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The pesticide adjuvant, Toximul™, alters hepatic metabolism through effects on downstream targets of PPARα

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Abstract

Previous studies demonstrated that chronic dermal exposure to the pesticide adjuvant (surfactant), Toximul™ (Tox), has significant detrimental effects on hepatic lipid metabolism. This study demonstrated that young mice dermally exposed to Tox for 12 days have significant increases in expression of peroxisomal acyl-CoA oxidase (mRNA and protein), bifunctional enzyme (mRNA) and thiolase (mRNA), as well as the P450 oxidizing enzymes Cyp4A10 and Cyp4A14 (mRNA and protein). Tox produced a similar pattern of increases in wild type adult female mice but did not induce these responses in PPARα-null mice. These data support the hypothesis that Tox, a heterogeneous blend of nonionic and anionic surfactants, modulates hepatic metabolism at least in part through activation of PPARα. Notably, all three groups of Tox-treated mice had increased relative liver weights due to significant accumulation of lipid. This could be endogenous in nature and/or a component(s) of Tox or a metabolite thereof. The ability of Tox and other hydrocarbon pollutants to induce fatty liver despite being PPARα agonists indicates a novel consequence of exposure to this class of chemicals, and may provide a new understanding of fatty liver in populations with industrial exposure.

Keywords: Pesticide adjuvant; PPARα; PPARα-null mice; MRNA and protein expression; Cyp4A; Peroxisomal fatty-acid oxidation

1. Introduction

Millions of tons of industrial surfactants (IS) are used annually by the textile, paint, cleaning supplies and agricultural/forestry industries, and use is increasing [1]. The agricultural/forestry industries rely on IS as adjuvants to emulsify water-insoluble pesticides to optimize the spreading, retention and uptake of active ingredients. These adjuvants can constitute up to 90% of pesticide formulations [2]. However, since they are considered by industry as 'inert ingredients', their use is largely unregulated and information regarding their composition is rarely available. Many of the Toximul™ (Tox) class of adjuvants are blends of structurally heterogeneous nonionic and anionic hydrocarbons (e.g., polyethylene glycol ethers, alkyl benzene sulfonates). The nonionic components are partially degraded to more toxic environmentally persistent metabolites, some of which (e.g., nonylphenol) are known endocrine disrupting chemicals [3].

A far less known consequence of environmental contaminant exposure is disruption of hepatic energy metabolism. During the past decade we have investigated the effects of Tox exposure on fat and carbohydrate metabolism in neonatal mouse livers as part of our long-term study of Tox potentiation of influenza B-induced mortality [2,4]. Mice exposed dermally to Tox daily for 12 consecutive days exhibit significant stimulation of peroxisomal fatty-acid β-oxidation (FAO) [5] and inhibition of

Abbreviations: IS, industrial surfactant(s); Tox, Toximul; pFAO, peroxisomal fatty-acid β-oxidation; mFAO, mitochondrial FAO; qPCR, quantitative polymerase chain reaction; PPARα, peroxisome proliferator-activated receptor alpha; PPARα(−/−), PPARα-null mice; PPARα(+/+), PPARα wild type mice; ACOX, acyl-CoA oxidase; L-PBE, peroxisomal bifunctional enzyme; pTHIO, peroxisomal 3-ketoacyl-CoA thiolase; Cyp4A, cytochrome P_{450}4A; CTRL, control (no Tox exposure)

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mitochondrial FAO [6], elevated synthesis of fatty acylcarnitines [7] and significant reductions in glycogen content [2]. In spite of these metabolic disturbances, the young mice have no apparent adverse health effects from Tox exposure.

Our previous efforts to elucidate the mechanism(s) underlying these metabolic derangements have been unsuccessful. However, a unifying mechanism has been described for the regulation of hepatic lipid homeostasis by many structurally diverse xenobiotic hydrocarbons (clofibrates, phthalates, pesticides) as well as endogenous metabolites (e.g., fatty acids, prostaglandins) [8]. Peroxisome proliferator-activated receptor alpha (PPARα) is a member of the superfamily of ligand-activated nuclear transcription regulators. When activated, PPARα is the 'master switch' that controls transcription of a host of genes involved in energy metabolism. The primary structural requirements for ligands of PPARα appear to be lipophilicity and a carboxyl functional group [9]. Recent in vitro studies demonstrated that perfluorooctane based chemicals, powerful IS used primarily in the paper and textile industries, can activate downstream targets of both human and mouse PPARα [10]. However no one has examined the effects of adjuvants commonly used in pesticide formulations, even though it is known that these chemicals can be more toxic than the active ingredients [11].

The purpose of this study was to determine whether chronic dermal exposure of young mice to Tox results in altered hepatic expression of PPARα and/or its target enzymes involved in lipid metabolism. To assess PPARα involvement in the Tox responses, we also determined the effects of the surfactant in adult PPARα-null mice (PPARα(−/−)) and their corresponding wild type (PPARα(+/+)) controls.

