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Kaempferol is an estrogen-related receptor α and γ inverse agonist

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ABSTRACT

Kaempferol is a dietary flavonoid that is thought to function as a selective estrogen receptor modulator. In this study, we established that kaempferol also functions as an inverse agonist for estrogen-related receptors alpha and gamma (ERRα and ERRγ). We demonstrated that kaempferol binds to ERRα and ERRγ and blocks their interaction with coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). Kaempferol also suppressed the expressions of ERR-target genes pyruvate dehydrogenase kinase 2 and 4 (PDK2 and PDK4). This evidence suggests that kaempferol may exert some of its biological effect through both estrogen receptors and estrogen-related receptors.

Structured summary:
MINT-6824653: *PGC-1 alpha* (uniprotkb:Q9UBK2) and *ERR gamma* (uniprotkb: P62508) *bind* (MI:0407) by *surface plasmon resonance* (MI:0107)

1. Introduction

Phytoestrogens are polyphenolic non-steroidal plant compounds that function at least in part as selective estrogen receptor modulators (SERMs) to influence the transcriptional response to estrogens in both context dependent and tissue selective manners [1,2]. Kaempferol (3,4′,5,7-tetrahydroxyflavone) is one of the most commonly found dietary flavonoid and has been isolated from tea, broccoli, grapefruit, and other plant sources [3]. Kaempferol has been suggested to play a role in preventing postmenopausal conditions, namely cardiovascular disease, hot flushes and osteoporosis [4]. Kaempferol can also induce growth arrest and apoptosis in colorectal, lung and prostate cancers through inhibiting DNA synthesis, inducing nuclear DNA degradation, and inhibiting kinase activities albeit at non-physiological concentrations [5–7].

Estrogen-related receptors alpha, beta and gamma (ERRα, ERRβ, and ERRγ) are members of the nuclear hormone receptor superfamily that were identified on the basis of their high levels of sequence identity to estrogen receptors (ERs) [8]. Like other nuclear hormone receptors, ERRs and ERs are comprised of N-terminal transcriptional activating domains (AF-1), centrally located DNA binding domains (DBD), variable hinge regions, and C-terminally located ligand binding domains (LBD) that harbor the ligand-dependent transcriptional activating function (AF-2) [9]. The LBDs of ERRs differ to that of ERs in primary sequences and that ERRs do not bind to estrogen 17β-estradiol; nonetheless, they share some common characteristics and abilities to bind to SERMs. Specifically, ER antagonist 4-hydroxytamoxifene (4-OHT) has also been shown to be an antagonist for both ERRβ and ERRγ [10,11]. Recent studies have also collectively suggested that natural or synthetic selective ligands for ERRα, ERRβ and ERRγ exist [12,13]. In this study, we explored if kaempferol would also exert some of its biological effects by modulating the activities of ERRs, employing a reporter-based activity screening system, a receptor-coactivator interacting assay and marker gene expression analysis, we identified kaempferol as an ERRα and ERRγ inverse agonist.

2. Materials and methods

2.1. Cell culture and reagents

HeLa and HepG2 cells were cultured according to ATCC instruction. Kaempferol was purchased from Shannxi Huike Botanical Development Co., Ltd. The BIAcore 3000 system, certified SA sensor chips, amine coupling reagents (EDC, NHS, and ethanolamine HCl), HBS and 0.5% SDS were obtained from BIAcore Inc.

Abbreviations: ERR, estrogen-related receptor; ER, estrogen receptor; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; LBD, ligand binding domains; 4-OHT, 4-hydroxytamoxifene; RU, resonance units; SPR, surface plasmon resonance; PDK, pyruvate dehydrogenase kinase

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2.2. Molecular clones

Expression vectors containing human cDNA of pCMX-hERRα, pCMX-hERRγ and pcDNA4-hPGC-1α were kindly provided by Dr. Hongwu Chen and Dr. Brian Lavan. PDK4-promoter was amplified and subcloned into pGL3 plasmid from HepG2 genomic DNA by primers 5′-TTGGAGTAAGGACATTTCGTACAGG-3′ and 5′-GCTCAGCAGCAAAGTGAACCC-3′ [14]. Expression vectors in pCMV-Gal4DBD and pET-30 a (+) were generated by subcloning the LBDs (ERRα: amino acids 144–423; ERRγ: amino acids 168–350) of the corresponding nuclear hormone receptors into the appropriate vectors. All plasmids were sequence verified.

