Biochemical and Biophysical Research Communications 444 (2014) 451–454

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A role for trans-caryophyllene in the moderation of insulin secretion

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ARTICLE INFO

Article history:
Received 16 November 2013
Available online 31 January 2014

Keywords:
Type 2 diabetes
Trans-caryophyllene
Glucose-stimulated insulin secretion
Type 2 cannabinoid receptor
Arf6

ABSTRACT

Glucose-stimulated insulin secretion (GSIS) is essential for the control of metabolic fuel homeostasis and its impairment is a key element in the failure of β-cells in type 2 diabetes. Trans-caryophyllene (TC), an important constituent of the essential oil of several species of plants, has been reported to activate the type 2 cannabinoid receptor (CB2R). The effects of TC on GSIS are still unknown. Our results demonstrate that administration of TC in MIN6 cells promotes GSIS in a dose dependent manner. However, inhibition of CB2R by a specific inhibitor or specific RNA interference abolished the effects of TC on GSIS, which suggests that the effects of TC on GSIS are dependent on activation of CB2R. Further study demonstrated that treatment with TC leads to the activation of small G protein Arf6 as well as Rac1 and Cdc42. Importantly, Arf6 silencing abolished the effects of TC on GSIS, which suggests that Arf6 participates in mediating the effects of TC on GSIS. We conclude from these data that TC has a novel role in regulating GSIS in pancreatic β-cells.

1. Introduction

Type 2 diabetes has been considered one of the most prevalent chronic diseases in the world. Insulin resistance is essential for the pathogenesis of this disease. Glucose-stimulated insulin secretion (GSIS) plays a critical role in the control of metabolic fuel homeostasis and its impairment is a key element involved in β-cell failure in type 2 diabetes [1]. Coordinated signaling events participate in regulating trafficking of insulin-laden secretory granules to plasma membranes for docking and fusion. Multiple lines of evidence have demonstrated that small G-proteins (e.g., Arf6, Cdc42, and Rac1) play a critical role in controlling such signaling events [2]. Arf6 is an important member of the Arf family which has been well-documented as a protein that plays positive modulatory roles in multiple cell types. Importantly, Arf6 has been verified to be expressed in islets and insulin-secreting RINm5f cells [3]. Up to now, a growing body of evidence has demonstrated that Arf6-regulated insulin secretion is mediated by glucose, GTPγS, and membrane depolarization. Importantly, Arf6 is considered to be the upstream signaling factor of Rac1 and CDC42 involved in the process of GSIS regulation [4]. However, the precise molecular and cellular mechanisms underlying GSIS still need to be elucidated.

Trans-caryophyllene (TC), an important constituent of the essential oils derived from several species of plants, has been reported to possess many pharmacological properties. Previous studies have demonstrated its antimicrobial [5] and analgesic effects [6]. In addition, it also possesses an anti-inflammatory effect [7]. Importantly, TC has been shown to be a specific agonist of the type 2 cannabinoid receptor (CB2R). Through activation of CB2R, TC is able to promote fatty acid oxidation in mouse myoblast cell line C2C12 [8] and reduce cerebral ischemic injury in rodents [9]. It is reportedly accepted that CB2R are present in pancreatic islets. However, the effects of TC in GSIS are still unknown. In this study, we investigated the effects of TC on GSIS and explored its underlying mechanisms.

2. Materials and methods

2.1. Cell culture

MIN6 β-cells (passage 22–30) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Determination of insulin secretion

In order to determine insulin secretion, MIN6 cells were preincubated in Krebs–Ringer HEPES buffer (KRHB) containing 0.1% BSA

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http://dx.doi.org/10.1016/j.bbrc.2013.11.136
0006-291X/

and 2 mmol/l glucose for 1 h at 37 °C. After that, cells were incubated for 1 h at 37 °C with KRHB containing either 2 or 20 mmol/l glucose. An aliquot of the buffer was taken and insulin release was measured by RIA.

