Abstract. Background: Geranylgeraniol (GGOH) is a C20 isoprenoid found in fruits, vegetables, and grains, including rice. As a food substance, GGOH is categorized as ‘Generally Recognized as Safe’. GGOH is an intermediate product in the mevalonate pathway and acts as a precursor to geranylgeranylprophosphate. Materials and Methods: C2C12 mouse myoblasts derived from muscle satellite cells were used. Quantitative reverse-transcriptase polymerase chain reaction, western blotting analysis, and immunocytochemical analysis were performed to respectively assess mRNA expression, protein levels, and the number of myofibers. Results: GGOH reduced the expression levels of skeletal muscle atrophy-related ubiquitin ligases in myofibers derived from C2C12 cells. GGOH induced myogenic differentiation of C2C12 cells via geranylgeranylation. GGOH did not adversely affect the proliferation of C2C12 cells. Conclusion: GGOH induces myoblast differentiation in C2C12 cells.

Satellite cells are skeletal muscle stem cells residing beneath the basal lamina that provide myonuclei for postnatal muscle growth, repair, and regeneration in adults. Satellite cells are activated in response to muscle injury, proliferate extensively and then differentiate into myoblasts. This is accompanied by the transcriptional up-regulation of myogenic differentiation (MYOD) and other myogenic differentiation marker genes, such as myogenin (MYOG), creatine kinase M-type (CKM), and myosin heavy chain (MYHC) (1-3). Insulin-like growth factors (IGFs) are essential for skeletal muscle development, regeneration, and hypertrophy – processes which all require satellite cell activation and differentiation (4-6). The autocrine action of IGF2 is especially critical for the differentiation of satellite cells in vitro (7). C2C12 cells are a murine myoblast cell line derived from satellite cells (8). C2C12 cells are commonly used as an in vitro model of muscle regeneration due to their ability to transition from a proliferative phase into differentiated myofibers, similar to satellite cells, upon adequate stimulus (3).

Statins act by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the first step of the isoprenoid biosynthetic pathway and the rate-limiting step of cholesterol biosynthesis (9). Statins are used as a frontline therapy for lowering plasma cholesterol and preventing cardiovascular disease (10-13). Statins are generally safe and effective. However, they may induce a variety of skeletal muscle-associated, dose-dependent adverse reactions that range from muscle pain to muscle cell damage and severe rhabdomyolysis (14-17). These statin-associated muscle disorders are likely due to inhibition of the synthesis of crucial intermediary molecules such as geranylprophosphate and geranylgeranylprophosphate (GGPP) (19-21). Treatment of C2C12 cells with GGPP was found to reverse the suppressive effect of statin on myotube formation (22). Geranylgeraniol (GGOH), a precursor to GGPP, reduced...
muscle damage induced by statin treatment in vitro (23). Thus, GGOH seems to have protective effects on skeletal muscle. However, the extent of this potentially beneficial effect remains unknown. In this study, the effect of GGOH on myogenesis in C2C12 cells was investigated.

Materials and Methods

Cell culture, transfection, and skeletal muscle differentiation. C2C12 murine myoblasts were purchased from American Type Culture Collection (Manassas, VA, USA). C2C12 cells were maintained as previously described (24) and cultured in the presence of 0, 5, 10, 50, or 100 μM GGOH (Sigma–Aldrich Chemicals, St. Louis, MO, USA) and 100 μM of the geranylgeranytransferase I inhibitor N-[4-[2(R)-amino-3-mercaptopropyl]amino-2-(1-naphtha-leny]benzoyl]-L-leucine methyl ester trifluoroacetate salt (GGTI-298) (Tocris Bioscience, Bristol, UK). Skeletal muscle differentiation in C2C12 cells was induced by culturing cells with Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum (Biosera, Kansas City, MO, USA) for 5-8 days (3).

Reverse transcription and quantitative polymerase chain reaction (qPCR) analysis. Total RNA was isolated from C2C12 cells using FastGene RNA Basic Kit (Nippon Genetics, Tokyo, Japan) and then reverse-transcribed into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). SYBR green-based qPCR was performed in 96-well plates using PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA, USA) with QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). Values were normalized to β-actin (Actb) using the 2−ΔΔCt method (25). The following primers were used for qPCR analyses: qPCR for murine Fbxo32: forward: agggagccgctacgata, reverse: gatcaacagcgcgctcaat; tripartite motif containing 63 (Trim63): forward: gaccaactcaagcagacgatc, reverse: gcccggcttcacacacggta; murine Myog: forward: ccctcaagctcaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat; murine Myod: forward: cctccttacccaacttcaggt, reverse: aagagatgagaagcacggccgctgtaatccatcat.