2.3. Transient transfection and luciferase assays

Cells were plated at a density of 10 000 cells/well (96-well plates) in phenol red free DMEM medium (Hyclone) supplemented with 10% (v/v) charcoal stripped fetal bovine serum. Cells were transiently transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were transfected with 20 ng per well expression plasmids of either Gal4-DBD-ERα-LBD, -ERβ-LBD, ERRα-LBD, or -ERRγ-LBD together with 20 ng per well luciferase reporter UAS-Luc and 5 ng per well control *Renilla* luciferase plasmid. For ERRα and ERRγ assays 10 ng per well pcDNA-PGC-1α expression vector was also added. Alternatively, HepG2 cells were cotransfected with 20 ng per well pGL3-PDK4-promoter-Luc and 20 ng per well pCMX-hERRα or pCMX-hERRγ, with or without 10 ng per well pcDNA-PGC-1α, and 5 ng per well *Renilla* luciferase plasmid. After cells were transfected for 6 h, compounds were then added for an additional 24 h. Luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega).

2.4. Interactions between coactivator and ERR-LBDs analyzed by BIAcore 3000

Interactions between hERRγ-LBD and PGC-1α coactivator peptides in the presence of kaempferol were analyzed using a BIAcore 3000 system. Human ERRγ LBD protein was expressed in *Escherichia coli* BL21(DE3) with 0.5 mM IPTG induction at 15 °C for 20 h and purified to more than 95% pure by a Ni-NTA column (QIAgene). Biotinylated PGC-1α NR2 EEPSLLKKLLLAPAN and negative NR2 EEPSLAKKAALAPAN peptides were captured onto streptavidin immobilized surface of a SA chip. After stabilization of surface by injection of 0.05% SDS, 30 nM of hERRγ-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 a quick 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.

2.5. Real-time PCR analysis

Total cDNA was used in a real-time PCR kit (Takara, Dalian, China) and the mRNA levels were detected by SYBR green fluorescence on ABI7300 machine. 18S rRNA was used as an internal control. Fold repression was calculated by 2^{DDCt}. Primers and conditions will be provided upon request.