2. Materials and methods

2.1. Neonatal mice

Male and female CD-1(ICR) outbred mice (Charles River, St. Constant, QU, Canada) were bred and newborn pups were pooled on postnatal day (P) one and divided randomly among nursing mothers. Twenty-four hours later, the litters were divided into two equal groups. In the control group (CTRL), minimal essential medium (MEM) was applied dermally to the abdomens of the pups. The remainder were painted with Tox 3409F (Stepan Company, Northfield IL) diluted in MEM (1:8, vol:vol)(~1 mg Tox/g/day). These treatments were repeated daily for 12 days (P2–P13). Body weights were recorded daily. On P13 the mice were killed by decapitation and the livers were excised, weighed and flash frozen for storage at −80 °C until assessed. In this study only female CD-1 mice were used, although preliminary experiments indicated that Tox-induced changes in mRNA expression were not gender specific in the pups on P13.

2.2. PPARα(+/+) and PPARα(−/−) mice

Age-matched (14–18 weeks) wild type (C57BL/6, *PPARα*(+/+)) female mice (22±1.3 g) and *PPARα*(−/−) mice (22.2±1.2 g) originated from the laboratory of Lee et al. [12] and were a gift to C. Sinal from Dr. F. Gonzalez, National Cancer Institute, National Institutes of Health, Bethesda, MD. Half of each group had Tox applied on their abdomens daily for 12 days at doses equivalent (wt/wt) to those to which the neonates were exposed. The remainder of each group received MEM. The mice were fed standard rodent diet *ad libitum* throughout the experiment. Body weights were recorded daily. After 12 days painting the mice were killed and their livers weighed and treated as described above. All mouse studies were carried out in compliance with the guidelines of the Canadian Council on Animal Care and approved by Dalhousie University's Committee on Laboratory Animals.

2.3. Quantitative PCR (qPCR) analysis

Total RNA was isolated from frozen livers using TRIzol reagent (Invitrogen Corporation, Burlington, Canada) and reverse-transcribed using a QuantiTect Reverse Transcription kit (Qiagen Inc., Mississauga, Canada) according to the manufacturer's instructions. cDNA products (2 μL) were amplified by qPCR using gene-specific primers (0.5 μM) and the QuantiTect SYBR Green PCR kit (Qiagen) in a total volume of 20 μL using a LightCycler 2.0 thermocycler (Roche Diagnostics, Laval, Canada). The primers used and their sources are shown in Table 1. Amplification consisted of a 15-min hot start (95 °C) followed by 35 cycles of denaturation (94 °C, 15 s), annealing (60 °C, 30 s) and elongation (72 °C, 30 s). Melting curves followed by separation of PCR products on a 2.5%, 0.5× TAE agarose gel were done to ensure the formation of a single product at the appropriate size. Relative gene expression, normalized to the reference gene RNA polymerase II (RPII), was calculated using the $-2^{\Delta\Delta CT}$ method [13]. RPII was used as the housekeeping gene as its expression is constant throughout development, is not gender specific and is not influenced by Tox exposure (not shown). To demonstrate the relative abundance of mRNAs, data for both CTRL and Tox-treated mice were expressed relative

Table 1
qPCR primers

Gene	GenBank accession number	PCR primer sequences (5′ to 3′)	PCR product size (bp)
[a]ACOX—acyl-Coenzyme A oxidase 1	NM_015729	Fw[b]: accgcctatgccttccactttc Rv[c]: gcaagccatccgacattcttcg	180
[d]Cyp4a10—cytochrome P450 4A10	NM_010011	Fw: ttccctgatggacgctcttta Rv: gcaaacctggaagggtcaaac	116
[a]Cyp4a14—cytochrome P450 4A14	NM_007822	Fw: gtctctcggggcaatatcg Rv: accaatccagggagcaaagaa	119
[a]L-PBE—L-specific peroxisomal bifunctional enzyme (*Ehhadh*)	NM_023737	Fw: aaagctagtttggaccatacgg Rv: atgtaaggccagtgggagatt	109
[a]RPII—RNA polymerase II largest subunit	U37500	Fw: ctggacctaccggcatgttc Rv: gtcatcccgctcccaacac	132
[d]pTHIO—peroxisomal 3-ketoacyl-CoA thiolase A and B (Acaa1a, Acaa1b)	NM_130864 NM_146230	Fw: gactgtacctttgtctacggtca Rv: tgccaatgtcataagacccattt	101

[a] Purchased from Invitrogen Corporation.
[b] Fw, forward primer.
[c] Rv, reverse primer.
[d] Purchased from Sigma-Aldrich Canada Ltd.

to the corresponding levels of RPII mRNA for each group of mice (CD-1, PPARα(+/+), PPARα(−/−)).

