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Neuritogenic activity of a genipin derivative in retinal ganglion cells is mediated by retinoic acid receptor β expression through nitric oxide/S-nitrosylation signaling

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Running title: IPRG001 induces axonal outgrowth *via* RARβ induction

Abbreviations:

c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide;

DAF-2DA, Diaminofluorescein–2 Diacetate;

DTT, dithiothreitol;

HDAC2, Histone diacetylase 2;

IOP, intraocular pressure;

IPRG001, (1R)-*iso*Propyloxygenipin;

KT5823, N-methyl-(8R, 9S, 11S)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-3,10-dihydro-8-11-epoxy-1H, 8H, 11H-2.7b,11a-triazadibenzo(a,g)cycloocta(cde) trinden-1-one

LE540, 4-(13H-10,11,12,13-tetrahydro-10,10,13,13,15-pentamethyldinaphtho[2,3-b][1,2-e]diazepin-7-yl) benzoic acid

MTT, 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide;

NAME, NG-nitro-L-arginine methyl ester;

NO, nitric oxide;

NOR1, (±)-(E)-4-Methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide;

nNOS, neural nitric oxide synthase;

PKG, cGMP-dependent protein kinase;

RARβ, retinoic acid receptor β

RGC, retinal ganglion cell;

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Abstract

Genipin, a herbal iridoid, is known to have both neuroprotective and neuritogenic activity in neuronal cell lines. As it is structurally similar to tetrahydrobiopterin, its activity is believed to be nitric oxide (NO)-dependent. We previously proposed a novel neuroprotective activity of a genipin derivative, (1R)-*iso*Propyloxygenipin (IPRG001), whereby it reduces oxidative stress in RGC-5, a neuronal precursor cell line of retinal origin through protein S-nitrosylation. In the present study, we investigated another neuritogenic property of IPRG001 in RGC-5 and retinal explant culture wherein we focused on the NO-cGMP-dependent and protein S-nitrosylation pathways. IPRG001 stimulated neurite outgrowth in RGC-5 and retinal explant culture through NO-dependent signaling, but not NO-dependent cGMP signaling. Neurite outgrowth with IPRG001 requires retinoic acid receptor β (RARβ) expression, which is suppressed by an RAR blocking agent and siRNA inhibition. Thereby, we hypothesized that RARβ expression is mediated by protein S-nitrosylation. S-nitrosylation of histone deacetylase 2 (HDAC2) is a key mechanism in chromatin remodeling leading to transcriptional gene activation. We found a parallelism between S-nitrosylation of HDAC2 and the induction of RARβ expression with IPRG001 treatment. The both neuroprotective and neuritogenic activities of genipin could be a new target for the regeneration of RGCs after glaucomatous condition.

Introduction

Genipin, a herbal iridoid, has been known to have both neuroprotective and neuritogenic activity in neural PC12h (Yamazaki et al., 1996, 2001a and 2004) and Neuro2a cells (Yamazaki and Chiba, 2005, Yamazaki et al., 2008).

We previously demonstrated the neuroprotective activity of genipin in PC12h and Neuro2a cells against 6-hydroxydopamine, hydrogen peroxide and serum-free conditions (Yamazaki et al., 2001b, 2008). The underlying toxicity of these insults is partly ascribed to reactive oxygen species. To study of the trophic effects of genipin *in vivo*, we produced (1R)-*iso*Propyloxygenipin (IPRG001), a more stable derivative to genipin (Suzuki et al., 2010). We recently proposed a novel antioxidative pathway involving IPRG001-induced neuroprotection against toxic insults in RGC-5 and retinal ganglion cells (RGCs) both *in vitro* and *in vivo* (Koriyama et al., 2010). Genipin induces NO generation in RGC-5 through its binding to neuronal nitric oxide synthase (nNOS, Ohkubo et al., 2004; Suzuki et al., 2007) and its structural similarity to tetrahydrobiopterin (Suzuki et al., 2010). NO S-nitrosylates kelch-like ECH-associated protein1 (Keap1), which facilitates NF-E2-related factor2 (Nrf2) translocation to the nucleus, where it binds antioxidative response elements (AREs) and induces the transcription of antioxidative protein genes, such as heme oxygenase-1 (HO-1). This was the first report of the activation of Nrf2/ARE signaling in antioxidative protection in neural tissue (Koriyama et al., 2010).

In previous studies, we proposed that nitric oxide (NO)-activated protein kinase activity is involved in genipin-induced neuritogenesis. NO donor and cGMP promoted neurite outgrowth in PC12h cells, whereas NO synthase inhibitor, NO scavenger and cGMP-dependent protein kinase (PKG) inhibitor all inhibited genipin activity (Yamazaki et al. 2001a, 2004). These results strongly indicate that the neuritogenic

activity of genipin involves NO-dependent PKG signaling.

The aim of this study was to investigate (i) whether genipin promotes neurite outgrowth in RGC-5 and retinal explant culture and (ii) whether the molecular mechanism behind its activity is dependent on the NO-cGMP pathway.

Herein, we report that, IPRG001 significantly promotes neurite outgrowth at 5-20 μM in RGC-5 cells and adult rat RGCs. The induction of neurite outgrowth by genipin in RGC-5 was not mediated by the NO-cGMP-PKG pathway, but did increase in retinoic acid receptor β (RARβ) expression and NO/protein S-nitrosylation. In this study, we propose a novel mechanism of neuritogenic RARβ expression through NO/protein S-nitrosylation signaling in RGC-5. We also suggest a candidate protein that may undergo S-nitrosylation and induce the expression of RARβ, thereby promoting neurite outgrowth. Both the neuritogenic and neuroprotective effects of IPRG001 that have been identified in our studies may have therapeutic potential for the treatment of neurodegenerative disorders, such as glaucoma.

Materials and methods

Chemicals

Genipin and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (1R)-*iso*Propyloxygenipin (IPRG001) was synthesized from genipin, as previously described (Suzuki et al., 2010), and dissolved in dimethyl sulfoxide. 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide sodium salt (c-PTIO) and (±)-(E)-4-Methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1), which were obtained from Dojindo (Japan). N-methyl-(8R, 9S, 11S)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-3,10-dihydro-8-11-epoxy-1H, 8H, 11H-2.7b,11a-triazadibenzo(a,g)cycloocta(cde) trinden-1-one (KT5823) was purchased from Biomol Research Laboratories (USA). NG-nitro-L-arginine methyl ester (L-NAME) and NG-nitro-D-arginine methyl ester (D-NAME) were purchased from Sigma-Aldrich (St. Louis, USA). 4-(13H-10,11,12,13-tetrahydro-10,10,13,13,15-pentamethyldinaphtho[2,3-b][1,2-e]diaz epin-7-yl) benzoic acid (LE540) was developed by one of the authors, Dr. Hiroyuki Kagechika (Umemiya *et al.* 1997).

Cell culture

RGC-5 cells were originally produced by Dr. N. Agarwal of University of North Texas Health Science Center and were received from a line maintained by Dr. H. Hara, at Gifu Pharmaceutical University. RGC-5 cells (number of passage, 23-24) were cultured in low-glucose Dulbecco's-modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, as previously described

(Koriyama et al., 2009b). Before the experiments, we checked the lot number of serum with observation of cell morphology, the response to staurosporine and assessment of the cell survival (data not shown). The cells were passaged by trypsinization every 3-4 days. RGC-5 cells (5×10^3 cells/ml) were cultured overnight prior to use. After washing with DMEM, cells were cultured in medium containing 1% FBS and 400 nM staurosporine to prevent over-proliferation and differentiation (Ganapathy et al., 2010). The effects of IPRG001 and other substances were observed by their addition to the culture medium for 24 h.

Neurite outgrowth

Neurite outgrowth of RGC-5 cells were observed by phase-contrast microscopy and assessed by NeuronJ imaging software (http://rsb.info.nih.gov/ij). To quantify neurite outgrowth, 20 random images were obtained per plate, and cells bearing processes 2-fold longer than the cell body were considered as positive. Over 100 cells were quantified and each data point corresponded to the average of five independent dishes (Iwasaki et al., 1999). The average neurite length from three independent experiments was expressed as the mean ± SEM. We did experiments with masking test by two co-authors.

