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Review article

Homeostasis of skeletal muscle function and a functional food ingredient: Citrus limonoid nomilin.

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Abstract

In an aging society, extending the healthy life expectancy of the elderly is an urgent issue. Since the healthy life expectancy is nothing less than maintaining physical function to live an independent life, there is a need to elucidate the mechanisms that increase muscle mass and strength. In the course of this research for functional analysis of bile acids, catabolic products of cholesterol, we have focused our research on the function of TGR5, a bile acid receptor. We sought to clarify the function of TGR5, especially in the skeletal muscle, by analysis of multiple species of genetically modified mice. As a result, TGR5 activation by bile acids in the skeletal muscle was found to lead to muscle hypertrophy and strengthening. Furthermore, we have established an evaluation system for the search of food ingredients that activate TGR5 instead of bile acids, and found that, in food ingredients, nomilin, a citrus limonoid, has a TGR5 agonist activity. In this review, new physiological functions of bile acids were discussed, relating to the possibility of maintaining skeletal muscle function by food ingredients.

***KEY WORDS*:** cholesterol, bile acid, TGR5, skeletal muscle, nomilin

Introduction

Those aged 65 and over of the overall population, aging rate, are soon accounting for 30%, and are estimated to be over 40% by the middle of this century. In order to prevent the caring costs from swelling with increasing population needed support/nursing care and above all things to realize a society consisting of active elderly, an extension of healthy life expectancy, especially maintaining physical function, is important. Since the healthy life expectancy means the life span in which you can live independently, it is desirable, by maintaining the skeletal muscle function, to keep the state that does not require support or nursing care for a long time. To maintain a functional skeletal muscle, appropriate eating habits and moderate exercise are required, however, exercise habits become difficult to continue with aging. In the time of hardships, it is expected to find food ingredients with an effect of increasing skeletal muscle function. During the process analyzing a function of the bile acid, a catabolized product from cholesterol, we paid attention to the role of TGR5, a specific receptor for bile acids, in skeletal muscle and proceeded with the research. As a result, the skeletal muscle TGR5, activated by binding to the bile acid as a ligand, has been shown to increase muscle mass and strengthen muscle [1]. Furthermore, aiming to maintain muscle mass in daily diet, we have established an evaluation system to search for food ingredients with TGR5 agonist activity. Among several hundreds of food ingredients, nomilin, a citrus limonoid, was finally found to have TGR5 agonist activity [2].

Healthy life expectancy and food function

In an aging society like Japan, we can say that it is an attempt with extremely high social demands to extend the healthy life expectancy of the elderly. The difference between average life expectancy and healthy life expectancy is about 9 years in males and 12-13 years in females, the reduction of this difference, which improves quality of life (QOL), is urgently needed. When looking at the reasons for the need for support and nursing care after a healthy life expectancy, cerebrovascular disease ranks first, however, it is reported that 35% are due to the locomotor disorders including frailty, joint disorders, falling down, and bone fractures ("Comprehensive Survey of Living Conditions in 2016" by the Ministry of Health, Labor and Welfare),

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indicating the importance of maintaining physical function in the elderly. Considering that it will be difficult, with aging, to maintain physical function by exercise habits, development of functional foods which maintain and improve locomotion function can be said to be an urgent issue. Concurrently, the development of next-generation agricultural, forestry, and fishery products having such a function may contribute to the promotion of Japanese agriculture considering export in their view.

The skeletal muscle

Our bodies are supported and protected by the skeletal muscle occupying 40 ~ 50% of the body weight. Simultaneously with this role, the skeletal muscle plays an important role as a metabolic tissue. When post-prandial hyperglycemia induces insulin secretin followed by reduction of blood glucose, nearly 75% of blood glucose is up taken into the skeletal muscle. Muscle mass decreases with aging, thus meaning that the locomotion function deteriorates and further weakens the metabolic control function mediated by blood glucose homeostasis. It is reported that the skeletal muscle mass decreases 1 ~ 2% per year in the elderly. Therefore, it leads the elderly to health maintenance by reserving the sound metabolic control function if they can protect their muscle loss, retain adequate muscle mass, and maintain the locomotion function which enables the independence activities. This may be the proper way of extending the healthy life expectancy.

