–481) was obtained by PCR from a mouse cDNA library and then inserted into pGEX-TEV using *Eco*RI and *Hind*III sites. Subsequently, T308/S473D mutations were introduced into the resulting vector to mimic the phosphorylated Akt. The anti-phospho-β-catenin (Ser33/37/Thr41) was purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Expression and purification of recombinant proteins

The catalytic domains of mouse CK1ε (residues 1–319) and GSK3β (residues 27–393) were prepared as hexahistidine-tagged proteins as described previously [28]. The N-terminal region (residues 1–133) of β-catenin was purified as described [28]. GST-fused active Akt catalytic domain, LRP-GSK, LRP-GSKmt and LRP6-motifC were expressed in *E. coli* and purified with GST-binding agarose and Q-anion exchange column. The final buffer was changed to 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and 2 mM β-mercaptoethanol for use with Centriprep (10 kDa cutoff; Millipore, Billerica, MA, USA).

2.3. In vitro kinase assay

To produce a substrate for GSK3β, the β-catenin N-terminal region (residues 1–133) was used, with Ser45 pre-phosphorylated by the catalytic domain of CK1ε as described previously [28]. Using the pre-phosphorylated β-catenin fragment as a substrate, the GSK3β activity was measured in buffer containing 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 1 mM ATP at 37 °C for 30 min using 10 ng of the GSK3β catalytic domain in the presence/absence of 5 μg of GST-fused LRP-GSK, LRP-GSKmt, or LRP6-motifC peptide. The reaction was stopped by addition of SDS–PAGE loading buffer and immediate boiling. To assess the effect of LRP-GSK, LRP-GSKmt, and LRP6-motifC phosphorylation, GST-fused LRP-GSK, LRP-GSKmt, and LRP6-motifC were prephosphorylated by CK1ε, GSK3β and/or Akt in buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM β-mercaptoethanol, and 10 mM ATP at 37 °C for 2 h, and then the kinases were removed by adding Ni-NTA agarose beads into the reaction mixture.

2.4. Peptide synthesis

Peptides for inhibition assays were prepared by solid-phase synthesis. The amino acid sequence of each peptide is shown in Fig. 1A. The N-terminus and C-terminus of all peptides were modified by FITC and amidation, respectively. The identity and purity of the synthesized peptides were confirmed by mass spectrometry.

2.5. Cell culture and protein analysis

Cell culture reagents were obtained from Life Technologies (Grand Island, NY, USA). Phospho-glycogen synthase (Ser641, 645) was purchased from Upstate Biotechnology (Lake Placid, NY, USA), and phospho-GSK3β (Ser9), phospho-Akt (Ser473) antibodies were from Cell Signaling Technology (Danvers, MA, USA). β-Actin antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA). HepG2 human hepatocellular carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and the culture was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in 5% CO_2.

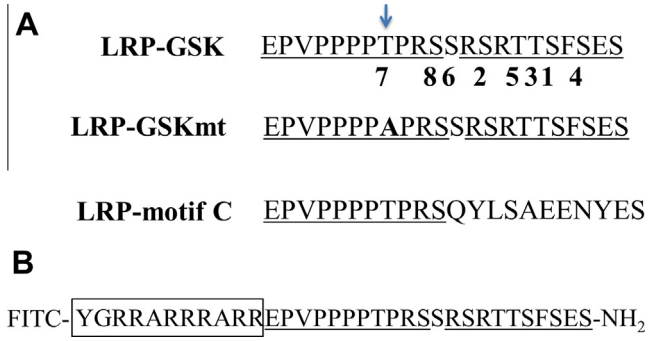

Fig. 1. Constructs of the chimeric peptides in this study. (A) Human LRP6 motif C was chosen as a direct GSK3β inhibitory sequence, and the C-terminus was designed as a sequence that can be phosphorylated by the indicated enzymes in different numbers. The underlined sequences in the left cluster were derived from LRP6 motif C and the sequences in the right cluster were from the GSK3β N-terminal region. The Arabic numerals under the sequence indicate the hypothetical order of phosphorylation by Akt (1), GSK3β (2, and 5–7) or CK1ε (3, 4 and 8). The arrow indicates the essential residue whose phosphorylation is most important in the direct inhibition of GSKβ. (B) The amino acid sequence of the synthetic peptide YGR-LRP-GSK. The N-terminus was modified by FITC and the C-terminus was modified by amidation (NH_2). The cell permeable sequence is boxed.

