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Structural activity relationship of flavonoids with estrogen-related receptor gamma

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ABSTRACT

In this study, we investigated the structural activity relationships (SARs) of flavonoids with estrogen-related receptor γ (ERRγ) and its coactivator peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α). Isoflavones (genistein and daidzein) and flavanones (naringenin and hesperetin) did not significantly affect ERRγ activity. Flavone apigenin and flavonol kaempferol directly suppressed the interaction between ERRγ and PGC-1α functioning as inverse agonists. In contrast, flavone luteolin suppressed PGC-1α activity in part through promoting the degradation of PGC-1α leading to suppressed ERRγ activity. Therefore, these flavones and flavonols utilize alternative mechanisms to influence the transcriptional activities of ERRγ and PGC-1α.

Structured summary:
MINT-7297297, MINT-7297435: *PGC-1 alpha NR2* (uniprotkb:Q9UBK2) *binds* (MI:0407) to *ERR gamma* (uniprotkb:P62508) by *surface plasmon resonance* (MI:0107)

1. Introduction

Flavonoids are natural polyphenolic products that can be found in a variety of plants and fruits [1]. Flavonoids can be further classified into flavones (2-phenylchromen-4-one), flavonols (3-hydroxy-2-phenylchromen-4-one), flavanones (2,3-dihydro-2-phenylchromen-4-one), and isoflavones (3-phenylchromen-4-one) (Fig. 1). Flavones such as apigenin and luteolin can be found in celery and green pepper, while flavanones such as naringenin and hesperetin are abundant in citrus fruits. Tea is a good source of flavonols such as kaempferol and quercetin; whereas, soybean is loaded with isoflavones such as genistein and daidzein. Since many of these flavonoids are anti-oxidants, high levels of dietary consumption are believed to confer a multitude of health benefits including reducing oxidative damages [2].

The mechanisms of action behind certain beneficial effects extend beyond just antioxidant activity. Estrogen receptors α and β (ERα and ERβ) are transcription factors that bind to specific hormone response elements located near their target genes and regulate their expression in a ligand-dependent manner. Genistein and other flavonoids are phytoestrogens, i.e. they function as selective estrogen receptor modulators (SERMs) [3]. It is hypothesized that these flavonoids modulate the endogenous activities of estrogen receptors to slow down or prevent the developments of breast and ovarian cancers [4].

Estrogen-related receptors α and γ (ERRα and ERRγ) together with their coactivator peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α) are key regulators of mitochondrial function [5]. ERRs share high degrees of similarity in the DNA binding domain (DBD) with ERs. On the other hand, the ligand binding domains (LBDs) of ERRs are divergent from that of ERs. Although ERRs are constitutively active transcription factors, we previously established that kaempferol can function as an ERRα and ERRγ inverse agonist [6]. In this study, we investigated into the differential abilities of flavones, flavonols, flavanones, and isoflavones to regulate the activities of ERRγ and PGC-1α by reporter-based assays in HeLa cells and direct interaction assay by surface plasmon resonance.

2. Materials and methods

2.1. Reagents

17β-Estradiol, 4-hydroxytamoxifen, daidzein, apigenin, and genistein were obtained from Sigma. Hesperetin, kaempferol, luteolin, naringenin, and quercetin were purchased from shanxi Huike Botanical Development Co., Ltd. DY-131 was obtained from Tocris. BIAcore 3000 system, certified SA sensor chips, and amine coupling reagents were obtained from BIAcore, Inc.

