Dysregulation of low-density lipoprotein receptor contributes to podocyte injuries in diabetic nephropathy

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Dysregulation of low-density lipoprotein receptor contributes to podocyte injuries in diabetic nephropathy. Am J Physiol Endocrinol Metab 308: E1140–E1148, 2015. First published April 28, 2015; doi:10.1152/ajpendo.00591.2014.—Dyslipidemia plays crucial roles in the progression of diabetic nephropathy (DN). This study investigated the effects of high glucose on lipid accumulation in podocytes and explored its underlying mechanisms. Male db/db and db/db mice were fed a normal chow diet for 8 wk. Immortalised mouse podocytes were treated with or without high glucose for 24 h. The changes to the morphology and ultrastructures of the kidneys in mice were examined using pathological staining and electron microscopy. Intracellular lipid accumulation was evaluated by Oil Red O staining and a free cholesterol quantitative assay. The expressions of the molecules involved in low-density lipoprotein receptor (LDLr) pathway and podocyte injury were examined using immunofluorescent staining, real-time PCR, and Western blot. There were increased levels of plasma lipid, serum creatinine, and proteinuria in db/db mice compared with db/m mice. Moreover, there was significant mesangial matrix expansion, basement membrane thickening, podocyte foot process effacement, and phenotypic alteration in the db/db group. Additionally, lipid accumulation in the kidneys of db/db mice was increased due to increased protein expressions of LDLr, sterol regulatory element-binding protein (SREBP) cleavage-activating protein, and SREBP-2. These effects were further confirmed by in vitro studies. Interestingly, the treatment with LDLr siRNA inhibited lipid accumulation in podocytes and decreased the protein expression of molecules associated with phenotypic alteration in podocytes. High glucose disrupted LDLr feedback regulation in podocytes, which may cause intracellular lipid accumulation and alteration of podocyte phenotype, thereby accelerating DN progression.

diabetic nephropathy; podocyte; low-density lipoprotein receptor

DIABETIC NEPHROPATHY (DN) is a major complication of diabetes mellitus and the leading cause of end-stage renal disease worldwide (2). Multiple mechanisms contribute to the development and outcome of DN, such as an interaction between hyperglycemia-induced metabolic and hemodynamic changes and genetic predisposition, which sets the stage for kidney injury (23). Dyslipidemia is an independent risk factor for the development and progression of DN (9). Recently, several lines of evidence have confirmed that the treatment of diabetic mice with a high-fat diet exacerbates albuminuria and glomerular lesions (10). Accordingly, the concept of synergistic toxicity caused by glucose and lipids, described as “glucolipotoxicity,” has emerged in recent years (9). Previous studies demonstrated that high glucose could induce lipid droplet deposition and, subsequently, extracellular matrix accumulation and fibrosis in the kidneys (20, 22). However, the underlying molecular mechanisms of this process have not been completely elucidated.

High glucose can induce macrophage expression of scavenger receptors such as CD36, class A scavenger receptor, and lectin-like oxidized LDL receptor-1, which subsequently promotes macrophage-derived foam cell formation (4, 6, 8). In the kidney, high glucose also increases mesangial cell expression of cholesterol influx regulators and suppresses cholesterol efflux modulators (19). Furthermore, Merscher-Gomez et al. (13) reported that cholesterol accumulation in podocytes plays a pathogenic role in DN and provided evidence of the beneficial effect of targeting this pathway.

The low-density lipoprotein receptor (LDLR) is the primary receptor and internalization mediator of plasma-derived LDL cholesterol and regulates plasma LDL concentrations (1, 7). It is widely expressed in both the liver and kidney. LDLR gene expression in mammalian cells is regulated predominantly by a negative feedback mechanism that depends on the mediation of intracellular cholesterol concentration by sterol regulatory element-binding proteins (SREBPs) and SREBP cleavage-activating protein (SCAP) (16). The current study was undertaken to provide an explanation for cholesterol accumulation in the kidney in diabetes by determining whether elevated plasma glucose concentrations affect LDLR expression in podocytes and subsequently exacerbate the podocyte injuries and glomerulosclerosis.

