Non-toxic sulfur enhances growth hormone signaling through the JAK2/STAT5b/IGF-1 pathway in C2C12 cells

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Abstract. Insulin-like growth factor-1 (IGF-1) regulates cell growth, glucose uptake and protein metabolism, and is required for growth hormone (GH) signaling-mediated insulin production and secretion. *IGF1* expression is associated with STAT5, which binds to a region (TTCNNNGAA) of the gene. Although sulfur is used in various fields, the toxicity of this element is a significant disadvantage as it causes indigestion, vomiting, diarrhea, pain and migraine. Therefore, it is difficult to conduct *in vitro* experiments to directly determine the effects of dietary sulfur. Additionally, it is difficult to dissolve non-toxic sulfur (NTS). The present study aimed to identify the role of NTS in GH signaling as a Jak2/STAT5b/IGF-1 pathway regulator. MTT assay was used to identify an optimum NTS concentration for C2C12 mouse muscle cells. Western blotting, RT-PCR, chromatin immunoprecipitation, overexpression and small interfering RNA analyses were performed. NTS was dissolved in 1 mg/ml DMSO and could be used *in vitro*. Therefore, the present study determined whether NTS induced mouse muscle cell growth via GH signaling. NTS notably increased STAT5b binding to the *Igf1* promoter. NTS also promoted GH signaling by upregulating GH receptor expression, similar to GH treatment. NTS enhanced GH signaling by regulating Jak2/STAT5b/IGF-1 signaling pathway factor expression in C2C12 mouse muscle cells. Thus, NTS may be used as a GH-enhancing growth stimulator.

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Introduction

Insulin-like growth factor (IGF) mediates the growth-promoting effects of growth hormone (GH), which is mainly produced in the liver (1-4). IGF-1 regulates growth, glucose uptake and protein metabolism, whereas the IGF-1-dependent effects of GH induce insulin secretion and lipolysis (5). The GH-liver axis provides signals related to growth and nutrient partitioning (6,7). GH also serves an important role in somatic cell growth.

Signal transducers and activators of transcription (STATs) are activated by various factors and cytokines and subsequently activate Jak tyrosine kinases, leading to receptor tyrosine phosphorylation (8). STAT5 signaling depends on the ability of this protein to translocate to the nucleus and bind to the nuclear response element of a target gene (9). *IGF1* expression is associated with STAT5, which binds to a region (TTCNNNGAA) in this gene (10,11). As a result, GH is stimulated by *IGF1* expression. This process begins with the stimulation of STAT5 via cell signaling, which increases *IGF1* mRNA expression by binding to the gene promoter (10-12).

Sulfur has long been used and collected from sulfur stones or emulsified minerals by heating, melting and obtaining liquid sulfur from the upper layer of the mineral. Pure sulfur is odorless and either colorless or pale yellow, and is not electrically conductive or water-soluble (13). Sulfur is a component of the essential amino acids cysteine and methionine. This element has been widely used. Historically, it was used in paints, gunpowder and weapons. At present, it is used in industries ranging from cosmetics to food supplements; ~85% of all sulfur is used to produce fertilizer (14). A number of studies have elucidated the functions of sulfur in the human body, namely as a component of skin, bones, hair and cartilage tissue (15,16). Sulfur is also essential for enzyme and immune reactions (17,18).

In humans, sulfur must be consumed indirectly; it is present in duck meat, as well as in garlic (*Allium sativum*), onions (*A. cepa*) and hooker chives (*A. hookeri*) (19,20). Direct administration of sulfur-containing natural compounds is generally toxic to humans and causes side effects, and thus sulfur-containing compounds are not used directly unless their toxic components are removed (21). The USA and other

countries use non-toxic forms of natural and dietary sulfur, e.g., methylsulfonylmethane (MSM) as growth enhancers, pain relievers and drugs for rheumatoid arthritis, depression, skin hardening, cancer and inflammatory disease (22-26). These applications indirectly demonstrate the efficacy of MSM; however, MSM is associated with high production costs and limited plant resources.

To utilize mineral sulfur, various substances (boiled pine tree or black soybean extract) are mixed with precipitated mineral sulfur, centrifuged and dehydrated to remove harmful components (27). The addition of non-toxic sulfur (NTS) to livestock feed has been demonstrated to improve immunity and meat quality (28). In addition, repeated oral administration of NTS did not induce toxicity in rats (29). However, to the best of our knowledge, no studies have addressed the underlying mechanisms of the effects of NTS on cell growth due to the inability to dissolve NTS. The present study aimed to describe a method for verifying the efficacy of NTS *in vitro*.

