Effect of intensive insulin therapy on β-cell function and glycaemic control in patients with newly diagnosed type 2 diabetes: a multicentre randomised parallel-group trial

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Summary

Background Early intensive insulin therapy in patients with newly diagnosed type 2 diabetes might improve β-cell function and result in extended glycaemic remissions. We did a multicentre, randomised trial to compare the effects of transient intensive insulin therapy (continuous subcutaneous insulin infusion [CSII] or multiple daily insulin injections [MDI]) with oral hypoglycaemic agents on β-cell function and diabetes remission rate.

Methods 382 patients, aged 25–70 years, were enrolled from nine centres in China between September, 2004, and October, 2006. The patients, with fasting plasma glucose of 7·0–16·7 mmol/L, were randomly assigned to therapy with insulin (CSII or MDI) or oral hypoglycaemic agents for initial rapid correction of hyperglycaemia. Treatment was stopped after normoglycaemia was maintained for 2 weeks. Patients were then followed-up on diet and exercise alone. Intravenous glucose tolerance tests were done and blood glucose, insulin, and proinsulin were measured before and after therapy withdrawal and at 1-year follow-up. Primary endpoint was time of glycaemic remission and remission rate at 1 year after short-term intensive therapy. Analysis was per protocol. This study was registered with ClinicalTrials.gov, number NCT00147836.

Findings More patients achieved target glycaemic control in the insulin groups (97·1% [133 of 137] in CSII and 95·2% [118 of 124] in MDI) in less time (4·0 days [SD 2·5] in CSII and 5·6 days [SD 3·8] in MDI) than those treated with oral hypoglycaemic agents (83·5% [101 of 121] and 9·3 days [SD 5·3]). Remission rates after 1 year were significantly higher in the insulin groups (51·1% in CSII and 44·9% in MDI) than in the oral hypoglycaemic agents group (26·7%; p=0.0012). β-cell function represented by HOMA B and acute insulin response improved significantly after intensive interventions. The increase in acute insulin response was sustained in the insulin groups but significantly declined in the oral hypoglycaemic agents group at 1 year in all patients in the remission group.

Interpretation Early intensive insulin therapy in patients with newly diagnosed type 2 diabetes has favourable outcomes on recovery and maintenance of β-cell function and protracted glycaemic remission compared with treatment with oral hypoglycaemic agents.

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Introduction

The UK Prospective Diabetes Study[1,2] has shown that β-cell function progressively deteriorates over time in people with type 2 diabetes mellitus, irrespective of lifestyle and existing pharmacological interventions. Notwithstanding, continued effort has been directed toward β-cell preservation or rejuvenation in an attempt to change or at least delay the natural course of type 2 diabetes.[3] Other studies have indicated that, in newly diagnosed patients, short-term intensive insulin therapies that target overall glycaemic control, such as multiple daily insulin injections (MDI) and continuous subcutaneous insulin infusion (CSII), could improve β-cell function and result in extended remissions in which only diet was needed to maintain normoglycaemia.[3–8] The results of our previous study[7] also suggest that the improvement of β-cell function, especially the restoration of the first-phase insulin secretion, could be responsible for the remission. Still unclear, however, is whether this disease-modifying effect is due to insulin therapy itself or the effects of simply eliminating glucotoxicity by achieving excellent glycaemic control, and which kind of initiation of early intensive therapy would be more beneficial.

We therefore did a multicentre, randomised, parallel-group trial to assess the efficacy of short-term intensive insulin therapy (including MDI and CSII) compared with oral hypoglycaemic agents on glycaemic control, remission rate, and β-cell function in patients with newly diagnosed type 2 diabetes. Several indicies were used for assessment of β-cell insulin-secretory capacity, including: HOMA B, which represents basal β-cell function; the first-phase insulin secretion after a glucose challenge, which represents the acute insulin response; and the ratio of plasma proinsulin to immunoreactive insulin (PI/IRI), which indicates β-cell secretory quality.[9]

Methods

436 patients with newly diagnosed type 2 diabetes, according to WHO diagnostic criteria (1999),[10] who had not received previous antihyperglycaemic therapy, were enrolled from nine centres in China between September, 2004, and October, 2006. The patients, aged 25–70 years, had levels of fasting plasma glucose between 7·0 mmol/L and 16·7 mmol/L. Patients were excluded if they had acute or severe chronic diabetic complications, severe intercurrent illness, or tested positive for glutamic acid decarboxylase antibody. Patients with maturity onset diabetes in youth and mitochondria diabetes mellitus were excluded.[11,12] There was a 3–7 days run-in period of diet alone. The protocol and informed consent document were approved by the research ethics board of the Sun Yat-Sen University. All patients gave written informed consent.

Study design

Patients were randomly assigned to one of three groups for antihyperglycaemic therapies: MDI, CSII, or oral hypoglycaemic agents. Sealed, opaque envelopes arranged in a computer-generated random order were prepared by the data-coordinating centre and distributed to each participating centre, where they were opened sequentially to determine the patients' treatment assignments.

Patients in the CSII group received human insulin (Novo Nordisk, Bagsværd, Denmark) with an insulin pump (H-Tron Plus V100; Disetronic Medical Systems). Patients in the MDI group were treated with pre-meal Novolin-R, and human insulin NPH (Novolin-N, Novo Nordisk) at bedtime. Initial insulin doses were 0·4–0·5 IU/kg and total daily doses were divided into 50% of basal and 50% of bolus injection in the CSII group and into 30%–20%–20%–30% in the MDI group. In the group treated with oral hypoglycaemic agents, patients with a body-mass index between 20 kg/m² and 25 kg/m² were initially treated with gliclazide (Servier, Tianjin, China) 80 mg twice a day, which was increased up to a maximum of 160 mg twice a day to achieve glycaemic control. Patients with a body-mass index of between 25 kg/m² and 35 kg/m² were initially treated with metformin (Glucophage, Bristol-Myers Squibb) 0·5 g twice a day and increased to a maximum of 2·0 g a day. A combination of gliclazide and metformin was used in patients who could not reach the glycaemic control goal with one oral hypoglycaemic agent or who had a fasting plasma glucose of 11·1 mmol/L or more at randomisation. The doses were titrated every day in the insulin groups and every 3 days in the hypoglycaemic agents group in order to attain the glycaemic goal. This goal was defined as a fasting capillary blood glucose of less than 6·1 mmol/L and capillary blood glucose at 2 h after each of three meals of less than 8·0 mmol/L. Treatments were maintained for 2 weeks after the glycaemic target was reached. Patients who did not achieve glycaemic control by CSII or MDI alone or by combined maximum dosage of oral hypoglycaemic agents within 2 weeks, or could not stand the side-effects of oral hypoglycaemic agents, were regarded as requiring additional or different therapy and were excluded from the efficacy analysis.

Fasting blood samples were collected for measurement of fasting plasma glucose, proinsulin, free fatty acids, and lipid profiles in all patients before and after treatment (2 days after insulin or hypoglycaemic agent cessation), immediately followed by an intravenous glucose tolerance test using 25 g of glucose (50 mL of 50% glucose), with serum samples obtained before and 1, 2, 4, 6, and 10 min after intravenous glucose load to measure insulin. 2-h postprandial (after breakfast) plasma glucose concentrations were assessed the day before the intravenous glucose tolerance test. The acute insulin response during the intravenous glucose tolerance test was used to assess the first-phase β-cell insulin secretion, which was calculated as the incremental trapezoidal area during the first 10 min. Homoeostasis model assessment was used to estimate basal β-cell function (HOMA B) and insulin resistance (HOMA-IR).[13] The following equations were used to calculate β-cell function and insulin resistance: HOMA B=20×fasting insulin/(fasting plasma glucose−3·5). HOMA-IR=fasting plasma glucose×fasting insulin/22·5. The PI/IRI ratio was also calculated.

Concentrations of insulin, proinsulin, and free fatty acids were assessed centrally at the Diabetes Center of First Affiliated hospital of Sun Yat-Sen University. Radioimmunoassay was used for measurement of insulin (3V Bio-engineering group, Weifang, China) and proinsulin (Linco Research, St Charles, MO, USA). Free fatty acids levels were assessed enzymatically with a Wako NEFA C test kit (Wako Chemicals, Dallas, TX, USA). Measurements of glycated haemoglobin A_{1c} (HbA_{1c}) levels and routine clinical laboratory tests were done in the central laboratory units of the nine participating centres. HbA_{1c} was assayed using the Bio-Rad Variant Hemoglobin A_{1c} assay.

After interventions were stopped, patients were instructed to continue diet and physical exercise only and were followed-up with glycaemic monitoring monthly during the initial 3 months and at 3-month intervals thereafter. Hyperglycaemia relapse was defined as either fasting plasma glucose of more than 7·0 mmol/L or 2-h postprandial plasma glucose of more than 10·0 mmol/L, which was confirmed 1 week later. The time of glycaemic remission was recorded, and patients with hyperglycaemic relapse were treated with oral hypoglycaemic agents or insulin, according to the guidelines of the International Diabetes Federation-Western Pacific Region. Patients who maintained optimum glycaemic control for at least 12 months without medication were defined as the remission group and those who relapsed during the 12 months of follow-up as the non-remission group. The above measures were repeated at 1-year follow-up.

The primary endpoint was the time of glycaemic remission and remission rate at 1 year after short-term intensive therapy in patients with newly diagnosed type 2 diabetes. The secondary endpoint was the effect of

different interventions including CSII, MDI, or oral hypoglycaemic agents on β-cell function in these patients.

Statistical analysis

As expected from our previous studies, 45% of patients who received insulin treatment (CSII or MDI) and 25% of patients treated with oral hypoglycaemic agents achieved long-lasting remission. With 89 patients in each group, the study had 80% power at 5% significance (2-sided) to detect a clinically significant difference (20%) in the remission rate between the insulin group and the group treated with oral hypoglycaemic agents. Since the average rate of not achieving glycaemic control by the intervention was about 20%, based on the results of previous studies, and the estimated dropout rate was 15%, at least 392 patients in total should be recruited. Efficacy analyses were done on the population who achieved 2 weeks of euglycaemic control during intensive intervention. Safety analyses were done on all randomly assigned patients who received study medication.

Data were analysed with the SPSS 11·0 program.[14] Normally distributed and continuous variables are presented as mean (SD), and non-normally distributed variables (triglycerides, acute insulin response, PI/IRI, HOMA B, and HOMA IR) expressed as median (IQR). For the assessment of differences between the treatment groups with regard to quantitative variables (ie, fasting plasma glucose, HbA_{1c}, lipid profile parameters, HOMA IR, PI/IRI), one-way analysis of variance (ANOVA) with multiple comparisons Scheffé post-test was used. The non-normally distributed variables have been log-transformed and then analysed with ANOVA. The comparison of β-cell function among the three groups was made using an ANCOVA model with treatment as fixed effects and sex, age, body-mass index, and baseline triglycerides as the covariates. A Kruskal-Wallis H or Friedman test was used to analyse the non-normally distributed variables (acute insulin response and the change of PI/IRI ratio from baseline to after interventions). Time-to-event distributions were summarised with Kaplan-Meier curves. The percentage reduction in risk was computed as 100×(1–hazard ratio), with the hazard ratio estimated from the Cox proportional-hazards model. χ^2 tests were done to analyse the differences of remission rates among the three intervention groups. Significance was defined as $p<0·05$.

This study was registered with ClinicalTrials.gov, number NCT00147836.

Role of the funding source

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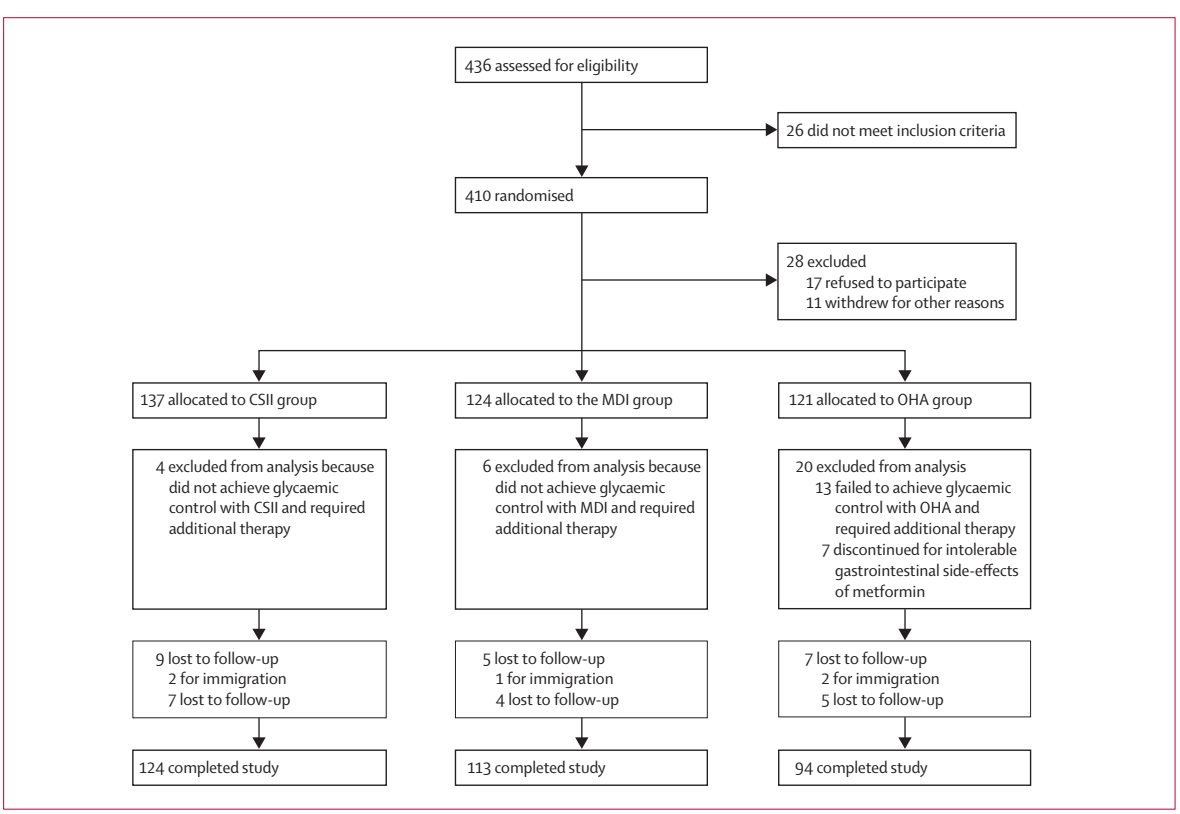

Figure 1: **Trial profile**
CSII=continuous subcutaneous insulin infusion. MDI=multiple daily insulin injections. OHA=oral hypoglycaemic agents.

sources had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

	CSII	MDI	Oral hypoglycaemic agents
Number	133	118	101
Men (n)	88	81	58
Age (years)	50 (11)	51 (10)	52 (9)
Body-mass index (kg/m²)	25·1 (3·0)	24·4 (2·7)	25·1 (3·3)
Fasting plasma glucose (mmol/L)			
Before therapy	11·3 (3·3)	11·5 (3·2)	10·8 (2·9)
After therapy*	6·6 (1·5)	6·8 (1·6)	6·5 (1·6)
2-h postprandial plasma glucose (mmol/L)			
Before therapy	16·1 (5·5)	17·5 (5·5)	16·6 (5·0)
After therapy*	7·5 (2·2) (n=113)	8·1 (2·9) (n=111)	8·2 (2·7) (n=90)
HbA_{1c} (%)			
Before therapy	9·8 (2·3)	9·7 (2·3)	9·5 (2·5)
After therapy*	8·0 (1·5)	8·0 (1·6)	7·9 (1·7)
Triglycerides (mmol/L)†			
Before therapy	1·7(1·4) (n=132)	1·7(1·4) (n=117)	1·8(1·1) (n=97)
After therapy*	1·3(0·7) (n=127)	1·3(0·8) (n=113)	1·4(1·0) (n=97)
Total cholesterol (mmol/L)			
Before therapy	5·2 (1·2) (n=128)	5·4 (1·2) (n=115)	5·4 (1·2) (n=100)
After therapy*	4·7 (1·0) (n=127)	5·0 (1·1) (n=113)	4·7 (0·9) (n=97)
HDL-C (mmol/L)			
Before therapy	1·2 (0·3) (n=132)	1·3 (0·4) (n=117)	1·3 (0·4) (n=96)
After therapy	1·2 (0·4†) (n=127)	1·3 (0·4) (n=113)	1·2 (0·4) (n=95)
p value	0·044	0·098	0·158
LDL-C (mmol/L)			
Before therapy	3·0 (1·0) (n=132)	3·2 (0·9) (n=117)	3·1 (0·8) (n=96)
After therapy	2·7 (0·8) (n=127)	2·8 (1·0) (n=113)	2·6 (0·7) (n=95)
p value	<0·0001	0·001	<0·0001
Free fatty acids (mmol/L)			
Before therapy	0·76 (0·39) (n=117)	0·70 (0·27) (n=101)	0·73 (0·22) (n=89)
After therapy	0·61 (0·20) (n=116)	0·62 (0·21) (n=99)	0·62 (0·19) (n=86)
p value	<0·0001	0·001	<0·0001
Acute insulin response (pmol/L per min)†			
Before therapy	−62 (421) (n=126)	−7 (347) (n=114)	−95 (452) (n=95)
After therapy*	889 (1087) (n=129)	793 (1150) (n=115)	736 (1289) (n=90)
PI/IRI (%)†			
Before therapy	23·8 (17·5) (n=123)	26·5 (22·6) (n=115)	28·4 (22·2) (n=95)
After therapy*	12·1 (11·8) (n=113)	16·8 (20·1) (n=105)	21·2 (20·7)‡ (n=90)
HOMA B†			
Before therapy	33·6 (45·6) (n=114)	38·3 (36·9) (n=108)	50·0 (60·6) (n=94)
After therapy*	87·5 (82·5) (n=113)	78·9 (65·2) (n=103)	102·3 (16·0) (n=90)
HOMA IR†			
Before therapy	6·0 (5·6) (n=114)	6·7 (5·7) (n=108)	6·9 (8·6) (n=94)
After therapy*	3·2 (2·8) (n=113)	3·1 (2·9) (n=103)	4·8 (4·8) (n=90)

Data are mean (SD) unless otherwise indicated. *p<0·0001 compared with before treatment. †Data are median (IQR). ‡p<0·05 compared with CSII.

Table 1: **Comparison of glucose control and β-cell function at baseline and after interventions**

Results

Of the 436 patients screened, 410 were eligible and were randomised. 28 patients withdrew before receiving interventions (figure 1). The remaining 382 patients (mean age 51 years [SD 10], body-mass index 25·0 kg/m² [3·0], and mean fasting plasma glucose 11·2 mmol/L [3·1]) were allocated to the CSII group (137), the MDI group (124), and the hypoglycaemic agents group (121).

Of these patients, 23 (four in the CSII group, six in the MDI group, and 13 in the oral hypoglycaemic agents group) did not reach the glycaemic control goals and seven patients in the oral hypoglycaemic agents group were withdrawn because of gastrointestinal side-effects of metformin. These 30 patients were therefore excluded from further analysis. Of the remaining 352 patients who completed transient intensive treatment, 331 completed 1-year visits and 21 (5·5%, nine in the CSII group, five in the MDI group, and seven in the oral hypoglycaemic agents group) dropped out because of immigration or were lost to follow-up (figure 1).

Clinical characteristics, glucose levels, and lipid profiles at baseline were similar between the three groups (table 1). 92·1% (352 of 382) of patients reached glycaemic goals in 7·9 days (SD 4·6) during the intervention period. More patients achieved target glycaemic control in the insulin groups (133 of 137 [97·1%] in CSII, and 118 of 124 [95·2%] in MDI) in less time (4·0 days [SD 2·5] in CSII and 5·6 days [3·8] in MDI) than those in the oral hypoglycaemic agents group (101 of 121 [83·5%], 9·3 [5·3] days; p<0·0001 *vs* CSII and p=0·01 *vs* MDI). The mean maximum daily doses were 0·68 IU kg^{-1} (SD 0·21) in the CSII group and 0·74 IU kg^{-1} (0·35) in the MDI group, and the median maximum daily doses in the oral hypoglycaemic agents group was gliclazide 160 mg plus metformin 1500 mg. 25 patients were treated with gliclazide alone and 27 with metformin alone.

Of the patients who reached glycaemic targets, the improvement of glucose control, represented by significant decrease of fasting plasma glucose, 2-h postprandial plasma glucose, and HbA_{1c}, did not significantly differ among the groups (table 1). Nor did the amelioration of lipid profile, indicated by decreased total cholesterol, LDL-C, triclycerides, and free fatty acid levels after intensive therapies.

The indicies of β-cell function and HOMA IR were similar between the three treatment groups before treatment (table 1). The acute insulin response was absent in all patients at that point. After 2–5 weeks of intensive treatment, the acute insulin response was partially restored and HOMA B was significantly increased in all patients (p<0·0001). The PI/IRI ratios were markedly decreased (p<0·0001), and HOMA IR also decreased (p<0·0001). When the data after therapy among three groups were compared, we found no significant difference in the improvement of acute

insulin response, HOMA B, and HOMA IR. But the decrease in the PI/IRI ratio was more obvious in both the insulin groups (median 8·7% [IQR 17·1%] in CSII, 10·8% [IQR 19·7%] in MDI) than in the oral hypoglycaemic agents group (4·1% [IQR 14·5%] *vs* CSII, p=0·038; *vs* MDI, p=0·011, respectively).

The remission rate at 1 year was 42·0% (148 of 352) in all patients who achieved glycaemic control during the intensive interventions. Figure 2 shows the remission rates at 1 year in the three groups: 51·1% (68 of 133) in the CSII group, 44·9% (53 of 118) in MDI group, and 26·7% (27 of 101) in the oral hypoglycaemic agents group. The remission rate was significantly higher in both insulin groups than in the oral hypoglycaemic agents group (p=0·0012). The risk of relapse was reduced by 44% (95% CI 0·40–0·78, p=0·001) with CSII and by 31% (95% CI 0·50–0·97, p=0·032) with MDI compared with oral hypoglycaemic agents.

The remission group had higher initial body-mass index, lower fasting plasma glucose and HbA_{1c}, and also achieved glycaemic control faster than the non-remission group (table 2). After short-term treatment, the remission group had a more marked reduction in fasting plasma glucose, 2-h postprandial plasma glucose, and HbA_{1c}.

The non-remission patients from different interventions were combined into one non-remission group in order to compare the acute insulin response between remission and non-remission patients. As a result, we found the increase of acute insulin response induced by CSII (median 1151 [IQR 1131] pmol/L per min), MDI (1065 [IQR 1158] pmol/L per min), or oral hypoglycaemic agents (968 [IQR 1752] pmol/L per min) was greater in the remission groups than in the non-remission group (601 [IQR 819] pmol/L per min; p<0·0001).

Among the remission groups, the increase of the acute insulin response was maintained after 1 year in CSII (809 pmol/L per min; p=0·235) and MDI groups (729 pmol/L per min; p=0·063) compared with immediately after the intervention. But it significantly declined in the oral hypoglycaemic agents group (335 pmol/L per min; p<0·0001). Acute insulin response in the oral hypoglycaemic agents group was significantly lower than that in the CSII group (p=0·006), whereas there was no difference between oral hypoglycaemic agents group and MDI group (p=0·097) at 1 year (figure 3).

There were no severe hypoglycaemic episodes—defined as an event requiring the assistance of another person to actively administer carbohydrate, glucagon, or other resuscitative treatments—during the short-term intensive interventions. The proportion of patients with one or more minor hypoglycaemic episodes—defined as having classical symptoms of hypoglycaemia or blood glucose level below 3·1 mmol/L and prompt recovery after the patient self-administered carbohydrate—was higher, although not significantly, in the CSII (31%, 42 of 137) and the MDI (28%, 35 of 124) groups than in the oral hypoglycaemic agents group (19%, 23 of 121). No pump-related side-effects or injection site reactions were reported. Diarrhoea and other gastrointestinal side-effects

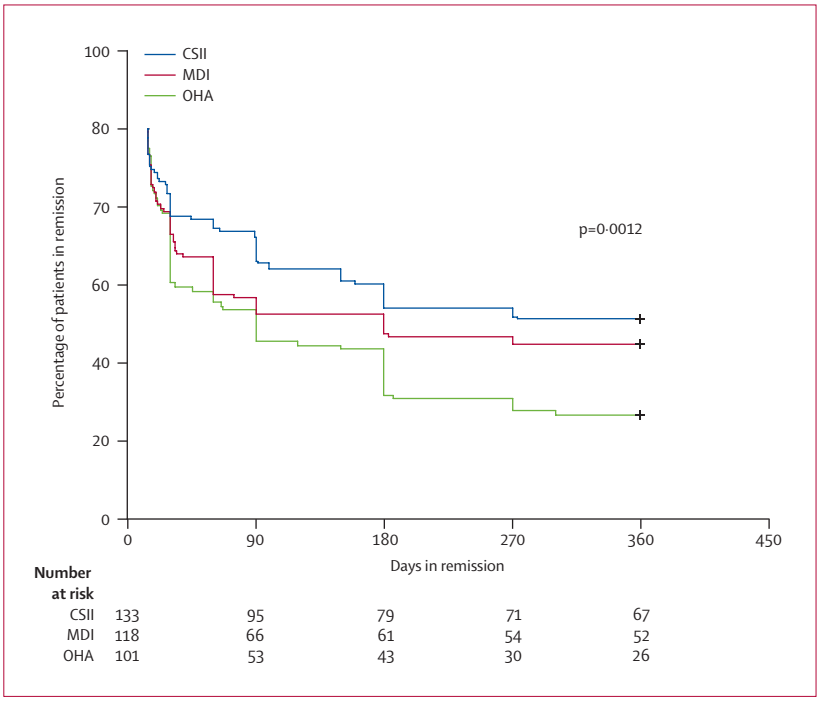

Figure 2: **Kaplan-Meier estimates of time to primary endpoint**

	Remission group	Non-remission group	p, compared between remission and non-remission group
Number	148	183	
Men (n)	104	110	0·055
Age (years)	50 (10)	52 (10)	0·0157
Body-mass index (kg/m²)			
Before treatment	25·5 (2·8)	24·3 (3·1)	<0·0001
Fasting plasma glucose (mmol/L)			
Before treatment	10·8 (3·2)	11·6 (3·1)	0·014
After treatment*	6·2 (1·0)	7·0 (1·9)	<0·0001
2-h postprandial plasma glucose (mmol/L)			
Before treatment	16·4 (5·6)	17·0 (5·1)	0·337
After treatment*	7·2 (2·0)	8·5 (2·8)	<0·0001
HbA_{1c} (%)			
Before treatment	9·2 (2·2)	10·0 (2·4)	0·004
After treatment*	7·7 (1·5)	8·1 (1·7)	0·029
Days of achieving euglycaemia	6·1 (4·1)	6·7 (3·5)	0·048

Data are mean (SD) unless otherwise indicated. *p<0·0001 compared with before treatment.

Table 2: **Comparison between remission group and non-remission group before and after treatment**

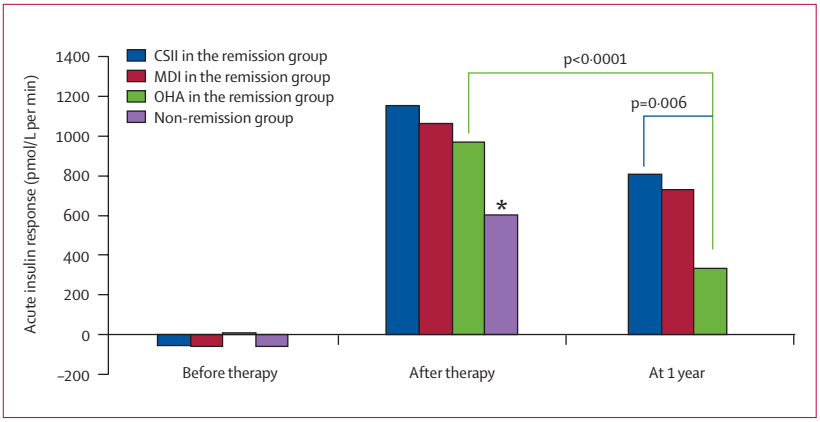

Figure 3: **Acute insulin response (shown as median) before and after different interventions and at 1 year**
*p<0·05 in the non-remission group compared with that in each intervention in the remission group (after treatment).

were reported in 14 patients in the hypoglycaemic agents group, all of whom were using metformin, and seven of these patients withdrew because of intolerance. The mean bodyweight of patients was unchanged after transient treatments in all groups. No other severe adverse events were reported during the study period.

Discussion

Our results show that excellent glycaemic control could be successfully achieved in 7·9 days (SD 4·6) in most patients with mean fasting plasma glucose of 11·2 mmol/L (SD 3·1), irrespective of the use of CSII, MDI, or oral hypoglycaemic intervention. Of those patients who reached glycaemic targets, both fasting plasma glucose and 2-h postprandial plasma glucose concentrations rapidly corrected to near physiological range after treatment, and HbA_{1c} was greatly improved after only 2–5 weeks.

In our study, the acute insulin response was absent before treatment and was restored partially in patients after short-term treatments. HOMA B was also significantly increased. The PI/IRI ratios were markedly decreased, indicating the improvement of qualitative insulin secretion. Additionally, the decline in HOMA-IR and amelioration of the lipid profile without the use of lipid-adjusting agents were also indicators of the reduction in glucotoxicity. Besides these improvements, 148 (42·0%) of 352 responsive patients achieved more than 1-year remission in which only diet control and exercise were necessary to maintain optimum glycaemic control. Hence, in patients newly diagnosed with type 2 diabetes, any kind of early intensive glycaemic control—either induced by insulin or oral hypoglycaemic agents—could rescue β-cell function and induce longterm glycaemic remission in almost half of patients, most likely through eliminating the effects of acute glucotoxicity and related pathogenesis factors.

The glucotoxicity generated by hyperglycaemia is commonly thought to be the fundamental acquired factor causing continuous decline of β-cell function in type 2 diabetes.[3,15] Thus, optimum metabolic control, especially early intensive glycaemic control, can eliminate the deleterious effects of hyperglycaemia and rescue injured β cells, avoiding irreversible loss of β-cell secretory function and β-cell mass that leads to the worsening of diabetes.

Comparing the effects of different interventions in those patients who achieved 2 weeks of euglycaemic control during intensive intervention, we found that more patients in the insulin groups achieved the glycaemic control goal earlier than did patients in the hypoglycaemic agents group. The average times to achieve euglycaemia were 4·0 days (SD 2·5) in the CSII group, 5·6 days (SD 3·8) in the MDI group, and 9·3 days (SD 5·3) in the oral hypoglycaemic agents group. These findings suggest that more patients would benefit from insulin treatment for near-normoglycaemic control, and would have a shorter period of antecedent glucotoxicity. The results are consistent with a previous study.[6]

Notably, insulin replacement could provide a type of β-cell rest and reduce excessive secretory demands on damaged β cells.[3,16] However, despite the similar improvement of glycaemic control and lipid profile and the magnitude of basal and stimulated insulin secretion among groups after treatments, the decrease in the PI/IRI ratio was more obvious in the two insulin groups than in the oral hypoglycaemic agents group, suggesting attenuated β-cell overstimulation with insulin treatment. In particular, the maintenance of improved acute insulin response was much better in the insulin groups, especially in the CSII group, than in the oral hypoglycaemic agents group at 1 year. A more profound β-cell rest by intensive insulin therapy could possibly have an extended beneficial effect. Other insulin effects such as anti-inflammatory[17] and anti-apoptosis effects,[18,19] and the more recent finding of normalised glucose-dependent insulinotropic polypeptide responsiveness by intensive insulin treatment, could also have contributed to the long-lasting beneficial effects of insulin.[20] Thus, all these distinct effects could reasonably be thought of as contributing to the large difference in 1-year remission rate between the groups. Compared with oral hypoglycaemic agents, insulin therapy, including CSII and MDI, has obviously increased remission rates (26·7% for hypoglycaemic agents *vs* 51·1% for CSII and 44·9% for MDI). The risk of hyperglycaemia relapse was reduced by 44% with CSII and by 31% with MDI compared with oral hypoglycaemic agents. From these observations, we conclude that early aggressive insulin treatment has unique effects on the recovery and maintenance of β-cell function and has more potential to induce glycaemic remission in patients with newly diagnosed type 2 diabetes than with oral hypoglycaemic agents.