MATERIALS AND METHODS

Animal model. Male db/m mice and db/db mice with a C57BL/KsJ genetic background were obtained from the National Model Animal Centre of Nanjing University (Nanjing, China) and were studied using protocols approved by the Ethics Committee of Southeast University, following the latest version of the Declaration of Helsinki. The mice were maintained under a constant 12-h photoperiod at temperatures between 21 and 23°C and allowed free access to food and water. Eight-week-old male mice were fed a normal chow diet containing 4% fat for 8 wk. Individual mice were placed in metabolic cages for 24-h urine collection. At the end of the experimental period, blood samples were obtained for the biochemical assays and kidney samples used for histological assessments.

Biochemical assays. After the experimental period, the mice were euthanized and blood samples obtained from the right ventricle for biochemical analysis using automatic analyzers (Hitachi). The concentrations of blood glucose (BG), blood urea nitrogen, and serum creatinine (Scr) were measured by glucose oxidase method, electrode method, and picric acid method, respectively. And the concentrations of triglyceride (TG) and total cholesterol (TC) were measured by gycerol kinase method and cholesterol oxidase method, respectively. The concentrations of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were determined by clearance method. The
quantitative analysis of 24-h urinary protein was determined using the Lowry assay.

Morphological analysis. The kidneys were either fixed in 10% buffered formalin and embedded in paraffin or fixed in 2.5% glutaraldehyde and embedded in Lowicryl K4M resin. Further procedures included periodic acid-Schiff (PAS) staining, Masson’s trichrome staining, and transmission electron microscopy. For the analysis of mesangial matrix expansion and glomerular sclerosis, 20 glomeruli were counted per slide and five slides counted per experimental group. The Image J analysis software was used to calculate the area of pink or red mesangial matrix deposition and blue fibrosis within each glomeruli and the entire glomeruli area. The ratio of pink or red area to the entire glomeruli area is the percentage of mesangial matrix deposition in a given glomeruli, and the ratio of blue area to entire glomeruli area is the percentage of fibrosis.

Immunohistochemical staining. After deparaffinization, sections were placed in citrate-buffered solution (pH 6.0) and heated for antigen retrieval. Subsequently, the sections were incubated with anti-mouse primary antibodies against LDLr, SREBP-2, and SCAP (Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with biotinylated secondary antibodies. Finally, a diaminobenzidine tetrahydrochloride substrate was used to develop the reaction. The results were observed under a light microscope (×400).

Immunofluorescent staining. The kidney sections were fixed with 4% paraformaldehyde and blocked in phosphate-buffered saline containing 10% bovine serum albumin for 60 min. The sections were incubated with rabbit, mouse, or goat anti-mouse primary antibodies against Wilms’ tumor 1 (WT-1), nephrin, LDLr, and α-smooth muscle actin (α-SMA) (Santa Cruz Biotechnology) overnight at 4°C, followed by goat anti-rabbit Fluor 488, donkey anti-rabbit Fluor 594, goat anti-mouse Fluor 594, and donkey anti-guoth Fluor 488 secondary fluorescent antibodies (Invitrogen), respectively. After washing, the slides were examined by laser confocal microscopy (×400).

Cell culture. The mouse conditionally immortalized podocyte cell line (kindly provided by Peter Mundel at the Mount Sinai School of Medicine) was cultured and induced to differentiate as described previously (15). In brief, the cells were cultured on dishes coated with type 1 collagen (BD Bioscience) and in RPMI 1640 medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 33°C in the presence of 10 U/ml mouse recombinant interferon-γ (Sigma). To induce differentiation, the podocytes were cultured under nonpermissive conditions at 37°C in the absence of interferon-γ for 10–14 days, as established previously. To determine the effect of high glucose, the cells were divided into three groups: the normal glucose group (5 mmol/l glucose), the mannitol group (15 mmol/l mannitol), and the high-glucose group (30 mmol/l glucose). Mannitol was used to exclude the osmotic effects of high glucose on the podocytes. The various assays were performed after 24 h of high glucose stimulation.

