The Stimulatory Activities of Polysaccharide Compounds Derived from Algae Extracts on Insulin Secretion in Vitro

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We prepared two series of polysaccharide compounds derived from algae extracts and investigated their stimulatory activity on insulin secretion in vitro using the rat pancreatic cell line, RIN-5F. Several of the compounds exhibited significant stimulatory activity in a dose-dependent manner without apparent cytotoxicity at concentrations above 10 µg. Glybenclamide, a commonly prescribed sulfonylurea (SU) against diabetes mellitus type II, was used as a positive control and showed moderate cytotoxicity in the cell culture assay system. Amylin (IAPP; islet amyloid polypeptide), an inhibitor for glybenclamide, did not inhibit the activity of the isolated compounds, suggesting that they act through a mechanism(s) different from glybenclamide. Algae-derived extracts could be candidates for a new class of anti-diabetic drugs.

Key words marine organism; polysaccharide; anti-diabetic; insulin secretion; algae-derived extract

The incidence and prevalence of diabetes mellitus have reached epidemic proportions.1,2) The number of people worldwide affected by diabetes mellitus is estimated to be more than 200 million and is expected to double by 2030. Most diabetes patients suffer from non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes).3,4) The insulin-stimulatory activities of sulfonylureas (SUs), common anti-diabetic drugs used to treat NIDDM patients, have been reported to decrease with time due to the gradual destruction of β cells.5,6) Furthermore, SUs are prescribed with many restrictions due to their side-effects, such as hypoglycemia. Therefore, an alternative anti-diabetic drug that shows low toxicity even after extended use is necessary.

In recent years, interest in the biological activities of compounds from marine organisms has intensified. Compounds derived from various marine organisms have been investigated and developed, and some have become commercially available drugs in China.7,8) Marine-derived compounds have been investigated as anti-diabetic drugs. For example, an amyllose compound extracted from Sargassum fusiforme (HARV.) Setchess, a type of algae, significantly lowered blood sugar levels in alloxan-induced diabetic mice and improved markedly their glucose tolerance.9)

We prepared two series of compounds from algae extracts. Some of these compounds have already been investigated for their ability to improve human immunologic and hepatic functions, to induce interferon synthesis, and to inhibit the replication of hepatitis B virus. The results of these studies will be reported elsewhere. Here, we report the anti-diabetic activities of these compounds.

MATERIALS AND METHODS

Cell Culture The RIN-5F cell line from RIN-m rat islets was purchased from the ATCC Global Bioresource Center. Cells were maintained by transfer to new 24-well plates every 3 d. RPMI 1640 medium with 25 mM HEPES and 200 mM GlutaMAX® and penicillin (10000 U/ml)–streptomycin (10000 µg/ml) solution, were purchased from Invitrogen® Gibco BRL (Grand Island, NY, U.S.A.), and fetal bovine serum (FBS) was purchased from Moregate BioTech Co., Ltd. (Bulimba, Australia).

Compounds from Algae The two series of compounds tested were supplied by The Institute of Marine Drug and Food, College of Medicine and Pharmaceutics, The Ocean University of China (Qingdao, China). Detailed methods for the preparation of these compounds will be published shortly elsewhere. Briefly, compounds A and B were prepared from different algal species by extraction with chloroform, ethyl acetate and n-butyl alcohol. The compounds were isolated by column chromatography on silica gel and Sephadex LH-20 columns, were purified on a macroporous absorption resin column, and then sulfonated with sulfuric acid. Compounds A1 to A6 and B1 to B6 were isolated from compound A (MW 30000—1000) and B (MW 40000—1000) equally according to differences in their molecular weight. The average MW of A1; 27600, A2; 19300, A3; 12800, A4; 8500, A6; 1900, B1; 37000, B2; 22000, B3; 18000, B4; 12000 B5; 5000 and B6; 1850 respectively. These molecular weights were used for calculations of molar concentration (µM).

Reagents for Assay Systems Rat insulin enzyme immunoassay (EIA) system (INSKR020, 96 assays) was obtained from Morinaga Biochemical Industries (Tokyo, Japan). CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega (Madison, WI, U.S.A.) was used to assay cytotoxicity. Absorbance at 490 nm was measured in a MTP-800 microplate reader (Corona Electric, Tokyo, Japan).

