Novel anti-diabetic effect of SCM-198 via inhibiting the hepatic NF-κB pathway in *db/db* mice

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**Synopsis**

There are reports of early evidence that suggest the involvement of chronic low-grade inflammation in the pathogenesis of Type 2 diabetes. Thus, substances that have effects in reducing inflammation could be potential drugs for Type 2 diabetes. Leonurine (4-guanidino-n-butyl syringate; SCM-198) is an alkaloid in HL (*Herba leonuri*), which was reported to possess anti-inflammatory properties. We hypothesize that SCM-198 may have beneficial effects on Type 2 diabetes.

In the present study, we attempted to test this hypothesis by evaluating the anti-diabetic effect of SCM-198 and the possible underlying mechanisms of its effects in *db/db* mice. SCM-198 (50, 100 and 200 mg/kg of body weight), pioglitazone (50 mg/kg of body weight, as a positive control) or 1% CMC-Na (sodium carboxymethylcellulose) were administered to the *db/db* or *db/m* mice once daily for 3 weeks. After 3 weeks, SCM-198 (200 mg/kg of body weight) treatment significantly reduced the fasting blood glucose level and increased the plasma insulin concentration in the *db/db* mice, meanwhile it significantly lowered the plasma TAG (triacylglycerol) concentration and increased the HDL (high-density lipoprotein)-cholesterol concentration. Moreover, the dysregulated transcription of the hepatic glucose metabolic enzymes, including GK (glucokinase), G6Pase (glucose-6-phosphatase) and PEPCK (phosphoenolpyruvate carboxykinase), was recovered by an Akt-dependent pathway. The pro-inflammatory mediators {such as TNFα (tumour necrosis factor α), IL (interleukin)-6, IL-1β, degradation of IκB [inhibitor of NF-κB (nuclear factor-κB)] α and thereafter activation of NFκB} were reversed by SCM-198 treatment in the *db/db* mice. The present study provides first evidence that SCM-198 exhibits anti-inflammatory activity and has an ameliorating effect on diabetic symptoms via inhibiting of NFκB/IKK (IκB kinase) pathway. Consequently, we suggest that SCM-198 may be a prospective agent for prevention and/or modulation of the progress of Type 2 diabetes.

**Key words:** anti-inflammatory, *db/db* mice, nuclear factor-κB (NFκB), SCM-198, tumour necrosis factor α (TNFα), Type 2 diabetes

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**INTRODUCTION**

Diabetes mellitus is a potentially life-threatening disease affecting approximately 3% of the population worldwide [1], and its incidence is increasing at an alarming rate with the trend of urbanization and lifestyle changes. Among the diabetes patients, approximately 90% are affected by Type 2 diabetes. This metabolic disorder is usually accompanied by serious complications causing damage to eyes, kidneys as well as the nervous and cardiovascular system [2,3]. Although there are several oral anti-diabetic drugs available on the market, many of them have drug-related side effects [4]. Therefore exploring new drugs with high efficacy and low side effects is desirable.

A body of evidence has documented the involvement of chronic low-grade inflammation in the development of insulin resistance and pathogenesis of Type 2 diabetes. The first molecular link between inflammation and insulin resistance – TNFα (tumour necrosis factor-α) – was identified by both Hotamisligil et al. [5] and Feinstein et al. [6]. Then elevated levels of other pro-inflammatory mediators, such as IL (interleukin)-6, IL-1β and CRP (C-reactive protein), were found in the blood of diabetic-...
and insulin-resistant subjects [7]. These cytokines are the key players in activating several intracellular pathways that promote the development of insulin resistance and Type 2 diabetes.

Several studies suggest the crucial role of NF-κB (nuclear factor κB)/IKK [IκB (inhibitor of NF-κB) kinase] pathway in counteracting insulin action. NF-κB is activated by many pro-inflammatory stimuli including cytokines such as TNFα, IL-6 and IL-1β, which in turn are also NF-κB-regulated products. As a molecular mediator of insulin resistance, the IKK/NF-κB pathway has become a pharmacological target for insulin sensitization and the anti-diabetic drugs discovery.