Materials and methods

Antibodies and cell culture reagents. DMEM was purchased from Gibco; Thermo Fisher Scientific, Inc. Penicillin-streptomycin solution and fetal bovine serum (FBS) were purchased from HyClone; GE Healthcare Life Sciences. Trypsin-EDTA (0.05%) was obtained from Gibco; Thermo Fisher Scientific, Inc. Antibodies specific for β-actin (cat. no. sc-47778), GH receptor (GHR; cat. no. sc-57161), IGF-1 receptor β (IGF-1Rβ; cat. no. sc-713), STAT5b (cat. no. sc-1656) and horseradish peroxidase-conjugated goat anti-mouse (cat. no. sc-516102) and anti-rabbit (cat. no. sc-2357) secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. An anti-IGF-1 antibody (cat. no. ab9572) was purchased from Abcam, and pIGF-1Rβ (cat. no. 3021s), pJak2 (cat. no. 3776s), Jak2 (cat. no. 3230s) and pSTAT5 (cat. no. 9314s) antibodies were purchased from Cell Signaling Technology Inc. Recombinant growth hormone (cat. no. 100-40) was purchased from PeproTech Inc. NTS was purchased from Nara Bio Co., Ltd.

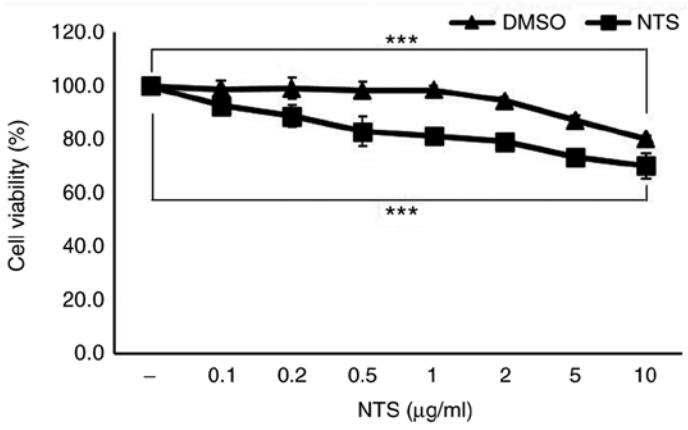
Cell culture and treatment. Mouse muscle C2C12 cells (ATCC CRL-1772) were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin at 37˚C in 5% CO_2. For each experiment, cells at 70-80% confluence were gently washed twice with PBS. For differentiation studies, cells at 85% confluence were transferred in DMEM supplemented with 2% horse serum (differentiation medium) and treated with NTS in fresh media. Unless otherwise specified, cells were treated with 0.2 μg/ml NTS in 99.9% DMSO (Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C.

Cell viability assay. Cell viability was assessed using an MTT assay (Sigma-Aldrich; Merck KGaA). The day before treatment, cells were re-suspended in DMEM at a density of $1x10^4$ cells/well in 24-well culture plates. The next day, the medium was replaced with fresh DMEM alone (vehicle control) or with different concentrations of NTS (0.1-10 μg/ml), followed by a 24 h incubation at 37˚C. Subsequently, MTT (5 mg/ml) was added and the cells were incubated at 37˚C for 4 h. The resulting formazan product was dissolved in DMSO, and the absorbance was measured at 560 nm using an Ultra Multifunctional Microplate Reader (Tecan US, Inc.). All measurements were performed in triplicate, and experiments were repeated at least thrice.

