Detection of Genipin/Geniposide-Target Molecules by a Geniposide Overlay Method Using Anti-Geniposide Antibody

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(Received December 17, 2003; Accepted January 14, 2004)

A geniposide overlay method for the detection of genipin/geniposide-binding proteins was attempted using a specific anti-geniposide antiserum. The specific anti-geniposide antiserum that recognized the genipin moiety was obtained from rabbits immunized with geniposide-rabbit serum albumin (RSA) conjugate as antigen. The results of the overlay method revealed a major geniposide binding protein with a molecular weight about 170 kDa in the cytosolic fraction of rat brain cortex and PC12h cells. The major protein band was comparable with the protein band (about 170 kDa) detected by simultaneous Western blot analysis using anti-neuronal nitric oxide synthase (nNOS) antiserum. Furthermore, geniposide activated NOS activity in a concentration-dependent manner. These results indicate that the antiserum obtained was useful for the detection of a genipin/geniposide-target molecule and strongly suggested that direct binding to and activating of nNOS cause the neuritogenic activity of genipin/geniposide in PC12h cells.

Key words —— genipin/geniposide, neuritogenic activity, target molecule, nitric oxide synthetase, overlay method, anti-genipin/geniposide antibody

INTRODUCTION

We have previously reported that geniposide and its aglucone, genipin, isolated from *Gardenia fructus*, have neuritogenic activity in PC12h cells[1] and suggested that the genipin-induced neurite outgrowth depends on activation of a nitric oxide synthase (NOS)-soluble guanylate cyclase-cyclic GMP-dependent protein kinase pathway.[2]

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To identify the target molecule for genipin/geniposide in the neuritogenic action, a geniposide overlay method was attempted using specific anti-geniposide antiserum.

MATERIALS AND METHODS

Materials —— Geniposide was generously donated by Toyo Food Color & Chemical Co. Ltd. (Japan). Gardenoside was obtained from Toray Techno Co., Ltd. (Japan). Genipin, aucubin, and glutaric anhydride were purchased from Wako Pure Chemical Industries Ltd. (Japan). N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-D-arginine methyl ester (D-NAME) were obtained from Research Biochemicals International (U.S.A.). Prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards (Broad Range) were obtained from BIO-RAD Laboratories (U.S.A.). Anti-rabbit IgG alkaline phosphatase conjugate and anti-rabbit IgG peroxidase conjugate were purchased from Sigma Chemical Co. (U.S.A.). The BCIP/NBT phosphatase substrate system was purchased from Kirkegaad & Perry Laboratory Inc. (U.S.A.).

Preparation of Cytosolic Fractions from PC12h Cells and Rat Brain Cortex —— The PC12h cells were grown on 35 mm culture dishes in high-glucose Dulbecco's modified Eagle's media (DMEM) supplemented with 5% (v/v) horse serum and 5% (v/v) precolostrum calf serum as previously reported.[3] The cells were treated by the freeze-thaw method with extraction buffer [phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Sigma Chemical Co.)] and the supernatant obtained at $10000 \times g$ was used as the cytosolic fraction. Wistar rats were sacrificed by decapitation under ether anesthesia and their brains removed and rapidly dissected on ice. The cerebral cortex was homogenized in ice-cold calcium, magnesium-free

(CMF)-PBS supplemented with protease inhibitor cocktail. The supernatant obtained by centrifugation at 100000 × *g* was used as rat brain cortical cytosol (RBCC).