2.4. Immunoblot analysis

For most Western blot analyses, frozen liver samples (~0.1 g) were homogenized in 0.5 mL of ice cold buffer containing 20 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 25 mM KCl, 1 mM dithiothreitol and a complete protease inhibitor cocktail (Roche Diagnostics). Triton X-100 (1%, v/v) was added and after 30 min incubation on ice, the homogenates were centrifuged (3000×*g*, 10 min, 4 °C) and supernatants collected. For PPARα analysis, nuclear extracts were prepared essentially as described by Gebel et al. [14]. Briefly, ~0.1 g of liver were homogenized in 0.3 mL of buffer (above), the nuclei were pelleted and resuspended in fresh buffer containing 0.4 M NaCl. The suspensions were mixed (4 °C, 30 min) and centrifuged (2000×*g*, 4 °C, 30 min), and supernatants (nuclear extracts) were collected. Protein was analyzed using a kit from Bio-Rad Laboratories (Mississauga, Canada) with bovine serum albumin as standard.

Liver homogenates or nuclear extracts (15 μg protein) were separated on 10% SDS-polyacrylamide gels, transferred to PVDF membranes and the membranes were blocked in TBST buffer (20 mM Tris–HCl [pH 7.5], 55 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk powder and incubated with primary antibody for 1–2 h. Primary antibodies and dilutions used were as follows: goat anti-β-actin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-murine PPARα (1:1000, Santa Cruz), rabbit anti-rat CYP4A (1:3000, Affinity BioReagents, Golden, CO), rabbit anti-rat ACOX 1 [15], and rabbit anti-rat pTHIO [16]. The last two were generous gifts from Dr. P.P. Van Veldhoven, K.U. Leuven, Belgium, and were used at 1:4000 dilutions. An antibody to L-PBE is not commercially available, which precluded analysis of this protein. TBST was used as wash buffer and antibody diluent. After washing 5×5 min, blots were incubated (45 min) with horseradish peroxidase-conjugated secondary antibodies, either anti-rabbit IgG (1:100,000, Chemicon, Temecula, CA) or anti-goat IgG (1:8000, Santa Cruz). Blots were given final washes (5×10 min) and antibody binding was detected on X-ray film by enhanced chemiluminescence (ECL Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK). The relative intensities were quantified using NIH Image software after films were scanned using Umax MagicScan32 software. Relative levels of protein in each group of mice are expressed relative to P13 CTRL=1. Data for the Tox-treated mice are expressed relative to the CTRL for its corresponding group.

2.5. Lauric acid hydroxylation assay

Cytochrome P450 ω-hydroxylase (Cyp4A) activity was determined using liver homogenates (1 mg protein) incubated (37 °C, 10 min) in 50 mM Tris–HCl, pH 7.4, containing 50 μM [1-^{14}C]lauric acid (11,000 dpm/nmol, Amersham Biosciences) and 1 mM NAPDH, in a final volume of 0.5 mL. Blank tubes contained all reactants except NAPDH. Reactions were terminated by addition of 0.5 mL acetonitrile and 200 mg of each of lauric and 12-hydroxylauric acids. The mixtures were extracted with diethyl ether and organic phases were pooled and dried under nitrogen. The residues were suspended in 25 μL methanol and reaction products were separated on silica gel on polyester plates (Sigma-Aldrich Canada Ltd., Oakville Canada) using the solvent system diethyl ether:petroleum ether:formic acid (70:30:1, vol:vol). Bands corresponding to lauric and 12-hydroxylauric acids were cut and radioactivity was quantitated using a scintillation counter. The data were expressed as nmol lauric acid hydroxylated/min/mg protein.

2.6. Pathology assessment

Thin (5 μm) sections were cut from mouse livers that were either fixed in formalin (10%, by vol) and embedded in paraffin, or flash frozen at the time of harvest. The paraffin sections were stained with hematoxylin and eosin, and the frozen sections were stained with Oil Red O. The sections were cut and stained in the Clinical Chemistry Laboratory of the IWK Health Centre. To evaluate liver fat content, images of the oil red O stained sections were analyzed using Adobe Photoshop CS2 after all non red stained portions were converted to white. The images were converted to grayscale, optimized for contrast, and the number of pixels derived from the red areas were counted (Image J, public domain software from the National Institutes of Health) and expressed as percent total pixel count.

2.7. Statistical analysis

The mRNA data for each set of animals are expressed relative to values for RPII (=1) for that set. Control protein levels for the PPARα(+/+) and PPARα(−/−) mice are expressed relative to those of the CD-1 controls (=1), and values for the Tox-treated mice are expressed relative to the corresponding controls. Unless indicated otherwise, data are the means±SEM of values from 4 to 12 mice. Statistical analyses were done using the two-tailed unpaired Student's *t* test.