Western blotting. Whole cell lysates were prepared from untreated or NTS-treated mouse muscle cells in Radioimmunoprecipitation assay buffer (EMD Millipore) containing phosphatase and protease inhibitors on ice. Cells were disrupted by aspiration through a 23-gauge needle, and the lysates were centrifuged at 18,300 x g and 4˚C for 10 min to remove cellular debris. Protein concentrations were measured using the Bradford method (Thermo Fisher Scientific, Inc.). Equal amounts of protein (100 μg/lane) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with 5% skimmed milk (BD Biosciences) in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1X Tween-20). The membranes were probed overnight at 4˚C with relevant primary antibodies diluted in 5% bovine serum albumin (EMD Millipore) or skimmed milk, washed with TBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. Visualization was performed using an Enhanced Chemiluminescence Plus detection kit (Amersham; GE Healthcare) and an ImageQuant LAS-4000 imaging device (Fujifilm) with LAS-4000 Control Software (Version 1.1; Fujifilm). The blots were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Inc.).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's protocol and quantified spectrophotometrically at 260 nm. Subsequently, RT-PCR analyses were performed to detect IGF-1 and GAPDH RNA expression. Briefly, cDNA was synthesized from total RNA at 42˚C for 1 h and 95˚C for 5 min using a first-strand cDNA synthesis kit (cat. no. K-2041; Bioneer Corporation) and oligo d(T) primers. A RT-PCR Premix kit (cat. no. K-2016; Bioneer Corporation) was used to amplify IGF-1 and GAPDH using specific primers (Bioneer Corporation): IGF-1 forward, 5'-CTACGCCAA TGTGGTGCTAT-3' and reverse, 5'-TCTGCCATTTGC CTGAAGTT-3' (409 bp); GAPDH forward, 5'-AAGGCC ATCACCATCTTCCA-3' and reverse, 5'-ACGATGCCA AAGTGGTCATG-3' (320 bp). The PCR conditions were as follows: 95˚C for 10 min; 32 cycles at 95˚C for 45 sec, 58˚C for 60 sec and 72˚C for 60 sec; and 72˚C for 10 min. The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized using ethidium bromide (Sigma-Aldrich; Merck KGaA). Davinch-K Gel Imaging System (Davinch-K Co., Ltd.) and Multi Gauge V3.1 (Fujifilm) were used for densitometry analysis.

Transfection and STAT5b overexpression analysis. C2C12 cells ($2x10^5$) were cultured in 6-well plates to 60% confluence. The cells were transfected with a STAT5b-pMX vector (22) (kindly provided by Dr Koichi Ikuta, Kyoto University, Japan) using the DharmaFECT transfection reagent (GE Healthcare Dharmacon, Inc.) for 24 h at 37˚C. Transfected cells were washed with ice-cold PBS and treated for 24 h with media



NTS (μg/ml)	DMSO (%)	NTS (%)
–	100.0	100.0
0.1	98.9	92.4
0.2	99.1	88.7
0.5	98.3	83.1
1	98.5	81.2
2	94.4	79.1
5	87.1	73.4
10	80.2	70.2

Figure 1. Effects of NTS on C2C12 cell viability. MTT-based evaluation of C2C12 cell viability revealed 8-17% growth inhibition at NTS concentrations of 0.1-0.5 μg/ml for 24 h. Data are representative of three independent experiments. ***P<0.001. NTS, non-toxic sulfur.

containing DMSO with or without NTS. Western blotting was used to isolate and analyze target protein and β-actin expression levels as described above.

Small interfering RNA (siRNA) transfection and analysis. C2C12 cells ($2x10^5$) were cultured in 6-well plates to 60% confluence and subsequently transfected with On-Target plus SMARTpool siRNA specific for STAT5b or non-targeting siRNA (cat. no. L-010539-00-0005; GE Healthcare Dharmacon) with FuGENE6 (Roche Diagnostics) according to the manufacturer's instructions. After 24 h, NTS was added for another 24 h at 37˚C. Proteins were isolated and subjected to western blotting for further analysis as described above.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed using the Imprint® Chromatin Immunoprecipitation kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Briefly, mouse muscle cells were fixed with 1% formaldehyde for 10 min at room temperature and quenched with 1.25 M glycine. Following washing with PBS, the cells were suspended in nuclear preparation buffer and shearing buffer and sonicated under optimized conditions (amplitude, 25%; pulse, 30 sec on/30 sec off for 20 min on ice). The sheared DNA was centrifuged at 21,000 x g for 10 min at 4˚C, and the cleared supernatant was subjected to protein/DNA immunoprecipitation. The clarified supernatant was diluted with the dilution buffer (ratio, 1:1), and 5 μl aliquots of the diluted samples were used as internal controls. The diluted supernatant was incubated with anti-STAT5b antibodies in Parafilm pre-coated wells for 90 min. The controls were incubated with normal goat IgG and anti-RNA polymerase II. Unbound DNA was removed using immunoprecipitation wash buffer, and bound DNA was collected using the cross-link reversal method with DNA release buffer containing proteinase K. The released DNA and internal control DNA were purified using the GenElute Binding Column G. DNA was then quantified using conventional quantitative PCR. The primer sequences were as follows: IGF-1 forward 5'-CCACACACACCTATTCACCC-3' and reverse, 5'-CCTGGAGCCATAGGGTATGA-3'. The qPCR conditions were as follows: 95˚C for 3 min; 40 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 40 sec; and 72˚C for 5 min.

