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 µM, 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 µM dose of norgalanthamine also increased phosphorylation of AKT, which was followed by an increase in glycogen synthase kinase 3β phosphorylation and nuclear translocation of β-catenin. In addition, LY294002, a phosphatidylinositol 3 kinase 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). 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. The anagen-activating signaling pathways have been investigated in DPCs. Norgalanthamine is a principal component of Crinum asiaticum var. japonicum, which has been shown to increase the hair-fiber length of vibrissa follicles as well as proliferation of DPCs. 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.

DPC Proliferation Assay Immortalized rat vibrissa DPCs were provided by the Skin Research Institute, Amore Pacific Corporation Research and Development Center, Korea. 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.). 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 µM norgalanthamine or 10 µM minoxidil (Sigma-Aldrich) in the presence or ab-
sence of 10 µM U0126 (Biosource, Camarillo, CA, U.S.A.) or 2.5 µM LY294002 (Biosource) for 96 h. After incubation, 50 µL 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 µL 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.).

Statistical Analysis
Experimental data were expressed as mean ± 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 µM norgalanthamine or 10 µM 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 µM U0126, an inhibitor of MEK 1/2 (Figs. 1A, B). Nevertheless that 10 µM norgalanthamine or 10 µM minoxidil was significantly attenuated by 10 µM U0126 alone scarcely affects the proliferation of DPC, DPC proliferation induced by norgalanthamine or minoxidil was also inhibited by 10 µM U0126 (Fig. 1C). On the other hand, 20 µM 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 µM norgalanthamine or 10 µM minoxidil and incubated for 6 h. Norgalanthamine and minoxidil both significantly increased the phosphorylation of AKT compared with vehicle-only controls (Fig. 2A, B). When DPCs treated with LY294002, an inhibitor of PI3K, we found that 2.5 µM LY294002 scarcely affects the proliferation of DPC, while 10 µM LY294002 showed cyto-
toxicity for DPCs (data not shown). When DPCs were treated with 0.1 µM norgalanthamine or 10 µM minoxidil in the presence or absence of 2.5 µM LY294002 for 96 h, 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 µM norgalanthamine or 10 µM minoxidil for 24 h, phosphorylation of GSK-3β 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 52 h 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/GSK3β/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.\(^13–19\) 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 control\(^9\) (Fig. 1). Undaria peterseniana extract, an herbal extract, and Rumex japonicus root 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 minoxidil\(^9\) (Fig. 2). On the other hand, phosphorylation of β-catenin by AKT can promote β-catenin transcriptional activity, and activation of AKT/GSK3β/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 root extract exert hair growth-promoting effects on hair follicle DPCs via activation of AKT and subsequent inactivation of GSK3β.\(^22,28\) In particular, to trigger the transition from telogen to anagen, Wnt/β-catenin signaling 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 GSK3β\(^9\) 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.\(^30\) 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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