Canavanine activates imidazoline I-2 receptors to reduce hyperglycemia in type 1-like diabetic rats

Chin-Hong Chang a, b, 1, Pin-Chun Chao c, 1, Ho-Shan Niu d, Gin-Chi Huang e, Li-Jen Chen d, Juei-Tang Cheng b, e, f, *

a Department of Neurosurgery, Chi-Mei Medical Center, Yong Kang, Tainan City 71003, Taiwan
b Department of Medical Research, Chi-Mei Medical Center, Yong Kang, Tainan City 71003, Taiwan
c Bachelor Program of Senior Services, College of Humanities and Social Sciences, Southern Taiwan University of Science and Technology, Yong Kang, Tainan City 71005, Taiwan
d Department of Nursing, Tzu Chi College of Technology, Hualien City 97005, Taiwan
e Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan City 70101, Taiwan
f Institute of Medical Sciences, Chang Jung Christian University, Guei-Ren, Tainan City 70101, Taiwan

ABSTRACT

Canavanine is a guanidinium derivative that has the basic structure of a ligand for the imidazoline receptor (I-R). Furthermore, canavanine is found in an herb that has been shown to improve diabetic disorders. Thus, the present study was designed to investigate the anti-hyperglycemic action of canavanine in rats with streptozotocin (STZ)-induced type 1-like diabetes. Canavanine decreased hyperglycemia in the STZ-induced diabetic rats, and this action was blocked by the antagonist specific to imidazoline I-2 receptors (I-2R), BU224, in a dose-dependent manner. Additionally, canavanine increased the plasma β-endorphin level, as measured using enzyme-linked immunosorbent assay (ELISA), and this increase was also blocked by BU224 in the same manner. Moreover, amiloride at a dose sufficient to block I-2AR attenuated the actions of canavanine, including the increased β-endorphin level and the anti-hyperglycemic effect. Otherwise, canavanine increased the radioactive glucose uptake into skeletal muscles isolated from the diabetic rats. Furthermore, canavanine increased the phosphorylation of AMPK measured using Western blot analysis in these isolated skeletal muscles in a dose-dependent manner. Additionally, the insulin sensitivity of the diabetic rats was markedly increased by canavanine, and this action was also blocked by BU224. Overall, canavanine is capable of activating imidazoline I-2R; I-2AR is linked to an increase in the plasma level of β-endorphin, and I-2BR is related to effects on the glucose uptake by skeletal muscle that reduces hyperglycemia in type 1-like diabetic rats. Therefore, canavanine can be developed as effective agent to treat the diabetic disorders in the future.

© 2015 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Canavanine was originally isolated from the jack bean, Canavalia ensiformis (L.) [1]. Additionally, Sutherlandia frutescens has the ability to reverse the insulin resistance and restore the pre-diabetic state to a normal state [2]. Canavanine is one of the active compounds in S. frutescens [3]. However, the effect of canavanine on blood glucose homeostasis is still unclear.

Imidazoline receptors, also known as imidazoline/guanidinium receptive sites, were shown to affect metabolism [4]. After the characterization of agmatine as an endogenous ligand of this receptor, three subtypes of imidazoline receptors were proposed; activation of I-1 receptors regulates the blood pressure [5], whereas I-3 receptors participate in insulin release [6], and activation of I-2 receptors (I-2R) increases the glucose uptake into muscle cells [7,8]. Pharmacological evidence supported the subdivision of I-2R into two subtypes, I-2AR and I-2BR, depending on the sensitivity to amiloride [9]. Furthermore, I-2AR regulates the secretion of β-
Subsequently, the muscle strips were further incubated with 1 ml 2-[14C]-deoxy-D-glucose (2-DG) (PerkinElmer Life Sciences, uptake was expressed as pmol/mg protein over 5 min, while the glucose uptake in skeletal muscles [10]. Moreover, compounds with guanidine-like structures may bind to I-2R [4] in a manner similar to the action of metformin [11]. Canavanine can activate I-3R to increase the insulin secretion both in vivo and in vitro [12]. Because canavanine is also a guanidinium derivative, the possible effect of canavanine on I-2R is particularly interesting.