3. Results and discussion

The long-term health effects of prolonged exposure to environmental pollutants, particularly xenobiotic hydrocarbons, is becoming of increasing concern to health care workers as well as the general public. Atypical hydrocarbons that accumulate in the environment or are present in human foods (e.g., phthalates, nonylphenol, perfluorooctanoate, oxidized frying fats) are known to alter hepatic lipid metabolism [9,17,18]. Only recently have several in vivo studies linked these alterations to effects on gene expression [9,19,20]. The objective of this study was to determine whether the widespread metabolic abnormalities that occur in young mice exposed to the pesticide adjuvant, Tox, were due to altered function of the transcription regulator, PPARα, and expression of its target genes involved in pFAO.

3.1. Tox effects on expression of pFAO enzymes

In vivo, activation of PPARα by clofibrate and Wy-16,643 increased expression of all three enzymes of the pFAO pathway (i.e., ACOX, L-PBE, pTHIO) [12]. Likewise, exposing the CD-1 mice to Tox increased expression of mRNAs coding for ACOX, L-PBE and pTHIO (2-, 4- and 1.7-fold, respectively) (Fig. 1A). ACOX protein levels were also elevated (1.6-fold) with Tox treatment, however those of pTHIO were not (Fig. 1B). The reason for the lack of change in pTHIO protein is unclear; perhaps it reflects an adaptive down-regulation of translation and/or rapid protein turnover. Livers of the adult PPARα(+/+) females exposed to Tox exhibited a similar pattern of change in pFAO enzyme expression, with significant increases in ACOX (mRNA and protein) and L-PBE mRNA. Levels of pTHIO mRNA also increased, however values did not reach statistical significance. This is consistent with the reported relative insensitivity of adult female mice to ligand-induced increases in pTHIO [21]. The fact that pTHIO protein was increased (~1.6-fold) in Tox-treated PPARα(+/+) mice may reflect a stable protein with a long half-life (Fig. 1B). Collectively, these data showing Tox-induced increases in expression of pFAO enzymes (protein and/or mRNA) in CD-1 pups and PPARα(+/+) mice are consistent with Tox effects being mediated by PPARα.

If Tox-mediated increases in pFAO activity [5] and enzyme expression are totally dependent on PPARα, these responses should not occur in PPARα(−/−) mice. As illustrated in Fig. 1, this was true of ACOX mRNA and protein expression. However,