Proliferation assay. C2C12 cells were cultured in the presence of GGOH at concentrations of 0, 5, 10, 50, or 100 μM for 1, 2, or 3 days. Proliferation of C2C12 cells was assessed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and then treated with or without (Ctrl) 50 μM GGOH for another 3 days. Total RNA was isolated, then F-box protein 32 (Fbxo32) (A) and tripartite motif containing 63 (Trim63) (B) mRNA levels were analyzed using quantitative polymerase chain reaction. All data are expressed as the means±SD (n=3). *Significantly different at p<0.01 versus vehicle-treated cells. Similar results were obtained by three independent experiments.

Statistical analysis. Comparisons were made using an unpaired Student’s t-test; the results are shown as the means±S.D. Statistical significance was defined as p<0.05.

Results

Firstly, it was confirmed whether GGOH protects skeletal muscle fibers derived from C2C12 cells from atrophy. Treatment of cells with 50 μM GGOH for 3 days reduced the expression levels of muscle atrophy-related ubiquitin ligase Fbxo32 (Figure 1A) and Trim63 (Figure 1B).

Next, we examined the effect of GGOH on skeletal muscle differentiation in C2C12 cells. qPCR analysis revealed that although 50 μM GGOH treatment for 2 days did not change the expression level of Myod (Figure 2A), it did enhance the induction of early-stage myogenic marker genes such as Myog and Ckm (Figure 2B and C). GGOH treatment also led to a dose-dependent increase in the protein level of MYOG (Figure 2F) as well as of the late myoblast marker MYHC (Figure 2G and H). In addition, GGOH dramatically stimulated the expression level of Igf2 in C2C12 cells (Figure 2D).
positive effect of GGOH on the induction of Myog was blocked by the addition of the geranylgeranyl transferase inhibitor GGIT-298 (Figure 2I), suggesting that the augmentative effect of GGOH on myogenic differentiation is via geranylgeranylation. GGOH did not adversely affect the proliferation of C2C12 cells (Figure 3).

Discussion

In the present study, we examined the effect of GGOH on fiber degradation, differentiation, and proliferation in C2C12 cells.

GGOH is a C20 isoprenoid found in fruits, vegetables, and grains, including rice. As a food substance, GGOH is categorized as ‘Generally Recognized as Safe’ (26). GGOH is an intermediate product in the mevalonate pathway and acts as a precursor to GGPP. In the cell, GGOH is thought to be subsequently converted into the pyrophosphate moiety, GGPP, by two successive monophosphorylation events (27). GGPP induces geranylgeranylation, which is necessary for the membrane localization of intracellular proteins, particularly the small GTP-binding proteins rat sarcoma virus oncogene (RAS), ras homolog family member (RHO), Rac family small GTPase (RAC) and Rap GTPase (RAP) (28). In our study, GGIT-298, an inhibitor of geranylgeranylation, eliminated GOOH-induced myoblast differentiation of C2C12 cells (Figure 2I), suggesting that GGOH-induced myogenesis requires geranylgeranylation of certain protein(s). Further experiments are needed to elucidate the mechanism that underlies GGOH-induced myoblast differentiation.

Inhibition of the isoprenoid biosynthetic pathway by statins may be involved in the occurrence of statin-associated...
Generally Recognized as Safe’ and can be taken orally (32), treatment, our data will provide the basis for a new potential product for the treatment of muscle atrophy. Myogenic differentiation. Since GGOH is categorized as a myogenic differentiating agent, it is expected to be useful in the treatment of muscle atrophy-related diseases. Skeletal muscle atrophy is associated with age-related sarcopenia, which can be prevented by skeletal muscle atrophy-related factors. In conclusion, we demonstrated that in C2C12 murine skeletal muscle myoblasts, GGOH reduced the expression of muscle atrophy-related genes and enhanced skeletal muscle mass and function.

In conclusion, we demonstrated that in C2C12 murine skeletal muscle myoblasts, GGOH reduced the expression of muscle atrophy-related genes and enhanced myogenic differentiation. Since GGOH is categorized as ‘Generally Recognized as Safe’ and can be taken orally (32), upon the establishment of a suitable protocol for GGOH treatment, our data will provide the basis for a new potential product for the treatment of muscle atrophy.

Conflicts of Interest

The Authors declare that they have no conflict of interests in regard to this study.

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