In the present study, we evaluated the antihyperglycemic action of canavanine and demonstrated that this action is mainly related to activation of I-2R in streptozotocin-induced type 1 -like diabetes rats (STZ-diabetic rats).

2. Materials and methods

2.1. Animals

Eight-week-old male Wistar rats were purchased from the animal center of National Cheng Kung University Medical College. Diabetes was induced in the rats by an intravenous injection (i.v.) of 65 mg/kg STZ into the Wistar rats. The diabetic state of the animals was confirmed using the blood glucose level (≥20 mM), polyuria and other characteristics of diabetes. The plasma insulin level in the STZ-diabetic rats declined to 2.8 ± 0.48 pmol/l (n = 8) from the increase of 162.4 ± 5.1 pmol/l (n = 8) in normal rats. The rats were maintained in a light-controlled holding facility. All experiments were conducted 2 weeks after STZ administration. All animal procedures were performed according to Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

2.2. Laboratory measurements

Blood samples were collected at the indicated times from the femoral vein of rats that were anesthetized with pentobarbital. The obtained blood samples were centrifuged at 12,000 × g for 3 min. The plasma glucose concentration was measured using a glucose kit by an automatic analyzer (Quik-Lab, Ames; Miles Inc., Elkhart, IN, USA). The plasma β-endorphin levels were determined using a commercial enzyme-linked immunosorbent assay kit (Peninsula Laboratories, Belmont, CA, USA). The difference between the two groups in response to a tested compound was also compared to evaluate the plasma glucose lowering activity (%); the difference was calculated as the percentage decrease of the initial glucose value according to the formula: (Gi - Gt)/Gi × 100%, where Gi is the initial glucose concentration, and Gt is the plasma glucose concentration after treatment with the tested compound.

2.3. Determination of glucose uptake

The glucose uptake assay in skeletal muscle was conducted as described in our previous study [13]. In brief, the soleus skeletal muscle isolated from a diabetic rat was incubated with canavanine or with porcine insulin monocomponent (Novo Industies; Bagsvaerd, Denmark) as a positive control at the indicated concentrations for 30 min at 37 °C with continuous shaking at 40 cycles/min. Subsequently, the muscle strips were further incubated with 1 μCi/ml 2-[14C]-deoxy-D-glucose (2-DG) (PerkinElmer Life Sciences, Boston, MA, USA) for 5 min. Uptake was terminated by the addition of ice-cold phosphate-buffer solution. Radioactivity was determined by lysing the samples in 1 N NaOH, and the neutralized aliquots were used to estimate the radioactivity using a scintillation counter (Beckman LS5000TA, Fullerton, California, USA). Nonspecific uptake was obtained by parallel determinations in the presence of 20 μmol/l cytochalasin B (Sigma–Aldrich). Specific 2-DG uptake was expressed as pmol/mg protein over 5 min, while the protein content was determined using the Bio-Rad protein dye-binding assay (Richmond, CA, USA).

2.4. Western blotting analysis

The muscle specimen were isolated for homogenization using ice-cold lysis buffer containing 300 mM NaCl, 20 mM Tris—HCl (pH 7.8), 2 mM EDTA, 2 mM dithiothreitol, 2% nonidet P-40, 0.2% sodium lauryl sulfate and a cocktail of protease inhibitors (Sigma–Aldrich). The protein extracts were obtained as previously described [14]. Thirty micrograms of the cell lyses was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The blots were then transferred to a polyvinyl difluoride membrane (Millipore, Billerica, MA, USA). After being blocked with 10% skim milk for 1 h, the blots were developed using primary antibody specific for phospho-AMPK and AMPK (Cell Signaling Technology, Beverly, MA, USA). The blots were subsequently hybridized using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Calbiochem, San Diego, CA, USA) and developed using a chemiluminescence kit (PerkinElmer). The optical densities of the bands were determined using Gel-Pro Analyzer software 4.0 (Media Cybernetics, Silver Spring, MD, USA).

