Chemical Research in Toxicology

Article

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Effects of Diisodecyl Phthalate on PPAR:RXR-Dependent Gene Expression Pathways in Sea Bream Hepatocytes

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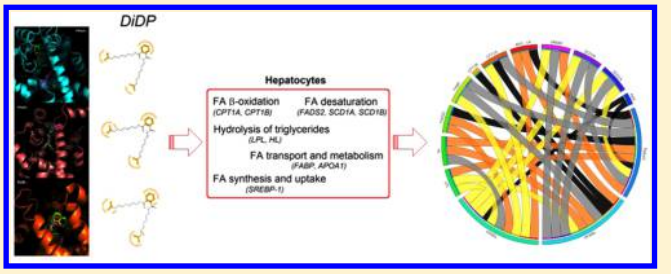
Supporting Information

ABSTRACT: Evidence that endocrine-disrupting chemicals (EDCs) may target metabolic disturbances, beyond interference with the functions of the endocrine systems has recently accumulated. Among EDCs, phthalate plasticizers like the diisodecyl phthalate (DiDP) are commonly found contaminants of aquatic environments and have been suggested to function as obesogens by activating peroxisome proliferator activated receptors (PPARs), a subset of nuclear receptors (NRs) that act as metabolic sensors, playing pivotal roles in lipid homeostasis. However, little is known about the modulation of PPAR signaling pathways by DiDP in fish. In this study, we have first investigated the ligand binding efficiency of DiDP to the ligand binding domains of PPARs and retinoid-X-receptor-α (RXRα) proteins in fish using a molecular docking approach. Furthermore, *in silico* predictions were integrated by *in vitro* experiments to show possible dose-relationship effects of DiDP on PPAR:RXR-dependent gene expression pathways using sea bream hepatocytes. We observed that DiDP shows high binding efficiency with piscine PPARs demonstrating a greater preference for RXRα. Our studies also demonstrated the coordinate increased expression of PPARs and RXRα, as well as their downstream target genes *in vitro*. Principal component analysis (PCA) showed the strength of relationship between transcription of most genes involved in fatty acid metabolism and PPAR mRNA levels. In particular, fatty acid binding protein (FABP) was highly correlated to all PPARs. The results of this study suggest that DiDP can be considered an environmental stressor that activates PPAR:RXR signaling to promote long-term changes in lipid homeostasis leading to potential deleterious physiological consequences in teleost fish.



INTRODUCTION

Evidence that endocrine-disrupting chemicals (EDCs) may target metabolic disturbances, beyond interference with the functions of the endocrine systems[1] has recently accumulated. To this regard, Grün and Blumberg[2] highlighted the involvement of the so-called environmental obesogens in misregulation of lipid metabolism and adipogenesis. EDCs are able to exert genomic effects by interacting with a wide variety of nuclear receptors (NRs) including peroxisome proliferator activated receptors (PPARs), a subset of NRs that act as metabolic sensors, playing pivotal roles in lipid homeostasis.[3] It has been demonstrated that binding of natural ligands (e.g., polyunsaturated fatty acids: PUFAs), as well as synthetic ligands (e.g., pharmaceutical and industrial compounds) can activate the PPARs.[4] Following ligand activation, PPARs heterodimerize with retinoid-X-receptor-α (RXRα), resulting in the recruitment of coactivators and loss of corepressors and modulating the expression of target genes.[5]

The research on the relationships among pollutants, drugs, obesogenicity, and PPARs have mostly focused on the activation of PPARγ isoforms for mediating obesogenic effects, particularly with organotins and phthalates.[6−9] In addition, *in vivo* and *in vitro* experiments have shown the obesogenic potential of PPARγ agonists such as rosiglitazone and pioglitazone that belong to a class of oral hypoglycemic drugs: the thiazolidinediones (TZDs).[10,11] On the contrary, fibrates are commonly identified as a class of drugs that exert their cholesterol-lowering effects by activating both PPARβ/δ and PPARα. Interestingly, it has been shown that bezafibrate (BZF) was a pan-PPAR activator, resulting in strong PPARα, β/δ, and weak PPARγ activation.[12] Among EDCs, phthalates are widely used as plasticizers and have been suggested to function as obesogens by activating both PPARα and PPARγ, and thus resulting in adipocyte differentiation and insulin sensitization.[9] Various studies have reported that phthalates, in particular di(2-ethylhexyl) phthalate (DEHP) and its metabolite mono (2-ethylhexyl) phthalate (MEHP), are able to

Received: December 22, 2014

DOI: 10.1021/tx500529x
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

produce toxic effects in the liver and reproductive organs of mammals by activating PPARs which in turn bind to peroxisome proliferator response elements (PPREs) located within the regulatory domains of target genes.[13] Interestingly, PPRE sequences can be found in both human and fish PPARα suggesting the existence of autoregulatory loop mechanisms. The potential for reproductive and developmental toxicity of phthalate esters (PEs) has also been investigated in fish focusing mainly on the interaction with estrogen receptors.[14,15] In addition, monoester phthalates such as monobenzyl phthalate and mono-(1-methyl)-hexyl phthalate were found to induce the transcription of piscine PPARα and PPARβ.[16] Recently, DEHP was listed under EU REACH regulation, as a substance of very high concern (SVHC) and currently substituted with C10 phthalates such as the diisodecyl phthalate (DiDP).[17] DiDP is defined as a high molecular weight general-purpose plasticizer widely used for manufacturing polyvinyl chloride (PVC) products. Exposure to DiDP is widespread as clearly demonstrated by human biomonitoring data based on the identification of phthalate metabolites in biological fluids.[18]

It is known that DiDP produced responses for peroxisome proliferation parameters.[19] In fact, recent molecular docking analyses demonstrated that DiDP efficiently binds to human PPAR and RXR subtypes showing high affinity values for human PPARγ,[20] but little is known about the binding affinity of DiDP to fish PPARs. It should also be noted that affinities of the ligands to PPAR isotypes may vary considerably among species, mainly due to differences in the ligand binding domain.[21] Despite this, there is only limited information available on the modulation of PPAR signaling pathways by DiDP in aquatic species.

Thus, the present study was designed first to investigate the ligand binding efficiency of DiDP to the ligand binding domains of PPARα, PPARγ, and RXRα proteins in fish, using a molecular docking approach. Second, *in silico* predictions were then complemented by *in vitro* experiments to show possible dose-relationship effects of DiDP on PPAR:RXR-dependent gene expression pathways using sea bream hepatocytes. In environmental toxicology research, fish cell cultures can be considered a valuable tool that is useful for studying toxic mechanisms allowing for subtle control of the experimental environment without the complex physiological conditions of *in vivo* approaches. In the present study, the mRNA transcription was analyzed for the three PPAR isoforms (α, β, and γ), RXRα, and 10 genes involved in various pathways of fatty acid (FA) metabolism that is controlled through the PPARs.

EXPERIMENTAL PROCEDURES

Caution: *This study involves chemicals that are hazardous to the aquatic environment and should be handled with care.*

Chemicals. DiDP and 3-aminobenzoic acid ethyl ester (MS-222) were purchased from Sigma (St. Louis, MO). Cell culture medium and serum [Leibovitz L-15 phenol red-free medium and fetal bovine serum (FBS)] were purchased from Life Technologies (Carlsbad, CA, USA).

