PPARγ antagonists induce aromatase transcription in adipose tissue cultures

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ABSTRACT

Aromatase is the rate-limiting enzyme in the biosynthesis of estrogens and a key risk factor for hormone receptor-positive breast cancer. In postmenopausal women, estrogens synthesized in adipose tissue promotes the growth of estrogen receptor positive breast cancers. Activation of peroxisome proliferator-activated receptor gamma (PPARγ) in adipose stromal cells (ASCs) leads to decreased expression of aromatase and differentiation of ASCs into adipocytes. Environmental chemicals can act as antagonists of PPARγ and disrupt its function. This study aimed to test the hypothesis that PPARγ antagonists can promote breast cancer by stimulating aromatase expression in human adipose tissue.

Primary cells and explants from human adipose tissue as well as A41hWAT, C3H10T1/2, and H295R cell lines were used to investigate PPARγ antagonist-stimulated effects on adipogenesis, aromatase expression, and estrogen biosynthesis. Selected antagonists inhibited adipocyte differentiation, preventing the adipogenesis-associated downregulation of aromatase. NMR spectroscopy confirmed direct interaction between the potent antagonist DEHPA and PPARγ, inhibiting agonist binding. Short-term exposure of ASCs to PPARγ antagonists upregulated aromatase only in differentiated cells, and a similar effect could be observed in human breast adipose tissue explants. Overexpression of PPARγ with or without agonist treatment reduced aromatase expression in ASCs.

The data suggest that environmental PPARγ antagonists regulate aromatase expression in adipose tissue through two mechanisms. The first is indirect and involves inhibition of adipogenesis, while the second occurs more acutely.

Abbreviations: 9cRA, 9-cis-retinoic acid; ANOVA, analysis of variance; ASC, adipose stromal cell; AU, arbitrary units; BMI, body mass index; cDNA, complementary DNA; DEHPA, di(2-ethylhexyl)phosphoric acid; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; DMEM, Dulbecco’s modified Eagle medium; DPhP, diphenyl phthalate; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; ESI, electrospray ionization; EV, empty vector; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; LBD, ligand-binding domain; LOD, limit of detection; LOQ, limit of quantification; ND, not detected; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PPARγ, peroxisome proliferator-activated receptor gamma; PPARγ2, peroxisome proliferator-activated receptor gamma 2; RT-qPCR, quantitative reverse transcription PCR; RXR, retinoid X receptor; SD, standard deviation; SNP, single nucleotide polymorphism; T3, triiodothyronine.

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1. Introduction

Breast cancer is the most commonly diagnosed cancer worldwide [1]. In women, the systemic levels of steroid hormones, not least estrogens, are major risk factors for breast cancer [2], and exposure to endocrine disrupting chemicals may contribute to breast cancer risk [3,4]. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) has been suggested to be a protective factor in breast cancer development [5], with a proposed mechanism being negative regulation of aromatase (encoded by CYP19A1) [6,7], the rate-limiting enzyme in the biosynthesis of estrogens. Notably, most studies investigating this link have focused on the effect of PPARγ activation on aromatase, while inactivation of PPARγ is less explored.

Numerous environmental and occupational compounds can target PPARγ by binding as agonists or antagonists in the binding pocket of the ligand-binding domain (LBD) [8,9]. PPARγ is highly expressed in adipose tissue, which is an important site of xenobiotic bioaccumulation and metabolic disruption [10]. Adipose tissue is an endocrine organ that plays important roles in the development and function of mammary epithelial cells but also contributes to development and progression of breast cancer [11]. Environmental PPARγ antagonists may potentially promote breast cancer development by inhibiting PPARγ function, leading to a derepression of aromatase expression and, consequently, increased production of estrogens acting locally on adjacent breast epithelial cells.

PPARγ is essential for adipogenesis [12]. It exists as two isomers, PPARγ1 and PPARγ2, transcribed from different promoters of the PPARγ gene. The PPARγ2 is almost exclusively expressed in the adipose tissue [13] and encompasses 30 additional N-terminal amino acids compared to PPARγ1. The variant allele of the common missense single nucleotide polymorphism (SNP) of PPARγ, Pro12Ala (rs1801282), is associated with lowered risk of alcohol-related breast cancer [14,15], as well as impaired adipogenesis [16]. The variant allele encodes a less active PPARγ2 [17], although its impact on metabolism is highly dependent on gene-environment interactions [18].

Since exposure to environmental chemicals may contribute to breast cancer development [19], it is important to fully understand the underlying mechanisms in order to identify potential interventions. The role of aromatase in promoting breast cancer development and progression is well established [20], however it is still unknown whether exposure to PPARγ antagonists affects aromatase expression in human adipose tissue cells. It is therefore hypothesized that PPARγ antagonism induces aromatase expression in the adipose tissue.

In this study, human adipose stromal cells (ASCs) were differentiated in the presence of previously identified PPARγ antagonists [9] to address the inhibitory effect of selected chemicals on PPARγ and to study the effect of impaired adipogenesis on aromatase expression. In addition, interactions between PPARγ and selected chemicals, alone or in combination, were determined using NMR spectroscopy. Finally, a more acute effect of PPARγ ligands on aromatase expression was studied by treating ASCs, differentiated ASCs, or adipose tissue explants with PPARγ antagonists.