Sulphonylurea drugs can exert negative effects by over-stimulating β-cells, offsetting its effects in

alleviating of glucotoxicity.[21,22] Alvarsson and colleagues[23] reported that early insulin versus glibenclamide treatment temporarily prolonged endogenous insulin secretion and promoted better metabolic control in a 2-year prospective study. Metformin is known to lower the fasting glucose level by reducing basal hepatic glucose production and can augment glucose-mediated glucose uptake to enhance the tissue sensitivity to insulin. Metformin has no stimulatory effects on insulin secretion.[24] We also found a slightly higher remission rate in the metformin group than in the sulphonylurea group. However, we could not draw a conclusion here because only a small proportion of patients with a mean fasting plasma glucose of 11·2 mmol/L (SD 3·1) achieved euglycaemia on one oral hypoglycaemic agent. Assessed together, the results of these studies suggest that early rigorous glycaemic control by intensive insulin intervention could have more persistent beneficial effects on β-cell function and protracted glycaemic control than treatment with oral hypoglycaemic agents, by affecting the metabolic memory, impeding the progression from metabolic abnormalities to irreversible cellular and epigenetic alterations. These effects might further alter the natural history of diabetes and ultimately prevent or reduce development and progression of diabetes-related complications.[25]

The characteristics of patients with type 2 diabetes who achieved near-normoglycaemic remission in our study were moderate obesity, had a shorter time to euglycaemia, and had relatively lower glucose levels at baseline than did non-remission patients. Those who went into remission had significant improvement in glycaemic control and greater recovery of acute insulin response after treatment than those who did not achieve remission during the 1-year study period. Many of these factors could indicate a shorter duration of diabetes. We should note that a new diagnosis of type 2 diabetes is not equivalent to a new onset of the disease. In this study, all patients were drug-naive and might have different duration of diabetes, although all were newly diagnosed. The patients who achieved near-normoglycaemic remission might possibly have had a shorter duration of diabetes. Due to uncertainty in disease duration, we could infer, but cannot conclude, that patients who did better had a shorter duration of diabetes.

In our study, several factors could limit the extent to which the results can be generalised. First, the range of fasting plasma glucose (7·0–16·7 mmol/L), age (25–70 years), and body-mass index (25·0 kg/m² [SD 3·0]) of our patients were appreciably wide, so the group was heterogeneous. However, the distribution in the three groups was even, and the subgroup analysis (fasting plasma glucose 7·0–11·1 mmol/L and 11·1–16·7 mmol/L) showed the same trends as the whole group (data not shown). Second, 28 patients withdrew before receiving interventions, resulting in imbalanced sample size among the three groups. Those 28 patients shared the same clinical characteristics with the whole group. We were concerned that some of the variations might have affected our results, but they had little effect on our conclusions.

In conclusion, early intensive insulin interventions in patients with newly diagnosed type 2 diabetes have favourable outcomes with regard to recovery and maintenance of β-cell function and prolonged glycaemic remission compared with treatment with oral hypoglycaemic agents. Our findings support the initiation of early transient intensive insulin treatment in those patients.

Contributors

YL wrote the first draft. JL, ML, and WX contributed to compilation of data and did the statistical analysis. JW designed and organized the study and co-wrote the first draft. YL, LS, DZ, ZZ, HT, ZL, L Yan, LZ, L Yang, WX, JL, QZ, YH, XY, XR, JX, FL, YC, and SY contributed to data collection, identification, and assessments of the primary data sources for each participating study centre. HC and ZF were members of the scientific committee for the study and contributed to manuscript discussion.

Conflict of interest statement

Sun Yat-Sen University has an unrestricted research grant from Novo Nordisk (China) and Roche Diagnostics (Shanghai). The authors declare that they have no conflict of interest. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. All authors have seen and approved the final text.

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Incidence of type 1 diabetes in China, 2010-13: population based study

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ABSTRACT

OBJECTIVE

To estimate the incidence of type 1 diabetes in all age groups in China during 2010-13.

DESIGN

Population based, registry study using data from multiple independent sources.

SETTING

National registration system in all 505 hospitals providing diabetes care, and communities of patients with diabetes in 13 areas across China, covering more than 133 million person years at risk, approximately 10% of the whole population.

PARTICIPANTS

5018 people of all ages with newly diagnosed type 1 diabetes and resident in the study areas from 1 January 2010 to 31 December 2013.

MAIN OUTCOME MEASURES

Incidence of type 1 diabetes per 100 000 person years by age, sex, and study area. Type 1 diabetes was doctor diagnosed and further validated by onsite follow-up. Completeness of case ascertainment was assessed using the capture mark recapture method.

RESULTS

5018 cases of newly diagnosed type 1 diabetes were ascertained: 1239 participants were aged <15 years, 1799 were aged 15-29 years, and 1980 were aged ≥30 years. The proportion of new onset cases in participants aged ≥20 years was 65.3%. The estimated incidence of type 1 diabetes per 100 000 persons years for all ages in China was 1.01 (95% confidence interval 0.18 to 1.84). Incidence per 100 000 persons years by age group was 1.93 (0.83 to 3.03) for 0-14 years, 1.28 (0.45 to 2.11) for 15-29 years, and 0.69 (0.00 to 1.51) for ≥30 years, with a peak in age group 10-14 years. The incidence in under 15s was positively correlated with latitude (r=0.88, P<0.001), although this association was not observed in age groups 15-29 years or ≥30 years.

CONCLUSION

Most cases of new onset type 1 diabetes in China occurred among adults. The incidence of type 1 diabetes in Chinese children was among the lowest reported in the study.

WHAT IS ALREADY KNOWN ON THIS TOPIC

The only available data on incidence of type 1 diabetes in China was provided by the DiaMond Project, reporting 0.51 and 0.59 per 100 000 person years among under 15s in 1985-94 and 1988-96, respectively

Most epidemiological studies worldwide have focused on onset of type 1 diabetes in childhood

A paucity of information thus exists on incidence of type 1 diabetes spanning all ages

WHAT THIS STUDY ADDS

The estimated incidence of type 1 diabetes per 100 000 persons years was 1.93 for 0-14 years, and 1.01 for all ages. Most new cases of type 1 diabetes in China are in adults

The incidence rates in under 15s were positively correlated with latitude

More resources should be provided to improve the care of people with onset of type 1 diabetes in adulthood

Introduction

International studies of type 1 diabetes,[1 2] such as the DiaMond (Diabetes Mondiale) Project and European Community Concerted Action Programme in Diabetes (EURODIAB) study have shown wide variation in the incidence of type 1 diabetes among children. According to the DiaMond Project, China had one of the lowest incidences of type 1 diabetes in children—0.51 per 100 000 person years during 1985-94.[3] A nationwide registry of type 1 diabetes in China does not exist.

The incidence of type 1 diabetes in children has been increasing worldwide.[2 4 5] Recent results of the SEARCH for Diabetes in Youth study from the United States suggest that environmental or behavioural factors, or both, play larger parts in the increased incidence of type 1 diabetes compared with decades ago.[5 6] The study of type 1 diabetes in a low incidence region such as China may help advance the understanding of the contribution of varying combinations of genetic and environmental factors to the development of the disease. Moreover, most epidemiological studies of type 1 diabetes focused on childhood onset type 1 diabetes. Although type 1 diabetes most often develops in children, it can occur at any age.[7 8] Our previous study also indicated that the onset of type 1 diabetes in adulthood is not rare in China.[9] Yet little is known about its incidence in adults aged more than 20 years.[10]

We carried out a nationwide, population based registry study (the Epidemiological Study of Type 1 Diabetes Mellitus in China (T1D China)) to investigate the incidence of type 1 diabetes in all age groups in China during 2010-13.

Methods

Our study is a population based multicentre observational study, with ascertainment of doctor diagnosed cases of type 1 diabetes. We identified new cases occurring during 2010 to 2013 in the resident population of 13 areas across China (Harbin, Shenyang, Beijing, Shanghai, Nanjing, Jinan, Wuhan, Changsha, Guangzhou, Chengdu, Xi'an, Lanzhou, Yinchuan; fig 1, and see supplementary table S1).

	Latitude	Population in 2010
North east China		
Haerbin	45.8	10 635 971
Shenyang	41.8	8 106 171
North China		
Beijing	39.9	19 612 368
North west China		
Yinchuan	38.5	1 993 088
Lanzhou	36.0	3 616 163
Xi'an	34.3	8 467 838
East China		
Jinan	36.4	6 813 984
Nanjing	32.1	8 003 744
Shanghai	31.1	23 019 196
South west China		
Chengdu	30.7	14 047 625
Central China		
Wuhan	30.5	9 785 388
Changsha	28.2	7 040 952
South China		
Guangzhou	23.2	12 701 948

Fig 1 | Thirteen study areas in China, 2010-13. The study was conducted in mainland China. Hong Kong and Macao not included

Study population

Our study population comprised residents of 13 study areas. These areas were chosen from the seven administrative regions of China (northeast, north, northwest, southwest, central, east, and south) according to geographical location, climate, culture, ethnicity, and population, and they are representative of the seven administrative regions. We selected at least one area from each of these administrative regions. Besides, a huge imbalance in population density exists in China: more than 90% of the population reside in the southeast. We also ensured that there was at least one study area every 5° of latitude (fig 1, and see supplementary table S1). In some of the administrative regions we therefore selected one or two additional areas. The study areas also consist of regions of different economic development levels, as represented by the gross domestic product in 2010.[11] The study areas covered the less developed, moderately developed, and well developed areas in China.

The study population (denominator) included residents of all ages in the 13 study areas during 2010 to 2013. According to the 2010 Chinese census,[12] we defined the resident population as: people whose registered address agreed with their primary address and who had stayed at their primary address for six months or more; people whose registered address disagreed with their primary address, but who had stayed at their primary address for six months or more; people whose registered address disagreed with their primary address, but who had stayed at their primary address for less than six months; and people in active military service and those whose primary address could not be specified—they were not included in the resident population.

The 2010 Chinese census[12] conducted by the National Bureau of Statistics of China provides precise information on China's mainland population, which is essential for the calculation of the denominator in a nationwide registry study. Our study period was 1 January 2010 to 31 December 2013, which allowed at least 18 months of a diagnostic time window for cases to be included in our study when we started the onsite data validation and inspection (see supplementary materials, section 4) in June 2015. We estimated the denominator yearly according to the 2010 Chinese census and annual government reports on natural population growth (see supplementary materials, section 2). Derivation of appropriate denominators is a multistep process, adjusted for sex categorisation and the natural growth rate of the local population. Our study population covered more than 133 million person years at risk between 2010 and 2013, which represents approximately 10% of the Chinese population, including 6% of those aged less than 15 years. This provided enough power to estimate the incidence over 1.0 per 100 000 person years.

The numerator included all newly diagnosed cases of type 1 diabetes in the resident population in the study areas from 1 January 2010 to 31 December 2013.

Data sources and collection

The data coordinating service provider collected and submitted data from four sources: the medical record databases from all the hospitals providing diabetes care in the 13 study areas (505 hospitals); outpatient based pharmacies in tertiary hospitals in the 13 areas (228 hospitals); government medical insurance databases; and patient self reports from online and offline communities of patients with diabetes spontaneously founded and maintained by patients with diabetes or their family members, or both, which were confirmed by medical records (see supplementary materials, section 2).

Collected data included identification markers and clinical information. Identification markers included mandatory and optional markers. For all validated cases, mandatory markers, including initials of each Chinese character of the name, sex, date of birth, registered address, primary address, date of diabetes

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diagnosis, date of insulin treatment initiation, hospitals where treated (with inpatient or outpatient status, or both, specified), and ethnicity, were collected, usually as part of the case validation process. For cases from communities of patients with diabetes, we collected mandatory information from the hospital where diabetes was first diagnosed. We excluded cases with missing mandatory information after onsite validation. Supplementary materials section 2 lists the optional markers. Clinical information was collected from medical record databases, as part of the case ascertainment process, including clinical presentations at onset (symptoms, occurrence of diabetic ketosis or ketoacidosis), family history, treatment for hyperglycaemia, C peptide level at onset or within 12 months after diagnosis, and presence of diabetes autoantibodies at any time.

The data coordinating service provider registered and anonymised the cases and sent the information to the data management committee. Mandatory information was available in 98.8% of cases. Data managers used identification markers for matching within the same source and across sources to identify potential duplicate records at area level (see supplementary materials, section 3, part 4)

Case ascertainment

Participating endocrinologists or paediatricians, or both, or an expert committee on type 1 diabetes established the diagnosis (see supplementary materials, section 1). The clinical diagnosis of type 1 diabetes was based on the American Diabetes Association descriptions of type 1 diabetes[8] and the World Health Organization reports on the classification of diabetes.[13] Diabetic ketoacidosis was defined based on guidelines[14 15] and data accessibility in China. Diabetic ketoacidosis was diagnosed in people with the following history and laboratory results: hyperglycaemia (glucose >11.1 mmol/L) or known diabetes ; ketonemia: raised levels of blood ketobodies as judged by participating investigators, or important ketonuria (>++ on standard urine dipsticks); and acidemia: bicarbonate ($HCO3^-$) <15.0 mmol/L or venous pH <7.3, or both.

Data managers examined all submitted information to identify participants with an uncertain diagnosis of type 1 diabetes. Uncertain results were sent back to the data coordinating service provider. This provider required the corresponding investigator to feedback on the diagnosis of type 1 diabetes and provide any previously absent data related to the diagnosis if available. The corresponding investigator would make the judgment of diagnosis based on the currently available data, and would follow-up the patient if necessary and then give feedback to the data coordinating service provider. We ensured that the diagnostic time window would be no less than 18 months for participants, as a report shows that 18 months is the usual duration from the time a patient first sees a doctor to the final diagnosis.[16] The data coordinating service provider informed the data managers of the feedback. Any cases of uncertain diagnosis were submitted to the expert committee for final judgment. We ascertained all cases with a final diagnosis of type 1 diabetes (see supplementary materials, section 3).

Statistical analysis

To assess the completeness of case ascertainment we used the capture mark recapture method[17] and a two-mode ascertainment model (see supplementary materials, section 5). We defined the percentage of completeness of ascertainment for each study area as the number of observed cases divided by the number estimated from the capture mark recapture method. Incidence by age group, sex, and area were calculated per 100 000 person years at risk, and we calculated 95% confidence intervals based on inverting the score test for a binomial proportion.[18] The model to estimate the national incidence of type 1 diabetes in China was developed from data collected in the 13 study areas and based on a Poisson distribution, in which factors potentially affecting the incidence of type 1 diabetes were added as covariates (see supplementary materials, section 6). Pearson's χ^2 analysis was applied to comparison of rates. Spearman correlation was used to analyse the relation between incidence and potentially related factors. For all statistical analyses we used SAS version 9.4 (SAS Institute, Cary, NC). We considered $P<0.05$ to be statistically significant.

Patient involvement

No patients were involved in setting the research question or the outcome measures, nor were they involved in developing plans for recruitment, design, or implementation of the study. No patients were asked to advise on interpretation or writing up of results. However, patient self reports were collected from online and offline communities of patients with diabetes spontaneously founded and maintained by patients with diabetes or their family members, or both. We contacted the founders and the people currently in charge of these communities and released a questionnaire asking for members of these communities to volunteer to self report. No patient or their family members were involved in the design of such a questionnaire, and the information collected from the questionnaire was anonymised. There are no plans to disseminate the results of the research to study participants.

Results

Our study population comprised 5018 people with type 1 diabetes newly diagnosed between 2010 and 2013 (fig 2): 1239 were aged 0-14 years, 1799 were aged 15-29 years, and 1980 were aged ≥30 years, and 2755 (54.9%) were male. Table 1 summarises the number of participants and the estimated annual incidence in each study area. Completeness of ascertainment was estimated to be more than 95% across the 13 areas. Completeness was relatively even among age groups, at 99.3% in the 0-14 years group, 98.4% in the 15-29

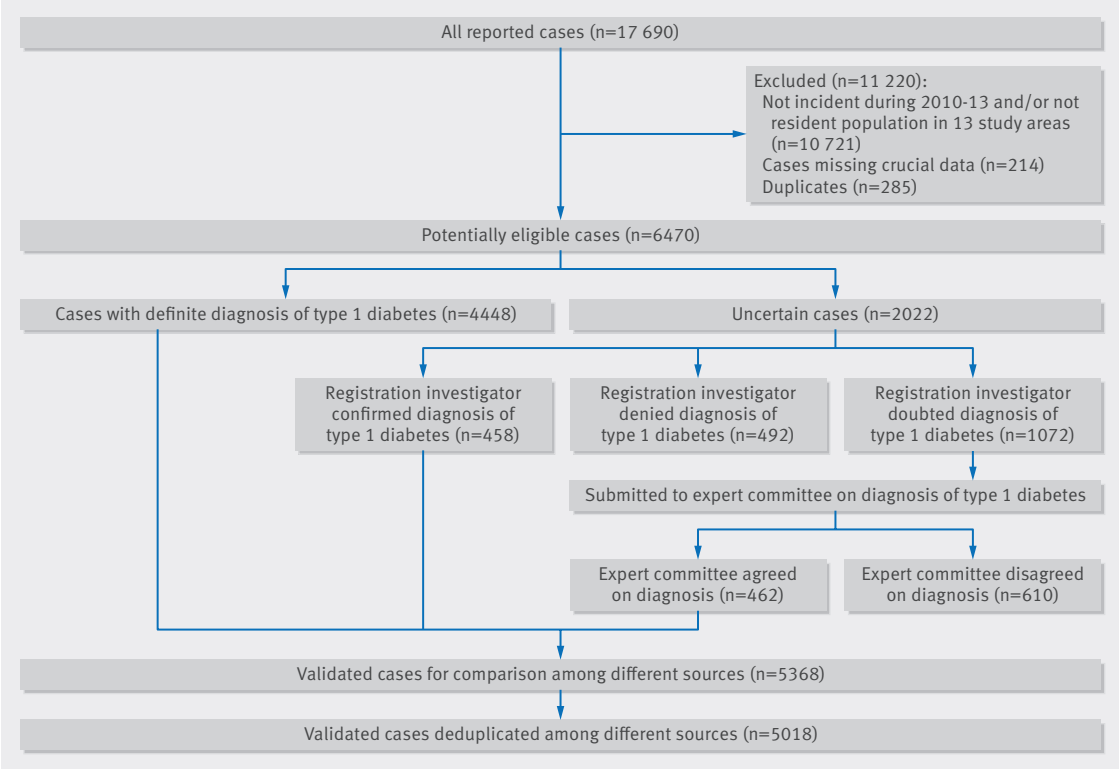

Fig 2 | Flow of participants through study

years group, and 98.8% in the ≥30 years group. (see supplementary table S3).

After adjustment for the denominator of annual change in population between 2010 and 2013, the overall estimated incidence of type 1 diabetes per 100 000 person years for all ages in the 13 study areas was 0.93 (95% confidence interval 0.90 to 0.95). There was a slight difference (P<0.001, χ^2=30.43) between males (0.95 per 100 000 person years, 0.91 to 0.99) and females (0.81 per 100 000 person years, 0.78 to 0.85; table 2). The incidence (per 100 000 person years) by age group was 1.90 (95% confidence interval 1.80 to 2.01) for 0-14 years, 1.02 (0.98 to 1.07) for 15-29 years, and 0.51 (0.49 to 0.53) for ≥30 years. The incidence per 100 000 person years among the 0-14 years group was 1.72 (1.58 to 1.86) among males and 2.21 (2.05 to 2.39) among females (P<0.001, χ^2=19.58). The incidence per 100 000 person years among the population aged ≥15 years was 0.92 (0.89 to 0.96) among males and 0.70 (0.67 to 0.74) among females (P<0.001, χ^2=68.22).

The estimated incidence of type 1 diabetes per 100 000 persons years for all ages varied from 0.52 (0.47 to 0.59) in Chengdu to 1.57 (1.38 to 1.79) in Lanzhou. For the 0-14 years group, the estimated incidence per 100 000 persons years varied from 1.14 in Chengdu (0.91 to 1.42) to 3.59 in Harbin (3.1 to 4.16). The incidence in the 0-14 years group was positively correlated with latitude (r=0.88, P<0.001). The estimated incidence per 100 000 person years for

Table 1 | Incidence of type 1 diabetes in different age groups in 13 areas of China

		No of cases				Incidence per 100 000 person years (95% CI)			
Study area	Latitude (°N)	0-14 years	15-29 years	≥30 years	Total No of cases	0-14 years	15-29 years	≥30 years	All
Harbin	45.8	179	265	190	634	3.59 (3.1 to 4.16)	2.43 (2.16 to 2.75)	0.75 (0.65 to 0.87)	1.54 (1.42 to 1.66)
Shenyang	41.8	96	123	118	337	2.48 (2.03 to 3.03)	1.44 (1.21 to 1.72)	0.58 (0.48 to 0.69)	1.03 (0.92 to 1.14)
Beijing	39.9	232	245	263	740	2.46 (2.16 to 2.79)	1.08 (0.95 to 1.22)	0.53 (0.47 to 0.6)	0.91 (0.84 to 0.97)
Yinchuan	38.5	23	31	14	68	2.43 (1.62 to 3.65)	0.14 (0.1 to 0.19)	0.03 (0.02 to 0.05)	0.98 (0.78 to 1.25)
Jinan	36.7	77	73	59	209	2.18 (1.75 to 2.73)	0.99 (0.78 to 1.24)	0.35 (0.27 to 0.46)	0.76 (0.66 to 0.87)
Lanzhou	36.0	40	77	106	223	2.22 (1.63 to 3.03)	0.71 (0.57 to 0.88)	0.42 (0.35 to 0.51)	1.57 (1.38 to 1.79)
Xi'an	34.3	78	107	115	300	1.82 (1.46 to 2.27)	1.13 (0.93 to 1.36)	0.56 (0.47 to 0.68)	0.88 (0.79 to 0.98)
Nanjing	32.1	85	115	135	335	2.23 (1.8 to 2.75)	1.28 (1.06 to 1.53)	0.69 (0.58 to 0.81)	1.03 (0.93 to 1.15)
Shanghai	31.1	130	236	417	783	1.19 (1.00 to 1.42)	0.92 (0.81 to 1.05)	0.72 (0.65 to 0.79)	0.83 (0.77 to 0.89)
Chengdu	30.7	78	104	114	296	1.14 (0.91 to 1.42)	0.69 (0.57 to 0.83)	0.33 (0.27 to 0.4)	0.52 (0.47 to 0.59)
Wuhan	30.5	77	171	186	434	1.61 (1.29 to 2.02)	1.51 (1.30 to 1.75)	0.77 (0.67 to 0.89)	1.08 (0.98 to 1.19)
Changsha	28.2	47	53	81	181	1.29 (0.97 to 1.72)	0.69 (0.53 to 0.9)	0.47 (0.38 to 0.59)	0.64 (0.55 to 0.73)
Guangzhou	23.2	97	199	182	478	1.55 (1.27 to 1.9)	1.36 (1.19 to 1.57)	0.6 (0.52 to 0.7)	0.94 (0.86 to 1.03)
Total		1239	1799	1980	5018	1.90 (1.80 to 2.01)	1.02 (0.98 to 1.07)	0.51 (0.49 to 0.53)	0.93 (0.90 to 0.95)

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Table 2 | Incidence of type 1 diabetes in different age groups in 13 areas of China

Variables	No of cases with type 1 diabetes	Population denominator (adjusted person years)*	Incidence rate per 100 000 person years (95% CI)
Total	5018	1 35 408 192.0	0.93 (0.90 to 0.95)
Age group (years):			
0-4	251	5 703 458.3	1.10 (0.97 to 1.24)
5-9	416	5 241 315.0	1.98 (1.80 to 2.18)
10-14	572	5 326 180.4	2.68 (2.47 to 2.91)
15-19	501	8 736 839.1	1.43 (1.31 to 1.56)
20-24	668	15 087 517.4	1.11 (1.03 to 1.19)
25-29	630	13 181 805.9	1.19 (1.11 to 1.29)
30-34	489	11 940 293.3	1.02 (0.94 to 1.12)
35-39	362	12 447 614.5	0.73 (0.66 to 0.81)
40-44	280	12 972 355.0	0.54 (0.48 to 0.61)
45-49	246	11 461 933.4	0.54 (0.47 to 0.61)
50-54	197	8 241 801.7	0.60 (0.52 to 0.69)
55-59	176	8 160 777.1	0.54 (0.47 to 0.62)
60-64	99	5 648 184.5	0.44 (0.36 to 0.53)
65-69	57	3 732 342.8	0.38 (0.29 to 0.49)
70-74	39	3 083 024.2	0.32 (0.23 to 0.43)
≥75	35	2 370 583.5	0.37 (0.27 to 0.51)
Male	2755	290 196 506	0.95 (0.91 to 0.99)
Female	2263	27 8391 771	0.81 (0.78 to 0.85)
Boys <15 years	586	34 082 195	1.72 (1.58 to 1.86)
Girls <15 years	653	29 531 351	2.21 (2.05 to 2.39)

*Estimated according to population data from 2010 to 2013.

the 15-29 years group varied from 0.14 in Yinchuan (0.1 to 0.19) to 2.43 in Harbin (2.16 to 2.75) and for the ≥30 years group varied from 0.03 in Yinchuan (0.02 to 0.05) to 0.77 in Wuhan (0.67 to 0.89). The latitude related correlations were not observed in the last two age groups (15-29 years: r=0.49, P=0.09; ≥30 years: r=0.03, P=0.92) (see supplementary table S4).

Table 2 summarises the incidence of type 1 diabetes in the different age groups. Among the under 15s, the incidence peak was observed in the 10-14 years group (incidence per 100 000 person years 1.10 (0.97 to 1.24), 1.98 (1.80 to 2.18), 2.68 (2.47 to 2.91), for age groups 0-4 years, 5-9 years, and 10-14 years, respectively). For groups aged more than 15 years, the incidence of type 1 diabetes decreased steadily with age (P<0.001, χ^2=989.54).

Based on previous reports[19-23] on factors affecting the incidence of type 1 diabetes and data availability, we performed correlation analysis and revealed that age group, latitude, and exposure to sunlight (as represented by average peak sunlight time (kWh/m^2/day)) was statistically significantly correlated with incidence (see supplementary materials, section 6). Using a Poisson model including these three factors as covariates and population size as weight, the annual incidence of type 1 diabetes across China based on the 13 study areas was 1.01 (0.18 to 1.84) per 100 000 person years. The incidence rates for ages 0-14 years, 15-29 years, and ≥30 years were 1.93 (0.83 to 3.03), 1.28 (0.45 to 2.11), and 0.69 (0.00 to 1.51) per 100 000 person years, respectively.

Table 3 summarises the characteristics of type 1 diabetes at diagnosis in different age groups from medical record databases. Overall, 4661 new cases of type 1 diabetes were ascertained from medical record databases, including 1143 people aged 0-14 years, 1664 aged 15-29 years, and 1854 aged ≥30 years. Insulin treatment was started immediately in 98.4% of participants in the 0-14 years group, 93.5% in the 15-29 years group, and 91.5% in the ≥30 years group (P<0.001, χ^2=59.76). The incidence rates of diabetic ketosis and diabetic ketoacidosis within six months of diagnosis were highest among the 0-14 years group (92.9%/51.4%), followed by the 15-29 years group (89.0%/43.0%), and lowest in the ≥30 years group (83.8%/30.8%) (diabetic ketosis: P<0.001, χ^2=57.71; diabetic ketoacidosis: P<0.001, χ^2=125.72). The proportions of cases with non-detectable

Table 3 | Clinical characteristics of participants with onset of type 1 diabetes

Characteristics	No of cases with information available	Overall %	No (%)		
			0-14 years	15-29 years	≥30 years
Total	4661	100.0	1143 (24.5)	1664 (35.7)	1854 (39.8)
Immediate initiation of insulin treatment at onset	4659	93.9	1125 (98.4)	1555 (93.5)	1696 (91.5)
Diabetic ketosis <6 months of diagnosis*	4652	87.7	1028 (92.9)	1415 (89.0)	1638 (83.8)
Diabetic ketoacidosis <6 months of diagnosis*	4453	40.1	546 (51.4)	694 (43.0)	547 (30.8)
At least one positive result for DAA <6 months of diagnosis†	173	60.7	25 (73.5)	44 (59.5)	36 (55.4)
Fasting C peptide ≤0.2 ng/mL	3475	24.4	191 (22.5)	245 (21.7)	411 (27.5)

DAA=diabetes autoantibody.
*Two deaths were identified in all eligible cases from medical record databases. Both were caused by diabetic ketoacidosis.
†DAAs including glutamate decarboxylase, the insulin antigen 2, islet cell antibody, insulin antibody, and the zinc transporter 8, were measured by radiobinding assay confirmed by the islet autoantibody standardization programme at the First Affiliated Hospital of Nanjing University or the Second Xiangya Hospital of Central South University.

fasting C peptide levels (<0.2 ng/mL) differed among the three age groups (22.5% *v* 21.7% *v* 27.5%, P=0.001, χ^2=13.88). Data were collected on diabetes autoantibodies, including glutamate decarboxylase, the insulin antigen 2, islet cell antibody, insulin antibody, and the zinc transporter 8, tested under standardised procedures. The proportion of patients with at least one positive test result for diabetes autoantibodies showed a non-significant tendency to decrease with age (0-14 years (73.5%), 15-29 years (59.5%), ≥30 years (55.4%), P=0.22, χ^2=3.16).

Discussion

China remains one of the countries with the lowest incidence of type 1 diabetes. In our study from 2010 to 2013 we found that although type 1 diabetes tends to develop in children, most of the new cases are diagnosed in adults. Furthermore, higher latitude is correlated with a higher incidence of type 1 diabetes in under 15s, but not in older population groups. We also noticed a high prevalence of diabetic ketoacidosis in participants ascertained within six months of diagnosis during our study period.

Low incidence of type 1 diabetes in China

Our study confirms that the incidence of type 1 diabetes remains low in China, even after the 3.8-fold increase of what was reported by the DiaMond Project two decades ago. Although the underlying mechanism is not completely known, the low incidence in China is probably attributed to genetic, environmental, and behavioural factors. A difference in prevalence of type 1 diabetes associated human leucocyte antigen genotypes has been reported to attribute to the difference in susceptibility to type 1 diabetes.[24] However, other genes and environmental factors could also have an influence. Our study population has a homogenous gene background: a population of Han people. Intriguingly, when we compared the northern areas with the southern areas we observed up to a threefold difference in incidence of type 1 diabetes in the 0-14 years group. Finland is a country of high latitude (around 60.1°N), which may contribute to a higher incidence of type 1 diabetes. But if we compare the incidence in Finland[25] with that in Harbin in northern China (45.8°N), it is still much lower (64.9 *v* 3.59 per 100 000 person years). This suggests that other factors, such as genetics, play a more important role in the incidence of type 1 diabetes.

