Phospho-GSK-3β is involved in the high-glucose-mediated lipid deposition in renal tubular cells in diabetes

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The phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) pathway mediates the high-glucose-induced lipid accumulation in the renal tubular cells in diabetes. Studies have revealed that the downstream target genes of phospho-Akt, BCL2-associated death promoter (Bad) and glycogen synthase kinase-3β (GSK-3β) are associated with lipid accumulation in hepatic cells and prediabetic. In the present study, it was revealed that the phospho-Akt, phospho-Bad, phosphor-GSK-3β contents and lipogenesis were increased in the renal tubular cells of diabetic rats. However, in high-glucose-treated human renal tubular cells, only the phospho-GSK-3β content increased without an alteration in the phospho-Bad content, which could be reversed by treatment with a short hairpin RNA vector aimed at Akt. Inhibiting GSK-3β activity using TWS119 increased the sterol regulatory element binding protein-1 (SREBP-1) content and lipogenesis in renal tubular cells. Furthermore, the exogenous expression of wild-type GSK-3β enhanced the level of phospho-GSK-3β in high-glucose-stimulated renal tubular cells, followed by increased SREBP-1 expression and lipogenesis. Moreover, exogenous expression of mutant GSK-3β (via vector 9S) prevented the increase in SREBP-1 expression and cellular lipogenesis by decreasing the phospho-GSK-3β content and increasing the GSK-3β activity in high-glucose-treated cells. These data suggested that phospho-GSK-3β is involved in the high-glucose-mediated increase of SREBP-1 expression and triglyceride content in renal tubular cells in diabetes.

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1. Introduction

Diabetic nephropathy is a severe complication of diabetes mellitus; the factors involved in the pathogenesis of this disease include hyperglycemia (Isoe et al., 2010), inflammatory cytokines (Ortiz-Munoz et al., 2010) and oxidative stress (Chang et al., 2011a). Diabetic nephropathy is characterized by hypertrophy of the renal mesangial cells (Dey et al., 2010), accumulation of extracellular matrix (Araoka et al., 2010), epithelial–mesenchymal transition (EMT) of the renal tubular cells and interstitial fibrosis (Hills and Squires, 2010). Among these, injury of the renal tubular cells is the most important factor in decreasing renal function. Previous studies demonstrated that high glucose could induce lipid droplet deposition and, subsequently, extracellular matrix accumulation and interstitial fibrosis in renal tubular cells (Sun et al., 2002; Wang et al., 2005). In our research, high glucose was found to be an effector leading to increased SREBP-1 and cellular triglyceride contents in renal tubular cells (Jun et al., 2009). Furthermore, we also found that activated Akt mediated the high-glucose-induced increase in SREBP-1 expression and lipogenesis; moreover, inhibition of Akt using a siRNA vector prevented the up-regulation of SREBP-1 expression and lipid droplet formation in renal tubular cells (Hao et al., 2011). However, it is well known that the PI3K/Akt pathway is multi-functional and is involved in cell functions such as growth, apoptosis, motility (Saudemont and Colucci, 2009), metabolism and transduction (Engelman et al., 2006). Furthermore, the PI3K/Akt pathway includes various downstream target genes, including Bad (Motoyama et al., 2009), Foxo1 (Leger et al., 2006), mTOR (Zoncu et al., 2011), PRAS40 (Dey et al., 2010) and GSK-3β (Takamatsu et al., 2010). It is unclear which one is involved in the PI3K/Akt pathway-mediated enhancement of SREBP-1 expression and lipogenesis in high-glucose-treated renal tubular cells. Exposing the human prediocyte cell line AML-1 to EPA (eicosapentaenoic acid) increased the accumulation of cytoplasmic lipids, as well as the accumulation of Bad and Akt (Hanada et al., 2011). In addition, Liu et al. found that serum withdrawal induced a marked decrease in the intracellular and plasma membrane cholesterol levels and in the levels of Akt inactivation and
Bad dephosphorylation in mouse embryonic fibroblasts (MEFs) obtained from [DHR24C(-/-)] mice lacking DHR24, which is the enzyme that catalyzes the last step of cholesterol biosynthesis ([Lu et al., 2006]). These studies suggested that Bad expression might be associated with lipid metabolism as a downstream target of Akt. Moreover, Chang et al. revealed that ER stress induced the expression of long chain acyl-CoA synthetase 3 (ACSL3) and the accumulation of lipids in HuH-7 cells. GSK-3β inhibitors attenuated the ACSL3 expression and lipid accumulation induced by ER stress. Similarly, shRNA that targets GSK-3β also inhibited the up-regulation of ACSL3 expression and lipid accumulation. The above data indicate that GSK-3β is a novel therapeutic target treatment of the lipid dysregulation caused by ER stress ([Chang et al., 2011b]). Therefore, we explored the potential function of Bad and GSK-3β in the abnormal renal lipid metabolism in diabetes.

