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Food factors that mimic bile acid functions

Food Factors That Mimic Bile Acid Functions

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ABSTRACT

TGR5 is a member of the G protein-coupled receptor family and is activated by bile acids. TGR5 is thought to be a promising food factor target for preventing metabolic diseases because the activation of TGR5 prevents obesity and hyperglycemia in mice fed a high-fat diet. In the present study, we established an evaluation system for the TGR5 ligand activity, and identified a limonoid nomilin as an activator of TGR5 from among approximate 150 purified food compounds. Unlike bile acids, nomilin did not exhibit the farnesoid X receptor ligand activity. Although the nomilin derivative obacunone was capable of activating TGR5, limonin (the most abundant limonoid in citrus seeds) was not a TGR5 activator. When male C57BL/6J mice fed a high-fat diet for 9 weeks were further fed a high-fat diet either alone or supplemented with 0.2% w/w nomilin for 77 days, nomilin-treated mice had lower body weight, serum glucose, serum insulin, and enhanced glucose tolerance. Our results suggest a novel biological function of nomilin as an agent having anti-obesity and anti-hyperglycemic effects that are likely to be mediated through the activation of TGR5.

Key words: limonoid, nomilin, TGR5, anti-obesity, anti-hyperglycemia

INTRODUCTION

The prevalence of metabolic disorders including obesity and type 2 diabetes is increasing at an alarming rate worldwide[1]. Since these metabolic disorders can cause cardiovascular disease and life-threatening complications including blindness, kidney failure, and heart disease, huge efforts are directed at finding new drugs and functional foods that can cure these disorders and/or prevent their development.

TGR5, a novel G protein-coupled receptor, is activated by bile acids (BAs), and its activation results in the elevation of intracellular cAMP levels[2,3]. TGR5 is expressed in brown adipose tissue (BAT) where its activation by BAs increases energy expenditure and attenuates diet-induced obesity in mice[4]. This metabolic effect of BAs is considered to be mediated by the induction of the cAMP-dependent thyroid hormone activating enzyme *type 2 iodothyronine deiodinase (DIO2)* in BAT[4]. Furthermore, TGR5 is expressed in enteroendocrine L cells, and its activation induces intestinal glucagon-like peptide-1 (GLP-1) release, thereby leading to improved liver and pancreatic function and enhancing glucose tolerance in obese mice[5]. These emerging data indicate that TGR5 is an attractive target for the potential prevention and treatment of metabolic disorders[6]. To date, several compounds including BA derivatives[7-10], oleanolic acid[11] present in *Olea europaea* leaves, and betulinic acid[12,13] that is widely distributed throughout the plant kingdom have been identified as TGR5 ligands.

To evaluate the TGR5 ligand activity of food factors, we aimed to generate a new assay system in which an increase in the cAMP level in response to the activation of TGR5 is detected using cultured cells with exogenously-expressed human TGR5. We identified a limonoid nomilin as an activator of TGR5 from among approximate 150 purified food compounds that are commercially available.

Limonoids are highly oxygenated triterpenoid compounds contained in *Citrus*. *Citrus* seeds contain approximately 1% (fresh weight percentage) limonoids. Although the relative composition of limonoids in *Citrus* seeds depends on the species, limonin and nomilin are the major limonoids[14]. Because limonin is responsible for the delayed bitterness of citrus juices, its occurrence lowers the quality and value of these juices[15]. Limonoids exhibit several pharmacological activities such as anti-cancer, anti-malarial, and anti-microbial activities[16,17]. However, the effect of limonoids on metabolic disorders is largely unknown.

MATERIALS AND METHODS

I. *Reagents*

Nomilin (purity, 98.09%) was purchased from Waterstone Technology. Obacunone (purity, 85.8%) was purchased from ChromaDex. Chenodeoxycholic acid (CDCA; purity, ≥ 98%) and limonin (purity, ≥ 90%) were purchased from Sigma.

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II. *Plasmid Constructs*

A pCRE-Luc reporter plasmid containing 4 copies of consensus sites for the cAMP response element (CRE) was obtained from Agilent Technologies. An expression plasmid for TGR5, pcDNA-TGR5, was constructed by inserting a 1.0-kb PCR fragment encoding full-length human TGR5 into a pcDNA3.1 vector (Invitrogen). Another reporter plasmid, pG5Luc, which contained 5 copies of GAL4 binding sites, has been described previously[18]. Moreover, an expression plasmid for the GAL4 DNA-binding domain(DBD)-farnesoid X receptor (FXR) fusion protein, pGAL4-FXR, has been described previously[19,20].