Leonurine (4-guanidino-n-butyl syringate; SCM-198) is an alkaloid present in the Chinese herb HL (Herba leonuri). HL has diverse pharmacological activities and has been widely used in Chinese folk medicine for more than 100 years. Several studies have provided support for HL applications in the treatment of inflammatory diseases, like arthritis. Islam et al. [8] reported that HL extract showed a potent anti-inflammatory activity against carrageenin-induced paw oedema in rats. In human mast cell line, HL is reported to inhibit the secretion of pro-inflammatory cytokines, like TNFα, IL-6 and IL-8 possibly by inhibiting NF-κB activation [9]. Lee [10] reported that HL could suppress TNFα-induced expression of adhesion molecules in HUVEC (human umbilical vein endothelial cells). Recently, colleagues in our group have also demonstrated that SCM-198 protected against TNFα-mediated inflammation in HUVEC [11]. This evidence indicates that HL may be helpful in regulating inflammatory diseases. In addition, our group has performed several studies of SCM-198, and found that it has potent cardio-cerebral protective effects in vitro and in vivo [12–15].

Given the reported anti-inflammatory activity of HL, we propose that the main alkaloid SCM-198 in HL may ameliorate Type 2 diabetes. This study was designed to test the anti-diabetic effect of SCM-198 in db/db mice, a spontaneous Type 2 diabetic animal model characterized by metabolic disturbances resembling diabetes mellitus in humans: hyperglycaemia, hyperinsulinaemia, polyuria, glycosuria and obesity [16]. The possible mechanisms of SCM-198 actions were explored further.

**MATERIALS AND METHODS**

Chemicals
SCM-198 (purity >98 %) was synthesized in our laboratory from syringic acid by carbonylation reaction with SCCl2 (thionyl chloride), and the Gabriel reaction. Pioglitazone (99.5 %) was purchased from JieCai Trading Co.

Animal and experimental protocol
Forty-two male C57BLKS/J db/db mice and five C57BLKS/J db/m mice (db/m mice were used as a normal control in this experiment) were provided by Shanghai ChemPartner. Mice were maintained under a 12 h light/12 h dark cycle, and fed a standard laboratory pellet chow and water ad libitum, and housed at a controlled temperature (23 ± 3°C) and humidity (approximately 60 %). All animal studies were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People’s Republic of China (14 November 1988) and the Institutional Review Committee for the use of Animals.

The db/db mice were randomly divided into five groups (n = 8–9 each) when they were 9 weeks old. SCM-198 (50, 100 and 200 mg/kg of body weight), pioglitazone (50 mg/kg of body weight, as a positive control) as suspension in freshly prepared 1 % CMC-Na (sodium carboxymethylcellulose) were orally administered to db/db mice respectively once daily for 3 weeks, whereas vehicle-treated db/db mice (n = 9) and db/m mice (n = 5) received 1 % CMC-Na for 3 weeks. The body weight was measured twice a week, and the fasting blood glucose level was measured once a week in all groups. At the end of the trial, after withholding food for 12 h, the fasting blood glucose concentration was determined. Then drugs and 1 % CMC-Na were given as usual, and 3 h after that blood samples for all the groups were collected to determine the plasma biomarkers. Then mice were killed, and the liver and epididymal white adipose tissue were removed, instantly soaked in liquid nitrogen and stored at −80°C until further analysis.

**Fasting blood glucose and OGTT (oral glucose tolerance test)**

Fasting blood glucose concentration was monitored in the venous blood taken from the tail vein using a glucometer (Johnson & Johnson) every week after a 12 h fasting period.

OGTT was performed after 2 weeks of treatment. Mice were given glucose solution (2 g/kg of body weight) after a 6 h fast, and blood glucose concentration was determined from the tail vein at 0 min (prior to glucose administration), 15, 30, 60 and 120 min.