Molecular Docking. The evaluation of the geometry and the strength of binding of a ligand/receptor complex is the main purpose of molecular docking. Owing to the lack of structural information on the three nuclear receptors taken into account (PPARα and γ from *Sparus aurata* and RXRα from *Danio rerio*), a homology modeling structure prediction was performed. The crystallographic structures of the corresponding receptors from humans (1I7G, 1I7I, and 3DZY, respectively)[22−24] were chosen as templates on the basis of their sequence identity with fish receptors (higher than 65%). Swiss PDB viewer (version 4.1) and Swiss-Model server[25] were used to preliminarily build the project files, modeling loops, and side chain, and to optimize and validate the models, respectively. All queried fish receptor sequences were obtained from UniProt Knowledge-base (http://beta.uniprot.org/). The obtained receptor models and the structures of BZF, diisodecyl phthalate (DiDP), and nafenopin molecules, built and minimized with Avogadro software (version 1.1.0. http://avogadro.openmolecules.net/),[26] were converted in the pdbqt format files and used in the docking procedure performed with Autodock Vina software (version 1.1.2)[27] on an Intel Core i7/Mac OS X 10.9-based platform. The docking zone was specifically set around the oxysterols-binding site with a dimension of 26 × 27 × 25 Å^3 considering also that no specific binding was shown outside this area. The resulting binding affinities, expressed as equilibrium dissociation constants, were obtained by the following formula calculated from the free binding energies:

$$K_d = e^{\Delta G_{\text{bind}} 1000/RT}$$

The final complexes were analyzed with LigandScout software (version 3.12)[28] to obtain the intermolecular interaction features and rendered by Mac PyMOL software (Python Molecular Graphics, version 1.3).

Experimental Animals and Hepatocyte Isolation. Gilthead sea bream juveniles *S. aurata* (15.5 ± 2.9 g initial weight) were provided by the hatchery of the Orbetello Pesca Lagunare srl., Orbetello (GR, Italy). Fish were acclimated for 2 weeks in 2.80 m × 1.00 m × 0.50 m tanks with constant aeration and natural photoperiod at Unità di Ricerca e Didattica of San Benedetto del Tronto (URDIS), University of Camerino in San Benedetto del Tronto (AP, Italy). Water quality parameters were monitored every 2 days, showing the following values: pH 7.9, O_2 = 5.5−7 ppm, and temperature = 21−23 °C, salinity 34 ± 1 ppt; the level of nitrites (NO^{2-}) and ammonia (NH_3) were undetectable. Fish were fed a commercial diet once a day during the acclimation period (Tetra Werke, Germany). Following the acclimation, fish were randomly anaesthetized using MS-222 (0.1 g L^{-1}) within 5 min after capture and sacrificed by decapitation. The liver tissue was aseptically harvested to obtain hepatocytes under a laminar flow hood, according to Centoducati et al.,[29] with slight modifications. Briefly, livers were carefully excised and rinsed with cold PBS plus antibiotic−antimycotic solution (100 U/mL) to remove blood cells. Livers were then pooled and digested after 20 min of incubation at 20 °C with PBS supplemented with a cocktail of four enzymes (0.1% collagenase type IV, 0.05% hyaluronidase type IV−S, 0.4% dispase type II, and 0.03% DNase type I). The digestion was blocked by adding Leibovitz L-15 phenol red-free medium (Life Technologies), prepared following the manufacturer's instructions, and supplemented with 10% FBS. The resulting cell suspension was filtered throughout a 40 μm nylon sieve and washed twice by centrifugation in cold PBS at 100 g at 4 °C for 5 min. Hepatocytes were purified on a Percoll gradient (90%−50%, 2:1, v/v) in PBS (10×) by centrifugation at 150*g* for 10 min at 4 °C. The hepatocytes layer was then isolated and further washed in cold PBS through two further centrifugations at 100*g* at 4 °C for 5 min. Following the isolation phases, purified hepatocytes were suspended in Leibovitz L-15 phenol red-free medium supplemented with 10% FBS, antibiotic−antimycotic solution (100 U/mL) and 10 mM HEPES. The cell density was estimated in a counting Burker Chamber, and the viability of hepatocytes used for experiments was always over 90%, as assessed with the Trypan blue exclusion assay.[30]

Primary Cell Culture and Exposure. Cells were seeded on 24-well Falcon Primaria culture plates (1 × 10^6 cells per well) in Leibovitz L-15 phenol red-free medium supplemented with 10% FBS, antibiotic-antimycotic solution (100 U/mL) and 10 mM HEPES. Cells were cultured for 24 h in an incubator (3% CO_2) at 23 °C before chemical exposure to allow attachment. Then, 50% of the L-15 phenol red-free medium culture medium was removed, and hepatocytes were exposed to medium containing the vehicle (ethanol, final concentration 0.01%) and 0.1, 1.0, or 10 μM of DiDP. Hepatocytes were incubated in an incubator (3% CO_2) at 23 °C for 48 h. After 24 h of culture, 90% of the medium was removed and replaced with fresh appropriate

DOI: 10.1021/tx500529x
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Table 1. Primer List

gene	primer sequence (5′-3′)	Genebank	ref
PPARα	GCAGCCTGTGAGTCTTGTGAGTGA	AY590299	Fernández et al.[35]
	CTCCATCAGGTCTCCACACAGC		
PPARβ	CGTGTTCGGGATTCGGGACT	AY590301	Fernández et al.[35]
	CACCCTGTCGTGCTGCTCTGTA		
PPARγ	CGGAGAGAGAAGCAAGAACAAGAA	AY590304	Fernández et al.[35]
	GAGGAGGAGGAGATGGAGGTGTA		
RXRα	GGGCTTCTTCAAGAGGACAGT	HS092100	Ribecco et al.[36]
	TGCACCGCTTCTCTCTTCAT		
LXRα	GCACTTCGCCTCCAGGACAAG	FJ502320	Benedito-Palos et al.[37]
	CAGTCTTCACACAGCCACATCAGG		
CPT1A	GTGCCTTCGTTCGTTCCATGATC	JQ308822	Pérez-Sánchez et al.[38]
	TGATGCTTATCTGCTGCCTGTTTG		
CPT1B	CAAGCCCCGACACAGACTCATACC	DQ866821	Boukouvala et al.[39]
	CCCATTTCCCAGCTGCGTTATTTT		
LPL	CGTTGCCAAGTTTGTGACCTG	AY495672	Benedito-Palos et al.[37]
	AGGGTGTTCTGGTTGTCTGC		
FADS2	GCAGGCGGAGAGCGACGGTCTGTTCC	AY055749	Benedito-Palos et al.[37]
	AGCAGGATGTGACCCAGGTGGAGGCAGAA		
SREBP-1	AGGGCTGACCACAACGTCTCCTCTCC	JQ277709	Benedito-Palos et al.[37]
	GCTGTACGTGGGATGTGATGGTTTGGG		
FABP	AAATGGTTGAGGCTTTCTGTGCTAC	HQ228170	Varó et al.[40]
	ATCGCTACTGTCGGCTTGGTG		
SCD1A	CGGAGGCGGAGGCGTTGGAGAAGAAG	JQ277703	Benedito-Palos et al.[37]
	AGGGAGACGGCGTACAGGGCACCTATATG		
SCD1B	GCTCAATCTCACCACCGCCTTCATAG	JQ277704	Benedito-Palos et al.[37]
	GCTGCCGTCGCCCGTTCTCTG		
APOA-I	GAATACAAGGAGCAGATGAAGCAGATG	AF013120	Varó et al.[40]
	TGGTGACGGAGGCAGCGATG		
18s	GCATTTATCAGACCCAAAACC	AY993930	Pérez-Sánchez et al.[41]
	AGTTGATAGGGCAGACATTCG		

medium. Concentrations of DiDP were chosen on the basis of molecular docking analysis and taking into account the doses of obesogens that are known to activate PPAR in the regulation of glucose and FA metabolism.[31−33] Exposure of primary sea bream hepatocytes was performed using 24-well plates, and six independent wells were setup for both the control and each concentration of DiDP. The entire experiment was repeated 3 times. After the end of exposure, all cell layers remained attached to the bottom of the plates. At this point, cell viability was again assessed by microscopic examination of the cell morphology and the Trypan blue exclusion test.