2. Materials and methods

2.1. Isolation of primary cells and explants

Primary cells were isolated from adipose tissue obtained from patients undergoing mastectomy, abdominoplasty, or reduction mammoplasty at Weill Cornell Medicine under IRB-approved protocol #20-0121391. Adipose tissue was finely minced, and 20–25 mL tissue was placed in a 50 mL centrifuge tube, which was filled with pre-warmed F-12 medium (10-080-CV, Corning) containing 10% fetal bovine serum (FBS; 35-010-CV, Corning) and 1% penicillin–streptomycin solution (PS; 15140122, Gibco). The tissue was incubated with 1 mg/mL collagenase (C0130, Sigma-Aldrich) and 0.01 mg/mL hyaluronidase (H3506, Sigma-Aldrich) for 1 h at 37 °C while rotating, followed by centrifugation at 500 x g for 15 min. The oil layer at the top was removed, and adipocytes were collected from the layer below. The cell pellet was resuspended in 20 mL culture medium and passed through 100 μm and 40 μm filters. The filtrate was centrifuged at 500 x g for 5 min, and the supernatant was removed. The cell pellet was resuspended in 1 mL Red Blood Cell Lysis Buffer (11814389001, Roche) and inverted periodically for 10 min. The tube was centrifuged at 500 x g for 5 min, supernatant was discarded, and the pellet was resuspended in culture medium or collected for lysis. The cell suspension was transferred to tissue culture flasks and incubated at 37 °C and 5% CO2 for 1 h before the medium was renewed. Information on the study participants is shown in Table 1.

Adipose tissue explants were obtained by cutting 2–5 mm3 sections of adipose tissue and incubating multiple pieces per well in F-12 culture medium with 10% FBS and 1% PS in 12-well plates at 37 °C. After a few hours, the medium was changed.

Primary adipocytes and ASCs collected for lysis were washed by adding phosphate-buffered saline (PBS) and inverting several times. ASCs were subsequently centrifuged for 5 min at 500 g. Adipocytes and ASCs were then frozen in liquid nitrogen and kept at −80 °C until gene expression analysis.

2.2. Cell culture and treatment

The human A41 ASCs cell line (hTERT A41hWAT-SVF, passage 8–19) [21] and the mouse C3H10T1/2 mesenchymal stem cell line (CCL-226, ATCC, passage 6–11) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; 41965-039, Gibco) containing 10% FBS (F7524, Sigma-Aldrich) and 1% PS solution (15070063, Gibco). The human NCI-H295R adrenocortical carcinoma cell line (CRL-2128, ATCC, passage 10–13) was cultured in DMEM/F-12 (11039, Thermo Fisher Scientific) supplemented with 2.5% Nu-Serum (355100, Corning) and 1% ITS+ (354352, Corning). All cells were cultured in humidified incubators at 37 °C and 5% CO2. Culture medium was changed every 2 or 3 days. Cells and explants were stimulated with the chemicals listed in Table 2: 9-cis-retinoic acid, Cosan 528, di(2-ethylhexyl)phosphoric acid (DEHPA), diphenyl phthalate, forskolin, GW9662, kresoxim-methyl, phorbol 12-myristate 13-acetate (PMA), prochloraz, pyraclostrobin, pyridaben, rosiglitazone, Violet Cibacert 2R, and zoxamide (all purchased from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). Solvent volumes were equal across all conditions for each experiment.

Adipose tissue explants were treated with chemicals for 48 h, then washed in PBS and lysed with QIAzol (Qiagen). For each biological sample, multiple technical replicate samples were collected for analysis. Undifferentiated or 12-day differentiated A41 cells in basal culture medium were treated for 24 h, while, in other experiments, primary ASCs, C3H10T1/2 cells, and A41 cells were treated throughout differentiation. Concentrations were selected based on previous reporter

| Participant number (#), surgery type, sex, age, body mass index (BMI), and PPARγ Pro12Ala genotype. |
|---|---|---|---|---|
| # | Surgery | Sex | Age | BMI |
| 1 | Mastectomy | Female | 37 | 35.51 |
| 2 | Mastectomy | Female | 46 | 28.70 |
| 3 | Mastectomy | Female | 40 | 26.20 |
| 4 | Mastectomy | Female | 56 | 27.20 |
| 5 | Mastectomy | Female | 65 | 19.49 |
| 6 | Mastectomy | Female | 45 | 30.70 |
| 7 | Abdominoplasty | Female | 55 | 36.28 |
| 8 | Abdominoplasty | Female | 39 | 38.37 |
| 9 | Mammaplasty | Female | 57 | 31.85 |
| 10 | Mammaplasty | Male | 18 | 23.95 |
| 11 | Mammaplasty | Female | 45 | 24.70 |
| 12 | Mammaplasty | Female | 35 | 32.34 |
| 13 | Mammaplasty | Female | 40 | 29.60 |
Table 2
The chemicals used for treatment of cells.