III. *0.2% Nomilin Feeding for 77 Day*

Mice (n = 8) were fed a pelleted HFD for 8 weeks and subsequently fed a powdered HFD for 1 week to allow adaptation to a powdered diet. The mice were then divided into 2 groups of similar average body weight and blood glucose levels; one group of mice was fed a HFD (HF; n = 4), and the second group was fed a nomilin supplemented HFD (HF + Nomilin; n = 4). Food intake was measured daily in the HF + Nomilin group, and the same amount of food was provided to the HF group. The mice were weighed every 4 d. At 66 d after the initiation of nomilin supplementation, an oral glucose tolerance test (OGTT) was performed. At the end of 77 d, the mice were fasted for 4 h and killed under anesthesia. BAT and epididymal white adipose tissue (WAT) were rapidly excised, frozen in liquid nitrogen, and stored at -80°C until further processing.

IV. *OGTT*

OGTT was performed at 66 d after the initiation of nomilin supplementation. After a 16 h period of food deprivation, glucose (2 g/kg body weight) in water was administered orally by gavage. Plasma glucose levels were measured from tail blood before and 15, 30, 45, 60, 90, 120, and 150 min after administration with a handheld glucometer (Ascensia Breeze 2, Bayer Diagnostic).

RESULTS

I. *Nomilin Is a TGR5 Agonist*

We performed reporter assays using a TGR5 expression plasmid and a CRE-driven luciferase reporter plasmid (Figure 1) and found nomilin to be a potent TGR5 agonist. Consistent with previous results[7], 100 μM CDCA increased ($p < 0.01$) the CRE-driven luciferase expression level only when TGR5 was expressed in HEK293 cells. Moreover, nomilin stimulated ($p < 0.01$) luciferase activity only in the presence of TRG5 in a dose-dependent manner, indicating that nomilin exhibits TGR5 ligand activity. Because BAs are dual agonists of TGR5 and FXR, we next examined whether nomilin stimulated FXR. Although CDCA activated ($p < 0.01$) the GAL4-DBD fused FXR, nomilin had no effect.

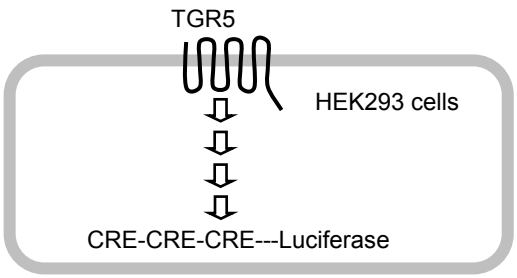

Figure 1. A reporter assay system to evaluate TGR5 ligand activities.

II. *Obacunone, But Not Limonin, Is a TGR5 Agonist*

Next, we examined whether other limonoids derived from nomilin, the most abundant limonoid limonin and obacunone, exhibited TGR5 ligand activity. Although 100 μM obacunone increased ($p < 0.01$) the CRE-driven luciferase expression level in the presence of TGR5 as well as nomilin, 100 μM limonin did not stimulate luciferase activity at all, indicating that obacunone, but not limonin, is another TGR5 agonist.

III. *Nomilin Supplementation with HFD Suppresses Diet-induced Obesity and Serum Glucose and Insulin Levels*

The mice were pair-fed either a HFD (HF) or HFD supplemented with 0.2% nomilin (HF + Nomilin) for 77 d. Compared with the HF group, the HF + Nomilin group had lower ($p < 0.05$) body weight at day 64 after nomilin supplementation, and thiseffect continued until sacrifice. Serum glucose ($p < 0.05$) and insulin ($p < 0.01$) levels as well as epididymal WAT weight ($p < 0.05$) were lowered by nomilin. An oral glucose tolerance test performed on day 66 after nomilin supplementation revealed that the HF + Nomilin group had enhanced ($p < 0.05$) glucose tolerance. Nomilin did not affect serum TG, ALT, and AST levels, whereas the serum NEFA level was lowered ($p < 0.01$). Contrary to our expectations, the expression of the key genes involved in energy expenditure, such as *DIO2*, *UCP1*, *CPT-1*, *ACO*, and *PGC-1β*, was unchanged, and *PGC1α* gene expression was reduced ($p < 0.05$) by nomilin in BAT.

