Note

Norgalanthamine Stimulates Proliferation of Dermal Papilla Cells *via* Anagen-Activating Signaling Pathways

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Norgalanthamine has been shown to possess hair-growth promoting effects, including increase in hair-fiber length in cultured rat vibrissa follicles and increase in dermal papilla cell (DPC) proliferation. However, the intracellular mechanisms that underlie the action of norgalanthamine in DPCs have not been investigated. In this study, we addressed the ability of norgalanthamine to trigger anagen-activating signaling pathways in DPCs. Norgalanthamine significantly increased extracellular signal-regulated kinase (ERK) 1/2 phosphorylation at 0.1 μ M, a concentration at which DPC proliferation was also induced. Furthermore, the increases in norgalanthamine-induced ERK 1/2 activation and subsequent DPC proliferation were suppressed by the mitogen-activated protein kinase/ERK kinase (MEK) 1/2 inhibitor, U0126. A 0.1 μ M dose of norgalanthamine also increased phosphorylation of AKT, which was followed by an increase in glycogen synthase kinase 3 β phosphorylation and nuclear translocation of β -catenin. In addition, LY294002, a phosphatidylinositol 3 kinase (PI3K) inhibitor, blocked the effect of norgalanthamine on DPC proliferation. These results suggest that norgalanthamine can stimulate the anagen phase of the hair cycle in DPCs *via* activation of the ERK 1/2, PI3K/AKT, and Wnt/ β -catenin pathways.

Key words norgalanthamine; anagen; dermal papilla cell; extracellular signal-regulated kinase 1/2; AKT; β -catenin

INTRODUCTION

Hair follicles undergo characteristic phases, collectively known as the hair cycle, which includes anagen (growth phase), catagen (regression phase), and telogen (resting phase).¹⁾ Dermal papilla cells (DPCs) are the key dermal component of the hair follicle and directly regulate hair cycle *via* the interaction with hair germ cells.^{2,3)} The anagen-activating signaling pathways have been investigated in DPCs of hair follicle.^{4–6)} The transition from telogen to anagen has been reported to be associated with activation of the Wnt/ β -catenin/Lef-1, sonic hedgehog, and signal transducer and activation of transcription 3 signaling pathways, and recent studies have shown that transient activation of β -catenin signaling was sufficient to stimulate the anagen phase of the hair cycle.^{4–6)} Minoxidil, one of hair regrowth drugs, increases the proliferation of DPCs through regulation of apoptosis-related proteins and activation of extracellular signal-regulated kinase (ERK)1/2.^{3,7)} Minoxidil also prolongs the anagen phase of the hair cycle in DPCs *via* activation of the Wnt/ β -catenin and phosphatidylinositol 3 kinase (PI3K)/AKT pathways.^{3,8)}

Alkaloids, isolated from the Amaryllidaceae family, have a galanthamine-derived backbone, and are potent acetylcholinesterase (AChE) inhibitors that have been used to treat the symptoms of Alzheimer's disease.^{9,10)} Norgalanthamine, a principal component of *Crinum asiaticum* var. *japonicum*, has been shown to increase the hair-fiber length of vibrissa follicles as well as proliferation of DPCs.¹¹⁾ In this study, we

addressed the intracellular mechanisms of norgalanthamine on the promotion of hair growth by investigating whether norgalanthamine can trigger anagen-activating signaling pathways, including ERK 1/2, AKT and β -catenin pathways, in DPCs.

MATERIALS AND METHODS

Materials Norgalanthamine was isolated and purified from the aerial parts of *Crinum asiaticum* var. *japonicum*, which were collected at the island of Jeju (Korea) during August 1998 and dried at room temperature. A voucher specimen (CNU 98105) was deposited at the herbarium in the College of Pharmacy, Chungnam National University, Korea. The structure of norgalanthamine was confirmed by comparison of spectroscopic and other physical properties, as previously reported.^{12,13)}

DPC Proliferation Assay Immortalized rat vibrissa DPCs were provided by the Skin Research Institute, Amore Pacific Corporation Research and Development Center, Korea.¹⁴⁾