Age and incidence

In our study the incidence of type 1 diabetes in under 15s during 2010 and 2013 was 1.93 (95% confidence interval 0.83 to 3.03) per 100 000 person years, presenting a 3.8-fold increase over that reported by the DiaMond study[3] in the 1990s, equal to a roughly 6.5% annual increase. This increase appears to be rapid compared with those reported by the EURODIAB (3.3%)[26] and SEARCH (1.8%) studies.[5] However, we did not measure the trends in incidence. In fact we compared our results with the incidence reported in China in 1998.[3] These results were from part of the DiaMond study, which may be underestimated as a result of incomplete ascertainment, as some of the participating centres reported small case numbers. Thus the apparent increase should be interpreted with caution. China remains one of the countries with the lowest incidence of type 1 diabetes globally, despite the increasing trend. None the less, considering its large population, China has the largest estimated number of new annual cases of type 1 diabetes in children, at 4271 of the estimated 10 000 in the West-Pacific region in 2015.[27]

In our study we found that most new cases of type 1 diabetes presented in adulthood. Indeed, approximately 65.3% of newly diagnosed cases were in participants aged more than 20 years. Our estimates for incidence of type 1 diabetes for all ages is 1.01 per 100 000 person years, and the incidence in the study areas for ages 20-29 years was 1.15 per 100 000 person years. Previous reported nationwide incidence rates at ages 20-29 years varies from 3.4 (Iran, 1990-94)[28] to 19.4 (Kronoberg, Sweden, 1998-2001)[29] globally. Although the paucity of type 1 diabetes incidence among all age groups makes comparison with other studies difficult, our estimates in both children and adults in China were among the lowest reported in the world. Nevertheless, we estimate that 9605 new cases occur annually in the population aged 15 or more years in China. This finding highlights the importance of the care of people with adult onset type 1 diabetes and that more resources should be provided to improve the care of this age group.

The age distribution of incidence of type 1 diabetes showed that the age of onset increases during childhood and then steadily decreases towards adulthood (table 2). The incidence rose to a peak of 2.68 per 100 000 person years in the 10-14 years group, consistent with rates reported by the DiaMond Project,[1] the USA,[5] and Japan[30] in different periods. Most epidemiology studies that reported incidence in both childhood and adult type 1 diabetes observed the peak appearing close to puberty. However, studies from the USA[31] and Europe[32] described bimodal incidences, with the first peak in ages 10-14 years and the second peak around age 50 years. Such a peak around age 50 years was not observed in our study.

Sex and incidence

Our data show that the incidence of type 1 diabetes was higher among girls aged 0-14 years, consistent with previous reports. Two international type 1 diabetes registries (EURODIAB and DiaMond)[24] showed that the overall sex ratio for incidence of type 1 diabetes is roughly equal in children, with a minor excess in males in regions with a high incidence (populations of European origin) and an excess in females in regions with a low incidence (populations of non-European origin) such as Asia and Africa. Several reports[33-35] indicate an excess in males among adults in populations of European origin. In contrast with the incidence of type 1 diabetes in children

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where an excess in girls was observed, we found that the incidence of type 1 diabetes in the population aged ≥15 years was greater in men than in women, consistent with previous findings. Different effects of environmental risk factors and lifestyle on incidence of type 1 diabetes in females and males were possible explanations for this difference.[33] Such a difference may also suggest that childhood onset and adult onset type 1 diabetes have different manifestations in the sexes.

Latitude and incidence

Our results showed that the incidence of type 1 diabetes among children aged 0-14 years was strongly correlated with latitude, with higher rates in the north and lower in the south, but such correlation was not observed in participants aged ≥15 years. Previously reported effects of latitude on incidence of type 1 diabetes among young people are inconsistent. The EURODIAB study reported that the incidence of type 1 diabetes in childhood was positively correlated with latitude,[36] whereas the SEARCH study did not show similar trends.[37] The correlation between latitude and incidence in the younger but not the older population has not been reported previously. The difference between childhood onset and adult onset type 1 diabetes regarding the latitude effects on incidence may suggest different triggers for these two disease forms.

Although gene pools of the populations, ultraviolet radiation level in different regions, and the prevalence of virus infections have been postulated to be responsible for the correlation between latitude and incidence of type 1 diabetes among under 15s,[19] the actual mechanism remains controversial. It has been reported that the Han population could be divided into three regional groups (northern, central, and southern) by variance in several loci of single nucleotide polymorphisms, including some in the human leucocyte antigen region previously reported to be related to psoriasis.[38] Although the association between this variance and the development of type 1 diabetes is yet unknown, it is worthy of further study to clarify whether such variance is related to the incidence of type 1 diabetes.

Prevalence of diabetic ketoacidosis in cases of type 1 diabetes

Compared with other studies, ranging from no more than 15% (Sweden) to around 30% (the SEARCH study and the EURODIAB study) in young people with a diagnosis of diabetes,[39] we observed a relatively high prevalence of diabetic ketoacidosis (40.1% in all age groups and 51.4% in under 15s) in participants with newly diagnosed type 1 diabetes during the study period. We collected the data on diabetic ketoacidosis occurring within six months of diagnosis. These episodes of diabetic ketoacidosis might occur at onset or after onset, reflecting different situations. Occurrence at onset of type 1 diabetes could be attributed to the acute onset of the disease or to the lack of awareness of the signs or symptoms of the disease by patients or healthcare providers. Diabetic ketoacidosis after onset but within six months of diagnosis could have been the result of insufficient education of the participant about diabetes or missing an injection of insulin. The high prevalence of diabetic ketoacidosis within six months of a diagnosis of type 1 diabetes in our study indicates that more effort and resources are needed to achieve earlier diagnosis, raise awareness, and improve education about type 1 diabetes in China.

Strengths and weaknesses of this study

We report the first population based registry study of incidence of type 1 diabetes in China in the past two decades. Our study is also the first nationwide study to provide incidence rates for type 1 diabetes in all age groups, covering a vast geographical area. These results should not only update the global map of type 1 diabetes in childhood, but also fill in the blank about the incidence of adult onset type 1 diabetes.

It has been a huge challenge to ascertain cases of type 1 diabetes. Although we were not able to utilise all the data from the medical insurance system owing to the inability to distinguish people with type 1 diabetes from those with type 2 diabetes, we had established a pharmacy based registry system and had utilised self report data from communities of patients with diabetes. Therefore, we believe that we have given as accurate an estimation as possible.

Several weaknesses of our study should be considered. The population we covered would provide enough power to estimate the incidence over 1.0 per 100 000 person years, but for the exceedingly low incidence rate of adult onset type 1 diabetes, especially in groups aged more than 40 years, a larger study population would be required to give a more accurate estimate. The study areas have a higher proportion of urban populations than that of the whole nation. This hindered us from studying the association between incidence of type 1 diabetes and environmental factors. However, correlation analysis showed no statistically significant association between incidence and the proportion of urban population (see supplementary table S4). Like most of the epidemiological studies in type 1 diabetes, the diagnosis in our study was a clinical one. We adopted a search strategy based on ICD (international classification of diseases, ninth and 10th revisions) codes and the diagnosis largely relied on our participating endocrinologists and paediatricians (see supplementary materials, section 3). This could result in missing cases or misdiagnosis. However, we took multiple steps to minimise such a possibility. We included all cases of diabetes in under 15s regardless of classification and diabetes coded as "unclassified" in all age participants when we searched with ICD codes to avoid any missing cases. Clinical information (presence of diabetes related symptoms, incidence of diabetic ketosis or diabetic ketoacidosis within six months of diagnosis, etc), and laboratory test results (including C peptide level and diabetic autoantibodies) were collected when available to assist the diagnosis.

Finally, we ensured that the diagnostic time window for cases included in our study was no less than 18 months.

Conclusions

We found a rapid increase in the incidence of type 1 diabetes in under 15s in the past two decades in China, a country with a low incidence for this disease. We estimated that more than 13 000 new cases of type 1 diabetes occur every year in China, with more than 9000 in people aged 15 or more. Furthermore, we found that the incidence of type 1 diabetes was positively associated with higher latitude in under 15s, but not in the older population.

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Contributors: JW, ZZ, and LG are joint first authors. JW designed and organised the study and cowrote the first draft of the manuscript. JY assisted JW to run the study, collect data in the study site, interpret the data, and cowrite the first draft. ZZ and LG assisted in the design and organisation of the study, and contributed in manuscript discussion. DZ, LJ, XL, YM, WJ, WY, HK, QL, YL, LY, XY, ZS, QJ, XR, JL, JZ, LC, LH, CG, LL, FL, and YX organised the study in their study regions and contributed to the data collection. LJ, DZ, ZS, and XL also contributed in data interpretation. HK, XJ, XR, and JL also contributed to data analysis. XZ, DY, and SL contributed to the data analysis, data interpretation, and manuscript discussion. EM advised on study design and contributed to the manuscript discussion. The corresponding author (JW) had full access to all the data in the study and had final responsibility for the decision on content and publication submission. All authors have seen and approved the final text. The funder of the study had no role in the study design; data collection, analysis, or interpretation; or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. JW, ZZ, and LG are the guarantors.

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Transparency: The lead author (JPW) affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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Supplementary material: Additional information

HEPATOLOGY

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SIRT1/HSF1/HSP Pathway Is Essential for Exenatide-Alleviated, Lipid-Induced Hepatic Endoplasmic Reticulum Stress

Xiaobin Zheng,* Fen Xu,* Hua Liang, Huanyi Cao, Mengyin Cai, Wen Xu, and Jianping Weng

Recent studies have indicated that lipid-induced endoplasmic reticulum (ER) stress is a major contributor to the progression of hepatic steatosis. Exenatide (exendin-4), a glucagon-like peptide-1 receptor agonist, is known to improve hepatic steatosis, with accumulating evidence. In this study, we investigated whether exenatide could alleviate lipid-induced hepatic ER stress through mammal sirtuin 1 (SIRT1) and illustrated the detailed mechanisms. Male C57BL/6J mice challenged with a high-fat diet (HFD) were treated with exenatide or normal saline by intraperitoneal injection for 4 weeks. We observed that HFD feeding induced hepatic ER stress as indicated by increased expression of glucose-regulated protein 78, phosphorylated protein kinase-like ER kinase, and phosphorylated eukaryotic initiation factor 2α, while these increases were significantly inhibited by exenatide. Exenatide notably decreased the liver weight and hepatic steatosis induced by HFD challenge. Consistently, in human HepG2 cells and primary murine hepatocytes, exendin-4 also significantly alleviated the ER stress and lipid accumulation induced by palmitate. Importantly, further studies showed that exendin-4 enhanced the binding of heat shock factor 1 to the promoter of heat shock protein (HSP) genes through SIRT1-mediated deacetylation, which then increased the expression of molecular chaperones HSP70 and HSP40 to alleviate hepatic ER stress. Finally, inhibition of SIRT1 by genetic whole-body heterozygous knockout or by lentiviral short hairpin RNA knockdown greatly diminished the effect of exenatide on deacetylating heat shock factor 1, increasing HSP expression and alleviating ER stress and hepatic steatosis in HFD-fed mice. *Conclusion*: The SIRT1/heat shock factor 1/HSP pathway is essential for exenatide-alleviated, lipid-induced ER stress and hepatic steatosis, which provides evidence for a molecular mechanism to support exenatide and incretin mimetics as promising therapeutics for obesity-induced hepatic steatosis. (HEPATOLOGY 2017;66:809-824)

Nonalcoholic fatty liver disease (NAFLD) is now recognized as the most common chronic liver disease worldwide.[1,2] It ranges from simple steatosis to more aggressive lesions including steatohepatitis, fibrosis, and cirrhosis.[3] Recent studies have demonstrated that NAFLD is considered to be the hepatic component of metabolic syndrome, which is closely associated with obesity, type 2 diabetes, and dyslipidemia.[4] Hepatic steatosis, characterized by excessive triglyceride accumulation in hepatocytes, is mainly associated with an overload of free fatty acids and serves as the key metabolic component of NAFLD.[5] It has been

Abbreviations: ChIP, chromatin immunoprecipitation; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GFP, green fluorescence protein; GLP-1, glucagon-like peptide-1; GRP78, glucose-regulated protein 78; H&E, hematoxylin and eosin; HFD, high-fat diet; HSF1, heat shock factor 1; HSP, heat shock protein; Lv, lentivirus; NAFLD, nonalcoholic fatty liver disease; p-, phosphorylated; PA, palmitate; PERK, protein kinase–like ER kinase; RNAi, RNA interference; SEM, standard error of the mean; shRNA, short hairpin RNA; SIRT1, sirtuin1; UPR, unfolded protein response; WT, wild-type.

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shown that excess free fatty acids in the liver, especially saturated free fatty acids, could induce an endoplasmic reticulum (ER) stress response and contribute to the pathogenesis of NAFLD.[6,7] In human beings and rodent models with obesity and NAFLD, enhanced hepatic ER stress was observed, indicating that ER stress may represent a common pathophysiological mechanism involved in systemic metabolic disorders.[8]

ER stress, characterized by accumulation of unfolded proteins in the ER lumen due to extracellular stress signals, promotes the dissociation of chaperone glucose-regulated protein 78 (GRP78) and leads to the activation of an adaptive program called the unfolded protein response (UPR) to reestablish equilibrium in the ER. The UPR is mediated by three transducers that are transmembrane proteins of the ER: protein kinase-like ER kinase (PERK), inositol-requiring enzyme 1α, and activating transcription factor 6. Each of these transducers activates specific pathways and collectively leads to decreased overall protein synthesis, enhanced ER folding capacity, and increased degradation of misfolded proteins, resulting in either recovery of ER homeostasis or cell death.[9] During chronic and unresolved ER stress, which is characteristic of obesity and type 2 diabetes, prolonged activation of UPR plays an important role in regulating expression of hepatic lipogenic genes and contributes to the development of hepatic steatosis.[10,11] Importantly, studies have shown that activation of PERK and subsequent phosphorylation of eukaryotic initiation factor 2α (eIF2α) appear to promote lipogenesis and thus the development of hepatic steatosis.[12,13]

Glucagon-like peptide-1 (GLP-1), an insulinotropic hormone released from the intestinal L cells in response to nutrient ingestion, has also been extensively shown to have extrapancreatic effects.[14] Studies have shown that exendin-4 or exenatide (synthetic exendin-4), a GLP-1 receptor agonist, ameliorated hepatic steatosis both *in vivo* and *in vitro*,[15,16] and this effect was found to be dependent on sirtuin1 (SIRT1), a highly conservative protein deacetylase in mammals.[17,18] However, it has not been clearly defined whether exenatide alleviates lipid-induced hepatic ER stress and whether this alleviation is SIRT1-dependent. A study revealed that hepatic overexpression of SIRT1 attenuated ER stress and hepatic steatosis in diet-induced and genetically obese mice.[19] Mechanistic studies also showed that the decreased SIRT1 expression could promote the greater acetylation of heat shock factor 1 (HSF1) and reduce its binding to the promoter of heat shock protein (HSP) genes, which then decreased the expression of molecular chaperone HSP and contributed to ER stress in neuronal cells and an experimental colitis model.[20-22] Thus, these findings revealed a potential link between the SIRT1/HSF1/HSP pathway and ER stress.

This study was aimed to investigate whether exenatide (exendin-4) alleviated lipid-induced hepatic ER stress through the SIRT1/HSF1/HSP pathway. We demonstrate that exenatide (exendin-4) increases HSP expression to alleviate lipid-induced ER stress and hepatic steatosis through SIRT1-mediated HSF1 deacetylation, which provides a novel mechanism to support the notion that exenatide and incretin mimetics act as promising therapeutics for obesity-induced hepatic steatosis.

Materials and Methods

ANIMAL MODELS

Seven-week-old male C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). After a 1-week acclimatization period, mice were randomly distributed into two initial groups fed with a chow diet (4% fat [wt/wt]; Guangdong Medical Laboratory Animal Center, Guangzhou, China) or a high-fat diet (HFD) (35.8% fat [wt/wt], D12331; Research Diets, New

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Brunswick, NJ). After 12-week diet intervention, each group of mice was then randomly subdivided to receive either exenatide (24 nmol/kg/d; Eli Lilly and Company, Indianapolis, IN) or normal saline as control by intraperitoneal injection during the light cycle with the respective diet feeding for 4 weeks. Body weight and daily food intake were monitored every 2 weeks. Glucose tolerance tests and insulin tolerance tests were carried out at the end of intervention as described in the Supporting Information.

The whole-body *Sirt1* heterozygous knockout (*Sirt1*$^{+/-}$) mice in a C57BL/6J genetic background were from J. Ye's laboratory at the Pennington Biomedical Research Center (Louisiana State University, Baton Rouge, LA)[23] and bred in the animal experimental center of our hospital. Eight-week-old male *Sirt1*$^{+/-}$ mice and their wild-type (WT) littermates were both challenged with an HFD for 12 weeks and then randomly subjected to either exenatide or normal saline for 8 weeks in the same way as described above.

A *Sirt1* knockdown mouse model was constructed in C57BL/6J mice by tail vein injection of lentivirus (Lv) expressing short hairpin RNA (shRNA) targeting *Sirt1*, which was designed and chemically synthesized by Shanghai GeneChem Co. Ltd. (Shanghai, China). The Lv vector expressing green fluorescence protein (GFP) only was used as the RNA inference (RNAi) control. Eight-week-old male C57BL/6J mice were randomly divided into two groups fed a chow diet or an HFD for 8 weeks. The HFD-fed mice were further divided into four groups: a normal saline–treated group (HFD), an exenatide-treated group (HFD+Exe), an Lv-GFP with exenatide–treated group (HFD+Exe+GFP), and an Lv-*Sirt1* RNAi with exenatide–treated group (HFD+Exe+*Sirt1* RNAi). One week after the injection of Lv (5×10^7 transfection units) or control solvent, mice were subjected to either exenatide or normal saline for 2 weeks as described above.

All animals received humane care, and all study procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University. Serum sampling and tissue collection, liver histology and immunohistochemistry, assays of serum and liver lipids, as well as serum adiponectin are detailed in the Supporting Information.

CELL CULTURE AND TREATMENT

Human HepG2 hepatocytes were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells at 70%-80% confluence were incubated in serum-free medium for 4 hours before treatment. Primary murine hepatocytes were isolated from 8-week-old male C57BL/6J mice using a two-step collagenase perfusion technique as described[24] and cultured on type I collagen–coated plates in Williams E medium supplemented with 2 mM L-glutamine, 100 nM dexamethasone, and antibiotics described above.

Thapsigargin and palmitate (PA) (Sigma-Aldrich, St. Louis, MO) were used as ER stress inducers. To examine the effect of exendin-4 (Sigma-Aldrich) on hepatic ER stress *in vitro*, hepatocytes were incubated in medium containing ER stress inducers with or without 100 nM exendin-4 for 24 hours. A SIRT1 inhibitor, EX-527 (Sigma-Aldrich), as well as two SIRT1 agonists, resveratrol (Sigma-Aldrich) and SRT1720 (Selleck, Houston, TX), were also included to investigate the role of SIRT1 in the effect of exendin-4 on PA-induced ER stress. For *Sirt1* knockdown *in vitro*, HepG2 cells were transfected with Lv *Sirt1* shRNA or scrambled shRNA at a multiplicity of infection (=10/20) according to the manufacturer's instructions. Transfected cells were then incubated in medium containing PA with or without exendin-4 for 24 hours.

IMMUNOPRECIPITATION

Total protein (500 μg) was incubated with 10 μL specific antibody against HSF1 (Millipore, Billerica, MA) overnight at 4 °C with continuous mixing. Then Protein A/G Mix Magnetic Beads (Millipore) were added, followed by incubation for 2 hours at 4 °C. After washing three times, the immunocomplexes were resuspended with denaturing elution buffer, heated at 100 °C for 5 minutes, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting with primary antibody against HSF1. For acetylation western blotting, 50 mM trishydroxymethylaminomethane (pH 7.5) with 10% (vol/vol) Tween-20 and 1% peptone was used for blocking, and 50 mM trishydroxymethylaminomethane (pH 7.5) with 0.1% peptone was used to prepare primary antibody against acetylated-lysine (Cell Signaling Technology, Danvers, MA) and secondary antibody.[25]

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation (ChIP) reactions were performed with an EZ-Magna ChIP HiSens kit

(Millipore) according to the manufacturer's instructions. Briefly, chromatin samples isolated from hepatocytes were sonicated, followed by incubation with 5 μL anti-HSF1 antibody (Millipore) or with control immunoglobulin G and ChIP A/G magnetic beads overnight at 4 °C with continuous mixing. Immunoprecipitated DNA and input genomic DNA were amplified with primers targeting the *Hsp70.1* promoter region containing heat shock element by real-time PCR as described above. Primers used for the *Hsp70.1* promoter were (forward) 5′-TGTCCCCTCCAG TGAATCCCA-3′ and (reverse) 5′-TATTCCAGG GTTTTCGCCTCC-3′. Results were normalized to reactions performed with 100% input samples.

STATISTICAL ANALYSIS

Data are presented as mean ± standard error of the mean (SEM). The unpaired two-tailed Student *t* test was used to determine significant differences between groups. $P < 0.05$ was considered statistically significant.

Additional materials and methods are described in the Supporting Information.

Results

EXENATIDE REDUCES BODY WEIGHT AND RESTORES SYSTEMIC AND HEPATIC GLUCOSE HOMEOSTASIS IN HFD-FED MICE

From the fourth week of intervention, the mice in the HFD group began to show a significant increase in body weight compared to the chow diet group, which lasted until the end of intervention (Fig. 1A). Interestingly, the HFD-induced mice subjected to 4-week exenatide treatment exhibited significant weight loss compared to the HFD group, especially in the first 2 weeks, which was accompanied by a decrease in daily food intake (Fig. 1A,B). Meanwhile, exenatide treatment showed beneficial effects on glucose tolerance and insulin sensitivity in HFD-challenged mice as indicated by glucose tolerance tests and insulin tolerance tests (Fig. 1C,D). To investigate the physiological mechanisms responsible for exenatide-restored systemic glucose homeostasis, the expression of hepatic glycometabolic genes was measured. As a result, the mRNA levels of gluconeogenic genes including *Pepck* and *Pck1* were increased in the liver by HFD challenge, and these changes were significantly reduced by exenatide. In contrast, the expression of a glycolytic gene, *Gck*, which was obviously reduced by HFD feeding, was restored by exenatide treatment (Fig. 1E). These findings demonstrate that exenatide reduces body weight and restores systemic and hepatic glucose homeostasis in HFD-fed mice.

EXENATIDE ALLEVIATES ER STRESS AND HEPATIC STEATOSIS IN HFD-FED MICE

To investigate whether exenatide alleviates lipid-induced hepatic ER stress in an HFD-fed mouse model, the protein levels of the ER stress marker GRP78 and the PERK/eIF2α arm of the UPR pathway were measured in the liver tissues. We found that HFD challenge strongly induced hepatic ER stress, as indicated by increased protein levels of GRP78, phosphorylated PERK (p-PERK) and p-eIF2α in the livers of the HFD group compared with the chow diet group. However, these increases were significantly inhibited by exenatide treatment (Fig. 2A). Similarly, both the liver weight and the ratio of liver to body weights in the HFD group were significantly decreased by exenatide (Fig. 2B). Consistently, histological analysis including hematoxylin and eosin (H&E) staining and oil red O staining revealed that exenatide greatly alleviated the lipid accumulation in the livers of HFD-fed mice, which was also indicated by the reduced liver triglyceride and cholesterol levels (Fig. 2C,D). The same trends were observed in the serum levels of triglyceride and cholesterol (Fig. 2E). Furthermore, the serum levels of adiponectin, an adipose-derived cytokine, were decreased by HFD challenge but reversed after exenatide treatment (Fig. 2E). The gene expression of two adiponectin receptors, *AdipoR1* and *AdipoR2*, was reduced in the livers of the HFD group. However, only the expression of *AdipoR2* was significantly increased by exenatide (Fig. 2F). Taken together, these results indicate that exenatide alleviates lipid-induced ER stress and hepatic steatosis in HFD-fed mice.

EXENDIN-4 ALLEVIATES PA-INDUCED ER STRESS AND LIPID ACCUMULATION IN HEPATOCYTES

To confirm whether exendin-4 alleviates lipid-induced hepatic ER stress *in vitro*, human HepG2

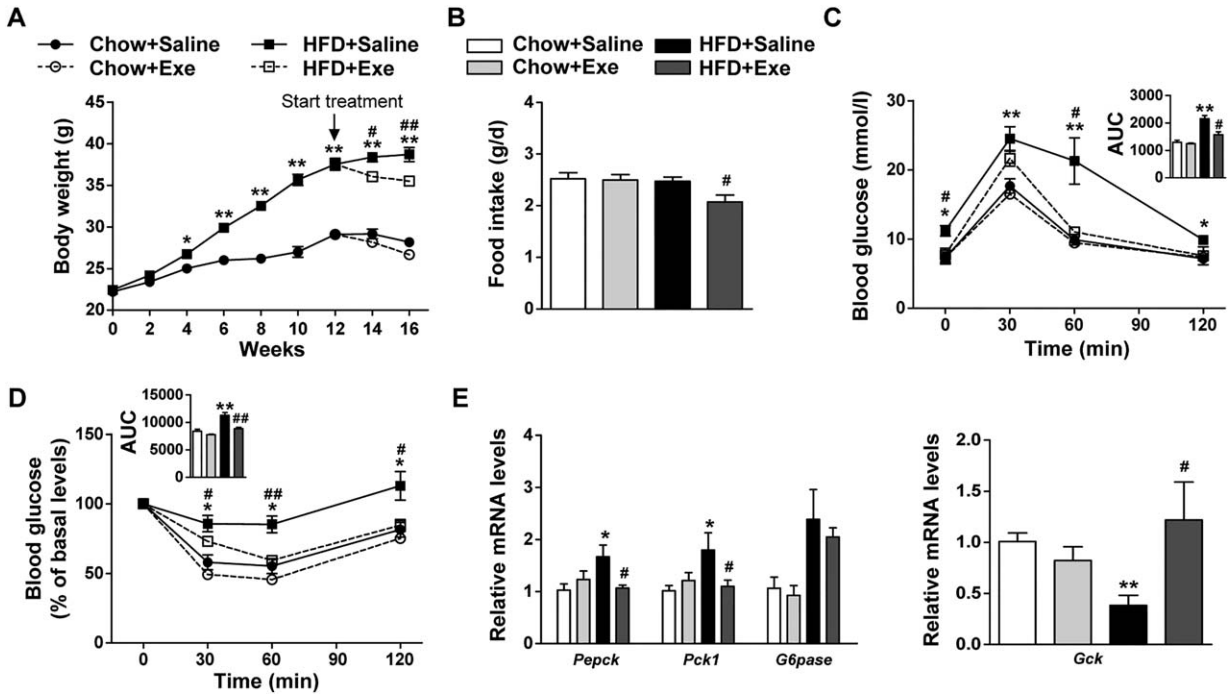

FIG. 1. Exenatide reduces body weight and restores systemic and hepatic glucose homeostasis in HFD-fed mice. C57BL/6J mice fed a chow diet or an HFD for 12 weeks were randomly assigned to either normal saline or exenatide treatment (24 nmol/kg) by intraperitoneal injection for 4 weeks with the diet intervention maintained ($n = 5$-6/group). (A) Body weight detected every 2 weeks. (B) Daily food intake during exenatide treatment. (C) Glucose tolerance tests after exenatide treatment and the area under curve. (D) Insulin tolerance tests after exenatide treatment and the area under curve. (E) Relative mRNA levels of gluconeogenic genes (*Pepck, G6pase*, and *Pck1*) and a glycolytic gene (*Gck*) by quantitative real-time PCR. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the chow diet group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the HFD group. Abbreviations: AUC, area under curve; Exe, exenatide.

cells and primary murine hepatocytes were introduced in our study. Thapsigargin, modifying Ca^{2+} concentration in the ER lumen by inhibiting Ca^{2+} adenosine triphosphatase, was served as a positive ER stress inducer. Here, thapsigargin exposure was observed to increase the protein levels of GRP78, p-PERK, and p-eIF2α in parallel with the lipid accumulation in HepG2 cells (Fig. 3B; Supporting Fig. S1), suggesting that ER stress could directly lead to lipid accumulation in hepatocytes. Both immunofluorescence staining and western blotting revealed that PA exposure also induced the protein levels of GRP78, p-PERK, and p-eIF2α in HepG2 cells, to a lesser extent than thapsigargin (Fig. 3A,B). Notably, the increased expression of these ER stress markers induced by thapsigargin and PA was significantly inhibited by exendin-4 treatment (Fig. 3A,B). Meanwhile, exendin-4 significantly alleviated the lipid deposition induced by thapsigargin and PA in HepG2 cells (Fig. 3C; Supporting Fig. S1). We also confirmed that exendin-4 reduced PA-induced lipid accumulation in primary murine hepatocytes (Fig. 3D). Immunofluorescence staining revealed that the enhanced positive staining of GRP78 located in the cytoplasm of primary murine hepatocytes induced by PA was substantially reduced by exendin-4 treatment (Fig. 3E). Likewise, the protein levels of GRP78 and p-eIF2α were increased by PA exposure alone and significantly decreased by exendin-4 (Fig. 3F). Collectively, these results suggest that exendin-4 alleviates PA-induced ER stress, which may contribute to its amelioration of lipid accumulation in hepatocytes.

