Bioorganic & Medicinal Chemistry Letters 19 (2009) 2062–2064

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Identification of nobiletin, a polymethoxyflavonoid, as an enhancer of adiponectin secretion

Kazuhiro Kunimasa [a], Sachi Kuranuki [b], Nobuyasu Matsuura [c], Nozomi Iwasaki [b], Megumi Ikeda [b], Akira Ito [d], Yutaka Sashida [d], Yoshihiro Mimaki [d], Masamichi Yano [e], Mayumi Sato [f], Yasuhiro Igarashi [g], Tsutomu Oikawa [b,*]

[a] Laboratory of Cell and Molecular Biology of Aging, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka, Shizuoka 422-8526, Japan
[b] School of Nutrition and Dietetics, Kanagawa University of Human Services, 1-10-1 Heisei-cho, Yokosuka, Kanagawa 238-8522, Japan
[c] Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama, Okayama 700-0005, Japan
[d] School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[e] Bio-oriented Technology Research Advancement Institution, 3-18-19 Toranomon, Minato-ku, Tokyo 105-0001, Japan
[f] Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan
[g] Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Kosugi, Toyama 939-0398, Japan

ARTICLE INFO

Article history:
Received 17 September 2008
Revised 15 January 2009
Accepted 2 February 2009
Available online 7 February 2009

Keywords:
Nobiletin
Polymethoxyflavonoid
Adiponectin
Lifestyle-related diseases
ST-13 preadipocyte

ABSTRACT

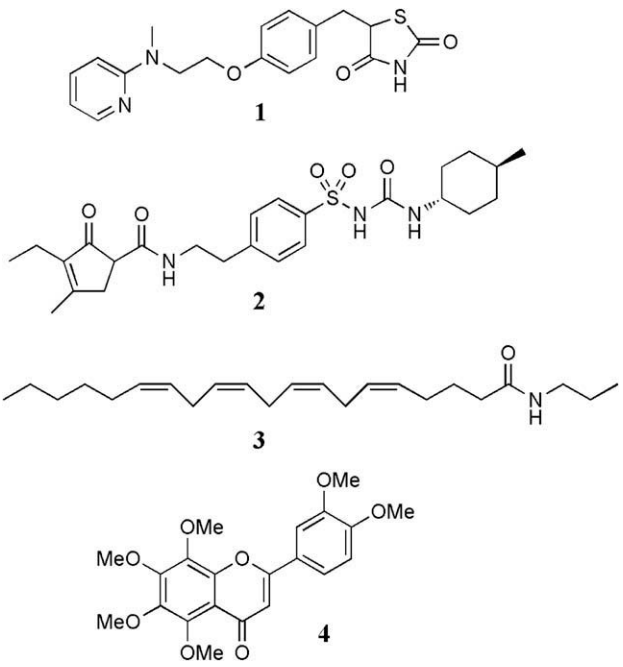
Adiponectin, an adipocyte-derived protein with insulin-sensitizing, anti-diabetic and anti-atherogenic activities, is known to be induced during adipocyte differentiation. Nobiletin, a citrus polymethoxy flavonoid, was found to induce the differentiation of ST-13 preadipocytes into mature adipocytes and enhance the production of adiponectin protein at a concentration of 10 μM.

Recent studies have revealed that in addition to functions as the major energy reservoir adipocytes play a role as endocrine cells that secrete different bioactive proteins, which are termed as adipocytokines, including leptin and adiponectin.[1–3] Among adipocytokines, the functions of adiponectin have been the focus of recent attention because accumulating evidence has shown that there are close relationships between circulating adiponectin levels and a variety of lifestyle-related diseases, including obesity, coronary artery disease, type 2 diabetes and metabolic syndrome, and adiponectin exhibits insulin-sensitizing, anti-diabetic and anti-atherogenic activities.[1–4] Collectively, it is reasonable to consider that adiponectin represents a useful target for treating or preventing insulin resistance, type 2 diabetes, metabolic syndrome and cardiovascular diseases. Adiponectin expression was found to be potently induced during adipocyte differentiation.[5,6] Thiazolidinedione analogs like rosiglitazone (**1**), which are synthetic ligands for peroxisome proliferator-activated receptor γ (PPARγ), have been shown to induce adipocyte differentiation and then enhance mRNA expression and secretion of adiponectin.[7,8] Most inducers of adiponectin expression and/or secretion, including sulfonylurea agents like glimepiride (**2**),[9] and anandamide (**3**),[10] have shown to induce adipocyte differentiation. These findings should imply the possibility that an inducer of adipocyte differentiation promotes the secretion of adiponectin protein. Based on this possibility, we searched an adipocyte differentiation inducer using a screening system involving a ST-13 preadipose cell line that we had previously established from adult mice,[11] and then examined the identified adipocyte differentiation inducers for their actions on the protein level of adiponectin.