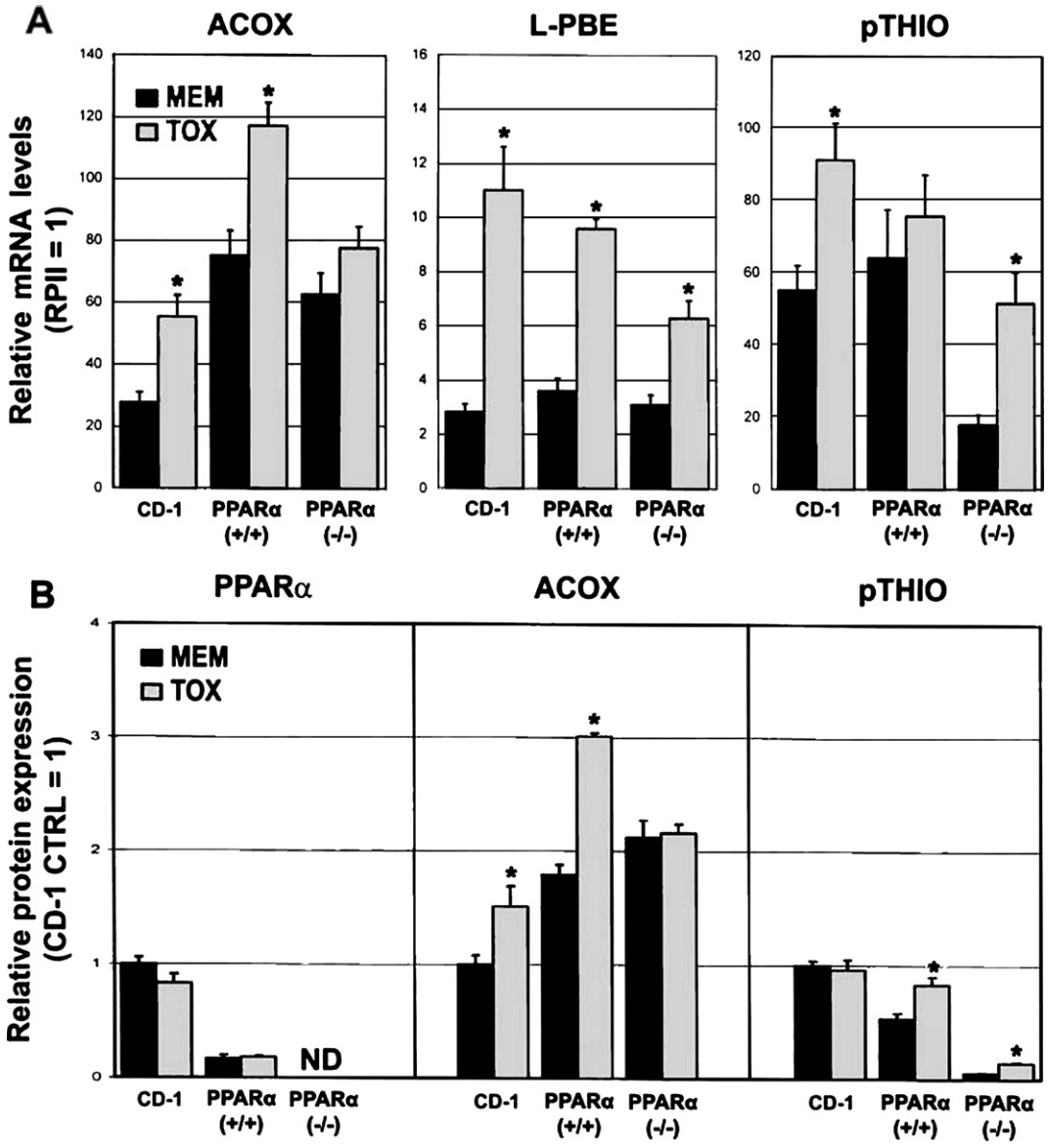

Fig. 1. Effect of Tox on expression of PPARα and peroxisomal fatty-acid β-oxidation enzymes. (A) mRNA expression Total RNA was extracted from mouse livers, reverse transcribed and analyzed by qPCR for expression of ACOX, L-PBE and pTHIO as described in Materials and methods. The data for each enzyme in each mouse group (CD-1, PPARα(+/+), PPARα(−/−)) are expressed relative to mean RPII levels in that group (RPII = 1). (B) Protein expression Levels of PPARα, ACOX and pTHIO protein were quantitated by Western blot analysis as described in Materials and methods. Raw data were standardized to β-actin and all values were standardized to those for the control CD-1 pups (CTRL = 1). Values represent the mean ± SEM (nS = 4–12 [CD-1, PPARα(+/+)]; $n = 2$ for PPARα(−/−) [PPARα, Cyp4A only]). *$p < 0.05$ – < 0.0001, relative to values for corresponding CTRL in that group of mice. ND, not detected.

expression of L-PBE (mRNA) was increased ~4-fold, and pTHIO mRNA and protein content increased almost 3-fold. This is one of few reports of a putative PPARα agonist increasing expression of L-PBE in PPARα(−/−) mice [22]. It should be emphasized that Tox-mediated increases in transcription may not translate into increases in protein that have tangible effects on metabolism. For example, pTHIO protein levels in the control PPARα(−/−) mice were < 10% of their PPARα(+/+) counterparts and although these levels increased with Tox exposure they did not exceed ~25% of the control PPARα(+/+) levels. Increases in pTHIO expression also occurred in PPARα(−/−) mice treated with clofibrate [12] or the branched-chain fatty-acid precursor, phytol [22]. These findings led the authors to conclude that these agonists can mediate responses by both PPARα-dependent and independent pathways. Gloerich et al. [22] did not speculate on the identity of the nuclear receptor(s) that mediate(s) PPARα-independent effects of phytol, but two potential candidates are liver X receptor α (LXRα) [23,24] and PPARγ [25]. The LXRα agonist, T0901317, upregulates all three pFAO enzymes in PPARα(−/−) mice. The natural ligands of LXRα are cholesterol and its derivatives, however other phenolic hydrocarbons are also potent agonists [26], and Tox may contain structurally similar molecules. The second candidate, PPARγ, is not highly expressed in PPARα(+/+) mouse liver, however feeding PPARα(−/−) mice high fat diets increased its expression by ~4-fold [25], with a concomitant upregulation of pFAO enzyme expression.

3.2. Tox effects on expression of PPARα

Western blot analysis of the P13 mouse livers showed that chronic dermal Tox exposure did not alter PPARα protein levels, a finding also observed in PPARα(+/+) mice (Fig. 1B, left panel). Predictably, PPARα protein was not detected in any PPARα(−/−) mice (Fig. 1B, left panel). The lack of change in PPARα protein in the pups and PPARα(+/+) mice was not unexpected, as others also have found its levels unchanged following exposure to PPARα agonists [9,12]. This may reflect its demonstrated rapid turnover rate and the fact that the ability of ligands to stabilize its expression is transitory [27].

