Genistein enhances the NGF-induced neurite outgrowth

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ABSTRACT

In the present report, we studied if an isoflavone, genistein, enhances the nerve growth factor (NGF)-induced neurite outgrowth of PC12 cells. Application of genistein enhanced the NGF-induced neurite outgrowth. Knockdown of Na+/K+/2Cl− cotransporter isoform 1 (NKCC1) abolished the stimulatory effect of genistein on the neurite outgrowth. These observations indicate that NKCC1 is essential for genistein to stimulate the NGF-induced neurite outgrowth, although genistein had no effect on the protein expression of NKCC1. On the other hand, genistein activates NKCC1 as shown in our previous study. Taken together, these observations indicate that genistein enhanced the NGF-induced neurite outgrowth in PC12 cells via activation of NKCC1.

Flavonoids are polyphenolic compounds widely contained in soybean, fruits, and vegetables. Recent studies have revealed that flavonoids modify various cellular functions such as proliferation (22), apoptosis (14), and gene expression (6). In addition, some flavonoids and their derivatives stimulate or inhibit various ion transporters (3–5, 7, 8, 29, 30, 36). We have recently reported that quercetin, a flavonoid, activates Na+/K+/2Cl− cotransporter isoform 1 (NKCC1) in human airway epithelial cells (4, 5). In addition, quercetin enhances nerve growth factor (NGF)-induced neurite outgrowth in rat pheochromocytoma PC12 cells, via activation of NKCC1 (27). Quercetin is categorized as a flavonol with a 3-hydroxyflavone backbone (3-hydroxy-2-phenylchromen-4-one) in flavonoids. On the other hand, another flavonoid, genistein belonging to an isoflavone with an isoflavone backbone (3-phenylchromen-4-one), also stimulates transepithelial Cl− transport by activating NKCC1 in the basolateral plasma membrane and cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel in the apical plasma membrane of renal and airway epithelial cells (29, 30). Therefore, in the present study, we tried to characterize the genistein action on the NGF-induced neurite outgrowth in PC12 cells based on the structure as a flavonoid; i.e., we studied whether genistein acts on the NGF-induced neurite outgrowth like quercetin based on the common structure of flavonoids or unlike quercetin based on the different structure between isoflavone and flavonol.

In the present study, we used PC12 cells (9) isolated from rat pheochromocytoma (a tumor of adrenal medulla), which Dr. M. Sano (Professor Emeritus, Kyoto Prefectural University of Medicine) kindly donated to us. We seeded the cells onto poly-L-lysine coated glass coverslips, and cultured the cells in D-MEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat inactivated horse serum (Invitrogen, Carlsbad, CA, USA), 5% heat inactivated fetal bovine serum (EQUITECH BIO, Kerrville, TX, USA), and 50 μg/mL gentamicin (Invitrogen). In neurite outgrowth assay, we treated the cells with genistein (10 μM, Sigma-Aldrich) or dimethyl sulfoxide (DMSO, 0.1%, as solvent control for genistein). At 30 min after application of genistein or DMSO alone, we added 2.5S NGF of 50 ng/mL (Alomone Lab, Jerusalem, Israel) to the culture.
medium, and subsequently cultured the cells for 5 days in the presence of NGF with genistein or DMSO alone. Then, we fixed the cells with 3.7% formaldehyde, stained them with Alexa488-phaloidin, and observed the neurites with a confocal microscope (FV1000; Olympus, Tokyo, Japan). We measured the length of each neurite by using FV1000 software or Image J software (NIH, Bethesda, MD, USA). To knock down NKCC1, we transfected two independent StealthTM small interfering RNA (Invitrogen) siRNA1660 and siRNA1921, respectively corresponding to nt 1660–1684 (TGTACT TCTGCA GCCTGCA ATTAA) or nt 1921–1945 (GAACCTCTTCGTGGTTACATTTAA) of open reading frame of rat NKCC1 to the cells, with Lipofectamine 2000 reagent (Invitrogen) according to manufacturer’s protocol. We used BLOCK-iT fluorescent oligo (Invitrogen) as control oligo. At 24 h after transfection, we treated the cells with 10 μM genistein or DMSO. At 30 min after application of genistein or DMSO, we added NGF to the culture medium, and subsequently cultured the cells for 5 days in the presence of NGF with genistein or DMSO alone. To observe the protein expression level, we cultured the cells in the presence of NGF with genistein or DMSO for 5 days, and lysed the cells with lysis buffer containing 150 mM NaCl, 25 mM HEPES-NaOH (pH 7.4), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL leupeptin and 2 μg/mL aprotinin. Equal amounts of proteins (10 μg) were separated by SDS-PAGE (8% gel), transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Billerica, MA, USA), and immunoblotted with a monoclonal anti-NKCC1 (T4) antibody (19) originally developed by C. Lytle and B. Forbush. The antibody (T4) was further developed by the Developmental Studies Hybridoma Bank (DSHB) under the auspices of the National Institute of Child Health and Human Development (NICHD), and was maintained by Department of Biological Science, the University of Iowa (Iowa City, IA, USA). We purchased a polyclonal anti-GAPDH antibody from Santa Cruz Biotechnology (Santa Cruz City, CA, USA). Genistein was prepared as 10 mM stock solution in DMSO, and used at 10 μM. All data are represented as means ± SEM. For statistical analysis, we used the ANOVA and Student’s t-test as appropriate. A P value < 0.05 was considered statistically significant.