The transfection of LDLr siRNA. The podocytes were cultured in six-well plates and transiently transfected with siRNA of LDLr and empty vector siRNA (Realgene), of which the final concentrations were 40 nmol/l using Lipofectamine RNAiMax (Invitrogen), following the manufacturer’s protocol. Briefly, 2 μl of LDLr siRNA and 5 μl of Lipofectamine RNAiMax were mixed with 100 μl of Opti-MEM medium (Gibco) at room temperature for 10 min before seeding in a well containing 900 μl of serum-free RPMI 1640. At the indicated time points, the cells were harvested and examined by Oil Red O staining, quantitative assay of intracellular cholesterol, immunofluorescent staining, and real-time PCR.

Observation of lipid accumulation. The lipid accumulation in cultured podocytes or mice kidneys was evaluated by Oil Red O staining. Briefly, samples were fixed with 4% paraformaldehyde solution and then stained with Oil Red O for 30 min. Finally, the samples were counterstained with hematoxylin for 5 min. The results were examined by light microscopy.

### Table 1. Mouse TaqMan primers for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
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<tbody>
<tr>
<td>Nephrin</td>
<td>Sense 5'GCCCAAGTGATACAGAAGCCGAC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'TCTGCTGACACAGCTTCAG-3'</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Sense 5'GGGANATGTTGGTAATGCAA-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'TGAAAATGATTCTGCT-3'</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Sense 5'ACCCAGTTGATGACATTTTC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'TCTTCCCGACCGACATAG-3'</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Sense 5'GCCCAATCAAGGCTACTCGG-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'GAAGGAGTTGCTGCT-3'</td>
</tr>
<tr>
<td>LDL receptor</td>
<td>Sense 5'GCCCAAGTGATACAGAAGCCGAC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'GACTTGTTGCTATGCTGTA-3'</td>
</tr>
<tr>
<td>SCAP</td>
<td>Sense 5'GCCGCTGACACAGCTTCAG-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'GGCTATTGCGGCCTTC-3'</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>Sense 5'GCCCAATCAAGGCTACTCGG-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'GAAGGAGTTGCTGCT-3'</td>
</tr>
<tr>
<td>β-ACTIN</td>
<td>Sense 5'GCCCAATCAAGGCTACTCGG-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'GACTTGTTGCTGCTGTA-3'</td>
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α-SMA, α-smooth muscle actin; SREBP-2, sterol regulatory element-binding protein-2; SCAP, SREBP cleavage-activating protein.
were evaluated for statistical significance using one-way analysis of variance followed by the Q-test, and correlations between variables were analyzed by Spearman rank-order correlation. A difference was considered statistically significant if the P value was <0.05.

RESULTS

Diabetic mice exhibited obesity and hyperlipidemia. As shown in Table 2, there were significantly increased plasma concentrations of BG, TG, and TC in the diabetic mice compared with the controls. In addition, db/db mice had higher body weights than db/m mice (P < 0.01) (Fig. 1).

Diabetic mice develop renal dysfunction, mesangial matrix expansion, and proteinuria. The levels of Scr were markedly higher in db/db mice than in db/m mice (Table 2). Compared with db/m mice, the glomeruli of db/db mice displayed increased positive PAS staining, which is a hallmark of mesangial expansion with higher extracellular matrix (ECM) protein contents (Fig. 2, A and C). Masson staining also demonstrated that there was a significant accumulation of collagen fibre (stained blue) in the glomeruli in the db/db mice, which was further confirmed by a quantitative analysis of positive staining using Image J software (Fig. 2, B and D). The transmission electron microscopy analysis demonstrated that db/db mice have a more significant degree of thickened glomerular basement membrane (GBM) and podocyte foot process fusion than db/m mice (Fig. 2E). The 24-h urine protein amount of the db/db group was increased significantly compared with db/m group, and it continued to elevate with diabetes progression (Fig. 2F), which usually is an early indicator of DN.