Control of muscle mass

The muscle mass depends on increase or decrease of protein amount in the skeletal muscle. The muscle mass decrease, for example, by a bed-ridden situation or staying in space with low gravity may be a result of enhanced proteolysis in these cases. The muscular proteolysis is intimately involved in degradation by proteasome after ubiquitin modification of proteins. When the feet of the experimental animals were casted with plaster, the muscle mass decreased significantly in about a week. In this process, the gene expression is elevated in several enzymes, *i.e.* E3 ligase, Atrogin-1, MuRF1, which bind ubiquitin to substrates, and ligase is also activated. Therefore, the food ingredients which inhibit gene expression or activation of E3 ligase can be expected to protect proteolysis, thus reducing muscular weakness. On the other hand, branched-chain amino acids (BCAA) are shown to be effective as a trial stimulating protein synthesis. BCAA content in skeletal muscle is high, the supply of BCAA, *i.e.* leucine, isoleucine, and valine, elevates protein synthesis in the skeletal muscle. It was also confirmed in the clinical trial that the administration of a large amount of ω3 fatty acids increased protein synthesis in the skeletal muscle [3].

New physical function of bile acids

In the human liver, about one gram of cholesterol is synthesized in a day. Regarding the food-derived cholesterol, the estimated absorption is about 150 ~ 200 mg. Although cholesterol is a lipid component, our body cannot burn or decompose cholesterol as an energy source. It is the only metabolic pathway that cholesterol is catabolized to bile acids by the liver. The structure of cholesterol is complicated with 27 carbon atoms. By the liver 3 carbon atoms are removed and catabolized to be bile acids with 24 carbon atoms, finally discharged to the stool; thus, cholesterol balance maintains equilibrium in the body. Bile acids, deposited in the gallbladder which contracts in response to the feeding stimulants, are secreted into the duodenum. By this way, food-derived fat-soluble ingredients are emulsified (micelle formation) and become able to be digested by lipase. Bile acids are transported down the small intestine to ileum while promoting fat digestion and absorption, then through the specific transporter, IBAT (ileal bile acid transporter), 95% of bile acids are re-absorbed into the portal vein. The bile acids are later transported to the liver, merging with newly catabolized bile acids, and again secreted into the small intestine. This form of enterohepatic circulation is repeated about 10 times, and then the bile acids are discharged with stool.

In the 2000s, a remarkable discovery was successive regarding the role of bile acids. Bile acid is an endogenous ligand of the nuclear receptor FXR (farnesoid X receptor), which is activated by binding to the bile acid and regulates gene expression of several proteins. Activated FXR increase the gene expression of SHP (small heterodimer partner), a response gene of FXR, resulting to decrease the gene expression of CYP7A1, a rate-limiting enzyme in bile acid synthesis. Once the bile acid synthesis is activated and enough amounts of bile acids turn back to the liver, catabolism from cholesterol to bile acids is suppressed as a negative feedback system [4].

Also, when bile acid is re-absorbed to the liver, a part of bile acids leak out into the systemic blood flow, thus present as a level of about 10 μM, and a bile-acid-binding receptor TGR5 (seven-pass transmembrane G protein-coupled receptor: GPCR) has been found [5, 6]. Once bile acid binds to TGR5, intracellular cAMP concentration elevates, protein kinase A activated in response, furthermore a transcription factor CREB (cAMP response element-binding protein) activated and downstream signals transmitted (***Fig. 1***). In the L cells in the small and large intestine, TGR5 stimulates secretion of GLP-1 (glucagon-like peptide-1) [7], one of incretin, which improves insulin sensitivity.

On the other hand, in the human skeletal muscle and rodent brown adipose tissue, TGR5 elevates the gene expression of DIO_2 (type II iodothyronine deiodinase), an enzyme converting thyroid hormone T_4 (inactive form) to T_3 (active form) in the cell. Besides, TGR5 elevates the gene expression of PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) and mitochondria activity; it was revealed that thermogenesis is promoted due to both effects [8]. In brief, bile acid improves insulin resistance by incretin actions through the bile acid-TGR5 axis, furthermore, exhibiting anti-obesity effects by elevated thermogenesis.