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<td>S36350</td>
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</table>

Cells were washed in PBS and lysed with Buffer RLT (Qiagen) containing 1 % β-mercaptoethanol. H295R cells were subcultured at approximately 50–60 % confluency (3 x 10⁵ cells) into 24-well plates and left overnight. Next day, culture medium was removed by aspiration and replaced with medium containing each chemical of interest. The plates were incubated at 37 °C and 5 % CO₂ for 48 h. Finally, medium was transferred to new 24-well plates and frozen at −80 °C until further processing to analyze hormone concentrations. Cells were washed with PBS and frozen at −80 °C for subsequent protein analysis. The experiment was performed in triplicates and repeated three times independently.

2.3. Adipocyte differentiation

Primary human ASCs were subcultured at high density to reach 100 % confluence. The cells were then washed twice with PBS and induced to differentiate by changing basal culture medium to serum-free adipogenic medium containing 0.1 or 2 μM rosiglitazone (day 0–4), 0.25 μM dexamethasone (day 0–6), 500 μM IBMX (day 0–6), 20 nM insulin, 0.2 mM T₃, 33 μM biotin, 17 μM pantothenic acid, 0.1 μM transferrin, and 10 μg/mL cortisol (all from Sigma-Aldrich). The cells were kept in adipogenic medium for 12 days and the medium was changed every 2 days.

A41 and C3H10T1/2 cells were grown to confluence, and differentiation was induced in serum-containing medium. For differentiation of A41 cells, the culture medium was supplemented with 1 μM rosiglitzone, 0.1 μM dexamethasone, 500 μM IBMX, 500 nM insulin, 2 nM T₃, 33 μM biotin, and 17 μM pantothenic acid. Differentiation of C3H10T1/2 cells was induced by supplementing the culture medium with 0.5 μM rosiglitzone, 1 μM dexamethasone (day 0–2), 500 μM IBMX (day 0–2), and 20 nM insulin (day 0–4). The adipogenic medium was changed every 2 or 3 days for C3H10T1/2 and A41 cells, respectively.

In experiments where cells were exposed to additional chemicals (Table 2) during differentiation, these chemicals were added together with the adipogenic factors every time the medium was replenished. Mature A41 cells used for acute chemical treatment were differentiated for 12 days and returned to regular growth medium for 2 days before 24 h treatment with chemical.

2.4. Lipid staining and quantification

Transparent 96-well plates (3596, Corning) were used for staining with Oil Red O. Cells were washed in PBS and fixed with 4 % formaldehyde (252549, Sigma-Aldrich) in PBS for 30 min at room temperature. The cells were then washed twice with water and incubated with 60 % isopropanol for 5 min. Afterwards, cells were incubated with sterile filtered 60 % Oil Red O (O0625, Sigma-Aldrich) solution for 20 min. Cells were washed 3 times with water and then viewed under the microscope. For quantification, cells were washed 3 times with 60 % isopropanol for 5 min. Oil Red O was then extracted with 50 μL 100 % isopropanol for 20 min. Finally, 40 μL of the extracted Oil Red O was transferred to a 384 well plate (3765, Corning). Absorbance was read at 518 nm in a Varioskan LUX Multimode Microplate Reader (Thermo Scientific), and 100 % isopropanol was used as a background control.

Fluorescent staining was carried out in black-walled 96-well plates (3603, Corning). Cells were washed in PBS and fixed with 4 % formaldehyde in PBS for 30 min at room temperature. The cells were then washed with PBS and incubated another 30 min with LipidTOX (H34477, Invitrogen; 1:5000 dilution) and Hoechst 33,342 (sc-495790, Santa Cruz; 1:10000 dilution). Cells were washed in PBS, and confocal imaging was performed using a Zeiss LSM 880 AxioObserver with a Plan-Apochromat 20x/0.8 M27 objective. Images (425.1 x 425.1 μm) were taken in random regions of each well to avoid selection bias. Image analysis was performed by batch processing to determine nucleus and lipid droplet number as well as lipid droplet diameter using Imaris 9.0.0 (Oxford Instruments). For quantification, batch analysis of images was carried out in a blinded and automated manner. In addition, LipidTOX and Hoechst were quantified in the plate reader by measuring fluorescence at 637/655 nm and 350/461 nm, respectively. Cell-free wells were subtracted as background.

2.5. Transient transfection

Human A41 cells were transiently transfected with pcDNA3.1 or pcDNA3.1 PPARG2 (kindly donated by Karsten Kristiansen, University of Copenhagen, Denmark) at about 80–95 % confluency in 6-well plates. For each well, 2 μg plasmid DNA and 6 μL TransIT-X2 (MR6000, Mirus Bio) were added to 200 μL Opti-MEM (31985070, Gibco). The mixture was incubated for 15–30 min and added to cell culture wells. The cells were incubated at 37 °C for 24 h, and then the medium was replaced with culture medium containing rosiglitazone or solvent. After an additional 24 h incubation, cells were lysed.

For reverse transfection, 200 μL of the mixture with TransIT-X2:DNA complexes were added to the culture wells and incubated for 25 min. Cell suspension was then transferred to the wells. Incubation, treatment, and lysis were performed as described above.

