Caulerpa lentillifera inhibits protein-tyrosine phosphatase 1B and protects pancreatic beta cell via its insulin mimetic effect

Bhesh Raj Sharma, Hyun Jung Kim¹, and Dong Young Rhyu*

Department of Oriental Medicine Resources and Institute of Korean Medicine Industry, Mokpo National University, Muan, Jeonnam 58554, Korea
¹College of Pharmacy, Mokpo National University, Muan, Jeonnam 58554, Korea

Received November 14, 2016
Revised December 28, 2016
Accepted January 12, 2017
Published online April 30, 2017

*Corresponding Author
Tel: +82-61-450-2664
Fax: +82-61-450-6643
E-mail: herb.mnu@gmail.com;
rhyudy@mokpo.ac.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2017

Abstract The aim of this study was to determine whether *Caulerpa lentillifera* extract (CLE) can protect pancreatic beta cells and enhance insulin signaling in adipocytes. We measured the protein tyrosine phosphatase (PTP)-1B inhibitory effect of CLE using an in-vitro enzyme assay. Proteins involved in the pancreatic beta-cell death and insulin signaling were measured by western blotting. Oil-red O staining was used to measure the insulin mimetic effect of CLE. CLE strongly inhibited the PTP1B enzyme. In rat insulinoma (RIN)-m5F cells, CLE decreased the activation of extracellular regulated kinase (ERK)-1/2, P38 mitogen activated protein kinase (P38), c-Jun NH2-terminal kinase (JNK), and nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB). Furthermore, CLE showed insulin-mimetic effect and enhanced the activation of insulin-signaling molecules including IRS, AKT, PI3K, and GSK-3β in 3T3-L1 adipocytes. Our results suggested that CLE-inhibited PTP1B, protected the pancreatic beta cells, and enhanced insulin sensitization in the adipocytes.

Keywords: *Caulerpa lentillifera*, protein tyrosine phosphatase (PTP)-1B, pancreatic beta cells, insulin signaling, adipocytes

Introduction

Pancreatic beta-cell failure and/or insulin-signaling reduction in metabolically active tissues are the root causes for defective glucose utilization in diabetes (1,2). Infiltration of inflammatory immune cells in and around the pancreatic islets generates excessive cytokines that leads to the activation of nucleus factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways to cause pancreatic beta-cell death (3). This finally modulates the glucose uptake in the insulin-sensitive tissues, leading to a defect in the insulin-signaling pathways (4). Therefore, an agent that prevents pancreatic beta-cell death and increases insulin signaling can prove to be a promising drug for the prevention and treatment of diabetes. Protein tyrosine phosphatase (PTP)-1B (PTP1B) inhibitors have recently been reported to have an effect on both pancreatic beta-cell modulation and glucose uptake (5,6). Therefore, interest in the possible therapeutic use of PTP1B inhibitors obtained through natural sources has been growing because natural remedies have fewer adverse effects compared with synthetic drugs and have multiple sites of action (7). However, the therapeutic use of most natural remedies has not been scientifically proven. *Caulerpa lentillifera* has been consumed as salads and vegetables in Japan, Korea, the Philippines, and other countries in South East Asia. This

plant has gained popularity in international food market because it contains high minerals, dietary fiber, vitamin A, vitamin C, and several essential unsaturated fatty acids. We recently discovered the effect of CLE on insulin secretion in RINm5F cells (8) and the mechanism of insulin resistance in *db/db* mice (9). Therefore, we hypothesized whether CLE may demonstrate PTP1B inhibitory effects that may regulate pancreatic beta-cell protection and insulin sensitization.

Materials and Methods

Reagents PTP1B (human, recombinant) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Interleukin (IL)-1β and interferon (IFN)-γ were purchased from R & D (Minneapolis, MN, USA). The antibodies to p-IRS-1, IRS, p-AKT, AKT, p-PI3K, PI3K, p-GSK3β, GSK3β, and GLUT4 were purchased from Cell Signaling Technology (Beverly, MA, USA). Nitrocellulose membranes and chemiluminescent reagents for Western blotting were purchased from Bio-Rad (Richmond, CA, USA) and Imegenex (San Diego, CA, USA), respectively. All solvents, chemicals, and reagents were of analytical grade and were purchased from Sigma-Aldrich unless otherwise specified.

Plant material *C. lentillifera* was purchased from Okinawa, Japan. The taxonomic identity of the plant was confirmed by Prof. Chan Seon Park and the sample was preserved for reference in the herbarium of the Department of Oriental Medicine Resources, Mokpo National University, South Korea. The dried *C. lentillifera* (1 kg) was extracted with ethanol at room temperature until the color disappeared. The extract was then filtered and evaporated under vacuum, followed by freeze-drying. The yield of the extract was 1% of the dried *Caulerpa lentillifera* (1 kg).