Measurement of NO production

4, 5-diaminofluorescein diacetate (DAF-2DA; Daiichi Pure Chemicals, Tokyo) was used as an indicator of NO production. RGC-5 cells were cultured and washed twice with Earle's balanced salt solution (EBSS), treated with IPRG001 for 1 h at 37°C, following which DAF-2DA (10 μM) was added to the culture medium for 1 h. The samples were then washed with EBSS and centrifuged at 100 g for 5 min at room

temperature. The supernatants were discarded and 500 μl of EBSS was added to the cell pellet. The fluorescence intensity of each cell suspension was measured by emission at 515 nm and excitation at 495 nm using a Fluoroskan Ascent plate reader (Labsystems, Finland).

Rat retinal explant culture

Sprague–Dawley male rats (body weight, 250–300 g) were used throughout this study. We certified that all experiments on animals were performed in accordance with the guidelines for animal experiments of Kanazawa University. Rat retinal explant cultures, were performed under anesthesia by sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan) before sacrificing, as previously described (Sugitani et al., 2006). Retinal explants were cut into small pieces (0.5 mm) and cultured in medium containing 45% DMEM, 45% Ham's F12 Nutrient Mixture (Sigma-Aldrich, St Louis, MO), 10% FCS and penicillin–streptomycin (100 U/ml) in collagen gel (Cellmatrix; Nitta Gelatin, Osaka, Japan) on a 35 mm culture dish. Neurite outgrowth from the retinal explants was assayed by measuring the length of neurites in each explant from a total of 30-40 explants per dish. Finally, the percentage of explants showing positive neurite outgrowth (>200 μm) was compared under various culture conditions. Four independent experiments were counted.

MTT assay

Cell survival was estimated using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. An aliquot (20 μl) of 2.75 mg/ml MTT in phosphate-buffered saline (PBS) was added to the culture medium as previously described (Koriyama *et al*. 2003). The reaction mixtures were incubated at 37°C for 3 h,

prior to adding 200 μl HCl/isopropanol. The resultant formazan was measured by its absorbance at 550 nm using a plate reader (Model 680, Bio-Rad Laboratories, USA). All experiments were repeated at least three times and compared with control conditions.

Subcellular fractions of RARβ protein

Cells were lysed by hypotonic lysis buffer containing 10 mM HEPES-KOH (pH7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatants were used as the cytoplasmic fraction and the pellets were incubated with a nuclear lysis buffer containing 20 mM HEPES-KOH (pH7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 5% glycerol, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) for 30 min on ice. The lysates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants contained the nuclear fraction. Immunoblotting analysis of β-actin and histone H4 was performed to ensure no contamination between cytoplasmic and nuclear fractions.

Western blot analysis

RGC-5 cells, cultured under various conditions, were extracted and 30 μg of protein were subjected to polyacrylamide gel electrophoresis using a 12.5% gel as previously described (Koriyama *et al*. 2009a). The separated proteins were transferred to a nitrocellulose membrane and incubated with primary and secondary antibodies (Santa Cruz Biotechnology, USA). Protein bands (nNOS, RARβ, HDAC2) were detected using a BCIP/NBT Kit (KPL, USA). An antibody against β-actin was used as an internal standard. Protein bands isolated from cells cultured under various conditions were

analyzed densitometrically using Scion Image Software (Scion, USA). All experiments were repeated at least three times.

siRNA for RARβ gene

Small interfering RNA (siRNA) for the target region of RARβ mRNA were as follows: 5'-GGAGCCGACUGCAAAUACAAG-3' (sense); 5'-UGUAUUUGCAGUCGGCUCCAA-3' (antisense) (Sigma-Aldrich, Japan); and randomly shuffled sequence: 5'-AGUCGUCGUAUACGGUAUAUC -3' (sense); 5'-UAUACCGUAUACGACGACUUC -3' (antisense). Transfection of siRNA into RGC-5 cells was performed using Lipofectamine 2000 (Invitrogen, USA). Knockdown of RARβ expression in RGC-5 cells was performed by incubating with siRNA (100 pmol) for 24 h.

S-nitrosylation analysis of HDAC2

S-nitrosylation of HDAC2 was assessed by a modified biotin switch assay (Jaffrey et al. 2001) using the S-nitrosylated Protein Detection Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). RGC-5 cells, exposed to 50 μM NOR1 or 20 μM IPRG001 for 1 h, were harvested and lysed with lysis buffer at 4°C. Free thiols were blocked by S-methyl methanethiosulfonate. Biotinylation of nitrosothiols was performed by maleimide-biotin. Biotinylated proteins were further purified by overnight incubation with Neutravidin-coupled agarose beads (Pierce-Thermo Scientific). Following incubation, beads were washed three times with PBS. Isolated proteins were recovered from beads by addition of Laemmli sample buffer, and heated at 85°C for 10 min. The quantity of S-nitrosylated HDAC2 protein in the samples was analyzed by western blot

using anti-HDAC2 antibody (Cell Signaling Technology, USA).

Statistics

All results were reported as means ± S.E.M for 3-5 experiments. Differences between groups were analyzed using ANOVA, followed by Dunnett's multi-comparison test with PASW Software (SPSS Inc., USA). P values < 0.05 were considered statistically significant.

Results ←-------- **Fig. 1**

IPRG001 induces neurite outgrowth from RGC-5 cells through nNOS/NO signaling

Firstly, we investigated whether genipin promotes neurite outgrowth in cultured RGC-5 cells. In this study, we used (1R)-*iso*Propyloxygenipin (IPRG001), a chemically-stable and long-acting derivative of genipin (Suzuki et al., 2007).

At 20 μM, IPRG001 significantly enhanced neurite outgrowth from RGC-5 cells within 24 h (Fig. 1b) compared to a no treatment control (Fig. 1a). At 5-20 μM, IPRG001 dose-dependently increased neurite outgrowth in RGC-5 cells *in vitro* (Fig. 1c). Furthermore, at 20 μM, it promoted a 1.5-fold increase in neurite outgrowth in RGC-5 cells as compared to the control.

Next, we investigated the induction of neural nitric oxide synthase (nNOS) protein expression by IPRG001. At 5-20 μM, IPRG001 significantly increased nNOS protein levels in RGC-5 cells within 1 h (Fig. 1d). At 20 μM, it increased nNOS protein levels 1.6-fold more than the control. The levels of β-actin protein did not change at any concentrations of IPRG001 used. We further investigated the effects of IPRG001 on NO production in RGC-5 cells. At 20 μM, it caused an increase in the fluorescent intensity of DAF-2DA, a fluorescent indicator of NO production, within 1 h (Fig. 1g) compared to the no treatment control (Fig. 1e). Figures 1f and 1h show RGC-5 cell nuclei counter-stained with DAPI. At 5-20 μM, IPRG001 dose-dependently increased the fluorescent intensity of DAF-2DA in RGC-5 cells within 1 h (Fig. 1i).

←-------- **Fig. 2**

Confirmation of NO-dependent neurite outgrowth by IPRG001 in the RGC-5 cells.

The neurite outgrowth induced by IPRG001 (Fig. 2b) was completely blocked by treatment with 10 mM of L-NAME, a NOS inhibitor, within 24 h (Fig. 2c), where neurite outgrowth was comparable to the no treatment group (Fig 2a). NO production

induced by 20 μM IPRG001 (Fig. 2e) was also suppressed with 10 mM L-NAME within 1 h (Fig. 2f) to similar levels as the control (Fig. 2d). Neurite outgrowth and NO production in the presence of NOS inhibitor are shown in Fig. 2g and 2h, respectively. At 10 mM D-NAME, a non-functional isomer of L-NAME, there was no inhibition of these activities. L-NAME or D-NAME alone did not affect either activity (Fig. 2g and 2h). Furthermore, 20 μM NOR1, a NO donor, significantly induced neurite outgrowth in RGC-5 cells within 24 h (Fig. 2j) compared to the control (Fig. 2i). Quantification of NOR1-promoted neurite outgrowth is shown in Fig. 2k.

←-------- **Fig. 3**

Involvement of cGMP/PKG signaling in neurite outgrowth induced by IPRG001.

To investigate the involvement of cGMP/PKG signaling on IPRG001-induced neurite outgrowth, we used adult rat retinal explant cultures. 1 mM 8Br-cGMP, a membrane-permeable cGMP, did not affect neurite outgrowth in retinal explants (Fig. 3b) as compared to the no treatment control (Fig. 3a). Furthermore, 1 μM KT5823, a PKG inhibitor did not suppress neurite outgrowth induced by 20 μM of IPRG001 (Figs. 3c and d). Quantification of neurite outgrowth from retinal explants is shown in Fig. 3e.

←-------- **Fig. 4**

nNOS/NO signaling-dependent induction of RARβ expression in RGC-5 cells by IPRG001.