2.6. Gene expression analysis

RNA from cultured cells or adipose tissue explants was extracted using RNeasy Kit (Qiagen) or RNeasy Lipid Tissue Mini Kit (Qiagen), respectively. Complementary DNA (cDNA) was synthesized from 1 μg RNA using iScript cDNA Synthesis Kit (1708891, Bio-Rad) for cell lines or from 1 μg, 0.2 μg, or 0.5 μg RNA using qScript cDNA Synthesis Kit (95047, Quantabio) for adipose tissue explants, directly isolated cells, and primary cell cultures, respectively. Quantitative reverse transcription PCR (RT-qPCR) was performed using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (600882, Agilent) and the CFX384 Real-Time PCR Detection System (Bio-Rad); Fast SYBR Green Master Mix (4385612, Applied Biosystems) and the 7500 Fast Real-Time PCR System (Applied Biosystems); or TaqMan Fast Advanced Master Mix (4444557, Applied Biosystems) and the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). Each biological sample was measured in technical triplicates and the 2^ΔΔCt method was used for relative quantification. Primers were purchased from TAG Copenhagen and are shown in Table 3. In addition, TaqMan assays (4331182, Applied Biosystems) were used for mouse Cyp19a1 (Assay ID: Mm00448409_m1) and Rps18 (Assay ID: Mm02601777_g1). The criteria for exclusion of outliers in the technical replicates was a standard deviation (SD) above 0.3 and a two-fold difference in the distance to the median.

2.7. Protein immunoblotting

Cells were washed with PBS and lysed using RIPA buffer (89900, Thermo Scientific) containing protease inhibitors (S8820, Sigma-
1. Aldrich). The lysates from wells of similarly treated cells were pooled. Lysates were then centrifuged for 10 min at 4 °C and 16,100 × g, supernatant was collected, and protein concentrations were determined using bicinchoninic acid assay. Samples were diluted in lysis buffer to obtain equal protein concentrations. Afterwards, Laemmli sample buffer was added, and samples were heated at 95 °C for 5 min. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. Immunostaining was performed using PPARγ (sc-7273, Santa Cruz; 1:500 dilution) and vinculin (1:2000 dilution) primary antibodies and HRP-conjugated secondary antibodies (1706515 and 1706516, Bio-Rad; 1:10000 dilution). Chemiluminescent imaging was performed using the ChemiDoc XRS + System (Bio-Rad).

2.8. Hormone analysis

Steroid hormone levels were analyzed by LC-MS/MS as previously described [22]. Deuterated internal standards were added to the cell culture supernatants, which were then centrifuged at 15,000 g for 10 min. Steroid hormones were separated, detected, and quantified by online SPE-LC-MS/MS using Waters Oasis HLB column (186002035, UIVISION Technologies; 2.1 x 100 mm, 15 µm) as the mobile phases. For 17β-estradiol and estrone analysis, a Kinetex C18 column (00D-4462-AN, Phenomenex; 2.1 x 20 mm, 15 µm) was used with an injection volume of 100 nL, measuring with negative electrospray ionization (ESI) and using methanol and 0.2 mM ammonium fluoride in water as the mobile phases (gradient flow rate was 0.4 mL/min). For the other hormones, an Ascentis Express C8 column (SU-53832-U, Supelco; 2.1 x 100 mm, 2.7 µm) was used with an injection volume of 100 µL, measuring with negative and positive ESI with acetonitrile and 0.1 % formic acid in water as the mobile phases (gradient flow rate was 0.25 mL/min). The following steroid hormones were measured: androstenedione, corticosterone, cortisol, dehydroepiandrosterone (DHEA), 11-deoxycortisol, dihydrotestosterone (DHT), epitestosterone, 17β-estradiol, estrone, 18-hydroxyoestrone, 17α-hydroxyprogesterone, pregnenolone, progesterone, and testosterone. The limit of quantification (LOQ) was 1.0 ng/mL for pregnenolone; 0.25 ng/mL for 11-deoxycortisol; 0.1 ng/mL for cortisol, DHEA, and DHT; 0.05 ng/mL for epitestosterone and hydroxyprogesterone; 0.02 ng/mL for androstenedione, corticosterone, 17β-estradiol, and testosterone; and 0.01 ng/mL for estrone, 18-hydroxycortisol, and progesterone. For quantification, external calibration standards were run before and after the samples at levels of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 10.0 and 20 ng/mL, with 4.0 ng/mL internal standards (testosterone-d2, methyltestosterone-d5, progesterone-c2 and estradiol-d3 from EURL Wageningen and cortisol-d4 and deoxycortisol-d8 from LGC Standards). For cortisol, deoxycortisol, estradiol, progesterone, and testosterone, dedicated internal standards were used. Furthermore, cortisol-d4 was used for hydroxyprogesterone; deoxycortisol-d8 was used for corticosterone; progesterone-c2 was used for pregnenolone; and estradiol-d3 was used for estrone. The limit of detection (LOD) and LOQ were estimated as the concentrations corresponding to three and ten times the signal-to-noise, respectively. Values below the detection limit were set to zero. The mass spectrometer was an EVOQ ELITE Triple Quadrupole instrument from Bruker (Bremen, Germany) and the UHPLC system was an UltiMate 3000 System with a DGP-3600RS dual-gradient pump. Data handling was done using the software MS Workstation version 8.2.1.