EXENDIN-4 ALLEVIATES PA-INDUCED HEPATIC ER STRESS IN A SIRT1-DEPENDENT MANNER

In HepG2 cells transfected with scrambled shRNA, PA exposure was observed to increase the protein levels

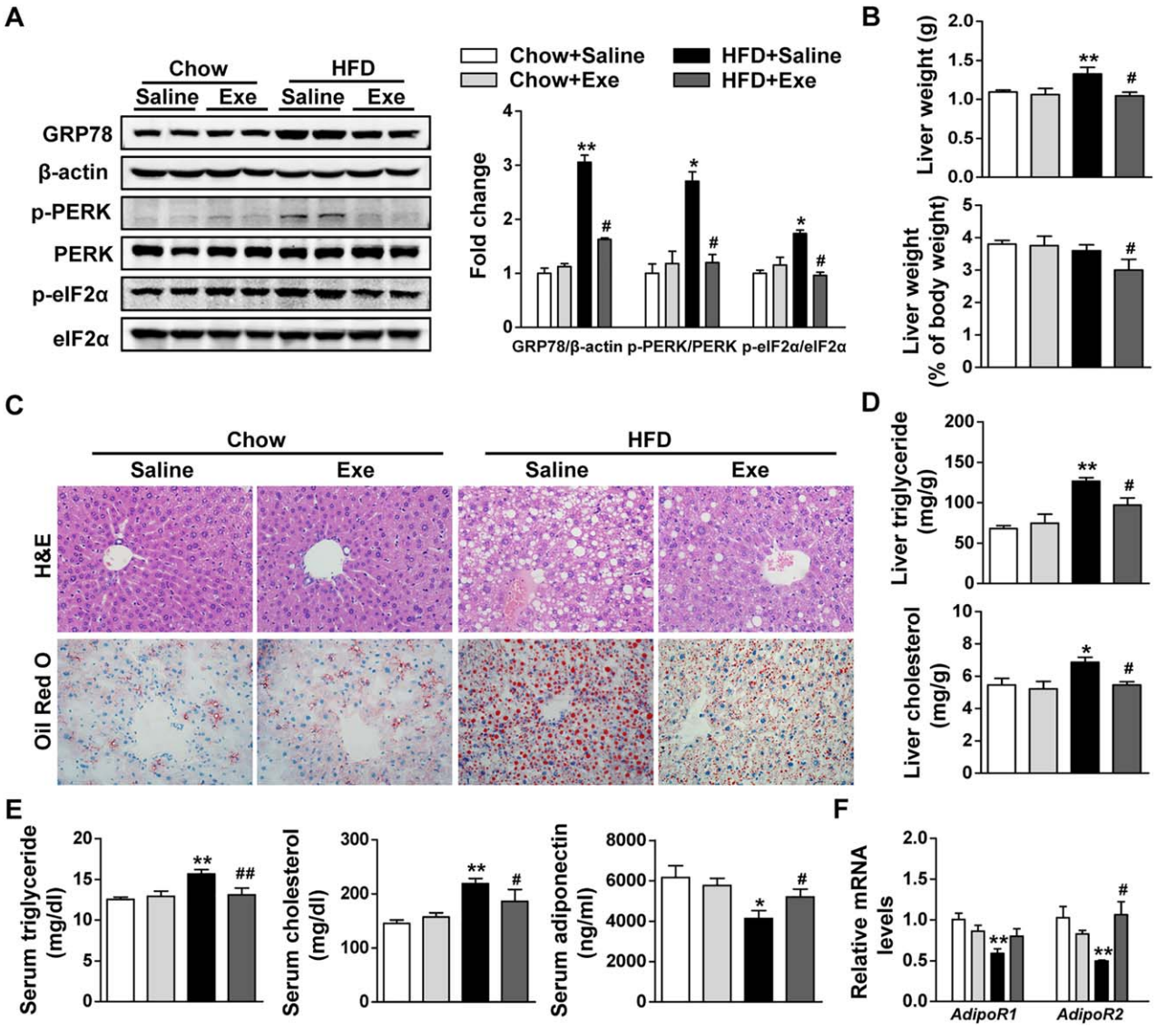

FIG. 2. Exenatide alleviates ER stress and hepatic steatosis in HFD-fed mice. C57BL/6J mice fed a chow diet or an HFD for 12 weeks were randomly treated with either normal saline or exenatide (24 nmol/kg/day) by intraperitoneal injection for 4 weeks with the diet intervention maintained (n = 5-6/group). (A) Western blot analysis of ER stress markers (GRP78, p-PERK, PERK, p-eIF2α, and eIF2α) in liver tissues and β-actin used as a loading control. (B) Liver weight and the ratio of liver weight to body weight. (C) Representative images of the liver, H&E staining and oil red O staining with ×400 magnification. (D) Liver triglyceride and cholesterol levels. (E) Serum triglyceride, cholesterol, and adiponectin levels. (F) Relative mRNA levels of *AdipoR1* and *AdipoR2* by quantitative real-time PCR. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the chow diet group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the HFD group. Abbreviation: Exe, exenatide.

of GRP78, p-PERK, and p-eIF2α in parallel with the reduced SIRT1 expression; and these changes were significantly reversed by exendin-4 treatment. By contrast, no obvious change in the expression of GRP78, p-PERK, and p-eIF2α was observed after exendin-4 treatment when *Sirt1* was knocked down by *Sirt1* shRNA (Fig. 4A). We noted that PA exposure induced a higher expression of GRP78 and p-eIF2α in HepG2 cells transfected with *Sirt1* shRNA than that with scrambled shRNA (Fig. 4A). We also observed that exendin-4 significantly decreased the mRNA expression of *Grp78* in PA-exposed HepG2 cells, but

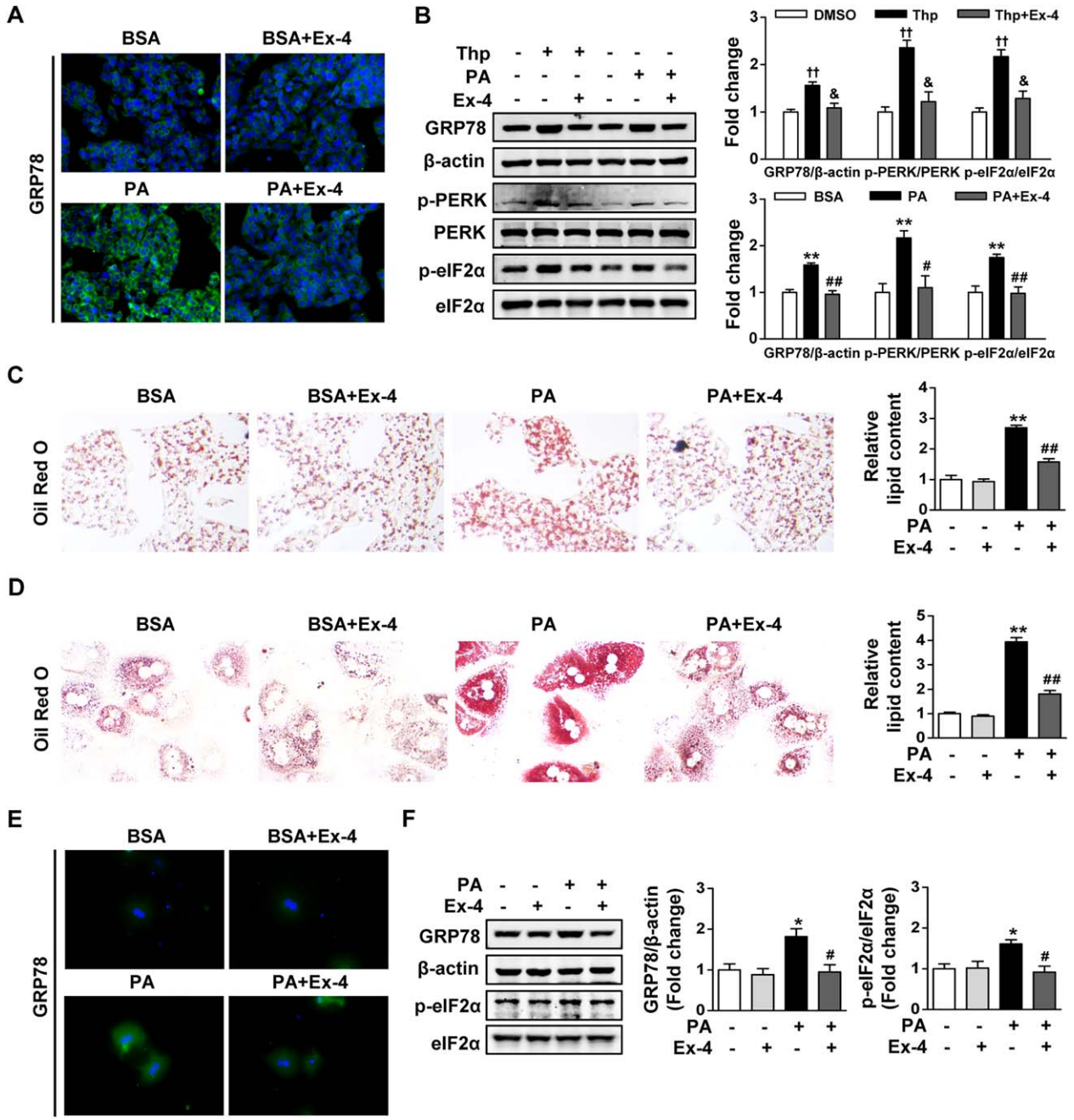

FIG. 3. Exendin-4 alleviates PA-induced ER stress and lipid accumulation in hepatocytes. HepG2 cells and primary murine hepatocytes exposed to thapsigargin (500 nM) or PA (300 μM) were treated with or without Ex-4 (100 nM) for 24 hours. (A) Representative images of immunofluorescence staining for GRP78 protein (green fluorescence) in HepG2 cells with × 400 magnification. (B) Western blot analysis of ER stress markers (GRP78, p-PERK, PERK, p-eIF2α, and eIF2α) in lysates from HepG2 cells, with β-actin as a loading control. (C,D) Representative images of oil red O staining were captured in HepG2 cells (C) and primary murine hepatocytes (D) with × 400 magnification. Absorbance of the oil red O content was determined at 510 nm with a spectrophotometer. (E) Representative images of immunofluorescence staining for GRP78 protein (green fluorescence) in primary murine hepatocytes with ×400 magnifications. (F) Western blot analysis of ER stress markers (GRP78, p-eIF2α, and eIF2α) in lysates from primary murine hepatocytes, with β-actin as a loading control. Data are expressed as mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the BSA group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the PA group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the DMSO group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with the Thp group. Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; Ex-4, exendin-4; PA, palmitate; Thp, thapsigargin.

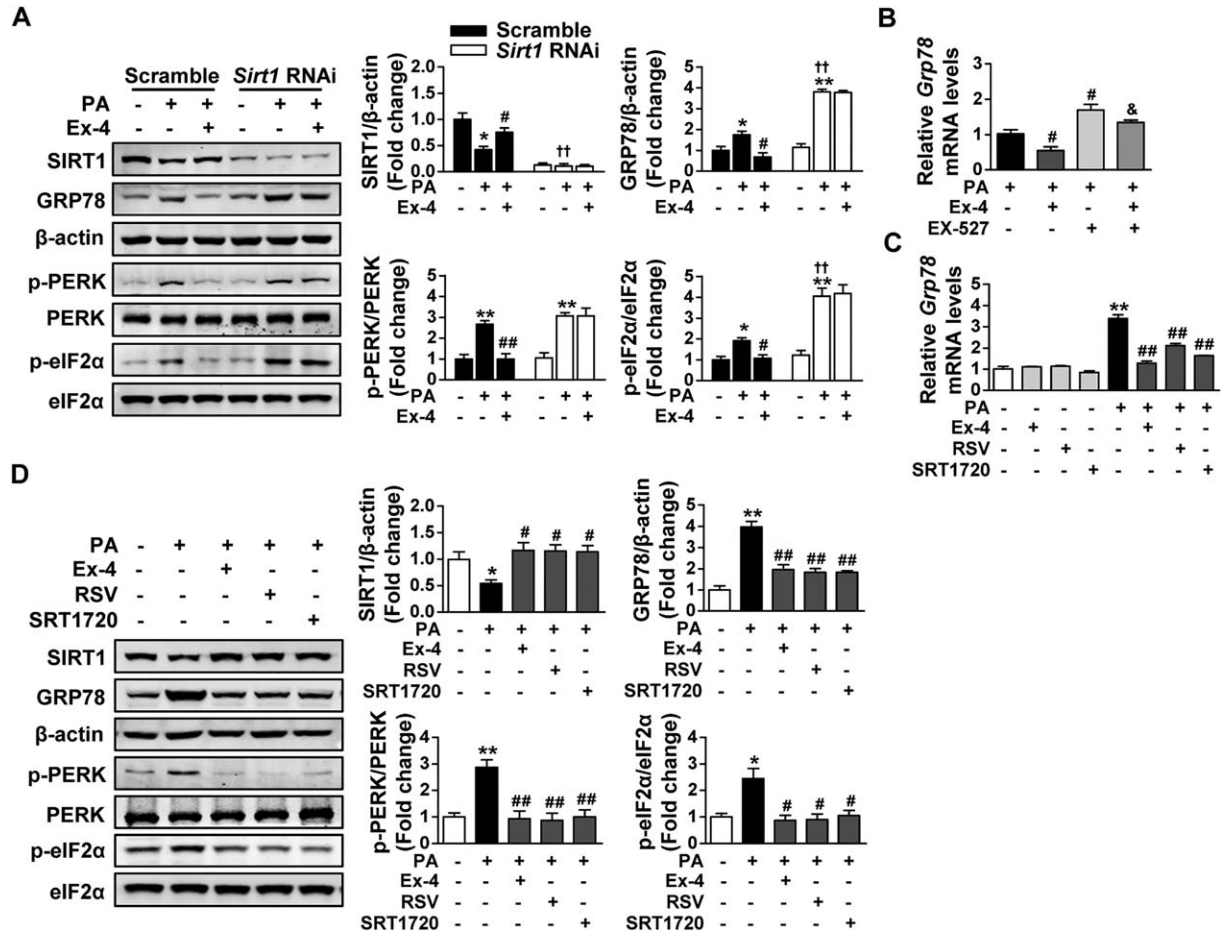

FIG. 4. Exendin-4 alleviates PA-induced hepatic ER stress in an SIRT1-dependent manner. (A) HepG2 cells were transfected with lentiviral *Sirt1* shRNA or scrambled shRNA and then incubated in medium containing PA (300 μM) with or without Ex-4 (100 nM) for 24 hours. Western blot analysis of SIRT1 and ER stress markers (GRP78, p-PERK, PERK, p-eIF2α, and eIF2α) was performed in the lysates, and β-actin was used as a loading control. Data are expressed as mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the corresponding BSA group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the corresponding PA group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the PA group transfected with scrambled shRNA. (B) HepG2 cells were pretreated with the SIRT1 inhibitor EX-527 (10 μM) for 1 hour and then incubated in medium containing PA (300 μM) with or without Ex-4 (100 nM) for 24 hours. Relative mRNA levels of *Grp78* were analyzed by quantitative real-time PCR. (C,D) HepG2 cells with or without PA (300 μM) were treated with Ex-4 (100 nM) or two SIRT1 agonists, RSV (50 μM) and SRT1720 (5 μM), for 24 hours. (C) Relative mRNA levels of *Grp78* by quantitative real-time PCR. (D) Western blot analysis of SIRT1 and ER stress markers (GRP78, p-PERK, PERK, p-eIF2α, and eIF2α) in lysates, with β-actin as a loading control. (B-D) Data are expressed as mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the BSA group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the PA group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with the PA with Ex-4–treated group. Abbreviations: BSA, bovine serum albumin; Ex-4, exendin-4; RSV, resveratrol.

this was greatly blocked by pretreatment with a SIRT1 inhibitor, EX-527 (Fig. 4B). In addition, the ability of exendin-4 to restore the reduced SIRT1 expression and the increased expression of ER stress markers induced by PA exposure was close to two SIRT1 activators, resveratrol and SRT1720 (Fig. 4C,D). These data demonstrate that exendin-4 alleviates PA-induced hepatic ER stress in a SIRT1-dependent manner.

EXENDIN-4-AMELIORATED PA-INDUCED HEPATIC ER STRESS IS ASSOCIATED WITH INCREASED HSP EXPRESSION THROUGH SIRT1-MEDIATED HSF1 DEACETYLATION

HSP, a large family of molecular chaperones that regulate protein homeostasis, prevent protein

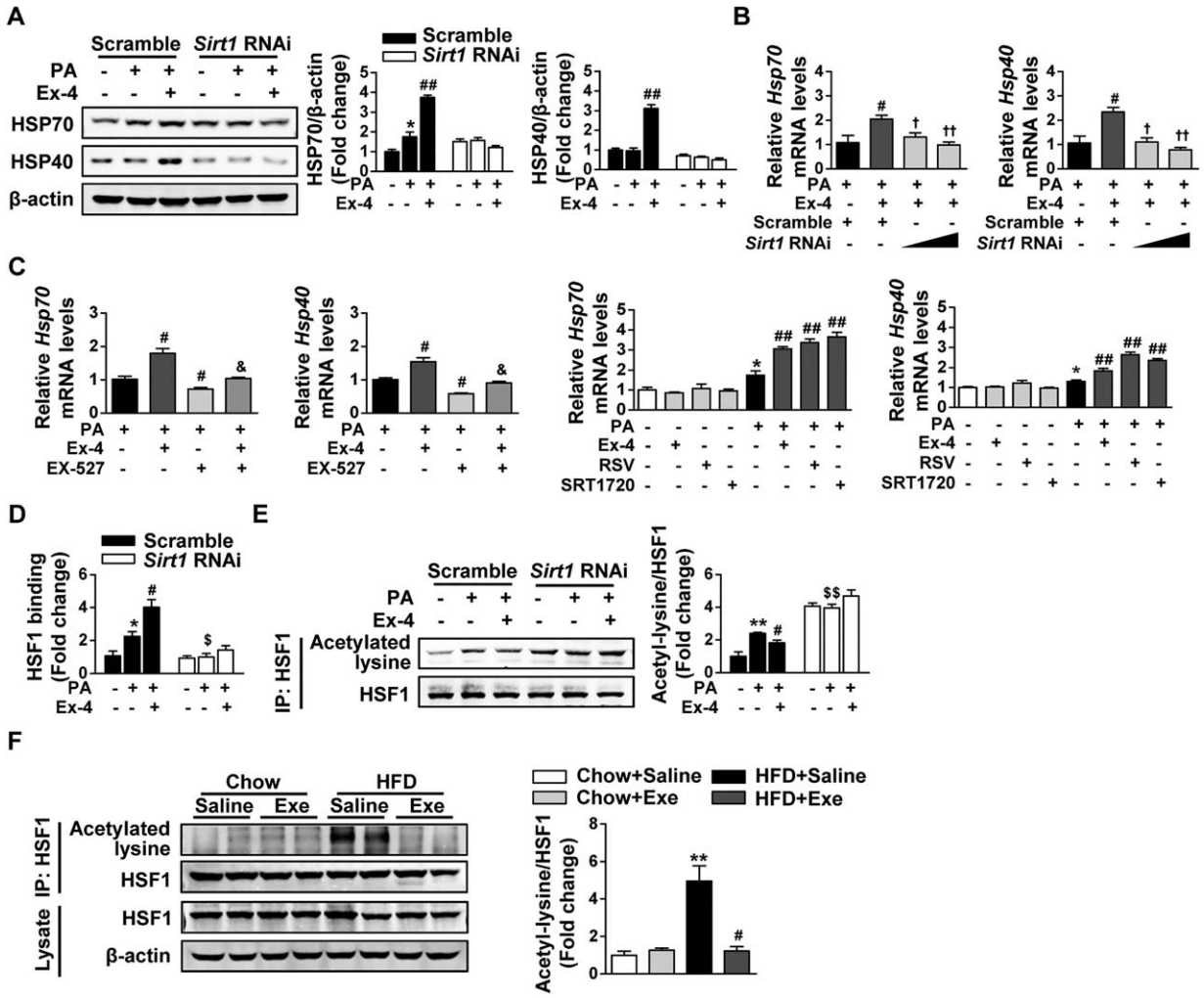

FIG. 5. Exendin-4-ameliorated, PA-induced hepatic ER stress is associated with increased HSP expression through SIRT1-mediated HSF1 deacetylation. (A,B,D,E) HepG2 cells were transfected with lentiviral *Sirt1* shRNA or scrambled shRNA and then incubated in medium containing PA (300 μM) with or without Ex-4 (100 nM) for 24 hours. (A) Western blot analysis of HSP (HSP70 and HSP40) in lysates, with β-actin as a loading control. (B) Relative mRNA levels of *Hsp70* and *Hsp40* by quantitative real-time PCR. (D) Relative HSF1 binding to the *Hsp70* promoter by ChIP and quantitative real-time PCR. (E) Acetylated lysine and total HSF1 expression by immunoprecipitation and western blot analysis. (A,B,D,E) Data are expressed as mean ± SEM from three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the corresponding BSA group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the corresponding PA group; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ compared with the PA with Ex-4–treated group transfected with scrambled shRNA; $^{\$}P<0.05$, $^{\$\$}P<0.01$ compared with the PA group transfected with scrambled shRNA. (C) HepG2 cells were pretreated with a SIRT1 inhibitor EX-527 (10 μM) for 1 hour and then incubated in medium containing PA (300 μM) with or without Ex-4 (100 nM) for 24 hours. In addition, HepG2 cells with or without PA (300 μM) were treated with Ex-4 (100 nM) or two SIRT1 agonists, RSV (50 μM) and SRT1720 (5 μM), for 24 hours. Relative mRNA levels of *Hsp70* and *Hsp40* were analyzed by quantitative real-time PCR. Data are expressed as mean ± SEM from three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the BSA group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the PA group; $^{\&}P<0.05$, $^{\&\&}P<0.01$ compared with the PA with Ex-4–treated group. (F) Acetylated lysine and total HSF1 expression in the livers of C57BL/6J mice described in Fig. 1 by immunoprecipitation and western blot analysis. Data are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ compared with the chow diet group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the HFD group. Abbreviations: BSA, bovine serum albumin; Ex-4, exendin-4; Exe, exenatide.

aggregation and participate in refolding or elimination of misfolded proteins in their capacity.[26,27] To investigate whether HSP were involved in exendin-4-ameliorated, PA-induced hepatic ER stress, we screened the expression of the HSP family in this system. As a result, exendin-4 treatment significantly increased the protein levels of HSP70 and HSP40 in PA-induced HepG2 cells. However, this effect disappeared when

Sirt1 expression was inhibited by *Sirt1* shRNA (Fig. 5A). The same trend was confirmed in the mRNA levels by quantitative real-time PCR (Fig. 5B; Supporting Fig. S2). Moreover, the effect of exendin-4 on gene expression induction of HSP was greatly blocked by the pretreatment with EX-527 (Fig. 5C). By contrast, exendin-4 was observed to up-regulate the gene expression of HSP70 and HSP40 comparable to resveratrol and SRT1720 (Fig. 5C). Taken together, these findings show that exendin-4-ameliorated, PA-induced hepatic ER stress is associated with increased HSP expression in a SIRT1-dependent manner.

Because HSF1 is a major transcription factor regulating the expression of HSP genes,[28] we performed a ChIP assay to investigate whether exendin-4 influenced the binding of HSF1 to the promoter of HSP genes in a SIRT1-dependent manner. As a result, exendin-4 significantly increased the binding of HSF1 to the *Hsp70* promoter in PA-induced HepG2 cells. However, no change was observed after exendin-4 treatment in *Sirt1* shRNA-transfected cells (Fig. 5D). Because SIRT1 functions as a protein deacetylase, the acetylation status of HSF1 was determined. In scrambled shRNA-transfected HepG2 cells, PA exposure increased the acetylation of HSF1, and this was significantly reversed by exendin-4 treatment. In *Sirt1* shRNA-transfected HepG2 cells, the acetylation of HSF1 was significantly higher and had no change regardless of exendin-4 treatment (Fig. 5E). We also confirmed that the acetylation of HSF1 was increased in the liver after HFD challenge, while this was significantly inhibited by exenatide (Fig. 5F). Altogether, these data indicate that exendin-4-ameliorated, PA-induced hepatic ER stress is associated with increased HSP expression through SIRT1-mediated HSF1 deacetylation.

EXENATIDE-INCREASED HSP EXPRESSION TO ATTENUATE ER STRESS AND HEPATIC STEATOSIS IS DIMINISHED IN HFD-FED *Sirt1*$^{+/-}$ MICE

To validate *in vitro* findings in animal studies, an HFD-fed *Sirt1*$^{+/-}$ mouse model was introduced in our study. We observed decreased SIRT1 expression in combination with an exaggerated ER stress response indicated by increased protein levels of GRP78 and p-PERK in the livers of *Sirt1*$^{+/-}$ mice compared to WT controls under HFD feeding (Fig. 6A). Not surprisingly, increased SIRT1 expression in parallel with decreased protein levels of GRP78, p-PERK, and p-eIF2α were observed after exenatide treatment in HFD-fed WT mice. However, no significant change was found in HFD-fed *Sirt1*$^{+/-}$ mice regardless of exenatide treatment (Fig. 6A). In addition, exenatide notably increased the protein levels of HSP70 and HSP40 in HFD-fed WT mice, while this effect was greatly diminished in HFD-fed *Sirt1*$^{+/-}$ mice (Fig. 6B). Histological analysis including H&E staining and oil red O staining showed more severe lipid accumulation in the livers of *Sirt1*$^{+/-}$ mice compared to WT controls under HFD feeding. The hepatic lipid accumulation induced by HFD challenge was dramatically improved by exenatide in WT controls. However, this effect was greatly weakened in *Sirt1*$^{+/-}$ mice (Fig. 6C). Consistently, the same tendency was observed in the triglyceride and cholesterol contents in both the liver and serum (Fig. 6D,E). These results suggest that exenatide-increased HSP expression to attenuate ER stress and hepatic steatosis is diminished in HFD-fed *Sirt1*$^{+/-}$ mice.

Lv *Sirt1* KNOCKDOWN ABOLISHES THE EFFECT OF EXENATIDE ON HSF1 DEACETYLATION, HSP INDUCTION, AND ALLEVIATION OF ER STRESS AND HEPATIC STEATOSIS

A *Sirt1* knockdown mouse model was also used to further confirm our conclusion. As a result, neither SIRT1 and p-eIF2α nor HSP70 and HSP40 displayed any change in protein expression after Lv-GFP injection compared to the HFD with the exenatide-treated group, while Lv-*Sirt1* RNAi injection dramatically decreased SIRT1 expression and abolished the effect of exenatide on increasing HSP expression and reducing p-eIF2α expression in the livers of HFD-fed mice (Fig. 7A,B). Consistent with the *in vitro* finding, we observed that exenatide-inhibited acetylation of HSF1 was greatly blocked by Lv-*Sirt1* RNAi injection (Fig. 7C). As shown by H&E staining and oil red O staining, 2-week exenatide treatment ameliorated the hepatic steatosis induced by 8-week HFD challenge, which was consistent with the results described above. Unlike Lv-GFP injection, Lv-*Sirt1* RNAi injection greatly diminished exenatide-ameliorated hepatic steatosis (Fig. 7D). Triglyceride and cholesterol quantification in the liver and serum displayed a similar tendency

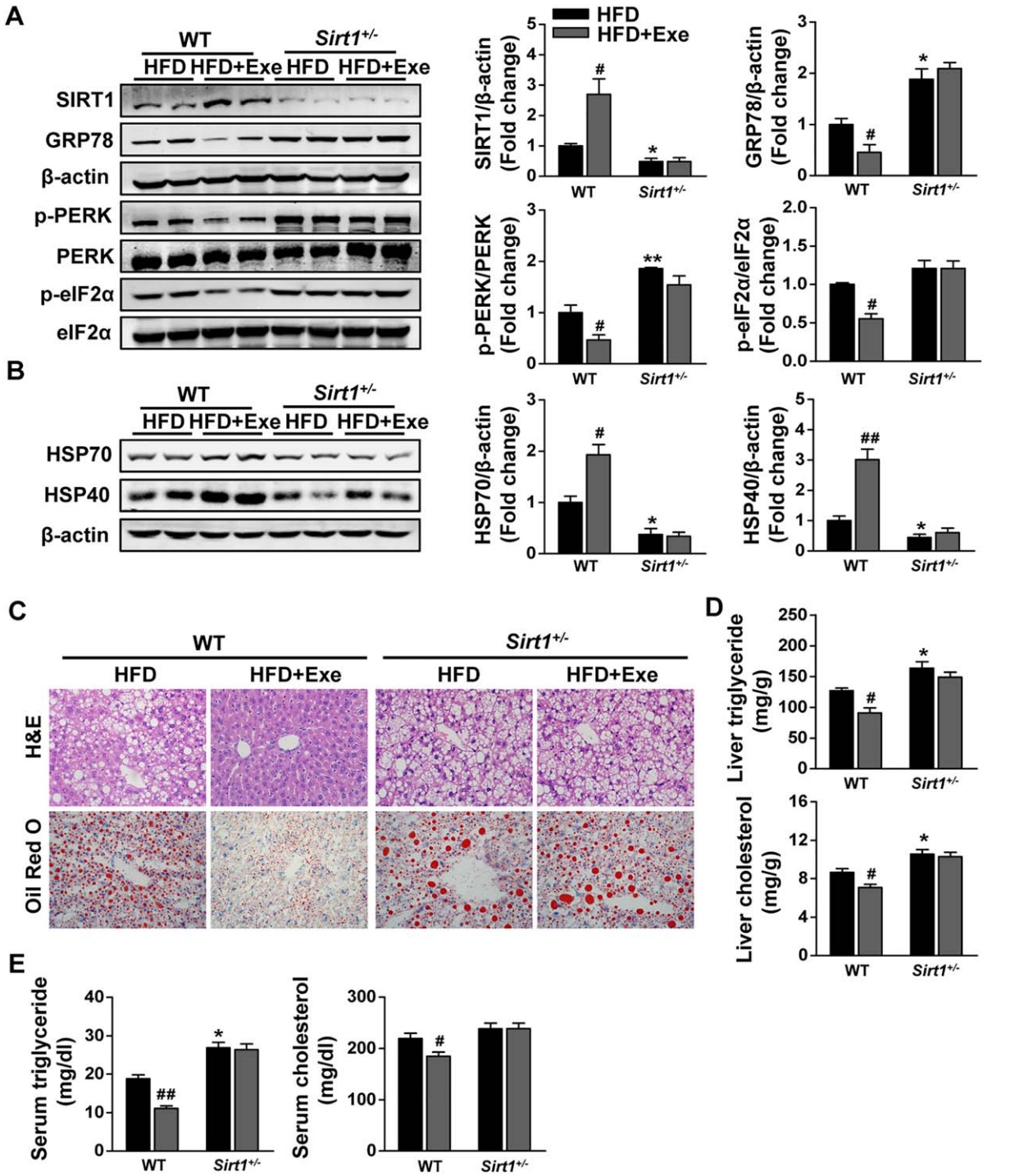

FIG. 6. Exenatide-increased HSP expression to attenuate ER stress and hepatic steatosis is diminished in HFD-fed *Sirt1*$^{+/-}$ mice. *Sirt1*$^{+/-}$ mice and their WT controls fed with an HFD for 12 weeks were randomly treated with either normal saline or exenatide (24 nmol/kg/day) by intraperitoneal injection for 8 weeks with the diet intervention maintained (n = 5-6/group). (A) Western blot analysis of SIRT1 and ER stress markers (GRP78, p-PERK, PERK, p-eIF2α, and eIF2α) in liver tissues, with β-actin as a loading control. (B) Western blot analysis of HSP (HSP70 and HSP40) in liver tissues, with β-actin as a loading control. (C) Representative images of liver H&E staining and oil red O staining with ×400 magnification. (D) Liver triglyceride and cholesterol levels. (E) Serum triglyceride and cholesterol levels. Data are expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the corresponding HFD group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the HFD group in WT mice. Abbreviation: Exe, exenatide.

with the histological analysis of liver (Fig. 7E,F). By contrast, the increased serum adiponectin levels and *AdipoR2* gene expression in the liver induced by exenatide treatment were not changed after Lv-*Sirt1* RNAi injection (Fig. 7F; Supporting Fig. S3A). In addition, exenatide-restored hepatic glucose homeostasis in HFD-fed mice was significantly weakened by Lv-*Sirt1* RNAi injection (Supporting Fig. S3B). Taken together, these findings verify again that *Sirt1* knockdown abolishes the effect of exenatide on HSF1 deacetylation, HSP induction, and alleviation of ER stress and hepatic steatosis.