Prior to treatment of the peptide, HepG2 cells were cultured in six-well plates in serum free DMEM overnight. Then, 10 μM YGR-LRP-GSK peptide in DMSO or DMSO was treated to the cell, and incubated for 4 h. Insulin (100 nM) was treated to cell, and then was harvested after 10 min. To analyze the phosphorylation of Akt, GSK3β, GS using Western blotting, cell pellets were resuspended in RIPA buffer and then separated by 15% reducing SDS–PAGE. After immunoblotting onto PVDF membrane in 20% methanol, 25 mM Tris and 192 mM glycine, the membranes were then blocked with 5% non-fat dry milk and incubated with the primary antibody overnight. The membranes were washed, incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase, rewashed and developed using an enhanced chemiluminescence system (GE Healthcare, Chalfont St Giles, UK).

2.6. Confocal microscopy

Cells were plated in six-well plates containing embedded glass cover slips and treated with 10 μM YGR-LRP-GSK (or DMSO as a control) for 30 min. Cells were fixed and stained with DAPI and observed using confocal microscopy. Confocal microscopy images were acquired using the FV10i Fluoview confocal microscope (Olympus, Tokyo, Japan).

3. Results

3.1. Design of Akt-activated GSK3β inhibitory peptide

We sought potent GSK3β inhibitory sequences, whose inhibitory activity was raised by phosphorylation. The well-known N-terminal region of GSK3β, which contains Ser9, might be a good candidate for this query. However, the inhibitory effect of the sequence was expected to be too weak to inhibit GSK3β when it was added separately (K_i = 700 μM) [22]. Recent findings have revealed that five iterative PPPSPxS motifs in the cytoplasmic domain of the Wnt co-receptor LRP6 act as a direct inhibitor of GSK3β when the motifs are phosphorylated by the membrane-located

GSK3β and CK1 [23]. The first serine residue is known to be phosphorylated by GSK3β and the second serine residue by CK1 in the PPPSPxS motif [24,25]; phosphorylation at the first serine residue plays a more important role in the inhibition of GSK3β [23,24]. Since the first serine residue is not a canonical substrate of GSK3β, the PPPSPxS motif alone was not able to be phosphorylated by GSK3β both in cells and *in vitro* [26]. The phosphorylation of the LRP6 PPPSPxS motifs is required for the Ser/Thr-rich motif in *cis* and another kinase CK1 in *trans* [23,26]. Biochemical study revealed that the inhibitory activity of the dually phosphorylated PPPSPxS motif (motif A; $K_i = 13$ μM) [23] is much stronger than that of the GSK3β N-terminal region [22]. An independent study reported that the third of the five PPPSPxS motifs of LRP6 (called motif C) showed the strongest ability to inhibit GSK3β activity [27], which suggests that the phosphorylated LRP6 motif C is the strongest inhibitory sequence. Thus we chose the LRP6 motif C as a candidate for GSK3β inhibition.

Efficient inhibition of GSK3β requires the phosphorylation of LRP6 motif C. In particular, the phosphorylation of the LRP6 motif C was triggered by Akt for the purposes of this study. We joined the LRP6 region (residues 1565–1575) containing motif C with the GSK3β N-terminal region (residues 4–13) containing the RxRxxS motif, which is a canonical Akt substrate sequence. One serine residue was added between the two regions in order to link the phosphorylation from the RxRxxS motif by Akt to the PPPSPxS motif (Fig. 1). The resulting sequence was "EPVPPPPTPRSSRSRTTSFSES" (designated LRP-GSK), where the PPPSPxS and RxRxxS motifs are underlined; this was generated as a GST-fusion protein. While GSK3β phosphorylation is primed by a phospho-serine or phospho-threonine at the $n+4$ position (S/T-X-X-X-pS/pT), CK1 phosphorylation is primed by a phospho-serine or phospho-threonine at the $n-3$ position (pS/pT-X-X-S/T). Once the serine residue at RxRxxS is phosphorylated by Akt, the other serine or threonine residues are mutually primed by CK1 and GSK3β, leading to phosphorylation at the serine residue of the PPPSPxS motif. The proposed order of phosphorylation of the chimeric peptide is shown in Fig. 1 and is analogous to the phosphorylation of APC [28]. Since the cellular activity of CK1 is constitutive, the Akt activity would be the rate-determining step in the phosphorylation of this chimeric peptide in a cell with hyperactivated GSK3β.