Figure 4a shows increased levels of retinoic-acid receptor-β (RARβ) protein in RGC-5 cells induced by IPRG001 at 20 μM within 6 h as compared to the no treatment control. The induction of RARβ by IPRG001 also depends on nNOS/NO production. The increased levels of RARβ protein were completely suppressed by 10 mM of L-NAME (Fig. 4b) and were dose-dependently increased by10-20 μM NOR1 (Fig. 4c).

←-------- **Fig. 5**

IPRG001-induced neurite outgrowth is mediated through RAR activation.

To investigate the involvement of RARβ on neurite outgrowth, we stimulated RGC-5 cells with LE540, a RAR antagonist, to inhibit its activity. Firstly, we determined the toxicity of LE540 on RGC-5 cells by MTT assay. At 0.1-1.0 μM, LE540 did not affect cell viability in this culture system (Fig. 5a). However, increasing concentrations of LE540 to 5-10 μM significantly decreased cell viability. Therefore, we used 0.1-1.0 μM LE540 throughout this study. Neurite outgrowth, induced by 20 μM IPRG001 (Fig. 5d), was completely inhibited by 1 μM LE540 within 24 h (Fig. 5e). LE540 alone did not have any effect on neurite outgrowth (Fig. 5c cf. Fig. 5b). Quantitative analysis is shown in Fig. 5f. LE540 dose-dependently inhibited neurite outgrowth induced by IPRG001.

←-------- **Fig. 6**

Confirmation of RARβ expression on neurite outgrowth induced by IPRG001 siRNA

To further investigate the involvement of RARβ expression on neurite outgrowth by IPRG001 in RGC-5 cells, we used siRNA to knockdown of RARβ expression. siRNA for RARβ mRNA completely suppressed the induction of RARβ protein levels by IPRG001 (Fig. 6a), while scrambled RNA did not have any effect within 6 h. Neurite outgrowth, induced by 20 μM IPRG001 (Fig. 6d), was completely suppressed by siRNA for RARβ (Fig. 6e) but not by scrambled siRNA (Fig. 6c). Quantification of neurite outgrowth is shown in Fig. 6f. siRNA for RARβ and scrambled siRNA alone had no effect on neurite outgrowth as compared to the no treatment control (Fig. 6b).

←-------- **Fig. 7**

Implication of HDAC2 S-nitrosylation on NO-dependent RARβ expression by IPRG001

We investigated the molecular mechanism behind RARβ expression induced by

IPRG001 through nNOS/NO signaling. As the cGMP/PKG system did not participate in neurite outgrowth by IPRG001 (Fig. 3), another NO-dependent signaling mechanism was proposed for this study. We tested the ability of protein S-nitrosylation by IPRG001 on RARβ expression. Dithiothreitol (DTT) is known to reverse SNO modifications of multiple proteins. (Hausladen et al., 1996, Kim et al., 1997, Xu et al., 1998, Pei et al., 2008) Firstly, we investigated the effect of DTT on IPRG001-induced RARβ expression by IPRG001. Induction of RARβ expression by IPRG001 was significantly blocked with 1 mM DTT (Fig. 7a). This indicates that IPRG001-induced RARβ expression may be implicated in protein S-nitrosylation. Next, we examined whether HDAC2 is S-nitrosylated by IPRG001. Indeed, there was 1.6-fold increase in HDAC2 S-nitrosylation by IPRG001 (20 μM) within 1 h, as compared to a no treatment control (Fig. 7b). This increase is nNOS/NO-dependent, as it was blocked by use of the NO scavenger, c-PTIO, at 100 μM and was induced by the NO donor, NOR1, at 50 μM (Fig. 7b). Furthermore, IPRG001-induced increases in S-nitrosylated HDAC2 proteins were significantly blocked by DTT at 1 mM (Fig. 7c).

←-------- **Fig. 8**

NO- and RARβ-mediated neurite outgrowth from adult rat retinal explants induced by IPRG001

In adult rat retinal explant cultures, 20 μM IPRG001 significantly induces neurite outgrowth within 5 days (Fig. 8b also see Fig. 3) as compared with a no treatment control (Fig 8a). This increase in neurite outgrowth by IPRG001 was completely suppressed by 10 mM L-NAME (Fig. 8c) or 1 μM LE540 (Fig. 8d). IPRG001 increased the number of explants with long neurites 2.6-fold compared to the no treatment control (Fig. 8e). L-NAME or LE540 alone did not have any effects on neurite outgrowth from rat retinal explants (Fig. 8e).

Discussion

IPRG001 induces neurite outgrowth from RGC-5 through nNOS/NO signaling

At concentrations of 5-20 μM, (1R)-isoPropyloxygenipin (IPRG001) dose-dependently stimulates neurite outgrowth from RGC-5 cells within 24 h. RGC-5 cells were treated with staurosporine to arrest cell growth and induce cell differentiation (Lieven et al., 2007). RGC-5 cells are known as neuronal precursor cells of retinal origin, not as retinal ganglion cell lines. Recently, Van Bergen et al. (2009) reported the re-characterization of RGC-5 cells. They reported that RGC-5 cells, cultured in succinyl concanavalin A (S Con A), no longer express the RGC marker protein Thy1.2. However, when differentiated by staurosporine, RGC-5 cells express the RGCs markers, Thy1 and the NMDA receptor (Frassetto et al., 2006, Hironaka et al., 2011). Van Bergen et al. also reported that staurosporine-induced RGC-5 cells resemble retinal ganglion cells (2009). Thus, we used staurosporine not S Con A as a reagent for RGC-5 differentiation in this study as a comparable *in vitro*.

IPRG001 (20 μM) induced neurite outgrowth by 1.6-fold compared to the non-treatment control in a nNOS/NO-dependent manner. IPRG001 directly induced nNOS and NO production, as revealed by DAF-2DA fluorescence within 1 h. This early induction of nNOS/NO signaling (0.5-1 h) is the first step in the neurotrophic activity of IPRG001 (Figs. 1 & 2). Furthermore, neurite outgrowth and NO production were completely blocked by L-NAME, whereas NOR1, a NO donor, stimulated neurite outgrowth within 24 h. We previously reported the stimulation of NO production in RGC-5 cells within 0.5-4 h after IPRG001 exposure (Koriyama et al., 2010). Because of the structural similarities between IPRG001 and tetrahydrobiopterin, a cofactor of nNOS enzymatic activity (Suzuki et al., 2007), genipin directly binds to nNOS and activates it. Although we do not know the exact mechanism behind genipin-induced

nNOS expression, we previously showed an increase in nNOS expression in PC12h cells within 4 h of exposure to genipin (Yamazaki et al. 2001a). We also have data showing that IPRG001 increases NADPH diaphorase staining and nNOS expression in adult rat RGCs (Y. Koriyama unpublished data).

Recently, there have been reports detailing different functions of genipin, including its use as a crosslinking reagent for gelatin and a bioadhesive wound dressing (Sung et al., 1999, Shen et al., 2011). Although, the crosslinking mechanism of genipin is not well understood, Touyama et al., (1994) reported that the reaction begins through a nucleophilic attack of the primary amine on the C3 carbon of genipin. Then, the dihydropyrane ring opens and the secondary amine attacks the resulting aldehyde group. However, IPRG001 is more stable than genipin (Suzuki et al., 2010) and the dihydropyran ring is hard to open. Thus, in this study, the possibility that IPRG001 induces neurite outgrowth by a crosslinking reaction with endogeneous molecules is low.

IPRG001 induces neurite outgrowth from RGCs through RARβ expression.

Firstly, we tested whether the NO-dependent neurite outgrowth is mediated through cGMP/PKG signaling, as we previously reported in PC12h and Neuro2a cells (Yamazaki et al., 2001a, Yamazaki and Chiba, 2005). Because staurosporine is non-specific protein kinase inhibitor including PKG, we used adult rat retinal explant culture which can ignore the effect of staurosporine. Even in the presence of staurosporine, IPRG001-induced neurite outgrowth in RGC-5 cells (Fig. 1b and C). In addition, the neurite outgrowth from rat RGCs was not mediated by cGMP/PKG signaling in retinal explant cultures (Fig. 3) unlike PC12h and Neuro2a cells. In contrast, neurite outgrowth in the RGC-5 cells was significantly dependent on RARβ expression.

