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Activation of Testicular Orphan Receptor 4 by Fatty Acids

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Summary

Nuclear receptors can be activated by chemicals, metabolites, hormones or environmental compounds to regulate gene expression. Bioassay-guided screening of mouse tissue extracts found that natural fatty acids of a certain carbon length and level of unsaturation could activate the mouse orphan nuclear receptor, testicular orphan receptor 4 (TR4). Subsequent experiments focused on γ -linoleic acid, a compound identified during screening of mouse tissues that exerts regulatory activity in TR4 transactivation assays. γ -linoleic acid positively modulates TR4 activity to promote the expression of downstream genes such as apolipoprotein E (ApoE) and phosphoenolpyruvate carboxykinase, and to activate a reporter carrying direct repeat 1 from the ApoE promoter. It also induced the interaction of TR4 with transcription coregulators such as RIP140 and PCAF. Comparisons of transactivation by TR4 and the metabolism-related peroxisome proliferator-activated nuclear receptors indicate that γ -linoleic acid regulation is specific to TR4. The data suggest that TR4 might exert its physiological function by sensing certain lipids. Identifying these compounds could be useful for examining the physiological pathways in which TR4 and its target genes are involved.

Keywords

TR4; linoleic acid; ApoE; PEPCK

Introduction

Nuclear receptors can be activated by various signals to regulate gene transcription [1]. Such receptor-activating compounds could potentially be used as drugs in therapeutic applications [2,3]. Testicular orphan receptor 4 (TR4; also known as TAK1 and Nr2c2) is an orphan nuclear receptor expressed in numerous tissues such as brain, testis, kidney and liver [4,5]. TR4 binds to the direct repeat (DR) AGGTCA sequence with variable spacer nucleotides, and can repress

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genes targeted by retinoic acid receptor, retinoid X receptor, peroxisome proliferator-activated receptors (PPARs), vitamin D3 receptor, and thyroid hormone receptor by competing at the DNA targets [4]. TR4 also interacts with diverse nuclear cofactors to exert transcriptional regulation [6,7].

Recently, animal studies showed that TR4 knockout mice exhibited abnormal glucose metabolism [8], ApoE [9] and Gata1 [10] gene regulation, spermatogenesis, female reproductive function, growth retardation, motor neuron coordination and cerebellar development [11-15]. Although these findings suggest the importance of TR4 in animals, the signaling pathways and mechanisms of action by which it functions remain elusive because of the lack of information regarding its activation by endogenous compounds. This study identifies natural fatty acids (FAs) of certain carbon lengths and degrees of unsaturation as activators of mouse TR4. In particular, γ -linoleic acid, which occurs endogenously in mouse liver, specifically activated endogenous ApoE and phosphoenolpyruvate carboxykinase (PEPCK). It also activated direct repeat 1 (DR1) reporter in a TR4-dependent manner, and enhanced TR4's interaction with transcription coregulators. Based on these findings, the use of certain specific natural lipids as seed compounds might contribute to the design and development of novel therapeutic compounds that exploit TR4 as a potential target in metabolic diseases.

Materials and Methods

The research was conducted in conformity with the PHS policy and studies were approved by the University of Minnesota Institutional Review Committee.

Chemicals and reagents

The following reagents were obtained from the indicated suppliers: organic solvents, WY-14643 (Sigma); ciglitazone, L-165041 (Calbiochem); FAs (Sigma and Calbiochem); free fatty acids (Wako Chemicals USA, Inc.); anti-TR4, anti-ApoE, anti-PCAF and anti-Actin antibodies (Santa Cruz Biotechnology); anti-RIP140 (Abcam).