2.3. Cellular transfection and knockdown

CB1R, CB2R, and Arf6 protein expression was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions [10]. Briefly, 24 h before transfection, cells (5×10^5) were plated in 0.5 ml cultural medium and allowed to grow overnight at 37 °C and 5% CO_2. The small interfering RNA (siRNA) transfection complex, formed by combining transfection reagent and 50 nM CB1R, CB2R, or Arf6 siRNA in serum-free Opti-MEM medium, was added to cells dropwise. The successful knockdown of CB2R or Arf6 was verified using Western blot analysis.

2.4. Quantitation of Arf6-GTP, Rac1-GTP and Cdc42-GTP in MIN6 cells

Active Arf6, Rac1, and Cdc42 were quantitated by pull-down assay. Recombinant protein PAK1-PBD-GST, which binds specifically to the GTP-bound forms of Rac and Cdc42, was prepared as described previously [11]. After having been washed with ice-cold PBS, cells were lysed with lysis buffer and lysate was then clarified by centrifugation at 16,000*g* at 4 °C for 15 min. For active Arf6, 400 μg protein was incubated with 100 μl of glutathione resin and 100 μg of GST-GGA3-PBD beads at 4 °C for 1 h with gentle rocking. For active Rac1 and Cdc42, 400 μg protein was incubated with PAK1-PBD-GST. The resulting proteins were separated by SDS–PAGE and activated Arf6, Rac1, and Cdc42 were identified using Western blot analysis.

2.5. Western blot analysis

Samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to polyvinylidene fluoride microporous membranes (Millipore, USA) as described previously [12]. Membranes were then blocked with 5% non-fat milk in TBST (150 mM NaCl, 25 mM Tris–HCl (pH 8.0), and 0.1% Tween 20) for 1 h at room temperature followed by incubation with Arf6, Rac1, and Cdc42 primary antibodies for 2 h at room temperature. After being washed 3 times, membranes were incubated with either HRP-conjugated secondary anti-mouse antibody or HRP-conjugated secondary anti-rabbit antibody for 1 h at room temperature. Blots were developed using the ECL technique (Santa CruzBiotechnology, USA) [13].

2.6. Statistical analysis

Data are represented as mean ± S.E.M. One way analysis of variance (ANOVA) was used to determine areas of statistical significance between different groups. A p-value < 0.05 was considered as the minimum level of statistical significance.

3. Results

One of the objectives of this study was to investigate the contributory roles of TC in GSIS. To address this, MIN6 cells were treated with TC at concentrations ranging from 0.1 to 1 μM. Insulin secretion was quantitated in these cells in the presence of 2.0 or 20 mM glucose. The molecular structure of TC is shown in Fig. 1A. Data in Fig. 1B shows that TC promoted GSIS in a dose dependent manner.

In previous studies, TC has been shown to be an important agonist of CB2R [9]. We next investigated whether the effects of TC on GSIS are dependent on CB2R. MIN6 cells were treated with either 1 μM CB2R inhibitor SR144528 or 1 μM CB1 inhibitor AM251. Results indicate that 1 μM CB2R inhibitor SR144528 abolished the effects of TC on GSIS. However, 1 μM CB1R inhibitor AM251 did not produce this effect (Fig. 2A). In order to further confirm this finding, expression of CB1R and CB2R was knocked down by being transiently transfected with CB1R and CB2R siRNA, respectively. Results indicating that the two proteins were successfully silenced are shown in Fig. 2B. Consistently, knockdown of CB2R but not CB1R abolished the effects of TC on GSIS (Fig. 2C). These findings suggest that the effects of TC on GSIS are dependent on CB2R.

Activation of CB2R has been reported to stimulate activation of small G proteins, including RhoA, Rac1, Rac2, and Cdc42 [14], activation of which is involved in GSIS. Recent studies identified activation of Arf6 as an upstream signaling event that leads to activation of Cdc42 and Rac1 [4]. Therefore, we investigated the possibility of TC playing a role in the activation of small G-proteins, specifically Arf6, in MIN6 cells. To address this, glucose-induced Arf6 activation was quantitated in cells that were treated with TC. The results listed in Fig. 3 indicate that administration of TC markedly promoted the ability of glucose to induce Arf6 activation, which was abolished by pretreatment with CB2R silence.