DPC proliferation was estimated by measuring metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (MTT, Sigma-Aldrich, St. Louis, MO, U.S.A.).¹⁵⁾ Briefly, DPCs were seeded onto 96-well plates at a density of 1×10^4 cells/mL, and cultured for 24 h under serum starvation with 1% fetal bovine serum (Hyclone, Logan, UT, U.S.A.). Cells were then treated with 0.1 μ M norgalanthamine or 10 μ M minoxidil (Sigma-Aldrich) in the presence or ab-

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Fig. 1. Norgalanthamine Induced Proliferation of DPCs via the Activation of ERK 1/2

A: DPCs were pretreated with or without 10 μ M U0126 (U) for 30 min then treated with 0.1 μ M norgalanthamine (N) or 10 μ M minoxidil (M) as a positive control, for 24 h. Whole cell lysates were subjected to immunoblotting with indicated antibodies. B: Data from the three independent experiments are presented as mean $\pm$ S.D. * p < 0.05, ** p < 0.01 compared with control (Con); ## p < 0.01, M treatment group vs. U + M co-treatment group; + p < 0.05, N treatment group vs. U + N co-treatment group. C: DPCs were pretreated with or without 10 μ M U0126 (U) for 30 min then treated with 0.1 μ M N or 10 μ M M as a positive control for 96 h. Data from the three independent experiments are presented as mean $\pm$ S.D. * p < 0.05, ** p < 0.01 compared with control group (Con); ## p < 0.01, M treatment vs. U + M co-treatment group; + p < 0.05, N treatment vs. U + N co-treatment group.

sence of 10 μ M U0126 (Biosource, Camarillo, CA, U.S.A.) or 2.5 μ M LY294002 (Biosource) for 96 h. After incubation, 50 μ L MTT (2 mg/mL stock solution) was added to each well, and cells were incubated at 37°C for 0.5 h. Media was carefully aspirated, and 200 μ L dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Absorbance at 540 nm was measured immediately using a microplate reader (BioTek Instrument Inc., Winooski, VT, U.S.A.). Results are expressed as a percentage of absorbance caused by treatment with norgalanthamine or minoxidil in the presence or absence of U0126 or LY294002, compared to vehicle-only controls (0.4 μ L DMSO-treated group). Minoxidil was used as a positive control.

Western Blot Analysis Western blot analysis was performed as previously described.¹⁶⁾ Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, then proteins were transferred onto polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies against phospho(ser473)-Akt, Akt, phospho(Thr202/Tyr204)-ERK 1/2, ERK 1/2, glycogen synthase kinase 3 β (GSK-3 β), phospho(ser9)-GSK3 β , c-Jun N-terminal kinase (JNK), phospho(Thr183/Tyr185)-JNK, phospho(Thr180/Tyr182)-p38, p38 (1:1000 dilution, Cell signaling, Beverly, MA, U.S.A.), lamin B1 (1:2000 dilution, Abcam, Cambridge, U.K.), α -tubulin (1:250 dilution), β -catenin (1:1000 dilution, Santa Cruz, CA, U.S.A.), or β -actin (1:5000 dilution, Sigma-Aldrich) at 4°C overnight. Blots were incubated with horseradish peroxidase-labeled anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG secondary antibodies (1:5000 dilution, Santa Cruz) at room temperature for 1 h. Blots were washed six times with Tween-20-TBS (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 30 min before being exposed to West-zol™ Plus reagent (iNtRon, Seoul, Korea). An enhanced chemiluminescent detection system was used according to the manufacturer's protocol, and immunoblots were exposed to X-ray film (AGFA, Mortsel, Belgium). To document loading controls, membranes were re-probed with primary antibodies against housekeeping pro-

teins, lamin B1, α -tubulin, and β -actin. Band intensities were quantified using NIH image software (<http://rsb.info.nih.gov/ij/>).

Statistical Analysis Experimental data were expressed as mean $\pm$ standard deviation (S.D.) from three independent experiments. The Student's *t*-test was used to determine the statistical significance of differences between experimental and control groups; p < 0.05 was considered statistically significant. SigmaStat (Systat Software Inc., CA, U.S.A.) was used for statistical analysis.