Quantitative Realtime PCR (q-PCR). After exposure, the medium was carefully removed, and cells were lysed by adding the TRIzol reagent (Invitrogen Life Technologies, Milan, Italy). Total RNA was isolated according to the manufacturer's specifications. DNase digestion (2 U, 30 min, 37 °C; Ambion, Austin, TX) was performed to eliminate genomic DNA contamination. RNA concentration and purity were assessed spectrophotometrically at an absorbance of 260/280 nm, and the integrity was confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide. The cDNA was synthesized from 1 μg of total RNA in 20 μL of total volume reaction using random hexamers (50 ng μL^{-1}) and 200 U of SuperScript III RT according to manufacturer's instructions (Invitrogen Life Technologies, Milan, Italy). SYBR green-based real-time PCR was used to evaluate expression profiles of PPARα, PPARβ, PPARγ, RXRα, carnitine palmitoyltransferase 1A-1B (CPT1A, CPT1B), lipoprotein lipase (LPL), hepatic lipase (HL), fatty acid desaturase 2 (FADS2), sterol regulatory element-binding protein 1 (SREBP-1), fatty acid binding protein (FABP), stearoyl-CoA desaturase 1A-1B (SCD1A, SCD1B), and apolipoprotein A-I (APOA-I) target genes. Analysis of the 18s rRNA gene expression confirmed that its expression was unaffected by exposure to phtalates (Table S1, Supporting Information), and thus, it is considered to be an appropriate reference gene for the qPCR analysis.[34] All of the primer sequences are reported in Table 1.[35−41]

The reaction included 12.5 μL of 2 × qSTAR SYBR Master Mix Kit (OriGene Technologies), 1 μL each of forward and reverse primers (both 10 μmol/L), 0.5 μL of cDNA template, and sterile distilled water to a final volume of 20 μL. The expression of individual gene targets was analyzed using the Mx3000P Real-time PCR system (Stratagene, La Jolla, CA, USA). Thermo-cycling for all reactions was for 10 min at 95 °C, followed by 40 cycle of 15 s at 95 °C, and 30 s at 59 °C. Fluorescence was monitored at the end of every cycle. Melting curve analysis demonstrated that a single peak was generated during the reaction indicating the production of a single product. Results were calculated using the relative $2^{-\Delta\Delta Ct}$ method[42] and expressed as normalized fold expression corrected for 18s rRNA and with respect to control levels. Values are the mean ± SD of three independent experiments.

Western Blotting Analysis. Total protein was extracted following the modified TRIzol protocol described in Simoes et al.[43] Total protein concentrations in the sample were determined according to the Bradford method[44] using bovine serum albumin (BSA) as standard. Western blotting was performed according to the standard protocol[45] before blotting as previously described.[46] Protein samples (10 μg) was separated using 12% separating SDS Mini-PROTEAN TGX precast PAGE (Bio-Rad Laboratories) electrophoresis. The gel was blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were washed in tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) and blocked for 30 min at room temperature with 5% nonfat milk powder dissolved in TTBS. A polyclonal PPAR antibody from Sigma-Aldrich that was produced using the C-terminal amino acid sequence IKK-TET-DMS-LHP-LLQ (amino acid sequence 484−498, molecular weight of 59 kDa) that is common to the mouse and rat PPARγ was used for protein detection.

DOI: 10.1021/tx500529x
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Using the amino acid sequence 484−498, we performed an alignment against *S. aurata* PPARs amino acid sequence, showing that the peptide is identical to the PPAR isoforms. Furthermore, the PPAR antibody used in this study has previously been shown to cross-react successfully with Atlantic salmon PPAR proteins.[47] Incubation with the diluted primary PPAR antibody (1:2000 in TTBS containing 5% nonfat milk) was performed overnight at 4 °C. After washing with TTBS, membranes were incubated with peroxidase conjugated goat antirabbit antibodies (GAR-HRP; Bio-Rad) diluted 1:3000 in TTBS containing 5% nonfat milk. The membrane was developed using an Immun-Star WesternC Chemiluminescent Kit (Bio-Rad) and visualized with Eastman KODAK Company's Molecular Imaging Systems (Rochester, NY, USA). Densitometric analysis was performed using ImageJ software for Windows.

Statistical Analysis. All statistical analyses were performed using R.[48] Data were first examined for their fit to a normal distribution and homogeneity of variance using Shapiro-Wilks and Levene median tests. A one-way analysis of variance (ANOVA) was used to compare results among exposure groups, followed by the Tukey post hoc test. Differences between means were considered significant when $P < 0.05$. For protein analysis, the Western blotting technique was mainly used for qualitative purposes to obtain a visual evaluation of protein expression after exposure to test compound.

Principal components analysis (PCA)[49] was employed to study the intercorrelations of the variables (gene expression data) by clustering them into common factors. The variables within each factor are highly correlated, and each factor explains a different part of the total variance in the data. Correlations between each PPAR and the various target genes are presented graphically using CIRCOS software.[50] Since the interpretation of principal components is not as clear-cut as that with other linear functions techniques,[49] a linear discriminant analysis (LDA) with a leave one out cross-validation (LOCV) evaluation function was used together with a stepwise subset selection procedure to perform a data-directed search of a subset of variables (i.e., genes) with a high discriminant power in terms of exposure groups.[49]

RESULTS AND DISCUSSION

Interaction of Fish Nuclear Receptors with DiDP. It is known that PPAR ligand binding domains exhibit promiscuity by binding to a variety of natural and synthetic ligands.[51] In addition, most of these ligands (e.g., BZF) can work as pan-agonists resulting in the activation of all three PPAR isoforms.[52] In the present study, the complexes between DiDP and a set of fish nuclear receptors were predicted and analyzed considering the geometry of binding and the equilibrium dissociation constants (calculated from free energy of binding), and compared with the complexes between the same receptors and two of their established ligands, namely, BZF and nafenopin. DiDP shows equilibrium dissociation constants in the range 20−0.2 μM with these nuclear receptors (Table 2), particularly with RXRα reporting the highest affinity ($K_d = 9.67 \times 10^{-07}$). These values predict the existence of specific interactions. According to the proposed models, the complexes are mainly stabilized by hydrophobic interaction between DiDP and the receptors, with a non-negligible contribution of a theoretical H-bond with Thr289 of PPARα. Sarath Josh et al.[20] demonstrated that diphthalates like DiDP show PPAR-binding efficiencies directly proportional to their chain length. This finding clearly suggests that the strength of interaction between PPARs and diphthalates is generally higher than that between PPARs and monophthalates. In addition, it has been shown that lipophilicity of the ligands may be related to the activation of receptors.[53]