Measurement of PTP1B activity CLE (3 μ L in DMSO) was added to a reaction mixture containing enzyme (2 μ L), reaction buffer [10 μ L, 50 mM citrate, pH 6.0, 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT)], water (35 μ L), and 50 μ L of 20 mM *p*-nitrophenylphosphate (*p*-NPP). The reaction mixture was placed in an incubator at 37°C for 30 min and the reaction was terminated with 1 M NaOH. The amount of *p*-nitrophenol produced was estimated by measuring the increase in absorbance at 405 nm.

Cell culture and cell viability assay Cell culture, cell viability, oil-red O staining, and western blotting were performed as per the methods described in previous studies (10,11).

Immunofluorescence microscopy Differentiated 3T3-L1 adipocytes, grown in a cover-glass-bottom dish, were incubated with CLE and insulin (IN) for 2 h at 37°C in 5% CO₂ atmosphere. Cells were washed with PBS and fixed with 4% formaldehyde for 20 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 15 min. Then, the cells were blocked with 5% BSA for 1 h, followed by overnight-incubation with an anti-glucose transporter type 4 (GLUT4, 1:50) antibody at 4°C. The next day, i.e., after 24 h, the cells were incubated with Goat anti-rabbit Immunoglobulin G-Isothiocyanate (IgG-FITC) secondary antibody after washing. Finally, the cells were examined using a fluorescence microscope.

Statistical analysis SPSS (SPSS, Inc. Chicago, IL, USA) was used for statistical data analysis. All data were presented as mean $\pm$ SEM. The means of all groups were compared using ANOVA, followed by Duncan's *post hoc* test of multiple comparisons. *p*-values less than 0.05 were considered statistically significant.

Results and Discussion

It has been reported that the increased PTP1B activity has been associated with pancreatic beta-cell death and defective glucose utilization, subsequently leading to type 2 diabetes and obesity (12). Therefore PTP1B inhibition can have dual effects on both the pancreatic beta cell-function and glucose utilization in metabolically active tissues (5,6). Davis *et al.* (13) recently reported that *Annona squamosa* hexane extract regulated the glucose metabolism in

Fig. 1. Effect of CLE on PTP1B inhibition. *Caulerpa lentillifera* extract (CLE) was used at the indicated concentrations (250, 500, and 1,000 μ g/mL). 30 μ M Ursolic acid (UA) was used as the positive control. Data displayed reflects the means $\pm$ SEM (*n*=6) of the three independent experiments. ****p*<0.001, *****p*<0.0001 versus control.

diabetic animals possibly by inhibiting the PTP1B enzyme. Therefore, we hypothesized whether CLE inhibits PTP1B and measured the PTP1B inhibitory activity using an in-vitro enzyme assay. We found that CLE inhibited PTP1B by 41.7% (Fig. 1), which is comparable with that of the positive control, ursolic acid. This led us to anticipate that CLE might protect pancreatic beta cells and demonstrate insulin mimetic effect.

PTP1B modulates β -cell mass in a cell-autonomous manner through the regulation of key signaling pathways involved in β -cell proliferation and apoptosis (5). NF- κ B plays an important role in the transcriptional regulation of cytokine-induced β -cell apoptosis. The role of NF- κ B, which can be activated by cytokines in type 1 diabetes, has been implicated as a key signaling mediator of pancreatic beta-cells death (4). Translocation of NF- κ B p65 from the cytosol to the nucleus of cytokine-stimulated cells is one of the measuring factors that drive pancreatic beta-cell death and insulinitis-provoking diabetes (14). Similarly, MAPKs are important modulators of protein synthesis through the phosphorylation of various crucial molecules. It has been suggested that three major molecules of the MAPK pathway, i.e., ERK1/2, JNK, and P38 play an important role in regulating the pancreatic beta-cell death in diabetes (3). Therefore, we investigated the effects of CLE on NF- κ B p65 translocation and MAPK activation. We found that CLE decreased the translocation of NF- κ B p65 subunit from the cytosol to the nucleus in a dose-dependent manner, which was confirmed by observing the decreased NF- κ Bp65 subunit expression in the nucleus and an increased NF- κ Bp65 subunit expression in the cytosol (Fig. 2A and 2B). MAPKs and Stat1 activation, which are crucial factors for both beta-cell failure and defective insulin signaling (14), were also found to decrease in the CLE-treated RINm5F cells (Fig. 2C and 2D, Fig. 3A and 3B) on reducing their phosphorylation in a dose-dependent manner by at least 40%. Our results agree with the recent findings in which glucose-lowering effect of CLE was found in streptozotocin-induced diabetic rats as an animal model of beta-cell damage (15). Since CLE exhibited both PTP1B-inhibitory and pancreatic beta-cell protective activities, we hypothesized whether CLE had insulin-like effect and can activate the