Diabetic rats and HKC cells were chosen to investigate the effect of Bad and GSK-3β on the PI3K/Akt-induced increase of SREBP-1 expression and lipogenesis. First, we determined the Bad, phospho-Bad, GSK-3β and phospho-GSK-3β levels in the renal tubular cells of diabetic rats and high glucose-stimulated HKC cells. Second, HKC cells were treated with TWS119 to determine the effect of activated GSK-3β on SREBP-1 expression and lipogenesis. Third, we constructed wild type GSK-3β vector and point mutant GSK-3β vector (S9A) that were transfected into high-glucose-treated HKC cells to further verify whether phospho-GSK-3β mediated PI3K/Akt activation-induced increased SREBP-1 expression and lipogenesis.

2. Materials and methods

2.1. Reagents and materials

The antibodies against Akt, phospho-Akt, Bad, phospho-Bad, GSK-3β and phospho-GSK-3β were purchased from Epitomics Inc. (Burlingame, CA, USA). The SREBP-1, ADRP and β-actin primary antibodies were purchased from Santa Cruz Biotechnology (CA, USA). The GSK-3β activity detection kit was purchased from Genmed Scientifics (Arlington, MA, USA). 4,6-disubstituted pyrrolo-pyrimidine (TWS119) was obtained from Selleck Chemicals (Houston, TX, USA).

2.2. Animals

Sixteen SD rats were purchased from the animal center of Hebei Medical University. The animal were fed the standard diet and housed at a room temperature of 22 ± 2°C. Ten rats were chosen for intraperitoneal STZ-injection to become diabetic models, and the rest were normal rats. One week after the injection the rats with serum glucose levels >16.7 mM were regarded as successful models. The level of blood glucose in the tail vein was determined weekly so that the rats regaining normal blood glucose could be discarded. The rats were raised for eight weeks, when they were sacrificed. At the end of the experiment, the rats’ blood was obtained from the abdominal aorta for determination of the renal function, blood glucose and blood lipids. The renal cortices were collected for the extraction of the total proteins or preparation of histological sections.

2.3. Cell line and cell culture

HKC cells were cultured as described previously ([Zhao et al., 2012]) and divided into different groups. The HKC cells were grown in medium containing 10% serum, 1% penicillin–streptomycin and 5% CO2. To determine the effect of high glucose, the cells were divided into the three following groups: the normal glucose group (5.5 mmol/L glucose), the high-glucose group (30 mmol/L glucose) and the mannitol control group (5.5 mmol/L glucose and 24.5 mmol/L mannitol). Mannitol was used to exclude the osmotic effects of high glucose on the HKC cells. The various assays were performed after 48 h of stimulation with high glucose. In addition, to explore the effect of Akt activation on GSK-3β, a shRNA vector aimed at Akt was used; HKC cells were divided into four groups: normal medium, high-glucose medium, high-glucose medium plus the sh-scramble vector and high-glucose medium plus the sh-Akt vector. Because it is the classic specific GSK-3β inhibitor, 10 μM TWS119 was used to explore the relationship between GSK-3β activity and SREBP-1 expression, as well as lipid metabolism, in HKC cells. Furthermore, to investigate the effect of the phosphorylation of GSK-3β on high-glucose-induced lipogenesis, HKC cells were transfected with a wild-type GSK-3β vector or a mutant GSK-3β vector (S9A).