Table 2. Predicted Equilibrium Dissociation Constants of the Ligand/Fish Nuclear Receptor Complexes

name	PPARα/K_d (M)	PPARγ/K_d (M)	RXRα/K_d (M)
DiDP	3.15×10^{-06}	1.71×10^{-05}	9.67×10^{-07}
BZF	1.78×10^{-07}	1.03×10^{-05}	6.89×10^{-07}
nafenopin	8.17×10^{-07}	2.66×10^{-06}	4.92×10^{-07}

In comparison with BZF and nafenopin, DiDP exhibits both similar positioning within the binding site of the receptors but nearly 10-fold higher equilibrium dissociation constants (Table 2). In fact, these two ligands can be further stabilized by additional H-bonds and aromatic−aromatic ring (or π-π) interactions (Figures 1 and 2) with the predicted affinities in the nanomolar range (Table 2). In this regard, several synthetic compounds (e.g., hypolipidemic agents WY-14,643 and clofibrate, PE plasticizers, herbicides, and a murine PPARα agonist, GW9578) have been reported to be specific PPARα activators.[51] Our results further demonstrate that DIDP and BZF showed similar K_d values for PPARγ. In accordance with this result, DiDP has previously been shown to have a higher G-score (9.99) than BZF (8.25) with hPPARγ.[20] Clinically, it has been shown that selective PPARγ agonists can cause water retention and weight gain.[54]

The DiDP preference for fish RXRα than PPARα/γ is another interesting result from the present study. This finding is not surprising because previous studies have demonstrated that other EDCs with putative obesogenic properties such as tributyltin (TBT),[6] bisphenol A (BPA), and DEHP[57] can activate the PPAR−RXR heterodimeric complex primarily through their interaction with RXR.[55,20] Overall, the potential binding of DiDP to each partner (i.e., PPAR and RXR) of the heterodimer can result in deregulated PPAR-RXR signaling pathways, which have been already observed following DEHP exposure.[56]

Gene Expression Profile of DiDP-Treated Sea Bream Hepatocytes. To validate key *in silico* predictions about the interaction of fish PPARs with DiDP, we performed gene expression studies using a sea bream hepatocyte *in vitro* model. Relative expression levels of PPARs (α, β, and γ) and their obligate heterodimeric partner RXRα are presented in Figure 3a. The mRNA expression of all genes examined increased significantly at low DiDP concentrations (0.1 to 1 μM), and this inductive effect diminished at 10 μM concentration; the concentration−response curve shows a monotonic, nonlinear response with the most efficacious concentration at the nanomolar range (Figure 3a).

It was previously demonstrated that PPAR ligands can regulate the transcription of their own receptors; both FA and agonist drugs (e.g., BZF) have been found to up-regulate PPARα mRNA in different cell lines.[58,59] However, Campioli et al.[33] showed a decrease in PPAR-γ transcript levels following MEHP treatment. In zebrafish hepatocytes, expression of PPAR subtypes was significantly induced by both DEHP and phthalic acid demonstrating that the PPAR subtype γ was less responsive to these treatments.[60] In accordance with our results, the highest increase in PPAR mRNA levels was observed with DEHP at 0.1 μM concentration. In addition, Ren et al.[61] have demonstrated that RXR expression can be directly modulated by DEHP exposure. It documented that the PPAR/RXR heterodimer binds to PPAR-responsive elements (PPREs) in target DNA[62] and that the PPRE sequence has also been reported in human PPARα.[63] In this regard, we previously showed that at least one putative PPRE sequence was identified as a direct repeat 1 (DR-1) element in PPARα and RXRα

DOI: 10.1021/tx500529x
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

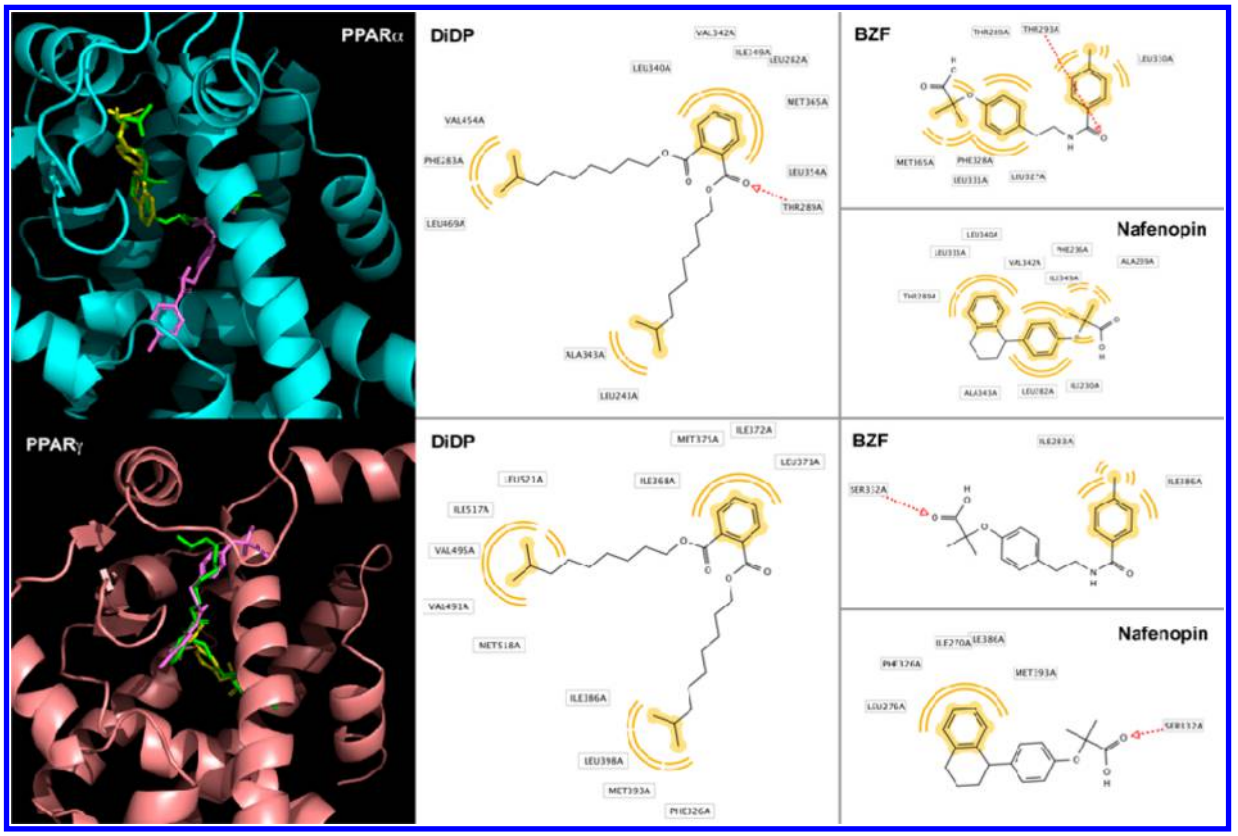

Figure 1. 3D and 2D predicted models of the complexes between each ligand and PPARα and PPARγ, obtained by molecular docking. In the 3D representations, DiDP, BZF and nafenopin are rendered as solid green, violet, and yellow stick, respectively. Predicted hydrophobic interactions (yellow areas) and H-bonds (red dotted lines) are shown in the 2D schemes.