Fig. 2. Effect of CLE on IL-1 β and IFN- γ -induced NF- κ Bp65 translocation and STAT1 activation in RINm5F cells. RINm5F cells (2×10^6) were pretreated with the indicated concentrations (50 and 250 μ g/mL) of CLE for 3 h, followed by stimulation with IL-1 β (2 ng/mL) and IFN- γ (100 U/mL) for 25 min. The NF- κ Bp65 translocation (A & B) and STAT1 activation (C & D) was determined by western blotting. The NF- κ Bp65 translocation was determined by measuring the expression of NF- κ Bp65 in both cytosolic and nuclear compartments. Each value represents the means $\pm$ SEM of the three independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control.

Fig. 3. Effects of CLE on IL-1 β - and IFN- γ -induced MAPKs activation in RINm5F cells. RINm5F cells (2×10^6) were pretreated with the indicated concentrations (50 and 250 μ g/mL) of CLE for 3 h, followed by stimulation with IL-1 β (2 ng/mL) and IFN- γ (100 U/mL) for 20 min. The MAPKs, p-P38, p-JNK1/2, and p-ERK1/2 (A & B) were determined by western blotting. Each value represents the means $\pm$ SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ versus control.

molecules involved in glucose-stimulated insulin-signaling pathway. As expected, we found that CLE showed an insulin-mimetic effect, differentiating adipocytes even in the absence of insulin (Fig. 4A and 4B), and increased the phosphorylated forms of insulin-signaling molecules such as IRS, PI3K, GSK-3 β , and AKT (Fig. 5A and 5B), which are major molecules involved on the insulin-signaling pathway in metabolically active tissues (16). Our results agree with those reported in the current literature suggesting that insulin-mimetic drugs can differentiate adipocytes even in the absence of insulin. Furthermore, CLE-treated adipocytes demonstrated a higher expression of GLUT4 positive adipocytes, revealing that CLE increased the adipose glucose uptake via the insulin-signaling pathway (Fig. 6A). This finding is further confirmed by an increased glucose-uptake in CLE-treated adipocytes (Fig. 6B). Consistent with our study, Gum *et al.* also reported that the reduction of PTP1B increases insulin-dependent glucose metabolism in *ob/ob* mice (12).

The major limitation in developing the PTP1B inhibitors is the identification of the specific inhibitor that inhibits only PTP1B, because most of the inhibitors developed till now, inhibit other tyrosine phosphatases as well. In the assay used in this study, we measured the effect of CLE on PTP1B inhibition and insulin-signaling molecules' expression as our objective was to later formulate CLE as a functional food and not a PTP1B-inhibiting drug. Future studies are required to explore the specificity of CLE on PTP1B inhibition. On the basis of our findings, we concluded that CLE might be useful as a candidate for PTP1B inhibition to prevent beta-cell death and activate insulin signaling in insulin-sensitive tissues. However, further studies are required to identify the possible active compounds that are responsible for the PTP1B inhibitory effect of CLE in an *in-vivo* system.

Disclosure The authors declare no conflict of interest.

Fig. 4. Insulin-mimetic effect of CLE on the differentiation of 3T3-L1 adipocytes. Confluent 3T3-L1 pre-adipocytes were induced to differentiate into adipocytes for 8 d (from day 0 to day 8) and treated with CLE and insulin and lipid droplets were stained with oil-red O dye. **Control:** Completely differentiated control adipocytes. **CLE-250:** adipocytes treated with 250 $\mu\text{g/mL}$ CLE, **Insulin:** adipocytes treated with 100 nm insulin. Representative images from the three independent experiments (A) and the content of oil-red O dye (B) are shown in the figure. Data were represented as the means $\pm$ SEM ($n=3$). *** $p<0.001$, **** $p<0.0001$ versus control.

Fig. 5. Effect of CLE on insulin-signaling proteins' (p-IRS, p-PI3K, p-AKT, and p-GSK3 β) expression in 3T3-L1 adipocytes (A & B). Confluent 3T3-L1 pre-adipocytes were induced to differentiate into adipocytes and treated with CLE and insulin. **Control:** Completely differentiated control adipocytes. **CLE-250:** adipocytes treated with 250 $\mu\text{g/mL}$ CLE, **Insulin:** adipocytes treated with 100 nm insulin. Data were represented as the means $\pm$ SEM ($n=3$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus control.