High glucose exacerbated the podocyte injury in vivo and in vitro. To examine the potential role of high glucose in podocyte injuries, we observed the podocyte phenotype and function in the kidneys of db/db mice and in high glucose-treated podocytes. As shown in Fig. 3, A and B, there is a significant decrease in the number of WT-1-positive cells in the glomeruli of db/db mice. Additionally, immunofluorescent staining revealed that there was increased α-SMA protein expression and decreased nephrin protein expression in the podocytes of db/db mice (Fig. 3C). These findings were in accord with those from the Western blot (Fig. 3, D and E). In the in vitro study, high glucose suppressed podocyte nephrin expression and increased α-SMA, fibronectin, and collagen I expression in podocytes, as shown by immunofluorescence staining (Fig. 3F), real-time PCR (Fig. 3G), and Western blotting (Fig. 3, H and I). These data strongly suggest that high glucose causes a phenotypic change that is reminiscent of epithelial-mesenchymal transi-

Fig. 1. Body weight change in 2 groups of mice. Eight-week-old male db/m and db/db mice (n = 10) were fed a normal chow diet containing 4% fat for 8 wk. The body weight change was measured each week. The values are represented as means ± SD *P < 0.01 vs. db/m group.
ing the translocation of the SCAP/SREBP-2 complex from the ER to the Golgi.

The increased LDLr expression was closely associated with podocyte injuries. We further analyzed the correlation between LDLr protein expression and podocyte injury using Western blotting. The results demonstrated that LDLr protein expression was negatively associated with nephrin protein expression (\( r = -0.937, n = 5, P < 0.05 \)). Moreover, a positive correlation was observed between LDLr and α-SMA protein expression (\( r = 0.748, n = 5, P < 0.05 \)). These findings suggest that the dysregulation of LDLr pathway in podocytes may be closely associated with podocyte injury and glomerulosclerosis.

Inhibition of LDLr pathway decreased lipid accumulation in podocytes and alleviated podocyte injuries. To explore whether the LDLr directly mediated the high glucose-induced lipid accumulation and phenotypic alteration in podocytes, an RNA interference-mediated knockdown of LDLr was done. Oil Red O staining showed that LDLr siRNA inhibited the high glucose-induced lipid accumulation in podocytes (Fig. 6A). This finding was in accord with the results from intracellular cholesterol quantitative assays (Fig. 6B). Furthermore, inhibi-

**Fig. 2.** Diabetic mice develop proteinuria and renal injuries. Eight-week-old male db/m and db/db mice (\( n = 10 \)) were fed a normal chow diet containing 4% fat for 8 wk. A and B: the histopathological changes were assessed by periodic acid-Schiff (PAS) staining (A) and Masson’s trichrome staining (B). C and D: the semiquantitative values for the positive areas are expressed as means ± SD from the mice in each group. E: changes to the glomerular ultramicrostructure were examined using electron microscopy (\( \times 10,000 \), arrows show the glomerular basement membrane, and stars show the foot process fusion). F: quantitative analysis of the 24-h urinary protein each week in the mice was performed using the Lowry assay. Values are represented as means ± SD. *\( P < 0.01 \) vs. db/m group.
Fig. 3. High glucose-induced phenotypic alterations and podocyte damage. Eight-week-old male db/m and db/db mice were (n = 10) were fed a normal chow diet containing 4% fat for 8 wk. A–E: the protein expression of Wilms’ tumor 1 (WT-1) (red; A), nephrin (green; B), and α-smooth muscle actin (α-SMA) (green; C) was measured using immunofluorescent staining (original magnification ×400) and Western blotting (D and E). The histogram represents means ± SD of the densitometric scans of the protein bands from mice normalized by comparison with β-actin. *P < 0.05 vs. db/m group. The podocytes were induced into a quiescent state using serum-free medium for 24 h and then maintained in serum-free medium containing 5 mmol/l glucose (NG), 5 mmol/l glucose and 25 mmol/l mannitol (NG + M), or 30 mmol/l glucose (HG) for 24 h. F: immunofluorescence analysis of nephrin, fibronectin, collagen I, and α-SMA expression in cells. G–I: real-time PCR (G) and Western blot (H and I) for nephrin, fibronectin, collagen I, and α-SMA expression in podocytes.
tion of LDLr mRNA significantly increased the expression of nephrin and decreased the expression of $\alpha$-SMA, fibronectin, and collagen I, as demonstrated by real-time PCR and immunofluorescent staining (Fig. 6, C and D). These data suggest that LDLr is involved in the lipid accumulation and phenotypic alteration induced by high glucose in podocytes.