A new function of TGR5 in the skeletal muscle

In the skeletal muscle, genes of multiple GPCR are expressed, and it is indicated that GPCR strengthen muscle by ligand binding followed by an increase of intracellular cAMP concentration [9]. Among GPCR, β_2 adrenergic receptor, relatively high expression in skeletal muscle, is known to induce muscle hypertrophy. Clenbuterol, a synthetic ligand, is

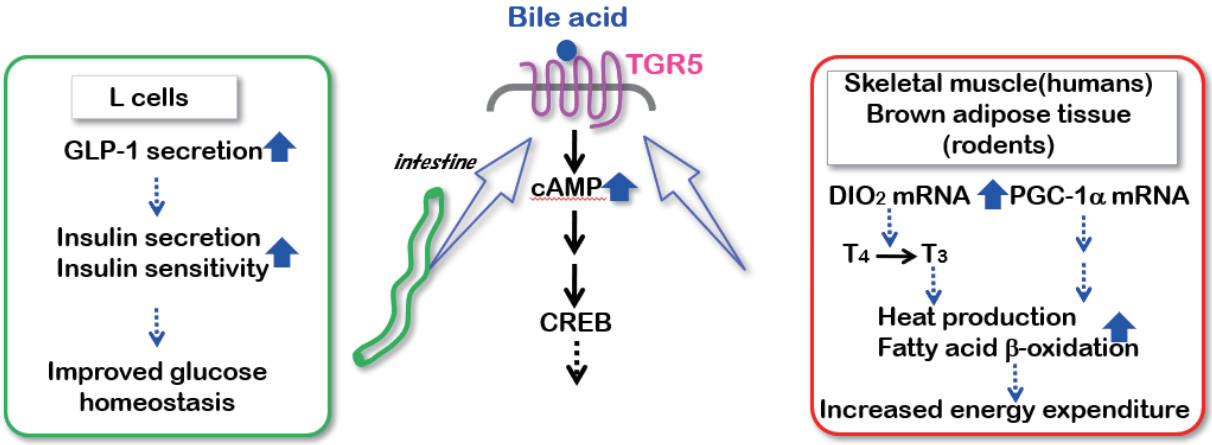

Fig. 1. **The elevation of insulin sensitivity and anti-obese effect by bile acid-TGR5 axis: An overview.**

confirmed to induce muscle hypertrophy when administered to experimental animals and humans[10]. Therefore, clenbuterol is designated as one of the doping banned drugs.

Since TGR5, expressed in the skeletal muscle, increases intracellular cAMP concentration like β_2 adrenergic receptor, it is expected to boost muscle mass. So, for the purpose of clarifying the function of a bile acid receptor TGR5 in the skeletal muscle, we have analyzed multiple genetically modified mice. First, we developed a transgenic (Tg) mouse overexpressing human TGR5 in the skeletal muscle and analyzed. Constant TGR5 expression increased muscle mass in gastrocnemius and quadriceps by 10～15%, and the muscle power appeared to have also strengthened significantly[1]. When prepared impaired glucose tolerance mice fed by a high-fat diet, the oral glucose tolerance test (OGTT) has shown that the postprandial hyperglycemia promptly ameliorated in the Tg mice with increased muscle mass and the glucose metabolism improved. Concurrently, the same analysis was conducted using TGR5 knockout mice which were obtained. On the contrary to the Tg mice, the muscle mass significantly decreased and the reduction of muscle strength confirmed. That is that, once the bile acid combines to TGR5 in the skeletal muscle, the signal transmitted, and the amount of muscle protein increases (***Fig. 2***).