2.4. Construction of plasmids

A plasmid encoding wild type GSK-3β was constructed according to standard protocols using pEGFP-N1 as the basic vector. First, a vector containing the complete CDS of the human GSK-3β gene was purchased from the Yiqiaoshenzhou Company (Beijing, China). The full-length sequence of the CDS was amplified by PCR using the vector as the template. The amplified product and the pEGFP-N1 vector were digested with EcoRI and BglII, respectively, for approximately 3 h at 37°C. The ligation of PCR segment and pEGFP-N1 was conducted using T4 ligase at 16°C overnight. The correct ligation was confirmed by double enzymes cutting and sequencing. The point mutation vector was obtained by PCR amplify and DpnI digestion. The digestion product was transfected into DH5α and the correct clone was confirmed by sequencing.

2.5. Transient transfection

Transient transfection was conducted using Lipofectamine 2000. In brief, the HKC cells were seeded in six-well plates. When the cells were approximately 80% confluent, transient transfection was performed as described previously. The mixture of 4.0 μg of vector DNA and 10 μl of Lipofectamine 2000 was added to 2 ml of serum-free DMEM medium. After 5 h, this medium was replaced with normal DMEM medium containing 10% fetal bovine serum (FBS) for 12 h. Then, to obtain the relevant data, the cells were cultured for 48 h in different media.

2.6. Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed according to the standard protocol described in the previous report, and the negative controls were obtained by replacing the specific antibodies with PBS. The details of the immunofluorescence procedure are as follows: the HKC cells were fixed with 10% formalin in 15 min at room temperature. After blocking with goat serum for 30 min at 37°C, the cells were incubated with anti-ADRP monoclonal antibody (1:100) overnight at 4°C. Then, the cells were observed after washing three times with PBS and incubation with FITC-labeled secondary antibody (Santa Cruz Biotechnology, CA, USA) for 60 min at 37°C.

2.7. Western blotting

Proteins were extracted from renal tissues or HKC cells according to the standard procedure. The proteins were blotted to PVDF membranes (Millipore Corporation, Bedford, MA) after electrophoresis. The membranes were blocked for 1 h at 37°C with Tris-buffered saline containing 0.05% Tween and 5% BSA and then incubated with primary antibodies (Bad 1:1000, phospho-Bad 1:1000; GSK-3β 1:1000; phospho-GSK-3β 1:1000; Akt 1:1000;
phospho-Akt 1:1000; β-actin 1:1000; or SREBP-1: 1:500) overnight at 4 °C. Subsequently, the membranes were rinsed three times with TBST and then incubated with a secondary antibody (1:5000) for 2 h at room temperature. After three rinses with TBST, the blots were treated with ECL solution (Pierce, Rockford, IL) and the labeled bands were detected using an Odyssey Infrared Imaging System.

2.8. Triglyceride quantification

The lipids were extracted from renal cortical tissues or HKC cells using the Folch method (Folch et al., 1957) employed in a previous study (Hao et al., 2012). The triglycerides were detected using a kit from Zhejiang Dongou Company.

2.9. GSK-3β kinase activity assay

To determine the effects of TWS119 and exogenously expressed wild-type GSK-3β or mutant GSK-3β (S9A) on the GSK-3β activity in HKC cells, a GSK-3β kinase kit (Genmed, Arlington, MA, USA) was used according to the manufacturer’s instructions. In brief, the cells were rinsed twice with reagent A and then treated with reagent B, the lysis buffer. After 30 min incubation on ice, the mixture was centrifuged at 12,000 × g for 5 min at 4 °C. The supernatant was collected and GSK-3β activity was detected using an enzyme activity kit by mixing 65 μl of reagent C, 10 μl of reagent D, 10 μl of reagent E and 10 μl of reagent F, followed by an incubation at 30 °C for 3 min. After adding 5 μl of the supernatant, the optical density at 340 nm was measured at 0 min and 5 min.

2.10. Statistical analysis

All of the data are presented as the mean values ± standard deviation (SD) and were analyzed using SPSS 13.0 for Windows software. Comparisons between two groups were conducted using an independent sample T-test and comparisons among multiple groups were conducted using a one-way analysis of variance (ANOVA). Furthermore, the Student–Newman–Keuls test was used to determine the statistical significance of the differences within and between the groups. P < 0.05 was statistically significant.