3.3. Tox effects on Cyp4A expression and activity

A hallmark response to activation of several nuclear receptors, including PPARα, by xenobiotic agents is an increase in expression of the P450 ω-hydroxylases, including Cyp4A [28]. In all three groups of mice in our study, mRNA levels of only two isoforms of Cyp4A (Cyp4A10 and Cyp4A14) were sufficiently abundant to reliably detect Tox-related changes. Characteristic of most strains of adult female mice [29], expression of Cyp4A12 was extremely low (≤0.2%) in both the pups and adults. Cyp4A10 and 14 are the isoforms highly inducible by PPARα ligands [12,25,30,31]. In this study, CD-1

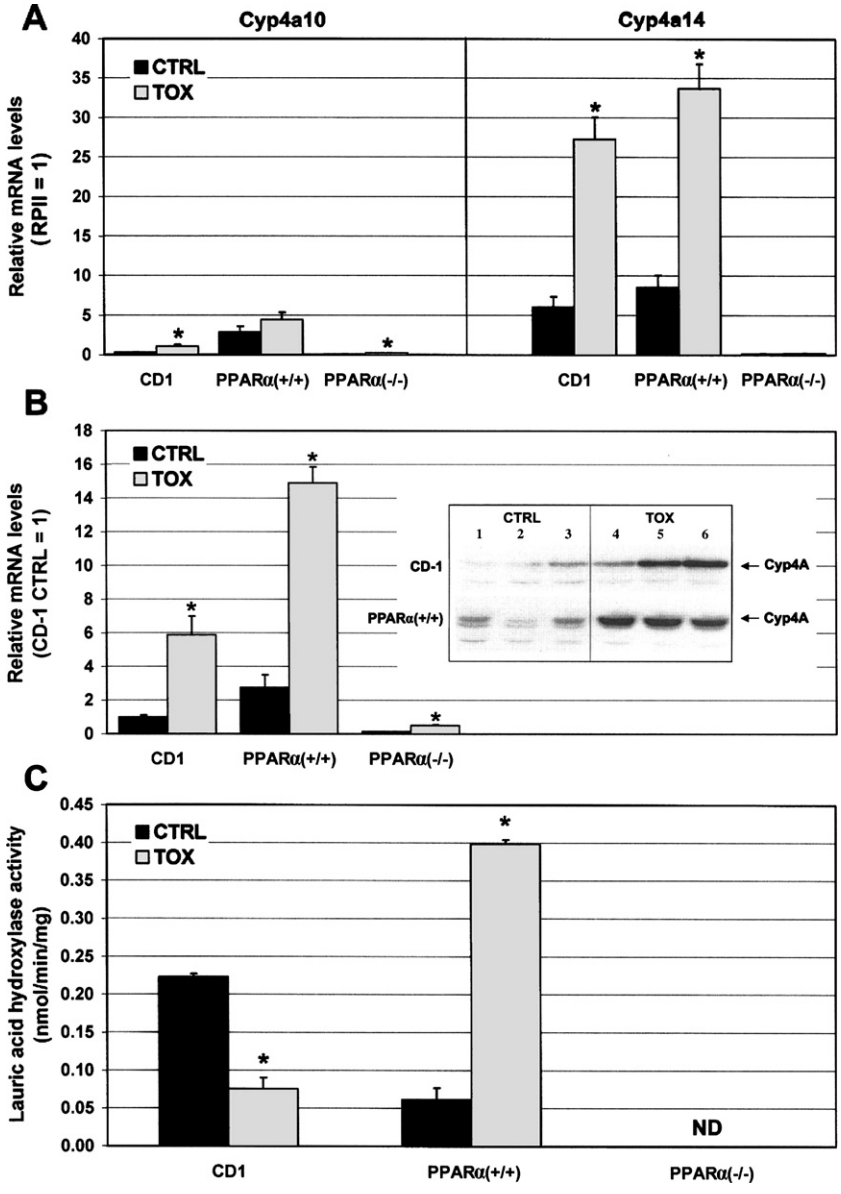

Fig. 2. Effect of Tox on hepatic Cyp4A expression and enzyme activity. (A) mRNA expression. Methods were as described in the legend to Fig. 1, using primers for Cyp4A 10 and 14; values were expressed relative to RPII values for the control group of corresponding mice. (B) Cyp4A protein content was quantitated as described in Fig. 1, using an antibody nonspecific for Cyp4A isoform. Data are expressed as described in Fig. 1B. The insert shows representative blots of CTRL and Tox-treated livers from CD-1 pups and PPARα(+/+) mice probed for total Cyp4A protein. (C) Lauric acid ω-hydroxylase activity was measured in livers of CD-1 and PPARα(+/+) mice as described in Materials and methods. The data are expressed as nmol lauric acid ω-hydroxylated/min/mg protein (mean ± SEM, $n=3$). Statistical analysis and data significance are as described in Fig. 1.