Growth hormone assay. Analysis of the growth hormone levels was conducted using Human Growth Hormone SimpleStep® ELISA kit (ab190811; Abcam). C2C12 cells ($2x10^5$) were cultured in 6-well plates to 60% confluence and treated with 99.9% DMSO and NTS for 24 h. The samples were centrifuged at 2,000 x g for 10 min, and the supernatants were collected. The samples were then added to 96-well plate along with equal amounts of antibody cocktail and incubated for 1 h at room temperature on a plate shaker at 400 rpm. The plates were washed with 1X wash buffer, and the development solution was added and incubated for 10 min in the dark on a plate shaker set to 400 rpm. Finally, the stop solution was added, and optical density was recorded at 450 nm using an Ultra Multifunctional Microplate Reader (Tecan US, Inc.). All measurements were performed in triplicate, and experiments were repeated at least thrice.

Statistical analysis. Data are expressed as the mean ± SEM of at least three experiments. Statistical analysis was performed using Student's t-test or one-way ANOVA with Tukey's post hoc test. Analyses were performed using the SAS 9.3 program (SAS Institute, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

NTS induces C2C12 cell death. NTS is not easily dissolved in common solvents such as water, ethanol or DMSO. Through a series of experiments involving normal and heated water, DMSO and ethanol, NTS was determined to dissolve completely in DMSO at a maximum concentration of 1 mg/ml (Table SI and Fig. S1). To evaluate the effects of NTS on C2C12 cell proliferation, MTT assay was conducted using 0.1-10 μg/ml NTS and DMSO alone (control). NTS induced significantly more cell death compared with an equal amount of DMSO (Fig. 1). For further experiments, NTS concentrations <0.5 μg/ml were selected, since they did not induce significantly greater cell death compared with the controls.

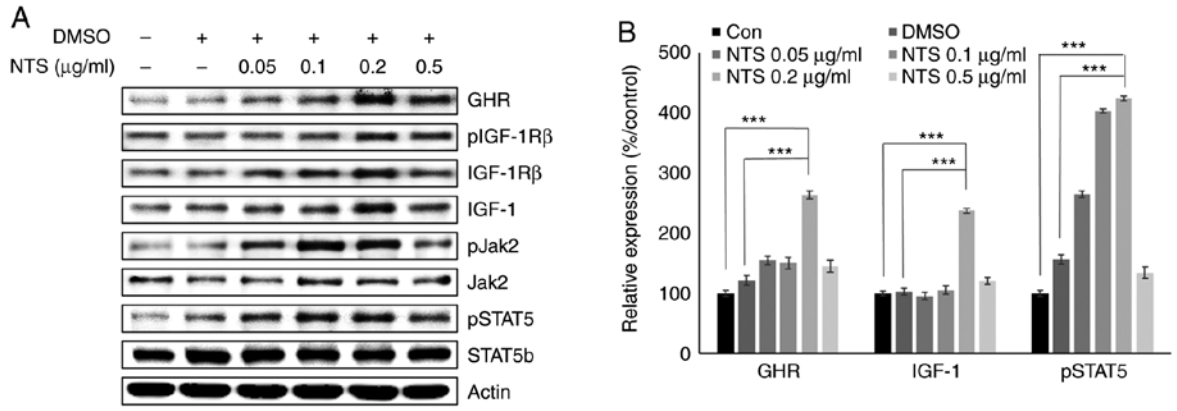


Figure 2. NTS increases the expression levels of GHR, IGF-1 and pSTAT5 in C2C12 cells. (A) NTS increased GHR, IGF-1 and pSTAT5 levels in C2C12 cells. Western blotting of C2C12 cell cytoplasmic proteins following 24 h NTS treatment. (B) Relative GHR, IGF-1 and pSTAT5 protein levels were determined by densitometry and normalized to β-actin. Data are representative of three independent experiments. ***P<0.001. NTS, non-toxic sulfur; Con, control; GHR, growth hormone receptor; IGF-1, insulin-like growth factor-1; IGF-1Rβ, insulin-like growth factor-1 receptor β; p, phosphorylated.