Control of gene expression of bile acid receptor TGR5

It has been clarified that the bile acid receptor plays a certain role in the control of the skeletal muscle from the analysis of TGR5 overexpressing Tg mice and TGR5 knockout mice as described above. Then, moving to another question, we proceeded with the analysis for possibilities whether any physical stimuli change TGR5 expression in the skeletal muscle, thus increasing or decreasing muscle mass. Using cultured myotube cells, C2C12, under various conditions using inhibitors or metabolic compounds, we monitored whether the endogenous TGR5 mRNA levels fluctuated. As a result, it was confirmed that, when the culture medium added with tunicamycin or thapsigargin, which inducing endoplasmic reticulum (ER) stress, TGR5 expression was significantly elevated[1]. When these two compounds were injected intramuscularly to the quadriceps of wild mice, then the muscle solubilized, followed by the Western blot analysis using TGR5 antibody, protein expression also was revealed to increase. In a previous research, the unfolded protein response (UPR), an ER stress response, was shown to be induced in the skeletal muscle when exercising mice transiently with moderate or higher load[11]. When exercising wild-type mice intermittently for about one hour using a treadmill, followed by sampling the gastrocnemius muscle for the analysis of TGR5 mRNA, the exercise, as expected, significantly increased mRNA levels.

ER stress is transmitted through the multiple pathways, among them, ATF6 (activating transcription factor 6) related gene expression pathway plays an important role. So, when scrutinizing the upstream of the transcription start point of the mouse TGR5 gene, a sequence similar to ATF6 response sequence was found. As a result of a reporter assay after forming reporter genes with mutation in this sequence, ATF6, activated in response to ER stress, was clarified to elevate TGR5 expression mediated by this sequence. Furthermore, we obtained ATF6−/− mice and exercise them side by side with wild-type mice. As a result, the elevation of TGR5 mRNA level was noted in the gastrocnemius after exercise, while the level was not changed in ATF6−/− mice. Sik1 (salt Inducible Kinase 1) and PGC-1αmRNA, known to elevate after exercise, were elevated in the both types of mice.

Bile acids are secreted into the small intestine by feeding response and it shows high blood levels after eating[12]. Therefore, we consider that the blood bile acid is one of the feeding signals. That is, the elevation of blood bile acid levels acts as a signal inducing skeletal muscle hypertrophy. Meanwhile, immediately after exercise, expression of a receptor TGR5 was elevated, bile acid-TGR5 signal worked, leading to the muscle hypertrophy. As the above- mentioned, a new functional coupling between blood bile acids and skeletal TGR5 can be clarified (***Fig. 2***). These findings clarify the physiological significance of bile acids that was previously unknown, as well as the importance of blood bile acid levels, and furthermore explain the effectiveness of food ingredients mimicking the bile acid function on muscle hypertrophy.