2.9. Protein production

Human PPARγ2 cDNA (residues 231 to 505, corresponding to the PPARγ-LBD) was cloned into a modified pET24a vector, encoding it with an N-terminal hexahistidine- and SUMO-tag (His-SUMO). The protein was produced in E. coli BL21(DE3) cells (New England BioLabs, Frankfurt, Germany) in auto-induction minimal medium [23], using 15NH4Cl as a nitrogen source for isotope labeling. Production was induced at OD600 of 0.8 by changing the temperature from 37 °C to 18 °C and was allowed to proceed for 24 h. Cells were harvested by centrifugation at 5,000 × g for 20 min and resuspended in lysis buffer (20 mM imidazole, 50 mM Tris pH 8, 200 mM NaCl, 10 % (v/v) glycerol). All buffers contained 5 mM β-mercaptoethanol. Cells were lysed using a cell disrupter (Constant Systems Ltd., Daventry, UK) at 25 kpsi and the lysate was cleared by centrifugation at 20,000 × g for 45 min. The supernatant was passed two times over 5 mL Ni-NTA resin (Qiagen, Hilden, Germany), pre-equilibrated with lysis buffer. Three wash steps were done, first with lysis buffer, then with lysis buffer containing 1 M NaCl and then with lysis buffer again, and the bound proteins finally eluted with elution buffer (lysis buffer with 500 mM imidazole). The protein was cleaved in 40 mM Tris, pH 8, 10 % (v/v) glycerol, 200 mM NaCl, and 5 mM β-mercaptoethanol overnight at 4 °C with ULP1-protease (in-house production, produced and purified as described in Reverter and Lima, 2009) [24]. The His-SUMO tag and ULP1 were removed by reverse Ni-NTA purification. The protein was further purified by ion exchange chromatography using a HiTrap QFF 5 mL column (Cytiva) and size exclusion chromatography using a Superdex 200 Increase 10/300 GL (Sigma-Aldrich). Ion exchange buffers were 25 mM bis-Tris pH 7.4, with the elution buffer containing additionally 1 M NaCl. The size exclusion buffer contained 40 mM Tris pH 8 and 500 mM NaCl.

2.10. Nuclear magnetic resonance (NMR) spectroscopy

NMR samples containing 80 µM 15N PPARγ LBD were changed into PBS buffer (pH 7.3, 137 mM NaCl, 10 % D2O, room temperature), and 0.7 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added as reference. Rosiglitazone, GW9662, and DEHPA were added at concentrations of 320 µM, 240 µM, and 1 mM, respectively. All NMR spectra were acquired at 298 K on a Bruker AVANCE III 750-MHz (1H) spectrometer equipped with a cryogenic probe. Free induction decays were transformed and visualized in TopSpin (Bruker BioSpin), and subsequently analyzed using the CcpNmr Analysis software [25]. Proton chemical shifts were internally referenced to DSS at 0.00 ppm, with heteronuclei referenced by relative gyromagnetic ratios. Assignments of PPARγ LBD were exported from BMRB entry 17975, as published by Hughes et al. 2012, and transferred to the spectra without ambiguities [26]. For each spectrum, intensities were normalized to the NMR peak of

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<th>Species</th>
<th>Sequence (forward)</th>
<th>Sequence (reverse)</th>
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E235, which was the most intense peak in every condition.

2.11. DNA sequence analysis

Cells were washed in PBS and DNA isolation was performed using QIAamp DNA Mini Kit (51304, Qiagen). A PPARG sequence containing the position of the rs1801282 SNP was amplified in a C1000 Touch Thermal Cycler (Bio-Rad) using Q5 Hot Start High-Fidelity 2X Master Mix (M0494S, New England Biolabs), forward primer (5'-GCCAATTCAAGCCAGTTCT-3') and reverse primer (5'-TTACATTAAATGGCCCATCGTCC-3'). The PCR products were run on an agarose gel to confirm that the amplicon size was correct. The QIAquick PCR Purification Kit (28104, Qiagen) was applied for isolation of the PCR products. The samples were sent to GENEWIZ (Azenta Life Sciences) for Sanger sequencing using the primers for PCR. The results are shown in Table 1.

2.12. Statistical analysis

Statistical significance was tested using the two-tailed (unpaired) Student’s t-test, Dunnett’s multiple comparison test, or one- or two-way analysis of variance (ANOVA), depending on the number of samples and variables. When there were more than two levels within a variable of the two-way ANOVA, Dunnett’s test for multiple comparisons was applied for the levels of that variable or for the means of the levels within each variable. Dunnett’s test was also performed following one-way ANOVA. When all values in a group were below the detection limit, a two sample Z test for proportions followed by Bonferroni correction for multiple comparisons was performed to compare the number of quantifiable values, including all technical replicates, in each group. For calculation of half-maximal inhibitory concentrations (IC50), concentration–response curves were fitted using the four-parameter logistic regression model with slope constraints of -1.5 to 0 and with initial value set to 1. Logistic regression could not be performed when there was a non-monotonic relationship between Oil Red O stain and chemical concentration. Data was normalized to the sum of values within each experiment and shown as the ratio of the test groups to a control group. Differences between groups were considered significant if p ≤ 0.05, and data were presented as means ± SD.

3. Results

Environmental PPARγ antagonists, previously identified by the Tox21 Program and verified in an orthogonal HEK293 cell-based reporter assay [9], were used in the present study to investigate PPARγ-mediated effects on the transcription of aromatase in ASCs, adipocytes, and adipose tissue explants.