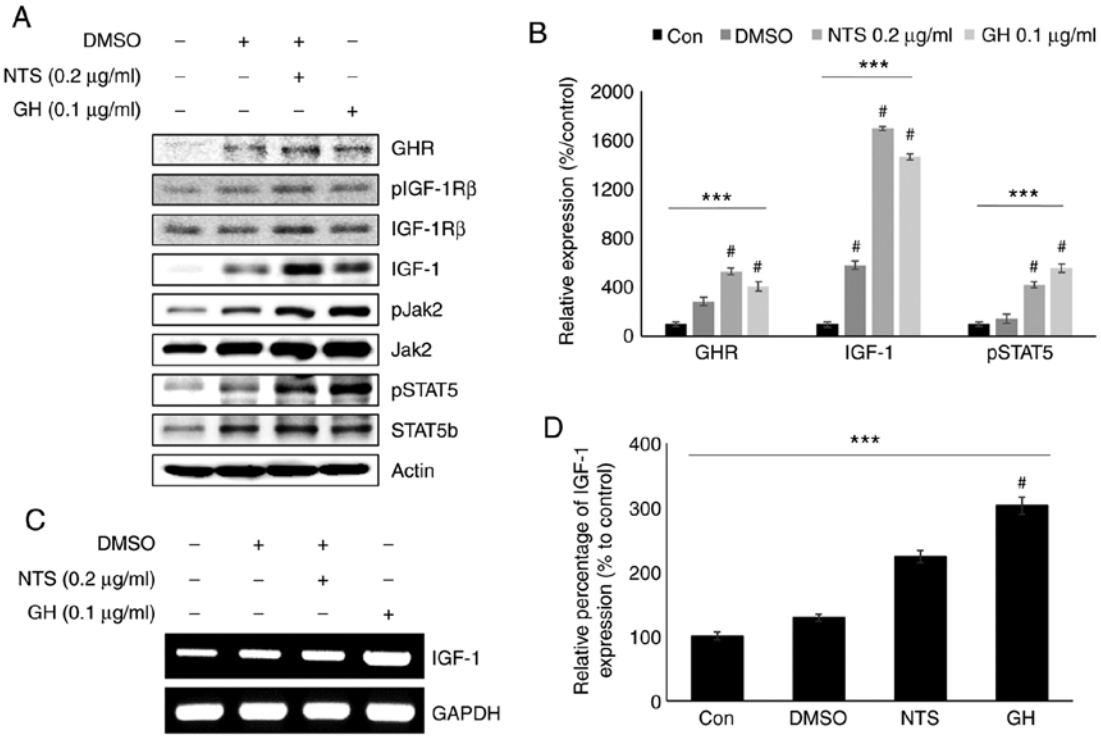


Figure 3. NTS and GH signaling similarly increase IGF-1 and pSTAT5 levels. (A) Western blotting of C2C12 cell cytoplasmic proteins after NTS (0.2 μg/ml) or GH (0.1 μg/ml) treatment revealed increases in GHR, IGF-1 and pSTAT5 protein levels. (B) Relative GHR, IGF-1 and pSTAT5 protein levels were determined by densitometry analysis and normalized to β-actin. Data are representative of three independent experiments. ***P<0.001. (C) NTS or GH signaling for 24 h enhances *Igf1* mRNA expression in C2C12 cells. (D) Relative *Igf1* mRNA expression was determined by densitometry and normalized to *Gapdh* mRNA. Data are representative of three independent experiments. ***P<0.001 among all groups; #P<0.001 vs. control. NTS, non-toxic sulfur; Con, control; GH, growth hormone; GHR, growth hormone receptor; IGF-1, insulin-like growth factor-1; IGF-1Rβ, insulin-like growth factor-1 receptor β; p, phosphorylated.

NTS increases the expression of GHR, pSTAT5 and IGF-1 in C2C12 cells. In the MTT assay, NTS concentrations ≤0.5 μg/ml induced <18% mortality in C2C12 mouse muscle cells. Western blotting was used to analyze translational expression of GHR, pSTAT5 and IGF-1 in C2C12 cells treated with 0.05-0.5 μg/ml NTS. The results demonstrated that compared with lower concentrations, 0.2 μg/ml NTS increased the expression of GHR, pIGF-1Rβ, pJak2, pSTAT5 and IGF-1 without inducing notable changes in total Jak2, STAT5b and IGF-1Rβ (Figs. 2A and S3). Slight decreases in the levels of GHR, pSTAT5 and IGF-1 proteins were observed at 0.5 μg/ml NTS, possibly due to increased cell death (Fig. 2B). To confirm the ability of NTS to induce growth hormone signaling, a growth hormone assay was performed in C2C12 cells in presence of NTS, and the results revealed increased levels of growth hormone in NTS compared with control cells (Fig. S2).