3. Results

3.1. Diabetic rats exhibited mild renal dysfunction

Diabetic rats maintained for approximately eight weeks were sacrificed and blood samples were taken for measurements of the blood glucose, blood lipids and the BUN. The results revealed that an increased blood glucose, decreased body weight and mild renal function injury were found in diabetic rats compared with normal rats (Table 1).

3.2. The phospho-Bad and phospho-GSK-3β levels were increased in the kidneys of diabetic rats

As shown in Supplementary Fig. S1, Bad, GSK-3β, phospho-Bad and phospho-GSK-3β was expressed in the cytoplasm of renal tubular cells. However, no marked difference in Bad and GSK-3β expression was found in the normal rats and diabetic rats.

3.3. The levels of phospho-Akt and SREBP-1 were increased in the renal tubular cells of diabetic rats

The expression of Akt, the key gene in the PI3K/Akt pathway and of genes relevant in lipid metabolism, including SREBP-1 and ADRP, was detected in the kidneys of diabetic rats and normal rats. As shown in Fig. 2, the contents of phospho-Akt and SREBP-1 were up-regulated in the kidneys of diabetic rats compared with normal control rats. Notably, the level of phospho-Akt was increased by approximately 2.98 times in diabetic rats. Similarly, the contents of the precursor and mature segments of SREBP-1 were increased by 2.55 times and 2.28 times, respectively, in diabetic rats.

3.4. Increased triglycerides in the kidneys of diabetic rats

Oil Red O staining revealed that the lipid deposits in the kidneys of diabetic rats were located in the tubular cells, seen as red-stained granules. ADRP, a marker of lipid droplets, was detected by immunohistochemistry and found in the same location, that is, the cytoplasm of renal tubular cells. The expression of ADRP was increased in diabetic rats compared with normal rats (Supplementary Fig. S2). Triglyceride analysis also confirmed that the renal triglyceride content was increased in diabetic rats (Table 2).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiocel.2013.07.007.

3.5. High glucose increased the level of phospho-GSK-3β in HKC cells

Stimulation with high glucose significantly increased the phospho-GSK-3β and phospho-Akt levels in HKC cells, without altering the phospho-Bad, Bad, GSK-3β and Akt levels. As shown in Fig. 3, the phospho-GSK-3β content was increased 2.21-fold in the high-glucose-treated HKC cells compared with normal control cells. In addition, there was no difference in phospho-GSK-3β levels of the normal glucose group and the mannitol control group. Furthermore, the level of phospho-Akt, the key regulator of the PI3K/Akt pathway, was also increased significantly in the high-glucose-stimulated HKC cells. In addition, as shown in Fig. 4, Oil Red O staining revealed obvious lipid droplets in the

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>Blood glucose (mmol/L)</th>
<th>Blood triglyceride (mmol/L)</th>
<th>Blood cholesterol (mmol/L)</th>
<th>BUN (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>425 ± 46.90</td>
<td>5.58 ± 0.42</td>
<td>1.18 ± 0.26</td>
<td>1.35 ± 0.13</td>
<td>6.43 ± 0.69</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td>271.43 ± 37.96</td>
<td>27.38 ± 2.70</td>
<td>3.17 ± 1.03</td>
<td>1.93 ± 0.41</td>
<td>9.72 ± 1.75</td>
</tr>
</tbody>
</table>

1 P < 0.05 vs. normal rats.

Table 2

The renal cortical triglyceride content in normal rats and diabetic rats (as ± s, mg/ml).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>6</td>
<td>588.92 ± 59.57</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td>6</td>
<td>771.17 ± 69.83</td>
</tr>
</tbody>
</table>

1 P < 0.05 vs. normal rats.
Fig. 1. Expression of phospho-Bad and phospho-GSK-3β was increased in the kidneys of diabetic rats. Western blotting was used to analyze the expression of phospho-Bad and phospho-GSK-3β in the kidneys of diabetic rats and normal rats. The levels of phospho-Bad, Bad, phospho-GSK-3β and GSK-3β were quantified by densitometry (mean values ± SD, n = 6). * Indicates P < 0.05 vs. normal rats.