Plasmids, transfection, and reporter assays

The ligand-binding domain (LBD) of TR4 cDNA (encoding amino acids 178–477) was amplified by PCR and subcloned into the *EcoRI* and *BamHI* sites of a pBD-GAL4 expression plasmid. Flag-RIP140 and Flag-PCAF constructs have been described previously [16]. The GST-TR4 was made by inserting the full-length TR4 cDNA into pGEX-2T vector. The GST-TR4 Δ LBD was made by deleting the ligand-binding domain of GST-TR4. The luciferase reporter assay [17] and the mammalian two-hybrid assay using full-length TR4 plasmids and plasmids containing truncated TR4 (i.e., lacking the LBD sequence) [7] were performed as reported previously, with the β -galactosidase gene (i.e., *lacZ*) as an internal control. For transactivation assays, cells transfected with the constructs of interest were grown in culture medium containing lipopolysaccharide-deficient or dextran-charcoal-treated fetal bovine serum.

Tissue extracts and HPLC

Fresh homogenized tissues (brain, testis, kidney and liver) collected from ten male mice were immediately subjected to organic solvent extraction with a 1:1 mixture of ethyl acetate and diethyl ether in the dark for 24 hours. The extracts were concentrated to dryness in a rotary evaporator under reduced pressure. Extracts from different tissues were weighed and dissolved in isopropanol for biochemical analysis. Fractionation was performed by reverse-phase HPLC (pump 126, detector 128; Beckman) on a C₁₈ column (4.6 $\times$ 250-mm; Beckman) using a

gradient of 0–90% acetonitrile (in 0.1% trifluoroacetic acid) over 30 minutes, followed by 100% acetonitrile for 15 minutes.

GC–MS

HPLC fractions capable transactivation were analyzed further by GC–MS to identify the activating compounds. Fractions were subjected to capillary GC using a Varian Saturn 3 system equipped with a DB-Wax column (0.5- μ M film) coupled to an electron impact ionization mass spectrometer (EI–MS; 70 eV, 51–400 m/z). The temperature program for the GC was: injector: 220°C; column: 50°C for 1 minute (initial), followed by a +5°C/minute ramp over a 32-minute run-time (i.e., 50–210°C, and 210°C for 15 minutes (final); detector: 200°C.

Real-time RT-PCR

RNA extraction and cDNA preparation were performed as described previously [16]. Real-time RT-qPCR was conducted as described [18] using a SYBR Green qPCR kit (Stratagene) and specific primers for ApoE (5'-TGTGGGCGCTGCTGTTGGTCAC-3' and 5'-TGCCTTGACACAGCTAGGCG-3'), PEPCK (5'-GAGACCAGACCCAGGCCTGAC-3' and 5'-CTTAGCCAGACGGCTGGCGATC-3'), TR4 (5'-CTATGGGGCTGTCAGTTGTG-3' and 5'-CTCCTCCACTGGTATCTATC-3') and actin (5'-TGGCCTTAGGGTGCAGGG-3' and 5'-GTGGGCCGCTCTAGGCACCA-3').

TR4 silencing by siRNA

TR4 silencing was conducted using triFECTa kit dicer substrate RNAi duplexes (Integrated DNA Technologies): (duplex 2: 5'-GCGGAUCCAAGGCUGAAACAAG-3'/3'-GGCGCCUAAGGUUCCGACUUUGUUCGG-5'; duplex 3: 5'-GCCUCACCUCAGCGCAUUCAGAUTG-3'/3'-AUCGGAGUGGAGUCGCGUAAGUCUAAC-5'). The duplexes were transfected into H2.35 cells using Lipofectamine-2000 (Invitrogen).

Statistical analyses

Experiments are performed with triplicates and repeated at least two times. Data are presented as means $\pm$ s.d. Statistical significance was determined using the two-tailed Student's *t*-test, where $P < 0.05$ was considered significant.

Results

Activation of TR4 by organic extracts of mouse tissues

To identify the factors stimulating the physiological function of TR4, organic extracts of various mouse tissues where TR4 is expressed abundantly were examined using a bioassay-guided fractionation and isolation protocol. Fresh tissue homogenates were extracted with organic solvents and the concentrated extracts were dissolved in isopropanol for assays using a standard Gal4-based transactivation system (Fig. 1A). The tissue extracts, particularly those from liver, substantially activated Gal4-TR4LBD in HEK293 cells. Extracts from brain, kidney and testis also showed activity, but were less potent than liver extract.