Fig. 6. Effect of CLE on GLUT4 expression (A) and glucose uptake (B) in 3T3-L1 adipocytes. Confluent 3T3-L1 pre-adipocytes were induced to differentiate into adipocytes and treated with CLE and insulin. **Control:** Completely differentiated control adipocytes. **CLE-250:** adipocytes treated with 250 $\mu\text{g/mL}$ CLE, **Insulin:** adipocytes treated with 100 nm insulin. Data were represented as the means $\pm$ SEM ($n=3$). *** $p<0.001$, **** $p<0.0001$ versus control.

References

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 33: S62-S69 (2010)

2. Kahn SE. The relative contributions of insulin resistance and beta cell dysfunction to the pathophysiology of type 2 diabetes. *Diabetologia* 46: 3-19 (2003)
3. Csorba TR, Lyon AW, Hollenberg MD. Autoimmunity and the pathogenesis of type 1 diabetes. *CRC Cr. Rev. Cl. Lab. Sc.* 47: 51-71 (2010)
4. Cerf ME. Beta cell dysfunction and insulin resistance. *Front. Endocrinol.* 4: 37 (2013)
5. Ruiz RF, Vieira E, Garcia-Roves PM, Gomis R. Protein tyrosin phosphatase 1B modulates pancreatic beta cell mass. *PLoS ONE* 9: e90344 (2014)
6. Johnson TO, Ernioloff J, Jirousek MR. Protein tyrosin phosphatase 1B inhibitors for diabetes. *Nat. Rev. Drug Discov.* 1: 696-709 (2002)
7. Cragg GM, Newman DJ. Natural products: A continuing source of novel drug leads. *Biochim. Biophys. Acta* 1830: 3670-3695 (2013)
8. Sharma BR, Rhyu DY. Anti-diabetic effects of *Caulerpa lentillifera*: Stimulation of insulin secretion in pancreatic beta cells and enhancement of glucose uptake in adipocytes. *Asian Pac. J. Trop. Biomed.* 4: 575-580 (2014)
9. Sharma BR, Kim HJ, Rhyu DY. *Caulerpa lentillifera* extract ameliorates insulin resistance and regulates glucose metabolism in C57BL/KsJ-db/db mice via PI3K/AKT signaling pathway in myocytes. *J. Transl. Med.* 13: 62 (2015)
10. Sharma BR, Kim MS, Rhyu DY. *Nelumbo nucifera* leaf extract attenuated pancreatic beta cells toxicity induced by interleukin-1 β and interferon- γ , and increased insulin secretion of pancreatic beta cells in streptozotocin-induced diabetic rats. *J. Tradit. Chin. Med.* 36: 71-77 (2016)
11. Park CH, Rhyu DY, Sharma BR, Yokazawa T. Inhibition of preadipocyte differentiation and lipid accumulation by 7-O-galloyl-D-sedoheptulose treatment in 3T3-L1 adipocytes. *Biomed. Prev. Nutr.* 3: 319-324 (2013)
12. Gum RJ, Gaede LL, Koterski SL, Heindel M, Clampit JE, Zinker BA, Trevillyan JM, Ulrich RG, Jirousek MR, Rondinone CM. Reduction of protein tyrosin phosphatase 1B increases insulin dependent signaling in *ob/ob* mice. *Diabetes* 52: 21-28 (2003)
13. Davis JA, Sharma S, Mittra S, Sujatha S, Kanaujia A, Shukla G, Katiyar C,

- Lakshmi BS, Bansa VS, Bhatnagar PK. Antihyperglycemic effect of *Annona Squamosa* hexane extract in type 2 diabetes animal model: Ptp1B inhibition, a possible mechanism of action? Indian J. Pharmacol. 44: 326-332 (2012)
14. Pirot P, Cardozo AK, Eizirik DL. Mediators and mechanisms of pancreatic beta cell death in type 1 diabetes. Arq. Bras. Endocrinol. Metab. 52: 156-165 (2008)
 15. Abouzid SF, Ahmed OM, Ahmed RR, Mahmoud A, Addella E, Ashour MB. Antihyperglycemic effect of crude extracts of some Egyptian plants and algae. J. Med. Food 17: 400-406 (2014).
 16. Mackenzie RW, Elliott BT. Akt/PKB activation and insulin signaling: A novel insulin-signaling pathway in the treatment of type 2 diabetes. Diabetes Metab. Syndr. Obes. 7: 55-64 (2014)