Preparation of Geniposide-Albumin Conjugates and Antiserum —— Referring to the method previously reported by Kanaoka *et al*.,[4] we prepared the conjugates as follows. A solution of geniposide and glutaric anhydride (molar ratio = 1 : 1) in pyridine was incubated for 4 hr at room temperature, and extracted with n-butanol/Dw (1 : 1). The butanol layer was evaporated and crystallized from absolute ethanol. The crude glutaryl-geniposide fraction (500 mg) obtained and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (70 mg; Dojindo, Osaka, Japan) were added to 10 mg of a carrier protein [rabbit serum albumin (RSA) or bovine serum albumin (BSA)] in 2 ml of acidified-DW (pH 4.5), and incubated for 2.5 hr at room temperature. The mixture was then centrifuged at 3000 rpm for 10 min and the supernatant was applied to a Sephadex G-50 column chromatograph. The protein fraction was collected and dialyzed against PBS. These conjugates with RSA and BSA are presented in this report as Gen/RSA and Gen/BSA, respectively. Gen/RSA was used to immunize the rabbits and Gen/BSA was used as the antigen in the enzyme-linked immunoadsorbent assay (ELISA). The antigen, Gen/RSA (2 mg), was emulsified with Freund's complete adjuvant. The emulsion was injected into domestic albino female rabbits at multiple sites on the back and boosted three times with 0.5 mg antigen at 2-week intervals. The blood was collected from an ear vein 2 weeks after the last injection and the titer of the antiserum was determined by ELISA using Gen/BSA as a solid phase antigen and EIA/RIA 8 well strips (COSTAR, U.S.A.) as microtiter plates.

The high titer antiserum obtained was applied to a competitive ELISA and the specificity to geniposide was determined using geniposide and the related iridoid compounds gardenoside, aucubin, and anthranyloyl-geniposide (Ant-Gen) as competitors.

Geniposide Overlay Method —— To detect geniposide-binding proteins, a geniposide overlay method was attempted using the specific anti-geniposide antiserum. Protein samples (RBCC or cytosolic fractions of PC12h cells) were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (Hybond-C; Amersham Pharmacia Biotech, Japan). The membranes were blocked with PBS containing 10% skim milk (Difco, U.S.A.) or 3% BSA (Nacalai Tesque, Japan) containing 0.01% NONIDATE P-40 (Sigma Chemical Co.) for 1 hr, and then incubated with 0.1 mg/ml geniposide solution. After 1 hr, the membranes were washed with washing solution (10 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 0.05% Tween 20), and incubated with anti-geniposide antiserum (× 500–2000) overnight. After washing the membranes, geniposide binding proteins were detected using an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma Chemical Co.) as secondary antibody and a BCIP/NBT phosphatase substrate system (KPL, U.S.A.). To compare the two bands detected by the geniposide-overlay method and Western blotting using anti-nNOS antiserum, the same samples were applied to one gel plate and SDS-PAGE was performed. After blotting the transferred membrane was cut into two separate sheets, and each membrane sheet was visualized using an anti-geniposide antiserum and a rabbit anti-nNOS antiserum (as primary antibody, × 500, Transduction Laboratories, U.S.A.) as mentioned above.

Assay of NOS Activity —— NOS activity was measured by thin-layer chromatography (TLC) according to the method of Kumar *et al*.[5] In the enzyme assay, a mixture (total 250 μl) of PBS (pH 7.4) containing 20 μM NADPH, 1 mM $CaCl_2$, 3 mM nicotinamide, 20 μM tetrahydrobiopterin, 1 mM dithiothreitol, 40 kBq of L-[^{14}C] arginine (Amersham Pharmacia Biotech, Japan), and RBCC (1 mg protein) was incubated for 2 hr at 37°C. To study the effects of geniposide on enzyme activity, a specified concentration of geniposide was added to the reaction mixture. The reaction was terminated by adding ice-cold methanol (650 μl), left on ice for 20 min, and then centrifuged at 20000 × *g* for 10 min at 4°C. The supernatant (20 μl) was directly spotted onto a silica gel TLC plate and developed with ammonium hydroxide : AcOEt : methanol : water (2 : 0.5 : 4.5 : 1). After development, the TLC plate was set on an imaging plate for [^{14}C] and analyzed by a fluoroimage analyzer (FLA-2000, Fujifilm, Japan). The radioactivity in each spot of ornithine and citrulline was quantified and shown as a relative value to the total radioactivity of each lane.