NTS increases the expression of pSTAT5 and IGF-1, similar to GH signaling. As NTS increased the levels of GHR, pSTAT5 and IGF-1, it was hypothesized that it may exert similar effects

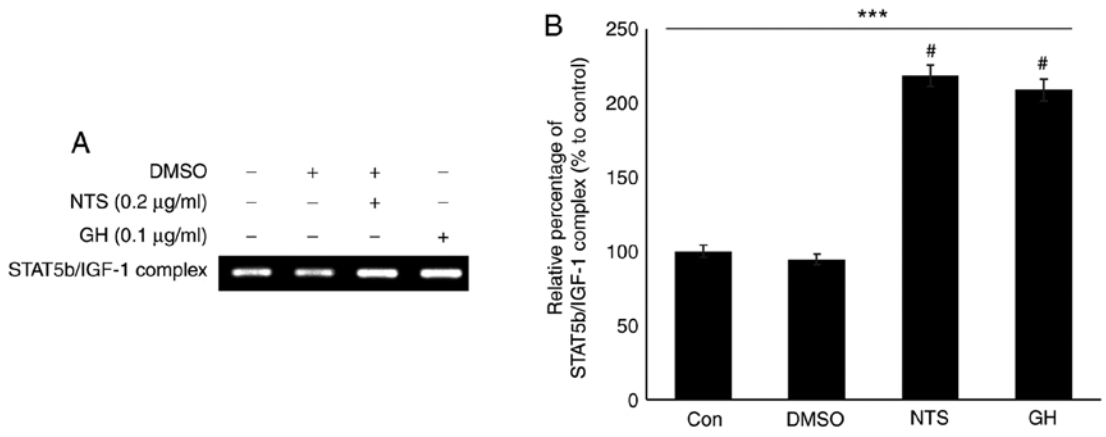


Figure 4. NTS increases STAT5b binding to *Igf1*. (A) ChIP analysis of STAT5b/*Igf1* complex formation in C2C12 cells following NTS treatment (0.2 μg/ml) for 24 h. Data were quantified using quantitative PCR. (B) Relative STAT5b/*Igf1* complex binding expressed as a percentage of the control. Data are representative of three independent experiments. ***P<0.001 among all groups; #P<0.001 vs. control. NTS, non-toxic sulfur; Con, control; GH, growth hormone; IGF-1, insulin-like growth factor-1.

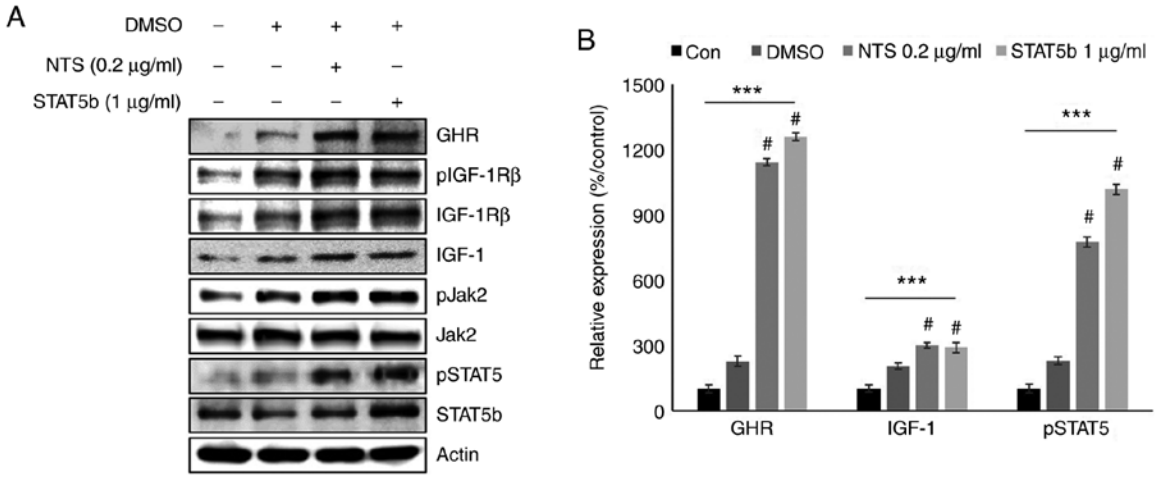


Figure 5. NTS increases the GHR and IGF-1 expression in a STAT5b-dependent manner. (A) STAT5b-overexpressing C2C12 cells and NTS-treated cells (0.2 μg/ml) exhibited similar increases in GHR, IGF-1 and pSTAT5 protein levels. (B) Relative GHR, IGF-1 and pSTAT5 protein levels were determined by densitometry and normalized to β-actin. Data are representative of three independent experiments. ***P<0.001 among all groups; #P<0.001 vs. control. NTS, non-toxic sulfur; Con, control; GHR, growth hormone receptor; IGF-1, insulin-like growth factor-1; IGF-1Rβ, insulin-like growth factor-1 receptor β; p, phosphorylated.