DISCUSSION

The results of the present study demonstrate that nomilin activated TGR5 in *in vitro* luciferase reporter assays. The mice fed a HFD supplemented with nomilin had a lower body weight and blood glucose levels as well as enhanced glucose tolerance compared with mice fed a nomilin-free HFD. These results clearly indicate that

dietary nomilin ameliorates some metabolic abnormalities in HFD-fed mice.

BAT is a major organ for cold- and diet-induced adaptive thermogenesis in rodents and primates. Adaptive thermogenesis is an energy dissipation process that occurs without accompanying ATP production[21]. Because obesity occurs when energy intake exceeds energy expenditure, acceleration of adaptive thermogenesis is considered to be an important target for combating obesity. BAs activate TGR5 that in turn elevates intracellular cAMP levels in BAT[4]. This elevation leads to an increase in the expression of key genes involved in energy expenditure, including *DIO2* that encodes an enzyme catalyzing the conversion of thyroxine into 3,5,3′-triiodothyronine and *PGC-1α*[4]. In a preliminary *in vivo* experiment in which the dietary nomilin content was gradually increased for 160 d, we observed that *DIO2* and *PGC-1α* gene expressions in BAT were enhanced by nomilin concomitantly with weight loss in HFD-fed mice. Taken together, these results suggest that dietary nomilin can enhance energy expenditure in BAT through TGR5 activation. However, nomilin feeding for 77 d in Expt. 2 did not increase the expression of the key genes involved in energy expenditure; instead, suppression of *PGC-1α* gene expression was observed despite nomilin-dependent weight loss in HFD-fed mice. Presently, the reason for this discrepancy is unclear. Some differences in the experimental conditions between the *in vivo* experiments (feeding period 160 d *vs.* 77 d; nomilin content gradually increased from 0.02 to 0.2% *vs.* 0.2%) may have been responsible for this difference. In addition, the serum NEFA level may have contributed to this difference.

Dietary nomilin (0.2% for 77 d) reduced the serum NEFA level in HFD-fed mice, whereas this decrease was not observed in mice fed a nomilin-containing HFD for 160 d. A number of studies have demonstrated that fatty acids increase *PGC-1α* gene expression[22,23], and *n-3* PUFAs including docosahexaenoic acid increase intracellular cAMP levels[24], which could lead to the elevated expression of the key genes involved in energy expenditure. Therefore, the activation of TGR5 by dietary nomilin may oppose the effects of decreased serum NEFA levels, thereby resulting in no significant increase in the expression of the key genes involved in energy expenditure in BAT after consumption of nomilin for 77 d. However, the reason why the serum NEFA levels differed in the two experiments remains unclear. Further studies will be required to elucidate these differences.

In the present study, dietary nomilin lowered blood glucose levels and brought about enhanced glucose tolerance in HFD-fed mice. At present, the means by which dietary nomilin impacts the blood glucose level and glucose tolerance is unclear. It is conceivable that dietary nomilin-mediated weight loss influences glucose metabolism. TGR5 ligands stimulate secretion of GLP-1, which is a known incretin hormone, from enteroendocrine L cells and improves insulin sensitivity and glucose tolerance in HFD-fed mice[5]. Thus, dietary nomilin may augment GLP-1 secretion through the activation of TGR5 in enteroendocrine L cells, thereby improving glucose metabolism. It is interesting to note that dietary nomilin has the potential to affect glucose metabolism in the gut before absorption and increase energy expenditure in BAT after absorption.

BAs are dual ligands for TGR5 and FXR[7], but triterpenoids such as oleanolic acid and betulinic acid are selective ligands for TGR5[11,12]. In addition, in the present study, we demonstrated that the highly oxygenated triterpenoid nomilin is a selective ligand for TGR5. Nomilin, obacunone, and limonin share a similar structure; their B- to D-rings and side chains are identical. Given that limonin did not activate TGR5, the presence of an A′-ring is likely to inhibit its binding to TGR5.