3.1. Effect of putative PPARγ antagonists on adipogenesis

Human ASCs were differentiated, using either 0.1 or 2 µM rosiglitazone, in the presence of seven different PPARγ antagonists, including the known PPARγ antagonist GW9662 that served as a positive control for inhibition of adipogenesis. Lipid staining with Oil Red O revealed that all seven chemicals inhibited lipid accumulation in the studied concentration ranges compared to solvent-treated cells (Fig. 1). The IC50 and maximal inhibition for each chemical is shown in Table 4. Lipid staining showed that 0.1 µM rosiglitazone resulted in significantly lower lipid accumulation compared to a concentration of 2 µM (results not shown). The inhibitory effect of GW9662, zoxamide, Cosan 528, and
DEHPA was greater when cells were differentiated using the lower concentration of rosiglitazone compared with the higher concentration.

Confocal microscopy was performed to analyze differences in lipid droplet number and size in response to PPAR\(\gamma\) antagonist treatment during differentiation (2\(\mu\)M rosiglitazone) of primary ASCs (Fig. 2A). Lipids and nuclei were stained with LipidTOX and Hoechst, respectively. Image analysis showed that treatment with GW9662, pyraclostrobin, Cosan 528, and DEHPA decreased the average number of lipid droplets per cell significantly compared to solvent-treated cells (Fig. 2B), and pyraclostrobin treatment also reduced lipid droplet size compared to solvent-treated cells (Fig. 2C). There was considerable variation between cells from the different study participants (Fig. 2B-2C). Fluorescence intensities of LipidTOX and Hoechst were measured in a plate reader, and the LipidTOX/Hoechst ratios were determined (Fig. 2D). Lipid accumulation normalized to cell number was decreased by treatment for all tested PPAR\(\gamma\) antagonists.

### 3.2. Aromatase expression during adipogenesis

To determine the influence of differentiation stage on aromatase expression, aromatase mRNA abundance was measured in ASCs and mature adipocytes. The adipocyte markers, PPAR\(\gamma\) and FASN, were used to confirm difference in cell types. Isolated human ASCs expressed higher levels of aromatase and lower levels of the adipocyte markers compared to mature adipocytes (Fig. 3A). Similarly, cultured undifferentiated A41 cells expressed higher level of aromatase and lower level of...
PPARGγ than fully differentiated A41 cells (Fig. 3B).

The effect of interruption of adipogenesis on aromatase expression was examined in A41 cells by removal of the adipogenic medium. On day 6 of differentiation, the adipogenic medium was renewed or kept in adipogenic medium for 2 days (n = 4). (D) A41 cells at day 6 (n = 3–4) or (E) at day 12 (n = 4) of differentiation were treated with 5 μM or 1 μM GW9662, respectively, during differentiation, and compared with a solvent control group (0.01 % DMSO or 0.002 %, respectively). (F) Mouse C3H10T1/2 cells were differentiated for 6 days in the presence or absence of 10 μM GW9662 or left undifferentiated. Gene expression analysis by RT-qPCR was performed for aromatase (Cyp19a1), adipocyte markers (Adipoq, Slc2a4, and Fasn) decreased, compared to the solvent control (D-E). For C3H10T1/2 cells shown in (F), asterisk indicates statistically significant difference compared to differentiated control cells using two sample proportion test with Bonferroni correction (p < 0.05).

3.4. Short-term regulation of aromatase by PPARγ

A more acute effect of PPARγ on aromatase expression was studied by treatment of undifferentiated (Fig. 5A) or differentiated (Fig. 5B) A41 cells with PPARγ ligands for 24 h. Stimulation of cells with rosiglitazone reduced aromatase expression in both undifferentiated and differentiated cells. Treatment with the PPARγ antagonists, GW9662, diphenyl phthalate, pyraclostrobin, DEHPA, or kresoxim-methyl, had no effect on aromatase expression in undifferentiated A41 cells. Treatment with the PPARγ agonist 9-cis-retinoic acid (9cRA) affected aromatase expression like rosiglitazone. Treatment of A41 cells with 9cRA caused a decrease in aromatase expression in undifferentiated cells (Fig. 5D) but had no effect on aromatase in mature A41 adipocytes (Fig. 5E).
Fig. 4. Direct interaction between DEHPA and PPARγ. (A) 15N-HSQCs of the PPARγ LBD with added chemical compounds. (B) Peak intensity profile of the rosiglitazone-bound PPARγ LBD compared to free PPARγ LBD in solvent (1% DMSO). (C) Peak intensity profile of the GW9662-bound PPARγ LBD compared to free PPARγ LBD in solvent (1% DMSO). (D) Peak intensity profile of the rosiglitazone-bound PPARγ LBD after GW9662 addition. (E) Peak intensity profile of the rosiglitazone-bound PPARγ LBD compared to rosiglitazone-bound PPARγ LBD after DEHPA addition. (F) Percentage of peaks visible and assignable depending on additives. (G) Cartoon representation of the crystal structure of PPARγ LBD bound to rosiglitazone (PDB: 1FM6). Rosiglitazone is shown as spheres in the binding pocket. Residues that are visible in the 15N-HSQCs when only rosiglitazone is bound are shown in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
It was tested if GW9662 also influenced aromatase expression in human breast adipose tissue explants (Fig. 5F). Aromatase expression was significantly increased in response to 48 h exposure to GW9662. In parallel exposures (n = 2), rosiglitazone stimulation decreased aromatase expression (results not shown since statistical analysis could not be performed), as observed in A41 cells and previously reported for troglitazone in primary adipose stromal cells [7].