We focused on RARβ protein expression for NO-dependent neurite outgrowth by IPRG001, as it is well-known that retinoic acid and RARβ play major roles in cell differentiation of human neuroblastoma cells (Cheung et al., 1996) and neuritogenesis in developing neurons (Corcoran et al., 2002, Agudo et al., 2010, Shiohara et al., 2010) and regenerating zebrafish retina after optic nerve injury (Nagashima et al., 2009). RARβ, which is involved in the developing rat retina, may have regenerative potential in optic nerve injury. However, this regenerative ability decreases with age (Kalil and Reh, 1979, Mori et al., 2001, Symonds et al., 2001). Our present study investigated the induction of RARβ in adult rat RGCs, further supporting its importance in neurite outgrowth (Fig. 8). Moreover, there is increasing evidence that overexpression of RARβ in injured spinal cord may promote the regeneration of CNS neurons (Corcoran et al., 2002, Wong et al., 2006, Yip et al., 2006). We preliminarily screened upregulated genes by microarray analysis of Neuro2a cells treated with genipin for 4 h (K. Chiba unpublished data). Among these results, RARβ was the more highly expressed gene. IPRG001 (20 μM) induced a 1.5-fold increase in RARβ protein expression within 6-24 h and this upregulation was NO-dependent. L–NAME clearly inhibited RARβ expression and NOR1 oppositely induced a significant RARβ expression with a slight reduced level as compared to IPRG001 (Fig. 4c). The pivotal function of RARβ on neurite outgrowth was further confirmed using a RAR blocker, LE540, and siRNA for RARβ (Figs. 5 and 6).

←-------- Fig. 9

IPRG001 induces RARβ expression associated with protein S-nitrosylation in RGC-5 cells

In the present study, we proposed a novel neuritogenic effect of IPRG001 in RGC-5 cells through the S-nitrosylation of a candidate protein, HDAC2. Inhibition of HDAC2

is well known to cause chromatin remodeling leading to transcriptional activation of genes, including RARβ (Qian et al., 2005, De los Santos et al., 2007, Watson et al., 2009). Furthermore, Nott et al. (2008) reported that S-nitrosylation of HDAC2 induced chromatin remodeling in neural cells similarly to HDAC2 inhibition. Owing to these findings, we hypothesized that S-nitrosylation of HDAC2 by IPRG001 through nNOS/NO signaling could induce RARβ expression in RGC-5 cells. IPRG001 (20 μM) induced a 1.6-fold increase in the S-nitrosylation of HDAC2 within 1 h compared to a no treatment control. The S-nitrosylation of HDAC2 was also NO-dependent. It was significantly blocked by a NO scavenger and was induced by a NO donor (Fig. 7). RARβ expression was blocked by the S-nitrosylation inhibitor, DTT (Fig. 7). We also have data showing that IPRG001 acetylates histone H3, within 90-120 min after treatment in RGC-5 cells (Y. Koriyama unpublished data). Together, this indicates that IPRG001 initially generates NO gas through nNOS/NO signaling within 0.5-1 h. S-nitrosylation of HDAC2 occurs within 1 h after IPRG001 exposure, and thus activates RARβ expression within 6 h and finally induces significant neurite outgrowth from RGC-5 cells within 24 h (Fig. 9). Neurite outgrowth by IPRG001 and RARβ expression was further confirmed in adult rat RGCs over 5 days of culture (Figs. 3 & 8). As cGMP/PKG signaling was not involved in IPRG001-induced neurite outgrowth from retinal explants, we developed an alternative hypothesis involving protein S-nitrosylation. cGMP/PKG signaling is activated by genipin in PC12h and Neuro2a cells, and fish RGCs during optic nerve regeneration to induce neurite outgrowth (Koriyama et al., 2009a). Therefore, further study is required to elucidate why murine RGCs differ from these other neural models. An increasing number of proteins have been identified as being activated by NO signaling-mediated S-nitrosylation, including cell survival ion channels and receptor-mediated signal transduction proteins (Ahern et

al., 2002, Hess et al., 2005). In this study, we reported for the first time that the neuritogenic RARβ protein was transcriptionally upregulated concurrently with chromatin remodeling following S-nitrosylation of HDAC2 by IPRG001 in murine RGC-5 and RGCs both *in vitro* and *in situ*.

In future, both the neuroprotective and neuritogenic actions of IPRG001 may be shown to have neuroregenerative activity for the treatment of RGC-5 and RGCs degeneration and glaucoma.

Acknowledgments

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Conflict of interest statement

The authors declare no conflicts of interests related to this work.

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Figure legends

Fig. 1 IPRG001-induced neurite outgrowth and nitric oxide (NO) production in RGC-5 cells

(a-b) Photomicrographs of neurite outgrowth after treatment with IPRG001. (a) No treatment. (b) 20 μM IPRG001. (c) Quantification of neurite outgrowth in RGC-5 cells. Scale = 20 μm. (n=100) (d) Expression of nNOS protein induced by various concentrations of IPRG001 in RGC-5 cells. (e-i) NO production levels by IPRG001 in RGC-5 cells were evaluated by DAF-2DA staining (green fluorescence). (e) No treatment. (g) 20 μM IPRG001. (f, h) RGC-5 cell nuclei were stained with DAPI. Scale = 20 μm. (i) Quantification of fluorescence intensity of DAF-2DA measured by fluorescence plate reader. *P < 0.05, **P < 0.01 vs no treatment (n=4).

Fig. 2 Nitric oxide (NO)-dependent neurite outgrowth by IPRG001

(a-c) Photomicrographs of neurite outgrowth promotion in RGC-5 cells. (a) No treatment. (b) 20 μM IPRG001. (c) 20 μM IPRG001 plus 10 mM L-NAME, Scale = 20 μm. (d-f) Photomicrographs of NO production measured by DAF-2DA (green). (d) No treatment. (e) 20 μM IPRG001. (f) 20 μM IPRG001 plus 10 mM L-NAME, Scale = 20 μm. (g) Quantification of neurite outgrowth by IPRG001. L-NAME completely inhibited neurite outgrowth promotion by IPRG001. *P < 0.01 vs no treatment. (n=100) (h) Quantification of NO production (intensity of DAF2DA). *P < 0.01 vs no treatment. (n=4) (i & j) Photomicrographs of NOR1-induced neurite outgrowth. (i) No treatment. (j) 20 μM NOR1. (k) Quantification of NOR1-induced neurite outgrowth. *P < 0.01 vs no treatment. (n=100)

Fig. 3 IPRG001-induced neurite outgrowth is not mediated by the cGMP/PKG pathway

in adult rat retinal explant culture

(a, b) Photomicrographs of neurite outgrowth in adult rat retinal explants. (a) No treatment, (b) 1 mM 8Br-cGMP. Scale = 100 μm. (c) 20 μM IPRG001, (d) 20 μM IPRG001 plus 1 μM KT5823, a PKG inhibitor. (e) Quantification of PKG inhibitor effects on neurite outgrowth by IPRG001 with cGMP-PKG-related substances.

Fig. 4 Characterization of RARβ expression by IPRG001 treatment in RGC-5 cells.

(a) Time course of RARβ expression after treatment with IPRG001. (open circles) No treatment (control); (solid circles) 20 μM IPRG001. *P < 0.01 vs no treatment. (b) RARβ expression by IPRG001 is dependent on NOS signaling. L-NAME significantly suppresses the expression of RARβ by IPRG001. *P < 0.01 vs no treatment, +P < 0.01 vs 20 μM IPRG001 (n=3). (c) RARβ expression by NOR1, a NO donor in RGC-5 cells. NOR1 dose-dependently stimulated RARβ expression. *P < 0.01 vs no treatment (n=3).

Fig. 5 RAR antagonist inhibited neurite outgrowth by IPRG001 in RGC-5 cells

(a) Cell viability of RGC-5 cells treated with LE540, a retinoic acid receptor antagonist. *P < 0.01 vs 0 μM LE540, (n=6). (b-e) LE540 inhibited neurite outgrowth by IPRG001. (b) No treatment. (c) 1 μM LE540. (d) 20 μM IPRG001. (e) IPRG001 plus 1 μM of LE540. Scale = 20 μm. (f) Quantification of neurite outgrowth with LE540. *P < 0.01 vs no treatment, +P < 0.01 vs 20 μM IPRG001 (n=100).

Fig. 6 RARβ-dependent neurite outgrowth by IPRG001 in RGC-5 cells.

(a) Decreased levels of RARβ proteins following treatment with RARβ-specific siRNA compared to no treatment (PBS only) or scrambled siRNA. *P < 0.01 vs. no treatment (n=3). (b-e) Photomicrographs of neurite outgrowth by IPRG001 following siRNA

knockdown of RARβ. (b) No treatment. (c) Scrambled siRNA. (d) IPRG001. (e) IPRG001 plus siRNA for RARβ. Scale = 20 μm. (f) RARβ-specific siRNA significantly inhibited neurite outgrowth by IPRG001 in RGC-5 cells. *P<0.01 vs. PBS treatment (n=100).