2.5. Effect of canavanine on insulin sensitivity

STZ-diabetic rats were used to investigate the response to exogenous insulin. These rats received an i.p. injection of long-acting human insulin at 1 IU/kg once daily to normalize the insulin sensitivity. Then, three days later, the STZ-diabetic rats were divided into three groups. One group received an intravenous injection of canavanine at 2.5 mg/kg, three times daily (t.i.d.), and another group received a similar treatment with the same volume of saline. The 3rd group received an injection of BU224 (1 mg/kg) 30 min before the injection of canavanine in the same manner. Injections of long-acting human insulin at 1 IU/kg were also continued once a day in each group of STZ-diabetic rats. After 5 days of treatment, all rats were challenged with exogenous insulin. Similar to our previous method [15], an intravenous insulin challenge test was performed by administrating short-acting human insulin at 0.1–1.0 IU/kg to STZ-diabetic rats. Blood samples (0.2 ml) from the femoral vein were drawn 30 min following the intravenous insulin challenge test to measure the plasma glucose concentrations.

2.6. Statistical analysis

Data are expressed as the mean ± standard errors mean (SEM). Statistical analysis was performed in Microsoft EXCEL. The statistical differences were evaluated using Student’s t-test, and the difference was considered significant at 2α = 0.05.

3. Results

3.1. Canavanine increases β-endorphin secretion to lower blood glucose in STZ-diabetic rats

In the preliminary experiments, the intravenous injection of canavanine into STZ-diabetic rats reduced the hyperglycemia in a time-dependent manner, and the change of hyperglycemia reached the plateau at 120 min later. Moreover, as show in Fig. 1, canavanine significantly decreased the blood glucose level of the STZ-diabetic rats in a dose-dependent manner parallel to the increase of plasma β-endorphin. At 120 min after injection, canavanine decreased the plasma glucose level and increased the plasma β-endorphin level to reach the maximal effect for the 2.5 mg/kg. Thus,
2.5 mg/kg canavanine was employed in the subsequent experiments of this study. These effects of canavanine on diabetic rats are similar to the actions of metformin, as described in our previous study [16], but the maximal action of canavanine was less than that of metformin.

3.2. Blockade of I-2 receptors by BU224 reverses the action of canavanine in STZ-diabetic rats

The role of I-2 receptors (I-2R) in the action of canavanine was investigated using BU224 at doses sufficient to block I-2R, as described previously [17]. The administration of BU224 to STZ-diabetic rats 30 min prior to the injection of canavanine significantly inhibited the canavanine-induced actions, both the decreased blood glucose and the increased \( \beta \)-endorphin secretion (Fig. 2). BU224 at the dose of 1 mg/kg abolished the increase in \( \beta \)-endorphin induced by canavanine. The plasma glucose-lowering action of canavanine was markedly reduced by BU224 in a dose-dependent manner while the increase of \( \beta \)-endorphin was totally inhibited by BU224 at the maximal dose (1 mg/kg). However, BU224 alone did not modify the basal plasma level of glucose or \( \beta \)-endorphin.

3.3. Characterization of the roles of I-2 receptor subtypes in the actions of canavanine in STZ-diabetic rats

Amiloride, an I-2AR blocker, was used to identify the role of I-2R subtypes in the actions of canavanine. When administered 30 min prior to the injection of canavanine, amiloride at a low dose diminished the increased plasma \( \beta \)-endorphin level induced by canavanine and markedly reduced but did not abolish the blood lowering-action of canavanine (Table 1). The blood glucose-lowering action of canavanine was completely blocked by amiloride at a higher dose (5 mg/kg). However, amiloride alone did not modify the basal plasma level of glucose or \( \beta \)-endorphin.