Large-scale fractionations of liver and kidney extracts were conducted by reverse-phase HPLC. Seven fractions of liver and kidney were prepared (Suppl. Fig. S1) and each fraction was tested for its ability to transactivate Gal4-TR4LBD (Fig. 1B–C). The HPLC fractions L4, L6 and L7 from liver extract (Fig. 1B) and K5 and K7 from kidney extract (Fig. 1C) transactivated Gal4-TR4LBD in HEK293 cells. Because activating fractions from both the kidney and liver extracts generated very similar results, it is likely that the activating compounds in these extracts are similar.

Identification of activating compounds in tissue extracts

Mass spectrometric (MS) analyses of active HPLC fractions using different mass ionization techniques (including electrospray ionization [ESI] and electron impact [EI] ionization) were conducted to determine the chemical identity of the activating compounds. Application of ESI-MS in soft ionization conditions using both positive and negative reflection modes failed to ionize the compounds (data not shown). However, EI ionization, a relatively harsh condition for mass analyses, generated a series of fragment ion peaks separated from each other by a 14-unit (i.e., $-\text{CH}_2-$) mass, suggesting a characteristic fragmentation pattern for long chain hydrocarbons. The EI mass provided very similar fragmentation patterns for all activating fractions, despite their variations in polarity.

To characterize the individual components present in the fractions, the liver fractions L4 and L7 were analyzed by gas chromatography coupled to EI-MS. GC-MS analyses of these fractions confirmed that the moderately hydrophobic fraction, L4, contained abundant free FAs, including palmitic acid (16:0), oleic acid (18:1), and linoleic acid (18:2), whereas the most hydrophobic liver fraction, L7, mainly contained FA methyl esters (Fig. 2A, top and middle). Some impurities, such as phthalate derivatives, were detected in activating fractions, possibly as a result of contamination by plasticizers from plastics or solvent. GC-MS analysis of the most hydrophobic kidney fraction, K7, indicated a chemical identity (Fig. 2A, bottom) similar to that of the fractions from liver.

The ability of commercially available pure FAs (Fig. 2B) to transactivate Gal4-TR4LBD was then examined (Fig. 2C). As expected, some FAs activated Gal4-TR4LBD in HEK293 cells. Among the FAs tested, ω -6 FAs such as γ -linoleic acid (18:2), γ -linolenic acid (18:3) and arachidonic acid (20:4) most strongly transactivated TR4, followed by monounsaturated oleic acid (18:1) and the ω -3 FA, docosahexaenoic acid (22:6). In general, the medium-length saturated FAs such as palmitic acid (16:0) and stearic acid (18:0) were much less active than the mono- and polyunsaturated FAs (PUFAs).

A fatty acid up-regulates expression of TR4 target genes

An 18:2 PUFA was found in the activating fractions of liver extract (Fig. 2A), and γ -linoleic acid (18:2) was an effective activator of TR4 (Fig. 2C). We wished to identify physiological components targeting TR4, and therefore subsequent experiments were designed to focus on γ -linoleic acid. A series of biochemical assays were performed to determine if γ -linoleic acid could regulate TR4 transcriptional activity. First, the direct interaction between TR4 and γ -linoleic acid was performed in an *in vitro* binding assay where GST-fused TR4 was used to pull down ^{14}C -labeled γ -linoleic acid. As shown in Fig. 3, γ -linoleic acid was significantly pulled down by the full-length TR4, but neither the TR4 deleted in its ligand-binding domain (TR4 Δ LBD) nor another negative control, the GST alone. This further supports the notion that γ -linoleic acid directly interacts with TR4 through its ligand-binding domain. Both ApoE and PEPCK are regulated by TR4 [8], and mouse liver H2.35 cells express these components endogenously. Treatment with γ -linoleic acid up-regulated the expression of both ApoE and PEPCK (Fig. 4A–B). Furthermore, up-regulation was abolished in TR4-silenced cells, indicating a requirement for TR4 in the γ -linoleic acid-induced activation of these genes. An interesting observation was that γ -linoleic acid up-regulated the protein level more significantly than the mRNA level of ApoE and PEPCK, suggesting that post-transcriptional events might be involved in the regulation of TR4 target genes by γ -linoleic acid. This will require further studies.

