Mechanism of Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) Transactivation by Hesperetin Glucuronides Is Distinct from That by a Thiazolidine-2,4-dione Agent

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Hesperidin, a flavanone glycoside present abundantly in citrus fruits, is predominantly metabolized to hesperetin-7-O-β-D-glucuronide (H7-OG) and hesperetin-3′-O-β-D-glucuronide (H3′-OG), which exhibit partial agonistic activity towards peroxisome proliferator-activated receptor gamma (PPARγ). Here, in order to understand the mechanism(s) of action of PPARγ transactivation elicited by hesperetin glucuronides, we compared the transactivation activities of PPARγ ligand-binding domain (LBD) mutants by hesperetin glucuronides and troglitazone, a thiazolidine-2,4-dione class PPARγ full agonist. The assay results indicated that the mechanisms of activation of PPARγ by hesperetin glucuronides and by troglitazone are distinct, probably due to a difference in the binding sites of these compounds on the PPARγ LBD. Flavanone-class PPARγ partial agonists, luteolin and hesperetin glucuronides, showed similar activation profiles of the PPARγ LBD mutants, even though they have different side chain functionalities.

Key words: hesperidin; hesperetin; hesperetin glucuronide; peroxisome proliferator-activated receptor gamma (PPARγ); PPARγ mutant

Hesperidin and its aglycone hesperetin, both of which are flavanone-type flavonoids abundantly present in citrus fruit, have been reported to exhibit various beneficial pharmacological activities, including antioxidant activity,5 antihypertensive activity,6 cholesterol-lowering activity,7 and anticancer activity.4 In humans, intake of hesperidin reduces the risk of chronic diseases such as cerebrovascular disease.5 Therefore, hesperidin and hesperetin intake from fruits and vegetables is very important from the viewpoint of preventive medicine. However, it is of great importance to note that orally ingested hesperidin is hydrolyzed by β-glucosidase to hesperetin and then absorbed into the blood as conjugated metabolites, such as hesperetin glucuronides.6,7 Indeed, the free aglycone concentration in circulating blood is undetectably low in humans.

Therefore, to understand the pharmacological activities elicited by orally ingested hesperidin and hesperetin, it is important to evaluate the effects of the major hesperetin metabolites, hesperetin-7-O-β-D-glucuronide (H7-OG) and hesperetin-3′-O-β-D-glucuronide (H3′-OG) (Fig. 1).

Hesperetin glucuronides (H7-OG and H3′-OG) were reported to decrease blood pressure in anesthetized spontaneously hypertensive (SHR) rats, to enhance endothelium-dependent vasodilation, and to decrease hydrogen peroxide-induced intracellular adhesion molecule-1 and monocyte chemotactant protein-1 mRNA expression in rat aortic endothelial cells.8 We also showed that not only hesperidin and hesperetin, but also H7-OG and H3′-OG exhibit partial agonistic activity towards peroxisome proliferator-activated receptor gamma (PPARγ) in vitro.9 PPARγ is a ligand-dependent transcription factor, and a master regulator of glucose and lipid homeostasis in humans.10 Therefore, the pharmacological activities elicited by hesperidin and hesperetin are likely to be mediated at least in part through hesperetin glucuronides. Although the in vitro PPARγ-agonistic activities of both hesperetin glucuronides are weak, these compounds significantly augmented expression of PPARγ target genes such as adiponectin and CCAAT enhancer-binding protein (C/EBP)α in 3T3-L1 cells, and accelerated 3T3-L1 adipocyte differentiation.9 We also found that these glucuronides showed an additive PPARγ-transactivating effect with troglitazone, a thiazolidine-2,4-dione class PPARγ full agonist previously used as an antidiabetic drug.9

We speculated that this additive effect might mean that hesperetin glucuronides activate PPARγ in a different manner from thiazolidine-2,4-dione derivatives, possibly by binding to the PPARγ ligand-binding domain (LBD) at a different site. Here, in order to test this idea, we compared the agonistic activity profiles of hesperetin glucuronides and thiazolidine-2,4-dione derivative, troglitazone, previously used as an antidiabetic drug, towards a series of mutant PPARγ LBDs.

Preparation of PPARγ LBD Mutants We prepared nine kinds of PPARγ LBD mutants: K265A, P269A, F287A, R288H, E291A, K301A, M329A, F363A and H466A. The function of these amino acids in the wild-type PPARγ LBD can be divided into three classes, i.e., 1) amino acids involved in ligand recognition and/or binding (R288 and F363), 2) amino acids involved in domain stabilization (K265, P269, F287, E291, M329), and 3) amino acids involved in co-factor recognition and/or recruitment (K301 and H466), and 3) amino acids involved in domain stabilization (K265, P269, F287, E291, M329). The positions of these key amino acids are depicted in Figs. 2B–E.

