Original Paper

Cannabinoid 2 Receptor Agonist Improves Systemic Sensitivity to Insulin in High-Fat Diet/Streptozotocin-Induced Diabetic Mice

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Key Words

Cannabinoid 2 receptor • Diabetes • Insulin secretion • Inflammation • Lipolytic

Abstract

Background/Aims: The endocannabinoid signalling (ECS) system has been known to regulate glucose homeostasis. Previous studies have suggested that the cannabinoid 2 (CB₂) receptor may play a regulatory role on insulin secretion, immune modulation and insulin resistance. Given that diabetes and insulin resistance are attributable to elevated inflammatory tone, we investigated the role of CB₂ receptor on glucose tolerance and insulin sensitivity in high-fat diet (HFD)/streptozotocin (STZ)-induced mice. **Methods:** Diabetes was induced in male ICR mice by HFD/STZ and exposed to a CB₂ receptor agonist, SER601, for 2- or 4-weeks via subcutaneous implantation of osmotic minipumps. Glucose and insulin tolerance tests were performed at the end of treatment. Islets were isolated for assessment of β -cell function. Pancreases and skeletal muscles were also obtained for histological analyses. **Results:** Despite a lack of impact on glucose tolerance, substantial improvement on insulin sensitivity was observed in SER601-treated mice, which could partly be attributed to improved islet β -cell function, shown as increased glucose-induced insulin secretion and insulin content. No changes on islet macrophage infiltration or skeletal muscle fat deposition were detectable from SER601-treated mice. However, a major decrease in body weight was recorded at the end of 4-week SER601 exposure, accompanied by a lack of epididymal adipose mass in SER601-treated mice. **Conclusion:** Our data suggest a lipolytic role of SER601 in HFD/STZ-induced diabetic mice, which results in significant improvement of systemic insulin sensitivity. Thus, the CB₂ receptor may be considered a promising target for therapeutic development against insulin resistance and obesity-related diabetes.

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Introduction

The endocannabinoid system (ECS) consists of the cannabinoid receptors, which are activated by their endogenous ligands called the endocannabinoids, and enzymes that are responsible for endocannabinoid synthesis and degradation. The ECS plays an essential role in regulating metabolism and glucose homeostasis [1]. Three types of cannabinoid receptors, the cannabinoid 1 (CB₁), cannabinoid 2 (CB₂) and atypical cannabinoid GPR55 receptors have been identified to date [1]. The *in vivo* activities of central CB₁ receptors have been most extensively investigated and rimonabant, a CB₁R antagonist, was briefly used clinically as an anti-obesity agent before its withdrawal as a result of adverse side-effects [2]. The hypothesis that ECS is overactive in obesity, which increases the risks of type 2 diabetes (T2D) and other related metabolic disorders has also been proposed [3, 4]. However, the notion that activation of the ECS *per se* is detrimental to disruption of systemic energy homeostasis is oversimplified.

Multiple studies have reported modulatory roles of the cannabinoid receptors on islet β -cell function [1, 5-15]. We have previously reported stimulatory impact of cannabinoid receptors activation in mouse and human islet insulin secretion *in vitro*, although contrary results regarding the activation of the CB₁ receptors, in particular, and in some cases the CB₂ receptors, were also demonstrated using isolated mouse islets and rodent β -cell lines [5-7, 11, 12]. The problem of non-specificity regarding the activities of current cannabinoid receptor ligands has been raised [5], which could explain the controversies concerning the *in vitro* effect of cannabinoid receptor activities in rodent and human islets. Other than its regulatory impact on insulin secretion, an additional role of the ECS on insulin resistance was also proposed since glucose deprivation induces changes on mRNA expression of the ECS in adipocytes and skeletal muscles [16].