Abbreviations: ERRγ, estrogen-related receptor γ; PGC-1α, peroxisome proliferators-activated receptor γ coactivator-1α; SAR, structural activity relationship

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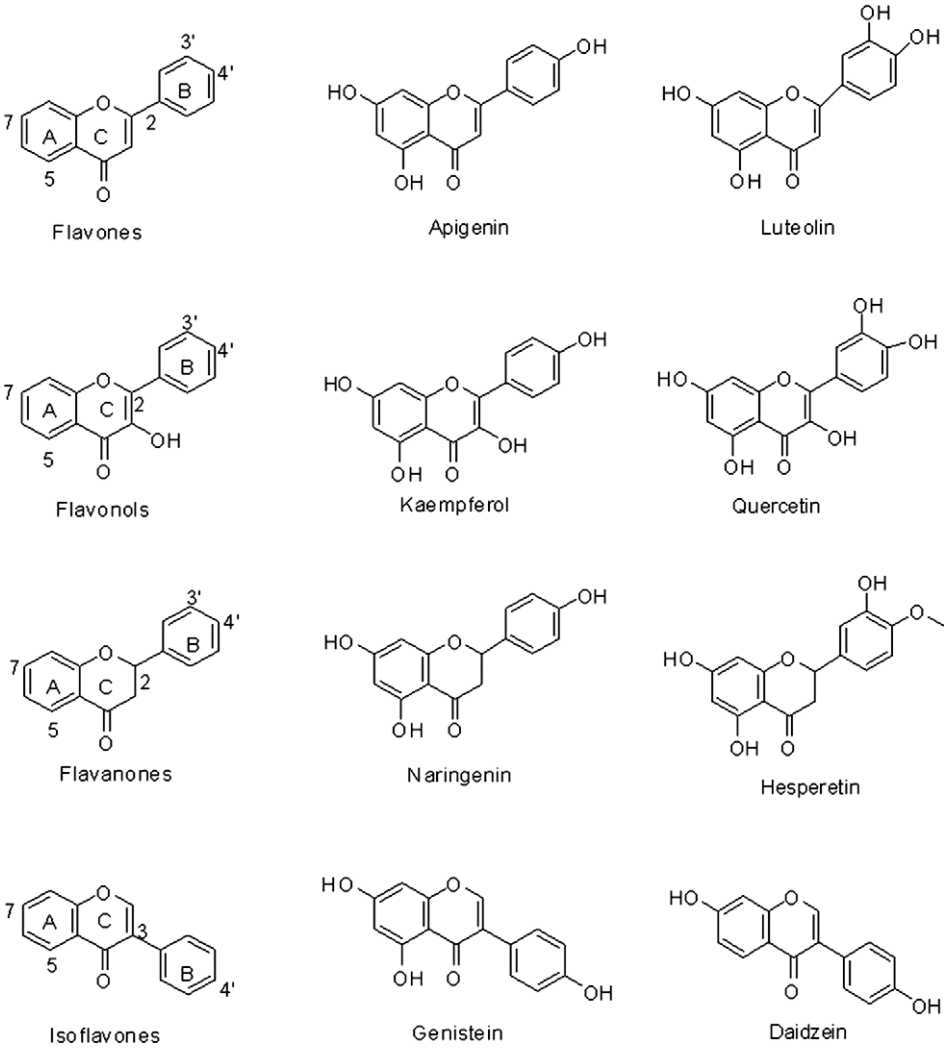

Fig. 1. Chemical structures of flavonoids. Structures of flavones, flavonols, flavanones, and isoflavones are shown with the A-, B-, and C-rings indicated.

2.2. Transfection and luciferase assays

A Gal4-DBD-PGC-1α fusion was cloned as described [7]. Transfection of HeLa cells by expression and reporter vectors as well as luciferase assays were performed as described [6]. All transfections were performed at least three times with the mean and SEM of these experiments shown in Figs. 2 and 4.