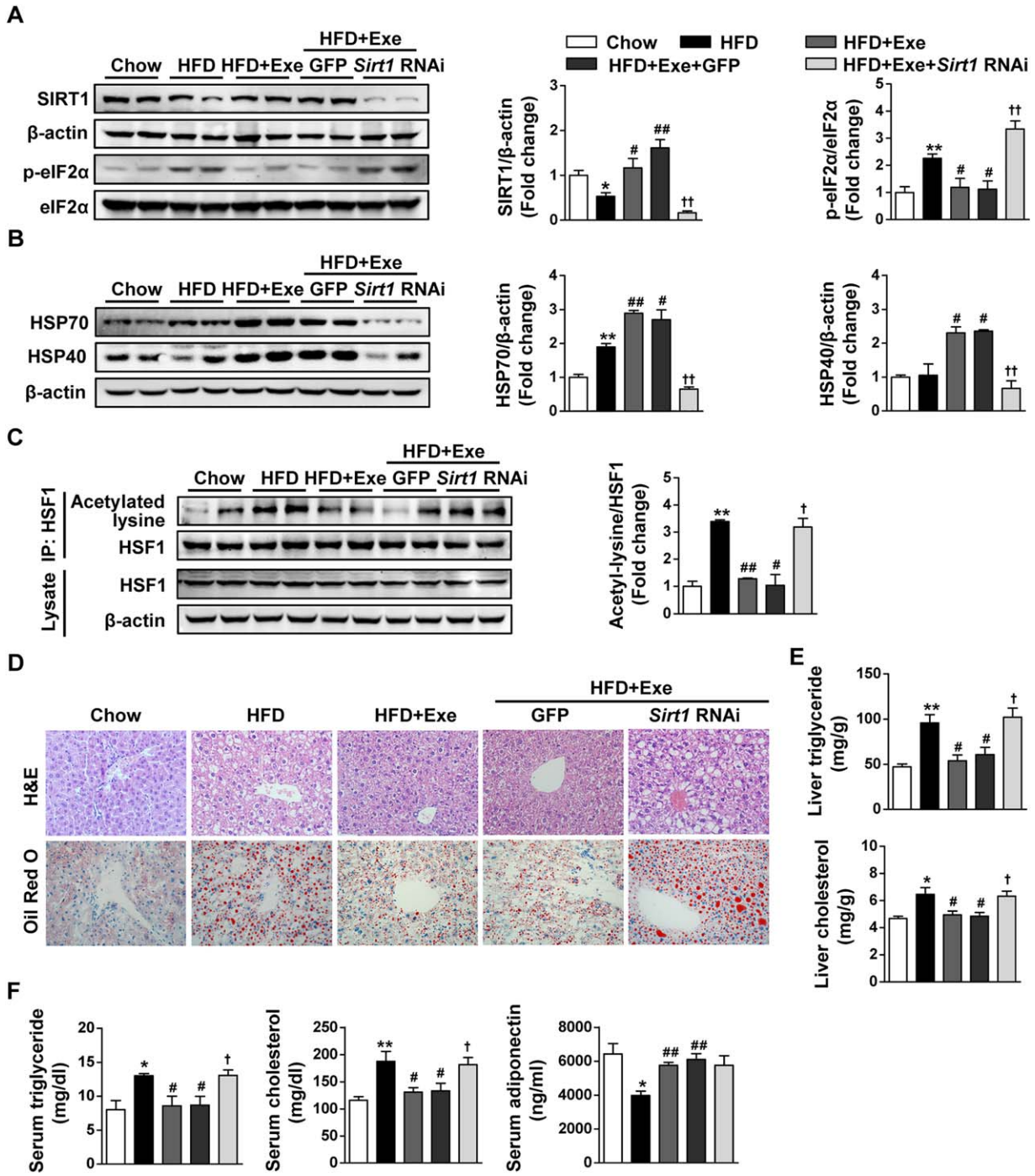

FIG. 7

Discussion

In the current study, we identify a mechanism that exenatide (exendin-4) increases HSP expression to alleviate obesity-induced ER stress and hepatic steatosis by up-regulating HSF1 deacetylation through SIRT1. It further supports the notion that exenatide and incretin mimetics act as promising therapeutics for obesity-induced hepatic steatosis.

GLP-1 receptor agonists, like exenatide and liraglutide, have been clinically licensed as antidiabetic agents. An early animal study by Ding et al. revealed the beneficial effect of exendin-4 on hepatic steatosis.[15] In addition, exenatide was observed to reverse fatty liver better than intensive insulin therapy in patients with obesity, NAFLD with elevated liver enzymes, and type 2 diabetes.[29] New clinical studies also demonstrated that liraglutide, which was safe and well tolerated, led to histological resolution of nonalcoholic steatohepatitis.[30,31] However, the mechanism involved in the beneficial effect of GLP-1 receptor agonists on hepatic steatosis has not been clearly defined. Accumulating evidence has indicated that the chronic and unresolved ER stress could contribute to the progression of hepatic steatosis through increased lipogenesis.[10,11] Genetic ablation studies revealed that the PERK/eIF2α arm of UPR appeared to promote the lipogenesis and development of hepatic steatosis.[12,13] In this study, we identified that exenatide (exendin-4) ameliorated hepatic lipid accumulation in a diet-induced obese mouse model and in PA-induced hepatocytes due to the alleviation of ER stress, which was indicated by significantly decreased expression of ER stress markers including GRP78, p-PERK, and p-eIF2α. This is the first time to prove that exenatide ameliorates lipid-induced hepatic ER stress to improve lipid deposition in hepatocytes both *in vivo* and *in vitro*. In support of our findings, a previous study showed that liraglutide protected against ER stress in the livers of HFD-induced insulin-resistant rats.[32] These findings collectively reveal the ability of GLP-1 receptor agonists to protect against ER stress and hepatic steatosis. Moreover, our study validated that exenatide (exendin-4) alleviated hepatic ER stress in a SIRT1-dependent manner by using genetic or pharmacological SIRT1 inhibition both *in vivo* and *in vitro*. This finding was supported by a study demonstrating that hepatic overexpression of SIRT1 attenuated ER stress and hepatic steatosis in obese mice.[19] Another study also revealed that SIRT1 activation could alleviate PA-induced ER stress in hepatocytes.[33] However, it has not been clearly defined how SIRT1 regulates hepatic ER stress.

SIRT1, the closest mammalian homolog of yeast silent information regulator 2, is now also recognized as a stress-response protein deacetylase.[34] HSF1, a stress-inducible transcription factor, translocates to the nucleus and binds to the promoter of HSP genes to up-regulate their transcription under a variety of stresses. Then the increased HSP serve as molecular chaperones to dampen proteotoxic stresses and maintain protein homeostasis through refolding of misfolded peptides and restraining protein aggregation.[28] A recent study demonstrates that SIRT1 deacetylates HSF1 and potentiates its binding to the promoter of HSP genes *in vitro*.[20] Thus, we infer that the SIRT1/HSF1/HSP pathway may serve as a mechanism involved in ER stress and metabolic disorders that are closely related with protein homeostasis. As a result, we found that exenatide (exendin-4) increased the deacetylation of HSF1 and HSP expression to alleviate lipid-induced ER stress and hepatic steatosis in a SIRT1-dependent manner. We identify an effect of regulation of the SIRT1/HSF1/HSP pathway on ER stress in hepatocytes, which is a new mechanism different from adenosine monophosphate–activated protein kinase activation that might account for exenatide-ameliorated hepatic steatosis.[17,18]

FIG. 7. Lentiviral *Sirt1* knockdown abolishes the effect of exenatide on HSF1 deacetylation, HSP induction, and alleviation of ER stress and hepatic steatosis. Male C57BL/6J mice were randomly divided into two groups fed a chow diet or an HFD for 8 weeks, and the HFD-fed mice were further divided into four groups: normal saline–treated group (HFD), exenatide-treated group (HFD+Exe), Lv-GFP with exenatide–treated group (HFD+Exe+GFP), and Lv-*Sirt1* RNAi with exenatide–treated group (HFD+Exe+*Sirt1* RNAi). One week after the injection of lentivirus or control solvent, mice were subjected to either normal saline or exenatide (24 nmol/kg/day) for 2 weeks (n = 5-6/group). (A) Western blot analysis of SIRT1 and ER stress markers (GRP78, p-eIF2α, and eIF2α) in liver tissues, with β-actin as a loading control. (B) Western blot analysis of HSP (HSP70 and HSP40) in liver tissues, with β-actin as a loading control. (C) Acetylated lysine and total HSF1 expression in the liver by immunoprecipitation and western blot analysis. (D) Representative images of liver H&E staining and oil red O staining with ×400 magnification. (E) Liver triglyceride and cholesterol levels. (F) Serum triglyceride, cholesterol, and adiponectin levels. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the chow diet group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the HFD group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the Lv-GFP injection group. Abbreviation: Exe, exenatide.

In addition, we observed that exenatide (exendin-4) up-regulated the expression of molecular chaperones including HSP70 and HSP40 to alleviate lipid-induced ER stress and hepatic steatosis both *in vivo* and *in vitro*, which emphasized the link between molecular chaperones and ER stress–related metabolic disorders. In support of our findings, studies have shown that activation of the inducible isoform of HSP70 protected against obesity-induced insulin resistance.[35,36] Hepatic overexpression of GRP78, an endogenous ER chaperone protein belonging to the HSP70 family, inhibited ER stress and sterol regulatory element binding protein 1c activation to reduce hepatic steatosis in mice.[37] Furthermore, chemical chaperones were found to reduce hepatic ER stress in a mouse model of type 2 diabetes and in PA-induced hepatocytes.[38,39] Taken together, these findings indicate that strategies targeting molecular chaperones may be novel therapeutics for the treatment of metabolic disorders involved with ER stress. A previous report showed that exendin-4 enhanced GRP78 expression but decreased ER stress–related cell death in fat-loaded hepatocytes.[40] By contrast, decreased GRP78 expression and increased HSP expression after exendin-4 treatment were observed simultaneously in our study. This inconsistency in GRP78 expression might reflect the different extent of ER stress existing in fat-loaded hepatocytes after exendin-4 treatment, which varied due to the different dosage and time course that both fatty acid and exendin-4 used. Because GRP78 is an ER chaperone protein belonging to the HSP70 family, we speculate that it is a direct transcriptional target of both UPR signaling and HSF1 signaling based on previous studies.[9,28] Therefore, the decreased GRP78 expression after exendin-4 treatment in our study might be mainly attributed to the down-regulated UPR signaling rather than the increased HSF1 deacetylation.

Recent publications have indicated a protective effect of adiponectin on hepatic steatosis and fibrosis.[41,42] AdipoR1 and AdipoR2 are two receptors of adiponectin, which are both expressed in the liver. AdipoR1 is primarily expressed in activated hepatic stellate cells, while AdipoR2 is primarily expressed in other liver cells such as hepatocytes. Adiponectin binds to its receptors and stimulates adenosine monophosphate–activated protein kinase in the liver, a crucial factor in glucose and lipid metabolism.[42,43] Thus, we also detected an effect of exenatide on adipose-derived adiponectin and its hepatic downstream signaling in this study. It showed that exenatide restored the reduced serum adiponectin levels and significantly up-regulated the mRNA expression of *AdipoR2* in the livers of HFD-induced obese mice, which indicated that the beneficial effect of exenatide-increased adiponectin was mainly mediated by AdipoR2 in the liver. Besides, our previous research verified that exenatide could up-regulate the adenosine monophosphate–activated protein kinase activity in the liver.[17] Taken together, our findings are consistent with a report indicating that exenatide increased serum adiponectin levels in obese mice,[15] which also provides another possible mechanism to explain the effect of exenatide on improving hepatic steatosis.

In our study, tail vein injection of viral shRNA was used for *in vivo* gene knockdown as described.[44,45] Due to the liver first-pass effect of virus, we infer that Lv-*Sirt1* shRNA by tail vein injection in our mouse model might primarily target to the liver. To confirm this conjecture, we examined SIRT1 expression in different organs with western blot analysis. As expected, liver was the organ that SIRT1 was knocked down the most (~90% reduction). There were no significant changes of SIRT1 expression in other insulin-sensitive organs including muscle, white adipose tissue (epididymal fat), and brown adipose tissue after Lv-*Sirt1* RNAi injection compared with Lv-GFP control (Supporting Fig. S4A). Meanwhile, immunohistochemical analysis of liver sections also revealed a marked decrease in SIRT1 expression after Lv-*Sirt1* RNAi injection (Supporting Fig. S4B). Consistently, the acetylation of p53 at Lys382, a specific deacetylation site of SIRT1,[19] showed a 2-fold to 3-fold increase in the liver after Lv-*Sirt1* RNAi injection (Supporting Fig. S4C). Studies have shown that SIRT1 regulates the production of adiponectin in adipocytes through forkhead box O1.[46,47] Importantly, we found no change in the serum adiponectin levels between Lv-*Sirt1* RNAi injection and Lv-GFP control. Thus, our result provided another credible proof that tail vein injection of Lv-*Sirt1* RNAi did not affect the white adipose tissue because adiponectin was primarily produced and secreted by white adipose tissue. Taken together, this Lv-*Sirt1* knockdown mouse model in our study could be considered as a proper model for studying the causal role of SIRT1 in mediating exenatide's effect on the liver. However, it should be noted that this mouse model has limitations due to the potential for Lv-*Sirt1* shRNA to target other non-insulin-sensitive organs and that the liver-specific *Sirt1* knockout mouse is the ideal mouse model for this study. Therefore, by combination with the two mouse

models of SIRT1 inhibition and *in vitro* models, our results provide strong biochemical evidence that the SIRT1/HSF1/HSP pathway mediates the beneficial effect of exenatide on lipid-induced ER stress and hepatic steatosis associated with obesity.

In summary, our study demonstrates that exenatide (exendin-4) increases HSP expression to alleviate lipid-induced ER stress and hepatic steatosis through SIRT1-mediated HSF1 deacetylation. This indicates that the SIRT1/HSF1/HSP pathway is essential for exenatide-alleviated, lipid-induced ER stress and hepatic steatosis, which provides a novel mechanism supporting exenatide and incretin mimetics as promising therapeutics for obesity-induced hepatic steatosis.

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Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29238/suppinfo.

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SIRT1 Mediates the Effect of GLP-1 Receptor Agonist Exenatide on Ameliorating Hepatic Steatosis

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GLP-1 and incretin mimetics, such as exenatide, have been shown to attenuate hepatocyte steatosis in vivo and in vitro, but the specific underlying mechanism is unclear. SIRT1, an NAD$^+$-dependent protein deacetylase, has been considered as a crucial regulator in hepatic lipid homeostasis by accumulated studies. Here, we speculate that SIRT1 might mediate the effect of the GLP-1 receptor agonist exenatide (exendin-4) on ameliorating hepatic steatosis. After 8 weeks of exenatide treatment in male *SIRT1*$^{+/-}$ mice challenged with a high-fat diet and their wild-type (WT) littermates, we found that lipid deposition and inflammation in the liver, which were improved dramatically in the WT group, diminished in *SIRT1*$^{+/-}$ mice. In addition, the protein expression of SIRT1 and phosphorylated AMPK was upregulated, whereas lipogenic-related protein, including SREBP-1c and PNPLA3, was downregulated in the WT group after exenatide treatment. However, none of these changes were observed in *SIRT1*$^{+/-}$ mice. In HepG2 cells, exendin-4–reversed lipid deposition induced by palmitate was hampered when SIRT1 was silenced by SIRT1 RNA interference. Our data demonstrate that SIRT1 mediates the effect of exenatide on ameliorating hepatic steatosis, suggesting the GLP-1 receptor agonist could serve as a potential drug for nonalcoholic fatty liver disease (NAFLD), especially in type 2 diabetes combined with NAFLD, and SIRT1 could be a therapeutic target of NAFLD.

Nonalcoholic fatty liver disease (NAFLD) is a burgeoning health problem that begins with the aberrant accumulation of triglyceride in the liver. It includes isolated fatty liver and nonalcoholic steatohepatitis (NASH), the latter of which can progress to cirrhosis and liver cancer in some individuals (1). In addition, NAFLD is mostly common in obesity and metabolic syndrome, both of which are strongly associated with insulin resistance. The most challenging problem is that no drug therapy has been approved for NAFLD so far (2).

GLP-1, an incretin hormone, is a gut-derived peptide secreted by intestinal L cells after a meal. It has pleiotropic functions in mammals to promote insulin secretion of pancreatic β-cells, suppress inappropriate glucagon secretion, slow gastric emptying, and induce insulin-mediated glucose uptake (3). As a new kind of antidiabetes drug, incretin mimetics, such as exenatide (exendin-4), increasing amounts of evidence have proved that they effectively improve lipid deposition in the liver (4–6). However, the specific underlying mechanism is little known. That the GLP-1 receptor is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro has been reported (5). A recent study found that exendin-4 could reduce inflammation in the liver by inhibiting macrophage recruitment and activation (7). Nevertheless, the exact mechanism of the signaling pathway of GLP-1 and its mimetics on improving hepatic steatosis is not fully understood.

SIRT1, mammalian sirtuin 1, is a kind of NAD$^+$-dependent protein deacetylase and is an important regulator of energy homeostasis in response to nutrient availability (8,9). We

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previously found that loss of SIRT1 leads to more serious liver steatosis in *SIRT1*$^{+/-}$ mice compared with wild-type (WT) mice after high-fat diet (HFD) induction (10). Another study also showed that hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation (11). However, hepatic overexpression of SIRT1 in mice attenuates endoplasmic reticulum stress and insulin resistance in the liver (12). Activating the SIRT1 signaling program by resveratrol or SRT1720 relieves fatty liver, with reduced lipid synthesis and an increased rate of fatty acid oxidation (13,14). A recent study reported that SIRT1 mediated the activation of FGF21, which could prevent liver steatosis caused by fasting (15). Another study demonstrated that hepatic deletion of SIRT1 promoted steatosis and inflammation in response to an ethanol challenge via lipin-1, a transcriptional regulator of lipid metabolism (16). All of the above indicate that SIRT1 is vital in the lipid homeostasis of the liver.

Because SIRT1 plays such an essential role in the lipid metabolism of the liver, whether the amelioration of hepatic steatosis by the GLP-1 receptor agonist exenatide is mediated by SIRT1 remains to be investigated. Here, we presume that exenatide improves liver steatosis via the SIRT1 pathway. In our study, *SIRT1*$^{+/-}$ mice and their WT littermates were challenged with an HFD, followed by exenatide treatment. We found that GLP-1 receptor agonist treatment could reverse liver steatosis in WT mice but not in *SIRT1*$^{+/-}$ mice, which indicates that loss of SIRT1 significantly impairs the effect of the GLP-1 receptor agonist. These results, for the first time to our knowledge, point out that SIRT1 is indispensable in mediating the effect of the GLP-1 receptor agonist exenatide on ameliorating hepatic steatosis.

RESEARCH DESIGN AND METHODS

Animals and Diets

SIRT1$^{+/-}$ mice in C57BL/6J gene background were a gift from Prof. Jianping Ye from Pennington Biomedical Research Center, Louisiana State University (10). C57BL/6J breeders (7–8 weeks old) were purchased from the Model Animal Research Center of Nanjing University, Nanjing, China. Male *SIRT1*$^{+/-}$ mice and their WT littermates were used in the study. The mice were maintained at 22 ± 2°C and 50 ± 5% relative humidity with a 12-h light/dark cycle. All mice had ad libitum access to rodent chow diet (5% fat wt/wt; Guangdong Medical Laboratory Animal Center) and water. The HFD (D12492; Research Diets), which contains 60% calories from fat, was used to induce obesity and fatty liver. After 12 weeks of the chow diet or the HFD challenge, mice were divided randomly into the following five groups: WT + chow diet, WT + HFD + saline, WT + HFD + exenatide, *SIRT1*$^{+/-}$ + HFD + saline, and *SIRT1*$^{+/-}$ + HFD + exenatide. Mice were treated with a daily intraperitoneal injection of exenatide (24 nmol/kg; Eli Lilly and Company, Indianapolis, IN) or normal saline control for 8 weeks. Food intake and body weight were monitored once every 2 weeks during this period. By the end of the 20th week, all the animals were fasted for 8 h, anesthetized with ether, and then killed for blood and tissue collection. All experiments were approved by the Sun Yat-Sen University Animal Ethics Committees.

Cell Culture and Treatments

HepG2 human hepatoma cells obtained from the American Type Culture Collection were cultured in minimum essential medium containing 10% (vol/vol) FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown to 70% confluence and incubated in serum-free medium for 4 h before treatments. To knock down SIRT1 in HepG2 cells, cells were transfected with a lentivirus vector expressing SIRT1 short hairpin (sh)RNA sequence and the control vector as scramble (Genechem, Shanghai, China). HepG2 cells were treated with palmitate (#P9767; Sigma-Aldrich) and exendin-4 (#E7144; Sigma-Aldrich) or resveratrol (#R5010; Sigma-Aldrich), if indicated. Cell lysates were collected for Western blot analysis.

Intraperitoneal Glucose Tolerance Test and Insulin Tolerance Test

For the intraperitoneal glucose tolerance test (IPGTT), mice were fasted overnight and administered with glucose (2.5 g/kg wt i.p.) the next morning. The intraperitoneal insulin tolerance test (IPITT) was conducted by an injection of insulin (0.75 units/kg wt i.p., Novolin R; Novo Nordisk) after 4 h fasting, as previously described. Tail vein blood glucose was measured at 0, 30, 60, and 120 min with the Optium Xceed glucometer (Abbott Diabetes Care, Inc., Alameda, CA) in IPGTT and IPITT.

Quantitative Real-Time PCR

Tissues were collected, kept in liquid nitrogen, and stored at −80°C. Total RNA was extracted from the liver using TRIzol reagent (Invitrogen, Shanghai, China). RNA was reverse transcribed to cDNA using the Prime Script RT Reagent Kit (Takara Bio, Shiga, Japan). The primers (Applied Biosystems, Foster City, CA) included F4/80 (Mm00802530_m1), tumor necrosis factor (TNF)-α (Mm00443258_m1), and monocyte chemoattractant protein (MCP)-1 (Mm00441242_m1). The quantitative real-time PCR was conducted with the LightCycler 480II Real-Time PCR System (Roche Diagnostics, Mannheim, Germany).

Western Blot

Livers were rinsed with ice-cold PBS and stored at −80°C until Western blot analysis. Liver tissues were homogenized in the whole cell lysis buffer. Antibodies included these against SIRT1 (#2496; Cell Signaling Technology, Danvers, MA), phosphorylated (p)-AMPK (Thr1724, #2535; Cell Signaling Technology), total-AMPK (#2603; Cell Signaling Technology), p-acetyl-CoA carboxylase ([ACC] #3661; Cell Signaling Technology), ACC (#3676; Cell Signaling Technology), PNPLA3 (ab81874; Abcam, Cambridge, U.K.),

SREBP-1 (#9874; Cell Signaling Technology), SREBP-1 (sc-367; Santa Cruz Biotechnology, Inc., Dallas, TX), and p-SREBP-1c (#9874; Cell Signaling Technology). β-Actin (#4970; Cell Signaling Technology) served as a loading control. The membranes were incubated with secondary antibodies (1:10,000, DyLight 800; Thermo Fisher Scientific, Waltham, MA) at room temperature for 1 h. The membranes were imaged with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band intensities were quantified by densitometry.

Liver Lipids Test

Liver tissues were homogenized in PBS (1 g/20 mL). The lipids were extracted from the liver tissue lysates using a chloroform/methanol (2:1) mixture (17). Triglyceride and glycerol were determined using the Serum Triglyceride Determination Kit (TR0100; Sigma-Aldrich). Cholesterol was determined with Cholesterol Reagent 80015 (Biovison, Milpitas, CA) according to the instructions by the manufacturer.

Hematoxylin and Eosin Staining

Fresh liver tissues were collected and fixed in 4% neutral buffered formalin solution (HT50-1-2; Sigma-Aldrich). The tissue slides were obtained through serial cross-section cutting at 6–8 μm thickness and processed with a standard procedure of hematoxylin and eosin staining.

Oil Red O Staining

Accumulation of triglyceride content in the liver and in the treated HepG2 cells was visualized by Oil Red O (Sigma-Aldrich) staining, as previously described (18). The lipids accumulation was photographed with a BX51WI microscope (Olympus, Tokyo, Japan).

Immunohistochemistry Staining

Fresh liver tissues were fixed in neutral buffered formalin, dehydrated, and embedded in paraffin. Thin tissue slides (3–5 μm) were deparaffinized, blocked, and incubated overnight at 4°C with antibody of mouse anti-rat F4/80 (sc-71087; Santa Cruz Biotechnology, Inc.). The immunoreactions were revealed using OneStep Polymer HRP anti-mouse/rat/rabbit Detection System (GTX83398; GeneTex, Inc., Irvine, CA) and using DAB as chromogen. Photomicrographs were taken under a DM 2500B microscope (Leica Microsystems, Wetzlar, Germany) with original magnification ×20 and ×40.

Statistical Analysis

The data are presented as the mean ± SEM from multiple samples (n = 5–12 for each group in the animal study). All of the in vitro experiments were replicated at least three times. The two-tailed, unpaired Student t test was used in the statistical analysis, with significance at $P \leq 0.05$.

RESULTS

The Effect of Exenatide on Reducing Body Weight and Maintaining Glucose Homeostasis Is Attenuated in *SIRT1*$^{+/-}$ Mice

First, *SIRT1*$^{+/-}$ mice and their littermate WT mice were induced with the HFD for 12 weeks and then treated with exenatide or saline as normal control for another 8 weeks. Body weight of WT mice on the HFD challenge was significantly higher than the body weight of mice fed the chow diet from 8 to 12 weeks. From 10 to 12 weeks, *SIRT1*$^{+/-}$ mice became statistically heavier than WT mice fed the HFD (Fig. 1*A*). No significant difference was observed in food intake (kcal/kg/h) between WT and *SIRT1*$^{+/-}$ mice fed the HFD (Fig. 1*B*).

After 4 weeks of exenatide administration, WT mice began to show a significant reduction of body weight compared with the saline control, which lasted until the end of the intervention period. However, this body weight–reducing effect diminished in *SIRT1*$^{+/-}$ mice, reflected by no significant difference observed between exenatide-treated mice and control mice (Fig. 1*C*). Food intake declined slightly after exenatide treatment in the WT and *SIRT1*$^{+/-}$ groups, yet still remained statistically comparable among all groups (Fig. 1*D*).

The IPGTT and IPITT were conducted after 8 weeks of exenatide administration. The fasting blood glucose (FBG) level of the HFD-challenged WT mice was higher than the level of those fed the chow diet and showed a significant reduction after exenatide treatment. *SIRT1*$^{+/-}$ mice had higher FBG compared with WT mice fed the HFD, and this difference was sustained after exenatide treatment in the two groups. However, the FBG level of *SIRT1*$^{+/-}$ mice did not show any statistical difference with or without exenatide treatment (Fig. 1*E*). After administration of glucose, all trends above were maintained or even stronger at 30 and 60 min (Fig. 1*E*). The area under the curve also revealed significantly improved glucose tolerance in WT mice after exenatide treatment. However, the impaired glucose tolerance of *SIRT1*$^{+/-}$ mice fed the HFD was not improved as much as that in the WT mice after exenatide treatment (Fig. 1*E*). During the IPITT test, administration of insulin led to a significant decrease of blood glucose levels in WT mice after the exenatide treatment but not in *SIRT1*$^{+/-}$ mice (Fig. 1*F*). The area under the curve showed statistically improved insulin sensitivity in WT mice after the exenatide treatment. However, no statistical improvement of insulin sensitivity was observed in *SIRT1*$^{+/-}$ mice fed the HFD after exenatide treatment (Fig. 1*F*).

All of the above results indicate that the effect of exenatide on reducing body weight and maintaining glucose homeostasis is attenuated in *SIRT1*$^{+/-}$ mice.

Exenatide-Improved Liver Weight, FBG, and Lipid Profile Are Weakened in *SIRT1*$^{+/-}$ Mice

After all of the treatments, we collected and weighed the livers, detected FBG and fasting insulin levels, and tested the lipids profile in these mice. As expected, the liver weight in WT mice fed the HFD was dramatically decreased after exenatide treatment compared with the saline control. However, this effect was weakened in *SIRT1*$^{+/-}$ mice. The same trend was observed in FBG change. The fasting insulin level did not show a significant

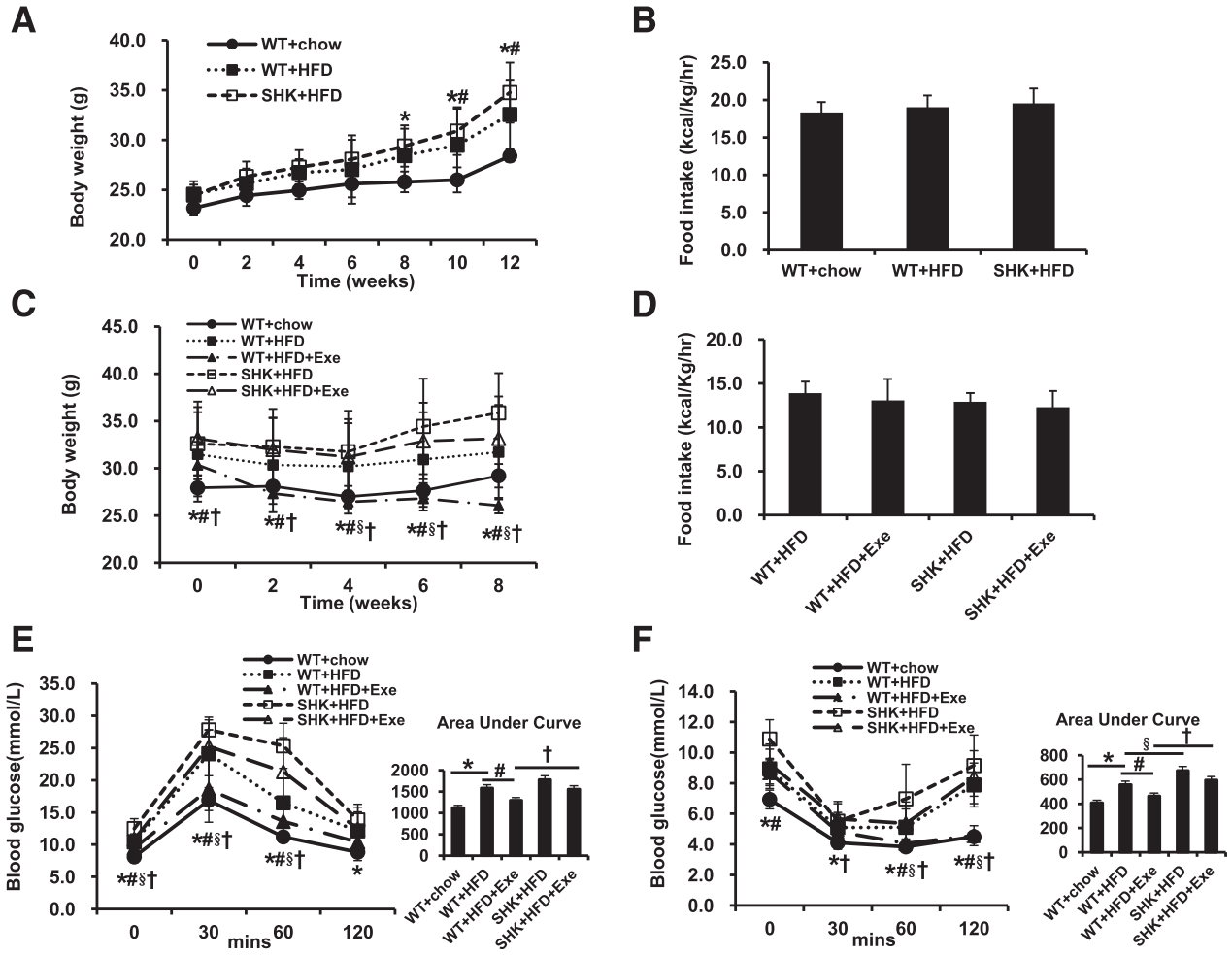

Figure 1—The effect of exenatide on reducing body weight and maintaining glucose homeostasis is attenuated in *SIRT1*$^{+/-}$ mice. *A*: Body weight during HFD feeding detected every 2 weeks. *P < 0.05 WT+HFD vs. WT+chow; #P < 0.05 SHK+HFD vs. WT+HFD. *B*: Average food intake during HFD feeding before exenatide treatment. *C*: Body weight during exenatide treatment detected every 2 weeks. *D*: Average food intake during exenatide treatment. *E*: IPGTT after exenatide treatment and area under the curve. *F*: IPITT after exenatide treatment and area under the curve. For *C*, *E*, and *F*, *P < 0.05 WT+HFD vs. WT+chow; #P < 0.05 SHK+HFD vs. WT+HFD; §P < 0.05 WT+HFD+Exe vs. WT+HFD; †P < 0.05 SHK+HFD+Exe vs. WT+HFD+Exe. Data are expressed as mean ± SEM (n = 5–12). Exe, exenatide; SHK, SIRT1 heterozygous knockout mice.

difference among these groups due to the big variation within groups (Table 1).

