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Saccharin enhances neurite extension by regulating organization of the microtubules

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ABSTRACT

Aims: In the present study, we found that saccharin, an artificial calorie-free sweetener, promotes neurite extension in the cultured neuronal cells. The purposes of this study are to characterize the effect of saccharine on neurite extension and to determine how saccharin enhances neurite extension.
Main methods: The analyses were performed using mouse neuroblastoma N1E-115 cells and rat pheochromocytoma PC12 cells. Neurite extension was evaluated by counting the cells bearing neurites and measuring the length of neurites. Formation, severing and transportation of the microtubules were evaluated by immunostaining and western blotting analysis.
Key findings: Deprivation of glucose increased the number of N1E-115 cells bearing long processes. And the effect was inhibited by addition of glucose. Saccharin increased the number of these cells bearing long processes in a dose-dependent manner and total neurite length and longest neurite length in each cell. Saccharin also had a similar effect on NGF-treated PC12 cells. Saccharin increased the amount of the microtubules reconstructed after treatment with nocodazole, a disruptor of microtubules. The effect of saccharin on microtubule reconstruction was not influenced by dihydrocytochalasin B, an inhibitor of actin polymerization, indicating that saccharin enhances microtubule formation without requiring actin dynamics. In the cells treated with vinblastine, an inhibitor of microtubule polymerization, after microtubule reorganization, filamentous microtubules were observed more distantly from the centrosome in saccharin-treated cells, indicating that saccharin enhances microtubule severing and/or transportation.
Significance: These results suggest that saccharin enhances neurite extension by promoting microtubule organization.

Introduction

Microtubules are one of the essential architectural elements for various functions such as cell polarization, cell division, and migration (de Forges et al., 2012). Microtubules are hollow tubular structures consisting of the heterodimers of α- and β-tubulin, which are nucleated at the γ-tubulin ring complex that is composed of γ-tubulin and other molecules (Jiang and Akhmanova, 2011). Microtubule structures dynamically change among growth, pause and shrinkage, depending on cellular states. These dynamics are indispensable for maintenance of cellular functions.

Neurons have a polarized morphology characterized by an axon and several dendrites. Microtubules form the skeleton of neuronal processes. Microtubules are newly formed in the centrosome, which is a microtubule-organizing center, and then are severed, followed by transportation into neuronal processes (Ahmad and Baas, 1995; Yu et al., 1993). In addition to the dynamics of pre-existing microtubules, the transportation process is strictly involved in effecting the length of neuronal processes (Baas et al., 2006). Therefore, the morphology of neuronal processes is largely dependent on the motility of microtubules.

It has been reported that several molecules promote neurite extension. Endogenous molecules such as long chain free fatty acids (Liu et al., 2008) and neurgulin-1β (Gerecke et al., 2004) or exogenous molecules such as the flavonoid isoquercitrin (Palazzolo et al., 2012) increase neurite extension by modulating functions of the molecules involved in their formation. Here, we report that an artificial sweetener, saccharin, promotes neurite extension. Saccharin was the first artificial sweetener marketed, and it has no calories. The sweetness of saccharin is 200 to 700 times higher than that of sugar (FDA Consum, 2006), and its toxicity has been reported (Arnold, 1983; Negro et al., 1994). However, Weihrauch and Diehl suggested that the risk seems to be negligible in a single generation, based on a review of more than 50 studies (Weihrauch and Diehl, 2004). The U.S. Food and Drug Administration (FDA) approved saccharin in 1970 and it has been used as a food additive in various foods such as jams, soft drinks and chewing gum (Whitehouse et al., 2008). In the present report, we demonstrated a

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novel effect of saccharin to enhance neurite extension by promoting microtubule organization.

Materials and methods

Reagents

Aspartame, D-fructose, D-glucose, D-galactose, sodium saccharin dihydrate (saccharin), D-sorbitol and other reagents were obtained from Wako (Osaka, Japan). Nerve growth factor 2.5S (NGF) was obtained from Life Technologies (Carlsbad, CA, USA).

Cell culture

N1E-115 cells or PC12 cells were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), penicillin G sodium (1×10^5 U/L), and streptomycin sulfate (1×10^5 g/L) in 5% CO_2 at 37 °C. D-glucose-free DMEM (glucose-free DMEM) was prepared from DMEM Base (Sigma Aldrich, St. Louis, MO, USA).

Immunostaining

After fixation with 4% paraformaldehyde in phosphate buffered-saline (PBS) for 10 min, the cells were rinsed with PBS. The cells were permeabilized with 0.1% Triton X-100 in PBS (TPBS) for 30 min and then blocked with BlockAce (Yukijirushi, Tokyo, Japan) in TPBS for 30 min. The cells were incubated with a rabbit antibody to β-tubulin III (1:5000, T2200, Sigma-Aldrich) at 4 °C overnight, followed by Alexa Fluor 488 goat anti-rabbit IgG antibody (1:5000, Life Technologies) at 4 °C for 2 h. After rinsed with PBS, the cells were incubated in PBS containing 1 μg/ml propidium iodide (Sigma-Aldrich) and 1 μg/ml RNase (Wako) at room temperature. Images were obtained with a confocal laser-scanning microscope (ECLIPS, Nikon, Japan).