DISCUSSION

Lipid-mediated renal injury is an important factor of glomerulosclerosis. Recently, several investigators have shown the presence of lipid deposits in the kidneys of experimental animals and diabetic patients. These researchers proposed that these lipid deposits may play an important role in the pathogenesis of diabetic kidney disease (14, 18). However, the potential mechanisms of lipid disorder in DN progression have not been elucidated. In this study, we investigated the effects of high glucose on lipid accumulation in DN podocytes.

First, using $db/db$ mice, a model of type 2 diabetes mellitus characterized by hyperglycemia, hyperlipidemia, and obesity, we observed that there was some evidence of DN, which was shown by an increased ratio of urine microalbumin to creatinine, increased matrix protein, mesangial expansion, and GBM thickening. Diabetes mellitus does not consist of isolated metabolic aberrations; rather, these aberrations are closely linked. The recognition that hypertriglyceridemia is associated with the alteration of multiple other lipoproteins has expanded the view of diabetic dyslipidaemia. Therefore, we investigated the effect of high glucose on lipid metabolism in podocytes and observed its role in the progression of renal injury using $db/db$ mice. A significant finding from our study is that there were excessive amounts of lipid deposits in the diabetic kidney, as shown by Oil Red O staining as well as an intracellular cholesterol quantitative analysis. These changes to cholesterol and triglyceride contents in diabetic kidneys were in accord with the observed renal functional and structural injuries. Previous studies demonstrated that high glucose increased lipid accumulation in renal tubular cells and mesangial cells in diabetic kidneys (11, 19). Our study demonstrated that high glucose enhanced lipid accumulation in podocytes. These changes may contribute to DN progression in conjunction with other risk factors (17).

Lipid accumulation in diabetic kidney cells may originate from an imbalance between lipid influx and efflux, including increased lipid uptake and biosynthesis or decreased lipid efflux from cells. The LDLr, whose expression is regulated predominantly by the intracellular cholesterol pool at the transcriptional level through a negative feedback mechanism, is an important cholesterol influx receptor. In this study, we found that LDLr expression in podocytes was increased in the diabetic mice compared with control mice. The results from the in vitro study also confirmed that high glucose significantly increased the expression of LDLr, SCAP, and SREBP-2 in podocytes. Further analysis showed that upregulated LDLr protein expression was mediated by increased protein expression of SREBP-2 and SCAP and enhanced SCAP/SREBP-2