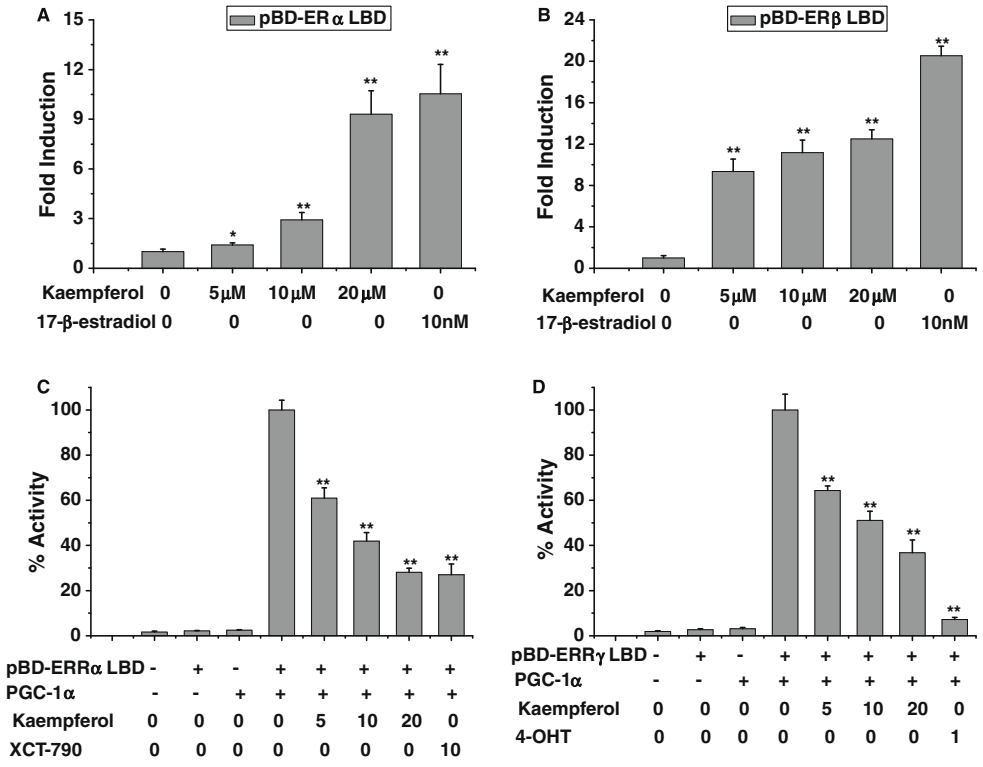

Fig. 1. Effects of kaempferol on the activities of ERs and ERRs. (A and B) HeLa cells were transfected with expression plasmids of either Gal4-DBD-ERα-LBD or ERβ-LBD together with luciferase reporter and control *Renilla* luciferase plasmid. Kaempferol or 17β-estradiol as a positive control was added for 24 h before luciferase assays. Fold induction indicates the activities of ERs under the influence of kaempferol compared to DMSO control set at 1. (C and D) HeLa cells were transfected with expression plasmids of Gal4-DBD-ERRα-LBD or ERRγ-LBD and pcDNA-PGC-1α together with reporter UAS-Luc and control *Renilla* luciferase plasmid. Kaempferol (0, 5, 10, 20 μM), XCT-790 (10 μM), or 4-OHT (1 μM) was added and enzyme assays were performed as in (A); % Activity indicates the normalized activities of ERRs under the influence of kaempferol compared to DMSO control set at 100%. Results represent mean ± S.D. $^{**}P \leqslant 0.01$.

3. Results

3.1. Kaempferol is an estrogen receptor agonist and estrogen-related receptor inverse agonist

We first investigated if kaempferol displayed agonistic or antagonistic activities on both ERs and ERRs in HeLa cells. HeLa were then tranfected with a luciferase reporter under the control of Gal4-DBD fusions, Gal4-DBD-ER or ERR-LBD expression vectors, and a *Renilla* luciferase vector as an internal control. Compared to 17β-estradiol as a positive control, we found that kaempferol enhanced the activities of both ERα and ERβ while showing a strong preference for ERβ over ERα (Fig. 1A and B), confirming kaempferol as a selective estrogen receptor modulator.

While the activities of estrogen receptors are stimulated by agonists, ERRs are constitutively active receptors that display strong transcriptional activities particularly in conjunction with their coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) [15]. We then asked if kaempferol would affect the activities of ERRα and ERRγ with cotransfection of a PGC-1α expression plasmid. Compared to two well established inverse agonists XCT-790 and 4-OHT of ERRα and ERRγ respectively [10,11,16], we found that kaempferol strongly suppressed ERRα and ERRγ activities in a dose dependent manner (Fig. 1C and D), strongly indicating that kaempferol acted as an inverse agonist of both ERRα and ERRγ.

3.2. Kaempferol binds to ERR-LBDs and blocks their interaction with coactivator

Although kaempferol functions as an inverse agonist in the cell based assay system, it remains to be established that kaempferol would bind directly to the LBDs of ERRs. We then employed an in vitro purified protein system to confirm if kaempferol can directly bind to the LBD of ERRγ. ERRγ-LBD was expressed as a His-tagged fusion protein in bacteria and purified over a Ni-NTA column to appropriately 95% in purity (data not shown). We then analyzed the interaction between the purified ERRγ-LBD and the LXXLL motif (nuclear receptor interacting box) of coactivator PGC-1α by a biophysical method. The purified ERRγ LBD was incubated with DMSO as a vehicle, an ERRγ synthetic agonist DY-131 [12], the ERRγ inverse agonist 4-OHT, or different doses of kaempferol. These ligand-bound LBDs were then allowed to pass through a sensor chip on which the LXXLL motif or control mutant peptides of coactivator PGC-1α were immobilized [17]. The specific interactions between the coactivator LXXLL peptides with that of LBDs were monitored by measuring the changes in surface plasmon resonance (SPR) with the non-specific interaction between the LBDs and mutant peptides deducted.