To explore how the PPARγ2 protein level affects aromatase expression, PPARG2 was overexpressed in undifferentiated A41 cells in combination with rosiglitazone or solvent treatment (Fig. 5G). Overexpression of PPARG2 or treatment with rosiglitazone reduced aromatase expression (results not shown since statistical analysis could not be performed), as observed in A41 cells and previously reported for troglitazone in primary adipose stromal cells [7].

Differentiated A14 adipocytes were reverse transfected with pcDNA3.1 PPARG2 and treated for 24 h with 5 μM rosiglitazone or solvent (0.02 % DMSO) for 24 h (n = 4). Differentiated A14 adipocytes were reverse transfected with pcDNA3.1 PPARG2 and treated for 24 h with 5 μM rosiglitazone or solvent (n = 3). The graphs present means ± SD. Asterisk (*) indicates statistically significant difference compared to solvent control group using t test or Dunnett’s test (p < 0.05). Hash (#) indicates statistically significant differences compared to control groups (solvent controls or EV controls) using two-way ANOVA (p < 0.05).

3.5. Effect of PPARG Pro12Ala polymorphism

Due to the potential effect of PPARγ Pro12Ala on the response to chemical treatment, the first coding exon of PPARG in the human primary ASCs was genotyped. Two of the study participants were heterozygous for the SNP (Table 1). However, cells from those persons did not appear to respond differently to treatments.

4. Discussion

This study demonstrated that previously identified PPARγ antagonists had an inhibitory effect on human adipocyte differentiation. The impaired adipogenesis resulted in an upregulation of aromatase expression, consistent with aromatase expression being higher in ASCs...
compared to mature adipocytes. In addition, a more acute negative effect of PPARγ activity on aromatase expression was demonstrated in mature adipocytes and adipose tissue explants exposed to environmental PPARγ antagonists.

4.1. PPARγ antagonism and adipogenesis

Adipogenesis in primary human ASCs during exposure to different putative PPARγ antagonists was studied to determine if the environmental chemicals would impact adipocyte differentiation. The non-monotonic concentration-response relationships for some of the PPARγ antagonists indicate that at high concentrations these chemicals likely targeted other pathways related to adipogenesis, abrogating the inhibitory effect on differentiation. Despite this, there was generally a greater inhibitory effect of PPARγ antagonists on adipogenesis at low rosiglitazone concentration (only statistically significant for GW9662, zoxamide, Cosan 528, and DEHPA), indicating competitive binding of antagonists and rosiglitazone to the ligand binding pocket of PPARγ. However, it cannot be excluded that PPARγ-independent mechanisms contributed to adipogenesis inhibition. Interestingly, the data suggest that the mechanisms affecting lipid content may be different across compounds, as some have more pronounced effects on either lipid droplet number or size, while others have effects on both. Overall, the data support previous results in HEK293 cells showing that these PPARγ antagonists inhibit rosiglitazone-induced activity of human PPARγ [9].

Higher aromatase expression in undifferentiated cells compared to mature adipocytes was observed in cells directly isolated from adipose tissue as well as in the A41 and C3H10T1/2 cell lines. Consistent with this, aromatase expression was previously reported to be higher in undifferentiated ASCs than in adipocytes [27,28]. Therefore, blocking adipogenesis by removal of PPARγ-inducing and -activating factors produced a strong elevation in aromatase mRNA. Likewise, inhibition of adipogenesis in response to PPARγ antagonist exposure led to increased expression of aromatase in both human A41 cells and mouse C3H10T1/2 cells, despite previous reports indicating distinct regulatory mechanisms of aromatase expression in mouse and human extragonadal tissues, such as adipose tissue, as a result of differences in the aromatase promoter region [29,30].

These results suggest that impaired adipogenesis in response to PPARγ antagonists may promote breast carcinogenesis by increasing the ratio of ASCs to adipocytes and thereby increasing the tissue expression of aromatase.

4.2. Direct interaction of antagonists with PPARγ LDB

Based on functional similarity to GW9662, DEHPA was selected for analysis by NMR spectroscopy to confirm direct interaction. To explore the interaction with PPARγ, the binding of DEHPA to rosiglitazone-
bound PPAR γ LBD was compared to the binding of GW9662. NMR studies on the isolated PPAR γ LBD have described that activating ligands, such as rosiglitazone, stabilize the LBD in the active state, homogenizing the ensemble of states resulting in more discernable peaks in the NMR spectra [31]. This was also observed here, and especially residues in helix 12 became visible in the rosiglitazone-bound state. Helix 12 is strongly connected to PPAR γ activity, where it is solvent exposed in the active state, while it is buried in the ligand binding pocket in the repressive state [32]. In addition, peaks originating from helix 3, helices 5 to 7, and the loops between them, as well as the C-terminal half of helix 10/11, became resolved. All these areas of PPAR γ LBD outline the ligand binding pocket and would be affected by helix 12 moving in or out. The NMR peak intensity profile of the rosiglitazone-bound PPAR γ LBD in presence of DEHPA lacked peaks corresponding to helix 12 and the structural elements forming the binding pocket. Thus, the NMR data confirmed direct repression of the active LBD state by DEHPA in a similar manner as observed for GW9662. The NMR peak profile looked very similar to the ligand-free or GW9662-bound states, which are both repressive states of PPAR γ LBD. This underscores the hypothesis that the observed aromatase induction follows a direct repression of PPAR γ activity by DEHPA. It would be highly relevant to apply NMR spectroscopy to investigate the interaction between the PPAR γ LBD and chemicals that were predicted to be PPAR γ antagonists in a recently developed PPAR γ antagonist QSAR model [9].