Fig. 2. Diabetic rats exhibited up-regulation of phospho-Akt, the precursor segment of SREBP-1 and the mature segment of SREBP-1 in the renal cortex. SREBP-1 was synthesized as the 1150-amino acid precursor protein that remains bound to the endoplasmic reticulum. Following a sequential two-step cleavage process, the NH2-terminal portion that is the mature segment of SREBP-1 was released. Western blot analysis was applied to detect the expression of phospho-Akt, Akt, the precursor segment of SREBP-1 and the mature segment of SREBP-1 (mean values ± SD, n = 6). * Indicates P < 0.05 vs. normal rats.

high-glucose-stimulated HKC cells; in contrast, lipid droplets were not observed in the cells in the normal control group or mannitol group. Similarly, ADRP was also found to be up-regulated in the HKC cells treated with high glucose. Finally, determination of the cellular triglycerides revealed that the cells in the high-glucose group contained more triglycerides than did the cells of the normal group or the mannitol group (Table 3).

Table 3 The triglyceride content in HKC cells stimulated with high glucose (¯x ± s, mg/ml).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Triglyceride content</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG</td>
<td>6</td>
<td>70.22 ± 8.65</td>
</tr>
<tr>
<td>HG</td>
<td>6</td>
<td>141.03 ± 8.31</td>
</tr>
<tr>
<td>Mannitol</td>
<td>6</td>
<td>70.11 ± 11.44</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. NG (normal glucose) group.
Fig. 3. High glucose increased the expression of phospho-GSK-3β, phospho-Akt and SREBP-1 in HKC cells. Western blotting was used to analyze the effect of high glucose on phospho-Bad, Bad, phospho-GSK-3β, GSK-3β, phospho-Akt, Akt and SREBP-1 protein contents in HKC cells. The levels of phospho-Bad, Bad, phospho-GSK-3β, GSK-3β, phospho-Akt, Akt and SREBP-1 were quantified by densitometry (mean values ± SD, n = 6); * indicates P < 0.05 vs. normal glucose group.

Fig. 4. High glucose increased ADRP expression and lipid droplet formation in HKC cells (400×). Lipid droplets were detected using with the method of Oil Red O staining. ADRP expression was evaluated by immunofluorescence.

3.6. Inhibition of Akt using the sh-Akt vector decreased the phospho-GSK-3β content of high-glucose-treated HKC cells

To investigate whether phospho-Akt mediates the phosphorylation of GSK-3β in high-glucose-treated HKC cells, a shRNA vector aimed at Akt (sh-Akt) was used to suppress the activation of Akt. As expected, the sh-Akt effectively decreased the expression of phospho-Akt. Furthermore, the high-glucose-mediated increase of the phospho-GSK-3β content was prevented, as shown in Fig. 5. Determination of the triglyceride content revealed that sh-Akt effectively decreased the cellular triglyceride content in the high-glucose-treated HKC cells, as shown in Table 4.

Table 4
The triglyceride content in HKC cells treated with sh-Akt vector (X ± s, mg/ml).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Triglyceride content</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG</td>
<td>6</td>
<td>69.69 ± 7.36</td>
</tr>
<tr>
<td>HG</td>
<td>6</td>
<td>141.59 ± 10.12</td>
</tr>
<tr>
<td>HG + sh-Scramble</td>
<td>6</td>
<td>141.48 ± 11.16</td>
</tr>
<tr>
<td>HG + sh-Akt</td>
<td>6</td>
<td>78.79 ± 8.75</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. HG (high glucose) group.
3.7. TWS119 caused the up-regulation of SREBP-1 expression and increased the level of cellular triglycerides in HKC cells

TWS119 was used to explore the direct effect of GSK-3β activity on SREBP-1 expression and lipid metabolism in HKC cells. We treated HKC cells with 10 μM TWS119 and the results demonstrated that it effectively suppressed the activity of GSK-3β via the GSK-3β kinase assay (Fig. 6A). In addition, compared with those of the normal control cells, the levels of SREBP-1 and ADRP were increased in the TWS119-treated HKC cells, as shown in Fig. 6B and C. Moreover, TWS119 treatment increased the cellular triglyceride content by 25.68%, compared with that of the normal control cells as shown in Table 5.