The sequence harboring an alanine substitution at the PPPSPxS motif was generated as a control and designated as LRP-GSKmt. In addition, the sequence consisting of only the LRP6 motif C region (residues 1565–1586) was also generated and was designated as LRP-motifC.

3.2. The chimeric protein is a direct inhibitor of GSK3β in a phosphorylation-dependent manner

We next tested whether the chimeric peptide showed GSK3β inhibitory activity depending on the phosphorylation state. The three GST-fusion proteins described in Fig. 1 were purified and then phosphorylated by a mixture of recombinant Akt, GSK3β, and CK1. After removing the kinases, we measured the GSK3β activity using the β-catenin N-terminal region as a substrate. To make the canonical substrate for GSK3β, the β-catenin fragment was prephosphorylated at Ser45 by CK1 as described previously [23]. When Ser45 is phosphorylated, the Ser37 and Ser33 are sequentially and efficiently phosphorylated by GSK3β [24]. We monitored the GSK3β activity by immunoblotting the β-catenin substrate with anti-phospho-β-catenin (S33/37).

As shown in Fig. 2A, the phosphorylated chimeric peptide (LRP-GSK) inhibited the enzymatic activity of GSK3β, whereas the alanine mutant (LRP-GSKmt) and LRP-motifC did not inhibit GSK3β activity (Fig. 2B and C). The upshift of the LRP-GSK band on the gel suggested that LRP-GSK was phosphorylated by the kinases

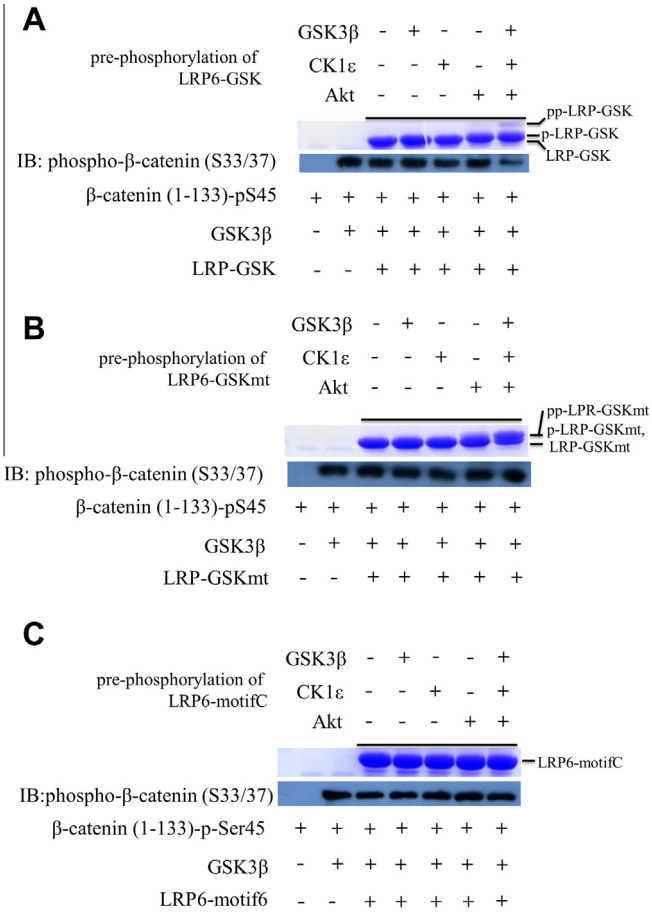

Fig. 2. Phosphorylated LRP-GSK sequence inhibits GSK3β activity on the β-catenin N-terminal region. (A) Pre-phosphorylated LRP-GSK was added to the reaction when β-catenin (1–133)-*p*-Ser45 was phosphorylated by GSK3β. SDS–PAGE was applied to analyze the effect of pre-phosphorylation of LRP-GSK by GSK3β, CK1ε, AKT or GSK3β/CK1ε/AKT (Top). The band shift suggests the phosphorylation states of LRP-GSK: p-LRP-GSK indicates phosphorylated LRP-GSK by Akt, and pp-LRP-GSK indicates phosphorylated LRP-GSK by Akt, CK1, and GSK3β. Phosphorylation of β-catenin (1–133)-*p*-Ser45 was detected with anti-phospho-β-catenin (S33/37) antibody (Bottom). The same experiments were performed in (B) and (C) with LRP6-GSKmt and LRP6-motifC, respectively.