Next, the lipids profile, including triglyceride, glycerol, and total cholesterol, in serum were tested. Exenatide treatment decreased the triglyceride, glycerol, and total cholesterol level by 37%, 29%, and 9% in HFD-induced WT mice compared with the saline control, respectively (Table 1). *SIRT1*$^{+/-}$ mice had significantly higher serum lipids levels than WT mice fed the HFD, and this difference was sustained after exenatide treatment in the two groups (Table 1). However, no statistical changes were observed in *SIRT1*$^{+/-}$ mice regardless of exenatide treatment (Table 1).

Exenatide-Ameliorated Liver Steatosis Disappears in *SIRT1*$^{+/-}$ Mice

Although a recent study showed a GLP-1 receptor agonist could relieve hepatic steatosis by upregulation of SIRT1 in C57BL/6J mice (19), whether exenatide-induced hepatic steatosis attenuation is directly mediated by SIRT1 is still unclear. To test this possibility, we examined the morphology, histology, and lipids content of the livers in *SIRT1*$^{+/-}$ mice and their littermate WT mice, with or without exenatide treatment. Photographs showed the livers exhibited bigger size and white coloring in *SIRT1*$^{+/-}$ mice irrespective of exenatide treatment (Fig. 2*A*). Hematoxylin and eosin staining and Oil Red O staining both showed a significant increase of lipid droplets in hepatocytes of *SIRT1*$^{+/-}$ mice compared with WT mice fed the HFD (Fig. 2*B*, original magnification ×40). The white coloring and lipid droplets in hepatocytes were dramatically improved in HFD-induced WT mice after exenatide treatment compared with the saline control; however, all of these effects disappeared in *SIRT1*$^{+/-}$ mice (Fig. 2*A* and *B*). Consistently, triglyceride, glycerol, and total

Table 1—Exenatide-improved liver weight, FBG, and lipid profile are weakened in *SIRT1*$^{+/-}$ mice

Assay	WT+chow	WT+HFD	SHK+HFD	WT+HFD+Exe	SHK+HFD+Exe
Liver weight (g)	1.16 ± 0.06	1.26 ± 0.06*	1.36 ± 0.07#	0.98 ± 0.05§	1.21 ± 0.06†
FBG (mmol/L)	7.60 ± 0.38	11.55 ± 0.58*	12.19 ± 0.61#	10.10 ± 0.51§	11.51 ± 0.58†
Insulin (pg/mL)	2,747.43 ± 393.71	2,379.88 ± 763.22	2,663.23 ± 859.33	2,173.86 ± 976.46	1,351.72 ± 602.80
Serum					
Triglyceride (mg/dL)	12.94 ± 0.65	20.82 ± 1.04*	28.86 ± 1.45#	13.10 ± 0.67§	28.40 ± 1.53†
Glycerol (mg/dL)	8.58 ± 0.43	14.79 ± 0.80*	21.96 ± 1.09#	10.52 ± 0.53§	21.10 ± 1.10†
Cholesterol (mg/dL)	157.23 ± 7.82	199.39 ± 9.97*	218.30 ± 10.92#	181.11 ± 9.06§	218.78 ± 10.88†

Exe, exenatide; SHK, SIRT1 heterozygous knockout mice. After 12 weeks of HFD induction and 8 weeks of exenatide treatment, mice were fasted overnight and tail vein blood glucose concentrations were measured the next morning. The mice were anesthetized before being killed. Blood was collected first, and then livers were collected and weighed. Serum levels of insulin, triglyceride, glycerol, and cholesterol were determined. Values are mean ± SEM (n = 5–12). *P < 0.05 WT+HFD vs. WT+chow. #P < 0.05 SHK+HFD vs. WT+HFD. §P < 0.05 WT+HFD+Exe vs. WT+HFD. †P < 0.05 SHK+HFD+Exe vs. WT+HFD+Exe.

cholesterol contents in the livers were decreased by 38%, 29%, and 13% in HFD-induced WT mice with exenatide compared with the saline control, respectively. But no change was observed in lipid contents in HFD-induced *SIRT1*$^{+/-}$ mice after exenatide treatment (Fig. 2*C–E*).

SIRT1 Is Required by Exenatide to Alleviate Inflammation in the Liver

As we know, activation of inflammatory processes was considered to be a consequence of fatty acids accumulation in liver (20). Our previous research showed that inflammatory genes expression was enhanced in the livers

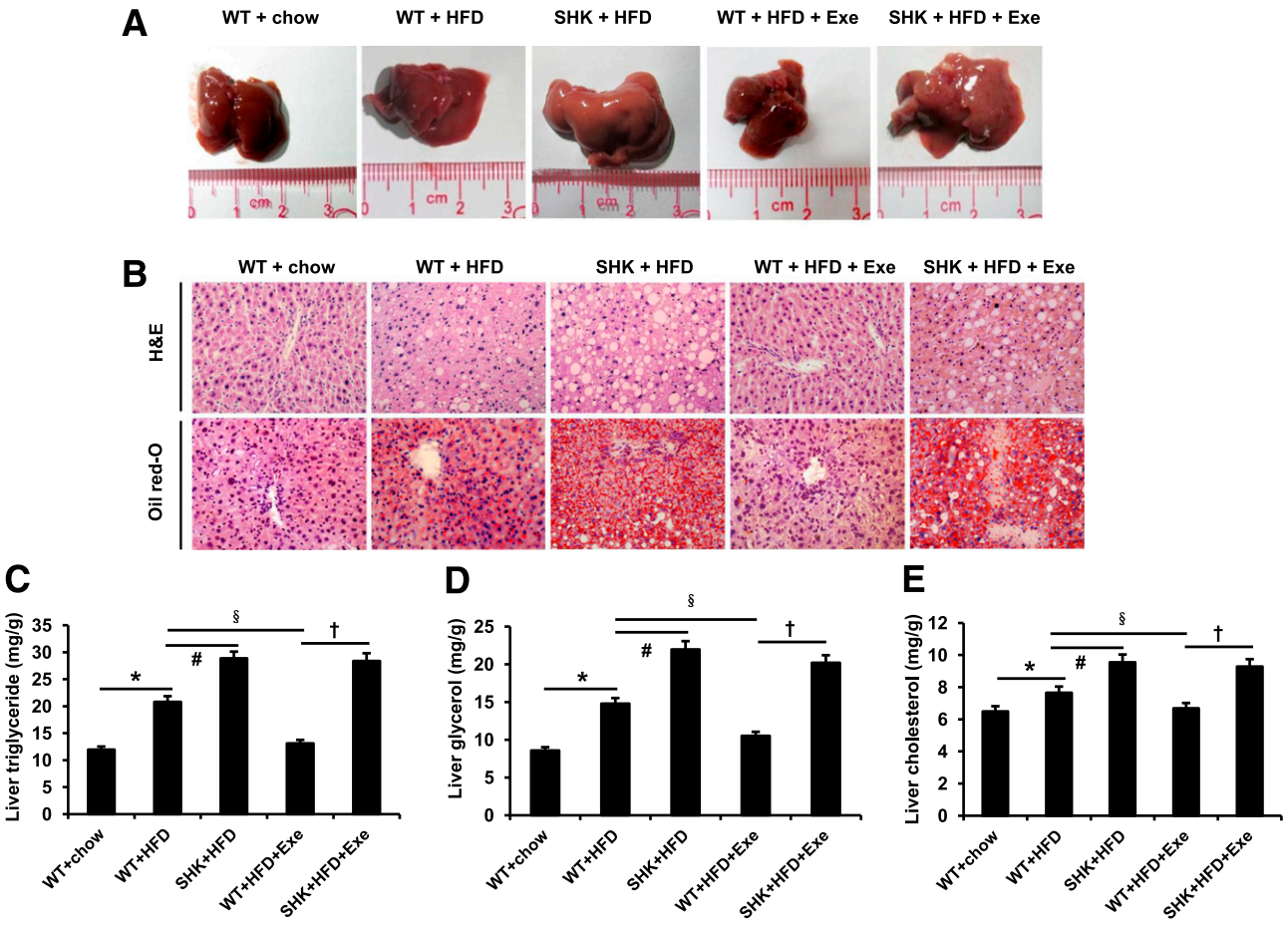

Figure 2—Exenatide-ameliorated liver steatosis disappears in *SIRT1*$^{+/-}$ mice. *A*: General photographs show liver size. *B*: Liver sections with hematoxylin and eosin (H&E) staining (*top row*) and Oil Red O staining (*bottom row*). Photomicrographs were taken using a microscope with original magnification ×40. Hepatic triglyceride (*C*), glycerol (*D*), and cholesterol (*E*) contents were determined. Data are expressed as mean ± SEM (n = 5–12). *P < 0.05 WT+HFD vs. WT+chow; #P < 0.05 SHK+HFD vs. WT+HFD; §P < 0.05 WT+HFD+Exe vs. WT+HFD; †P < 0.05 SHK+HFD+Exe vs. WT+HFD+Exe. Exe, exenatide; SHK, SIRT1 heterozygous knockout mice.

of mice fed the HFD, indicated by F4/80 and TNF-α gene expression (10). Here, the results demonstrated that exenatide could reduce inflammatory gene expression, including TNF-α, F4/80, and MCP-1, in the livers of HFD-induced WT mice. However, no obvious change was observed in the expression of those genes in *SIRT1*$^{+/-}$ mice, with or without exenatide treatment (Fig. 3*A*–*C*). Macrophage infiltration was also determined using F4/80 protein expression in the liver. Immunohistochemical staining of F4/80 suggested that the F4/80 protein was remarkably reduced in the livers of WT mice after exenatide treatment compared with saline control mice, but not in *SIRT1*$^{+/-}$ mice (Fig. 3*D*). The data suggest that exenatide SIRT1 is required by exenatide to relieve inflammation in liver.

Exenatide-Reduced Hepatic Steatosis Depends on the SIRT1/AMPK Pathway

AMPK is a metabolic fuel gauge that regulates lipid metabolism through phosphorylation by sensing changes in the intracellular AMP-to-ATP ratio, especially in the liver (21). To test whether the actions of exenatide are mediated by SIRT1 through AMPK, protein expression of SIRT1 and AMPK was examined. In accordance with the upregulation of SIRT1 by exenatide in HFD-induced WT mice, p-AMPK expression was also remarkably increased compared with saline control; however, no change was observed in the livers of *SIRT1*$^{+/-}$ mice, irrespective of exenatide injection (Fig. 4*A*–*C*).

We then verified the above hypothesis in the HepG2 cell line in vitro. Because exenatide is a synthetic version of exendin-4, we used exendin-4 for the treatment in HepG2 cells to exclude the influence of auxiliary material. Intracellular lipid detection by Oil Red O staining showed that exendin-4 could reverse palmitate-induced lipid accumulation in HepG2 cells (Fig. 4*D*). In addition, exendin-4 (20 nmol/L and 200 nmol/L, respectively) increased SIRT1 and p-AMPK protein levels significantly in palmitate-induced HepG2 cells (Fig. 4*E*). The phosphorylation of ACC, a substrate enzyme of AMPK, was upregulated in parallel with p-AMPK (Fig. 4*E*). After silencing SIRT1 using SIRT1 RNA interference (RNAi), the effect of exendin-4 (20 nmol/L) was attenuated sharply (Fig. 4*F*). Resveratrol (50 μmol/L), an SIRT1 activator, was provided to compare with the effect of exendin-4 on palmitate-induced HepG2 cells. As shown, the effect of exendin-4 on activating SIRT1 and p-AMPK was comparable with resveratrol (Fig. 4*G*).

Because SIRT1 is NAD^+ dependent, whether exendin-4 acts by altering the level of NAD^+ or the NAD^+-to-NADH ratio were further investigated in HepG2 cells in vitro. The results showed that exendin-4 did reverse the palmitate-reduced NAD^+ level and the NAD^+-to-NADH ratio in

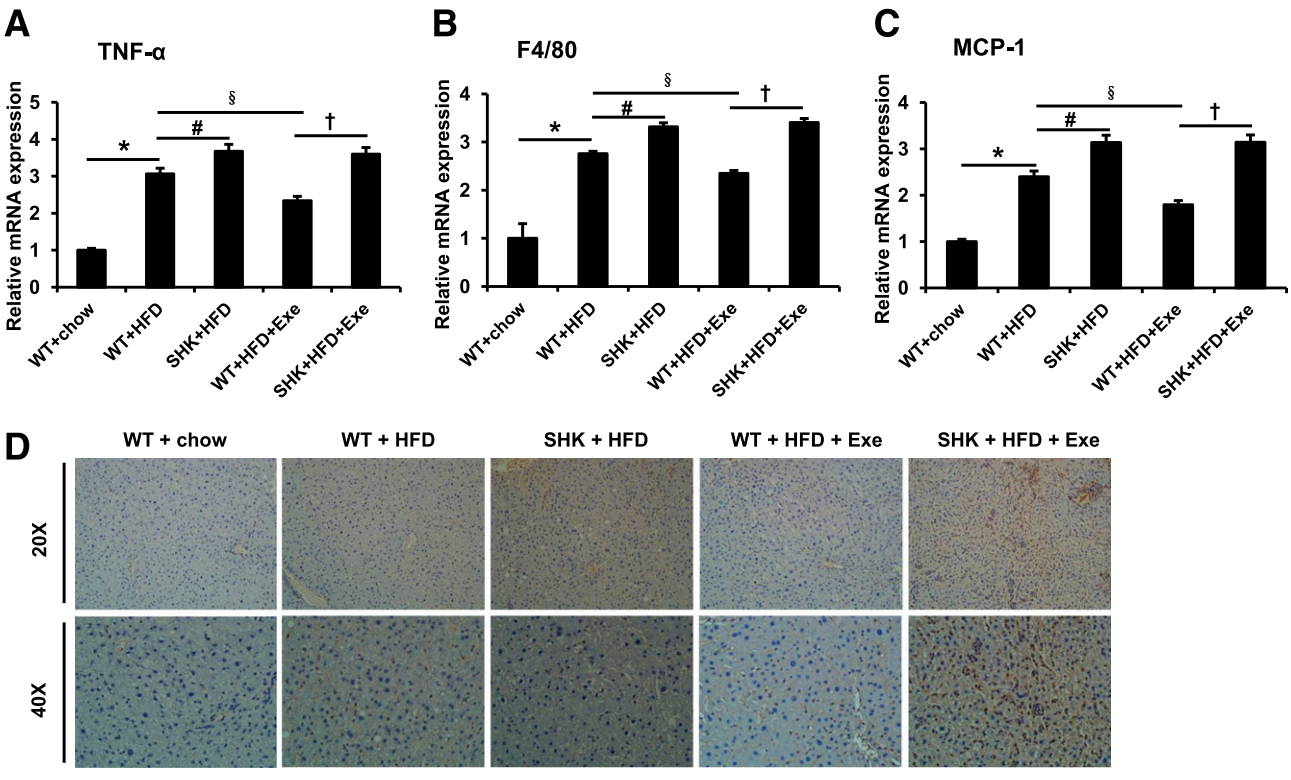

Figure 3—SIRT1 is required by exenatide to relieve inflammation in liver. Relative mRNA expression is shown for TNF-α (*A*), F4/80 (*B*), and MCP-1 (*C*) in the liver. *D*: Immunohistochemical staining with macrophage marker F4/80 in the liver. Data are expressed as mean ± SEM (n = 5–12). *P < 0.05 WT+HFD vs. WT+chow; #P < 0.05 SHK+HFD vs. WT+HFD; §P < 0.05 WT+HFD+Exe vs. WT+HFD; †P < 0.05 SHK+HFD+Exe vs. WT+HFD+Exe. Exe, exenatide; SHK, SIRT1 heterozygous knockout mice.

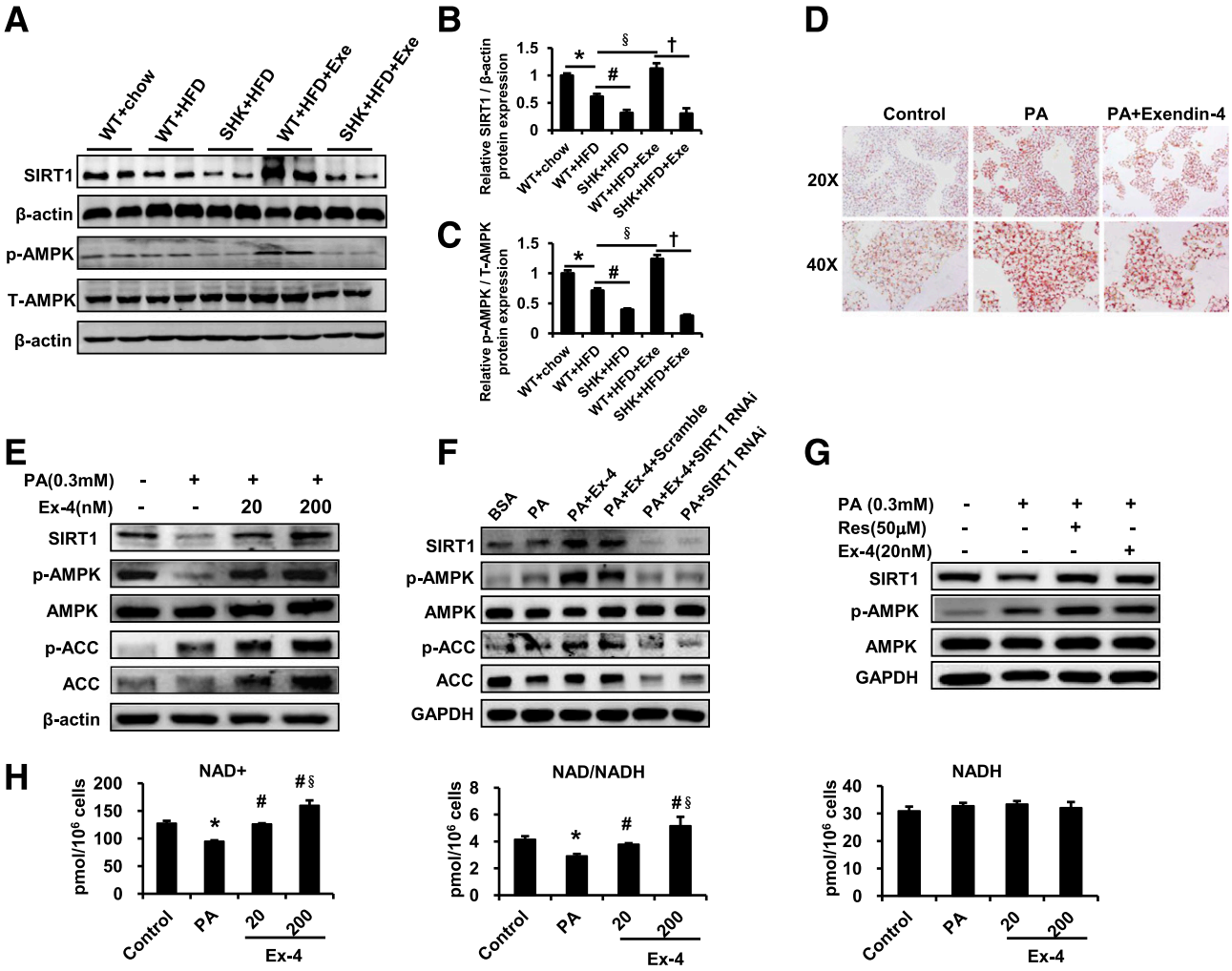

Figure 4—Exenatide-reduced hepatic steatosis depends on the SIRT1/AMPK pathway. *A*: Total protein extracted from liver lysates was used in Western blot. SIRT1, p-AMPK, and total (T-)AMPK were detected with specific antibodies. Ratios of SIRT1 to β-actin (*B*) and p-AMPK to T-AMPK (*C*) were quantified in three independent experiments per condition. Data are expressed as the mean ± SEM (n = 3). *D*: Oil Red O staining of HepG2 cells treated with PA (0.3 mmol/L) and Ex-4 (20 nmol/L) as indicated for 24 h. *E*: HepG2 cells were treated with PA (0.3 mmol/L) and Ex-4 (20 nmol/L and 200 nmol/L, respectively) as indicated for 24 h. *F*: HepG2 cells were transfected with lentiviral vectors expressing SIRT1 RNAi for 12 h and the medium was changed, and cells were cultured for another 48 h. Then, transfected cells were treated with PA (0.3 mmol/L) and Ex-4 (20 nmol/L) as indicated for 24 h. *G*: HepG2 cells were treated with PA (0.3 mmol/L) and resveratrol (50 μmol/L) or Ex-4 (20 nmol/L), as indicated, for 24 h. *H*: Intracellular levels of the NAD^+-to-NADH ratio, NAD^+, and NADH in HepG2 cells treated with PA (0.3 mmol/L) and Ex-4 (20 nmol/L and 200 nmol/L, respectively), as indicated, were quantified in three independent experiments per condition. Data are expressed as the mean ± SEM (n = 3). For *H*, $^*P < 0.05$ vs. control; $\#P < 0.05$ vs. PA; §$P < 0.05$ vs. Ex-4 (20 nmol/L). For *B* and *C*, $^*P < 0.05$ WT+HFD vs. WT+chow; $\#P < 0.05$ SHK+HFD vs. WT+HFD; §$P < 0.05$ WT+HFD+Exe vs. WT+HFD; †$P < 0.05$ SHK+HFD+Exe vs. WT+HFD+Exe. Exe, exendin; Ex-4, exendin-4; PA, palmitate; SHK, SIRT1 heterozygous knockout mice.

HepG2 cells in a dose-dependent way (Fig. 4*H*), which suggest exendin-4 could induce not only the upregulation SIRT1 of protein expression but also its activation through increasing the NAD^+-to-NADH ratio.

These data support our speculation that exenatide-reduced hepatic steatosis depends on the SIRT1/AMPK pathway.

Exenatide Requires SIRT1 to Ameliorate Lipogenesis Through Inhibiting SREBP-1 in the Liver

SREBP-1c is one of the master transcription factors of de novo lipogenesis in the liver (22). PNPLA3, a target gene of SREBP-1c, also plays a role in lipogenesis in the mouse liver (23). These two factors were both examined in our study. The results showed a significant decrease of SREBP-1 and PNPLA3 protein expression in HFD-challenged WT mice with exenatide treatment compared with saline control. Not surprisingly, again, neither SREBP-1 nor PNPLA3 protein expression changed in *SIRT1*$^{+/-}$ mice, whether with or without exenatide treatment (Fig. 5*A*–*C*). In vitro, exendin-4 (20 nmol/L and 200 nmol/L) significantly decreased SREBP-1 protein expression in palmitate-induced HepG2 cells (Fig. 5*D*). After knocking down SIRT1 in HepG2 cells, the effect of exendin-4 (20 nmol/L) on inhibiting SREBP-1 expression

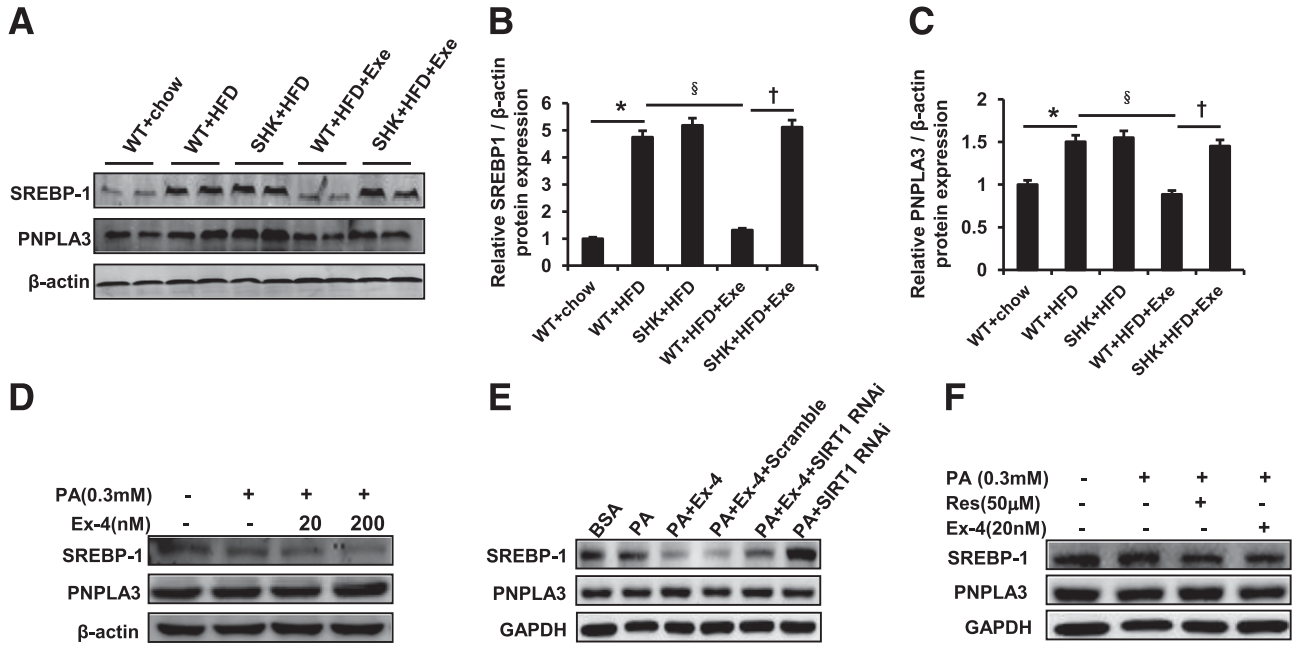

Figure 5—SIRT1 is required by exenatide to ameliorate lipogenesis through inhibiting SREBP-1 in the liver. *A*: Total protein extracted from liver lysates was used in Western blot. SREBP-1 and PNPLA3 were detected with specific antibodies. Ratios of SREBP-1 to β-actin (*B*) and PNPLA3 to β-actin (*C*) were quantified in three independent experiments per condition. Data are expressed as the mean ± SEM (n = 3). *D*: HepG2 cells were treated with PA (0.3 mmol/L) and Ex-4 (20 nmol/L and 200 nmol/L, respectively), as indicated, for 24 h. *E*: HepG2 cells were transfected with lentiviral vectors expressing SIRT1 RNAi for 12 h, the medium was changed, and cells were cultured for another 48 h. Transfected cells were then treated with PA (0.3 mmol/L) and Ex-4 (20 nmol/L), as indicated, for 24 h. *F*: HepG2 cells were treated with PA (0.3 mmol/L) and resveratrol (50 μmol/L) or Ex-4 (20 nmol/L), as indicated, for 24 h. *P < 0.05 WT+HFD vs. WT+chow; §P < 0.05 WT+HFD +Exe vs. WT+HFD; †P < 0.05 SHK+HFD+Exe vs. WT+HFD+Exe. Exe, exendin; Ex-4, exendin-4; PA, palmitate; SHK, SIRT1 heterozygous knockout mice.

was weakened dramatically (Fig. 5*E*). Besides, resveratrol (50 μmol/L) was provided to treat with palmitate-challenged HepG2 cells. As shown, the effect of exendin-4 on inhibiting SREBP-1 was comparable with resveratrol (Fig. 5*F*).

These results indicate that exenatide requires SIRT1 to ameliorate lipogenesis via inhibiting SREBP-1 in liver.

DISCUSSION

NAFLD has become a worldwide health concern because the global incidence of obesity has increased. Epidemiological studies showed that NAFLD is strongly associated with type 2 diabetes mellitus (T2DM)—each is highly predictive of the other (24,25). The coincident occurrence of hepatic steatosis and insulin resistance also leads to the hypothesis that excess triglyceride in the liver causes insulin resistance, which contributes to T2DM (2). Because the ideal treatment for NAFLD has not been established, novel approaches aimed at reducing lipotoxicity and inhibiting proinflammatory cytokines are urgently needed. The incretin hormone GLP-1 and its mimetics, a new kind of antidiabetes drugs, show pleiotropic functions in pancreatic β-cells and in extrapancreatic organs in mammals (26,27). Accumulated evidence demonstrate that it could improve lipid deposition and inflammation in liver effectively (6,7,28), which indicates GLP-1 and its mimetics could be a potential drug for the treatment of NAFLD, especially in NAFLD combined with T2DM (29,30). Here, we report a new mechanism of a GLP-1 receptor agonist on improving liver steatosis, which demonstrates that SIRT1 mediates the effect of exenatide (exendin-4) on ameliorating hepatic steatosis.

SIRT1 plays a vital role in hepatic lipid metabolism by deacetylation of acetylated lysine residues on histones and various transcriptional regulators (8). Complete deletion of the SIRT1 gene leads to developmental defects and postnatal lethality (31,32), which implies that *SIRT1*$^{-/-}$ mice are not appropriate for the study for medication intervention. *SIRT1*$^{+/-}$ mice are normal in development and postnatal growth (31,32). As our animal experiments were proceeding, a study was reported that the GLP-1 receptor agonist exendin-4 attenuated fatty liver through activation of SIRT1 in HFD-induced C57BL/6J mice (19). However, whether the effect of the GLP-1 receptor agonist on improving fatty liver is mediated by SIRT1 has never been proved in a genetic model of SIRT1 knockout, and the exact underlying mechanisms remain elusive. Our data showed that hepatic steatosis, which was improved dramatically in the WT group, diminished in *SIRT1*$^{+/-}$ mice after exenatide treatment (Fig. 2*B–D*). This indicates that exenatide-improved lipid deposition in the liver is indeed mediated by SIRT1. We inferred the

reason steatosis was not improved in response to exendin-4 in *SIRT1*$^{+/-}$ mice was that the effect of exenatide on improving liver steatosis might require not only the existence of SIRT1 but also a certain amount of SIRT1 expression. More than 50% loss of SIRT1 protein level was not able to exert the effect of exenatide on hepatocytes. After knocking down SIRT1 by RNAi in HepG2 cells (Fig. 4*F*), there was still very low SIRT1 expression, although much less compared with scramble control, but exendin-4 was not able to upregulate SIRT1 and its downstream factors. This indicates that low expression of SIRT1 is not enough to mediate the effect of exendin-4.