RESULTS

To determine whether norgalanthamine increases the proliferation of DPCs through activation of mitogen activated protein (MAP) kinases, such as ERK 1/2, p38 and JNK, DPCs were treated with 0.1 μ M norgalanthamine or 10 μ M minoxidil and incubated for 24 h under 1% serum conditions. Norgalanthamine and minoxidil both increased the level of phosphorylated ERK 1/2 (Figs. 1A, B), but did not alter levels of phosphorylated p38 and JNK (data not shown). In addition, the increase in ERK 1/2 phosphorylation by norgalanthamine or minoxidil was significantly attenuated by 10 μ M U0126, an inhibitor of MEK 1/2 (Figs. 1A, B). Nevertheless that 10 μ M U0126 alone scarcely affects the proliferation of DPC, DPC proliferation induced by norgalanthamine or minoxidil was also inhibited by 10 μ M U0126 (Fig. 1C). On the other hand, 20 μ M U0126 alone reduced the proliferation of DPC (data not shown).

To determine whether norgalanthamine stimulates the PI3K/AKT signaling pathway, a known anagen-promoting signaling pathway, DPCs were treated with 0.1 μ M norgalanthamine or 10 μ M minoxidil and incubated for 6 h. Norgalanthamine and minoxidil both significantly increased the phosphorylation of AKT compared with vehicle-only controls (Figs. 2A, B). When DPCs treated with LY294002, an inhibitor of PI3K, we found that 2.5 μ M LY294002 scarcely affects the proliferation of DPC, while 10 μ M LY294002 showed cyto-

Fig. 2. Norgalanthamine Induced Proliferation of DPCs via Activation of the PI3K/AKT Pathway

A: DPCs were treated with 0.1 μ M norgalanthamine (N) or 10 μ M minoxidil (M) as a positive control, for 6h, and levels of phosphorylated AKT were measured. B: Data from the three independent experiments are presented as mean $\pm$ S.D. * p < 0.05, ** p < 0.01 compared with control group (Con). C: DPCs were pretreated with or without 2.5 μ M LY294002 (L) for 30 min then treated with 0.1 μ M N or 10 μ M M as a positive control for 96h. Data from the three independent experiments are presented as mean $\pm$ S.D. * p < 0.05, ** p < 0.01 compared with control group (Con); # p < 0.05, M treatment vs. L + M co-treatment group; ++ p < 0.01, N treatment vs. L + N co-treatment group.

Fig. 3. Norgalanthamine Increased Nuclear β -Catenin via the Increased Phosphorylated GSK-3 β

A–B: DPCs were treated with 0.1 μ M norgalanthamine (N) or 10 μ M minoxidil (M) as a positive control, for 24h and levels of phosphorylated GSK-3 β were measured. C–E: DPCs were treated with 0.1 μ M N or 10 μ M M for 52h, and whole cell lysates were separated into nuclear and cytoplasmic fractions. Levels of β -catenin in the nuclear and cytoplasmic fractions were determined using immunoblotting. α -Tubulin and lamin B1 were used as a loading control in the cytoplasmic and nuclear fractions, respectively. Data from the three independent experiments are presented as mean $\pm$ S.D. * p < 0.05, ** p < 0.01 vs. control (Con).

toxicity for DPCs (data not shown). When DPCs were treated with 0.1 μ M norgalanthamine or 10 μ M minoxidil in the presence or absence of 2.5 μ M LY294002 for 96h, DPC proliferation was significantly inhibited (Fig. 2C).

We next examined whether norgalanthamine activates the Wnt/ β -catenin pathway, which is crucial for the proliferation of DPCs and hair growth. When DPCs were treated with 0.1 μ M norgalanthamine or 10 μ M minoxidil for 24h, phosphorylation of GSK-3 β was significantly increased compared with that in vehicle-only controls (Figs. 3A, B). Norgalanthamine and minoxidil also significantly increased the nuclear translocation of β -catenin after 52h treatment, compared with that in vehicle-only controls (Figs. 3C–E). Furthermore, we assessed if the activation of PI3K/AKT pathway induced by norgalanthamine or minoxidil increases the nuclear β -catenin level using LY294002. LY294002 attenuated norgalanthamine-induced increase of nuclear beta-catenin, which suggests that

norgalanthamine could activate AKT/GSK3 β / β -catenin signaling (data not shown).