(Fig. 2A). Thus, this result shows that the phosphorylated LRP-GSK is a phosphorylation-dependent inhibitor of GSK3β. The intact PPPSPxS motif is required for inhibition of GSK3β [24], but the LRP-GSKmt peptide harbored the alanine substitution at the critical serine residue in the PPPSPxS motif. As expected, the LRP-motifC was not phosphorylated by the kinases because the peptide sequence does not contain the Ser/Thr-rich cluster or the non-canonical phosphorylation site [26]. Since the cellular CK1 activity is constitutively present, these results suggest that LRP-GSK can regulate GSK3β activity in response to insulin stimulation in the presence of high GSK3β levels.

3.3. The peptide decreases the cellular GSK3β activity only when cells are treated with insulin

To see the effect of the designed peptide in cell, we chose HepG2 cell, which is derived from human liver cells. To deliver the peptide to the cytoplasm of the cells, a cell permeable sequence was fused to the N-terminus of the peptide (YGR-LRP-GSK). To monitor whether the peptide is penetrated into the cell, the peptide was labeled with FITC. The cellular activity of GSKβ was monitored through Ser641/645 phosphorylation of GS using the

phosphorylation-specific antibody. Prior to treatment of insulin, the peptide with the cell-permeable sequence was pretreated to the cells, and incubated for 4 h. Cells were treated with insulin, and then the phosphorylation (activation) of the cellular Akt, the Ser9 phosphorylation of GSK3β, and the cellular GSK3β activity was measured by the Western blotting analysis using the phosphorylation-specific antibodies.

As expected, the phosphorylation of Akt and the phosphorylation of GSK3β were increased in response to insulin (Fig. 3). The pretreatment of the peptide significantly reduced the phosphorylation of GS (or the cellular activity of GSK3β) only when the insulin was treated, whereas the peptide did not affect the phosphorylation of GS in the absence of insulin stimulation. However, the peptide did not affect the phosphorylation levels of Akt and GSK3β regardless of the insulin treatment. We verified that the peptide is transported into the cytosol by the fluorescence microscope. These results indicate that the peptide inhibited the activity of GSK3β in response to insulin without regulation of the activities of Akt and the phosphorylation at Ser9 of GSK3β. Our observations are consistent with the results using the purified system, and further indicate that the designed peptide is activated in response to insulin through the activated Akt, leading to additional inhibition of GSK3β.

4. Discussion

Besides type 2 diabetes, GSK3β is involved in several other diseases, including inflammation, bipolar disorder, Alzheimer's disease, and other neurodegenerative diseases [29]. In the process of the downregulation of the TLR-mediated inflammation signaling, the downregulation of the proinflammatory signals is retarded when the GSK3β activity is efficiently inhibited by the Akt [30]. Herein we designed a novel peptide by combining the PPPSPxS motif from the LRP6 C-terminal region and the RxRxxS motif from the GSK3β N-terminal region, since the PPPSPxS motif can inhibit GSK3β and the RxRxxS motif can be phosphorylated by Akt. *In vitro* phosphorylation assays suggested that the phosphorylation triggered by Akt can propagate into the PPPSPxS motif by the mutual priming action of GSK3β and CK1, as expected. *In vitro* experiment using the purified proteins showed that the phosphorylated peptide was capable of inhibiting GSK3β, as expected. Consistently, the peptide significantly inhibited the cellular GSK3β activity only in the presence of insulin stimulation when a cell permeable sequence is attached to the peptide. This inhibition of GSK3β was independent on the inhibitory phosphorylation of GSK3β (Ser9), indicating the additive inhibition of the GSK3β.