Fig. 7 IPRG001 stimulates RARβ expression and S-nitrosylation of HDAC2. (a) DTT significantly inhibited RARβ expression by IPRG001. *P < 0.01 vs. no treatment; +P < 0.01 vs 20 μM IPRG001 (n=3). (b) S-nitrosylation of HDAC2 by IPRG001. RGC-5 cells were exposed to 20 μM IPRG001 for 1 h and analyzed by biotin-switch assay. Biotinylated proteins were mixed with avidin-beads, eluted and analyzed by western blot with anti-HDAC2 antibody. NOR1, but not c-PTIO, S-nitrosylates HDAC2 protein *P < 0.01 vs. no treatment (n=3). (c) IPRG001-induced S-nitrosylation was inhibited by DTT. *P < 0.01 vs. no treatment (n=3).

Fig. 8 IPRG001 promotes neurite outgrowth in adult rat retinal explants culture. (a) Neurite outgrowth after 5 days without treatment; scale = 100 μm. (b) 20 μM IPRG001. (c) 20 μM IPRG001 plus LE540. (d) IPRG001 plus 10 mM L-NAME. (e) Graphical representation of neurite outgrowth. *P < 0.01 with no treatment, +P < 0.01 vs 20 μM IPRG001 (n=6).

Fig. 9 Schematic diagram for neurite outgrowth by IPRG001 in RGC-5 cells. IPRG001 intially induces nitric oxide (NO) production, followed by S-nitrosylation of HDAC2 and RARβ upregulation. IPRG001 accelerates neurite outgrowth through RARβ induction.

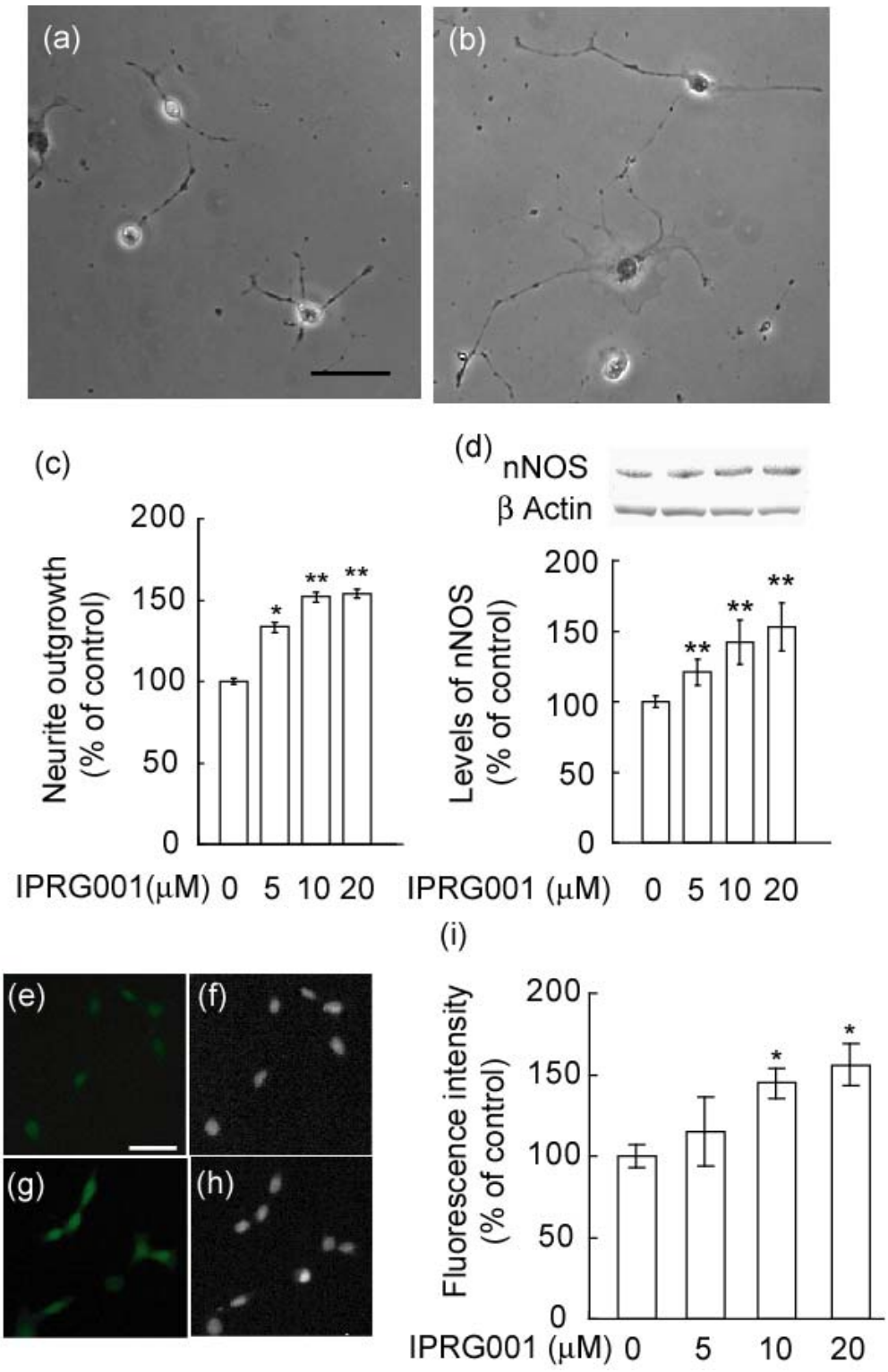
(a)
(b)
(c)
Neurite outgrowth (% of control)
200
150
100
50
0
*
**
**
IPRG001(μM) 0 5 10 20
(d)
nNOS
β Actin
Levels of nNOS (% of control)
200
150
100
50
0
**
**
**
IPRG001 (μM) 0 5 10 20
(e)
(f)
(g)
(h)
(i)
Fluorescence intensity (% of control)
200
150
100
50
0
*
*
IPRG001 (μM) 0 5 10 20

Fig.1

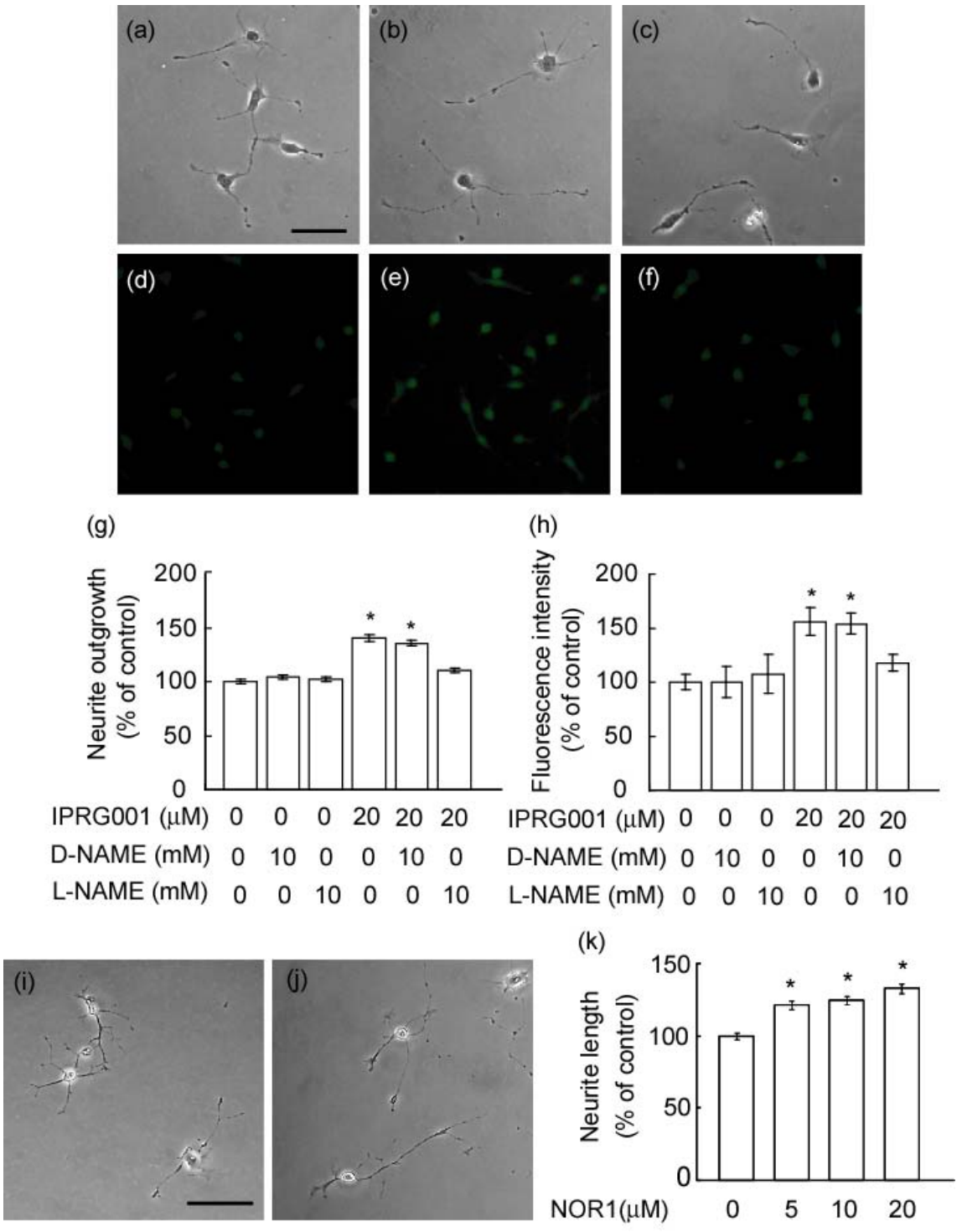
(a)
(b)
(c)
(d)
(e)
(f)
(g)
Neurite outgrowth (% of control)
200
150
100
50
0
*
*
IPRG001 (μM) 0 0 0 20 20 20
D-NAME (mM) 0 10 0 0 10 0
L-NAME (mM) 0 0 10 0 0 10
(h)
Fluorescence intensity (% of control)
200
150
100
50
0
*
*
IPRG001 (μM) 0 0 0 20 20 20
D-NAME (mM) 0 10 0 0 10 0
L-NAME (mM) 0 0 10 0 0 10
(i)
(j)
(k)
Neurite length (% of control)
150
100
50
0
*
*
*
NOR1(μM) 0 5 10 20