3.4. Canavanine-induced glucose uptake is abolished by BU224 in isolated soleus muscle

Canavanine induced a marked increase in the glucose uptake into isolated soleus muscle (Fig. 3). The action of canavanine was significantly reduced by 0.1 \( \mu \)M BU224. Furthermore, treatment with a higher concentration of BU224 completely abolished the increased glucose uptake induced by canavanine (Fig. 3). These results indicate that canavanine affects glucose uptake in a manner similar to that of metformin.
3.5. Increase of AMPK phosphorylation by canavanine in isolated skeletal muscle

Our previous report [7] showed a possible link between the activation of imidazoline I-2BR and the phosphorylation of AMPK. Therefore, we measured this signal to further support the effect of canavanine on imidazoline I-2BR. In the present study, canavanine increased the phosphorylation of AMPK in isolated skeletal muscles in a concentration-dependent manner (Fig. 4). With metformin as a positive control, as shown in Fig. 4, the increased phosphorylation of AMPK by 10 μM canavanine was similar to that induced by 2 mM metformin.

3.6. Increase in insulin sensitivity by canavanine in STZ-induced diabetic rats

We performed the insulin challenge test in STZ-induced diabetic rats. The advantage of using STZ-induced diabetic rats for this challenge is the negligible effect of endogenous insulin. The obtained results can thus be used to demonstrate insulin sensitivity. The plasma glucose-lowering activity of the exogenous insulin in the STZ-diabetic rats that received repeated canavanine treatments was significantly higher than that of the control group receiving the same volume of vehicle (Fig. 5). Additionally, the canavanine-induced increase in insulin sensitivity was blocked by BU224 (Fig. 5), showing the involvement of imidazoline I-2 receptors.

4. Discussion

In the present study, we found that canavanine is capable of activating I-2 receptors (I-2R). Activation of both I-2R subtypes by canavanine was associated with a reduction in hyperglycemia in type 1-like diabetic rats; I-2AR mediated an increase in the plasma β-endorphin level, and I-2BR connected AMPK to enhanced glucose utilization.

At first, we observed that an intravenous injection of canavanine in STZ-diabetic rats decreased the blood glucose level in a dose-dependent manner that is similar to the action of allantoin [13]. This effect seems reasonable because canavanine and allantoin are belonged to guanidinium derivatives that can activate I-2R [7].

### Table 1

<table>
<thead>
<tr>
<th>Effect of amiloride on canavanine treated STZ-diabetic rats.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Basal level</td>
</tr>
<tr>
<td>+ Canavanine (2.5 mg/kg)</td>
</tr>
<tr>
<td>+ Amloride</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM (n = 8 per group). *P < 0.05 and **P < 0.01 as compared with basal level.

Additionally, we used an antagonist specific to I-2R, as indicated in a previous report [18]. BU224 at a dose sufficient to block I-2R significantly inhibited the actions of canavanine in STZ-diabetic rats, implying the involvement of I-2R in the antihyperglycemic action of canavanine. Therefore, we characterized the effect of canavanine on I-2R in vivo.

Essentially, I-2R has been divided into two subtypes, I-2AR and I-2BR, depending on the sensitivity to amiloride [9]. I-2AR regulates the secretion of \(eta\)-endorphin from rat adrenal glands [8], and I-2BR stimulates glucose uptake in skeletal muscles [10]. Using the blockade of I-2R by BU224, the involvement of I-2R in the plasma \(eta\)-endorphin level-increasing action of canavanine has been demonstrated. An increase in \(eta\)-endorphin secretion is known to lower plasma glucose levels [13] because the released \(eta\)-endorphin may enhance the glucose uptake into skeletal muscles and inhibit hepatic gluconeogenesis in animals [19–21]. Metformin also improved hyperglycemia via increased plasma \(eta\)-endorphin in human subjects [22]. Additionally, the adrenal gland is responsible for the increase in the plasma \(eta\)-endorphin levels in STZ-diabetic rats [23]. Adrenocortical dysfunction also plays an important role in obesity and metabolic syndrome [24]. Although the presence of I-2R in mitochondria was reported [9], I-2R has also been found in the cell membrane [25,26]. Moreover, I-2R is a trans-membrane G protein-coupled receptor [27]. Therefore, canavanine seems mainly to act through I-2R in the cell membrane, and this view has not been mentioned previously.