pups chronically exposed to Tox had ≥4-fold elevations in mRNA coding for both Cyp4A10 and 14 (Fig. 2A); total Cyp4A protein content (Fig. 2B) and enzyme activity (Fig. 2C) were also increased (~6- and >3-fold, respectively). It should be noted that the Cyp4A antibody does not distinguish between isoforms. Consistent with earlier reports [30], there were no gender-specific differences in the responses of the P13 pups to Tox (not shown). PPARα(+/+) adult females treated with Tox also had increased levels of Cyp4A14 mRNA, total Cyp4A protein and enzyme activity (4-, 5- and >6-fold, respectively) (Fig. 2). Cyp4A10 mRNA was increased, however values did not reach statistical significance. One possible explanation is that upregulation of this isoform does not occur except with very long exposure, as seen with dioxin treatment [28]. PPARα(−/−) mice had predictably low constitutive levels of Cyp4A10 and 14, and no change in Cyp4A14 mRNA occurred with Tox exposure (Fig. 2A). Unexpectedly, Cyp4A10 content was increased ~4-fold with Tox treatment. However, Anderson et al. recently determined that Cyp4A10 was the one Cyp4A isoform whose expression was regulated independent of PPARα [24]. They provided evidence that this could occur by binding of the ligand (e.g., a component of Tox) to one of the retinoid X receptors that are the mandatory heterodimeric partners for activation of most nuclear receptors, including PPARα, LXR and PPARγ.

3.4. Effects of Tox on liver weights and pathology

Chronic dermal exposure to Tox did not have obvious adverse health effects on any of the mice, nor were body weights affected (Table 2). Relative liver weights were significantly increased in Tox treated CD-1 and PPARα(+/+) mice (12% and 37%, respectively) (Table 2), a response typically seen in wild type rodents exposed to the prototype PPARα agonists, clofibrate and Wy-16,643 [12]. This effect of the latter drugs is commonly attributed to their ability to increase hepatocyte number and/or size, and does not occur in PPARα(−/−) mice. In contrast, relative liver weights in Tox-treated PPARα(−/−) mice were elevated significantly (Table 2). Pathologic assessment of stained sections of livers from each group of mice showed vacuoles in the PPARα(+/+) mice, a feature more prominent in the PPARα(−/−) animals. There were no obvious structural abnormalities in the CD-1 mice, and there was no evidence of inflammation or increased numbers of mitotic figures in any Tox-treated mice. The possibility that the vacuoles were due to glycogen storage was ruled out as several sections stained with periodic acid schiff were negative which indicates that the vacuoles did not contain glycogen (data not shown). This was consistent with our earlier findings that Tox exposure significantly reduces hepatic glycogen [2]. Oil red O staining showed that vacuoles in the Tox-treated livers were positive for fat (Table 2) in PPARα(−/−) and PPARα(+/+), indicating lipid accumulation that could be endogenous fat and/or Tox components. The absence of a statistical difference in oil red O staining of Tox exposed P13 mice may reflect insensitivity of this assay at lower levels of quantification, as focal areas of staining were present. There was correlation between increases of relative liver weight and percentage oil red O staining (Fig. 3), which supports Tox related liver weight increases are predominantly lipid accumulation. The finding that exposure to petroleum-derived hydrocarbons increases relative liver weights in both PPARα(+/+) and PPARα(−/−) mice is not unique, as Yang et al. reported the same result in PPARα(−/−) mice fed perfluorooctanoic acid for 7 days [32]. These authors concluded that the effect on relative liver weights was independent of PPARα. A more likely explanation for the discrepancy between the effects of clofibrates and Tox on relative liver weights in PPARα(−/−) mice is the marked difference in the substrates being catabolized. Treatment with clofibrate stimulates oxidation of endogenous fatty acids, primarily to ketone bodies that are rapidly cleared from the liver. By contrast, Tox is a complex mixture of linear and branched-chain hydrocarbons, some of which likely have cyclic and/or substituted (e.g., sulfated, methylated) components. As with naturally occurring fatty acids, xenobiotic hydrocarbons that reach the liver have the potential to undergo structural modifications, as well as be oxidized and/or incorporated into triglyceride for export to the periphery [33]. There is strong evidence that xenobiotics with abnormal structures are not completely catabolized and fail to be esterified to form triacylglycerols, with resultant hepatic accumulation. We observed this earlier in livers of Tox-treated mice [34]. We propose that in both PPARα(+/+) and PPARα(−/−) mice, the Tox components that get into the bloodstream, either via transdermal transport or by ingestion during grooming, are transported to the liver. Their presence in the PPARα(+/+) mice would activate PPARα, as this is a primary route for at least partial degradation and clearance of xenobiotic hydrocarbons. This pathway is inactive in the PPARα(−/−) mice, with the result that Tox components accumulate in even higher quantities. The fact that fatty liver also occurred, albeit to a lesser extent, in the CD-1 pups is likely

Table 2
Effects of 12 days of dermal exposure to Tox 3409F on body and liver weights and on Oil red O staining for fat