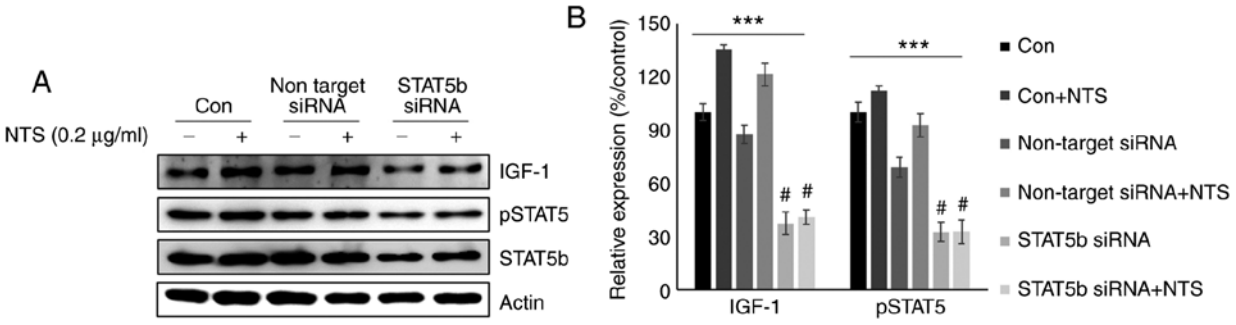


Figure 6. NTS regulates IGF-1 expression through STAT5b. (A) On-target STAT5b inhibition decreased IGF-1 expression in NTS-treated C2C12 cells. (B) Relative pSTAT5 and IGF-1 protein levels were determined by densitometry and normalized to β-actin. Data are representative of three independent experiments. ***P<0.001 among all groups; #P<0.001 vs. control. NTS, non-toxic sulfur; Con, control; IGF-1, insulin-like growth factor-1; siRNA, small interfering RNA; p, phosphorylated.

to those of GH signaling. Western blotting was used to analyze cells treated with 0.1 μg/ml recombinant GH (Fig. 3A). The patterns of GHR, STAT5b, pSTAT5, Jak2, pJak2 and IGF-1 expression were similar between cells treated with NTS and

GH (Figs. 3B and S4). pIGF-1Rβ and IGF-1Rβ expression did not exhibit any differences compared with the control. *Igf1* mRNA levels in the presence of 0.2 μg/ml NTS or 0.1 μg/ml GH were also evaluated (Fig. 3C). Similar to the protein levels, increased *Igf1* mRNA expression was observed in response to NTS and GH, suggesting that these factors induce similar signaling pathways (Fig. 3D).

NTS and GH increase the binding of STAT5b to the Igf1 promoter. As NTS and GH signaling exhibited similar effects, the effects of NTS on the binding of STAT5b to the *Igf1* promoter region were evaluated using 0.1 μg/ml recombinant GH for comparison. The primer was designed to cover the *Igf1* promoter region. The results of ChIP with a STAT5b antibody demonstrated that 0.2 μg/ml NTS increased the binding activity of STAT5b to the *Igf1* promoter and thus may have initiated transcription (Fig. 4A). The increased formation of the STAT5b/*Igf1* complex in response to GH signaling also suggested similarity with NTS activity (Fig. 4B).

NTS increases the expression of GHR and IGF-1 similar to STAT5b signaling. Considering the similarities between NTS activity and GH signaling, the role of STAT5b was compared with NTS treatment by overexpressing STAT5b. Western blotting analysis of NTS-treated and STAT5b-overexpressing cells exhibited similar increases in the levels of GHR, IGF-1 and pSTAT5 (Fig. 5A). NTS also upregulated pJak2, which suggested that it may induce GH signaling by regulating Jak2/STAT5b/IGF-1 signaling. Similar upregulation of GHR in response to NTS treatment and STAT5b overexpression also suggested an association between these signaling axes (Fig. 5B).