Chronic inflammation contributes substantially to the onset of insulin resistance, T2D and obesity [17]. The CB₂ receptors were initially identified in the immune system [18]. Immunomodulatory property of cannabinoids via the CB₂ receptor has later been discovered, which fuelled investigations of CB₂ receptor agonists as potential therapeutic candidates for autoimmune diseases and ischaemic organ injuries [19-22]. Thus, administration of delta-9-tetrahydrocannabinol, a derivative of cannabinoid, significantly inhibited atherosclerosis progression in mice [23]. Neuroprotective effect of JWH133, a CB₂ agonist, was also demonstrated in mice with ischaemic cerebral damage [24]. A recent report suggested a beneficial role of the CB₂ receptor in allograft protection [25]. Given that obesity, insulin resistance and T2D are all considered consequences of impaired immune responses [17], the CB₂ receptor may be regarded as novel therapy targets to ameliorate inflammation via which normalisation of fatty acid and glucose metabolism may be achieved. Thus, to examine the role of CB₂ receptor activation on glucose tolerance and insulin sensitivity, a highly selective CB₂ receptor agonist, SER601, was administered in mice with high-fat diet (HFD)/streptozotocin (STZ)-induced diabetes via subcutaneous implantation of osmotic minipump. Effects of CB₂ receptor activation on β -cell function, islet macrophage infiltration and systemic fat deposition were also assessed.

Methods and Materials

Materials

All tissue culture reagents were purchased from Gibco (Beijing, China). SER601 was and the micro-osmotic minipumps were from Tocris (Bristol, UK) and ALZET® (Cupertino, CA, USA), respectively. Collagenase V, DAPI, streptozocin, LPS and Histopaque® were obtained from Sigma-Aldrich (Beijing, China). Antibodies against insulin, glucagon, BrdU and CD68 were obtained from Abcam (Shanghai, China). Alexa 594 anti-guinea pig and anti-rabbit Alexa 488-conjugated secondary antibody were from Invitrogen (Beijing, China). The insulin and glucagon ELISA kit was purchased from Merck Millipore (Shanghai, China) and R&D Systems (Beijing, China), respectively. The TNF- α , INF- γ , IL-4 and IL-10 ELISA kits were from eBioscience (San Diego, CA, USA) and the Annexin V/PI apoptosis kit was purchased from Dojindo (Shanghai, China). All animals were from the Laboratory Animal Centre of the Academy of Military Medical Sciences (Beijing, China). Procedures performed in the present study were in compliance with the regulations of the Tianjin

Table 1. Composition of high-fat diet and standard chow diet

Nutrient information	Standard chow diet (3.29 kcal/g)		High-fat diet (4.78 kcal/g)	
	% g	% kcal from	% g	% kcal from
Protein	20.0	24.4	19.77	16.53
Fat	4.5	12.3	29.18	54.91
Carbohydrate	52.0	63.3	34.15	28.56

Committee of Use and Care of Laboratory Animals and the overall project protocol was approved by the Animal Ethics Committee of the Chinese Academy of Medical Science.

LPS-induced islet cell apoptosis

Islets were isolated by collagenase digestion and purified via Histopaque® gradient according to protocols detailed previously [6]. Groups of 200 islets were then handpicked and cultured under standard tissue culture condition in LPS (1 µg/mL)-supplemented RPMI 1640 media in the presence or absence of SER601 (5 nmol/L) for 3 or 7 days. Cells were then dissociated using cell dissociation solution for 12 minutes at 37°C, labelled using an Annexin V/PI apoptosis kit according to the manufacturer's instruction and analysed by flow cytometry (BD Accuri C6, Beijing, China).

Animals

Male ICR mice (4-week-old) were put on a high-fat diet (HFD, Table 1) for 6 weeks followed by repeated intraperitoneal (i.p.) injection of low dose streptozotocin (STZ, 40 mg/kg body weight, every other day, 3 times in total) to induce T2D as previously reported [26]. Mice were then treated with SER601 (20 ng/kg body weight, equivalent to approximately 1 nmol/L per mouse with an average body weight of 35 g), a CB₂R agonist, via subcutaneous implantation of osmotic minipumps which release the agent in a continuous manner for a total of 2- or 4 weeks. Concentration of the SER601 treatment was selected according to an earlier study demonstrating that the binding affinity of SER601 towards the CB₂ but not CB₁ receptors when level of the compound ranges between 1-5.3 nmol/L [27]. Body weights were measured weekly. Animals were sacrificed at the end of treatment and serum insulin and glucagon levels of both SER601-treated and the control groups and age-matched non-diabetic animals were quantified by ELISA. For β-cell proliferation assessment, mice were treated with BrdU incorporated drinking water (1 mg/mL, freshly prepared daily) for 7 consecutive days during week 4.