Fig.2

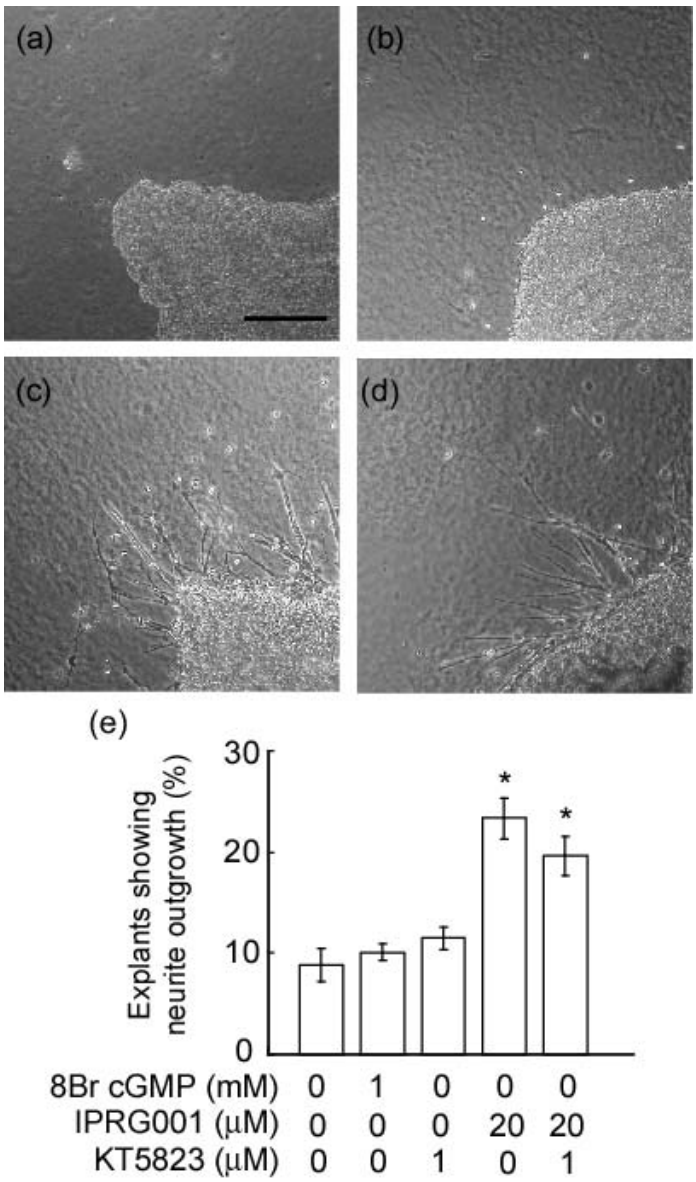
(a)
(b)
(c)
(d)
(e)
Explants showing neurite outgrowth (%)
30
20
10
0
*
*
8Br cGMP (mM) 0 1 0 0 0
IPRG001 (μM) 0 0 0 20 20
KT5823 (μM) 0 0 1 0 1

Fig.3

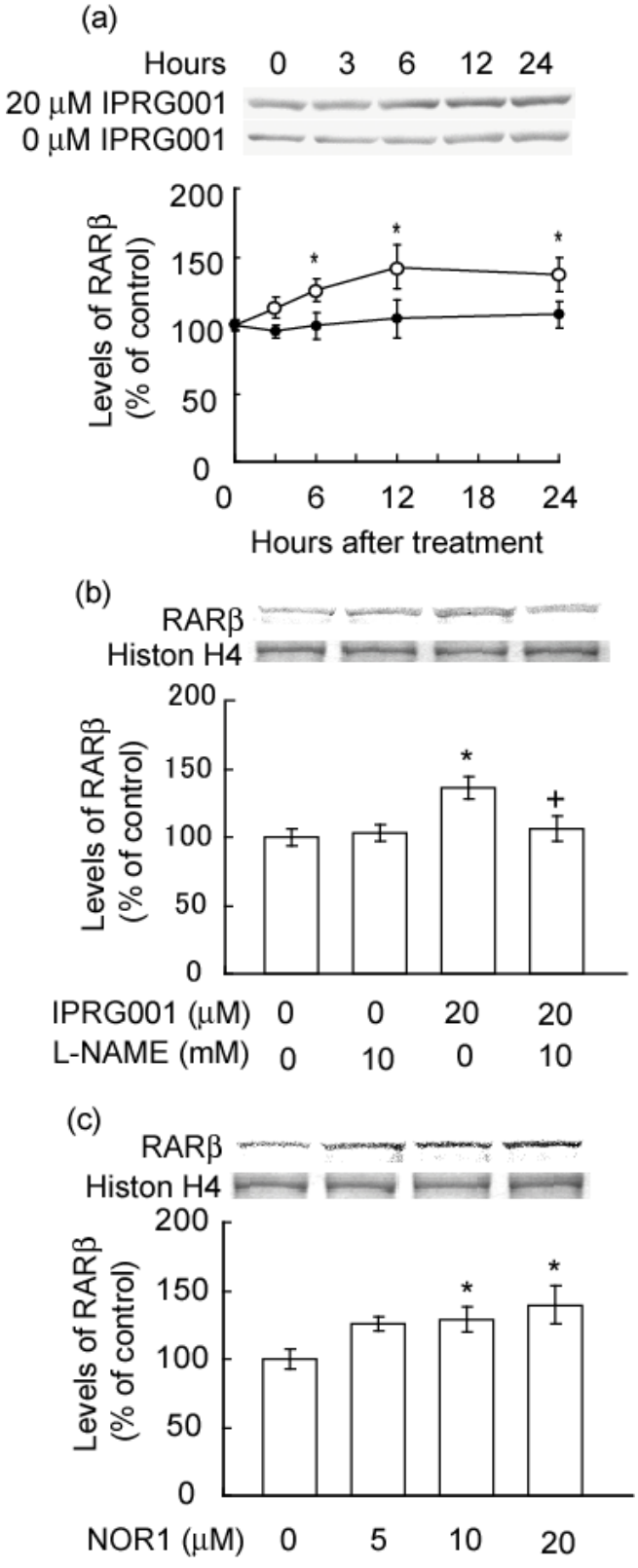
(a)
Hours 0 3 6 12 24
20 μM IPRG001
0 μM IPRG001
Levels of RARβ (% of control)
200
150
100
50
0
0 6 12 18 24
Hours after treatment
(b)
RARβ
Histon H4
Levels of RARβ (% of control)
IPRG001 (μM) 0 0 20 20
L-NAME (mM) 0 10 0 10
(c)
RARβ
Histon H4
Levels of RARβ (% of control)
NOR1 (μM) 0 5 10 20