We first examined if genistein enhances the NGF-induced neurite outgrowth in PC12 cells. As shown in our previous report (27), quercetin of 10 μM enhances the NGF-induced neurite outgrowth in PC12 cells. Therefore, we studied if genistein of 10 μM shows its action on the NGF-induced neurite outgrowth in PC12 cells. Genistein of 10 μM enhanced the NGF-induced neurite outgrowth (Fig. 1A) similar to quercetin as shown in our previous report (27). We also applied genistein of 100 μM to PC12 cells, however genistein of 100 μM showed its toxicity leading to cell death (data not shown). Distribution of neurite lengths in genistein- or DMSO-treated cells (Fig. 1B) shows that genistein increased the percentage of long neurites. We further studied if genistein affects the morphology and number of neurites. Genistein did not affect the morphology (data not shown) or number of neurites (genistein, 3.83 ± 0.19 / cell (n = 59); DMSO, 3.76 ± 0.20 / cell (n = 66); not significant difference).
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neurite outgrowth (23–25), and 2) quercetin (a flavonoid) enhances the NGF-induced neurite outgrowth in PC12 cells through activation of NKCC1 (27). Therefore, to study if the stimulatory effect of genistein on neurite outgrowth of PC12 cells is mediated via an NKCC1-dependent pathway, we next examined if the knockdown of NKCC1 diminishes the stimulatory effect of genistein on neurite outgrowth. Figs. 2A and 2B show that transfection of siRNA successfully reduced the expression level of NKCC1 protein. The expression level of NKCC1 protein in siRNA1660-transfected or siRNA1921-transfected cells was approximately 15% of mock transfected or control oligo-transfected cells. Fig. 2C indicates that down regulation of NKCC1 protein expression abolished the genistein-enhanced neurite outgrowth. On the other hand, in control cells (Mock or Control oligo), genistein stimulated the neurite outgrowth (Fig. 2C).

As described above, our recent studies (23–25) have revealed that the NKCC1 is essential for NGF-induced neurite outgrowth in PC12 cells and PC12D cells by participating in transport of Cl−, which plays various roles in cellular functions (10, 11, 20, 26, 31, 32, 37). NGF treatment increased protein expression of NKCC1 (27), and the knockdown of NKCC1 diminished the NGF-induced neurite outgrowth (Fig. 2). Therefore, we speculate if genistein enhances the NGF-induced neurite outgrowth by increasing protein expression of NKCC1. However, genistein had no effect on the protein expression of NKCC1 (Fig. 3).

Activity of solute carrier 12 family proteins including NKCC1 (scl12a2) is regulated by the phosphorylation/dephosphorylation (2, 18, 33, 35). Ste20-related proline-alanine-rich kinase (SPAK) and oxidative-stress-responsive kinase 1 (OSR1),

Fig. 2  Role of NKCC1 in the stimulatory effect of genistein on NGF-induced neurite outgrowth. (A) Representative Western bolts. * indicates non-specific protein bands. (B) Statistical results of relative NKCC1 protein expression. Values are indicated as means ± SEM. *, P < 0.05 vs. mock. n = 3. (C) The statistical results of neurites length. Values are indicated as means ± SEM. *, P < 0.01 vs. DMSO. NS, no significant difference vs. DMSO. n = 86–94.