Intraperitoneal glucose and insulin tolerance tests

Glucose and insulin tolerance tests were performed at week 2 (*n*=6) and 4 (*n*=6) during SER601 treatment using 8-hour fasted mice following single intraperitoneal (i.p.) injection of glucose (2 g/kg body weight) or insulin (0.75 U/kg body weight). Tail vein plasma glucose levels were measured with a glucose meter at time 0, 14, 30, 45, 60, 75, 90, 105 and 120 minutes. To assess the potential effect of SER601 on *in vivo* insulin secretion, i.p. glucose challenge was performed in the presence and absence of SER601 (20 ng/kg body weight) using HFD/STZ-induced diabetic mice and age-matched non-diabetic mice. Serum samples were obtained from tail vein blood collected at 0 and 30 min. Plasma glucose levels were measured with a glucose meter and serum insulin levels were quantified by ELISA.

Immunofluorescence and red oil O staining

At the end of 4-week treatment, mouse pancreases were dissected and fixed in 4% paraformaldehyde for paraffin embedding. Sections (5 µm thickness) were cut onto microscopic slides and co-immunofluorescence staining was performed using antibodies against insulin (1:100) and glucagon (1:100) or antibodies against insulin (1:100) and CD68 (1:80) or antibodies against insulin (1:100) and BrdU (1:100) as previously reported [6, 28]. Briefly, the sections were dewaxed, rehydrated and permeabilised with 0.1% (vol./vol.) Triton x-100/0.25% BSA in PBS and incubated overnight at 4°C with primary antibodies. For staining of BrdU⁺ cells, the sections were pre-treated as detailed before followed by primary antibody incubation [29, 30]. Alexa Fluor 594 and 488-conjugated secondary antibodies (1:200) were then added to the designated

sections and incubated overnight. Excessive secondary antibodies were washed off and the sections were stained with DAPI before visualisation under a laser con-focal microscope (Leica TCS SP8, Germany). Mouse skeletal muscles were also obtained and OCT embedded for red oil O staining, which was examined under a light microscope (Leica, Germany).

Measuring islet insulin secretory capacity and insulin content

Islets were isolated from SER601 and control animals as previously detailed [6]. Groups of 30 isolated islets were exposed to 2 or 20 mmol/L glucose-supplemented physiological salt solution [5]. Insulin levels of the supernatant were measured by an insulin ELISA kit according to manufacturer's instruction. The islet pellets were then sonicated (10 μ , 10-20 s) for quantification of insulin content.

Results

Administration of SER601 ameliorated insulin resistance in HFD/STZ-induced diabetic mice

As demonstrated in Figure 1 a&c, results from IPGTT tests showed no change on glucose tolerance in mice that have been treated with 2- or 4-week SER601 (closed circles) compared to controls (open circles). However, both 2- and 4-week exposure to SER601 resulted in significant improvement of insulin sensitivity (area under curve $52 \pm 13\%$ and $62 \pm 20\%$ respectively, $P < 0.05$ vs control; Fig. 1b&d), implicating a beneficial impact of CB₂ receptor activation on overall insulin sensitivity in diabetic mice.

Administration of SER601 improves β -cell function but could not prevent islet disruption

Islets extracted from the SER601-treated animals also exhibited better insulin secretory capacity in response to 20 mmol/L glucose (0.82 ± 0.06 vs 0.28 ± 0.01 ng per islet per hour, $142 \pm 29\%$ over control, $P < 0.05$; Fig. 2a), accompanied by increased insulin content (27.15 ± 0.77 vs 21.49 ± 0.76 ng per islet, $126 \pm 14\%$ over control, $P < 0.05$; Fig. 2b), indicating a direct effect of SER601 on β -cell function. Consistent with a stimulatory effect of the SER601 on *in vivo* β -cell

Fig. 1. Effect of SER601 administration on diabetic mice. (a&c) Average plasma glucose concentrations after an i.p. glucose challenge in mice after 2-week (a) and 4-week (c) treatment with SER601 (closed circles). (b&d) Average plasma glucose concentrations after an i.p. insulin challenge in mice after 2-week (b) and 4-week (d) treatment with SER601. Untreated control mice were labelled as open circles. Data are shown as means $\pm$ standard errors, $n=6$, * $P < 0.05$, ** $P < 0.01$ compared to controls.