STAT5b regulates IGF-1 expression in C2C12 cells following NTS treatment. To confirm the role of STAT5b in the NTS-mediated regulation of IGF-1, *Stat5b* expression was silenced using siRNA. Western blotting analysis of on-target STAT5b inhibition revealed increases in the levels of pSTAT5 and IGF-1 following NTS treatment (Fig. 6A). The relative levels of pSTAT5 and IGF-1 suggested that STAT5b may be a key mediator of the effects of NTS in C2C12 mouse muscle cells (Fig. 6B). These results suggested that NTS may act as a GH mimic to regulate STAT5b and IGF-1 signaling.

Discussion

Sulfur is the third-most abundant mineral element present in the human body after calcium and phosphorous, and it is used in metabolism (30). As direct sulfur administration is generally toxic, this element is normally derived from dietary protein sources. NTS is important as it enables non-toxic sulfur supplementation that can be delivered via non-dietary methods. However, the research on NTS is preliminary, and a suitable solvent for NTS is uncertain. In the present study, NTS was successfully dissolved in DMSO ≤1 mg/ml.

Sulfur is essential for growth and development. The sulfur-containing amino acids methionine and cysteine are required for protein synthesis and optimal growth (31). Sulfur exerts antibacterial effects against acne-causing bacteria (32) and facilitates the shedding of skin, and thus could be used to treat certain skin conditions (33). External sulfur therapy may be an effective growth-promoting option in a system that cannot produce sulfur-containing amino acids. The present study hypothesized that NTS may act a as growth factor by mimicking GH signaling. GH is an important promoter of stem cell activation, cell growth and differentiation (34). GHR is a class 1 receptor to which GH binds to promote cell growth (35). A previous study demonstrated that MSM could enhance GH signaling by regulating the Jak2/STAT5b pathway (36). Therefore, it was hypothesized in the present study that NTS may enhance GH activity in C2C12 mouse muscle cells. The results demonstrated an increase of GH signaling by NTS in C2C12 cells, which indicated the ability of NTS to enhance GH activity.

GH promotes cell growth by regulating Jak2 and STAT5b activity and subsequently inducing IGF-1 expression (37). STAT5b acts as a transcription factor for IGF-1 (11). GH binds to GHR to promote cell proliferation, whereas GHR knockdown prevents the phosphorylation of STAT5 by Jak2, thus inhibiting growth (38). In the present study, NTS upregulated the expression of GHR, pSTAT5 and IGF-1, suggesting a role in growth enhancement similar to that of GH. Therefore, the effects of NTS were compared with those of recombinant GH in C2C12 cells; the results demonstrated that both NTS and GH upregulated the levels of GHR, pSTAT5, pJak2 and IGF-1. These results confirmed that NTS may act similarly to GH or mimic GH signaling to promote cell growth. NTS and GH also increased the formation of the STAT5b/*Igf1* complex, which further confirmed that enhanced STAT5b signaling may be mediated by IGF-1.

STAT5b serves an important role in cell growth, as GH promotes cell growth by regulating IGF-1 production via the GHR/STAT5b cascade (39). IGF-1 production also depends on Jak2-dependent STAT5b phosphorylation during cell growth and development (40). The present study evaluated the role of STAT5b in NTS-mediated cell growth enhancement by comparing the effects of NTS and STAT5b overexpression. Of note, cells treated with NTS or overexpressing STAT5b exhibited increased levels of GHR, pJak2, pSTAT5 and IGF-1. These results confirmed that NTS may enhance GH signaling by regulating the Jak2/STAT5b/IGF-1 signaling cascade. STAT5b is a major molecule associated with sulfur-containing compound-mediated growth enhancement (41). Combined STAT5b knockdown and NTS treatment increased the expression of IGF-1, thus confirming the role of STAT5b in mediating the effect of NTS on GH signaling.

In conclusion, NTS may act to enhance GH signaling by upregulating the expression of GHR. Specifically, NTS enhanced GH signaling by regulating the Jak2/STAT5b/IGF-1 signaling pathway in C2C12 mouse muscle cells. STAT5b may also serve a vital role in the ability of NTS to enhance GH signaling.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

DYK and NS conceived, designed and performed the experiments and wrote the manuscript. YMY and K-JJ contributed in designing the experiments and data analysis. ESJ, HDK, IHK and SWB analyzed the data. All authors contributed to revising the manuscript and approved the final version for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

Hyoung Do Kim is affiliated with Nara Bio Co., Ltd., which provided funding for this study and supplied non-toxic sulfur. The remaining authors declare that they have no competing interests.

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