The peptide designed in this study requires both the Akt, and GSK3β to be activated. Because the activity of GSK3β is suppressed by Akt, the two kinases could be barely activated simultaneously in cells except for the pathological state. Thus our peptide would give a limited effect on the normal cells. Therefore, the peptide developed in this study is expected to have reduced side effects in diverse types of normal cells, compared to constitutively active GSK3β inhibitors.

Release of insulin from the pancreatic β-cell is stimulated by an elevated blood glucose level, and the insulin release is stopped when the blood glucose level is back to the normal [17]. However, insulin or the conventional (or constitutively-active) GSK3β inhibitor can or could cause the hypoglycemic shock because they could lower the blood glucose level even when the blood glucose concentrations fall below a normal level. By contrary, our peptide could lower the blood glucose level only in the hyperglycemic state because the peptide is activated in response to insulin, and thus the peptide is unlikely to cause the hypoglycemic shock.

We carried out animal test to see whether the peptide exhibits the blood glucose level only in the presence of insulin that is secreted when the blood glucose level is higher than the normal state. Unfortunately, the significant effect was not observed when the peptide contained the cell permeable sequence *in vivo* (data not shown). Probably the peptide does not have sufficient efficacy, stability, or cell membrane permeability, which are necessary properties to see the *in vivo* effects. However, if new techniques for overcoming these drawbacks are developed in the future, our

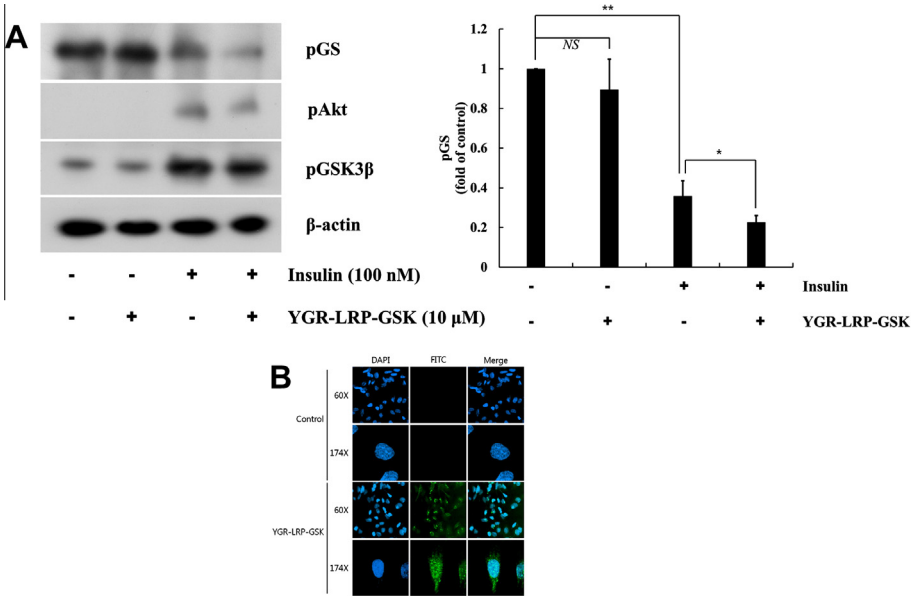

Fig. 3. Insulin-dependent inhibition of GSK3β in HepG2 cells by the peptide (YGR-LRP-GSK). (A) Serum-starved HepG2 cells were pretreated with DMSO (−) or 10 μM YGR-LRP-GSK solubilized in DMSO (+). After 4 h incubation, the insulin was treated to the cell, and then the phosphorylation of Akt (Ser473), GSK3β (Ser9), and GS (Ser641/645) was assessed by the Western blotting. Three independent experiments were carried out and a representative blot is shown in the left. The quantified results are displayed in the right panel. The band intensities were measured with the program ImageJ (NIH). Data are expressed as means of triplicates ± standard error of the measurements ($^{*}p < 0.05$, $^{**}p < 0.01$ by Student's *t*-test). (B) The confocal microscope images. The HepG2 cells were incubated with the FITC-labeled peptide (YGR-LRP-GSK), and then stained with DAPI. In the control cell, DMSO was treated to the cells instead of the peptide.

peptide would be a good candidate as an anti-diabetes drug that does not cause a hypoglycemic shock. In conclusion, our results represent a conceptual advance in the drug design for type 2 diabetes mellitus and other diseases with abnormal GSK3β activity.

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