We first established that ERRγ-LBD constitutively bound to PGC-1α LXXLL motif in a dose dependent manner (Fig. 2A). This constitutive interaction was enhanced by ERRγ agonist DY-131 but was reduced by ERRγ inverse agonist 4-OHT (Fig. 2B). We found that kaempferol reduced the interaction between ERRγ-LBD and PGC-1α LXXLL motif in a dose dependent manner (Fig. 2C). In addition, we also found that kaempferol reduced the interaction between ERRα-LBD and PGC-1α by this method (data not shown). Since this system used purified LBDs, it is therefore likely that kaempferol directly bound to ERRs and suppressed their interaction with coactivators thus functioning as an inverse agonist.

3.3. Kaempferol decreases the expressions of ERR-regulated target genes

While we established that kaempferol was able to function as an inverse agonist of ERRα and ERRγ through their respective LBDs,

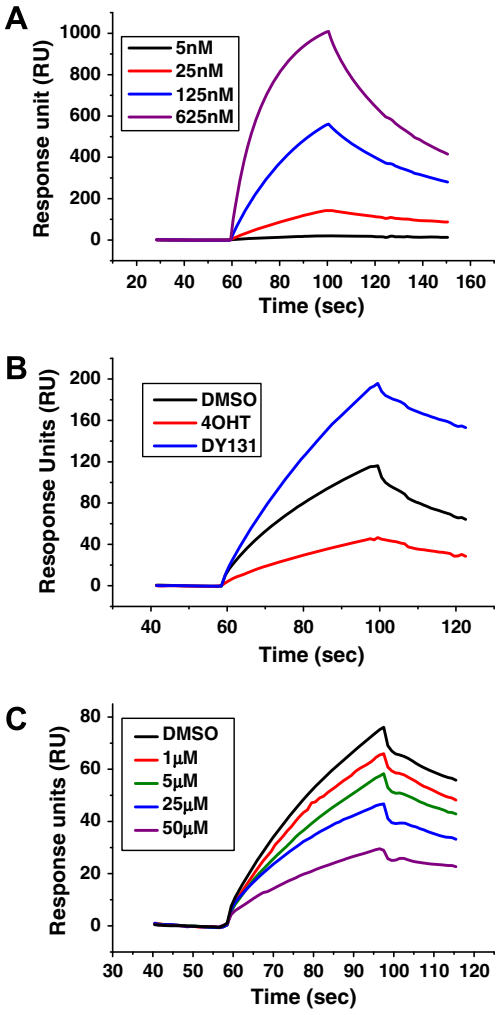

Fig. 2. Kaempferol directly binds to ERR-LBDs and blocks coactivator interaction. (A) Purified ERRγ-LBD was tested for its interaction with PGC-1α LXXLL motif as described in materials and methods. (B) DMSO, 10 μM DY-131, or 1 μM 4-OHT was incubated with 30 nM purified ERRγ-LBD before analysis as in (A). (C) Different doses of kaempferol were tested as in (B).

whether kaempferol would affect the expression levels of ERR-target genes remain to be investigated. Pyruvate dehydrogenase kinase 2 and 4 (PDK2 and PDK4) are ERRα and ERRγ target genes [14,18]. We first cloned the 2 kb upstream promoter of PDK4 containing ERR response elements into a luciferase reporter vector pGL3. Cotransfection of HepG2 cells with this reporter together with expression vectors of ERRα or ERRγ and coactivator PGC-1α stimulated the expression of this reporter (Fig. 3A and B). We found that kaempferol dose dependently suppressed the inductions of PDK promoter by both ERRα and ERRγ and the coactivation mediated by PGC-1α (Fig. 3A and B). Importantly, we found that kaempferol suppressed the expression levels of endogenous PDK2 and PDK4 mRNA measured by quantitative real-time PCR (Fig. 3C). Collectively, these results suggested that kaempferol functioned to suppress the activities of ERRs.