Adipocytes, but not preadipocytes, show accumulation of lipid droplets in their cytoplasms. So it is easy to distinguish adipocytes and preadipocytes by a microscopic examination. Using this examination, we screened different samples, including chemical agents and crude extracts derived from plants, microorganisms and marine sponges, for their differentiation-inducing activity against ST-13 preadipocytes.[12] Among the tested samples, nobiletin (**4**; 5,6,7,8,3′,4′-hexamethoxyflavone), a polymethoxy flavonoid from *Citrus depressa*, was found to be one of the most potent inducers of ST-13 preadipocyte differentiation. Figure 1A shows the observa-

* Corresponding author. Tel.: +81 46 828 2660; fax: +81 46 828 2661.
E-mail address: oikawa-t@kuhs.ac.jp (T. Oikawa).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.02.002



tions in representative experiments. The cells treated with nobiletin showed visible deposits of Oil red-stained lipid droplets, compared to the vehicle-treated cells. When ST-13 preadipocytes were treated with the indicated concentrations of nobiletin, they differentiated into lipid-accumulating adipose cells within 11 days after cell seeding. Troglitazone, a positive control, also showed such an activity. The triglyceride determination in the nobiletin-treated cells showed that its effect was concentration-dependent up to 10 μM (Fig. 1B). The ability of nobiletin to induce adipocyte differentiation of ST-13 cells was confirmed by molecular biologi-

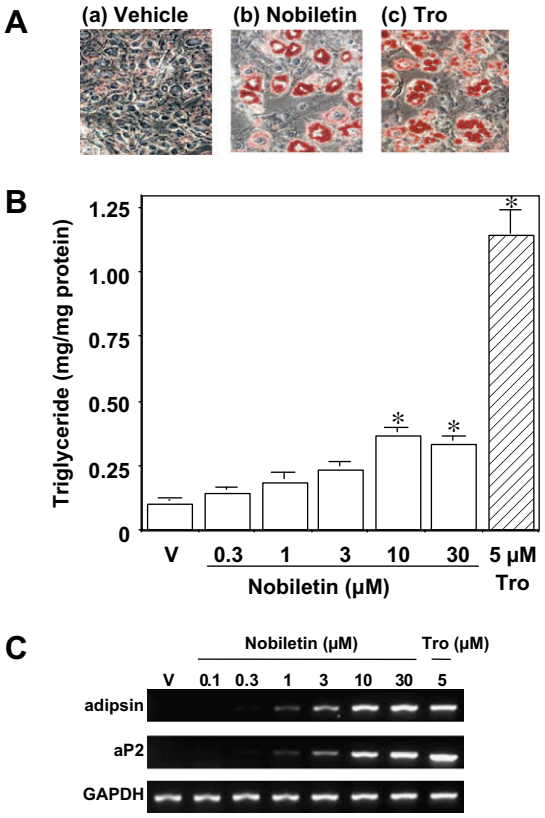


Figure 1. Effects of nobiletin on adipose differentiation of ST-13 cells. ST-13 preadipocytes were cultured in the presence of vehicle (V, 0.1% dimethylsulfoxide), nobiletin, and troglitazone for 11 days. (A) Oil red O staining of ST-13 cells at day 12 in vehicle medium (a) or in medium supplemented with 10 μM nobiletin (b), or 5 μM troglitazone (c). Original magnification × 100. (B) Triglyceride contents were determined by the method of Fletcher.[18] Data are expressed as means ± SD (n = 3). $^{*}P < 0.05$, versus vehicle (V). Tro = troglitazone. (C) mRNA levels of adipsin and adipocyte P2 (aP2), which are genes specific for differentiated adipocytes, were analyzed by semi-quantitative RT-PCR.