TR4 can also bind to the DR1 element located in the hepatic control region-1 of the ApoE gene [9]. This element was used to generate a reporter that could be regulated by TR4 [7]. This construct was then used to determine if FAs affect the TR4-dependent activation of the DR1

reporter. Silencing TR4 by transfecting TR4 siRNA into H2.35 cells (which express TR4 endogenously) abolished γ -linoleic acid-induced DR1 reporter activity in this cell line (Fig. 5A). In contrast, when COS-1 cells (which have relatively low endogenous TR4) were transfected with a TR4 expression plasmid, γ -linoleic acid further activated the DR1 reporter (Fig. 5B). However, γ -linoleic acid was unable to activate the DR1 reporter in COS-1 cells transfected with TR4 lacking the LBD, confirming the specific requirement of LBD domain on FA-induced activation (Fig. 5C).

Fatty acids enhance coregulator recruitment of TR4

Nuclear receptors recruit certain coregulators to modulate target gene expression [19-21]. The effects of FAs on TR4's interaction with the known cofactors PCAF and RIP140 [7,22,23] were examined by coimmunoprecipitation using Flag-RIP140 or Flag-PCAF, and by mammalian two-hybrid interaction tests using Gal4-TR4LBD and VP16AD-fused RIP140 or PCAF (Fig. 6). In the presence of γ -linoleic acid, the interactions between TR4 and both PCAF and RIP140 were enhanced significantly in both systems, further supporting the regulatory effect of FAs on TR4 activity.

Comparison of transactivation by TR4 and PPARs

PPARs regulate multiple metabolism-related transcriptional events on binding their ligands. Transactivation assays were performed using selected PPAR ligands: WY-14643 for PPAR- α , L-165041 for PPAR- δ , and ciglitazone for PPAR- γ . All three ligands failed to activate TR4 at concentrations capable of activating Gal4 constructs containing their respective PPARs (Fig. 7A). Surprisingly however, γ -linoleic acid was able to activate PPAR- α and TR4 to a similar degree. Therefore, PPAR- α activation of the DR1 reporter was compared to DR1 reporter activation mediated by TR4. PPAR- α failed to activate DR1 reporter even in the presence of γ -linoleic acid, contrasting with the activation of DR1 reporter by TR4 and the enhancement of that activation induced by γ -linoleic acid (Fig. 7B).

Discussion

This study establishes for the first time that certain endogenous PUFAs are capable of stimulating TR4-dependent transcriptional activity in multiple cell lines. Although γ -linoleic acid was able to interact directly with TR4 (Fig 3), it remains possible that γ -linoleic acid might also act through other pathways to regulate TR4 activity, such as through other post-transcriptional mechanisms as suggested from data shown in Fig. 4. This is an interesting subject for future studies. Further, γ -linoleic acid was also able to weakly activate PPARs, suggesting that γ -linoleic acid might have more general effects toward certain nuclear receptors.

γ -linoleic acid belongs to a group of essential fatty acids, called omega-6 fatty acids. It is an intermediate metabolite during the production of arachidonic acid from linoleic acid. The Δ -6-desaturase converts linoleic acid to γ -linoleic acid. Studies have shown that diabetic patients require higher intake of linoleic acid due to the lower level of γ -linoleic acid produced in these patients, which is believed to be caused by impaired Δ -6-desaturase activity in the patients [24]. Decrease in γ -linoleic acid in diabetic patients could be a physiological, protective mechanism to limit TR4 activity in order to improve insulin sensitivity and to reduce gluconeogenesis, because TR4 knockout mice showed insulin hypersensitivity [8] and PEPCK could contribute to diabetes-induced hyperglycemia[25,26]. It will be an interesting topic in the future to evaluate whether γ -linoleic acid contributes to the regulation of insulin sensitivity through TR4.