4. Discussion

As an ERβ selective agonist at sub-micromolar concentrations, the physiological function of kaempferol has been attribute to it being a selective estrogen receptor modulator [1,2]. In addition, kaempferol has been studied as an antagonist of aryl hydrocarbon

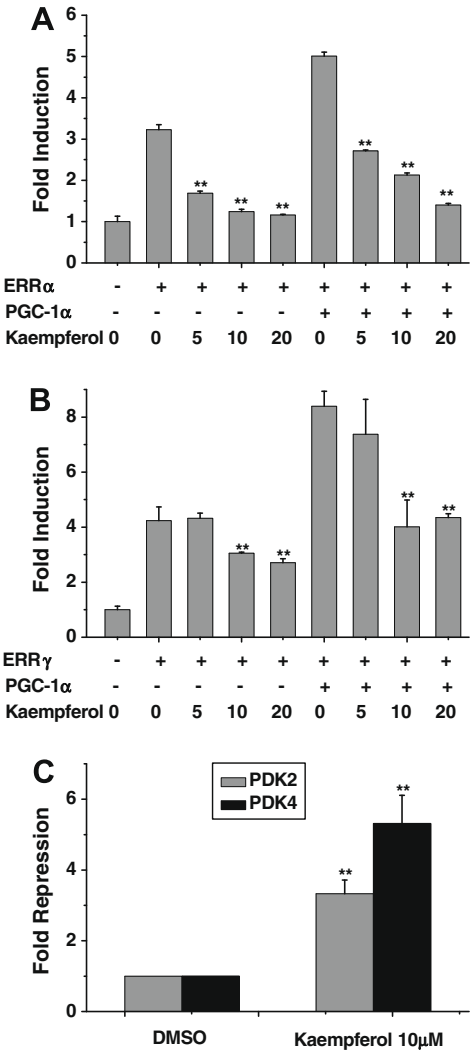

Fig. 3. Effects of Kaempferol on the ERRs target gene expression. (A and B) HepG2 cells were cotransfected with pGL3-PDK4 promoter-Luc, expression vectors for ERRα or hERRγ, with or without an expression vector for PGC-1α, and *Renilla* luciferase plasmid. Kaempferol (0, 5, 10, 20 μM) addition and enzyme assays were performed as in Fig. 1. Fold induction indicates the activities of PDK4-promoter under the influence of ERRs and PGC-1α compared to empty expression vector control set at one. Results represent mean ± S.D. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$. (C) HepG2 cells were treated with kaempferol at 10 μM for 24 h before RNA collection. The expression levels of ERR-target genes PDK2 and PDK4 were measured by real time PCR with 18S rRNA used as a control. The relative expression level of gene with DMSO as control was set at one.

receptor (AhR) [19]. Using a cell based Gal4-DBD-ERR-LBD screening assay, an in vitro purified LBD-coactivator interaction assay, and target gene expression analysis; we established that kaempferol functioned as an inverse agonist of both ERRα and ERRγ. Therefore, depending on the cellular concentrations, kaempferol can modulate the activities of some or all of these receptors.

It appears that the level of suppression may be modified by the ligand independent transcriptional activities (AF-1) located at the N-terminal regions of ERRs. Kaempferol suppresses the activities of both ERRα-LBD and ERRγ-LBD to similar extents in the Gal4-DBD assays (Fig. 1C and D). However, kaempferol suppresses the full length ERRα-mediated induction of PDK4 expression more avidly than ERRγ (Fig. 3A and B). Since the overall activities of full-length ERRs are combinations of their N-terminal AF-1s and LBD-located AF-2s, the strong activating function of ERRγ AF-1 may partially overcome the suppressive effect of kaempferol on ERRγ-LBD transcriptional activity, allowing ERRγ to still function as a constitutive transcription factor. This effect is more obvious at lower concentrations like 5 μM at which kaempferol preferentially activates ERβ (Fig. 1B) while suppresses ERRα (Fig. 3A). At higher concentrations like 20 μM, kaempferol additionally activates ERα (Fig. 1B) and suppresses ERRγ (Fig. 3A). Since the doses of kaempferol used in this study are still relatively high compared to physiological concentrations [7], a more extensive investigation using animal models would further shed lights on the importance of kaempferol as an ERR inverse agonist in vivo.

ERRs play important roles in oxidative phosphorylation, mitochondrial biogenesis, and energy metabolism [20,21]. Additionally, ERRα has been identified as prognostic markers in breast, prostate, colon cancers [22]. Our observation that kaempferol functions as an ERRα and ERRγ inverse agonist and that higher level of ERRα expression is positively related to adverse clinical outcome raises an interesting possibility that kaempferol may exert its anti-cancer activity at least in part through suppressing ERRα activity.

Acknowledgements

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