**Measurement of plasma parameters**
The blood was collected in a heparin-coated tube and centrifuged at 3000 g for 15 min at 4°C. Plasma insulin and TNFα level were determined by the ELISA method using a commercial kit (Crystal Chem Inc., eBioscience). The plasma TAG (triacylglycerol), TC (total cholesterol), HDL (high-density lipoprotein)-cholesterol and LDL (low-density lipoprotein)-cholesterol as well as the hepatic functional parameters ALT (alanine aminotransferase) and AST (aspartate aminotransferase) levels were examined on a Hitachi 7080 automatic biochemistry analyser.

**RT–PCR (reverse transcription–PCR)**
Total RNA of the liver was extracted using TRIzol® Reagent (Takara) and quantified by spectrophotometric absorbance at 260 nm according to the method described by Wang et al. [17]. Total RNA (2 μg) of each sample was reverse transcribed into cDNA using random primers (Takara).

PCR was then performed using Taq DNA polymerase (Takara) for each set of specific primers. Primer sets are summarized in
Table 1. β-Actin was used as an internal control. PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide. The absorbance of the bands was visualized and analyzed with Fluor Chem SP system, and the results were expressed relative to the corresponding intensity of the β-actin bands from the same RNA sample.

Western blot analysis
Total protein of the liver was extracted and quantified by employing the method described by Gong et al. [18]. The proteins were then separated by SDS/PAGE and transferred on to a PVDF membrane. The membrane was then incubated with a primary antibody against total Akt (1:1000 dilution), phospho-Akt (1:1000 dilution), TNFα (1:1000 dilution), phospho-NF-κB p65 (1:1000 dilution), IκBα (inhibitor of NF-κB; 1:1000 dilution), phospho-Foxo1 (forkhead box O1) (1:1000 dilution) (all the above antibodies were from Cell Signaling Technology) and β-actin (1:2000 dilution; Sigma). Following incubation, the secondary HRP (horseradish peroxidase)-conjugated anti-rabbit IgG antibodies (1:5000–1:8000 dilution; Santa Cruz Biotechnology) and secondary HRP (horseradish peroxidase)-conjugated anti-mouse IgG antibodies (1:5000–1:8000 dilution; Santa Cruz Biotechnology) were added. Immunoreactive proteins were then visualized using enhanced chemiluminescence. The signals were quantified by densitometry using a Western blotting detection system (Alpha Innotech).

Statistical analysis
Results are represented as means ± S.E.M. The statistical significance of difference between two groups was determined using two-tailed Student’s t test. A probability value of P < 0.05 was considered significant.

RESULTS

Effects of SCM-198 on body weight gain, liver index and epididymal white adipose tissue index
We studied the effects of SCM-198 on the changes of body weight, liver index and epididymal white adipose tissue weight after 3 weeks of treatment. As shown in Table 2, there was no significant difference in body weight gain, liver index and epididymal white adipose tissue weight index between the vehicle and SCM-198 treated db/db mice group. However, pioglitazone-treated db/db mice showed a significant increase in body weight gain which was consistent with that reported previously [19].

Effects of SCM-198 on fasting blood glucose level and OGTT
Figure 1(A) shows the effects of SCM-198 on fasting blood glucose level. After 3 weeks of treatment, the fasting blood glucose levels in the SCM-198 treated groups (50, 100 and 200 mg/kg of body weight) were lowered by 39.6, 24.5 and 64.0% respectively compared with that of the vehicle db/db mice. However, only the group receiving SCM-198 at 200 mg/kg of body weight showed a significant decrease in the blood glucose levels. The administration of SCM-198 also slightly improved the glucose tolerance of the db/db mice, although it only showed a modest decrease in the plasma glucose concentration at 120 min after the administration of glucose solution (Figure 1B), with a smaller AUC (area under the curve) of the blood glucose concentration in the group receiving SCM-198 at 200 mg/kg of body weight compared with that of the vehicle db/db mice (Figure 1C), indicative of an improvement in glucose tolerance to a certain degree.