Assay of Insulin Secretion Activity The RIN-5F cell line, derived from rat pancreatic β cells, was used to evaluate insulin secretion activity. Cells at a concentration of 2.0 × 10³ cells/well in 24-well plates were incubated with conditioned RPMI 1640 medium supplemented with 10% FBS and penicillin–streptomycin (100 U/ml and 100 µg/ml final concentration, respectively) at 37 °C under a 5%
CO₂ atmosphere. After incubation for 72 h, the medium in each well was replaced with fresh medium and the cells were incubated for another 24 h.

The medium in the wells was then removed and the cells were washed with fresh conditioned medium to remove any released insulin. Next, 360 µl of conditioned medium, with or without 20 µl of glucose solution (20 mg/ml; 16.7 mM vs. 11.1 mM final concentration, respectively) and 20 µl of the test compound solution at various concentrations (20 µM — 20 mM) were pipetted into the wells, except for the blank and no-drug controls. After 3 h incubation, aliquots of medium in all wells were withdrawn and centrifuged to remove the cells. The concentration of insulin in the supernatants was determined using the EIA system. The absorbance at 490 nm was measured using a microplate reader. The activities of the compounds tested were evaluated by the increase in concentration of released insulin compared with the high-glucose control in the absence of test compounds. Each experiment was done in triplicate, and the results are presented as means±S.D. Groups of data were compared using Student’s t-test.

**Assay of Cytotoxicity** Cytotoxicity was determined by the CellTiter 96₆ AQ₃eous One Solution Cell Proliferation Assay. Cells at a concentration of 2.0×10⁴ cells/well in 96-well plates were inoculated and incubated with conditioned RPMI 1640 medium at 37 °C under a 5% CO₂ atmosphere. After incubation for 72 h, the RIN-5F cells were treated with or without active (i.e., A or B series) compounds for 24 h, then 20 µl of the CellTiter 96₆ AQ₃eous One Solution Cell Proliferation Assay solution was pipetted into all 96 wells and the cells were further incubated for 1 h. The absorbance at 490 nm was measured using a microplate reader.

**RESULTS AND DISCUSSION**

We investigated the stimulatory activities of the above compounds on insulin secretion *in vitro* in order to estimate their anti-diabetic potential. A rat insulin enzyme-immunoassay system is commercially available for evaluation of *in vitro* activities. Activities were estimated from the concentration of insulin released from pancreatic RIN-5F cells into the medium by adding two series (A and B) of test compounds. The results are shown in Fig. 1. No-drug (negative) control and high-glucose (HG) control wells without added test compounds were included in each experiment. As a positive control, we used glybenclamide, an established anti-diabetic agent known to stimulate pancreatic cells and cause insulin secretion. As shown in Fig. 1A, compounds A, A1, A4 and A5 elicited significant insulin secretion at a concentration of 100 µM in comparison with the HG control. In particular, compound A exhibited almost 10 times higher insulin secretion than the HG control. The activity of compound A is comparable to that of Glybenclamide. In contrast, compounds A2, A3 and A6 showed no significant activity. In Fig. 1B, compounds B2, B4 and B5 stimulated significant insulin secretion at a concentration of 100 µM with compound B5 exhibiting almost 9 times higher insulin secretion than the HG control. Compounds B, B1, B3 and B6 showed no significant activity. The opposite profile of activities between unseparated compounds (A or B) and separated compounds (A1, A5 for A series and B4, B5 for B series) was observed.

The reason for the different profile is presently unknown, and the mechanism of insulin release remains to be explored.

Next, we performed dose–response experiments using glybenclamide and the statistically significant (*p<0.01*) active compounds (A, A1, A5, B4 and B5) shown in Figs. 1A and
B. The dose–response curves are shown in Fig. 2. All compounds tested showed simple sigmoidal dose–response curves, suggesting a single mechanism for each agent. The concentrations at 50% efficacy (ED50) were similar, around 50 μM, suggesting that their affinities for an assumed receptor are almost the same. Since their maximal activities are different, their efficacy characteristics such as agonist, partial agonist or antagonist would be different, indicating the necessity for further investigation.

Toxicity profiles of significantly potent compounds including glybenclamide in the cell cultures were determined from 1 to 1000 μM by the CellTiter 96® AQCell Proliferation Assay. Compounds tested did not show apparent cytotoxicity up to 100 μM (Fig. 3). In contrast, it seemed that glybenclamide was more toxic than the compounds prepared from algae. Cell proliferation during the 3-h incubation period of the insulin secretion assay was insufficient for detecting cytotoxicity, (e.g., inhibition of cell growth), therefore, the cultures were incubated for 24 h with the compounds to test for cytotoxicity. Since the insulin secretion assay is a 3-h test whereas the toxicity assay is conducted after 24-h, the toxicity profile in the toxicity assay is exaggerated compared with the presumed toxicity in the insulin secretion assay. Therefore, the activities of compounds prepared from the algae were not due to their toxicity.