Fig.4

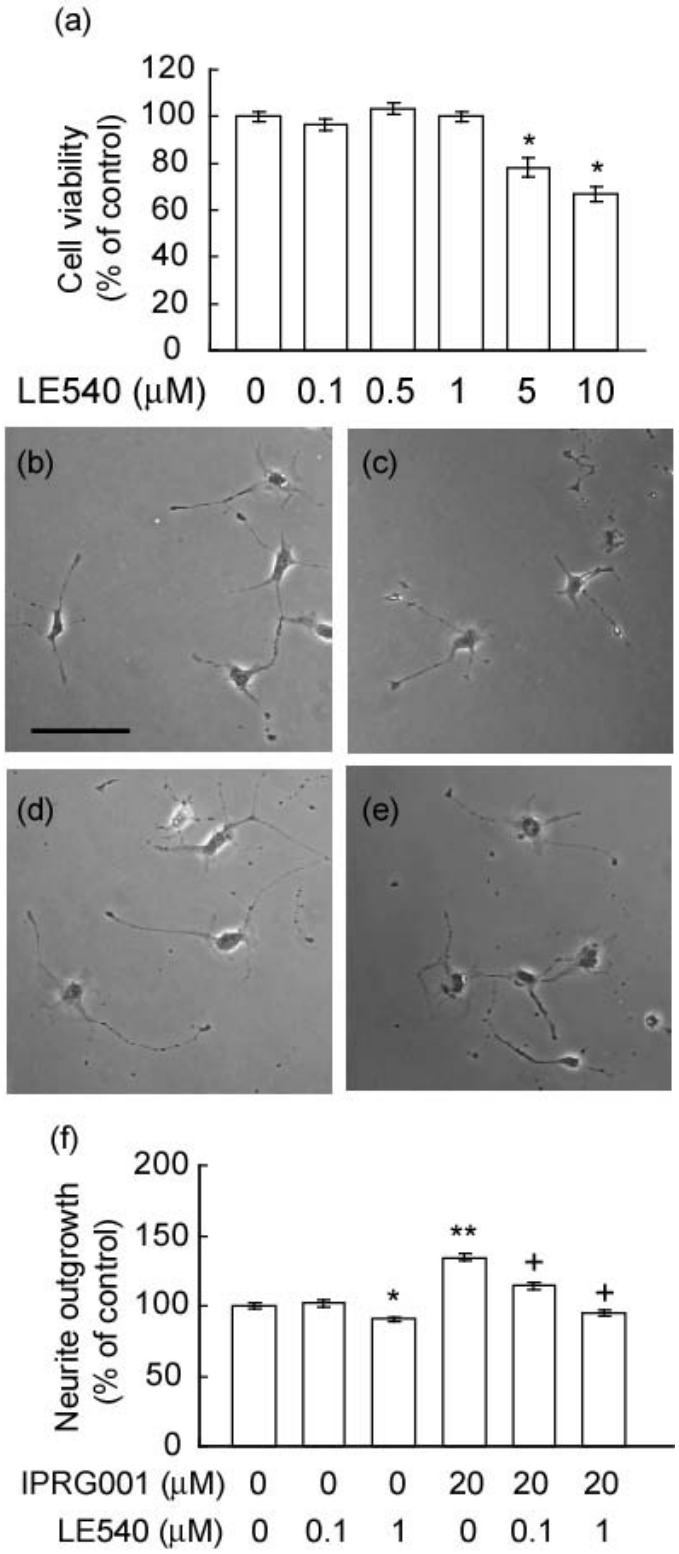
(a)
Cell viability (% of control)
120
100
80
60
40
20
0
*
*
LE540 (μM) 0 0.1 0.5 1 5 10
(b)
(c)
(d)
(e)
(f)
Neurite outgrowth (% of control)
200
150
100
50
0
*
**
+
+
IPRG001 (μM) 0 0 0 20 20 20
LE540 (μM) 0 0.1 1 0 0.1 1

Fig.5

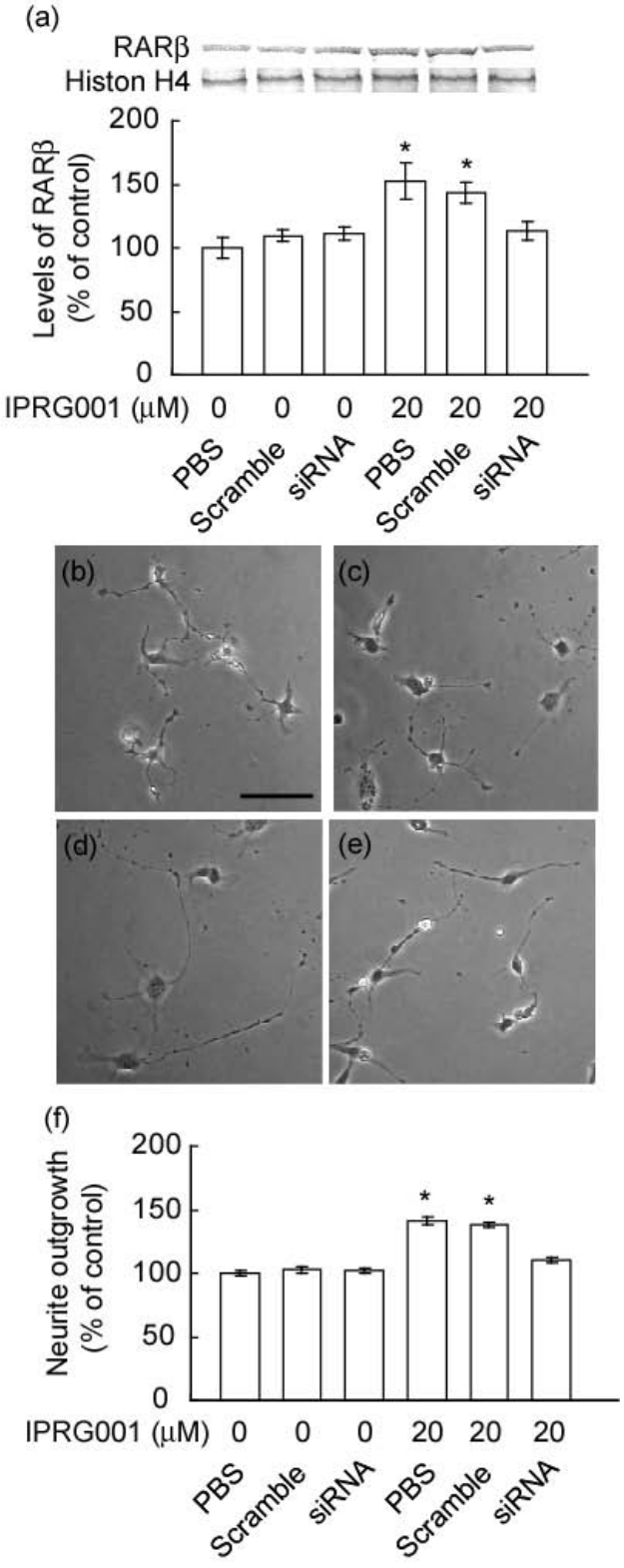
(a)
RARβ
Histon H4
Levels of RARβ (% of control)
200
150
100
50
0
*
*
IPRG001 (μM) 0 0 0 20 20 20
PBS
Scramble
siRNA
PBS
Scramble
siRNA
(b)
(c)
(d)
(e)
(f)
Neurite outgrowth (% of control)
200
150
100
50
0
*
*
IPRG001 (μM) 0 0 0 20 20 20
PBS
Scramble
siRNA
PBS
Scramble
siRNA

