Growth hormone-releasing peptide-biotin conjugate stimulates myocytes differentiation through insulin-like growth factor-1 and collagen type I

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Based on the potential beneficial effects of growth hormone releasing peptide (GHRP)-6 on muscle functions, a newly synthesized GHRP-6-biotin conjugate was tested on cultured myoblast cells. Increased expression of myogenic marker proteins was observed in GHRP-6-biotin conjugate-treated cells. Additionally, increased expression levels of insulin-like growth factor-1 and collagen type I were observed. Furthermore, GHRP-6-biotin conjugate-treated cells showed increased metabolic activity, as indicated by increased concentrations of energy metabolites, such as ATP and lactate, and increased enzymatic activity of lactate dehydrogenase and creatine kinase. Finally, binding protein analysis suggested few candidate proteins, including desmin, actin, and zinc finger protein 691 as potential targets for GHRP6-biotin conjugate action. These results suggest that the newly synthesized GHRP-6-biotin conjugate has myogenic stimulating activity through, at least in part, by stimulating collagen type I synthesis and several key proteins. Practical applications of the GHRP-6-biotin conjugate could include improving muscle condition. [BMB Reports 2015; 48(9): 501-506]

INTRODUCTION

The importance of circulating hormones in the aging process has been discussed in various reports (1). Clinical trials have used growth hormone (GH) to investigate the effects of the hormone in preventing the aging process and improving aging symptoms. Virtually all tissues in the human body are responsive to GH and GH-involved functions may be impaired by age-related downregulation of GH expression (2). Some studies have used GH secretagogues (GHS), peptide and non-peptide compounds that stimulate the secretion of GH, to modulate GH secretion (3). One of the most potent compounds, GH releasing peptide (GHRP)-6, stimulates GH release via a unique pathway independent of GH releasing hormone (GHRH) (4). Efforts to develop a small molecule with GH releasing activity, most of which were based on peptidomimetic approaches to GHRP-6, have resulted in a few preclinical candidate compounds (3, 5). The positive effects of GHS on skin, in part due to increased collagen synthesis, highlight the possible cosmetic application of GHRP-6 and its derivatives (1). However, insufficient stability of topical formulations and low bioavailability, due to low permeability of peptide compounds via the skin, make them impractical for topical application. Recently, we reported the use of a GHRP-6 derivative as a cosmetic ingredient. To improve the permeation of GHRP-6 through skin as well as to improve stability in the formulation, several GHRP-6 derivatives conjugated with biotin (vitamin B7) were synthesized and their effects on skin-derived cells were investigated. Significant increases in collagen synthesis were observed with select GHRP-6 biotin conjugate compounds. In addition to cosmetics, effects of GH on muscle formation through stimulation of collagen synthesis are also well-known. Studies have shown that certain genes involved in myocyte differentiation, such as myogenin, MRF-4, and MEF2c, are regulated in part by GH (6). Based on these results, in this study, we investigated the effects of a GHRP-6-biotin conjugate compound on myocyte differentiation. We show here stimulation of myogenesis via application of a GHRP-6-biotin conjugate compound, as indicated by increased expression of myogenesis marker proteins and collagen synthesis. Further investigation into the underlying signaling pathways revealed the potential involvement structural proteins, such as desmin and actin.

Keywords: Biotin, Collagen, Growth-hormone releasing peptide, Insulin-like growth factor, Myogenesis

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RESULTS

GHRP-6-biotin conjugate stimulated differentiation of cultured myoblasts

Synthesis of the GHRP-6 hexapeptide (His-(D-Trp)-Ala-Trp-(D-Phe)-Lys-NH2)-biotin conjugate was achieved by linking the carboxyl group of biotin with an amine group, in either the histidine residue at the N-terminal or the lysinamide residue at the C-terminal of GHRP-6 by a solid-phase peptide synthesis method. A preliminary in vitro cytotoxicity evaluation of the two conjugate forms showed that the lysinamide-biotin conjugate was more cytotoxic than the histidine-biotin conjugate form (data not shown). For further evaluation, the histidine-biotin conjugate form (conventional name: Myotide) was chosen and the biological activity was assessed using cultured myocytes and compared with the native GHRP-6 and biotin.