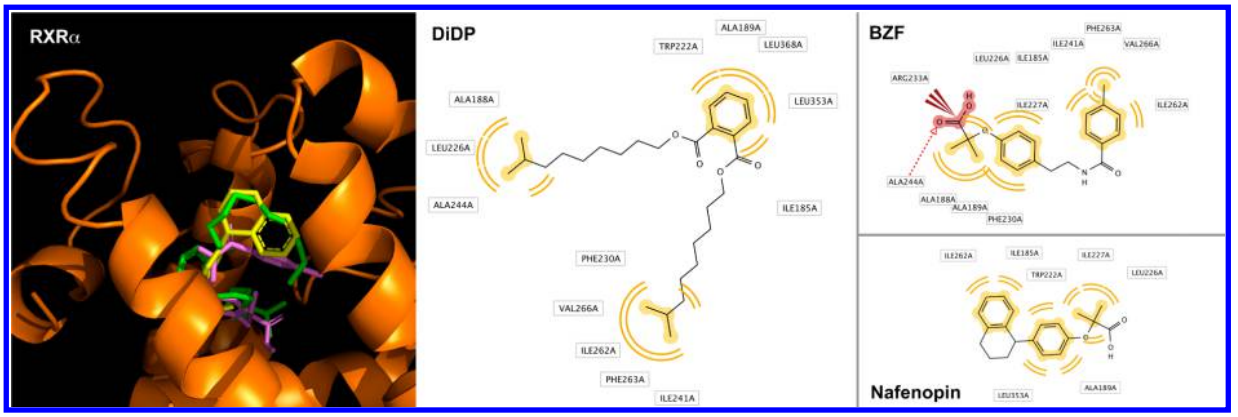

Figure 2. 3D and 2D predicted models of the complexes between each ligand and RXRα, obtained by molecular docking. In the 3D representations, DiDP, BZF, and nafenopin are rendered as solid green, violet, and yellow stick, respectively. Predicted hydrophobic interactions (yellow areas), H-bonds (red dotted lines), π-π or aromatic−aromatic ring interactions (blue circle and bold arrows), and negative ionizable areas (dark red areas) are shown in the 2D schemes.

sequences of different teleost species including *S. aurata*.[64] Overall, these findings suggest potential autoregulation of PPARα and RXRα expression.

Western Blotting Analysis of PPAR Protein in *S. aurata* Hepatocytes. In parallel to transcriptomic analysis, we investigated the expression of PPAR at the protein level. The effect of DiDP on PPAR isotype mRNAs and protein expression patterns did not change in parallel. In fact, Western blotting analysis revealed a different PPAR pattern induced by the different DiDP doses. In particular, the DiDP10 dose caused a clear decrease in PPAR (50 kDa) protein expression with respect to the control group (Figure 3b,c). In a recent study, PPARα protein expression was increased in DEHP-treated rat cardiomyocytes suggesting the induction of PPAR-mediated metabolic remodeling processes.[65] However, differences in PPAR mRNA and protein expression after DiDP exposure may have several explanations. For example, this finding could be due to a modulation of genes involved in protein folding or protein degradation. In this regard, Ismail and Nawaz[66] demonstrated that there is a functional link between transcriptional activation of nuclear receptors and their protein degradation by the ubiquitin−proteasome pathway. In fact, several studies suggest that many downstream events, including phosphorylations and acetylation, are critical for

DOI: 10.1021/tx500529x
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

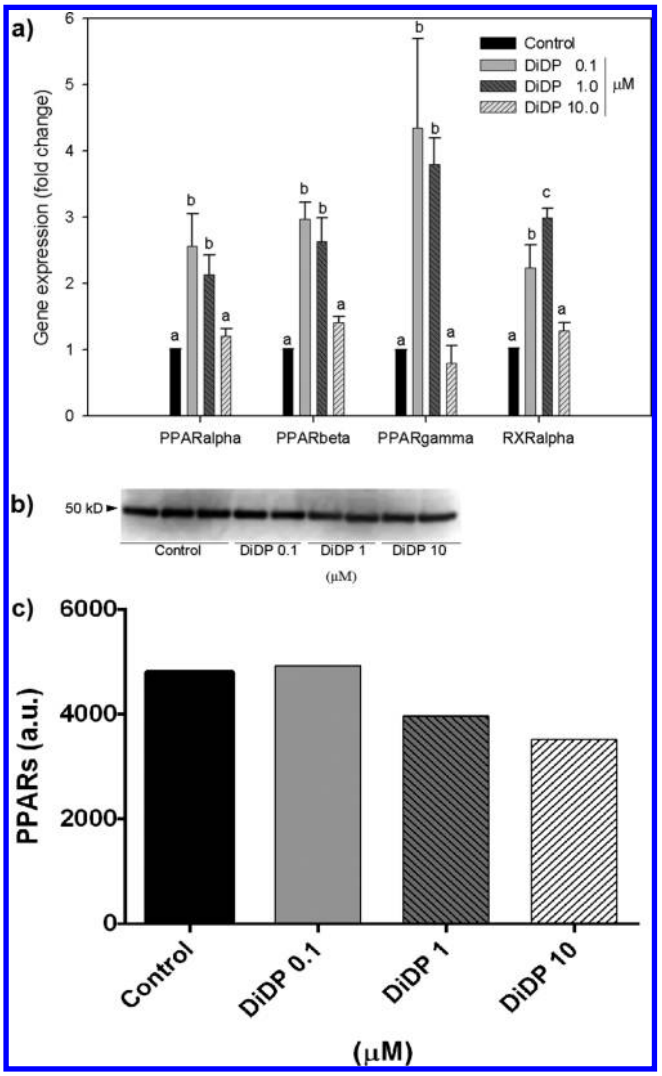

Figure 3. Peroxisome proliferator-activated receptor (PPARα, PPARβ, and PPARγ) mRNA (a) and 50 kDa PPAR protein (b,c) levels in *Sparus aurata* hepatocytes exposed to different doses (μM) of DiDP. q-PCR results are expressed as normalized fold expression corrected for 18s rRNA and with respect to control levels. Values are the mean ± SD of three independent experiments. Different letters indicate significant differences among the groups (Tukey, $p < 0.05$). Western blot results are in arbitrary units (a.u.).

nuclear receptor degradation after its activation following exposure to EDCs.[67,68] Many endogenous pathways are activated to restore cellular homeostasis following chemical exposure, including the removal of damaged or excess proteins through proteolysis. In a recent study, the expression of genes involved in proteome maintenance (e.g., chaperonins and ubiquitin degradation pathway) were induced after exposure to higher concentrations of 7 different peroxisome proliferator chemicals, including WY-14,643, clofibrate, fenofibrate, valproic acid, DEHP, perfluorooctanoic acid, and perfluorooctanesulfonate.[61]

Expression of FA Metabolism and Regulation-Related Genes after DiDP Treatment. The PPARs are involved in the positive regulation of target gene expression and pathways associated with metabolic status. Thus, exogenous compounds (e.g., phthalates)[69] that are able to activate PPARs may affect metabolic homeostasis.[70] Herein, we demonstrated that genes involved in multiple parts of the FA pathway were up-regulated by low concentrations (0.1 to 1 μM) of DiDP (Figure 4). These genes included FA β-oxidation (CPT1A and CPT1B), FA desaturation (FADS2, SCD1A, and SCD1B), hydrolysis of triglycerides and phospholipids (LPL and HL), FA transport and metabolism (FABP and APOA-I), and FA synthesis and uptake (SREBP-1). Conversely, the expression of each gene examined was not modulated by the highest concentration (10 μM) of DiDP.