Fatty acids are ubiquitous biological molecules that are used as metabolic fuels, as covalent regulators of signaling molecules, and as essential components of cellular membranes. It is thus logical to predict that FA levels should be closely regulated. Indeed, some of the most common medical disorders in westernized societies (e.g., cardiovascular disease, hyperlipidemia, obesity, insulin resistance) are characterized by altered levels of FAs or their metabolites. PUFAs, for instance, improve insulin sensitivity, lower triglyceride, increase plasma HDL and decrease hepatic lipogenesis. The mechanisms by which PUFAs regulate lipid metabolism have been studied extensively in recent years. Several nuclear receptors, such as PPAR- α and liver X receptor (LXR), mediate the biological activities of PUFAs [27]. For example, PUFAs decrease the expression of the sterol regulatory element binding protein-1c (SREBP-1c) by inhibiting LXR binding to the LXR response element [28]. Suppression of SREBP-1c expression could contribute to reduced lipid synthesis. However, the molecular details by which dietary fatty acids regulate genes are not yet fully understood. The identification of FAs as upstream signals for TR4 is an initial step in enhancing our understanding of these mechanisms, as well as of the potential crosstalk among various nuclear receptors and of cell signaling pathways. Of particular interest is the finding that TR4 is minimally activated by FAs when it is phosphorylated on specific MAP kinase sites [7]. TR4 activation by FAs might therefore require an appropriate cellular environment in which the TR4 is posttranslationally modified in a specific fashion.

The present study also suggests that FA treatment might induce TR4 activation of one of its targets, ApoE. The ApoE gene is an important player in cardiovascular and nervous systems. It is involved in cholesterol homeostasis and regression of atherosclerosis, increases cholesterol efflux from foam cells, prevents atherosclerotic lesions in vascular walls and protects against eventual death from myocardial infarction and hypertension. ApoE also is involved in adipocyte differentiation, as well as neuronal regeneration and degeneration processes [29, 30]. The ApoE allele e4 has been linked genetically to Alzheimer's disease (AD), whereas alleles e2 and e3 might exert a neural-protective effect and reduce the progression of familial and sporadic AD. Interestingly, diets enriched with PUFAs prevented neuronal defects in a transgenic mouse model of AD, and essential fatty acids in food increase the secretion of ApoE alleles e2 and e3 and reduce the onset of progression of AD in people, relative to subjects fed a diet without essential fatty acids [31,32]. The TR4 activation and the up-regulation of the ApoE gene induced by certain essential fatty acids in the present study are consistent with these reports, and shed some light on the potential mechanisms involved in neuronal degenerative diseases, metabolic diseases, and atherosclerosis-related cardiovascular disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effects of tissue extracts on TR4 transactivation

(A) Transactivation of mouse Gal4-TR4LBD by organic extracts of mouse tissues. HEK293 cells were incubated with 200 $\mu\text{g/mL}$ of the various extracts for 24 hours. Relative luciferase activity was normalized against *lacZ* expression. C: control; B: brain; T: testis; K: kidney; L: liver. Experiments were repeated three times. (B–C) Transactivation of Gal4-TR4LBD by HPLC fractions from liver (B; L1–L7) or kidney (C; K1–K7) extracts. HEK293 cells were incubated with 100 $\mu\text{g/mL}$ of the various extracts for 24 hours.

Figure 2. Identification of endogenous activators for mouse TR4

(A) Characterization of activating liver and kidney subfractions by GC–MS analysis. Spectrographs of liver subfractions L4 (top) and L7 (middle), and kidney subfraction K7 (bottom). Free FAs identified in the fractions included palmitic acid (16:0; peak d), palmitoleic acid (16:1), stearic acid (18:0; peak e), oleic acid (18:1) and linoleic acid (18:2). Methyl ester (ME) derivatives of palmitic acid (16:0 ME; peak a), stearic acid (18:0 ME; peak f), oleic acid (18:1 ME; peak b) and linoleic acid (18:2 ME; peak c) were also identified. ‘X’ peaks: phthalate derivatives (e.g., plasticizers); ‘U’ and ‘u’ peaks: unknown phenolic compounds (as determined by a chemical library search of GS–MS data.) (B) Structures of fatty acids used for transactivation experiments. 1: palmitic acid (16:0); 2: palmitoleic acid (16:1); 3: stearic acid (18:0); 4: oleic acid (cis isomer; Z-18:1); 5: oleic acid (trans isomer; E-18:1); 6: γ -linoleic acid (18:2); 7: γ -linolenic acid (18:3); 8: arachidonic acid (20:4); 9: docosahexaenoic acid (22:6). (C) Transactivation of Gal4-TR4LBD by natural FAs. HEK293 cells were incubated with 200 nM of each of the compounds in (B) or with 10 nM all-*trans* retinoic acid (at-RA) for 24 hours.