As an I-2AR-sensitive antagonist, amiloride shows high affinity for I-2R in the rabbit cortex but low affinity in other tissues or species [27]. Additionally, the high (human placenta) or low affinity (human brain) of amiloride for I-2R has been documented [28,29]. Therefore, low concentrations (under 0.1 M) of amiloride are widely used to block I-2AR, as described previously [29]. In the present study, the canavanine-induced secretion of \(eta\)-endorphin was abolished by pretreatment with a low dose of amiloride 30 min prior to the injection of canavanine in STZ-diabetic rats. Therefore, we suggested that canavanine can activate I-2AR to increase the secretion of \(eta\)-endorphin in STZ-diabetic rats. Moreover, the antihyperglycemic action of canavanine in STZ-diabetic rats was abolished by amiloride only at a dose higher than that used to block I-2AR. This finding suggested the involvement of I-2BR [6] in the antihyperglycemic action of canavanine. This view was further supported by the canavanine-induced glucose uptake in isolated skeletal muscles. Additionally, activation of I-2BR may connect to the increased phosphorylation of AMPK [7]. We also observed that canavanine dose-dependently increased the phosphorylation of AMPK in isolated skeletal muscle, which is known to be a specific site of I-2BR [11]. Therefore, both I-2A and I-2B receptors are involved in the antihyperglycemic action of canavanine. The distribution of I2R in rat peripheral tissues has been demonstrated using positron emission tomography [30]. Therefore, effects of canavanine on these sites shall be investigated in the future.

The effect of canavanine on changes in plasma insulin was not determined in this study because the effect of endogenous insulin is negligible in STZ-induced diabetic rats [16]. However, we used STZ-induced diabetic rats to perform the insulin sensitivity challenge because the plasma glucose lowering response is directly caused by the effect of exogenous insulin in these rats. We found that canavanine enhances the hypoglycemia induced by exogenous insulin in STZ-induced diabetic rats. An increase in insulin sensitivity by canavanine can thus be considered. Additionally, this action of canavanine was also blocked by BU224 indicating the involvement of I-2 receptors. However, the detailed integrations of the cellular signals leading to this result require clarification in the future.

Taken together, our data provide the first demonstration of the potential mechanism(s) for the antihyperglycemic action of canavanine in rats with type 1-like diabetes. However, diabetic disorders involve many parameters, including incretin [31], leptin [32] and others [33]. The limitation of this report is that the results mainly focused on the changes in type 1-like diabetic rats. Additionally, a downstream effect of hyperglycemia status is liver diseases [34]. Therefore, more investigations of the actions of canavanine in another diabetic model related to these substances are required in the future.

5. Conclusion

Canavanine is capable of activating imidazoline I-2 receptors and can increase \(eta\)-endorphin secretion through activation of I-2A receptors while also enhancing glucose uptake via activation of the I-2BR receptors in the skeletal muscle of STZ-diabetic rats. Both actions of canavanine may facilitate the reduction of hyperglycemia in STZ-diabetic rats. Thus, canavanine, as a ligand of I-2R, could be developed into a treatment for decreasing hyperglycemia in the future.

Acknowledgments

We thank Miss MJ Wang for her skillful assistance in the experiments. The present study was partly supported by a grant from Chi-Mei Medical Center, Yong Kong, Tainan City, Taiwan (CMFHTI0301).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.cbi.2015.08.019.

References


W. Creutzfeldt, The incretin concept today, Diabetologia 16 (1979) 75–85.

T.M. Rizk, E. Sharif, Leptin as well as free leptin receptor is associated with polycystic ovary syndrome in young women, Int. J. Endocrinol. 2015 (2015) 927805.