In our study, low concentrations of DIDP produced an increase in FADS2, SCD1A, and SCD1B expression after 48 h of exposure. SCD enzymes are considered ubiquitously expressed in all living organisms including fish, where mRNA expression levels of these key regulatory enzymes is strictly regulated by ration size.[37] In teleost species, duplicate copies of SCD1-type (SCD1a and SCD1b) were identified as a result of the fish specific genome duplication.[71] Two SCD1 isoforms were also found in the liver of common carp.[72] Overall, regulation of SCD gene expression is under the control of multiple influences such as dietary, thermal, and hormonal treatment.[73] In this regard, the mRNA expression of SCD1 was significantly increased in the liver of mice exposed to DEHP and Aroclor 1254.[74] Recently, SCD expression has been proposed as molecular markers of lipogenesis in both humans and rodents suggesting that low SCD mRNA levels can be associated with a reduced risk of developing insulin resistance and metabolic syndrome.[75,76]

An additional level in the regulation of tissue FA composition is related to the role of endogenous extracellular triacylglycerol lipase (TAGL), which acts in the metabolism and transport of lipids. Different lipase expression patterns have been found in fish tissues suggesting that HL is more expressed in the liver, while LPL appears to be ubiquitously expressed.[77] Both enzymes drive the allocation patterns of body fat stores in fish and show distinct regulation mechanisms.[78] Relevant to this, LPL activity and mRNA levels were down-regulated in the adipose tissue during fasting.[79,80] In addition, recent studies have demonstrated the ability of phthalates to modulate hepatic LPL expression resulting in the stimulation of FA uptake and TAGL synthesis.[81] In the same study, significantly increased levels of LPL mRNA were observed following exposure to troglitazone, a well-known PPARγ agonist. Interestingly, troglitazone was also able to upregulate the expression level of Srebf1 mRNA, which is involved in regulating adipogenic gene trascription and generating endogenous PPARγ ligands.[82,83] In contrast, exposure to a high dose of dibutyl phthalate (DBP) decreased SREBP2 expression in rat fetal Leydig cells leading to reduction in the activity of several lipid metabolic pathways.[84] However, this DBP-induced reduction of lipid metabolism gene expression was found to be species-specific since the same genes were induced after DBP exposure in a mouse model.[84] Previous studies have localized a DR1element in the human promoter of SREBP1c suggesting the direct involvement of PPARα in transcriptional regulation of SREBP1c.[85] Furthermore, conserved regulatory elements for PPARα and SREBP1 have also been reported in the promoter region of SCD enzymes.[73]

In the present study, APOA-I expression was increased after DiDP exposure. APOA-I is known to be one of the most abundant components of high-density lipoprotein (HDL) and participates in reverse cholesterol transport. PPARα agonists increase hepatic mRNA expression of APOA-I in humans by interacting with functional PPREs identified in the promoter of the human gene.[86,87] Conversely, APOA-I mRNA is down-

DOI: 10.1021/tx500529x
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

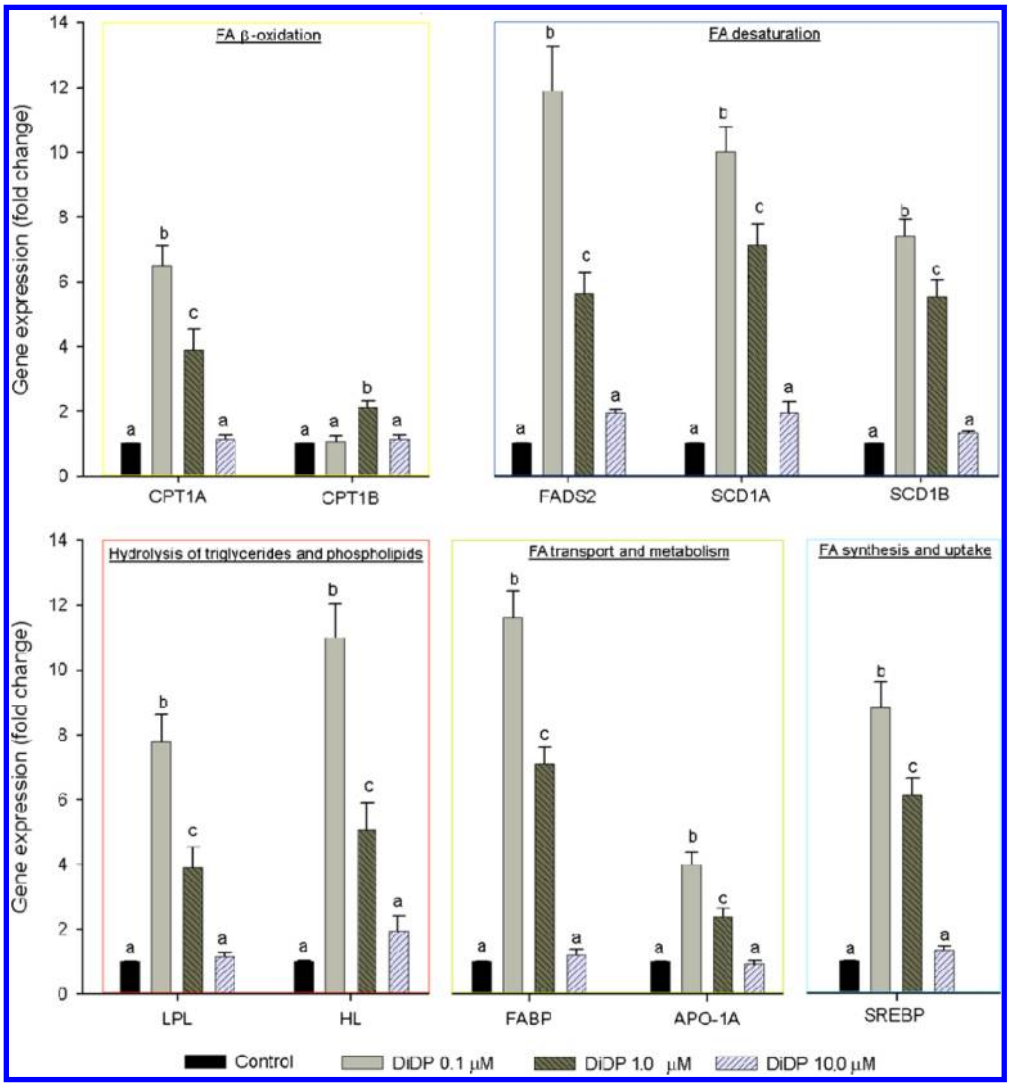

Figure 4. Transcription level of genes involved in FA β-oxidation (CPT1A and CPT1B), FA desaturation (FADS2, SCD1A, and SCD1B), hydrolysis of triglycerides and phospholipids (LPL and HL), FA transport and metabolism (FABP and APOA-I), and FA synthesis and uptake (SREBP-1) in *Sparus aurata* hepatocytes exposed to various concentrations (0.1, 1, and 10 μM) of DiDP. Results are expressed as normalized fold expression corrected for 18s rRNA and with respect to control levels. Values are the mean ± SD of three independent experiments. Different letters indicate significant differences among the groups (Tukey, $p < 0.05$).

regulated by PPARα agonists in rodents through an indirect pathway involving the transcription factor REV-ERBα.[88] It has been reported that polychlorinated biphenyls (PCB) induced coordinated increases in APOA-I gene expression and cholesterol synthesis in the liver of rats resulting in hypercholesterolemia.[89]