Fig.6

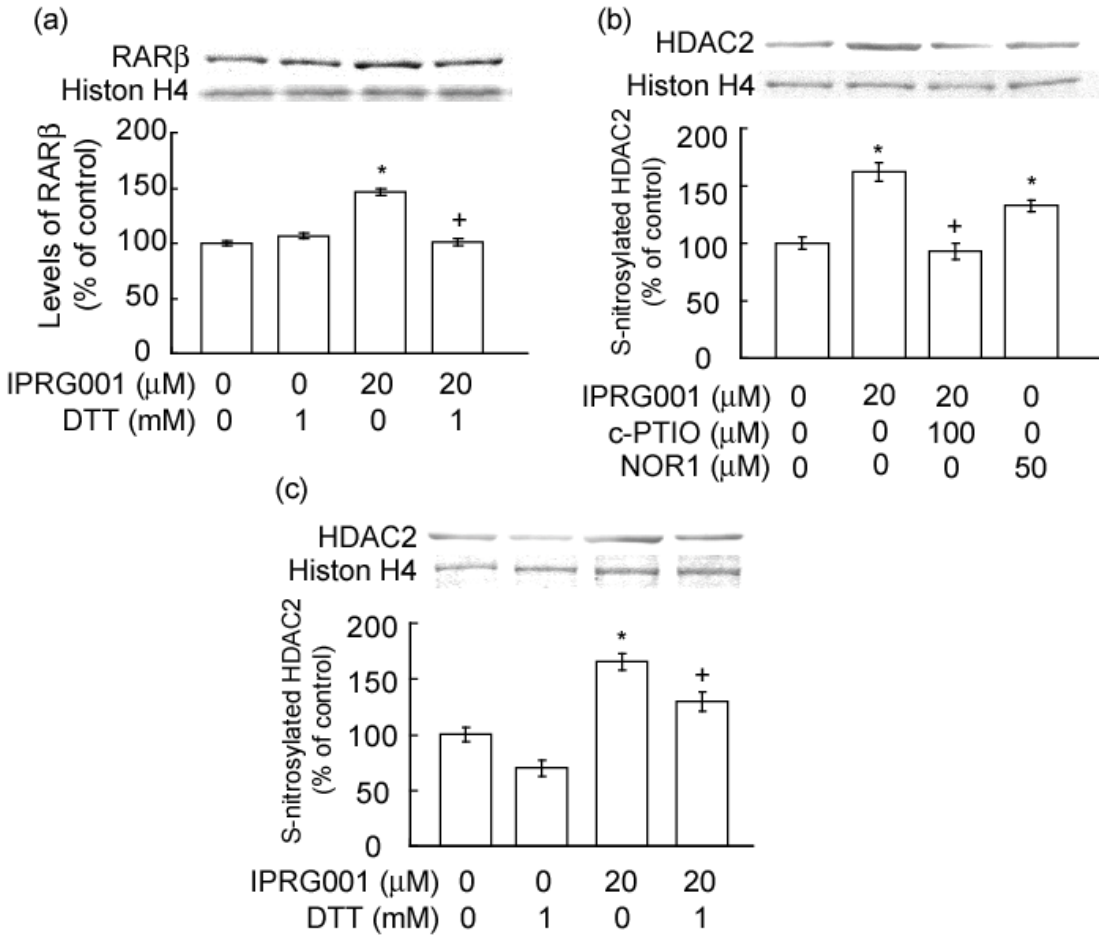
(a)
RARβ
Histon H4
Levels of RARβ (% of control)
200
150
100
50
0
*
+
IPRG001 (μM) 0 0 20 20
DTT (mM) 0 1 0 1
(b)
HDAC2
Histon H4
S-nitrosylated HDAC2 (% of control)
200
150
100
50
0
*
+
*
IPRG001 (μM) 0 20 20 0
c-PTIO (μM) 0 0 100 0
NOR1 (μM) 0 0 0 50
(c)
HDAC2
Histon H4
S-nitrosylated HDAC2 (% of control)
200
150
100
50
0
*
+
IPRG001 (μM) 0 0 20 20
DTT (mM) 0 1 0 1

Fig.7

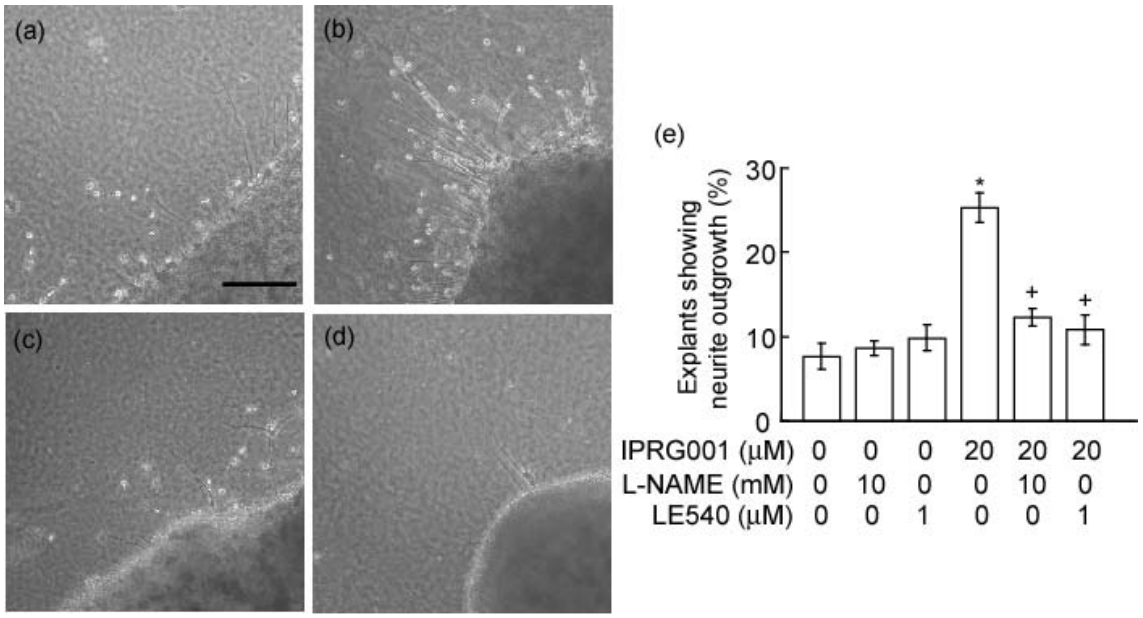
(a)
(b)
(c)
(d)
(e)
Explants showing neurite outgrowth (%)
30
20
10
0
*
+
+
IPRG001 (μM) 0 0 0 20 20 20
L-NAME (mM) 0 10 0 0 10 0
LE540 (μM) 0 0 1 0 0 1

Fig.8

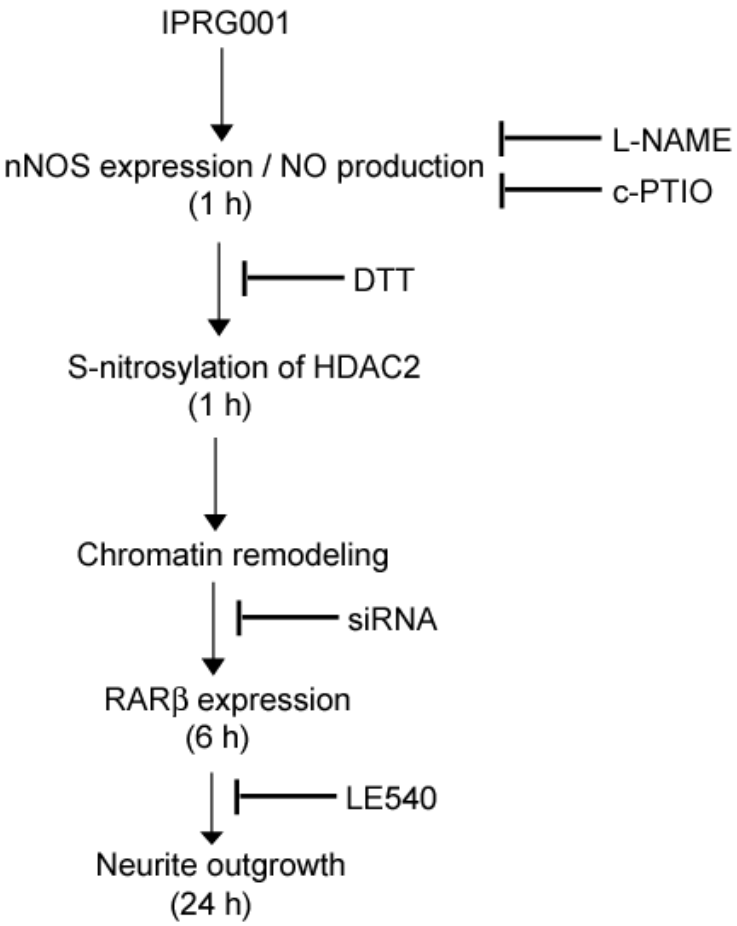
IPRG001
nNOS expression / NO production
(1 h)
L-NAME
c-PTIO
DTT
S-nitrosylation of HDAC2
(1 h)
Chromatin remodeling
siRNA
RARβ expression
(6 h)
LE540
Neurite outgrowth
(24 h)

Fig.9