Fig. 2. SER601 administration stimulates glucose-induced insulin secretion and insulin content. (a) Levels of glucose-stimulated insulin secreted from islets obtained from SER601-treated and control animals. (b) Insulin contents of islets obtained from SER601-treated and control animals. Data are shown as means $\pm$ standard errors, $n=6$.

Fig. 3. SER601 administration ameliorates islet disruption. (a) Representative images of double immunofluorescence staining of pancreases obtained from SER601-treated and control animals. Insulin and glucagon-positive cells are shown in green and red, respectively. Nuclei were stained with DAPI and shown in blue. Scale bar=50 μ m. (b) Representative image of double immunofluorescence staining of pancreas obtained from non-diabetic mice. Insulin and glucagon-positive cells are shown in red and green, respectively. Scale bar=50 μ m. (c) Average islet area within pancreatic sections of SER601-treated, control and non-diabetic mice. Serum insulin (d) and glucagon (e) levels of SER601-treated, control and age-matched non-diabetic mice. Data are shown as means $\pm$ standard errors, $n=6$.

function, fasting serum insulin levels of the SER601-treated mice were significantly higher than controls (Fig. 3d). More importantly, normalisation of fasting serum glucagon concentration was also observed from SER601-treated diabetic animals after 4-week exposure of SER601 (Fig. 3e). Given that imbalance of glucagon secretion is also characteristic of patients with T2D [31], this result may be implicative of a beneficial impact of CB₂ receptor activation on islet hormone regulation during hyperglycaemia. In addition, it is also worth noting that we did not detect any

Fig. 4. Acute SER601 administration does not affect *in vivo* insulin secretion nor β -cell proliferation, but SER601 exposure exerts moderate effect on LPS-induced islet cell death. (a-d) Potential impact of SER601 on *in vivo* insulin secretion was assessed in HFD/STZ-induced diabetic mice (a-b) or age-matched non-diabetic mice (c-d). Serum insulin levels during an i.p. glucose challenge in HFD/STZ-induced diabetic mice (a) or non-diabetic mice (c). Blood glucose levels during an i.p. glucose challenge in HFD/STZ-induced diabetic mice (b) or non-diabetic mice (d). Open circles: non-treated controls; Closed circles: SER601-treated animals. Data are shown as means $\pm$ standard errors, $n=7-10$. (e) Immunostaining images of pancreases obtained from SER601-treated and control mice. Positive BrdU signals are shown as green. Insulin was co-stained red. Nuclei were visualised by DAPI staining and shown in blue. Upper panel scale bar=50 μ m. Lower panels: higher magnitude, scale bar=20 μ m. Data are representative of 6 animals, $n=6$. (f-g) Islets were exposed to LPS (1 μ g/mL) for 3 days (a) and 7 days (b) in the presence (black column) or absence (white column) of SER601. Islet cell apoptosis was quantified by flow cytometry after Annexin V/PI double labelling. Significant reduction in Annexin only positive cells could be observed from the LPS+SER601 group. Data are representative of 3 independent experiments and presented as means $\pm$ standard errors $n=6$.

direct impact of acute administration of SER601 on *in vivo* insulin secretion in HFD/STZ-induced diabetic mice (Fig. 4a&b) or age-matched non-diabetic mice (Fig. 4c&d), which may partly explain the lack of effect on overall blood glucose levels of chronic SER601 treatment in diabetic mice despite increased insulin content and *in vitro* glucose-stimulated insulin secretion (Fig. 2 a&b).