The FABP is another gene that increased after DiDP treatment of sea bream hepatocytes. FABP is a member of a family of proteins involved in the regulation of mechanisms related to lipid transport and storage.[90] In fish, FABPs are also associated with stress response.[91] In fact, increased expression of liver FABP was observed in fish subjected to handling and crowding procedures.[92] This finding is not surprising given that FABP may mediate lipid mobilization for coping with the increased energetic requirements due to stress. Recently, it has been reported that FABP can directly interact with PPARα in cultured primary hepatocytes.[93] Studies indicate that FABPs act as a shuttle for both FA and lipophilic xenobiotics to the nucleus resulting in the delivery of activator ligands directly to PPARα.[94,95] Velkov[96] found that liver FABP is able to bind PPAR subtype selective agonists with high affinity and to activate a channeling mechanism facilitating the induction of PPAR transcriptional activity. Taken together, these studies demonstrate high affinity and structural molecular interaction between FABP and PPARα suggesting a potential role of the former in regulating PPARα signaling pathways. In this context, our results showed that exposure to low DiDP concentration may activate the functional interaction between FABP and PPARα resulting in the modulation of FA metabolism and xenobiotic responses.

PPARs can be considered key mediators in FA β-oxidation processes during which the uptake of long-chain FA to the mitochondrion is mediated by the CPT system.[97] Relevant to this, it has been demonstrated that CPT1 mRNA levels are increased by PPARs in mammals.[98] Interestingly, mRNA levels of both PPARα and CPT1 were increased in parallel with triacylglycerol content in the liver of rats exposed to a high fat diet.[99] In fish, correlated expression patterns of CPT1B and PPARβ were found in both the white muscle and liver of sea bream suggesting the key role of PPARβ isoform in controlling

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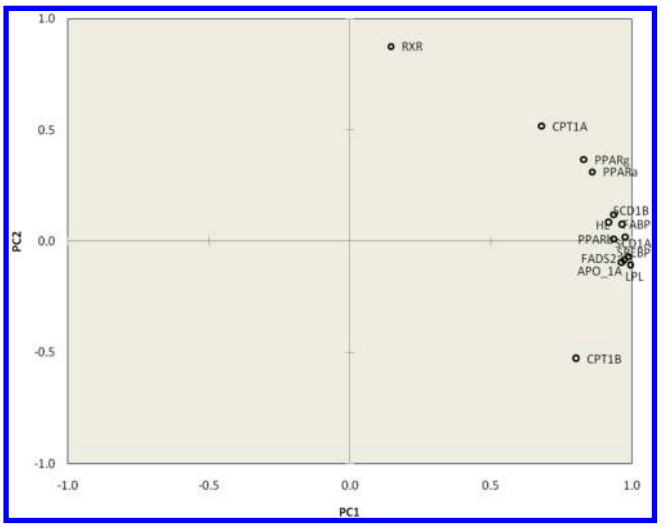

Figure 5. Transcriptomic effects of DiDP on sea bream hepatocytes. PCA was conducted using expression data from all genes examined. Rotation method was Oblimin with Kaiser normalization.

FA metabolism through CPT1B activation.[39] A previous study in salmon *in vitro* hepatocytes has reported that exposure to clofibric acid and BZF produced PPARγ-mediated increase in FA β-oxidation.[100] Overall, our findings suggest the potential activation of a compensatory mechanism for counteracting the disturbance of lipid metabolism (e.g., increased expression of adipogenic genes) induced by DiDP exposure.

Table 3. Wilks' Lambda Summary

eigenvalues	C.V. importance	Roys' statistic	Wilks' lambda	*F*	*P*-value
156.2084	0.8016	0.9936	0.0001	68.8642	0.0000
38.1092	0.1956	0.9744	0.0164	25.3088	0.0000
0.5612	0.0029	0.3595	0.6405	2.5254	0.0452

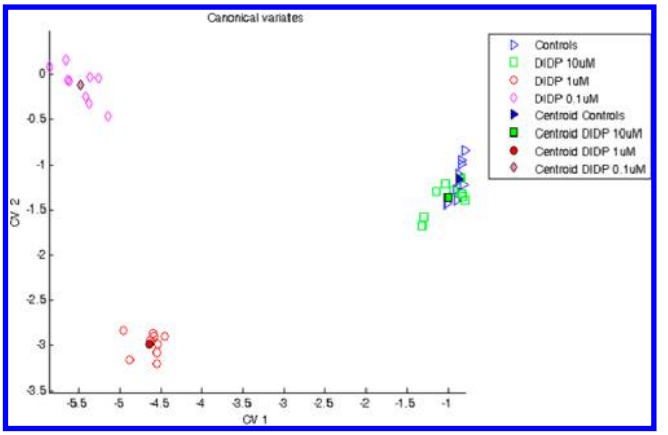

Figure 7. Plot of the canonical variates.

PCA and LDA. In the current study, PCA was used to evaluate the correlation patterns of the observations and of the variables. PCA of the data showed that 90.7% of all variance could be described using the first two principal components,

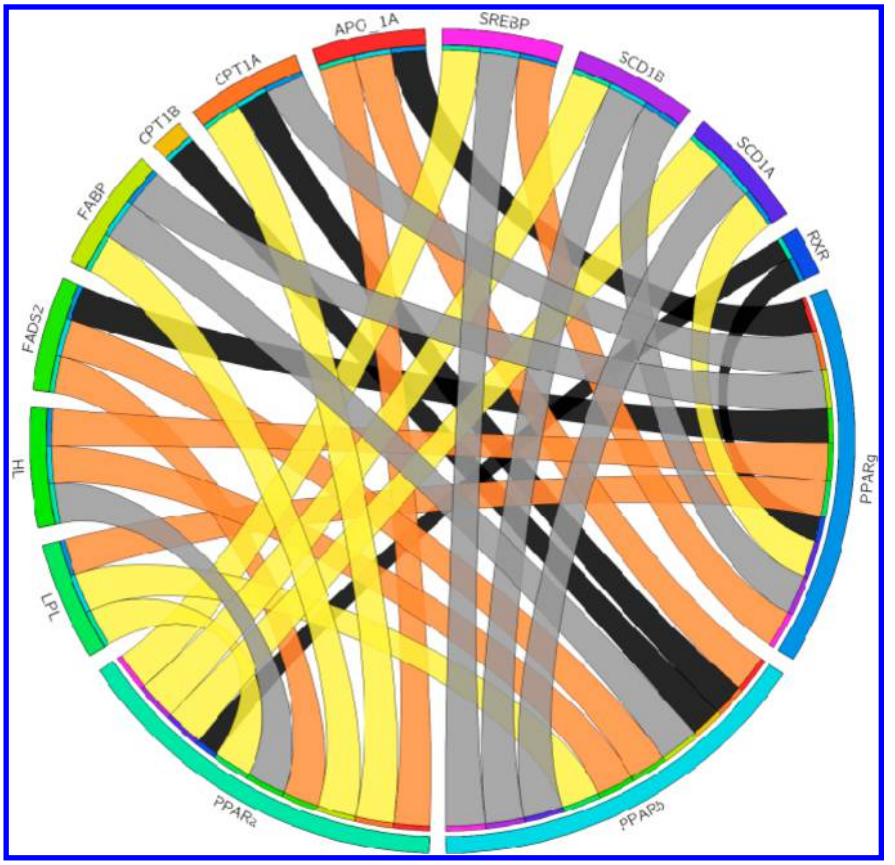

Figure 6. Circos diagram showing correlations between each PPAR and the various target genes. The degree of correlation is set by the ribbon color in 4 levels corresponding to 4 quartiles, from the lowest to the highest correlated: Q1, quartile ribbons are black; Q2 quartile ribbons are orange; Q3, quartile ribbons are yellow; and Q4, quartile ribbons are gray.