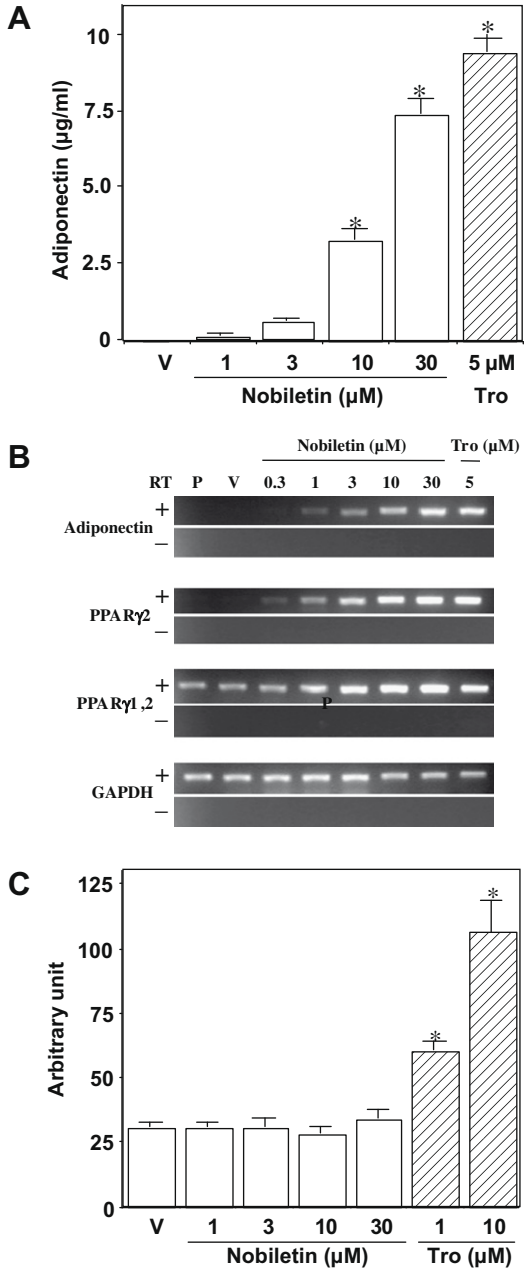


Figure 2. Effect of nobiletin on secretion of adiponectin protein (A), mRNA expression of adiponectin in ST-13 cells (B), and activation of PPARγ in luciferase ligand assay system (C). (A) The conditioned medium of nobiletin-treated ST-13 cells for 11 days was determined for its adiponectin amounts by ELISA. Data are expressed as means ± SD (n = 3). $^{*}P < 0.05$, versus vehicle (V). Tro = troglitazone. (B) The nobiletin-treated ST-13 cells were subjected to semi-quantitative RT-PCR. RT = reverse transcriptase. Tro = troglitazone. PPAR = peroxisome proliferator activated receptor. P = ST-13 preadipocytes. V = vehicle. (C) Data are expressed as means ± SD (n = 3). $^{*}P < 0.05$, versus vehicle (V). Tro = troglitazone.

cal analyses; namely, the compound induced the mRNA expression of adipsin and aP2, both of which are adipose-specific genes,[13] as shown in Figure 1C. These observations prompted us to evaluate the effect of nobiletin on the level of adiponectin protein. So the conditioned media of the ST-13 cells treated with nobiletin were examined for their adiponectin content by an ELISA kit according to the manufacturer's directions (mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo, Japan). Nobiletin promoted adiponectin secretion in a concentration-dependent manner (Fig. 2A). It stimulated the adiponectin secretion by 340% at a concentration of 10 μM, compared to the vehicle-treated control. A structure/activity relationship analysis showed that 5,7,8,4′-tetramethoxyflavone had a small effect on adiponectin secretion, suggesting that dimethoxy groups of nobiletin at C6 and C3′ were responsible for its activity.

To examine whether the increased secretion of adiponectin protein by nobiletin was associated with an increase in mRNA level of adiponectin, a semi-quantitative RT-PCR was performed. As shown in Figure 2B, nobiletin potently increased adiponectin mRNA expression, compared to the vehicle-treated control. Such increases seemed to be concentration-dependent. Saito et al. reported that nobiletin upregulated adiponectin mRNA levels in 3T3-L1 cells.[14] However, they did not evaluate the protein level of adiponectin. Yamada et al. showed that telmisartan upregulated the mRNA expression of adiponectin, but did not increase the production of adiponectin protein.[15] Thus, our study first provides evidence that nobiletin elevates the production of adiponectin protein. Nobiletin also stimulated the mRNA levels of aP2, adipsin and PPARγ2, biomolecular markers specific for differentiated adipocytes.

Some substances like thiazolidinediones have found to induce adiponectin expression through direct activation of PPARγ.[9,10] So we examined whether nobiletin showed PPARγ agonist activity using a luciferase ligand assay system.[16] The luciferase reporter assay showed that nobiletin exhibited little or no PPARγ agonistic activity at a concentration of 30 μM, whereas troglitazone, a PPARγ ligand, potently and concentration-dependently activated reporter gene (Fig. 2C). This might imply that the adiponectin-inducing action of mechanism of nobiletin might differ from those of thiazolidinediones like troglitazone which upregulate adiponectin expression via activation of PPARγ. Like nobiletin, cyanidin without PPARγ agonist activity has been reported to induce adiponectin mRNA expression and secretion.[17]

In summary, the present study identified both the PPARγ nonagonist (nobiletin) and the PPARγ agonist (thiazolidinediones) as an enhancer of adiponectin protein production. This implies that the system can also detect an inducer of adiponectin protein that has an action of mechanism distinct from those of the PPARγ agonists. These findings indicate that our screening system is useful for discovering an adiponectin inducer.