To investigate the effects of the GHRP-6-biotin conjugate on the differentiation of cultured myoblasts, C2C12 cells were treated with 50 μM GHRP-6-biotin conjugate and expression of myogenic differentiation marker proteins, myosin heavy chain I (MyHC I), myogenin, MG53 (mitsugumin 53), and caveolin-3, were quantified by Western blotting (7). As shown in Figs. 1A and S1, treatment with the GHRP-6-biotin conjugate increased the expression of all measured proteins up to day 3. Furthermore, a dose-dependent increase in protein expression was also observed after 48 h of treatment (Figs. 1B, S2). As shown in Figs. 1C and S3, the nuclei number of MyHC-positive cells increased ~3-fold in 3-day-differentiated myotubes and satellite cells. Treatment with biotin or GHRP-6 alone did not induce significant change in myogenesis, suggesting that the conjugated compound may stimulate signaling pathway(s) different from those that are affected by single-compound treatment (Fig. S4). Further investigations into the underlying pathways induced by single and conjugate compounds are currently underway.

GHRP-6-biotin conjugate induced IGF-1 expression and collagen synthesis

Prior studies have suggested a potential relationship between GHRP-6 and insulin-like growth factor-1 (IGF-1) (8). Thus, we next investigated the effects of the GHRP-6-biotin conjugate on IGF-1 expression. As shown in Fig. 2A, treatment with the...
GHRP-6-biotin conjugate compound increased IGF-1 expression in a dose-dependent manner up to 100 μM. However, biotin did not induce any significant changes at the concentrations tested and treatment with GHRP-6 downregulated the expression of IGF-I in cultured myoblast cells, consistent with the results in Fig. S4.

Studies have also reported on the importance of collagen in myocyte differentiation. To investigate whether the collagen is also involved in myogenic differentiation induced by the GHRP-6 biotin conjugate, we monitored collagen type I content levels 24 h post treatment in myocytes. The results showed that collagen type I protein quantity in conjugate cultured medium and intracellular collagen production increased in a dose-dependent manner (Fig. 2B). Preliminary experiments with human skin fibroblasts also showed increased expression of collagen I with the GHRP-6-biotin conjugate compound treatment (data not shown). Treatment with biotin or GHRP-6 alone did not affect collagen type I production in cultured C2C12 myoblasts (Fig. 2C). To clarify whether the increased collagen production directly mediated myogenic differentiation of C2C12 cells, effects of extracellular matrix protein-coated plates on cellular responses were tested. Cells incubated using collagen type I-coated plates showed the highest expression levels of myogenic marker proteins (Fig. 2D, E).

**GHRP-6 biotin conjugate increases the energy metabolites for muscle function**

To further investigate the effects of the GHRP-6-biotin conjugate at the cellular level, changes in cytosolic ATP, lactate concentrations, enzymatic activities of creatine kinase (CK), and lactate dehydrogenase (LDH) were measured. Treatment with the GHRP-6-biotin conjugate resulted in increased cytosolic ATP and lactate concentrations, and increased activity of both CK and LDH (Fig. 3). As shown in Fig. 3E, skeletal α-actin protein expression, which is closely related to muscle contraction was increased significantly with GHRP-6-biotin treatment. Together, these data suggest that the GHRP-6-biotin conjugate may improve energy metabolism, which is important for muscular function, by stimulating production of energy-rich products and increasing skeletal α-actin expression.

**Identification of binding partners of the GHRP-6-biotin conjugate**

To better understand the specificity of GHRP-6-biotin conjugate's effects on myoblasts, we investigated the involvement of possible binding partners for the GHRP-6-biotin conjugate in myoblasts. To explore potential candidate proteins as binding partners, co-precipitated proteins were analyzed by MALDI-TOF. Our results identified desmin, actin, and zinc finger protein 691 as possible binding partners (Fig. 4, Table S1). While the important role of desmin and actin for forming the intermediate filaments between adjacent myofibrils and cytoskeleton are well accepted, the role of zinc finger protein 691 as a transcription factor is not yet known and needs further investigation. Quantitative enzyme-linked immunosorbent assays
(ELISAs) revealed that the GHRP-6-biotin conjugate interacted directly with desmin in a dose-dependent manner (Fig. 4C).