Larger average islet area was also observed in SER601-treated mouse pancreases as shown by double immunofluorescence staining for insulin and glucagon (151 $\pm$ 61% over control, $P<0.05$; Fig. 3a left panels & Fig. 3b). However, SER601 exposure did not appear to prevent islet disruption completely. It can be observed from Figure 3a, islets from the SER601-treated animals have lost the typical islet architecture which is generally composed of a β -cell core surrounded by α - and other islet cells. Instead, the α - and β -cells were mixed up, particularly in islets of the control animals, indicating an ameliorative role of CB₂ receptor activation in islet disintegration during diabetes progression. Furthermore, immunofluorescence staining for BrdU incorporation revealed virtually no BrdU⁺ cells within the endocrine islets for both SER601-treated and control animals (Fig. 4e). Parallel *in vitro* analysis showed that SER601 exposure resulted in a slight inhibition of LPS-induced islet cell apoptosis, shown as significant reduction of Annexin only positive cells in the SER601-exposed group (Fig. 4 f&g), suggesting that the positive role of

Fig. 5. SER601 administration showed no effect islet macrophage infiltration. (a) Representative images of double immunofluorescence staining of pancreases obtained from SER601-treated and control animals. CD68 and insulin-positive cells are shown in green and red, respectively. Nuclei were stained with DAPI and shown in blue. Scale bar=50 μm. (b) Average CD68 expression was quantified by measuring fluorescence intensity of FITC. Data are shown as means ± standard errors, $n=6$.

Fig. 6. SER601 administration showed a lipolytic effect but did not change fat deposition in the skeletal muscles. (a) Representative images of red oil O staining of skeletal muscles obtained from SER601-treated, control and non-diabetic overweight mice. Nuclei were stained with haematoxylin. Scale bar=50 μm (upper panels) & 100 μm (lower panels). (b) Average body weight of mice during 2-week SER601 treatment. (c) Average body weight of mice during 4-week SER601 treatment. Untreated control mice were labelled as open circles and SER-treated mice were labelled as closed circles. Data are shown as means ± standard errors. $n=6$, $*P<0.05$ compared to controls. (d) Representative images of SER601-treated and control mice following dissection. Red circle indicates epididymal adipose mass in the control animal. Data are representative of 6 animals, $n=6$.

SER601 on maintaining islet structure was independent from SER601-induced endogenous islet cell proliferation, but more likely due to SER601-inhibited islet cell apoptosis.

SER601 administration did not ameliorate islet macrophage infiltration

A modest yet statistically insignificant reduction of islet CD68 macrophage infiltration could be detected after 4-week exposure of SER601 (Fig. 5). Thus, substantial amount of CD68⁺ cells could be seen within the endocrine islets from both SER601-treated and control animals. Expression of CD68 was moderately elevated in the control group, quantified by fluorescence intensity of FITC, however no statistical significance was achieved (Fig. 5b). In addition, diffused nuclei were clearly detectable in islets from the controls, indicating *bona fide* islet cell death.

Effect of SER601 administration on skeletal muscle fat deposition

To examine fat deposition in metabolic active organs, red oil O staining was employed. As shown in Figure 6a, no difference in skeletal muscle fat deposition between SER601-treated and control mice (Fig. 6a). Furthermore, when compared to obese but non-diabetic mice (average body weight <45g, average fasting blood glucose <6.5 mmol/L), both control and SER601 group exhibited less fat deposition within the skeletal muscles. Intriguingly though, virtually no epididymal adipose mass could be seen from the SER601-treated animals during dissection (Fig. 6d). Average body weight of the SER601-treated animals was also markedly lower than the control animals at the end of 4-week treatment (85±16% over control, *P*<0.05; Fig. 6c), which could be partly due to the lack of epididymal adipose tissue in SER601-treated mice.

Discussion

The association of obesity and diabetes has been firmly established by previous studies demonstrating increased risk of developing diabetes in non-diabetic obese individuals [32]. Intentional weight loss through introducing healthier dietary options and regular physical exercises has been recommended to prevent or delay the onset of obesity-related T2D and clinical measures for weight reduction were also investigated. Current anti-diabetic options mainly include insulin replacement therapy, insulin secretagogues that stimulate endogenous insulin secretion and insulin sensitizers, but clinical use of most anti-diabetics such as insulin, sulphonylurea and thiazolidinediones is accompanied by excessive weight gain and increased fat deposition [33]. We demonstrate here that administration of a CB₂ receptor agonist, SER601, could significantly improve insulin sensitivity in HFD/STZ-induced diabetic mice.