Acknowledgements

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan and a fund from School of Nutrition & Dietetics, Kanagawa University of Human Services. We are grateful to Dr. S. Kato (University of Tokyo) for donating plasmids. We also thank Miku Takahata, Minako Takayama, Aiko Namikawa and Chisa Masio for their help with the study.

References and notes

1. Lihn, A. S.; Pedersen, S. B.; Richelsen, B. *Obes. Rev.* **2005**, *6*, 13.
2. Matsuzawa, Y. *Arterioscler. Thromb. Vasc. Biol.* **2006**, *580*, 2917.
3. Kadowaki, T.; Yamauchi, T.; Kubota, N.; Hara, K.; Ueki, K.; Tobe, K. *J. Clin. Invest.* **2006**, *116*, 1784.
4. Hug, C.; Lodish, H. F. *Curr. Opin. Pharmacol.* **2005**, *5*, 129.
5. Scherer, P. E.; Williams, S.; Fogliano, M.; Baldini, G.; Lodish, H. F. *J. Biol. Chem.* **1995**, *270*, 26746.
6. Hu, E.; Liang, P.; Spiegelman, B. M. *J. Biol. Chem.* **1996**, *271*, 10697.
7. Maeda, N.; Takahashi, M.; Funahashi, T.; Kihara, S.; Nishizawa, H.; Kishida, K.; Nagaretani, H.; Matsuda, M.; Komuro, R.; Ouchi, N.; Kuriyama, H.; Hotta, K.; Nakamura, T.; Shimomura, I.; Matsuzawa, Y. *Diabetes* **2001**, *50*, 2094.
8. Combs, T. P.; Wagner, J. A.; Berger, J.; Doebber, T.; Wang, W. J.; Zhang, B. B.; Tanen, M.; Berg, A. H.; O'Rahilly, S.; Savage, D. B.; Chatterjee, K.; Weiss, S.; Larson, P. J.; Gottesdiener, K. M.; Gertz, B. J.; Charron, M. J.; Scherer, P. E.; Moller, D. E. *Endocrinology* **2002**, *143*, 998.
9. Bouaboula, M.; Hilairet, S.; Marchand, J.; Fajas, L.; Le Fur, G.; Casellas, P. *Eur. J. Pharmacol.* **2005**, *517*, 174.
10. Fukuen, S.; Iwaki, M.; Yasui, A.; Makishima, M.; Matsuda, M.; Shimomura, I. *J. Biol. Chem.* **2005**, *280*, 23653.
11. Hiragun, A.; Sato, M.; Mitsui, H. *In Vitro* **1980**, *16*, 685.
12. ST-13 preadipocytes were plated at a density of 5×10^3 cells per cm^2 in dishes containing a basal medium (0.5 ml per cm^2) containing DME/HD, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin on day 1. Moreover, the fresh medium containing different samples was replaced on day 2, 5, and 8. On day 12, preadipocyte differentiation-inducing activity assay, oil red O stain assay, triglyceride assay, total RNA isolation, or the collection of conditioned medium for ELISA was performed.
13. Sandouk, T.; Reda, D.; Hofmann, C. *Am. J. Physiol.* **1993**, *264*, C1600.
14. Saito, T.; Abe, D.; Sekiya, K. *Biochem. Biophys. Res. Commun.* **2007**, *357*, 371.
15. Yamada, S.; Ano, N.; Toda, K.; Kitaoka, A.; Shiono, K.; Inoue, G.; Atsuda, K.; Irie, J. *Hypertens. Res.* **2008**, *31*, 601.
16. An expression plasmid containing the ligand binding domain of human PPARγ fused to the GAL4 DNA binding domain (pPPARγ-GAL4), and the luciferase reporter plasmid, 17m2G TATA Luc (p17m2G), were kindly donated by Dr. S. Kato (University of Tokyo, Tokyo, Japan). We transiently transfected COS-1 cells (6×10^5 cells) with pPPARγ-GAL4 (0.25 μg) and p17m2G (1 μg) using Effectene Transfection Reagent (QIAGEN, Tokyo, Japan). Transfections were performed in triplicate in 24-well plates according to the manufacture's instructions. After 16 h, the transfected cells received the indicated concentrations of nobiletin (or troglitazone), and were cultured for additional 24 h at 37 °C in a 5% CO_2 incubator. Then cells were lysed and luciferase activities were measured by a Steady-Glo® Luciferase Assay System (Promega, Madison, WI) according to manufacturer's instructions.
17. Tsuda, T.; Ueno, Y.; Aoki, H.; Koda, T.; Horio, F.; Takahashi, N.; Kawada, T.; Osawa, T. *Biochem. Biophys. Res. Commun.* **2004**, *316*, 149.
18. Fletcher, M. J. *Clin. Chim. Acta* **1968**, *22*, 393.