Effects of SCM-198 on the plasma parameters
The plasma insulin levels were significantly higher in the db/db mice receiving SCM-198 at 100 and 200 mg/kg of body weight compared with the vehicle db/db mice. In addition, we investigated the effects of SCM-198 on the plasma lipid profiles. SCM-198 (200 mg/kg of body weight) significantly lowered the plasma TAG concentrations by 65%, but increased the plasma HDL-cholesterol concentration 1.2 times. However, it seemed that SCM-198 had no effects on the plasma TC and LDL-cholesterol concentrations during this treatment period. The results of ALT and AST, which are liver function parameters, indicated that SCM-198 itself did not cause or aggravate liver toxicity in db/db mice. All the above results are shown in Table 3.
Table 2 Effects of SCM-198 on body weight, liver index and epididymal white adipose tissue (EWAT) index

Animals were administered SCM-198 (50, 100 and 200 mg/kg of body weight), pioglitazone (Pio; 50 mg/kg of body weight) or 1 % CMC-Na once daily for 3 weeks. At the end of the experiment, the liver and epididymal white adipose tissue were excised and weighed. Results are expressed as means ± S.E.M. (n = 8–9 for db/db mice; n = 5 for db/m mice). *P < 0.05 compared with vehicle db/db mice, as determined using a Student’s t test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg of body weight)</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Body weight gain (g/3 weeks)</th>
<th>Liver (mg/g of body weight)</th>
<th>EWAT (mg/g of body weight)</th>
</tr>
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<tbody>
<tr>
<td>db/db</td>
<td>Vehicle –</td>
<td>39.61 ± 1.06</td>
<td>46.23 ± 1.05</td>
<td>6.62 ± 0.94</td>
<td>38.92 ± 3.39</td>
<td>52.84 ± 2.49</td>
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<tr>
<td></td>
<td>Pio 50</td>
<td>38.27 ± 1.45</td>
<td>49.14 ± 1.73</td>
<td>10.87 ± 0.58*</td>
<td>46.70 ± 1.69</td>
<td>52.73 ± 5.38</td>
</tr>
<tr>
<td></td>
<td>SCM-198 50</td>
<td>37.53 ± 1.68</td>
<td>45.16 ± 1.55</td>
<td>7.63 ± 0.84</td>
<td>41.56 ± 1.85</td>
<td>48.64 ± 3.70</td>
</tr>
<tr>
<td></td>
<td>SCM-198 100</td>
<td>38.10 ± 1.45</td>
<td>45.54 ± 1.44</td>
<td>7.44 ± 0.38</td>
<td>40.44 ± 1.15</td>
<td>53.40 ± 1.62</td>
</tr>
<tr>
<td></td>
<td>SCM-198 200</td>
<td>38.59 ± 1.21</td>
<td>47.34 ± 0.88</td>
<td>8.74 ± 0.40</td>
<td>41.60 ± 1.34</td>
<td>54.18 ± 0.87</td>
</tr>
<tr>
<td>db/m</td>
<td>–</td>
<td>25.27 ± 0.57*</td>
<td>28.15 ± 0.52*</td>
<td>2.88 ± 0.41*</td>
<td>36.81 ± 2.49</td>
<td>16.08 ± 2.19*</td>
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</table>

Effects of SCM-198 on the mRNA expression of the hepatic glucose metabolic enzymes

As shown in Figure 2, the hepatic mRNA expression of GK (glucokinase) was significantly lower in the vehicle db/db mice than in the db/m mice; however, with SCM-198 treatment. GK expression was increased in a dose-dependent manner compared with the vehicle db/db mice. On the other hand, SCM-198 suppressed the mRNA expressions of hepatic G6Pase (glucose-6-phosphatase) modestly and PEPCK (phosphoenolpyruvate carboxykinase) significantly when compared with the vehicle db/db mice.

Effects of SCM-198 on the expression of total Akt and phosphorylation of Akt and Foxo1

Because Akt is a necessary component in insulin signalling and its phosphorylation regulates the transcription of hepatic glucose metabolic enzymes, we investigated the effects of SCM-198 on the expression of total Akt and the level of Akt phosphorylation. The total Akt did not differ between the groups, but the phosphorylation of Akt decreased by 21.7 % compared with the db/m mice. However, as shown in Figure 3, administration of SCM-198 at 200 mg/kg of body weight increased the phosphorylation of Akt by 32.7 %.