Fig. 3  Effect of genistein on NKCC1 protein expression. (A) Representative results. * indicates non-specific protein bands. (B) Statistical results of relative NKCC1 protein expression. Values are indicated as means ± SEM. NS, no significant difference vs. DMSO. n = 4.
serine/threonine kinases, activate NKCC1 by phosphorylating NKCC1 (2). On the other hand, certain protein phosphatases, such as protein phosphatase type1/type2 (PP1 and PP2), attenuate the activity of NKCC1 via dephosphorylation of NKCC1 (18, 33). Genistein may affect activity of these kinases and/or phosphatases, modulating indirectly NKCC1 activity.

Genistein, as an isoflavone, is well known to show an estrogen-like action with its binding potential to estrogen receptor (ER) (13, 38). Genistein competes with 17β-estradiol in binding to ER. Kupper et al. (15) have reported that genistein is bound to both ERα and ERβ, and that genistein has much higher affinity for ERβ than ERα. In addition, Lehraiki et al. (16) have reported that genistein inhibits production of testosterone in fetal testis via ERα. On the other hand, estrogen protects neuronal cells from various apoptotic stresses, and stimulates neuronal differentiation (21). In addition, a citrus fruit flavanone, hesperetin, protects PC12 cells against oxidative stresses via an ER/TrkA (specific receptor for NGF)-mediated mechanism (12). Therefore, the genistein action on the NGF-induced neurite outgrowth shown in the present study may be mediated via an ER/TrkA-mediated pathway similar to hesperetin (a flavonoid) as described above.

Genistein is known as an inhibitor of protein tyrosine kinase (PTK) in a broad range (1), and inhibits both receptor-type and non-receptor-type PTKs, such as epidermal growth factor (EGF) receptor (receptor-type) and Src kinase (non-receptor-type). Activity of TrkA as PTK is essential for NGF signaling (17). TrkA is dimerized and activated by NGF binding to NGF receptor, and phosphorylates tyrosine residue(s) of downstream molecules, such as phosphatidylinositol 3-kinase (17). On the other hand, recent studies unexpectedly have revealed that genistein activates PTK-mediated signaling pathways in some cases; e.g., Nakamura et al. (28) have reported that genistein activates the EGF receptor signaling, stimulating cell proliferation in a human prostate cancer cell line via enhancement of PTK activity of EGF receptor and downstream Src kinase. Neurotrophin receptors such as TrkA belong to receptor-type PTK family. Genistein may show its stimulatory action on NGF-induced neurite outgrowth via an increase in PTK activity of TrkA followed by activation of TrkA downstream signaling cascades. Further studies are necessary to clarify if genistein activates or inhibits TrkA signaling cascade in PC12 cells.

As mentioned above, quercetin belongs to a flavonol subcategory that possesses a 3-hydroxyflavone backbone (3-hydroxy-2-phenylchromen-4-one). On the other hand, genistein belongs to an isoflavone subcategory that has an isoflavone backbone (3-phenylchromen-4-one). Isoflavone differs from flavonol in location of the phenyl group, which is located at the position 2 in flavonol, and at the position 3 in isoflavone. Furthermore, flavonol possesses a hydroxyl (-OH) group in their position 3. As shown in the present study and our previous study (27), both quercetin (a member of flavonol) and genistein (a member of isoflavone) stimulate NGF-induced neurite outgrowth of PC12 cells. These observations suggest that the core structure “chromen-4-one” is important to show the stimulatory action of flavonoids, rather than the phenyl- or hydroxy-group at the position 2 or 3. The clarification of molecular mechanisms underlying how flavonoids regulate the activity of NKCC1 is an interesting subject for future studies.

Plants such as soybean, vegetables, and fruits widely contain flavonoids including genistein and quercetin with relatively low toxicity compared with other plant compounds (e.g. alkaloids) (34). We ingest flavonoids of significant quantities in diet. Therefore, our present and previous (27) studies suggest that intake of foods containing flavonoids could be valuable for facilitating recovery from nerve injury, and further studies on the action of food containing flavonoids are very interesting in terms of facilitating recovery from nerve damage.

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