Characterization of neurite formation

Two thousand of cells were seeded in 10% FBS DMEM per well on a 96-well culture plate. For N1E-115 cells, the medium was changed to 10% FBS DMEM, DMEM or glucose-free DMEM with or without 5.56 mM saccharin after 24 h. For PC12 cells, 50 ng/ml NGF with or without 5.56 mM saccharin was added to the medium after 24 h. Then, neurite formation was evaluated in each cell after 48 h. The cells bearing shorter or longer neurites were counted according to separate definitions for each cell line, as follows. For N1E-115 cells, neurites 2 or 5 times greater than the diameter of a cell body were defined as shorter or longer neurites, respectively. For PC12 cells, neurites 2 or 4 times greater than the diameter of a cell body were defined as shorter or longer neurites, respectively. Numbers of neurite-bearing cells were expressed as a percentage of the 100 cells randomly selected in each well for quantitation. Each experiment was performed independently at least 3 times. The length of neurites was measured in 100 cells selected randomly using DS-L1 (Nikon, Japan) in three independent tests. The number of neurites per cell was counted in 100 cells selected randomly in the photomicrographs obtained in three independent tests.

Transfection of small interfering RNA

siRNAs against mouse Tas1r2 (Mm_Tas1r2_6724), Tas1r3 (Mm_Tas1r3_1168), and negative control siRNA (SIC-001) were obtained from Sigma Aldrich. Twenty thousand of N1E-115 cells were seeded on a 35-mm dish. The next day 3 nM of each siRNA was transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Next day the medium was changed to glucose-free DMEM with or without 5.56 mM saccharin, and then neurite formation was evaluated after 48 h.

Image analysis of microtubule reconstruction

These assays were performed according to a previous report (Ahmad and Baas, 1995), with some modifications. Fifty thousand of cells were grown on a poly-L-lysine-coated coverslip. N1E-115 cells were cultured in 10% FBS DMEM, DMEM or glucose-free DMEM with or without 5.56 mM saccharin for 24 h, and PC12 cells were treated with 50 ng/ml NGF in the presence or absence of 5.56 mM saccharin for 24 h. In the experiments shown in Fig. 5, N1E-115 cells were treated with both 50 ng/ml NGF and 5.56 mM saccharin in the presence or absence of 1 μM dihydrocytochalasin B for 24 h. Then, each cell was treated with 10 μM nocodazole (Sigma Aldrich) for 3 h. After washout, cells were cultured in the same conditioned medium as before treatment with nocodazole for 30 min in the experiments shown in Figs. 4 and 5. In contrast, N1E-115 cells or PC12 cells were cultured in the same conditioned medium as before treatment with nocodazole for 15 min or 30 min, and then followed by additional culture for 15 min in the presence of 3 μM vinblastine sulfate (Wako) in Fig. 6e and f. The cells were rinsed once in a microtubule-stabilizing buffer, PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9), and then treated with 0.1% Triton X-100 in PHEM containing 10 μM paclitaxel (Cayman Chemical, MI, USA) for 3 min at 37 °C in order to remove unpolymerized tubulin while preserving microtubules. After fixation with 4% paraformaldehyde in PBS for 10 min, the cells were rinsed with PBS, and treated with 10 mg/ml sodium borohydride in PBS 3 times for 5 min each to reduce autofluorescence. Then, the cells were subjected to the immunostaining with the procedures described above. Primary antibodies were a mouse antibody to β-actin (1:5000, AC-15, Sigma-Aldrich), a rabbit antibody to β-tubulin III (1:5000, T2200, Sigma-Aldrich) or a mouse antibody to γ-tubulin (1:5000, GTU-88, Sigma-Aldrich). Secondary antibodies were Alexa Fluor 546 goat anti-rabbit IgG antibody (1:5000, Life Technologies) or Alexa Fluor 488 goat anti-mouse IgG antibody (1:5000, Life Technologies). The distance from the centrosome, which is marked by γ-tubulin, to the distal edge of the microtubules, marked by β-tubulin, was measured.

Quantitative analysis of microtubule reconstruction

To evaluate the effect of saccharin on cell proliferation, five thousand of PC12 cells were seeded in 10% FBS DMEM per well on a 96-well culture plate. Next day, the medium was changed to 10% FBS DMEM containing 50 ng/ml NGF with or without 5.56 mM saccharin. After 24 h, the numbers of cells were estimated by the MTT assay using Cell Proliferation Kit I (Roche, Penzberg, Germany).

One hundred thousand of PC12 cells were grown on a poly-L-lysine-coated coverslip soaked in a 35 mm dish. This method prevents detachment of cells during subsequent treatments. After treatment with NGF in the presence or absence of 5.56 mM saccharin for 24 h, the cells were subjected to microtubule reconstruction according to the procedures described above. Following treatment with 0.1% Triton X-100 in PHEM containing 10 μM paclitaxel for 3 min at 37 °C, the cells attached to the coverslip were collected in 100 μl cell lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After 20 min on ice, the lysates were centrifuged at 3000 g for 5 min and the supernatants were collected. The supernatants added to an equal volume of SDS sample buffer containing 250 mM Tris–HCl (pH 6.8), 5% (w/v) SDS, 6% (v/v) β-mercaptoethanol and 4% (v/v) glycerol were subjected to western blotting.

Collection of total cell lysates

The cells were seeded at a density of 1×10^5 cells/dish onto 35-mm dish. N1E-115 cells were grown in glucose-free DMEM with or without