3.8. The wild-type GSK-3β vector and mutant GSK-3β vector (S9A) were successfully constructed

The full-length human GSK-3β gene was amplified from the gene clone using primers that included restriction enzyme sites. After the pEGFP-N1 vector was digested with the appropriate enzymes, the GSK-3β segment was ligated into it. The recombinant vector was identified by restriction enzyme digestion. In addition, the successful construction of the vector was proven by sequencing. The mutant plasmid was constructed by complementary primer amplification and DpnI digestion. Sequencing confirmed the mutant plasmid was valid; the results are shown in Supplementary Fig. S3.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.07.007.

3.9. Mutant GSK-3β effectively prevented the high-glucose-induced increase in SREBP-1 expression and lipogenesis

As shown in Fig. 7, transfection of the wild-type GSK-3β vector increased the expression of exogenous GSK-3β in HKC cells cultured in normal glucose medium, compared to that of untransfected cells. However, the level of phospho-GSK-3β did not differ among the three groups. Similarly, the GSK-3β kinase activity assay revealed that the exogenous expression of wild-type GSK-3β did not affect the GSK-3β activity of HKC cells cultured in medium with normal glucose (Fig. 7B). Correspondingly, the exogenous expression of wild-type GSK-3β also did alter SREBP-1 expression in any of the three groups of HKC cells.

HKC cells in the high-glucose plus wild-type GSK-3β vector group showed higher expression of exogenous GSK-3β and exogenous phospho-GSK-3β (Ser9). In addition, GSK-3β activity was decreased by wild-type GSK-3β vector compared with that in the HKC cells in the high-glucose untransfected group. In contrast, the up-regulation of exogenous phospho-GSK3β (Ser9) due to transfection of the wild-type GSK-3β vector was prevented completely by transfection of the mutant GSK-3β vector (S9A). Similarly, GSK-3β activity was significantly increased by transfection of the mutant GSK-3β vector (S9A) (Fig. 8A). Consistently, transfection with the wild-type GSK-3β vector increased the expression of SREBP-1 and the deposition of cellular lipid droplets compared with that in high-glucose-treated untransfected HKC cells. However, transfection of the mutant GSK-3β vector (S9A) suppressed the increase in SREBP-1 expression and increased the level of cellular triglycerides in HKC cells stimulated with high glucose (Figs. 8B and C, Table 6).

4. Discussion

PI3Ks are a family of related intracellular signal transducer enzymes capable of phosphorylating the 3-position hydroxyl group of the inositol ring of phosphatidylinositol. Through activating protein kinase B, also called Akt, PI3-kinases have been linked to an extraordinarily diverse group of cellular functions, including cell

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**Table 5**
The triglyceride content in HKC cells treated with TWS119 (x±s, mg/ml).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Triglyceride content</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG</td>
<td>6</td>
<td>69.50 ± 7.50</td>
</tr>
<tr>
<td>NG+TWS119</td>
<td>6</td>
<td>87.35 ± 5.80</td>
</tr>
</tbody>
</table>

*P<0.05 vs. NG (normal glucose) group.
Fig. 6. TWS119 enhanced the cellular expression of ADRP by inhibiting GSK-3β activity. A: GSK-3β kinase activity was evaluated in HKC cells in the control group and the TWS119-treated group. *Indicates $P<0.05$ vs. control group. B: Western blotting analysis was used to clarify the effect of 10 μM TWS119 on SREBP-1 expression (mean values ± SD, n = 6). *Indicates $P<0.05$ vs. blank control group. C: Immunofluorescence was applied to detect the expression of ADRP in the control and TWS119-treated HKC cells.

growth, proliferation, differentiation, motility, survival and intracellular trafficking (Engelman, Luo, 2006; Saudemont and Colucci, 2009).