Fig. 4. High glucose increased lipid deposition in podocytes. Eight-week-old male $db/m$ and $db/db$ mice ($n = 10$) were fed a normal chow diet containing 4% fat for 8 wk. A: kidneys were examined for lipid accumulation by Oil Red O staining. B: cholesterol ester components in the kidneys were measured as described in MATERIALS AND METHODS. Results are represented as means ± SD. *$P < 0.05$ vs. $db/m$ group. C: podocytes were induced into a quiescent state using serum-free medium for 24 h and then maintained in serum-free medium containing NG, NG + M, or HG for 24 h. Cells were examined for lipid accumulation by Oil Red O staining. D: free cholesterol and cholesterol ester were assayed as described in MATERIALS AND METHODS. Results are represented as means ± SD. *$P < 0.05$ vs. NG group.
Fig. 5. High glucose increased LDL receptor (LDLr) expression and sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP)/SREBP-2 complex translocation from the endoplasmic reticulum (ER) to the Golgi. Eight-week-old male db/m and db/db mice (n = 10) were fed a normal chow diet containing 4% fat for 8 wk. A–C: the colocalization of LDLr (green) and WT-1 (red) was measured using immunofluorescence staining (original magnification, ×400; A), and the protein expression of LDLr, SCAP, and SREBP-2 was measured by Western blotting (B and C). Histogram represents means ± SD of the densitometric scans of the protein bands from mice normalized by comparison with β-actin. *P < 0.05 vs. db/m group. Podocytes were induced into a quiescent state using serum-free medium for 24 h and then maintained in serum-free medium containing NG, NG+M, HG for 24 h. D: the translocation of the SCAP/SREBP-2 complex from the ER to the Golgi in podocytes was detected by confocal microscopy. E–G: real-time PCR (E) and Western blot for nephrin, fibronectin, collagen I, and α-smooth muscle actin (α-SMA) expression in podocytes (F and G). Results are represented as means ± SD. *P < 0.05 vs. NG group.
complex translocation from the ER to the Golgi. However, after treatment with LDLr siRNA, high glucose-induced lipid accumulation in podocytes was significantly decreased. Similarly, Sun et al. (20) found that in diabetes mellitus, hyperglycemia directly upregulates renal expression of the transcription factor SREBP-1, which causes increased fatty acid synthesis and triglyceride accumulation. Studies from Proctor et al. (17) showed that CXCL16 is one of the main receptors in human podocyte mediating the uptake of oxidized LDL during diabetic nephropathy onset. Additionally, high concentrations of plasma glucose cause lipid accumulation in mesangial cells by altering the expression of uptake molecules scavenger receptor A1 and CD36 or efflux molecules adenosine triphosphate-binding cassette (ABC)A1 and ABCG1. Our study showed that the LDLr pathway is an important main cholesterol influx regulator in podocytes in diabetes. These results suggest that there are differences in lipoprotein receptor expression in podocytes compared with other cells in response to high glucose.

Podocyte injury is an important event during the onset and development of DN. Despite remarkable advances in our understanding of the pathogenesis of podocyte injury and proteinuria (3, 21), how high glucose and lipid deposition cause podocyte damage in DN remains poorly understood. It has been accepted that phenotypic alteration of podocytes leads to functional impairment and the progression of chronic kidney disease (12). Phenotypic alteration would certainly cause podocytes to lose their multifaceted morphological architecture and relinquish their highly specialized functions, which undoubtedly impair the integrity of the glomerular filtration barrier and the synthesis of ECM components such as collagen, laminin, and fibronectin leading to the onset of proteinuria. Our results indicate that α-SMA, collagen I, and fibronectin in podocytes are increased in response to high glucose. The correlation analysis further showed that LDLr protein expression was positively associated with α-SMA protein expression and negatively associated with nephrin. Interestingly, LDLr inhibition with LDLr siRNA reversed the high glucose-induced podocyte phenotypic alteration. These observations suggest that high glucose accelerates DN progression by disrupting the LDLr feedback pathway, which is closely associated with the induction of podocyte phenotype conversion.
In summary, our findings demonstrated that LDLr is a major receptor mediating lipid uptake in podocytes, and high glucose disrupted the feedback regulation of the LDLr pathway. Subsequently, this dysregulation led to lipid accumulation in podocytes, thereby accelerating DN progression. Diabetic dyslipidemia may represent a main drug target for therapeutic intervention to protect podocytes from damage and subsequently alleviate the excessive burden of DN.

Limitations. The db/db mouse, lacking the hypothalamic leptin receptor, is a model of type 2 diabetes mellitus that exhibits hyperglycemia, hyperinsulinemia, and hyperleptinemia associated with the hyperphagia and obesity. However, the main limitation of this model is that the mice are a model of the early changes in human DN but fail to recapitulate later and morphologically advanced features of DN. It is difficult to observe the pathological changes of mesangiolysis or nodular mesangial sclerosis and progressive renal insufficiency, which are the characteristic manifestations of DN.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Y.Z., J.L., Y.W., Z.B.H., and L.L. performed experiments; Y.Z., J.L., and K.L.M. conception and design of research; Y.Z. drafted manuscript; K.L.M. performed experiments; Y.Z., J.L., and K.L.M. analysis and interpretation of data; Y.Z., J.L., and Y.W. drafting/rewriting; K.L.M. approved final version of manuscript.

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