Then we examined the phosphorylation of Foxo1, as it is a downstream target of Akt and it directly regulates the transcription of G6Pase and PEPCK. In accordance with the results for Akt, we found that the phosphorylation of Foxo1 in the vehicle db/db mice was lower than 25.1 %, whereas the treatment with SCM-198 (200 mg/kg of body weight) gave a 91.3 % increase in the phosphorylation of Foxo1, suggesting its inactivation (Figure 3). The above results suggest that SCM-198 regulates transcription of hepatic glucose metabolic enzymes through the Akt pathway.

Effects of SCM-198 on the mRNA expression of the inflammatory mediators

We measured the mRNA expression of several inflammatory mediators, including TNFα, iNOS (inducible nitric oxide synthase), COX-2 (cyclo-oxygenase 2), IL-6 and IL-1β in the liver by RT-PCR analysis. The mRNA expression of TNFα, iNOS, COX-2, IL-6 and IL-1β were significantly up-regulated in the liver of db/db mice compared with the db/m mice. Nevertheless, the administration of SCM-198 caused a pronounced down-regulation of expression of these genes (Figure 4).

Effects of SCM-198 on the plasma TNFα level and hepatic protein expression of TNFα

We detected the plasma TNFα concentration using the ELISA method, and found that the vehicle db/db mice showed a significantly higher concentration of TNFα, however, SCM-198 (100 and 200 mg/kg of body weight) treatment reduced the up-regulated secretion of TNFα (Figure 5). At the same time, the expression of hepatic TNFα protein was determined by Western blotting. Consistent with the plasma concentration of TNFα, the vehicle db/db mice showed a significant increase in the protein expression of TNFα compared with the db/m mice (Figure 6). However, after treatment with SCM-198, the TNFα protein expression was significantly lowered when compared with the db/db mice (Figure 6).

Effects of SCM-198 on the NF-κB/IKK pathway

To further explore the mechanism underlying the inhibitory effect of SCM-198 on the production of TNFα and other pro-inflammatory mediators, the NF-κB/IKK pathway, a pro-inflammatory pathway, was detected by Western blotting. IκBα directly binds to NF-κB to inhibit its nuclear translocation. As soon as IKK is activated, IκBα is phosphorylated and subsequently degraded, thus releasing NF-κB that translocates to the nucleus. In the present study, the abundance of IκBα, as an indicator of IKK activity was first examined. The protein level of the IκBα was significantly decreased in the vehicle db/db mice, compared with the db/m mice. Accordingly, the subsequent NF-κB p65 phosphorylation was significantly up-regulated in the db/db mice compared with the db/m mice (Figure 6). However, we observed that SCM-198 inhibited the IκBα degradation and the subsequent phosphorylation of NF-κB p65 in the liver of db/db mice (Figure 6).
DISCUSSION

In the present study, we evaluated the anti-diabetic effect of SCM-198 in db/db mice. Our results show that administration of SCM-198 for 3 weeks did not alter the body weight, whereas pioglitazone significantly increased the body weight gain of the db/db mice, which was consistent with previous reports [19,20] (Table 2). At the same time, we observed that SCM-198 (200 mg/kg of body weight) significantly decreased the fasting blood glucose level in the db/db mice (Figure 1A). Meanwhile, the plasma insulin concentration was significantly increased in the SCM-198 (100 and 200 mg/kg of body weight) treated groups (Table 3). Moreover, we observed that there was a significant decrease in TAG level and an increase in HDL-cholesterol level compared with the vehicle db/db mice after SCM-198 treatment (200 mg/kg of body weight) (Table 3). The above results imply that SCM-198 has anti-diabetic effects in db/db mice.

The liver is an important organ for maintaining glucose and lipid homoeostasis. Abnormal hepatic glucose metabolism is a major characteristic of Type 2 diabetes and increased rate of hepatic glucose production is largely responsible for the development of overt hyperglycaemia [21]. The liver maintains the glucose homoeostasis primarily through controlling glucose utilization and glucose release depending largely on some glucose metabolic enzymes, among which the most important ones are GK, G6Pase and PEPCK.