Mice	Treatment	Body weight (g)	Absolute liver weight (g)	Relative liver weight (%)	Oil Red O % positive stain
CD-1 (13-day-old)	Control (n=9)	6.48±0.24	0.188±0.010	2.90±0.09	0.00±0.00
	Toximul 3409 (n=9)	7.01±0.15	0.227±0.005*	3.24±0.04*	0.00±0.00
PPARα+/+(adult)	Control (n=5)	21.58±0.76	0.825±0.038	3.84±0.23	0.61±0.33
	Toximul 3409 (n=5)	23.02±0.68	1.210±0.027***	5.26±0.09**	4.05±1.51***
PPARα−/− (adult)	Control (=4)	21.92±0.78	0.878±0.031	4.00±0.04	1.60±0.38
	Toximul 3409 (n=4)	22.12±0.37	1.228±0.030**	5.56±0.14***	9.10±2.91***

Values are expressed as mean±SEM. Data were analyzed by the unpaired Student's t test for significant differences between the Toximul 3409F-treated groups and their respective controls. *p<0.05; **p<0.001; ***p<0.0001.

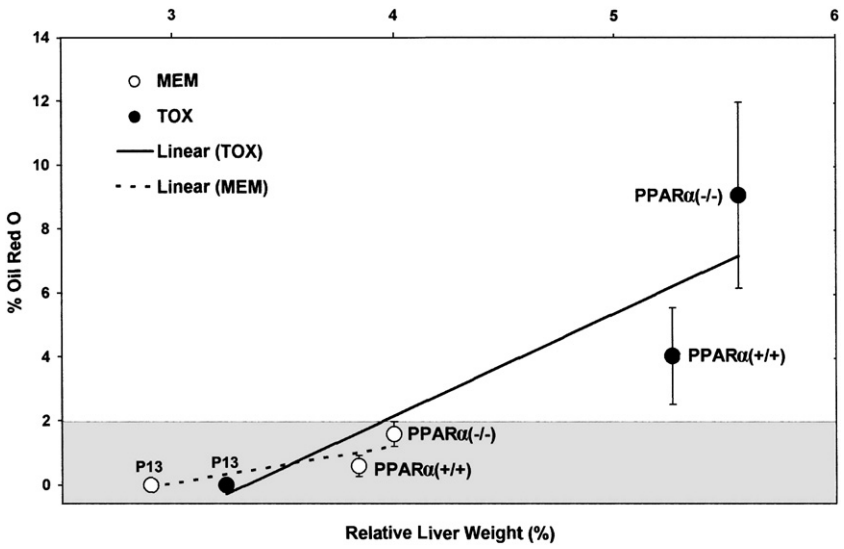

Fig. 3.

due to age- or strain-dependent differences in the pharmacokinetic properties of the Tox components, a phenomenon observed by others [35]. Of particular interest to this study, several groups have reported that unmetabolized xenobiotic hydrocarbons are more potent in activating PPARα than are endogenous ligands [36,37].

3.5. Relevance

Pesticides are ubiquitous in the environment, and concern that exposure can pose adverse health risks is growing, particularly as these xenobiotics are stored and accumulate for a very long time in humans [38]. Very few active pesticide ingredients appear to exert health risks in vivo by activation of PPARα [31]. The 'other' ingredients in pesticide formulations, the xenobiotic hydrocarbons, are often more toxic than the active ingredients [11], yet have received very little attention. This is the first study to obtain evidence that subclinical dyslipidemia in young mice dermally exposed to a pesticide adjuvant involves upregulation of select lipid metabolizing enzymes. These effects occur despite several factors that could attenuate the effective dose. Since Tox is a complex mixture of structurally heterogeneous components, some components may not penetrate skin, and those that do likely have variable metabolic fates and liver clearance. As well, only select components or metabolites of Tox may be responsible for the changes in gene expression. From our data with the PPARα(−/−) mice it appears that Tox components may serve as ligands for more than one nuclear receptor. Whether effects on enzyme expression are mediated through direct or indirect activation of a nuclear receptor(s) remains to be determined. Nevertheless, our results suggest that exposing high-risk populations, particularly those in the petrochemical and agricultural industries, to this class of persistent organic chemicals has the potential to predispose them to significant perturbations in energy metabolism and development of fatty liver. We believe that this study has far broader implications than exposure to pesticides, as similar effects are elicited by the wide range of structurally diverse industrial chemicals to which humans have been exposed since the onset of industrialization.

In summary, this study has demonstrated that chronic dermal exposure to the currently used pesticide adjuvant, Tox, significantly increases expression of select lipid metabolizing enzymes in livers of young and adult mice. The changes in expression most likely to result in meaningful effects on hydrocarbon β-oxidation (e.g., ACOX) or ω-oxidation (Cyp4A) did not occur in PPARα(−/−) mice, suggesting involvement of PPARα. A significant consequence of Tox exposure that occurred despite the absence of PPARα was increased relative liver weights and development of fatty liver (Fig. 3). Evidence suggests that other xenobiotic hydrocarbons (e.g., perfluorooctanoic acid) elicit a similar effect [32]. This is a very important distinction between effects of the classic PPARα agonists and environmental pollutants, and the reason we should be even more fearful of these chemicals.

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