2.3. Interaction between ERRγ and PGC-1α analyzed by surface plasmon resonance assay

Human ERRγ-LBD protein was expressed and purified as described [6]. Interactions between ERRγ-LBD and PGC-1α coactivator peptides in the presence of various compounds were analyzed using a BIAcore 3000 system. Biotinylated PGC-1α peptides: EEPSLAKKAALAPAN (NR2 negative control); ENEANLLAVLTETLD (NR1); EEPSLLKKLLLAPAN (NR2); and RPCSELLKYLTTNDD (NR3) were captured onto streptavidin immobilized surface of SA chip in flow cells 1, 2, 3, and 4, respectively. ERRγ-LBD incubated with various ligands in running buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.002% NP-40, 0.2 mM DTT) were injected into flow cells for 3 min at a flow rate of 20 μL/min at 25 °C. Changes in resonance units (RU) were monitored simultaneously in all flow cells. Surface was regenerated at the end of each cycle by an injection of 0.05% SDS. Sensorgrams were generated by BIAcontrol software 4.1 using double-referencing to eliminate responses from the reference surface and buffer-only control. Specific changes in RU were generated with non-specific interaction between LBD and NR2 negative control mutant peptide deducted.

2.4. Western blot analysis

Cells were lysed using RIPA reagent (Shanghai Shenneng) according to the manufacturer's protocol and protein extracts were analyzed by 10% SDS–PAGE and blotted onto PVDF membrane. Membranes were incubated with rabbit anti-human-PGC-1α antibody generated in house or mouse anti-β-actin antibody (Boster) followed by horseradish peroxidase-conjugated secondary antibody (Amersham) and developed with BeyoECL Plus reagent (Beyotime).

3. Results

3.1. Effects of flavonoids on ERβ and ERRγ

We aimed to study the structural activity relationship (SAR) of flavonoids with ERRγ. We first confirmed the abilities of these flavonoids to act as ERβ agonists. By measuring the activity of a reporter gene luciferase under the control of a Gal4-DBD-ERβ-LBD