**DISCUSSION**

Since the discovery of the first potent growth hormone (GH)-releasing hexapeptide, GH releasing peptide (GHRP)-6, many studies have sought to develop more potent and less toxic GH secretagogues (3, 9). Therapeutic benefits of GHRP-6 have been reported in various diseases, including type 1 diabetes (10), age-dependent cerebellar cell death (8), and acute myocardial infarction (11). Recently, the intranasal delivery of GHRPs has been introduced (12) and the development of topical formulations for peptides has also been reported (13).

Myogenic differentiation for muscle formation is a tightly regulated process, involving cell cycle withdrawal, fusion of myoblast cells into multinucleated myotubes and subsequent differentiation and expression of myogenic regulatory factors, including MyoD and Myf5 (14-16). Genes controlling myogenic differentiation are exquisitely regulated by hormones and growth factors (17). Among these growth factors, the insulin-like growth factors (IGFs) control myoblast proliferation, myogenic differentiation, and skeletal muscle hypertrophy (18). Previous studies have also suggested that systemic administration of GHRP-6 may increase expression of both IGF-1 mRNA and protein levels in various organs, including skeletal muscle, cardiac muscle, brain, and hypothalamus (19, 20). GHRP-6 increased IGF-1 mRNA levels in the cerebellum of aged rats (8) and GHRP-6 also stimulated IGF-1 mRNA expression in C2C12 cells (20). However, whether the effect of GHRPs in muscle differentiation and development is mediated through IGF-1 remains unclear. GHRP-6 did not affect plasma IGF-1 levels or IGF-1 mRNA levels in intact hearts and the myocardial IGF-1 mRNA level was not enhanced by GHRP-6 treatment (11). In this study, we showed that GHRP-6 down-regulated the expression of IGF-1 in myoblasts and inhibited myocyte differentiation (Fig. S4). However, treatment with the GHRP-6-biotin conjugate induced myogenesis and increased IGF-1 expression, suggesting that conjugation of biotin with GHRP-6 changes the biological properties of GHRP-6. While further investigation is needed, a change in the antioxidant properties of GHRP-6 by conjugation with biotin may be responsible for the difference seen in IGF-1 expression inducing activity.

In addition to upregulating IGF-1 expression, the GHRP-6-biotin conjugate treatment also stimulated the synthesis of collagen type I proteins in cultured C2C12 cells. While the exact role of extracellular matrix molecules in myogenesis is not yet fully understood, previous studies have reported that collagen may be involved in satellite cell self-renewal, muscle regeneration, muscle differentiation, and wound healing. Recently, Zhou et al reported that collagen synthesis significantly increases during wound healing in muscle tissue after surgery (21). Additionally, collagen IV knockout mice (Col6a1−/−) show impaired muscle regeneration with satellite cell defects. Restoration of collagen IV synthesis rescued the self-renewal capability of the satellite cells and maintained muscle structure (22). Moreover, studies have also reported that integrin/collagen mediates the myogenesis process by regulating myogenic gene expression and myoblast fusion (23, 24). Given this background, collagen synthesis may play a critical role in muscle differentiation and regeneration.

In this study, we showed that treatment with the GHRP-6-biotin conjugate stimulated myogenic differentiation. Furthermore, this differentiation was mediated by collagen synthesis through, at least in part, IGF-1-mediated signaling. However, both GHRP-6 and biotin, as non-conjugated forms, did not show IGF-1 stimulating activity. To explore binding molecules specific for GHRP-6-biotin conjugate compounds, MADLI-TOF analysis after coprecipitation was performed (Fig. 4). Among the proteins identified was desmin, a muscle-specific protein and a key subunit of the intermediate filament in cardiac, skeletal, and smooth muscle. It has been suggested that desmin plays a critical role in the maintenance of the structural and mechanical integrity of the contractile apparatus in muscle tissue (25). Skeletal muscle of desmin knockout mice shows higher susceptibility to mechanical injury (26) and inhibiting desmin protein expression via anti-sense RNA treatment resulted in a blockade of myotube formation in cultured myoblast C2C12 cells (27). In a skeletal muscle atrophy model, desmin is also a target molecule for muscle-specific ubiquitin ligase, Atrogin1, and tripartite motif (RING, B-box, coiled-coil) containing Trim32 (28, 29). These results suggest that the GHRP-6-biotin conjugate compound might prevent the degradation of desmin by ubiquitin ligases through direct binding. While further investigation is needed to clarify the role of the GHRP-6-biotin conjugate and its effects on myogenesis, our results suggest that the newly synthesized GHRP-6-biotin conjugate has stimulatory effects on myogenesis, at least in part, through up-regulating collagen type I and IGF-1 expression.