DISCUSSION

We previously reported that *Crinum asiaticum* var. *japonicum* possesses hair-growth promoting activities, and norgalanthamine, a principal component of *C. asiaticum* var. *japonicum*, can increase hair-fiber length of vibrissa follicles via proliferation of DPCs.¹¹⁾ To the best of our knowledge, this study is the first to report that norgalanthamine stimulates the anagen phase of the hair cycle via activation of ERK 1/2, PI3K/AKT and Wnt/ β -catenin pathways in DPCs. These data therefore reveals the mechanism of action of norgalanthamine on hair-growth activity.

DPCs, mesenchymal derived fibroblasts that reside in the hair bulb, play a pivotal role in regulation of the hair cycle.²⁾

Hair growth depends on the length of the anagen phase, which is affected by an increase in DPC proliferation.⁸⁾ The role of ERK 1/2 in mitogenesis or cell growth has been described in several cell types, including DPCs.^{17–19)} In the study, we found that norgalanthamine significantly increased DPC proliferation through ERK 1/2 activation, in a manner similar to that of minoxidil, a positive control³⁾ (Fig. 1). *Undariopsis peterseniana* extract, an herbal extract, and *Rumex japonicus* HOUTT. extract are known to stimulate DPC proliferation via ERK 1/2 activation.^{20–22)} However, we did not observe activation of p38 or JNK in our experiments. Whether minoxidil stimulates p38 or JNK activation has not been reported.

AKT is known to play a critical role in mediating survival signals.^{23,24)} Growth factors and cytokines activate AKT via the PI3K pathway.²⁵⁾ In the study, norgalanthamine induces DPC proliferation via AKT activation, in a manner similar to minoxidil³⁾ (Fig. 2). On the other hand, phosphorylation of β -catenin by AKT can promote β -catenin transcriptional activity, and activation of AKT/GSK3 β / β -catenin signaling is known to be involved in the survival of neurons after traumatic brain injury in rats.^{26,27)} Sinapic acid and *Rumex japonicus* HOUTT. extract exert hair growth-promoting effects on hair follicle DPCs via activation of AKT and subsequent inactivation of GSK3 β .^{22,28)} In particular, to trigger the transition from telogen to anagen, Wnt/ β -catenin signals from the dermal papilla are transmitted to hair follicle stem cells and/or hair germ cells.²⁾ The expression of nuclear β -catenin was found to be increased in the dermal papilla and upper matrix of anagen hair follicles.²⁹⁾ Mammalian target of rapamycin (mTOR), a serine/threonine kinase, can regulate cell proliferation and cell cycle progression during the hair cycle.^{30,31)} A previous study showed that hair cycle initiation was delayed after treatment with an mTOR inhibitor, and ERK and AKT signaling pathways function through parallel mechanisms to promote mTOR complex 1 (mTORC1).^{31,32)} Results of this present study showed that norgalanthamine-induced AKT activation triggered DPC proliferation via the inactivation of GSK3 β and an increase in nuclear β -catenin translocation (Fig. 3). On the other hand, ERK 1/2 and AKT pathways are activated in hepatocellular carcinoma cells via acetylcholine.³³⁾ Suppression of AChE activity increases cell proliferation, which is involved in inhibition of p27 and cyclins in Huh-7 and HepG2 cell lines.³⁴⁾ In addition, eserine, an AChE inhibitor, induces cell proliferation and carcinogenesis that takes place in the rat mammary gland.³⁵⁾ However, lycorine, an AChE inhibitor, is known to exert anticancer effects via suppression of Wnt/ β -catenin signaling in non-small cell lung carcinoma cells.³⁶⁾ Taken together, our results suggest that norgalanthamine can stimulate the anagen phase via activation of the ERK 1/2, PI3K/AKT and Wnt/ β -catenin pathways in DPCs, and thus it may have a pivotal role in hair growth.

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Conflict of Interest The authors declare no conflict of

interest.

Supplementary Materials The online version of this article contains supplementary materials.

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