4.3. Short-term regulation of aromatase by PPAR γ

In addition to the effect occurring through inhibition of adipogenesis, a short-term effect of PPAR γ on aromatase expression was demonstrated. Reduced aromatase expression has been observed in response to thiazolidinediones in human ASCs [6,7] and other human cell types such as ovarian granulosa cells [33] and endometrial stromal cells [34]. In the present study, the effect of PPAR γ antagonists was adipocyte-specific, supporting the hypothesis that it is mediated by PPAR/2, as adipocytes express much higher levels of PPAR γ than ASCs. It has been demonstrated that the antagonist GW9662 reverses rosiglitazone-induced aromatase repression in rat granulosa cells [35], consistent with the present results. Treatment of breast adipose explants with GW9662 resembled the effects observed in A41 adipocytes more closely than those in A41 pre-adipocytes. This is in accordance with the high expression level of PPAR γ in adipose tissue as well as a large proportion of the adipose tissue consisting of fully developed adipocytes.

Since PPAR γ is a heterodimeric partner with RXR, the effect of the RXR agonist 9cRA on aromatase expression was expected. Activation of RXR was previously shown to repress aromatase expression or activity, both alone and in combination with PPAR γ activation [33,35–37]. In the current study, treatment with 9cRA had a similar effect as rosiglitazone in undifferentiated cells but had no effect in differentiated cells, suggesting involvement of a PPAR γ-independent mechanism. The combined treatment of ASCs with forskolin and PMA has been shown to induce aromatase expression and activity in human breast ASCs [7,28]. This treatment also resulted in decreased PPAR γ mRNA level, suggesting that forskolin/PMA treatment may exert its effect on aromatase partially through downregulation of PPAR γ. This would also explain the strong repressive effect of troglitazone on forskolin/PMA-induced aromatase expression observed previously [7].

Overexpression of PPAR γ2 resulted in reduced aromatase expression, consistent with a study in which PPAR γ overexpression decreased FSH-induced aromatase expression in KGN cells [38]. Surprisingly, there was no synergy between PPAR γ2 overexpression and rosiglitazone treatment. This could suggest that the effect of rosiglitazone is independent of PPAR γ or, more likely, that the limit of aromatase repression via PPAR γ-dependent pathways was reached. This was supported by the observation that rosiglitazone treatment in PPAR γ-overexpressing A41 adipocytes reduced aromatase expression by less than 50%, suggesting that aromatase transcription cannot be further reduced through alteration of the PPAR γ level or activity. The human H295R adrenocarcinoma cell line has been used previously to study effects of PPAR γ ligands on steroidogenesis [39], however this cell model was not suitable for studying PPAR γ-mediated effects due to the low expression level of PPAR γ, which most likely explains the lack of response to rosiglitazone and GW9662.

4.4. Relevance of the findings to adverse health effects

The influence of the Pro12Ala variant of PPAR γ on the response to PPAR γ antagonists was difficult to assess because of the low number of study participants carrying the minor allele as well as the large biological variation between cells isolated from different persons. In relation to breast cancer, PPAR γ Pro12Ala has been reported to modify the effect of alcohol intake such that only homoyzous major allele carriers were at increased risk of breast cancer when drinking alcohol, while variant allele carriers were not at increased risk [14,15].

Studies of PPAR γ antagonists as endocrine and metabolic disruptors may provide new insights into potential health risks presented by various environmental and occupational exposures. Dysregulation of PPAR γ signaling is connected to various adverse outcomes such as type 2 diabetes, obesity, and cancer [40,41]. This study connects lowered PPAR γ activity with increased aromatase transcription in adipose tissue, and thereby supports that PPAR γ antagonists may act as breast carcinogens. In addition, PPAR γ antagonists inhibit adipocyte differentiation, an effect that may lead to hypertrophy, ectopic fat deposition, local inflammation, and insulin resistance.

CRediT authorship contribution statement

Jacob Ardenkjær-Skinnerup: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. Daniel Saar: Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. Patricia S.S. Petersen: Investigation, Writing – review & editing. Mikael Pedersen: Methodology, Resources, Writing – review & editing. Terje Svingen: Methodology, Resources, Writing – review & editing. Birthe B. Krage-Lund: Formal analysis, Methodology, Resources, Supervision, Writing – review & editing. Niels Hadrup: Conceptualization, Supervision, Writing – review & editing. Gitte Ravn-Haren: Conceptualization, Supervision, Writing – review & editing. Brice Emanuelli: Methodology, Resources, Supervision, Writing – review & editing. Kristy A. Brown: Investigation, Methodology, Resources, Supervision, Writing – review & editing. Ulla Vogel: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.
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