GK exerts dominant control on glycogen synthesis and glycolysis via regulating the rate of synthesis of G6Pase, while PEPCK and G6Pase are mainly responsible for the control of glucose release through glycojenolysis and gluconeogenesis [22]. Accumulating evidence has shown that in several animal models of diabetes the mRNA expression of GK is reduced while those of the G6Pase and PEPCK are increased [23–25]. In this study, we observed that the SCM-198 treatment significantly increased the mRNA expression of GK, and reduced that of PEPCK. This indicated that the SCM-198 might cause the hypoglycaemic effect partly by regulating the transcription of hepatic glucose metabolic genes and thus reducing hepatic glucose production.

In the liver, expression of GK is strictly dependent on the presence of insulin. Insulin induction of GK mRNA was abrogated by inhibitors of Akt activation [26,27]. More importantly, co-transfection of plasmids encoding constitutively active PI3K (phosphoinositide 3-kinase) or Akt stimulated GK promoter activity [28]. Akt signalling also regulates glycogen synthesis and glycolysis through stimulating the association of GK with the mitochondria, where they phosphorylate glucose more readily [29]. Moreover, insulin signalling through Akt can inhibit the transcription of G6Pase and PEPCK as well. Foxo1 interacts with the consensus IREs (insulin response elements) of G6Pase and PEPCK present in their promoters, thus regulating the expression of these two genes. When Akt is activated by insulin signalling, it can phosphorylate Foxo1 at three conserved sites (Thr24, Ser253 and Ser316), resulting in its translocation out of nucleus, thereby, preventing Foxo1 from activating its target genes.
Table 3 Effects of SCM-198 on plasma parameters

Animals were administered SCM-198 (50, 100 and 200 mg/kg of body weight), pioglitazone (Pio; 50 mg/kg of body weight) or 1 % CMC-Na once daily for 3 weeks. At the end of the experiment, blood samples were collected to determine the plasma biomarkers. Results are expressed as means ± S.E.M. (n = 5–9). *P < 0.05 compared with vehicle db/db mice, as determined using a Student’s t test. HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg of body weight)</th>
<th>Insulin (ng/ml)</th>
<th>ALT (units/l)</th>
<th>AST (units/l)</th>
<th>TC (mmol/l)</th>
<th>TAG (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
</tr>
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<tbody>
<tr>
<td>db/db</td>
<td>−</td>
<td>6.64 ± 1.25</td>
<td>186 ± 51</td>
<td>189 ± 35</td>
<td>3.13 ± 0.21</td>
<td>0.85 ± 0.19</td>
<td>2.69 ± 0.16</td>
<td>0.43 ± 0.5</td>
</tr>
<tr>
<td>Pio</td>
<td>50</td>
<td>7.28 ± 0.58</td>
<td>127 ± 14</td>
<td>119 ± 4</td>
<td>3.37 ± 0.10</td>
<td>0.26 ± 0.06*</td>
<td>2.98 ± 0.07</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>SCM-198</td>
<td>50</td>
<td>8.48 ± 0.93</td>
<td>77 ± 15</td>
<td>168 ± 32</td>
<td>3.42 ± 0.08</td>
<td>0.52 ± 0.19</td>
<td>2.68 ± 0.43</td>
<td>0.51 ± 0.06</td>
</tr>
<tr>
<td>SCM-198</td>
<td>100</td>
<td>10.10 ± 0.93*</td>
<td>112 ± 14</td>
<td>142 ± 22</td>
<td>3.47 ± 0.20</td>
<td>0.54 ± 0.13</td>
<td>3.00 ± 0.17</td>
<td>0.56 ± 0.11</td>
</tr>
<tr>
<td>SCM-198</td>
<td>200</td>
<td>9.55 ± 0.84*</td>
<td>120 ± 17</td>
<td>189 ± 61</td>
<td>3.64 ± 0.16</td>
<td>0.30 ± 0.03*</td>
<td>3.27 ± 0.15*</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>db/m</td>
<td>−</td>
<td>0.76 ± 0.49</td>
<td>45 ± 2*</td>
<td>132 ± 8</td>
<td>2.17 ± 0.08*</td>
<td>0.40 ± 0.06</td>
<td>1.81 ± 0.09*</td>
<td>0.39 ± 0.01</td>
</tr>
</tbody>
</table>