Figure 3. Ligand binding domain of TR4 is required for direct interaction of TR4 with γ -linoleic acid
 ^{14}C -labeled γ -linoleic acid is pulled down by GST, GST-TR4 or GST-TR4 deleted in the ligand binding domain (GST-TR4 Δ LBD). *: $P < 0.05$. GST proteins are shown on the right. Predicted bands of GST proteins are marked by arrows.

Figure 4. γ -linoleic acid up-regulates TR4 target genes

H2.35 cells were transfected with control (siCon) or TR4 (siTR4) siRNA and then incubated with 200 nM γ -linoleic acid (18:2) for 24 hours. ApoE and PEPCK expression was assessed by (A) real-time RT-PCR and (B) immunoblots. Actin expression was used as an internal control, and TR4 silencing was verified by real-time RT-PCR or probing immunoblots with anti-TR4 antibody. *: $P < 0.05$.

Figure 5. γ -linoleic acid up-regulates DR1 reporter in a TR4-dependent manner

A schematic of the construct containing the DR1 TR4-binding sequence is shown above each graph. (A) Effect of γ -linoleic acid (18:2) on DR1 reporter activation in H2.35 cells with or without TR4 silencing. Knockdown efficiency of TR4 (as measured by RT-PCR) is shown above the graph. (B) Effect of γ -linoleic acid on DR1 reporter activation in COS-1 cells transfected with empty vector (CMV) or a full-length TR4 expression plasmid (TR4). (C) Effect of γ -linoleic acid on DR1 reporter activation in COS-1 cells transfected with empty vector (Ctrl), full-length TR4 expression plasmid (TR4) or TR4 plasmid lacking the LBD (TR4- Δ LBD). *: $P < 0.05$.

Figure 6. γ -linoleic acid enhances interaction between transcription cofactors and TR4

(A) H2.35 cells were transfected with Flag-PCAF or Flag-RIP140 and incubated with (+) or without (-) 200 nM γ -linoleic acid (18:2) for 24 hours. Samples were coimmunoprecipitated (IP) with anti-TR4 and immunoblotted (IB) with anti-Flag. One-eighth of the input from each sample was also immunoblotted. (B) The effect of γ -linoleic acid on transcription cofactor recruitment by TR4 in a mammalian two-hybrid assay using Gal4BD-TR4LBD and VP16AD-RIP140 or VP16AD-PCAF. COS-1 cells were incubated with 200 nM γ -linoleic acid for 24 hours. TR4 interactions with PCAF and RIP140 were enhanced ~12- and ~23-fold, respectively, as compared to basal activation of Gal4-TR4LBD alone (~5-fold). *: $P < 0.05$.

Figure 7. Comparison of the transactivation activity of TR4 and PPARs

(A) Transactivation in COS-1 cells transfected with Gal4-TR4LBD (for TR4) or Gal4 reporter (for PPARs α , δ , and γ) and treated with γ -linoleic acid (18:2) or PPAR-specific ligands. Clz: ciglitazone (5 μ M); Wy: WY-14643 (5 μ M); L16: L-165041 (10 nM). (B) γ -linoleic acid (18:2) significantly enhances DR1 reporter expression in a TR4-dependent manner. PPAR- α was unable to activate the DR1 reporter, even when stimulated with γ -linoleic acid. *: $P < 0.05$.