In order to ensure that the effects of TC on GSIS are indeed mediated by Arf6, cells were transfected with Arf6 siRNA. Successful knockdown of Arf6 is shown in Fig. 4A. Importantly, GSIS was significantly inhibited in Arf6 knockdown cells (Fig. 4B). In addition, TC treatment also promoted Cdc42 activity as well as Rac1 activity, which was abolished by knockdown of Arf6 (Fig. 4C and D). These data suggest that the effects of TC on GSIS are mediated by Arf6.

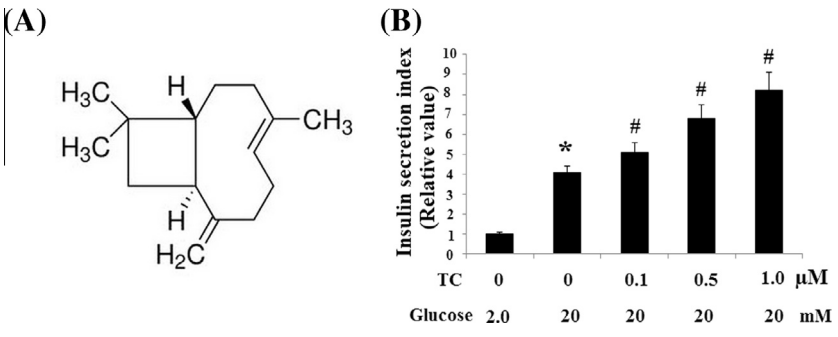

Fig. 1. Impact of Trans-caryophyllene (TC) on insulin secretion in MIN6 cells. (A) Molecular structure of Trans-caryophyllene (TC); (B) MIN6 cells were pretreated with TC at a variety of concentrations for 24 h. Cells were challenged with either 2.0 or 20 mM glucose. Insulin secretion determination displayed that TC promoted glucose-stimulated insulin secretion (GSIS) in a dose-dependent manner (*p < 0.01 vs. 2 mM Glucose treated group; #p < 0.01 vs. 20 mM Glucose only treated group).

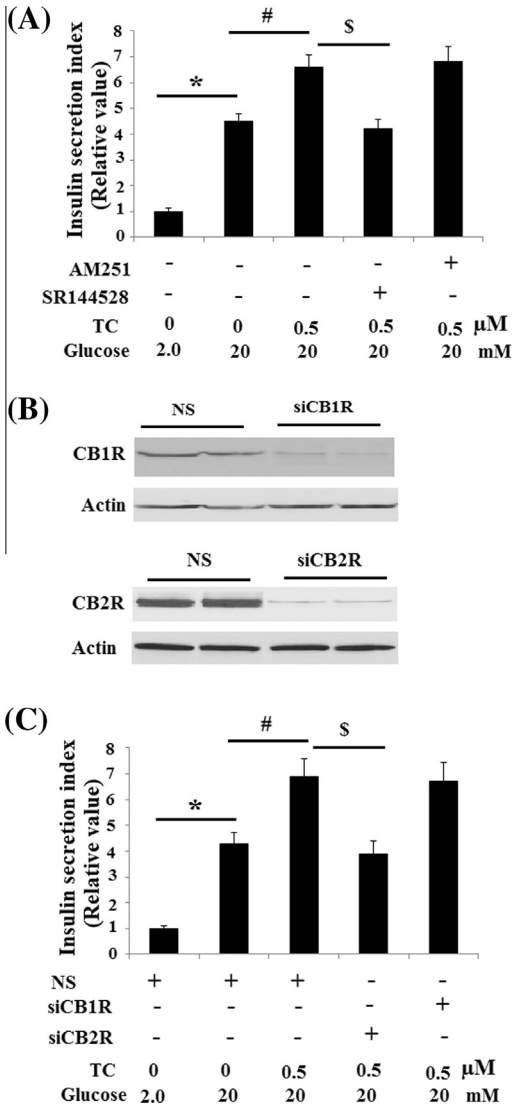

Fig. 2. Effects of TC on glucose-stimulated insulin secretion (GSIS) is dependent on Cannabinoid receptor 2 (CB2R). (A) MIN6 cells were pretreated with DMSO, 1 μM CB2R inhibitor SR144528, or 1 μM CB1R inhibitor AM251. Pretreatment with 1 μM CB2R inhibitor SR144528 significantly inhibited the effects of TC on GSIS ($^{*,\#,\$}p < 0.01$); (B) MIN6 cells were transfected with non-specific RNA (NS), CB1R siRNA (siCB1R), and CB2R siRNA (siCB2R). Western blot analysis revealed successful knockdown of CB1R and CB2R; (C) Knockdown of CB2R abolished the effects of TC on GSIS ($^{*,\#,\$}p < 0.01$).