Figure 2 Effects of SCM-198 on the mRNA expressions of glucose metabolic enzymes in liver

(A) RT–PCR results of different glucose metabolic enzymes genes. The relative absorbance was normalized to β-actin. (B) GK, (C) G6Pase and (D) PEPCK mRNA expression. S-50, SCM-198 (50 mg/kg of body weight), S-100, SCM-198 (100 mg/kg of body weight), S-200, SCM-198 (200 mg/kg of body weight). Results are expressed as means ± S.E.M. (n = 3). *P < 0.05 compared with vehicle db/db mice as determined using a Student’s t test.

Because of the above analysis, we presume that the transcription of these genes altered by SCM-198 treatment in this study might act through the Akt pathway. Thus, we first examined the phosphorylation of Akt using the phosphospecific (Ser473) antibody, and correspondingly revealed that impaired Akt signalling in the liver of the db/db mice is recovered by treatment with SCM-198. Then we further detected the phosphorylation of Foxo1, which is the downstream target of Akt, and found that SCM-198 treatment promoted the phosphorylation of Foxo1 (Figure 3), suggesting that SCM-198 may exert its anti-diabetic effect through regulating hepatic glucose metabolic enzymes in an Akt-dependent manner.
Some evidence has suggested a state of chronic low-grade inflammation is involved in the development of insulin resistance and the pathogenesis of Type 2 diabetes, characterizing altered levels of several circulating inflammatory factors such as TNFα, MCP-1 (monocyte chemoattractant protein-1), IL-6, IL-1β and other biological markers of inflammation [7]. It has been clearly demonstrated that pro-inflammatory cytokines like TNFα and IL-6 are able to interfere with the insulin signalling pathway, and decrease insulin action. In vitro studies showed that treatment of cultured 3T3-L1 and HepG2 cells with TNFα can induce serine phosphorylation of IRS-1 (insulin receptor substrate 1) to attenuate insulin signalling, finally leading to insulin resistance [31]. As for IL-6, both in vitro and in vivo studies showed that it can inhibit insulin receptor signal transduction [32,33]. When TNFα/IL-6 are neutralized with a specific antibody in obese animals, the tyrosine kinase activity in fat and muscle or liver is restored, as well as the insulin sensitivity [34,35].

As previous studies [8,9] have shown, HL and SCM-198 exert potent anti-inflammatory properties. In our present study, we propose that the anti-diabetic effect of SCM-198 may be correlated with its anti-inflammatory properties. Therefore we examined the mRNA expression profiles of inflammatory mediators. Consistent with our hypotheses, the increased mRNA levels of inflammatory cytokines, such as TNFα, IL-6 and IL-1β, as well as inflammatory enzymes COX-2 and iNOS, in the liver of db/db mice were reduced significantly by SCM-198.

We further examined the plasma TNFα concentration and hepatic TNFα protein expression and found that SCM-198 could reduce both of them. Shin et al. [9] reported that HL inhibits the secretion of pro-inflammatory cytokines, such as TNFα, IL-6 and IL-8, possibly by inhibiting NF-κB activation, and the NF-κB/IKK pathway is widely involved in the development of insulin resistance and Type 2 diabetes. Hence to explore further the possible molecular mechanism underlying the hypoglycaemic action of SCM-198, we investigated the NF-κB/IKK pathway by Western blotting.