RESULTS AND DISCUSSION

Specificity of Anti-Geniposide Antiserum

The specificity of anti-geniposide raised against gen/RSA conjugates was tested by competitive

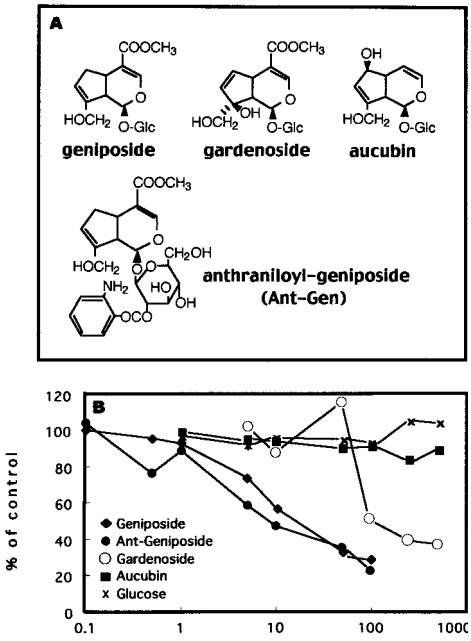

Fig. 1. A: Chemical Structures of Genipin and the Related-Iridoids Used as Competitors, B: Cross-Reactivity of Anti-Geniposide Antiserum to Several Iridoids

The cross-reactivity was checked by ELISA using Gen-BSA as a solid phase antigen according to the assay procedure described in the "MATERIALS AND METHODS." Data are expressed as percentages of control(- competitors).

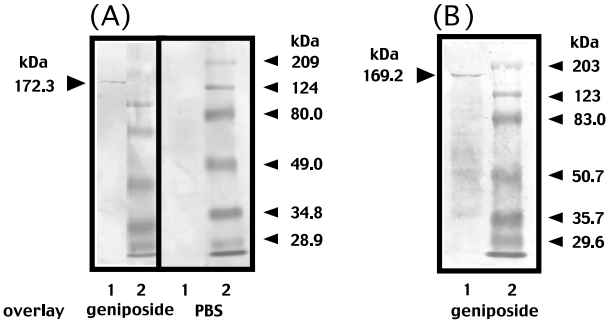

Fig. 2. Detection of Geniposide Binding Proteins in RBCC and in Cytosol of PC12h Cells

RBCC (A , 60 μg protein/lane) or cytosol of PC12h cells (B, 50 μg protein/lane) was applied to each lane, separated by SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was incubated for 1 hr with 0.1 mg/ml geniposide in PBS or PBS only. Geniposide binding proteins were detected by the geniposide overlay method as described in the "MATERIALS AND METHODS." Lane 2 in each membrane indicates molecular weight standards.

ELISA with various closely-related iridoid compounds, aucubin, gardenoside, and geniposide and its derivatives as competitors (Fig. 1A). As shown in Fig. 1B, in the case of geniposide and Ant-Gen rather high-cross reactions were observed, but the other competitors showed less cross reactivity than geniposide. Thus, the antiserum obtained had higher specificity for the genipin moiety of geniposide and Ant-Gen than aucubin, gardenoside, and the other compounds.

Detection of Geniposide Binding Proteins

As shown in Fig. 2A, the main geniposide-binding protein band with about 172 kDa and several minor bands in RBCC were observed under geniposide overlay, however, it was not observed under PBS overlay (Fig. 2B, right lane). In the cytosolic fraction of PC12h cells, a main protein band with similar molecular weight (about 169 kDa) was also observed (Fig. 2B). These main protein bands observed in RBCC and PC12h cells were not observed when overlay was performed by PBS and when nonimmune rabbit serum was used as primary antiserum (data not shown). Therefore, it seems that the geniposide overlay method is specific and useful for the detection of geniposide binding proteins.