**MATERIALS AND METHODS**

**Cell culture and antibodies**

The mouse myoblast cell line C2C12 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Invitrogen, Grand Island, NY, USA) supplemented with 1% penicillin/streptomycin and 10% FBS in a 5% CO2 incubator at 37°C according to a previously described method (30). Briefly, C2C12 myoblasts at 90-100% confluency were differentiated into myotubes by growth medium with differentiation medium (DMEM supplemented with 1% penicillin/streptomycin and 2% horse serum). Every 48 h, the differentiation medium of the myotubes was replaced.

Anti-myogenin and caveolin-3 antibodies were purchased from BD Transduction Laboratories (San Jose, CA, USA), and anti-beta-actin and alpha-actin antibodies were from Pierce...
Materials
GHRP-6 was synthesized by Anygen Co., Ltd. (Gwangju, Korea) and biotin and other reagents used for the conjugation were purchased from Sigma-Aldrich. The GHRP-6-biotin conjugate compound was synthesized by a FMOC solid-phase peptide synthesis method using a glass reactor vessel. The reaction mixture was purified by reverse-phase high-performance liquid chromatography (HPLC) using a Vydac Everest C18 column. Elution was carried out with a water-acetonitrile linear gradient (10-75% (v/v) acetonitrile) containing 0.1% (v/v) trifluoroacetic acid. The molecular weight of the purified compound was confirmed by LC/MS.

Protein measurements and enzyme activity assay
To measure protein expression and enzyme activity in vitro, C2C12 myoblast cells were treated with test compounds in differentiation media for 24 h. The expressions of IGF-1 and collagen 1 in the culture medium were measured using an IGF-1 ELISA kit (Abcam, Cambridge, MA, USA) and a collagen type I ELISA kit (Chondrex Inc., Redmond, WA, USA), respectively, according to the manufacturers’ instructions. For measuring the energy-metabolism related enzyme activities and metabolites, cell were incubated with serum-free medium containing test samples for 16 h. After washing with ice-cold PBS three times, cells were collected and lysed with assay buffer. Cell lysates were centrifuged (12,000 g, 10 min). The supernatant was collected and creatine kinase (CK) and lactate dehydrogenase (LDH) activities were measured by appropriate activity assay kits (Abcam), after filtering the supernatant with a 10kDa spin column (Abcam). ATP and lactate concentrations in the supernatant were also measured using an ATP assay kit and a lactate assay kit (Abcam), respectively.

Western blotting and silver staining
Western blotting was performed as described previously (31). Briefly, the proteins in the whole cell lysates were separated on SDS-polyacrylamide gels and transferred to a PVDF membrane. The antigens were visualized by sequential treatment on SDS-polyacrylamide gels and transferred to a PVDF membrane. The antigens were visualized by sequential treatment on SDS-polyacrylamide gels and transferred to a PVDF membrane.

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis
To search the binding proteins of the test sample, MALDI-TOF analysis was performed. Briefly, proteins were subjected to in-gel trypsin digestion and excised gel spots were destained with 100 μl of destaining solution (50% methanol/distilled water) with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate solution for 20 min. The gel pieces were dehydrated with 100 μl of acetonitrile and dried in a vacuum centrifuge. Then, 50 μl of 10 mM DTT in 0.1 M ammonium bicarbonate solution was added to the residue and incubated for 30 min at 56°C with shaking. After spin down, the supernatant was removed and acetonitrile was added to shrink the protein. After adding 50 μl of 55 mM of iodoacetamide in 0.1 M ammonium bicarbonate solution, the mixture was incubated for 20 min in the dark. Washing with acetonitrile and 0.1 M ABC solution treatment was performed three times. The dried gel pieces were rehydrated with 20 μl of 50 mM ammonium bicarbonate solution containing modified trypsin (Promega, Madison, WI) for 45 min on ice. After removal of the solution, 30 μl of 50 mM ammonium bicarbonate solution was added and the digestion reaction was performed overnight at 37°C. The peptide solution was desalted using a ZipTip C18 column and analyzed using an UltraFlex III MALDI-TOF/TOF system (Bruker Corp., Fremont, CA, USA).

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