An HFD containing 0.2% nomilin ingested for 77 d did not alter serum ALT and AST levels, indicating that nomilin is not toxic to mice although it does reduce food intake. This reduction is believed to be due to the bitter taste of nomilin. Limonoid glucosides are tasteless, and limonin is detected at nanomolar levels in the plasma of healthy humans 6 h after administration of limonin glucoside (2.0 g in 200 mL of buffered water)[25]. Therefore, it is possible that the administration of nomilin glucoside exerts the same effect as nomilin but without the bitter taste.

CONCLUSIONS

Our study demonstrates that dietary nomilin suppressed diet-induced obesity and hyperglycemia in mice, and this function is mediated, at least in part, by the activation of TGR5[26]. Because we used purified nomilin, it is essential to determine whether nomilin glucosides and crude extracts of citrus fruits exhibit the same effect as purified nomilin. This issue is now under investigation.

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REFERENCES

1. Zimmet, P., Alberti, K. G. and Shaw, J. 2001. Global and societal implications of the diabetes epidemic. Nature 414: 782-787.
2. Maruyama, T., Miyamoto, Y., Nakamura, T., Tamai, Y., Okada, H., Sugiyama, E., Itadani, H. and Tanaka, K. 2002. Identification of membrane-type receptor for bile acids (M-BAR). Biochem. Biophys. Res. Commun. 298: 714-719.
3. Kawamata, Y., Fujii, R., Hosoya, M., Harada, M., Yoshida, H., Miwa, M., Fukusumi, S., Habata, Y., Itoh, T., Shintani, Y., Hinuma, S., Fujisawa, Y. and Fujino, M. 2003. A. G protein-coupled receptor responsive to bile acids. J. Biol. Chem. 278: 9435-9440.