4. Discussion

Loss of glucose sensing in pancreatic β-cells is a key hallmark of T2DM. The current study investigated the role of TC in regulating glucose responsiveness in islet MIN6 cells. Our results demonstrate that TC promotes GSIS in a dose dependent manner. In addition, the effects of TC on GSIS are dependent on activation of CB2R. Inhibition of CB2R by either specific inhibitors or RNA interference abolished the effects of TC on GSIS. More importantly, Arf6 was proven to participate in mediating the effects of TC on GSIS. We conclude from these data that TC plays a novel role in regulating GSIS in pancreatic β-cells.

As a natural product extracted from several species of plants, caryophyllene has been reported to possess many pharmacological effects. Importantly, β-caryophyllene selectively binds to CB2R and acts as a full agonist [15]. Upon binding to the CB2R, β-caryophyllene inhibits adenylate cylcase, leads to intracellular calcium transients and weakly activates the mitogen-activated kinases Erk1/2 and p38 in primary human monocytes. In addition, β-caryophyllene inhibits lipopolysaccharide (LPS)-induced proinflammatory cytokine expression in peripheral blood and attenuates LPS-stimulated Erk1/2 and JNK1/2 phosphorylation in monocytes [16]. It is well-established that cannabinoid receptors play an important role in regulating energy balance. Both of the two cannabinoid receptors, CB1R and CB2R, have been shown to be present in pancreatic islets [17]. However, the potential role of such receptors in the cross-talk between adipose tissue (obesity) and A-cell function in normal and pathological conditions is not completely clear as of yet. Importantly, endogenous cannabinoid AEA, an agonist of the two cannabinoid receptors, enhanced GSIS in normal rat islets [18]. However, our data display that the effects of TC on GSIS are dependent on CB2R but not on CB1R. CB2R has been reported to be localized in α- and β-cells [19,20], including MIN6 cells [21]. CB2R, which unlike CB1R does not induce central side effects, has been shown to be a promising therapeutic target [15]. Activation of small G-proteins has been considered an essential event in GSIS [22]. Meanwhile, it is well-documented that CB2R agonists lead to activation of small G-proteins, including RAC1 and Cdc42 [23]. In this study, we found that Arf6 is involved in the effects of TC on GSIS promotion. Effects of TC on Arf6 activation are mediated by CB2R. Notably, inhibition of CB2R abolished the effects of TC on Arf6 activation as well as GSIS. Arf6 is considered to be the upstream signal of Rac1 and Cdc42 due to findings demonstrating that GSIS involves sequential activation of Arf6, Cdc42, and Rac1 [4]. Although there is compelling evidence showing that Arf6 is involved in numerous functions that take place in many cell types, little is known regarding regulatory factors for Arf6 in β-cells.

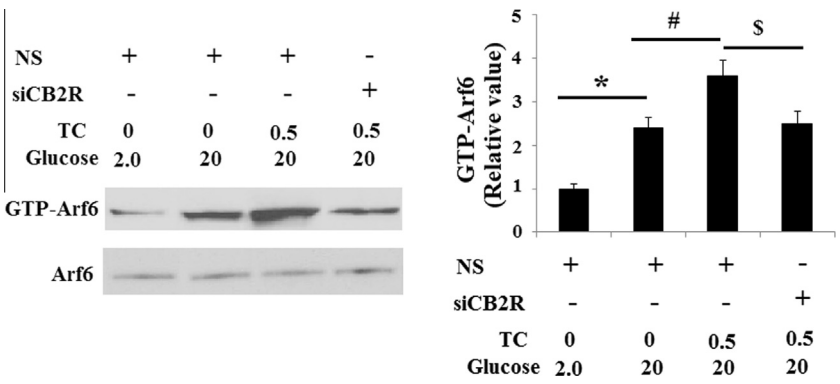

Fig. 3. Changes in Arf6 activity levels in TC treated MIN6 cells. The GTP-bound forms of Arf6, were isolated from MIN6 cells by pull-down assay and analyzed by Western blot analysis. The figure shows the level of GTP-Arf6 is increased by treatment with TC and high concentration Glucose, the effect of which is abolished by knockdown of CB2R ($^{*,\#,\$}p < 0.01$).