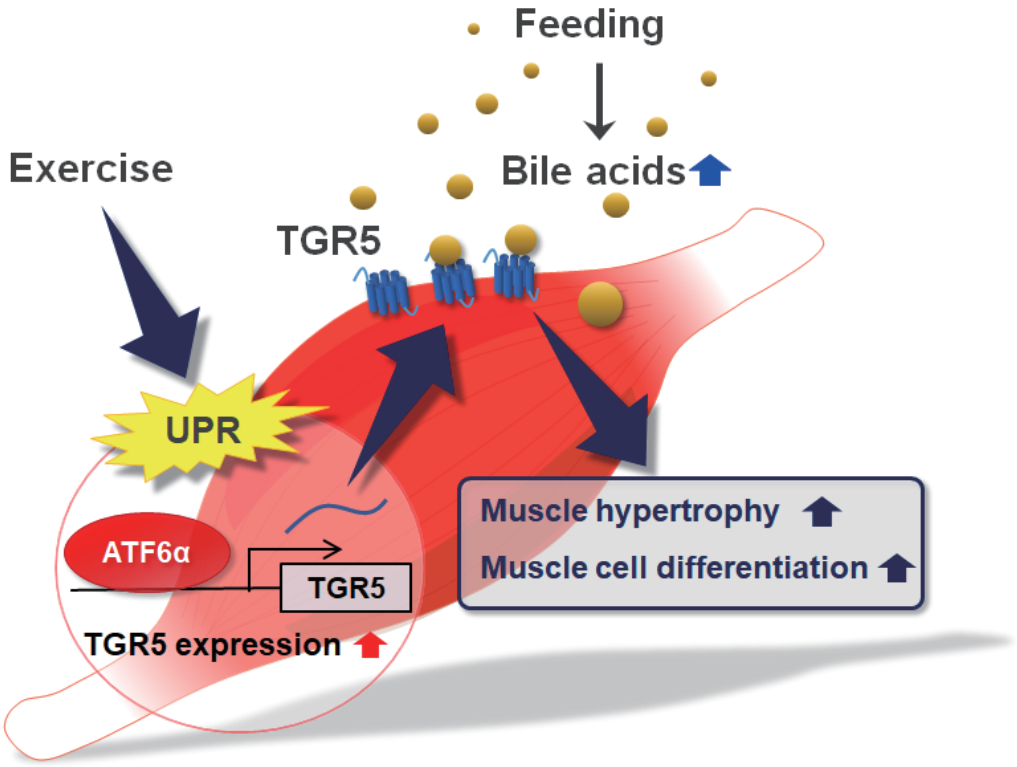

Fig. 2. **The muscle hypertrophy and the promotion of muscle differentiation by bile acid-TGR5 axis in the skeletal muscle.**

UPR, unfolded protein response.

New motor functional food ingredients inducing muscle hypertrophy

Aiming to activate TGR5 by food ingredients, we have started searching for active food ingredients with TGR5 agonist activity like bile acids. The addition of bile acids to foods is not approved, that is also the reason for searching for alternative compounds. We have purchased a compound, refined specimen, contained in food as a reagent, and created a library of about 140 compounds. About 300 kinds of fragrance ingredients were obtained, approximately 500 compounds in total were used in the evaluation system and agonist activities were quantified. Specifically, TGR5 was expressed on the cultured cell surface with an expression vector incorporated human TGR5 gene, the assay system was established where luciferase protein expression can be elevated when ingredients are added to the medium bind to TGR5 and intracellular cAMP are increased, followed by the measurement of activities. This assay is highly sensitive where the luciferase activity several tens of times higher than that of the control is detected once agonist activities are found. As a result, we have found nomilin (***Fig. 3***), one of the citrus limonoids [2]. Nomilin and the other citrus limonoids have been reported to show antibacterial activity, anti-HIV (human immunodeficiency virus) activity, and cancer prevention effect [13-15], and have been evaluated as functional food ingredients. When C57BL/6 mice were bred for 77 days fed by a high fat diet containing 0.2% nomilin, a strong inhibitory effect on weight gain was confirmed. The OGTT revealed that the rapid disappearance of hyperglycemia was observed with the addition of nomilin. Namely, the expected anti-obesity and glucose metabolism improving effects were verified. The TGR5 agonist activity has also been identified in other food triterpene components including betulinic acid and oleanolic acid [16]. Regarding to the muscle hypertrophic actions, in mice bred for 4 weeks fed by a diet containing 0.1% obacunone, a product produced in citrus after nomilin undergoes metabolic conversion, the muscle mass in quadriceps and gastrocnemius was found to be significantly increased [17].

Furthermore, by computer simulation, the connecting style was attempted to estimate how bile acids and nomilin, the primary ligands, bind to TGR5. Based on the results, amino acids, predicted to be involved in binding, were sequentially mutated to Ala residue, and the involvement in binding was checked [18]. As a result, it was confirmed that, the natural ligand, bile acid, binds embeddedly in the transmembrane pocket formed in 7 transmembrane regions of TGR5, while nomilin, which is not the original ligand, enters one end of the molecule into the transmembrane pocket and the other end binds to two amino acid residues (Gln77 and Arg80) located at the extracellular domain of TGR5 molecule (a loop part connecting between the second [TM2] and third [TM3] transmembrane area) with a form protruding out of the cell membrane (***Fig. 3***).