AMPK is a metabolic fuel gauge that regulates lipid metabolism through phosphorylation by sensing changes in the intracellular AMP-to-ATP ratio, especially in the liver (21). One recent study demonstrates that SIRT1 plays an essential role in the ability of moderate doses of resveratrol to stimulate AMPK and improve mitochondrial function (33). SIRT1 also mediates the effect of α-lipoic acid on regulating lipid metabolism through activation of AMPK (34). In the current study, we tested whether the actions of exenatide were mediated by SIRT1 through the activation of AMPK. Our data showed that exenatide upregulated SIRT1 and p-AMPK in the livers of HFD-induced WT mice but not in *SIRT1*$^{+/-}$ mice (Fig. 4*A*–*C*). After silencing SIRT1 by lentivirus expressing SIRT1 RNAi in palmitate-induced HepG2 cells, the effect of exendin-4 on increasing SIRT1 and p-AMPK protein levels was attenuated sharply as well (Fig. 4*E* and *F*). The results support our speculation that the role of exenatide in hepatic steatosis alleviation is mediated by SIRT1 through AMPK.

Hepatic steatosis could result from an increase of de novo lipogenesis. SREBP-1c is one of the master lipogenic transcription factors of de novo lipogenesis in the liver (22). PNPLA3, a target gene of SREBP-1c, also participates in lipogenesis in the mouse liver (23). In our study, SREBP-1 and PNPLA3 protein expression were both decreased remarkably in HFD-challenged WT mice after exenatide treatment, whereas no changes were observed in *SIRT1*$^{+/-}$ mice with exenatide treatment (Fig. 5*A*–*C*). In vitro, the effect of exendin-4 on inhibiting SREBP-1 was mediated by SIRT1 using the RNAi method to knock down SIRT1 expression in palmitate-induced HepG2 cells (Fig. 5*E*). We also found that exendin-4 reduced the precursor of SREBP-1 (-P) (Supplementary Fig. 1*A*) and that this effect was mediated by SIRT1 (Supplementary Fig. 1*B*). Meanwhile, exendin-4 inhibited the nuclear translocation of SREBP-1 (Supplementary Fig. 2). Besides, SREBP-1 Ser 372 phosphorylation did not change under the concentration of 20 nmol/L exendin-4 and was upregulated with the concentration of 200 nmol/L (Supplementary Fig. 1*A*). Our data indicated that exendin-4 inhibited the synthesis of the precursor SREBP-1 (-P) and the nuclear translocation of SREBP-1 instead of affecting its proteolytic processing. Previous study verified SREBP-1c is an in vivo target of SIRT1 and that SIRT1 deacetylates and inhibits SREBP-1c activity in the regulation of hepatic lipid metabolism (35). Our results indicate that exenatide required SIRT1 to ameliorate lipogenesis via inhibiting the synthesis of the precursor of SREBP-1c in the liver. Whether exenatide affects the acetylation/deacetylation of SREBP-1c through SIRT1 remains to be investigated.

NAFLD includes isolated fatty liver and NASH. Hepatic steatosis is usually accompanied with chronic inflammation, indicated by inflammatory cell infiltration during NAFLD progression according to liver histology findings (2). A recent study found that exendin-4 reduced inflammation in liver by reducing macrophage recruitment and activation (7). In T2DM patients, administration of liraglutide, a GLP-1 analog, improved liver inflammation and altered liver fibrosis (28). Here in our study, inflammation was reduced by exenatide in the livers of WT mice, and this effect disappeared in the livers of *SIRT1*$^{+/-}$ mice (Fig. 3). Recent studies have demonstrated SIRT1's properties in anti-inflammation (36–38). SIRT1 knockdown led to enhanced inhibitor of κB kinase phosphorylation and nuclear factor-κB activation in adipocytes stimulated by lipopolysaccharide and also resulted in increased expression of proinflammatory cytokines such as TNF-α, interleukin-1β, and interleukin-6 (36). In line with SIRT1's role of anti-inflammation, that SIRT1 is required by exenatide to alleviate inflammation in liver in our study is not hard to understand, although the exact mechanism remains to be investigated further.

In conclusion, the current study demonstrates that SIRT1 mediates the effect of the GLP-1 receptor agonist exenatide on relieving liver steatosis. The actions of exenatide in ameliorating lipogenesis mediated by SIRT1 are through activation of the AMPK pathway and the inhibition of SREBP-1c simultaneously; moreover, exenatide requires SIRT1 to alleviate inflammation in liver as well. Our study indicates, for the first time to our knowledge, that SIRT1 is essential for the GLP-1 receptor agonist exenatide to reduce hepatic steatosis in mice, which suggests that the GLP-1 receptor agonist could serve as a potential drug for NAFLD, especially in T2DM combined with NAFLD and that SIRT1 might be a therapeutic target of NAFLD.

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Author Contributions. F.X. contributed to the study design, to the acquisition and interpretation of data, and to writing the manuscript. Z.L. researched data, performed animal studies, and analyzed data. X.Z. researched data, performed cell culture and lentivirus transfection, and contributed to the

data analysis. H. Liu contributed to the performance of the animal studies. H.Lia. contributed to the study design and data analysis. H.X. contributed to the data interpretation and to writing the manuscript. Z.C. contributed to the performance of the animal studies. K.Z. contributed to the acquisition of data. J.W. contributed to the study design, acquisition of data, revision of the manuscript, and approval of the version to be submitted. F.X. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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GUIDELINES

Standards of care for type 2 diabetes in China

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Epidemiology of T2DM in China

Epidemiology of T2DM

The past 30 years have witnessed significant increases in the prevalence of type 2 diabetes mellitus (T2DM) in China. A 1980 epidemiological survey that included 30 000 people from 14 provinces and cities nationwide indicated that the prevalence of diabetes was 0.67% [1]. A 1994–1995 epidemiological survey that included 210 000 people from 19 provinces and cities found that the prevalence of diabetes was 2.5% among individuals who were 25–64 years old (with a population standardized rate of 2.2%) and that the prevalence of impaired glucose tolerance was 3.2% (with a population standardized rate of 2.1%) [2]. A national nutrition survey conducted in 2002, showed that the prevalences of diabetes were 4.5% and 1.8% among people over 18 years in the urban and rural areas, respectively [3]. In 2007–2008, the Chinese Diabetes Society (CDS) performed an epidemiological survey in 14 provinces and cities nationwide. After adopting a weighted analysis that took into account factors such as gender, age, rural and urban distributions and regional differences, the estimated prevalence of diabetes was 9.7% in adults over 20 years of age in China [4], accounting for 92.4 million adults with diabetes (43.1 million rural residents and 49.3 urban residents) (Table 1).

In summary, the current epidemic of diabetes in China shows the following characteristics:

1. T2DM accounts for more than 90% of the overall population with diabetes in China; type 1 diabetes mellitus (T1DM) accounts for approximately 5.0%, and other types of diabetes account for only 0.7% [5]. Notably, due to lack of reliable data on T1DM incidences and prevalences over the past years in China, further investigation has to be conducted to report the proportion.
2. The prevalences of diabetes appear to be correlated with degree of economic development: in the 1994 survey, the prevalence of diabetes among the high-income group was 2–3 times higher than that of the low-income group [2]. A latest study showed that the prevalence of diabetes in developed regions was still significantly higher than that in under-developed regions, and the prevalence rate in cities was higher than those in rural areas [4].
3. A large proportion of diabetes is undiagnosed: in the 2007–2008 national survey among adult population over 20 years, patient with newly diagnosed diabetes accounted for 60% of total diabetes population.
4. Male gender and low-education levels are risk factors of diabetes: in the 2007–2008 survey, after adjusting for other risk factors, the risk for men were found increased by 26% compared with that for women, and risk of diabetes among people without college education was 57% higher than those with college or higher education [4].
5. Phenotypic characteristics: the average body mass index (BMI) of China's T2DM population is approximately 25 kg/m^2, whereas the average BMI of Caucasian diabetes population is generally higher than 30 kg/m^2. In China, there is a larger proportion characterized by postprandial hyperglycaemia. Further, postprandial hyperglycaemia alone accounts for nearly 50% of the overall newly diagnosed population [6].

Table 1. Summary of five nationwide epidemiological surveys of diabetes in China

Year of survey (diagnostic criteria)	Number of surveyed people (10 000)	Age (years)	Prevalence of diabetes (%)	Prevalence of impaired glucose tolerance (%)	Screening method
1980[a] [1] (Lanzhou standard)	30	Entire population	0.67	—	Urine glucose + 2h PG (steamed bread tolerance test) for screening the high risk subjects
1986 [49] (WHO 1985)	10	25–64	1.04	0.68	2h PG (steamed bread tolerance test) for screening the high risk subjects
1994 [2] (WHO 1985)	21	25–64	2.5	3.2	2h PG (steamed bread tolerance test) for screening the high risk subjects
2002 [3] (WHO 1999)	10	≥18	4.5 (urban) 1.8 (rural)	IFG 2.7 IFG 1.6	FPG screening of the high-risk group
2007–2008 [4] (WHO 1999)	4.6	≥20	9.7	15.5[b]	One-step OGTT method

1 mmol/L = 18 mg/dL.
FPG: fasting plasma glucose; WHO: World Health Organization; IFG: impaired fasting glucose; OGTT: oral glucose tolerance test; 2 hPG, 2-h postprandial blood glucose; —, no data.
[a]Diagnostic criteria are FPG ≥130 mg/dL and/or 2 hPG ≥200 mg/dL and/or more than three items on the OGTT curve that are above the diagnostic criteria [0′ 125, 30′ 190, 60′ 180, 120′ 140, and 180′ 125 mg/dL, in which 0′, 30′, 60′, 120′ and 180′ are time points (min), and 30′ or 60′ is one time point; the glucose measurement uses the o-toluidine method with 100 g of glucose].
[b]Prediabetes, including IFG, IGT or both (IFG/IGT).

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6. Cardiovascular diseases are common among diabetic patients. Because diabetes population in China shows a shorter disease duration late chronic complications such as diabetic retinopathy and diabetic nephropathy may pose great challenges in the future.

Diagnosis and classification of diabetes mellitus

Diagnosis of diabetes

This guideline recommends the World Health Organization's (WHO) (1999) the criteria for diagnosis and classification of diabetes, and classification of metabolic status (Table 2): either the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value after a 75-g oral glucose tolerance test (OGTT) can be used alone for epidemiological investigations or mass screenings [7]. However, the data in China include only the FPG levels, resulting in a larger proportion of diabetes being missed. The ideal investigation should simultaneously check FPG and 2-h PG after the glucose load; blood glucose levels at other time points after the OGTT are not used as diagnostic criteria.

Individuals with impaired fasting glucose should undergo the OGTT to reduce the number of missed diabetes diagnoses.

The issue of using HbA_{1c} for diabetes diagnosis

The 2010 American Diabetes Association guidelines added glycated haemoglobin (HbA_{1c}) ≥6.5% as a diagnostic criterion for diabetes [8]. In 2011, the WHO also recommended that wherever conditions permit, countries and regions may consider adopting this cut-off point for diabetes diagnosis [9]. However, given that the HbA_{1c} test is not yet commonly applied in China, the insufficient degree of standardization, and the fact that the instruments and quality control for measuring HbA_{1c} are currently unable to meet the current diagnostic standard for diabetes, this guideline does not recommend the use of HbA_{1c} for diagnosis of diabetes in China. Nevertheless, for hospitals that use a standardized HbA_{1c} assay with a normal reference value of 4.0–6.0% and strict quality control, HbA_{1c} ≥6.5% can be used as a reference when diagnosing diabetes.

Table 2. Diagnostic criteria for diabetes and prediabetes

Diagnostic methods	Venous plasma glucose level (mmol/L)
(1) Typical symptoms of diabetes (polydipsia, polyuria, polyphagia and weight loss) plus random blood glucose testing	≥11.1
or	
(2) Fasting plasma glucose	≥7.0
or	
(3) 2 h after the glucose load test Individuals who do not present diabetes symptoms should be re-tested on a separate day.	≥11.1

The fasting state refers to not eating for at least 8 h. Random blood glucose refers to the blood glucose level at any time of day regardless of the time of the last meal, which cannot be used to diagnose impaired fasting glucose or impaired glucose tolerance.

Classification of diabetes mellitus

This guideline adopts the diabetes aetiology classification system proposed by the WHO (1999), which divides diabetes into four major categories based on aetiological evidence, that is, T1DM, T2DM, gestational diabetes mellitus (GDM) and special types of diabetes.

Primary, secondary and tertiary diabetes prevention

Primary, secondary and tertiary prevention of T2DM

The goal of primary prevention is to prevent the occurrence of T2DM. Secondary prevention aims to prevent diabetic complications in patients with T2DM. Tertiary prevention aims to delay the progression of diabetic complications, to reduce morbidity and mortality and to improve the patients' quality of life.

Strategies for the primary prevention of T2DM

Risk factors and intervention strategies for T2DM
The risk of T2DM depends primarily on the patient's number and degree of risk factors. Some of these factors cannot be changed, whereas others can (Table 3).

Diabetes screening of high-risk populations
Primary prevention efforts for T2DM should adopt hierarchical management approaches based on the differences between the high-risk population and general population. It is not feasible either to screen prediabetes in the entire Chinese population or to systematically identify high risk groups by blood glucose tests, considering the huge

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Table 3. Risk factors for type 2 diabetes mellitus

Unchangeable risk factors	Changeable risk factors
Age	Prediabetes (impaired glucose tolerance or combined impaired fasting glucose), the most important risk factor
Family history or genetic predisposition	Metabolic syndrome
Ethnicity	Overweight, obesity and depression
History of gestational diabetes mellitus or women with history of delivery of a baby weighing ≥ 4 kg	Excess dietary caloric intake, sedentary or physically inactive
Polycystic ovary syndrome	Use of drugs that can increase the risk of diabetes
Intrauterine growth retardation or premature birth	Social environments that can cause obesity or diabetes

population in China. Therefore, the identification of high-risk groups relies primarily on opportunistic screening (e.g. screening that occurs during routine physical examinations or during treatment for other diseases).

Screening of diabetes benefits the early diagnosis of diabetes and improves the prevention and treatment of diabetes and its complications. Therefore, when conditions permit, high-risk groups should be targeted for diabetes screening.

Definition of the high-risk diabetes group among adults are as follows: adults (>18 years) with one or more of the following diabetes risk factors: (1) age ≥40 years, (2) history of impaired glucose regulation, (3) overweight (BMI ≥24 kg/m^2) or obesity (BMI ≥28 kg/m^2) and/or central obesity (male waist circumference ≥90 cm and female waist circumference ≥85 cm), (4) sedentary lifestyle, (5) first-degree relatives with T2DM, (6) women who delivered a baby weighing ≥4 kg) or were diagnosed with GDM (7) hypertension [systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg (1 mmHg = 0.133 kPa)] or on therapy for hypertension, (8) dyslipidemia [high-density lipoprotein cholesterol (HDL-C) ≤0.91 mmol/L (≤35 mg/dL) and triglycerides ≥2.22 mmol/L (≥200 mg/dL)] or on therapy for hyperlipidemia, (9) atherosclerotic cardiovascular disease, (10) a transient history of steroid diabetes, (11) polycystic ovary syndrome and (12) long-term use of antipsychotics and/or antidepressant treatment. Of the aforementioned factors, impaired glucose regulation is the most important high-risk factor: approximately 5%–10.0% of patients with impaired glucose tolerance progress to T2DM annually [10].

Diabetes screening age and frequency. For adults in the high-risk group, diabetes screening should be performed as early as possible, regardless of age; for populations with no diabetes risk factors other than age, screening should begin at ≥40 years of age. For children and adolescents at a high risk for diabetes, screening should begin at age 10 years; however, for individuals with an earlier onset of puberty, this guideline recommends that screening starts at puberty. Those whose initial screening results are normal are recommended to undergo screening again at least once every 3 years.

Diabetes screening strategy. At medical institutions with a qualified laboratory, diabetes screening is recommended for high-risk patients during their visits or physical examinations.

Diabetes screening method. The fasting blood glucose test is a simple diabetes screening method that should be used for routine screening, albeit there's risk of missing diagnosis. When conditions permit, the OGTT (both FPG and 2-h PG after glucose load) should be performed as often as possible. HbA_{1c} testing is not currently recommended as a routine screening method.

Diabetes screening of the general population. To improve the effectiveness of diabetes screening for the general population, targeted diabetes screening should occur according to the individual's degree of diabetes risk.

T2DM prevention through intensive lifestyle intervention

Multiple randomized and controlled studies have shown that people with impaired glucose tolerance can be delayed or prevented from developing to T2DM, through appropriate lifestyle interventions, [11–13]. In a study conducted in Daqing, China, patients in the lifestyle intervention group were asked to increase vegetable intake and reduce intake of alcohol and monosaccharides, and those who were defined as overweight or obese (BMI >25 kg/m^2) were encouraged to lose weight, increase intensity of physical activity by performing at least 30 min of moderately intense activity per day. After a 6-year lifestyle intervention, the cumulative incidence of T2DM risk for the subsequent 14 years decreased by 43% [14]. The lifestyle intervention groups in the Finnish Diabetes Prevention Study [15] and the American Diabetes Prevention Program [16] also demonstrated that the intervention could significantly reduce the risk of developing T2DM among patients with impaired glucose tolerance.

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This guideline recommends that patients with prediabetes lower the risk of diabetes through diet control and exercise; that patients should receive regular follow-up that provides psychosocial support to ensure patients' long-term adherence to a healthy lifestyle; that blood glucose levels should be regularly tested; that the cardiovascular disease risk factors (such as smoking, hypertension and dyslipidemia) should be closely monitored; and that appropriate intervention measures should be provided. The specific objectives are (1) the BMI of overweight or obese patients should be lowered to approximately 24 kg/m^2 or weight loss of at least 5–10% should be achieved, (2) the patients' total daily caloric intake should be reduced by at least 400–500 kcal (1 kcal = 4.184 kJ), (3) the patients' saturated fatty acid intake should be less than 30% of their total fatty acid intake and (4) the patients should be encouraged to engage in moderate-intensity physical activity for at least 150 min/week.

T2DM prevention through medical intervention

Drug intervention trials in a pre-diabetic population showed that the oral administration of hypoglycaemic agents, such as metformin, α-glucosidase inhibitors, thiazolidinediones (TZDs), metformin combined with TZDs, the diet pill orlistat and traditional Chinese herbal medicine (Tianqi capsules), reduced the risk of diabetes [13,17–21]. However, because there is no sufficient evidence showing that drug interventions have long-term efficacy and/or health economics benefits, the clinical guidelines developed by various countries have not widely recommended medical interventions as the primary prevention for diabetes. Given that economic development in China is still in the preliminary stage and significant regional imbalances exist and that diabetes prevention-related health care is currently unsophisticated and imperfect, this guideline currently does not recommend the use of drug interventions to prevent diabetes.

Strategies for the secondary prevention of T2DM

Blood glucose control

The clinical trials on intensive glucose control, such as the Diabetes Control and Complications Trial (DCCT) [22], the United Kingdom Prospective Diabetes Study (UKPDS) [23] and the Kumamoto Study in Japan [24], found that among patients in the early stage of diabetes, intensive glucose control can significantly reduce the risk of diabetic microvascular diseases. The UKPDS study also showed that in obese or overweight populations, the use of metformin was correlated with a significant decrease in the risk of myocardial infarction and death [25]. The long-term follow-up studies of the DCCT and UKPDS patient populations indicated that early intensive glycaemic control was correlated with a reduction in diabetic microvascular diseases and a significant decrease in the risks of myocardial infarction and death [26,27]. These results provide evidence that intensive blood glucose control during the early stages of T2DM can reduce the risks of diabetic macrovascular and microvascular diseases.

This guideline recommends that for newly diagnosed diabetes patients and early T2DM patients, strict glycaemic control strategies should be adopted to reduce the risk of diabetic complications.

Blood pressure control, lipid control and aspirin use

The UKPDS study showed that in patients newly diagnosed with diabetes, intensive blood pressure control not only significantly reduced the risk of diabetic vascular diseases but also the risk of microvascular diseases [28]. An analysis of a subgroup in a trial of hypertensive optimization therapy and other clinical trials of anti-hypertensive therapy also showed that intensive blood pressure control reduced the risk of cardiovascular diseases in diabetic patients without significant vascular complications [28,29]. The British Heart Protection Study–subgroup analysis of diabetic patients [30], the Collaborative Atorvastatin Diabetes Study [31] and other large-scale clinical studies [32] indicated that the use of statins to lower low-density lipoprotein cholesterol (LDL-C) could reduce the risk of cardiovascular diseases in diabetic patients without causing significant vascular complications. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study showed that the combination of statins and lipid-lowering drug did not achieve additional cardiovascular benefits, as compared with statins alone [32]. The results of clinical trials using aspirin for the primary prevention of cardiovascular diseases in diabetic patients varied [33,34]; therefore, whether aspirin has a protective effect in the primary prevention of cardiovascular diseases in diabetes patients remains unclear. Nevertheless, a systematic review of multiple clinical trials demonstrated that among patients with T2DM and cardiovascular disease risk factors, aspirin showed a certain cardiovascular protective effect [35].

This guideline recommends that for T2DM patients without significant diabetic vascular complications but with risk factors for cardiovascular diseases, controlling blood glucose, lowering blood pressure and adjusting lipids (mainly to reduce LDL-C) and aspirin therapy are all useful methods to prevent cardiovascular diseases and diabetic microvascular diseases.

Strategies for the tertiary prevention of T2DM

Blood glucose control

The clinical findings in intensive glucose control trials such as DCCT, UKPDS, Kumamoto, The Action in Diabetes

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and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE), and the Veterans Affairs Diabetes Trial (VADT) suggest that intensive glucose control reduced the progression of diabetic microvascular diseases (e.g. background diabetic retinopathy and microalbuminuria) [22,24,28,36,37].

Among patients who have already developed severe diabetic microvascular diseases, relevant clinical evidence is still necessary to verify whether intensive glucose control measures can reduce the risks of blindness, kidney failure and amputation.

The results of clinical trials such as ADVANCE, ACCORD and VADT all suggest that for patients with a longer duration of diabetes, who are older in age and who have multiple cardiovascular risk factors or cardiovascular diseases, the use of intensive glucose control measures does not reduce the risks of cardiovascular diseases and death. Conversely, the ACCORD study showed that in the aforementioned described population, intensive glucose control was correlated with an increased risk of all-cause mortality [38].

This guideline recommends that for patients who are older and who have a longer diabetes duration and cardiovascular diseases, the pros and cons of adopting intensive glucose control must be cautiously evaluated. In addition, an individualized strategy should be used and, a patient-centred diabetes management system should be developed to determine glycaemic control targets.

Blood pressure control, lipid control and aspirin use

There is sufficient clinical evidence that in patients with T2DM who have had cardiovascular diseases, lowering blood pressure, lowering lipids, or the proper use of aspirin therapy alone or in combination can reduce the risk of cardiovascular disease recurrence and death [35,39–43]. In patients with diabetic nephropathy, the use of blood pressure-lowering agents, particularly the use of angiotensin-converting enzyme inhibitor or angiotensin II receptor antagonist drugs, significantly reduced the risk of diabetic nephropathy progression [43].

This guideline recommends that for older patients who have had a long diabetes duration and cardiovascular disease, in terms of individualized glycaemic control, measures such as lowering blood pressure, adjusting lipids (mainly to reduce LDL-C) and taking aspirin should be used to reduce the risk of recurrent cardiovascular diseases and death and to reduce the risk of diabetic microangiopathy.

Diabetes education and management

The risks of microvascular and macrovascular diseases in diabetic patients are significantly higher than in non-diabetic patients, and reducing these risks in diabetic patients depends not only on controlling high blood glucose but also on addressing other cardiovascular disease risk factors and improving lifestyle. In addition to drug therapy, diabetes control must also monitor blood glucose and other cardiovascular risk factors so as to determine whether the control reaches the target or whether the treatment must be adjusted. Moreover, as diabetes is a lifelong disease, the patient behaviour and self-management ability are keys to successful diabetes control; further, diabetes control is not a treatment in the traditional sense but a management approach in nature.

Objectives of integrated T2DM control and treatment options for high blood glucose

Objectives for comprehensive T2DM control

The ideal comprehensive control of T2DM varies according to the age, comorbidities and complications of patients (Table 4). A treatment that does not achieve the control targets should not be viewed as a failure because any improvement in the control indicators confers benefits to the patient and reduces the risks associated with complications; for example, reductions in HbA_{1c} are closely

Table 4. Targets for the integrated control of type 2 diabetes mellitus in China

Indicator	Target value
Blood glucose (mmol/L)[a]	
Fasting	4.4–7.0
Non-fasting	<10.0
Glycated haemoglobin (%)	<7.0
Blood pressure (mmHg)	<140/80
Total cholesterol (mmol/L)	<4.5
High-density lipoprotein cholesterol (mmol/L)	
Male	>1.0
Female	>1.3
Triglycerides (mmol/L)	<1.7
Low-density lipoprotein cholesterol (mmol/L)	
Not complicated with coronary heart disease	<2.6
Complicated with coronary heart disease	<1.8
Body mass index (kg/m^2)	<24.0
Urinary albumin/creatinine ratio [mg/mmol (mg/g)]	
Male	<2.5 (22.0)
Female	<3.5 (31.0)
Urinary albumin excretion rate [μg/min (mg/dL)]	<20.0 (30.0)
Active aerobic activity (min/week)	≥150.0

[a]Capillary blood glucose.

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correlated with reductions in microvascular complications and neuropathy.

The primary principle for determining the targets for integrated T2DM control is individualization management, which should comprehensively consider age, disease duration, life expectancy, severity of complications or comorbidities and other relevant factors of patients.

Hypertension is a common complication of diabetes. Younger patients and those with a shorter disease duration may not require much treatment to reduce blood pressure to 130/80 mmHg or less. The target blood pressure value for elderly patients may be adjusted to 150/90 mmHg.

T2DM blood glucose control strategy and treatment options

T2DM is a progressive disease. The blood glucose tends to increase gradually as the disease duration increases; therefore, the intensity of hyperglycaemia control treatment should be increased accordingly. Lifestyle intervention is the basis for T2DM treatment and should be applied throughout the diabetes treatment process. When lifestyle change alone is unable to reach blood glucose target, drug treatment should be initiated. The preferred first-line drug for T2DM is metformin. If no contraindications are present, metformin should remain part of the diabetes treatment regimen. Patients who could not take metformin may use α-glucosidase inhibitors or insulin secretagogues. When metformin alone is unable to achieve blood glucose target, insulin secretagogues, α-glucosidase inhibitors, dipeptidyl peptidase IV (DPP-4) inhibitors or TZDs (a second-line treatment) can be added. Patients who could not take metformin may undergo combination therapy with other oral medicines. When a combination therapy of two types of oral medicines still unable to achieve blood glucose target, insulin may be added (once-daily basal insulin or once-daily or twice-daily premixed insulin), or a combination of three types of oral medicines may be initiated. Glucagon-like peptide-1 (GLP-1) receptor agonists can be used as a third-line treatment. When basal insulin or premixed insulin combined with other oral medications is still unable to achieve blood glucose target, the regimen should be adjusted to include multiple daily injections of insulin (basal insulin plus prandial insulin or thrice-daily premixed insulin analogues). When treating with premixed insulin and multiple insulin injections, insulin secretagogue use should be discontinued.

Based on the principles mentioned above, and the recommendations of International Diabetes Federation (IDF) [44], the American Diabetes Association (ADA) [45] and National Institute for Health and Clinical Excellence (NICE) [46], the treatment pathways for hyperglycaemia in T2DM are proposed and shown in Figure 1.

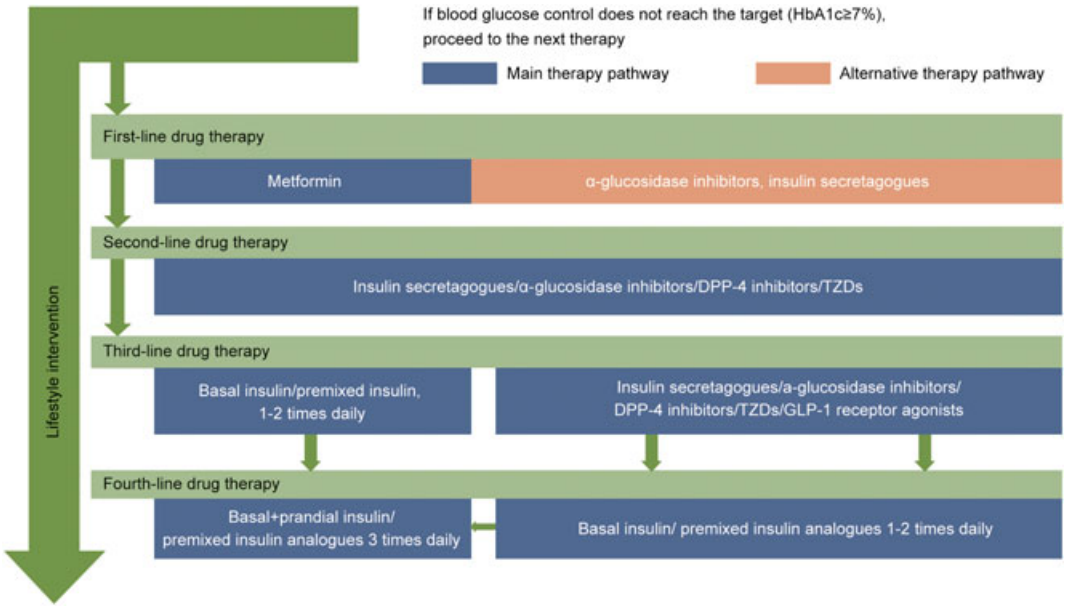

Figure 1. The treatment algorithm for high blood glucose in T2DM. The blue paths are the recommended primary drug treatment paths based on comprehensive considerations, including clinical evidence of the drug's health economics, efficacy and safety and China's national conditions. These paths are similar to the drug treatment pathways recommended by most international diabetes guidelines. The orange paths are alternative paths for the corresponding blue paths. HbA_{1c}, glycated haemoglobin; DPP-4, dipeptidyl peptidase IV; TZD, thiazolidinedione; GLP-1, glucagon-like peptide-1

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Medical nutrition therapy for T2DM

Principles of nutrition therapy

Patients with diabetes or prediabetes require individualized medical nutrition therapy. Such treatment should be provided under the guidance of a dietician or an integrated management team (including a diabetes educator) who is familiar with diabetes treatment. To achieve the metabolic control objective for patients and satisfy his or her dietary preference, reasonable quality objects should be established. In order to control the total energy intake and distribute various nutrients in a reasonable and balanced manner, the nutrition status should be evaluated before setting reasonable quality objectives. For overweight or obese patients, this guideline recommends moderate weight loss measures combined with physical exercise and behavioural changes to maintain weight loss outcomes.

Objectives of medical nutrition therapy

1 Maintaining a proper body weight: the weight loss goal for overweight/obese patients is 5–10% of body weight in 3–6 months. People who are underweight should recover and maintain an ideal body weight over the long term via a sound nutrition plan.
2 Providing balanced nutritious meals.
3 Achieving and maintaining an ideal blood glucose level and reducing the HbA_{1c} level.
4 Reducing the risk factors for cardiovascular disease, dyslipidemia and hypertension.
5 Reducing insulin resistance and pancreatic β-cell load.