RBCC (40 μg protein) was applied to the gel, separated by SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was cut into two halves prior to incubation of one half with anti-nNOS antibody (lane 1) and the other with 0.1 mg/ml geniposide (lane 2) and detected by Western blotting (lane 1) and by geniposide overlay (lane 2), respectively, as described in the "MATERIALS AND METHODS."

We have previously indicated that the neuritogenic activity of genipin/geniposide was almost completely suppressed by NOS inhibitors.[2] It seems that genipin/geniposide may directly bind to and activate NOS. To clarify the possibility that the target molecule of genipin/geniposide is NOS, we examined whether the main bands (170 kDa) observed in RBCC corresponded with nNOS. In the comparative experiment described in the "MATERIALS AND METHODS," the main band detected by geniposide overlay corresponded closely to the band of nNOS detected by Western blotting (Fig. 3). This suggests that one of the target molecules for genipin/geniposide is nNOS.

Effect of Geniposide on NOS Activity

Since it has been suggested that geniposide directly binds to nNOS, we investigated the effect of geniposide on NOS activity by TLC with RBCC as the enzyme sample. The relative densitometric values of the citrulline spot liberated from arginine were increased in a concentration-dependent manner (Fig. 4A, closed bars) and the relative values were

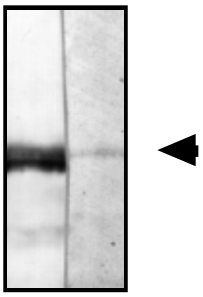

Fig. 3. Detection of nNOS and Geniposide Binding Protein in RBCC

RBCC (40 μg protein) was applied to the gel, separated by SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was cut into two halves prior to incubation of one half with anti-nNOS antibody (lane 1) and the other with 0.1 mg/ml geniposide (lane 2) and detected by Western blotting (lane 1) and by geniposide overlay (lane 2), respectively, as described in the "MATERIALS AND METHODS."

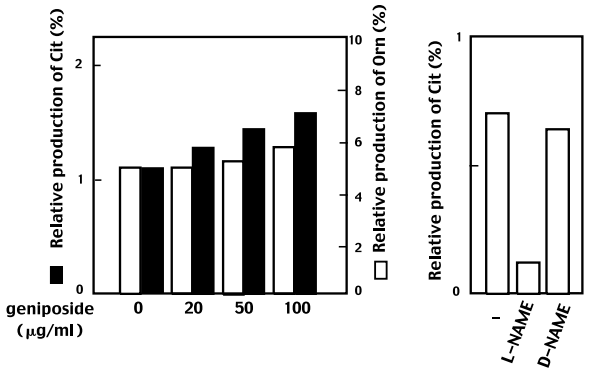

Fig. 4. Effect of Geniposide on NOS Activity

NOS assay was performed with RBCC (1 mg protein) and various concentrations of geniposide at pH 7.4 as described in the "MATERIALS AND METHODS." (A) Values of the relative production of ornithine (Orn., open bar) and citrulline (Cit., closed bar) were calculated from each TLC pattern. (B) Values of the relative production of Cit. were obtained in the absence (PBS) or presence of 10 mM L-NAME and D-NAME.

selectively depressed by a specific NOS inhibitor (L-NAME) but not by an inactive isomer (D-NAME) (Fig. 4B). Since NOS and arginase use L-arginine as a common substrate, competition between both enzymes for the substrate may be involved in the modulation of NOS activity.[6] However, geniposide did not inhibit arginase activity evaluated by the relative ornithine spot (Fig. 4A, open bars). These observations, together with the results of geniposide overlay, suggest that geniposide directly binds to NOS molecules, probably nNOS, and activates the enzyme. Although further examination is required to obtain an understanding of the detailed mechanisms of NOS activation by binding of genipin/geniposide, the results strongly suggest that NOS is an important candidate of the target molecules for the neuritogenic activity of these iridoids.

Acknowledgements We would like to thank Dr. Ogura (National Cancer Center Research Institute, Japan) for the supply of anti-NOS antibody. This work was supported in part by grants of special research fund of Hokuriku University.

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