The PI3K/Akt pathway was proven to be involved in lipid metabolism in a few tissues and cultured cells. Some researchers reported a relationship between SREBP-1 expression and activation of the PI3K/Akt pathway in hepatitis patients (Jackel-Cram et al., 2010; Park et al., 2009). Our previous research demonstrated that the PI3K/Akt pathway mediated the high-glucose-induced increase in SREBP-1 expression and the accumulation of cellular triglycerides (Hao et al., 2011). However, the exact mechanism involved in the phospho-Akt-induced SREBP-1 up-regulation in the high-glucose-stimulated HKC cells is unknown.

In the present study, we first investigated Bad and GSK-3β, downstream targets of phospho-Akt, in the kidneys of diabetic rats. The results revealed that the levels of phospho-Bad and phospho-GSK-3β were increased in the renal tubular cells of diabetic rats compared with those in normal rats, accompanied by an increase in the phospho-Akt content, SREBP-1 expression and lipogenesis. These data suggested that as the downstream effectors of phospho-Akt, phospho-Bad and phospho-GSK-3β might mediate the renal lipid metabolic pathology of diabetic rats. Similarly, Chang et al. found the GSK-3β inhibitors attenuated the long-chain acyl-CoA synthetase 3 (ACSL3) expression and the lipid accumulation induced by endoplasmic reticulum stress in hepatocytes, which suggested that GSK-3β is a novel therapeutic target for lipid
Fig. 7. The effect of the wild-type GSK-3β vector on the expression of phospho-Akt, Akt and SREBP-1 in HKC cells cultured with normal glucose medium. A: Expression of endogenous phospho-GSK-3β, exogenous GSK-3β, endogenous GSK-3β, phospho-Akt, Akt and SREBP-1 were determined by Western blot analysis and quantified by densitometry (mean values ± SD, n = 6) in HKC cells transfected with the control pEGFP-N1 vector or the wild-type GSK-3β vector. * Indicates P<0.05 vs. the untransfected group. B: GSK-3β kinase activity in HKC cells cultured with normal glucose medium.

Dysregulation caused by ER stress (Chang et al., 2011b). In another study, the status of GSK3β was examined in vivo in the renal cortex of db/db mice with type 2 diabetes at 2 weeks and 2 months of diabetes. Diabetic mice exhibited increased phosphorylation of the renal cortical GSK3β and decreased phosphorylation of elf2Bepisol at two weeks, and increased laminin beta1 and fibronectin protein contents at two months, which correlated with renal hypertrophy (Mariappan et al., 2008).

In addition, in vitro cultured HKC cells, high glucose enhanced the level of phospho-GSK-3β without changing that of phospho-Bad, and subsequently up-regulated SREBP-1 expression and lipid accumulation. These findings suggest that high glucose, the main characteristic of diabetes, might lead to lipogenesis in renal tubular cells via the phosphorylation of GSK-3β, not the phosphorylation of Bad. Then, increase in the kidneys of diabetic rats is most likely involved in pathological responses other than lipogenesis. Therefore, factors other than high glucose mediate the phosphorylation of Bad in the lipid metabolic pathology of diabetic nephropathy. These hypotheses warrant further research. Mariappan et al. reported that both high glucose and high insulin induced Ser-9 phosphorylation and inactivation of GSK3β at 2 h that lasted for up to 48 h. This was associated with the dephosphorylation of elf2Bepisol and elf2, and increased phosphorylation of 4E-BP1 and elf4E (Mariappan et al., 2008). Thus, high glucose is a stimulator that leads to the phosphorylation of GSK-3β, that is, inactivation of GSK-3β.

GSK-3β, a downstream target of phospho-Akt, was phosphorylated in HKC cells treated with high glucose. The phosphorylation of GSK-3β was inhibited when the activity of Akt was suppressed using a shRNA vector aimed at Akt. These data suggested that GSK-3β was regulated by phospho-Akt in HKC cells. Notably, the same regulatory mechanism was also found in gliomas (Atkins et al., 2012), airway epithelial cells (Wang et al., 2012) and cardiomyocytes (Ishikawa et al., 2012).