4. Watanabe, M., Houten, S. M., Mataki, C., Christoffolete, M. A., Kim, B. W., Sato, H., Messaddeq, N., Harney, J. W., Ezaki, O., Kodama, T., Schoonjans, K., Bianco, A. C. and Auwerx, J. 2006. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 439: 484-489.
5. Thomas, C., Gioiello, A., Noriega, L., Strehle, A., Oury, J., Rizzo, G., Macchiarulo, A., Yamamoto, H., Mataki, C., Pruzanski, M., Pellicciari, R., Auwerx, J. and Schoonjans, K. 2009. TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metab. 10: 167-177.
6. Tiwari, A. and Maiti, P. 2009. TGR5: an emerging bile acid G-protein-coupled receptor target for the potential treatment of metabolic disorders. Drug Discov. Today 14: 523-530.
7. Sato, H., Macchiarulo, A., Thomas, C., Gioiello, A., Une, M., Hofmann, A. F., Saladin, R., Schoonjans, K., Pellicciari, R. and Auwerx, J. 2008. Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. J. Med. Chem. 51: 1831-1841.
8. R. Pellicciari, H. Sato, A. Gioiello, G. Costantino, A. Macchiarulo, B. M. Sadeghpour, G. Giorgi, K. Schoonjans, J. 2007. Auwerx. Nongenomic actions of bile acids. Synthesis and preliminary characterization of 23- and 6,23-alkyl-substituted bile acid derivatives as selective modulators for the G-protein coupled receptor TGR5. J. Med. Chem. 50 : 4265-4268.
9. Rizzo, G., Passeri, D., De Franco, F., Ciaccioli, G., Donadio, L., Orlandi, S., Sadeghpour, B., Wang, X. X., Jiang, T., Levi, M., Pruzanski, M. and Adorini, L. 2010. Functional characterization of the semisynthetic bile acid derivative INT-767, a dual farnesoid X receptor and TGR5 agonist. Mol. Pharmacol. 78: 617-630.
10. Pellicciari, R., Gioiello, A., Macchiarulo, A., Thomas, C., Rosatelli, E., Natalini, B., Sardella, R., Pruzanski, M., Roda, A., Pastorini, E., Schoonjans, K. and Auwerx, J. 2009. Discovery of 6alpha-ethyl-23(S)-methylcholic acid (S-EMCA, INT-777) as a potent and selective agonist for the TGR5 receptor, a novel target for diabesity. J. Med. Chem. 52: 7958-7961.
11. Sato, H., Genet, C., Strehle, A., Thomas, C., Lobstein, A., Wagner, A., Mioskowski, C., Auwerx, J. and Saladin, R. 2007. Anti-hyperglycemic activity of a TGR5 agonist isolated from Olea europaea. Biochem. Biophys. Res. Commun. 362: 793-798.
12. Genet, C., Strehle, A., Schmidt, C., Boudjelal, G., Lobstein, A., Schoonjans, K., Souchet, M., Auwerx, J. Saladin, R. and Wagner, A. 2010. Structure-activity relationship study of betulinic acid, a novel and selective TGR5 agonist, and its synthetic derivatives: potential impact in diabetes. J. Med. Chem. 53: 178-190.
13. Genet, C., Schmidt, C., Strehle, A., Schoonjans, K., Auwerx, J., Saladin, R., Wagner, A. 2010. Redefining the TGR5 triterpenoid binding pocket at the C-3 position. Chem. Med. Chem. 5: 1983-1988.
14. Hasegawa, S., Bennett, R. D., Verdon, C. P. 1980. Limonoids in citrus seeds: origin and relative concentration. J. Agric. Food Chem. 28: 922-925.
15. Hasegawa, S. and Miyake, M. 1996. Biochemistry and biological functions of citrus limonoids. Food Rev. Int. 12: 413-435.
16. Roy, A. and Saraf, S. 2006. Limonoids: overview of significant bioactive triterpenes distributed in plants kingdom. Biol. Pharm. Bull. 29: 191-201.
17. Manners, G. D. 2007. Citrus limonoids: analysis, bioactivity, and biomedical prospects. J. Agric. Food Chem. 55: 8285-8294.
18. Misawa, K., Horiba, T., Arimura, N., Hirano, Y., Inoue, J., Emoto, N., Shimano, H., Shimizu, M. and Sato, R. 2003. Sterol regulatory element-binding protein-2 interacts with hepatocyte nuclear factor-4 to enhance sterol isomerase gene expression in hepatocytes. J. Biol. Chem. 278: 36176-36182.
19. Nakahara, M., Furuya, N., Takagaki, K., Sugaya, T., Hirota, K., Fukamizu, A., Kanda, T., Fujii, H., Sato, R. 2005. Ileal bile acid-binding protein, functionally associated with the farnesoid X receptor or the ileal bile acid transporter, regulates bile acid activity in the small intestine. J. Biol. Chem. 280: 42283-42289.
20. Sato, R., Miyamoto, W., Inoue, J., Terada, T., Imanaka, T. and Maeda, M. 1999. Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. J. Biol. Chem. 274: 24714-24720.
21. Lowell, B. B., Spiegelman, B. M. 2000. Towards a molecular understanding of adaptive thermogenesis. Nature 404: 652-660.
22. Zhang, P., Liu, C., Zhang, C., Zhang, Y., Shen, P., Zhang, J. and Zhang, C. Y. 2005. Free fatty acids increase PGC-1alpha expression in isolated rat islets. FEBS Lett. 579: 1446-1452.
23. Staiger, H., Staiger, K., Haas, C., Weisser, M., Machicao, F. and Haring, H. U. 2005. Fatty acid-induced differential regulation of the genes encoding peroxisome proliferator-activated receptor-gamma coactivator-1alpha and -1beta in human skeletal muscle cells that have been differentiated in vitro. Diabetologia 48: 2115-2118.
24. Tai, C. C., Chen, C. Y., Lee, H. S., Wang, Y. C., Li, T. K., Mersamm, H. J., Ding, S. T., Wang, P. H. 2009. Docosahexaenoic acid enhances hepatic serum amyloid A expression via protein kinase A-dependent mechanism. J. Biol. Chem. 284: 32239-32247.
25. Manners, G. D., Jacob, R. A., Breksa, A. P., Schoch, T. K. and Hasegawa, S. 2003. Bioavailability of

citrus limonoids in humans. J. Agric. Food Chem. 51: 4156-4161.
26. Ono, E., Inoue, J., Hashidume, T., Shimizu, M. and Sato. R. 2011. Anti-obesity and anti-hyperglycemic effects of the dietary citrus limonoid nomilin in mice fed a high-fat diet. Biochem. Biophys. Res. Commun. 410: 677-681.