DOI: 10.1021/tx500529x
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Table 4. Confusion Matrix

	groups	control	DiDP 10 μM	DiDP 1 μM	DiDP 0.1 μM
training set	control	7	2	0	0
	DiDP 10 μM	2	7	0	0
	DiDP 1 μM	0	0	9	0
	DiDP 0.1 μM	0	0	0	9
	APER	0.89			
cross-validatation	control	7	2	0	0
	DiDP 10 μM	2	7	0	0
	DiDP 1 μM	0	0	9	0
	DiDP 0.1 μM	0	0	0	9
	LOCVER	0.89			

Table 5. Standardized Coefficients for the Discriminant Functions

variables	functions		
	1	2	3
SCD1A	−0.9398	−0.6142	−0.0357
FADS2	−0.3912	0.9832	0.4112
PPARγ	−1.6872	−0.5733	1.3349
SREBP	−0.8521	0.0586	−0.3437
PPARβ	0.1463	−0.6463	−1.1448
RXR	−0.4217	−2.5078	−0.3457
CPT1B	−1.2117	−3.3509	1.1470
LPL	0.7411	1.5124	−0.2920

PC1 and PC2 (Figure 5). The first component (PC1) explained most (80.2%) of the total variability observed with almost all variables lying in this dimension. Moreover, the second component PC2 describes a little part of the total variation (10.5%) with RXR localized very close to PC2 in comparison to other genes and CPT1 isoforms, which seem to be located quite far away from PC1 with CPT1A above and CPT1B below. Correlation of the transcription of PPARs with that of the other genes is represented in Figure 6. The analysis showed that almost all genes were correlated to PPAR isotypes. In particular, FABP was highly correlated to all PPARs. In contrast, RXR was not significantly correlated to PPARβ, and in addition, CPT1B was not correlated to both PPARα and PPARγ.

In the present article, we also applied a stepwise LDA. The results indicate that, using a stepwise subset selection procedure, it was possible to selected a set of eight variables (SCD1A, FADS2, PPARγ, SREBP, PPARβ, RXR, CPT1B, and LPL) to have a good discrimination of the four exposure groups. These genes could represent the specific genomic signature of our sea bream hepatocyte *in vitro* system induced by low doses of DiDP. Performing an LDA procedure on the selected subset of data, we first tested the statistical significance of differences between means in different groups (Table 3). The *p*-values show the significance of the test, indicating that the differences within the group mean discriminant scores are greater than what could be attributed to sampling error. The first eigenvalue accounts for a substantial proportion of the total. In fact, the importance of the first root is 0.8016; thus, the mean vectors lie largely in the first dimension. The proportion of the second eigenvalue on the total is 0.1956. The squared canonical correlations between each of the two discriminant functions and the grouping (dummy) variables are 0.9936 and 0.9744, respectively. The canonical variables produced by the model are shown in Figure 7; the subjects seem to cluster in 4 groups. The LDA gives only a 0.11 estimate both of the apparent error rate (APER) and the LOCV error rate computed with the cross-validation procedure, with an 89% correctly classified cases. Specifically, Table 4 shows the confusion matrix computed for the training set and for the LOCV procedure. It can be seen that only two values belonging to the 10 μM DiDP group are wrongly classified. This finding is not surprising given that DiDP changed the gene expression signature of the hepatocytes: low concentrations had the highest effect on gene expression, while the high concentration did not show any significant effect.

Table 5 shows the estimated standardized coefficients of the discriminant functions that provide information on the relative impact of the predictors on the dependent variables. In particular, we remark that the higher (in magnitude) standardized coefficients correspond to PPARγ and CPT1B in the first function. Stepwise LDA shows that a data-directed search of a subset of variables with a high discriminant power in terms of exposure groups can be used in metabolic profiling of DiDP-mediated effects. This procedure reduces the complexity of large data sets and increases the accuracy in the selection of optimal gene subsets that can provide a genomic signature that characterizes the molecular effects of DiDP on lipid homeostasis.

CONCLUSIONS

In summary, DiDP exposure was shown to activate PPARs and RXRs signaling pathways in an innovative fish *in vitro* system at low concentrations. Although PPARs are modulated by phthalates, little is known in fish, particularly in the gilthead sea bream. DiDP binds to piscine PPARs with an affinity comparable to that observed for human PPARs (data not shown). This interaction should be strong enough to generate peroxisome proliferation pathway dysregulation after a long period of exposure to environmental DiDP. Our studies also demonstrate the coordinate increased expression of PPARs and RXRα, as well as their downstream target genes, suggesting potential alteration of lipid homeostasis. Taken together, these data further confirm the strength of the relationship between the transcription of most genes involved in FA metabolism and PPARs mRNA levels. Therefore, phthalates such as DiDP can be considered environmental stressors that activate PPAR:RXR signaling to promote long-term changes in lipid homeostasis leading to potential deleterious physiological consequences. The novel findings presented herein will contribute to a better understanding of *in vivo* molecular responses of fish to DiDP in an aquatic environment.

ASSOCIATED CONTENT

Supporting Information

Selection of suitable housekeeping genes for expression analysis using quantitative RT-PCR. This material is available free of charge via the Internet at http://pubs.acs.org.

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DOI: 10.1021/tx500529x
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Funding
This work was supported by MIUR, PRIN 2010/2011.

Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS

ANOVA, analysis of variance; APER, apparent error rate; APOA-I, apolipoprotein A-I; BPA, bisphenol A; BSA, bovine serum albumin; BZF bezafibrate; CPT1A, carnitine palmitoyltransferase 1A; CPT1B, carnitine palmitoyltransferase 1B; DBP, dibutyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DiDP, diisodecyl phthalate; DR-1, direct repeat 1; EDCs, endocrine-disrupting chemicals; FA, fatty acids; FABP, fatty acid binding protein; FADS2, fatty acid desaturase 2; FBS, fetal bovine serum; GAR-HRP, peroxidase conjugated goat antirabbit antibodies; HDL, high-density lipoprotein; LDA, linear discriminant analysis; LOCV, leave one out cross-validation; LPL, lipoprotein lipase; MEHP, mono (2 ethylhexyl) phthalate; MS-222, 3-aminobenzoic acid ethyl ester; NRs, nuclear receptors; PCA, principal component analysis; PCB, polychlorinated biphenyls; PEs, phthalate esters; PPARs, peroxisome proliferator activated receptors; PPREs, peroxisome proliferator response elements; PUFAs, polyunsaturated fatty acids; PVC, polyvinyl chloride; PVDF, polyvinylidene difluoride; RXRa, retinoid-X-receptor-α; SCD1A, stearoyl-CoA desaturase 1A; SCD1B, stearoyl-CoA desaturase 1B; SREBP-1, sterol regulatory element-binding protein 1; SVHC, substance of very high concern; TAGL, triacylglycerol lipase; TBS, tris-buffered saline; TBST, tris-buffered saline with 0.05% Tween 20; TZDs, thiazolidinediones

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