Inhibiting GSK-3β activity with TWS119 increased SREBP-1, ADRP expression and lipid deposition in HKC cells cultured in normal glucose medium, confirming the direct effect of GSK-3β activity on lipid metabolism in renal tubular cells. GSK-3β has been proven to be involved in lipid metabolism in many types of cells and animals. Diaz-Velasquez CE et al. reported that staurosporine rapidly commits 3T3-F442A cells, a type of pre-adipocyte, to the formation of adipocytes by activating GSK-3β (Diaz-Velasquez et al., 2008). A stable zebrafish transgenic line, Tg(krt4Hsa.myrAkt1)(cy18), displays a severely obese phenotype, a rapid increase in body weight, hyperplastic growth of adipocytes and an abnormal accumulation of fat tissues, accompanied by the activation of the endogenous downstream targets of mTOR, GSK-3α/β and 70S6K (Chu et al., 2012). Mice lacking the essential mTORC2 component rictor in their livers [Lirictor(KO)] fail to develop hepatic steatosis on a high-fat diet and manifest half-normal serum cholesterol levels. This condition is accompanied by lower levels of expression of SREBP-1c and SREBP-2, as well as decreased phosphorylation of Akt and its substrates FoxO, GSK-3β, PRAS40, AS160, and Tsc2 (Yuan et al., 2012). Therefore, we suggested that phospho-GSK-3β mediated high-glucose-induced lipogenesis in renal tubular cells.

In this study, we constructed a wild-type human GSK-3β vector and a mutant human GSK-3β vector (S9A). Transfection of high-glucose-treated HKC cells with the wild-type GSK-3β vector increased their GSK-3β expression. However, due to the inhibitory effects of high glucose, transfection with the wild-type GSK-3β vector did not lead to increased GSK-3β activity. Similarly, the content of phospho-GSK-3β (Ser9), which is the inactivated form of
Fig. 8. Effect of the wild-type GSK-3β vector and the mutant GSK-3β vector (S9A) on phospho-Akt, Akt, SREBP-1 and the cellular lipogenesis in HKC cells cultured in high-glucose medium. A: GSK-3β kinase activity was evaluated in HKC cells cultured in high-glucose medium. B: Normalized by β-actin, the levels of expression of exogenous phospho-GSK-3β, endogenous phospho-GSK-3β, exogenous GSK-3β, endogenous GSK-3β, phospho-Akt, Akt and SREBP-1 were determined by western blotting analysis and quantified by densitometry in HKC cells transfected with the pEGFP-N1 vector, wild-type GSK-3β vector or mutant GSK-3β vector (S9A). * Indicates P < 0.05 vs. untransfected group. # Indicate P < 0.05 vs. the wild-type GSK-3β vector-transfected group. C: Cellular lipid droplet formation was detected using the method of Oil Red O staining in HKC cells transfected with the pEGFP-N1 vector, wild-type GSK-3β vector or mutant GSK-3β vector (S9A) (400×).
GSK-3β was increased in high glucose-treated HKC cells transfected with the GSK-3β wild-type vector, subsequently resulting in the over-expression of SREBP-1 and lipid accumulation. However, these effects could be prevented by GSK-3β with a mutation in its phosphorylation site (Ser9), which increased GSK-3β activity by preventing the inhibitory effect of high glucose on GSK-3β activity. These results indicated that phosphorylated GSK-3β (Ser9) was an important effector of lipid metabolism in HKC cells treated with high glucose, but GSK-3β was not. Therefore, combined with Eldar-Finkelman’s finding that serine-9 is a key regulatory site of GSK-3 inactivation and that cells expressing the 9MA mutant present decreased glycosyn synthase activity (50%) [Eldar-Finkelman et al., 1996], it is possible to conclude that treatment with the point mutant might be an effective method to prevent the high-glucose-induced renal lipogenesis resulting from the phosphorylation of GSK-3β.

In conclusion, GSK-3β mediated the PI3K/Akt-induced up-regulation of SREBP-1 expression and lipid deposition in HKC cells treated with high glucose through phosphorylation by phospho-Akt. Phosphorylated GSK-3β, the inactivated form of GSK-3β, promoted the up-regulation of cellular triglycerides in HKC cells.

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References