NF-κB is a nuclear transcription factor consisting of homo- or hetero-dimers containing Rel-domain protein, namely Rel A (p65), Rel B, c-Rel, p50 (NF-κB1) and p52 (NF-κB2) among which is a prototypical NF-κB complex comprising a p65 and p50. In a resting state, the inhibitors of NF-κB (IκB) directly bind to the NF-κB complex to retain it in the cytoplasm by masking its nuclear-translocating sequences. However, when IκB is phosphorylated at Ser32 and Ser36 by IKK, which consists of three subunits, namely IKKα, IKKβ and IKKγ, it is subsequently ubiquitinated and degraded by the 26S proteasome complex, thus releasing NF-κB to translocate into the nucleus, where it can activate pro-inflammatory and anti-apoptosis genes by binding to κB sites in their promoter regions [3,36].

Several studies have suggested the involvement of NF-κB/IKK axis in the development of insulin resistance and Type 2 diabetes. Genetic disruption of NF-κB signalling pathways has been shown to improve insulin resistance. Heterozygous IKK-β+/− mice under high-fat diet regimen or mated with ob/ob obese mice were protected against insulin resistance [37]. Additionally, it has been reported that pharmacological inhibition of IKK activity by high...
dose of salicylates ameliorates insulin resistance in vitro [31] and in vivo [38,39].

IKK activity was monitored using IκBα protein abundance as an indicator in the present study. The protein level of IκBα was significantly lowered in the vehicle db/db mice than in the vehicle db/m mice, although the degradation of IκBα in the db/db mice was reversed by the SCM-198 treatment. Accordingly, the subsequent phosphorylation of the p65 subunit of

Figure 4 Effects of SCM-198 on the mRNA expression of the inflammatory mediators
(A) RT-PCR results of different inflammatory genes. The relative absorbance was normalized to β-actin. (B) TNFα, (C) COX-2, (D) iNOS, (E) IL-1β and (F) IL-6 mRNA expression. S-50, SCM-198 (50 mg/kg of body weight), S-100, SCM-198 (100 mg/kg of body weight), S-200, SCM-198 (200 mg/kg of body weight). Results are expressed as means ± S.E.M. (n = 3). *P < 0.05 compared with vehicle db/db mice as determined using a Student’s t test.
NF-κB was inhibited by the SCM-198 treatment. Taken together with the decrease in the above pro-inflammatory mediators, it can be concluded that SCM-198 inhibited the NF-κB/IKK pathway, which in turn repressed the induction of pro-inflammatory mediators, including TNFα, IL-6, IL-1β, COX-2 and iNOS.

The above data provide evidence in support of a sequence of cellular and molecular events mediating the anti-diabetic effect of SCM-198 in the liver of the db/db mice. The NF-κB/IKK pathway was activated in the liver of db/db mice, which induced the expression of NF-κB target pro-inflammatory cytokine genes, such as TNFα, IL-6 and IL-1β. These cytokines are additionally reported to interfere with the insulin signalling, especially the tyrosine phosphorylation of the insulin receptor and IRS [31–33], thereby inhibiting their downstream molecular function, like Akt activation. Inactivated Akt is incapable of regulating the transcription of glucose metabolic enzymes, including GK, G6Pase and PEPCK, thus leading to increased hepatic glucose production and hyperglycaemia. Nevertheless, in the present study, SCM-198 strongly inhibited the NF-κB/IKK pathway, and decreased the expression of relative pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β, thus restoring insulin signalling, therefore causing hypoglycaemic effect via promoting glucose utilization and inhibiting hepatic glucose production by regulating the expression of glucose metabolic enzymes.

In summary, the present study evaluates the anti-diabetic effect of SCM-198 in the db/db mice, a typical Type 2 diabetes animal model, for the first time. We found that SCM-198 exhibits anti-inflammatory activity and an ameliorating effect on diabetic symptoms via inhibiting the NF-κB/IKK pathway. Consequently, we suggest that SCM-198 may be a prospective agent for preventing and/or moderating the progress of Type 2 diabetes.
AUTHOR CONTRIBUTION
Yizhun Zhu designed the research; Hui Huang, Yajun Xu, Danyi Wen and Yahua Zhang designed and performed the animal experiments; Hui Huang, Hong Xin and Xinhua Liu conducted the molecular biology research; and Hui Huang, Hong Xin, Xinhua Liu and Yizhun Zhu wrote the paper.

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