PPARγ-Agonistic Activities of a Series of PPARγ Agonists towards PPARγ LBD Mutants The PPARγ-transactivating activities of troglitazone, H7-OG, H3′-OG, and a structurally similar flavanone-type flavonoid, luteolin, which was reported to be a PPARγ partial agonist,11 are summarized in Fig. 2. The concentrations of these PPARγ agonists used were 10 µM, 250 µM, 250 µM, and 25 µM, those were enough

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Fig. 2. PPARγ-Transactivating Activities of Troglitazone, H7-OG, H3'-OG and Luteorin towards a Series of PPARγ LBD Mutants, and Sites of Mutation of the PPARγ LBD in This Study

(A) The concentration of the test compounds used were troglitazone: 10 µM, H7-OG: 250 µM, H3'-OG: 250 µM and luteolin: 25 µM. *p<0.01, significantly different from cells adding troglitazone 10 µM of F287A. #p<0.01, significantly different from cells adding troglitazone 10 µM of H466A. (B) Secondary structure of PPARγ LBD (from residues 207 to 477). The amino acids mutated in this study are depicted as open circles. (C)–(E) PPARγ LBD structures. The amino acids mutated in this study are depicted as red, blue and brown cylinder models. (Color images were converted into gray scale.)
concentrations to elicit their maximum transactivation activities (EC_{50} (E_{max}) values of these agonists were troglitazone: 4 \mu M \ (1.00), H7-OG: 100 \mu M \ (0.69), H3'-OG: 100 \mu M \ (0.63), luteolin: 5 \mu M \ (0.73)). LBD mutants K265A, P269A, F363A, R288H, and E291A were activated similarly by all PPAR\gamma agonists tested, as compared to the vehicle control. On the other hand, F287A is effectively activated by only flavonoid type PPAR\gamma partial agonists (H7-OG, H3'-OG, and luteolin). Further, only troglitazone activated H466A.

The residues K301, M329 and H466 appear to be critically important for PPAR\gamma transactivation, because mutation of these amino acids to alanine substantially abolished the PPAR\gamma transactivation ability, except in the case of troglitazone with H466A. The reason why troglitazone activated this mutant is unknown.

Thus, there were three patterns of response of the mutants to the PPAR\gamma agonists examined, i.e., 1) responsive to all PPAR\gamma agonists tested, 2) responsive to flavonoid-type PPAR\gamma partial agonists but not troglitazone, and 3) responsive to troglitazone but not flavonoid-type PPAR\gamma partial agonists. These results support the idea that the mechanism of transactivation elicited by hesperetin glucuronides is distinct from that of troglitazone.

It is noteworthy that all three flavonoid-type PPAR\gamma partial agonists tested, i.e., luteolin, H7-OG, and H3'-OG, showed similar transactivation profiles. This suggests that the binding modes and their mechanisms of action are similar.

The three-dimensional structure of luteolin has been reported. In the luteorin–PPAR\gamma LBD complex, the LBD takes a partial-agonist form and the N-terminal AF2 portion is incompletely folded into \alpha-helical conformation. We speculate that H7-OG and H3'-OG bind to the PPAR\gamma LBD in a similar manner to luteolin, even though these compounds have the hydrophilic glucuronic acid moiety at different positions of the flavanone framework. Further study is ongoing.

**Conclusion**

We evaluated the PPAR\gamma transactivation activities elicited by hesperetin glucuronides, luteolin and troglitazone in a series of LBD mutants. The assay results indicate that the mechanism of transactivation by hesperetin glucuronides is distinct from that by the previously clinically used thiazolidine-2,4-dione PPAR\gamma full agonist, troglitazone. This may imply that the two types of agents have different binding sites on the LBD.

**Experimental**

**Chemicals** Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Troglitazone was obtained from Cayman Chemicals (MI, U.S.A.). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Pasching, Austria). Other reagents were obtained from Nacalai Tesque (Kyoto, Japan). Hesperetin-7-O-\beta-D-glucoside (H7-OG) and hesperetin-3'-O-\beta-D-glucuronide (H3'-OG) were prepared according to the literature methods.12,14

**Mutagenesis** GAL4 fusions of mutant hPPAR\gamma LBDs were prepared using a QuickChange® Site-Directed Mutagenesis Kit (Stratagene). The expression vector pCMX-GAL4N-hPPAR\gamma was used as a template. The presence of the desired mutation was confirmed by DNA sequencing.

**Cell Culture and Transactivation Assays** COS-1 cells were obtained from RIKEN Biosource Center (Ibaraki, Japan). COS-1 cells were cultured in DMEM containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 in air. Transfections were performed by effectene transfection reagent (QIAGEN). Twenty four hours after transfection, ligands (troglitazone: 10 \mu M, H7-OG: 250 \mu M, H3'-OG: 250 \mu M and luteolin: 25 \mu M) were added. Cells were harvested approximately 24h after the treatment, and luciferase and secreted embryonic alkaline phosphatase (SEAP) activities were assayed using a luminometer and a microplate reader. Luciferase data were normalized to an internal SEAP control and reported values are the means of triplicate assays.

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