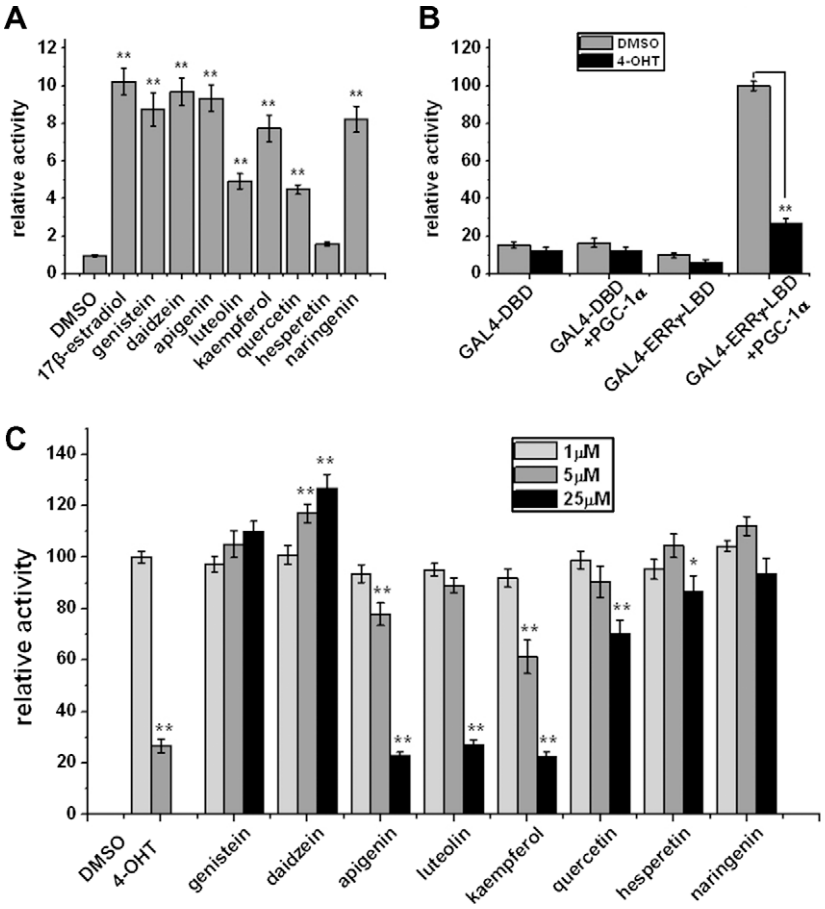

Fig. 2. Effects of flavonoids on the activities of ERβ and estrogen-related receptor γ (ERRγ). (A) HeLa cells were transfected with an expression plasmid of Gal4-DBD-ERβ-LBD together with a luciferase reporter and a control *Renilla* luciferase plasmid. About 10 nM 17β-estradiol as a positive control or different flavonoids at 5 μM were added for 24 h before luciferase assays. Fold induction by compounds were calculated and shown compared to dimethyl sulfoxide (DMSO) as a vehicle. (B) HeLa cells were transfected with expression plasmids of Gal4-DBD control or Gal4-DBD-ERRγ-LBD with or without pcDNA-peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α) together with a luciferase reporter and a control *Renilla* luciferase plasmid. DMSO or 10 μM 4-hydroxytamoxifen (4-OHT) was added and assays performed as in (A). (C) Transfection were performed as in (B) with different flavonoids (1, 5, and 25 μM); % activity indicates the normalized activities of ERRγ under the influences of flavonoids compared to DMSO control set at 100%. (A–C) Results represent mean ± S.E.M. $^{**}P < 0.01$.

fusion, we found that these flavonoids at 5 μM acted as ERβ agonists in HeLa cells except for hesperetin (Fig. 2A).

The activity of ERRγ is strongly enhanced in the presence of co-expressed PGC-1α while an inverse agonist 4-hydroxytamoxifen suppresses this activity (Fig. 2B) [8]. We then examined the effects of these flavonoids on ERRγ in this system and found that isoflavone genistein did not significantly affect whereas daidzein very modestly enhanced ERRγ activity (Fig. 2C). Flavanones naringenin and hesperetin did not show any dose-dependent effects (Fig. 2C). In contrast, flavone apigenin and flavonol kaempferol suppressed ERRγ activity in dose-dependent manners with kaempferol being more potent (Fig. 2C). Flavone luteolin fully and flavonol quercetin partially suppressed ERRγ activity at 25 μM (Fig. 2C). We then asked if the abilities of these flavonoids to suppress ERRγ activity are due to reduced cellular viabilities. Total cellular level of ATP is reflective of viability. We measured total cellular ATP content upon flavonoid treatments and found that only quercetin partially reduced viability correlating to its ability to suppress ERRγ activity (Supplementary Fig. S1). This result also implies that the abilities of other flavonoids to function as ERRγ activity modulators rely on mechanisms other than reducing viability.

3.2. Apigenin interferes with the interaction between ERRγ and PGC-1α

ERRγ activity is dependent on its interaction with PGC-1α through the receptor interacting (NR) motifs of PGC-1α with a strong preference for NR2 over NR1 and NR3 (Fig. 3A). We then examined this in vitro interaction in the presence of daidzein, apigenin, luteolin, and kaempferol. Among these modulators, we previously demonstrated that kaempferol functions as an ERRγ inverse agonist by directly blocking this interaction measured by a surface plasmon resonance based assay [6]. Using this assay, we found that daidzein and luteolin failed to enhance or interfere with this interaction compared to positive controls with DY-131 as an agonist and 4-hydroxytamoxifen as an inverse agonist (Fig. 3B) [8,9]. On the other hand, apigenin dose-dependent suppressed this interaction apparently functioning as an ERRγ inverse agonist (Fig. 3C). Genistein, naringenin, and hesperetin did not affect the interaction between ERRγ and PGC-1α (data not shown).