Nomilin is mainly contained in seeds and peels in

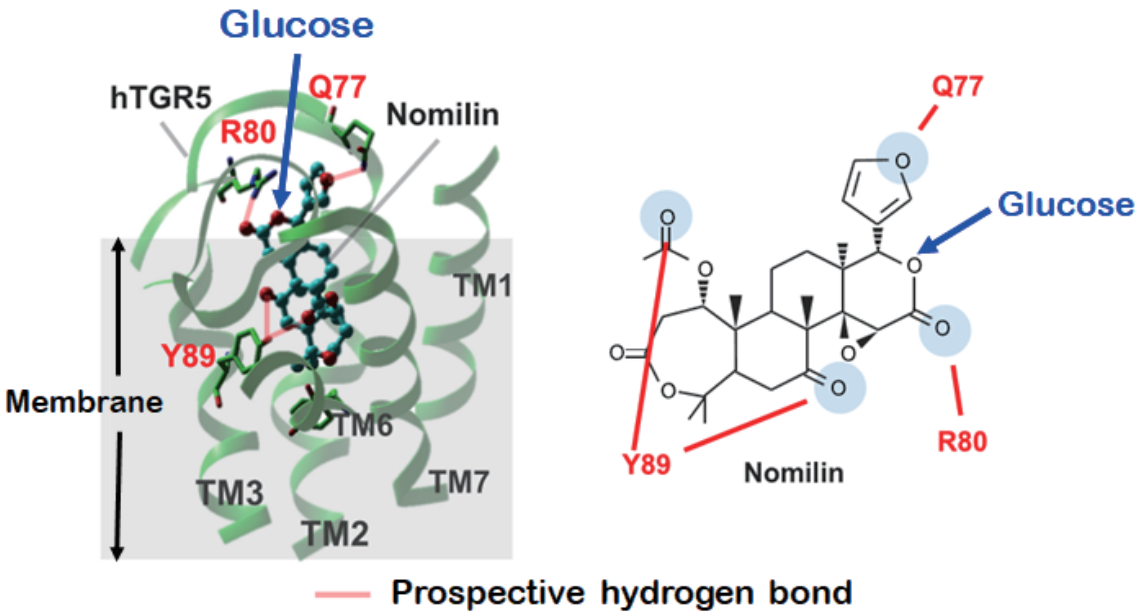

Fig. 3. **The binding pattern between nomilin and TGR5: A diagram.**

The Nomilin tip enters the transmembrane region and the other end, located extracellularly, binds to TGR5. Once the glycosides added glucose to portions with arrows, the binding of TGR5 to Q77 (Gln 77) and R80 (Arg 80) is predicted to be interrupted.

citrus plants, while, the pulp part, which we usually eat, contains a glycoside form where nomilin is bound to sugar (glucose). The fruit pulp area is an aqueous environment and the addition of sugar imparts hydrophilicity to nomilin. Then, the glycoside-type nomilin was purified from the citrus pulp and its TGR5 agonist activity was compared with the aglycone-type nomilin without sugar. As a result, glycosides were found to have significantly lower agonist activity. Therefore, if we habitually ingest citrus flesh, the probability of functioning as a TGR5 agonist is expected to be low. Although the possibility that the sugar moiety is cleaved and absorbed in the intestine cannot be ruled out, nomilin is known to be low in absorption and incidence into the blood. Judging from the connecting style between TGR5 and nomilin mentioned above, glucose located extracellularly is likely to interfere with nomilin when binding to the two essential parts of amino acid residues in the extracellular domain of TGR5. For the above reasons, glycosides can be understood as having low agonist activity (***Fig. 3***).

Conclusion

In the super-aged society coming in the middle of the twenty-first century, attempts to maintain skeletal muscle function utilizing the food power will become increasingly important in the future. According to the forecast of demographic changes over the next 40 years in Japan, elderly people aged 65 and over keep 35-39 million and will be almost unchanged in a declining society. Over the next few decades, it is expected that many people hope the development of functional foods, worthy of trust, with a healthy life extension effect. We hope that a healthy life extension can be achieved, by leveraging the potential of food, with maintaining skeletal muscle function.

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Conflict of Interest Statement

The authors claim no conflict of interest in this study.

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