Thus, SER601 was administrated by subcutaneous implantation of osmotic minipump that releases the CB₂ receptor agonist in a continuous manner and the HFD/STZ-induced diabetic mice were exposed to SER601 for 2 or 4 weeks. Substantial increase of systemic insulin sensitivity was recorded from the SER601-treated mice after 2- or 4-week exposure, which could be attributable to several factors.

Increased islet area, insulin content and more robust insulin secretory capacity in response to glucose were observed from islets obtained from the SER601-treated mice, implicating a beneficial impact of CB₂ receptor activation on β -cell function, which is consistent with multiple previous studies reporting stimulatory impact of CB₂ receptor activation on *in vitro* insulin secretion [1, 5-8]. In addition, substantial elevation of fasting serum insulin level was also recorded from the SER601 animals, accompanied by normalised serum glucagon concentration, supporting a role of the CB₂ receptor activation on maintaining islet hormone secretion. However, no direct impact on *in vivo* insulin secretion was demonstrated following acute administration of SER601, which could be due to the complexity and precision of the ways in which plasma insulin and glucose levels are regulated. This may also explain the lack of significant effect of SER601 on glucose tolerance after 4 weeks SER601 treatment. However, considering the beneficial actions of the CB₂ receptor agonist on maintaining islet integrity and β -cell function, a prolonged course of SER601 exposure may possibly lead to improved fasting plasma glucose level and glucose responsiveness of HFD/STZ-induced diabetic mice.

The CB₂ receptor was first identified in the immune cells and an immune regulatory role of the receptor was initially proposed [18]. Subsequent studies have since confirmed CB₂ receptor-dependent immune modulatory actions. Indeed, it has been shown that CB₂ receptor activation ameliorated cerebral ischaemia by inhibiting neutrophil recruitment [24]. CB₂ receptor knockout mice were more prone to acute rejection of during allogeneic heart transplantation by hampering pro-inflammatory cytokine production [25]. In contrast, other studies demonstrated that exposure to 2-arachidonylglycerol, an endogenous endocannabinoid, and certain CB₂ receptor agonists, JWH015 and JWH133, induced migration of B lymphocytes [34], natural killer cells [35], eosinophils [36] and monocytes [37]. Given that diabetes is also now considered an inflammatory disorder, we also assessed the potential impact of SER601 administration on local and systemic inflammation of diabetic mice. Somewhat surprisingly, SER601 administration did not result in substantial changes on islet macrophage infiltration. Moreover, despite a significant reduction of serum INF- γ level observed from SER601-treated mice ($3.24 \pm 17.86\%$ over untreated control, $p < 0.01$), no differences could be detected regarding the levels of pro-inflammatory cytokine TNF- α and anti-inflammatory cytokines IL-4 and IL-10 in the sera. Similarly no differences of the all 4 tested cytokine concentrations were recorded in the pancreatic lysates of SER601-treated and control animals (data not shown). However, a recent publication reported that the CB₂ receptors are not involved in mediating macrophage migration in response to pharmacological agents that are traditionally known as CB₂ receptor agonists [38], which may offer a partial explanation to the lack of effect of SER601, a highly selective for the CB₂ receptors, on islet macrophage infiltration.

We subsequently examined potential difference of fat deposition between SER601-treated and control mice since a significant decrease of average body weight was recorded from the SER601-treated animals towards the end of 4-week SER601 treatment. Despite no difference in skeletal muscle fat deposition as revealed by red oil O staining, virtually no epididymal adipose mass could be detected from the SER601-treated mice, implicating a lipolytic property of the CB₂ receptor agonist. Thus, given the lipolytic property of SER601 and its beneficial role on β -cell function, CB₂ receptor could be considered a promising target for therapeutic development against diabetes, particularly obesity-related diabetes.

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Disclosure Statement

No conflicts of interest are declared by the authors.

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