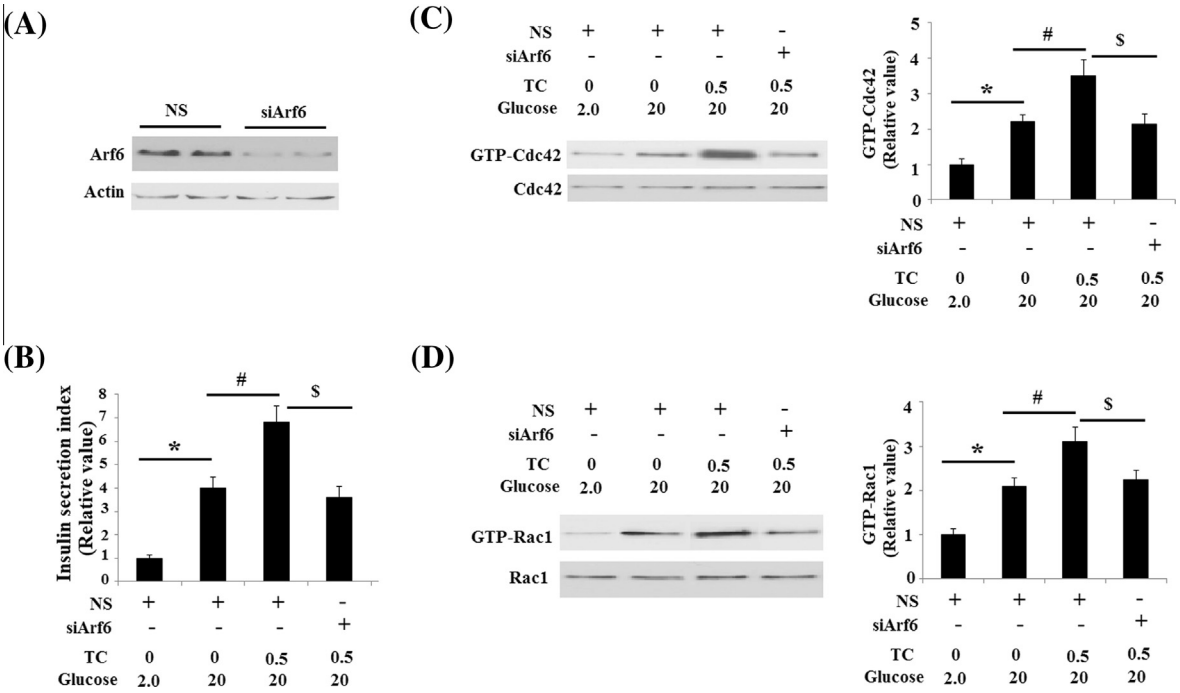

Fig. 4. Inhibition of expression of Arf6 abolished the effects of TC in glucose-stimulated insulin secretion (GSIS). (A) MIN6 cells were transfected with non-specific RNA (NS) and Arf6 siRNA (siArf6). Western blot analysis revealed successful knockdown of Arf6; (B) Arf6 silencing abolished the effects of TC on GSIS ($^{*,\#,\$}p < 0.01$). (C) Pull down and Western blot analysis revealed that Arf6 silencing abolished the effects of TC on Cdc42 activity ($^{*,\#,\$}p < 0.01$). (D) Pull down and Western blot analysis revealed that Arf6 silencing abolished the effects of TC on Rac1 activity ($^{*,\#,\$}p < 0.01$).

The results of this study have helped to expand our understanding of Arf6 in GSIS.

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