It is known that insulin secretion from pancreatic β cells is stimulated by SUs, and the mechanisms by which insulin is released by these drugs have been elucidated. SUs bind to SU receptors, causing closure of ATP sensitive K+ channels in the pancreatic β cell plasma membrane which results in membrane depolarization, calcium influx, and insulin secretion.10) In addition, SUs act by direct interaction with the secretory machinery. 10) Although the precise mechanisms of SU action are not clear, islet amyloid polypeptide (IAPP, amylin) inhibits insulin secretion by glybenclamide.5,6,11) In this paper, two potent compounds in series A and B, namely compounds A and B5, stimulated insulin secretion as much as the SU, glybenclamide. Amylin had no influence on the insulin secretion effects of compounds prepared from algae on RIN-5F cells (Fig. 4), suggesting that the stimulatory effects of glybenclamide and the compounds tested arise from different mechanisms.

The stimulatory effects of selected test compounds together with glybenclamide were also investigated. As shown in Fig. 5, the effect of the combination of 50 μM A and 50 μM glybenclamide was almost the same as the effect of 50 μM A plus the effect of 50 μM glybenclamide; the effect of the combination of 50 μM B5 and 50 μM glybenclamide was also almost the same as the effect of 50 μM B5 plus the effect of 50 μM glybenclamide. Although the results per se suggest an additive effect of these drugs, the results do not suggest any mechanism for the activity of the compounds tested.

Collectively, the compounds extracted from algae used in this study have stimulatory activities on insulin secretion by RIN-5F cells (the RIN-m rat islet cell line), comparable to the activity of glybenclamide, a typical SU drug. However, the mechanism(s) of the activity of these compounds appears to be different from that of glybenclamide.

Physiological insulin secretion would be regulated by many intrinsic factors such as free fatty acids, amino acids and newly identified hormones (GLP-1; glucagon-like polypeptide-1, PACAP; pituitary adenylate cyclase activating polypeptide, and others). Intracellular signal transduction including KATP channel dependent and independent mechanisms, and extracellular CaR (calcium-sensing receptor)
mechanism including MAPK (mitogen-activated protein kinase) cascade for insulin secretion has been recently elucidated. Insulin secretion is also regulated by exocytosis mechanism depending on several molecules. Therefore, polysaccharide compounds prepared from algae could have one or number of mechanisms mentioned above. Ganoder lucidum polysaccharide, however, is reported to have insulin-releasing effect through Ca\(^{2+}\) influx into pancreatic \(\beta\) cells,\(^{12}\) which suggests that compounds extracted from algae reported here may have a similar mechanism to that of Ganoderma lucidum polysaccharide.

Since \textit{in vivo} pancreatic \(\beta\) cells might be different from the RIN-5F cell line in terms of insulin secretion mechanisms, and thus may respond differently to these compounds, experiments using other insulin-producing cell lines and primary culture \(\beta\) cells would provide additional insights. Moreover, a compound could have multiple \textit{in vitro} and \textit{in vivo} activities on pathological conditions such as hyperlipidemia and/or atherosclerosis.\(^{5,13}\) Some natural ingredients of herbal medicines have several biological activities in common; likewise, the compounds tested in this study could have several activities. Systems using different types of cells (e.g., vascular endothelium) and animal \textit{in vivo} experiments are necessary for determining other anti-diabetic activities.

NIDDM is an intricate chronic disease that may require a multi-pronged approach for its control, such as diet, exercise and multi-faceted drugs. Therefore, the compounds investigated in this study are potentially useful as anti-diabetic drugs, but further studies aimed at drug development are required.

CONCLUSION

In this paper, we described the stimulatory effects of two series of compounds derived from algae extracts on \textit{in vitro} insulin secretion by rat pancreatic RIN-5F cells. Several compounds significantly promoted insulin-release, in particular compounds A and B5. Therefore, effective biological agents can be obtained from natural compounds, especially marine organisms, and their efficacy \textit{in vivo}, and well as their mechanism of action, should be studied further.

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