Exercise therapy for T2DM

Exercise plays an important role in the comprehensive management of T2DM. Regular exercise increases insulin sensitivity, helps control blood glucose, reduces cardiovascular risk factors, reduces weight and improves overall well-being [47,48]. Moreover, exercise has a remarkable primary preventive effect on populations at high risk of diabetes [49]. Epidemiological studies have shown that the regular exercise of more than 8 weeks reduced the HbA_{1c} level by 0.66% and that the mortality of diabetes patients who adhered to regular exercise for 12–14 years significantly decreases [47].

Smoking cessation

Every diabetic smoker should be advised to stop smoking or using tobacco products. Patients' smoking status and the extent of nicotine dependence should be assessed. Brief consultations and hotlines for quitting should be provided, and if necessary, medications should be prescribed to help patients quit smoking.

Drug treatments for hyperglycaemia

Oral antihyperglycaemic medications

Medical nutrition therapy and exercise treatment are basic for controlling high blood glucose in T2DM. When diet and exercise cannot effectively control the blood glucose level, medication therapy, including oral medications, should be provided in a timely manner.

T2DM is a progressive disease. During the natural course of T2DM, pancreatic β-cell function gradually decreases, meanwhile insulin resistance undergoes less change. Thus, as T2DM progresses, the reliance on exogenous glycaemic control measures gradually increases. Clinical treatment often requires the use of oral medication and a combination of oral medication and injectable anti-diabetic medications (e.g. insulin and GLP-1 receptor agonists).

Metformin

Metformin hydrochloride is the primary biguanide medication currently used in medical practice. The major pharmacological effect of biguanides is lowering blood glucose by reducing the hepatic glucose output and improving peripheral insulin resistance. The diabetes treatment guidelines of many countries and international organizations recommend metformin as the basic medication among the first-line medications and combinations for control of hyperglycaemia in T2DM [44,45,50]. Systematic reviews of clinical trials have shown that metformin can reduce HbA_{1c} by 1.0–1.5% and can also reduce body weight [51]. The efficacy of metformin has been shown to be separate from the body weight reduction. The UKPDS study results showed that metformin also decreased the likelihood of cardiovascular events and death in obese patients with T2DM [25]. In China, randomized controlled clinical trials have been conducted to investigate the effect of metformin and sulfonylureas on recurrent cardiovascular events in patients with T2DM combined with coronary heart disease, and the results showed that metformin treatment was correlated with a significant reduction of major cardiovascular events. Metformin alone did not cause hypoglycaemia, but the combination of metformin and insulin or insulin secretagogues increased the risk of hypoglycaemia. The main side effect of metformin was gastrointestinal reactions. Starting with a small dose and gradually increasing the dosage was an effective way to reduce adverse

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reactions. The efficacy of metformin was unaffected by body weight [52]. The relationship between biguanides and lactic acidosis risk is uncertain [53].

Biguanides are contraindicated in patients with renal insufficiency [serum creatinine >132.6 μmol/L (1.5 mg/dL) for men, >123.8 μmol/L (1.4 mg/dL) for women or estimated glomerular filtration rate (eGFR) <45 mL/min], liver dysfunction, serious infections, hypoxia or those undergoing major surgery. Metformin should be temporarily discontinued for patients undergoing angiography with iodinated contrast agents.

Sulfonylureas

Sulfonylureas are insulin secretagogues, and their main pharmacological effect is increasing the insulin level by stimulating insulin secretion from pancreatic β-cells, therefore lowers the blood glucose level [54]. Clinical trials have shown that sulfonylureas can reduce HbA_{1c} by 1.0–1.5% [55]. At present, sulfonylureas are the primary medications recommended in the diabetes treatment guidelines of many countries and international organizations. Prospective and randomized clinical studies have shown that the use of sulfonylureas was correlated with reduced risks of diabetic microvascular and macrovascular diseases [28]. Currently, the main commercially available sulfonylureas in China are glyburide, glimepiride, gliclazide, glipizide and gliquidone. Sulfonylureas, if used improperly, can lead to hypoglycaemia, particularly in elderly patients and in those with liver and kidney dysfunctions; sulfonylureas may also cause weight gain. Patients with mild renal insufficiency should use gliquidone. Patients who exhibit poor compliance can take sulfonylurea drugs once a day. Xiao Ke Wan is a fixed dose combination drug containing glibenclamide and various traditional Chinese medicines (TCM) that have a antihyperglycaemic effect similar to that of glyburide. Compared with glyburide, Xiao Ke Wan carries a lower risk of hypoglycaemia and yields a more pronounced improvement of diabetes-related TCM symptoms [56].

TZDs

Thiazolidinediones decrease blood glucose primarily by increasing the target cells' sensitivity to the action of insulin. Currently, the main commercially available TZDs in China are rosiglitazone and pioglitazone. Clinical trials have shown that TZDs can decrease HbA_{1c} by 1.0–1.5% [55].

Thiazolidinediones do not cause hypoglycaemia when used alone, but they may increase the risk of hypoglycaemia when used in combination with insulin or insulin secretagogues. Weight gain and oedema are common side effects of TZDs, and these side effects are more remarkable when TZDs are used in combination with insulin. TZD use has been correlated with increase risk of fractures and heart failure [57]. Patients with heart failure (New York Heart Association heart function classification class II and above), active liver disease, transaminase elevations exceeding 2.5 times the upper limit of normal, and severe osteoporosis and fractures should not take TZDs.

Glinides

Glinides are non-sulfonylurea insulin secretagogues. The currently available glinides in China are repaglinide, nateglinide and mitiglinide. This class of medications reduces postprandial blood glucose by stimulating insulin secretion in the early phase, and they can lower HbA_{1c} by 0.5–1.5% [55]. These medications must be taken immediately before a meal and can be used separately or in combination with other anti-diabetic medications (except sulfonylurea). The systematic reviews of clinical studies conducted on T2DM patients in China showed that in terms of reducing HbA_{1c}, repaglinide was superior to placebo and sulfonylureas and was equivalent to α-glucosidase inhibitors, nateglinide, metformin and TZDs. A systematic review of clinical studies of Asian populations with T2DM, including Chinese people, showed that in terms of reducing HbA_{1c}, nateglinide worked better than α-glucosidase inhibitors and was similar to sulfonylureas, repaglinide and mitiglinide [58]. For newly diagnosed T2DM patients, combination therapy using repaglinide with metformin reduced HbA_{1c} more significantly than repaglinide alone but with a significantly increased risk of hypoglycaemia [59].

Common side effects of glinides are hypoglycaemia and weight gain, but the risk and degree of hypoglycaemia are lower with glinides than with sulfonylureas. Glinides can be used in patients with renal insufficiency.

α-Glucosidase inhibitors

α-Glucosidase inhibitors reduce postprandial blood glucose by inhibiting carbohydrate absorption in the upper small intestine. They are suitable for patients who consume carbohydrates as their main food ingredient and experience postprandial hyperglycaemia. In China, commercially listed α-glucosidase inhibitors include acarbose, voglibose and miglitol. Systematic reviews of clinical studies conducted on the T2DM population, including Chinese patients, showed that α-glucosidase inhibitors could reduce HbA_{1c} by 0.50% and cause weight loss [60]. Clinical studies of Chinese people with T2DM showed that the hypoglycaemic effect of a daily dose of 300 mg of acarbose was equivalent to that of a daily dose of 1500 mg of metformin [61]. α-Glucosidase inhibitors can be combined with biguanides, sulfonylureas, TZDs or insulin.

Common adverse reactions to α-glucosidase inhibitors are gastrointestinal reactions, such as abdominal distension and flatulence. Starting with a small dose and gradually increasing the dosage are effective way to reduce adverse effects. The use of this class alone usually does

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not lead to hypoglycaemia and may reduce the risk of preprandial reactive hypoglycaemia; no adjustments in medication dosage and frequency are necessary for elderly patients, no increase in the incidence of hypoglycaemia occurs and this medication is well tolerated. When patients using combination therapy with α-glucosidase inhibitors manifest hypoglycaemia, glucose or honey can be used as treatments; dietary sucrose and starchy foods have a poor ability to correct hypoglycaemia.

DPP-4 inhibitors

Dipeptidyl peptidase IV (DPP-4) inhibitors enhance endogenous levels of GLP-1 by reducing the deactivation of GLP-1 *in vivo* through inhibition of DPP-4. GLP-1 enhances insulin secretion in a glucose concentration-dependent manner and inhibits glucagon secretion. Currently, the commercially available DPP-4 inhibitors in China include sitagliptin, saxagliptin, vildagliptin, linagliptin and alogliptin. Clinical trials in T2DM patients in China showed that sitagliptin, saxagliptin and vildagliptin can reduce HbA_{1c} by 0.70–0.90%, 0.40–0.50% and 0.50%, respectively [62–64]; a comparison study showed that the HbA_{1c}-lowering effect of vildagliptin was similar to that of acarbose [64] and that linagliptin and alogliptin can reduce HbA_{1c} by 0.68% and 0.57–0.68%, respectively. Notably, the HbA_{1c}-lowering extent of DPP-4 inhibitors is related to the patient's baseline HbA_{1c} level, that is, the higher the baseline HbA_{1c} level, the much it will be reduced by DPP-4 inhibitors. The use of DPP-4 inhibitors alone does not increase the risk of hypoglycaemia. DPP-4 inhibitors have a neutral effect on body weight or may increase it. Saxagliptin, alogliptin and sitagliptin do not increase the risk of cardiovascular disease, pancreatitis and pancreatic cancer. When sitagliptin, saxagliptin, alogliptin or vildagliptin is prescribed for patients with renal dysfunction, the dosage must be reduced according to the instructions of medication. When using linagliptin in patients with liver or renal insufficiency, dosage adjustments are unnecessary.

GLP-1 receptor agonists

Glucagon-like peptide-1 (GLP-1) receptor agonists reduce blood glucose by activating GLP-1 receptors. They enhance insulin secretion and inhibit glucagon secretion in a glucose concentration-dependent manner and can delay gastric emptying, thus reducing food intake via central appetite suppression. Currently, in the Chinese domestic market, the available GLP-1 receptor agonists are exenatide and liraglutide, both require subcutaneous injection. GLP-1 receptor agonists effectively lower blood glucose; and also significantly reduce body weight and improve triglycerides and blood pressure. GLP-1 receptor agonists alone do not significantly increase the risk of hypoglycaemia. Clinical trials of patients with T2DM, including Chinese patients, showed that the HbA_{1c}-lowering effect of liraglutide was similar to that of glimepiride, leading to a body weight loss of 1.8–2.4 kg and a decrease in systolic blood pressure of approximately 3 mmHg [65]; additionally, exenatide reduced HbA_{1c} by 0.8% and body weight by 1.6–3.6 kg [66]. GLP-1 receptor agonists may be used alone or in combination with other oral antihyperglycaemic agents. A number of clinical studies have shown that when used after the failure of an oral antihyperglycaemic agent (metformin or sulfonylurea), GLP-1 receptor agonists showed better efficacy than the active control drug [67]. Common side effects of GLP-1 receptor agonists are gastrointestinal symptoms (e.g. nausea and vomiting), which occur mainly in the initial stage of treatment and gradually diminish with treatment time increased.

Insulin

Initial treatment with insulin

Basal insulin or premixed insulin can be used to initiate insulin therapy.

Short-term intensive insulin therapy programme for newly diagnosed T2DM patients.

For newly diagnosed T2DM patients with HbA_{1c} >9.0% or FPG >11.1 mmol/L and with hyperglycaemic symptoms, short-term intensive insulin therapy may be implemented [68–71]. The appropriate treatment duration is 2 weeks–3 months, with a therapeutic target of 3.9–7.2 mmol/L for fasting blood glucose and ≤10.0 mmol/L for non-fasting blood glucose, without considering the HbA_{1c} target as treatment objective. Intensive insulin therapy should be combined with medical nutrition, exercise therapy and diabetes education. Intensive insulin treatment regimen include a basal-prandial insulin regimen [multiple subcutaneous insulin injections or continuous subcutaneous insulin infusion (CSII)] or premixed insulin injections two or three times a day.

For patients who fail to achieve treatment goals after short-term intensive insulin therapy, the decision to continue insulin therapy or to switch to another medication should be based on the patient-specific conditions as determined by a diabetes specialist. For patients have reached the therapy target, regular (e.g. every 3 months) follow-up monitoring should be planned; if blood glucose increases again (i.e. FPG >7.0 mmol/L or 2-h PG >10.0 mmol/L), the medication should be re-initiated.

Intensive insulin therapy programme

Multiple subcutaneous insulin injections.

CSII. CSII is a form of intensive insulin therapy delivered via an insulin pump. The main appropriate populations

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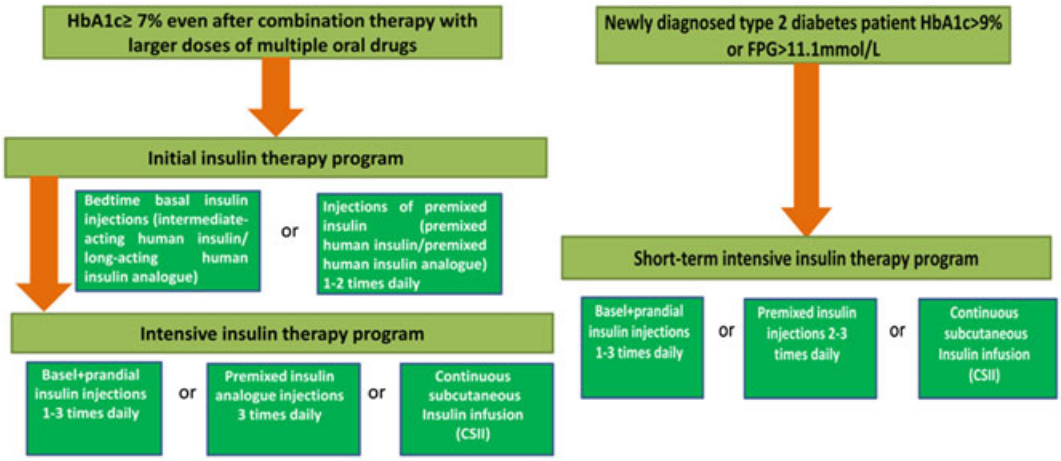

Figure 2. Insulin treatment paths for T2DM. HbA_{1c}, glycated haemoglobin; FPG, fasting plasma glucose; CSII, continuous subcutaneous insulin infusion

are T1DM patients, women with diabetes who are pregnant or expect to become pregnant, pregnant women who require insulin therapy and patients with T2DM who require intensive insulin therapy.

The insulin treatment paths are shown in Figure 2.

Hypoglycaemia

During treatment, patients may experience hypoglycaemia, which may cause discomfort and can be life-threatening. Hypoglycaemia poses a major obstacle to reaching the blood glucose target and warrants special attention.

Bariatric surgery to treat T2DM

Indications for bariatric surgery

Patients with T2DM who are 18 to 60 years old are generally in good condition, have a low surgical risk and are difficult to control the disease or concomitant diseases (HbA_{1c} >7.0%) after lifestyle interventions and various drug treatments and who meet the following conditions may consider bariatric surgery.

1. Indications: gastrointestinal bariatric surgery is feasible if the patient has a BMI ≥32 kg/m^2 with or without diabetic complications.
2. Precautions: bariatric surgery could be considered with caution for T2DM patients with a BMI of 28–32 kg/m^2, particularly in the presence of other cardiovascular risk factors.
3. Not recommended: patients with a BMI 25–28 kg/m^2, with diabetic complications and central obesity (waist circumference >90 cm in men and >85 cm in women) and at least two additional metabolic syndrome components: high triglycerides, low HDL-C and high blood pressure. Surgery should be conducted in strict accordance with study protocol with the patient's informed consent. The operation should be regarded as pure for clinical research and must be approved by the Medical Ethics Committee in advance; currently, evidence is insufficient, and surgery is not recommended as a clinical routine treatment.

Contraindications for bariatric surgery

1 Patients abuse drugs, addict to alcohol or have a mental illness that is difficult to control, and who are lack the ability to understand the risks, benefits and expected consequences of bariatric surgery.
2 Patients with confirmed, diagnosed T1DM.
3 T2DM patients who have a clear failure of pancreatic β-cell function.
4 Contraindications for surgery.
5 BMI <25 kg/m^2.
6 GDM and other specific types of diabetes.

Chronic complications of diabetes

Diabetic nephropathy

Approximately 20–40% of diabetic patients suffer from diabetic nephropathy, which is the main cause of renal failure in diabetes patients [72,73].

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Diagnosis

Diagnosis of diabetic nephropathy: T1DM-induced renal damage is divided into five stages, which are also used for T2DM-induced renal damage: stage I, elevated glomerular filtration rate and increased renal size; stage II, intermittent microalbuminuria; stage III, early diabetic nephropathy with persistent microalbuminuria; stage IV, clinical diabetic nephropathy with overt albuminuria; and stage V, renal failure. Diabetic nephropathy is an important type of chronic kidney disease; for diabetic nephropathy patients, the eGFR should be calculated using the Modification of Diet in Renal Disease Study equation or the Cockcroft–Gault formula (Table 5) [74–77].

Treatment

1. Lifestyle changes: reasonable weight control, diabetic diet, smoking cessation, proper exercise and so on
2. Low-protein diet.
3. Control blood glucose.
4. Control blood pressure.
5. Correct dyslipidemia.
6. Control proteinuria: starting from the early stages of diabetic nephropathy (microalbuminuria) with or without hypertension, renin-angiotensin system inhibitors (angiotensin-converting enzyme inhibitor or angiotensin II receptor antagonist drugs) are the preferred drugs for reducing urinary albumin [78–80]. Because these drugs may also lead to a short-term decline in the GFR in the first 1–2 weeks, serum creatinine and potassium concentrations must be monitored. Renin-angiotensin system inhibitors are not recommended for patients with serum creatinine levels >265.2 μmol/L (3 mg/dL).
7. Dialysis therapy and transplantation: when the eGFR is less than 60 mL/(min · 1.73 m^2), the potential complications of chronic kidney disease should be assessed and treated. Diabetic patients with kidney failure who require dialysis or transplant treatments should undergo these procedures as soon as possible. Generally, when the GFR drops to 15–20 mL/min or the serum creatinine level is higher than 442 μmol/L (5 mg/dL), dialysis, either peritoneal dialysis or haemodialysis, should be prepared. When conditions permit, a kidney or pancreas-kidney transplant could be performed.

Table 5. Stages of renal function in CKD

CKD stage		Feature description	eGFR [mL/(min · 1.73 m^2)]
1		Increased GFR or normal GFR with kidney damage[a]	≥90
2		Slightly decreased GFR with kidney damage[a]	60–89
3	3a	Mild to moderate GFR decrease	45–59
	3b	Moderate to severe GFR decrease	30–44
4		Severe GFR decrease	15–29
5		Kidney failure	<15 or dialysis

CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate.
[a]Kidney injury is defined as an abnormality in pathological, urine, blood or imaging examinations.

Diabetic retinopathy

Diabetic retinopathy is the most common cause of new onset blindness among adults aged 20–74 years.

Screening

Patients with non-proliferative diabetic retinopathy and macular oedema may have no obvious clinical symptoms; therefore, in terms of preventive treatment, regular fundus examinations are particularly important.

Follow-up frequency: diabetic patients without retinopathy are recommended to undergo follow-up check-up once every 1–2 years; patients with mild retinopathy should be checked once a year, and patients with severe retinopathy should be checked once every 3–6 months. The frequency of check-up should be increased for pregnant women.

Diagnosis

Diabetic retinopathy is graded according to the observable indicators after dilation under ophthalmoscope. The international clinical grading standard for diabetic retinopathy is shown in Table 6.

Table 6. International clinical grading standard for diabetic retinopathy (2002)

Disease severity	Observation after dilation under ophthalmoscope
No obvious diabetic retinopathy	No abnormality
NPDR	
Mild	Diabetic microaneurysm only
Moderate	Diabetic microaneurysm with mild or moderate NPDR
Severe	Any of the following, but without PDR 1. More than 20 intraretinal haemorrhages in any one quadrant 2. Retinal venous beading in two or more quadrants 3. Intraretinal microvascular abnormalities in one or more quadrants
Proliferative diabetic retinopathy	One or more of the following: new vessels at the optic disc, vitreous haemorrhage or preretinal haemorrhage

NPDR, non-proliferative diabetic retinopathy.

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Treatment
Good control of blood glucose, blood pressure and lipids may prevent or delay the progression of diabetic retinopathy [81,82].

1. Patients with sudden blindness or retinal detachment require an immediate referral to an ophthalmologist; diabetic patients with any degree of macular oedema, severe non-proliferative diabetic retinopathy or any proliferative diabetic retinopathy should be referred to an ophthalmologist with extensive experience in diagnosing and treating diabetic retinopathy.
2. Laser photocoagulation therapy may reduce high-risk proliferative diabetic retinopathy, clinically significant macular oedema and the risk of blindness in some patients with severe non-proliferative diabetic retinopathy [83].
3. Anti-vascular endothelial growth factor therapy may be used to treat patients with diabetic macular oedema [84].
4. Retinopathy is not a contraindication for aspirin therapy; aspirin therapy does not increase the risk of retinal haemorrhage.
5. Fenofibrate may slow the progression of diabetic retinopathy and decrease the need for laser treatment.

Diabetic neuropathy

Diabetic neuropathy is one of the most common chronic complications of diabetes. Neuropathy may affect the central nervous system or, more commonly, the peripheral nerves [85].

Diabetic peripheral neuropathy refers to peripheral nerve dysfunction-related symptoms or signs in diabetic patients that cannot be attributed to other causes. Distal symmetric polyneuropathy is a typical diabetic neuropathy. The diagnosis of other asymptomatic diabetic neuropathies relies on the screening of clinical signs or electrophysiological examination [86].

Prevention
(1) General treatment: good blood glucose control, correction of dyslipidemia and hypertension control. (2) Regular disease screening and evaluation: all patients should undergo screening for diabetic peripheral neuropathy at least once a year after the diagnosis of diabetes. For patients with a long course of diabetes or microvascular complications, such as retinopathy and nephropathy, check-up should occur every 3–6 months. (3) Increased foot care: patients suffering from peripheral neuropathy should receive education about foot care to reduce the incidence of foot ulcers [87].

Etiological therapy
(1) Glycaemic control. (2) Nerve repair: commonly used medications, such as methylcobalamin and growth factors, may be useful. (3) Anti-oxidative stress: commonly used medications, such as lipoic acid, may be useful. (4) Improved microcirculation: commonly used medications include prostaglandin E1, beraprost natriuretic peptide, cilostazol, pentoxifylline, pancreatic kallikrein, calcium antagonists and blood circulation-promoting TCM [88].

Symptomatic treatment
Medications for the treatment of painful diabetic neuropathy include anticonvulsants (pregabalin, gabapentin, valproate and carbamazepine), antidepressants (duloxetine, amitriptyline, imipramine and citalopram), opioids (tramadol and oxycodone) and capsaicin [87,88].

Lower extremity vascular disease

Lower extremity vascular disease mainly refers to peripheral artery disease; although it is not a complication specific to diabetes, the risk of peripheral artery disease in patients with diabetes significantly increases compared with patients without diabetes. In addition, patients with diabetes also have an earlier age of onset and increased severity of lower extremity vascular disease, as well as more extensive pathology and worse prognoses [82].

Lower extremity arterial disease
Lower extremity arterial disease (LEAD) is a component of peripheral artery disease that manifests as lower extremity arterial stenosis or occlusion.

Screening for diabetic LEAD
For diabetes patients over age of 50 years, LEAD screening should be conducted routinely [89,90]. For diabetes patients with LEAD-associated risk factors (e.g. cardiovascular disease, dyslipidemia, hypertension, smoking or a diabetes duration of more than 5 years) should be screened at least once a year.

For diabetes patients with foot ulcers and gangrene, regardless of their age, a comprehensive examination and evaluation of arterial disease should be conducted.

Diagnosis of diabetic LEAD
(1) If the patient has a resting ABI ≤ 0.90, regardless of the presence of lower limb discomfort, a LEAD diagnosis should be considered. (2) For a patient who experiences discomfort upon moving and has a resting ABI ≥ 0.90: if ABI decreases by 15–20% after a treadmill test, a LEAD diagnosis should be considered; (3) if the patient has a resting ABI < 0.40, or ankle arterial pressure < 50 mmHg or toe arterial pressure < 30 mmHg, a critical limb ischaemia diagnosis should be considered.

Treatment of diabetic LEAD
The therapeutic approach to LEAD includes the prevention of systemic atherosclerotic disease progression, the

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prevention of cardiovascular events, the prevention of ischaemic-induced ulcers and gangrene, the prevention of amputation or the reduction of the amputation level and the improvement of the functional status of patients with intermittent claudication. Therefore, the standard treatment for diabetic LEAD consists of three parts: primary prevention (to prevent or delay the occurrence of LEAD), secondary prevention (to relieve symptoms and delay LEAD progression) and tertiary prevention (to promote revascularization and reduce amputation and cardiovascular events).

Prevention and treatment of cardiovascular and cerebrovascular diseases in patients with T2DM

Diabetes is an independent risk factor for cardiovascular and cerebrovascular diseases. Patients with diabetes have 2–4 times higher risk of cardiovascular and cerebrovascular diseases [91–93] compared with patients without diabetes. FPG and postprandial hyperglycaemia are correlated with an increased risk of cardiovascular and cerebrovascular diseases, even when they do not reach the diagnostic criteria for diabetes. Diabetic patients often present important risk factors for cardiovascular and cerebrovascular diseases, such as dyslipidemia and hypertension [94,95].

Clinical evidence suggests that strict glycaemic control in patients with T2DM has a limited effect on reducing the risks of cardiovascular and cerebrovascular diseases and death from those causes, particularly among patients with a longer disease duration, who are older, and who have a history of cardiovascular diseases or multiple cardiovascular risk factors [38]. However, the comprehensive management of multiple risk factors can significantly decrease the risk of cardiovascular and cerebrovascular diseases and death from those

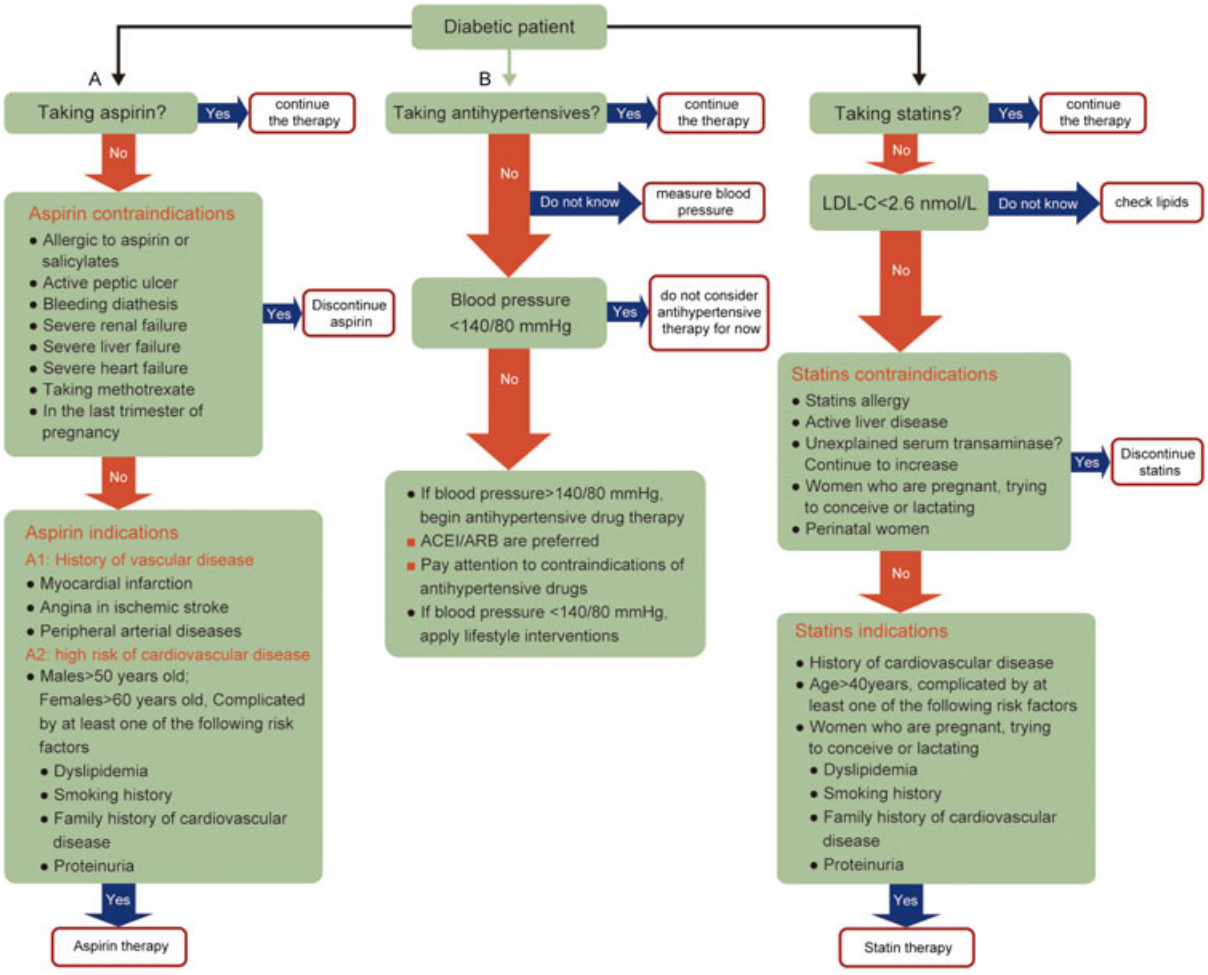

Figure 3. The clinical decision-making paths for screening and the standard lipid-lowering, antihypertensive and antiplatelet treatments for patients with T2DM. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor antagonist; LDL-C, low-density lipoprotein cholesterol

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causes in patients with diabetes. Therefore, the prevention of diabetic vascular diseases requires the comprehensive assessment and control of cardiovascular disease risk factors (e.g. high blood glucose, hypertension and dyslipidemia) and appropriate antiplatelet therapy.

At present, the incidence of cardiovascular risk factors is high among T2DM patients in China, and they are insufficiently controlled. Among outpatients with T2DM, only 5.6% achieved all triple therapeutic goals for HbA_{1c}, blood pressure, and total cholesterol [96]. The use of aspirin has also been low. Clinically, more active screening and treatment of cardiovascular risk factors and an increased rate of aspirin therapy are recommended.

The clinical decision-making paths for screening and the lipid-lowering, antihypertensive and antiplatelet treatments for patients with T2DM are shown in Figure 3.

Metabolic syndrome

Diagnostic criteria for metabolic syndrome

According to an epidemiological analysis of metabolic syndrome in the current Chinese population, this guideline has revised the quantitative indicators of the metabolic syndrome components based on the CDS's 2004 recommendations [97]. The diagnostic criteria are as follows: (1) abdominal obesity: waist circumference: men ≥90 cm and women ≥85 cm, (2) high blood glucose: fasting blood glucose ≥6.1 mmol/L or glucose at 2 h after glucose load ≥7.8 mmol/L and/or diabetes diagnosis and treatment, (3) high blood pressure: blood pressure ≥130/85 mmHg and/or diagnosed and on antihypertension therapy, (4) fasting TG ≥1.70 mmol/L and (5) fasting HDL-C < 1.04 mmol/L. Patients with three or more of the aforementioned characteristics are diagnosed with metabolic syndrome.

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