3.3. Luteolin suppresses the transcriptional activating function within PGC-1α

Since luteolin modulated ERRγ activity without affecting its interaction with PGC-1α, we examined if it would directly affect the transcriptional activating function within PGC-1α. When fused to a Gal4-DBD, PGC-1α displays a strong transcriptional activating function on a reporter under the control of Gal4-DBD [7]. We found that luteolin affected PGC-1α activity mirroring its modulation on ERRγ activity (Fig. 4A). We next examined if luteolin suppressed PGC-1α activity by alerting its protein stability. We found that

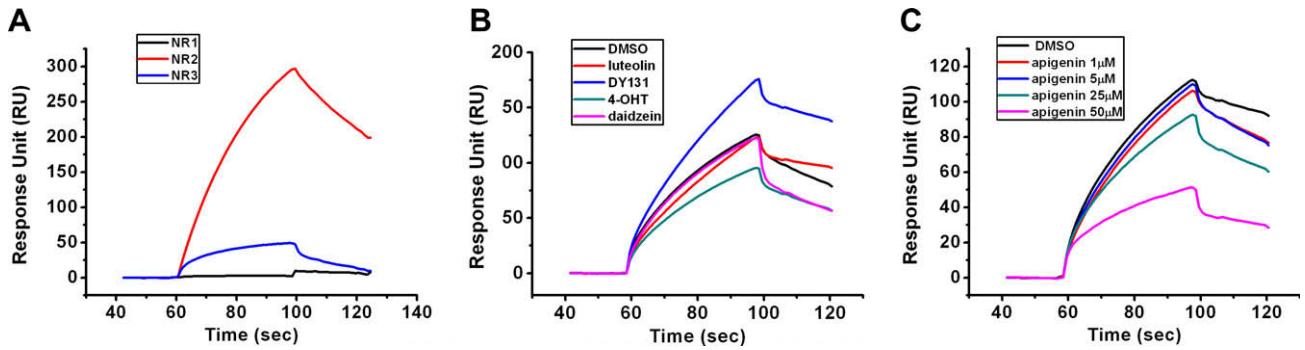

Fig. 3. Apigenin directly blocks the interaction between ERRγ and PGC-1α. (A) 125 nM of purified ERRγ-LBD was tested for its interaction with NR1, NR2, and NR3 motifs as described [6]. (B) DMSO, 1 μM 4-OHT, 5 μM DY-131, 25 μM daidzein, or 25 μM luteolin was incubated with 50 nM ERRγ-LBD for 1 hr before interaction analysis with NR2 as in (A). (C) Different doses of apigenin were tested as in (B).

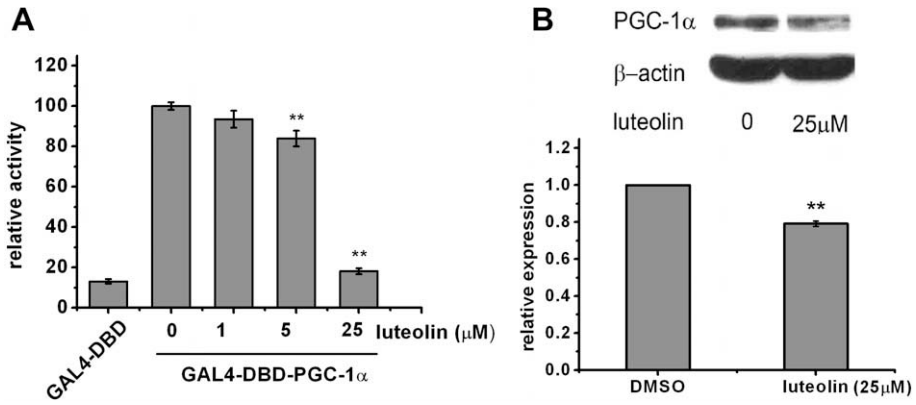

Fig. 4. Effects of flavonoids on the activity of PGC-1α. (A) HeLa cells were transfected with an expression plasmid of Gal4-DBD or Gal4-DBD-PGC-1α together with a luciferase reporter and a control *Renilla* luciferase plasmid. DMSO or 1, 5, and 25 μM luteolin were added for 24 h; % activity indicates the normalized activities of PGC-1α compared to DMSO control set at 100%. (B) HeLa cell extracts pre-treated with DMSO or 25 μM luteolin was probed with anti-PGC-1α or β-actin antibodies for Western analysis. PGC-1α protein levels normalized to β-actin from three independent experiments were quantified. Results represent mean ± S.E.M. $^{**}P < 0.01$.

luteolin modestly reduced PGC-1α protein level by Western analysis (Fig. 4B).

4. Discussion

Flavonoids share a common core phenylchromen-4-one structure consists of A-, B-, and C-rings (Fig. 1). Both flavones and isoflavones function as ERβ agonists, suggesting that the position of the B-ring is not critical (Fig. 2A). However, substitutions on the B-ring play a key role. Although apigenin, luteolin, kaempferol, and quercetin function as ERβ agonists, luteolin and quercetin with additional hydroxyl groups at the 3'-position of the B-ring are slightly less potent (Fig. 2A). Importantly, hesperetin with a methoxy group at the 4′-position of the B-ring instead of a hydroxyl group as in naringenin, fails to function as an ERβ agonist (Fig. 2A).

Several flavonoids, specifically, isoflavones genistein, daidzein, biochanin A and flavone 6,3′,4′-trihydroxyflavone were reported to function as ERRα and ERRγ agonists based on virtual ligand screening and reporter-based assays [10]. However, in this study we did not observe any effect of genistein on ERRγ by in vitro purified ERRγ-LBD interaction with PGC-1α NR2 motif and luciferase reporter assays (Figs. 2 and 3). Independently, another group also failed to reproduce these effects [11]. On the other hand, we identified in this and a previous study that apigenin and kaempferol block the interaction between ERRγ and PGC-1α and suppress the expression of ERRγ target gene pyruvate dehydrogenase kinase 4 (PDK4) (Supplementary Fig. S2) as bone fide ERRγ inverse agonists (Fig. 3) [6].

Based on these results, we conclude that isoflavones with their B-rings at position 3 of the C-ring do not block the in vitro ERRγ interaction with PGC-1α. On the contrary, certain flavones and flavonols such as apigenin and kaempferol with their B-rings at position 2 are capable of interfering with this interaction. While apigenin and kaempferol function as ERRγ inverse agonists, luteolin and quercetin do not affect the ERRγ interaction with PGC-1α (Fig. 3). Comparing the structures among these flavonoids suggests that a hydroxyl group at position 3 of the C-ring can be accepted but a hydroxyl group at position 3′ of the B-ring is not tolerable (Fig. 1). Although flavanones such as naringenin and hesperetin have their B-rings at position 2 of the C-rings, they fail to affect the interaction due to their 2,3-dihydro substitutions on the C-rings.

Disrupting the interaction between ERRγ and PGC-1α is not the only mechanism by which certain flavones and flavonols function to suppress ERRγ activity. Despite failing to block the ERRγ interaction with PGC-1α (Fig. 3), luteolin down-modulates ERRγ activity by suppressing the transcriptional activating function within PGC-1α in part through reducing its protein level (Fig. 4). PGC-1α is subjected to degradation by the ubiquitin-proteasome pathway [12] which is regulated by glycogen synthase kinase 3β (GSK3β) and p38MAPK [13]. Luteolin may affect the activities of these kinases or the expression of E3 ubiquitin ligases to reduce PGC-1α protein level through promoting its degradation. Since luteolin is more potent in suppressing PGC-1α activity than reducing its protein level (Fig. 4), luteolin may also influence other post-translational modifications of PGC-1α to affect its activity [14]. In all, we established in this study a SAR between flavonoids and ERRγ

activity; namely, certain flavones and flavonols utilize alternative mechanisms to suppress ERRγ activity through either disrupting the interaction between ERRγ and PGC-1α or suppressing PGC-1α activity through promoting PGC-1α protein degradation. These alternative mechanisms may be further subjected to cell type specific regulation; thus, more extensive analysis of the influences of flavonoids on metabolic regulation mediated by ERRγ and PGC-1α is warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.11.026.

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