1   **Original**

2   **Role of peroxisome proliferator-activated receptor-α in hepatobiliary**

3   **injury induced by ammonium perfluorooctanoate in mouse liver**

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12   Short title: Hepatobiliary injury induced by PFOA

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17

17   Abstract

18   Peroxisome proliferator-activated receptor- α (PPARα) has been suggested to protect

19   against chemically induced hepatobiliary injuries in rodents. This function could mask

20   the potential toxicities of Perfluorooctanoic acid (PFOA) that is an emerging

21   environmental contaminant and a weak ligand for PPARα. However the function has

22   not been clarified. In this study, PFOA was found to elicit hepatocyte and bile duct

23   injury in *Pparα* -null mice after 4 week treatment with PFOA ammonium salt (0, 12.5,

24   25, 50 μmol/kg/day, gavage), suggesting that PPARα protects from bile duct injury. In

25   wild-type mice, PFOA caused major hepatocellular damage dose-dependently and

26   minor cholangiopathy observed only at 25 and 50 μmol/kg. In treated *Pparα*-null mice,

27   PFOA produced marked fat accumulation, severe cholangiopathy, hepatocellular

28   damage and apoptotic cells especially in bile ducts. Oxidative stress was also increased

29   4-fold at 50 μmol/kg and *TNF*-α mRNA was upregulated more than 3-fold at 25

30   μmol/kg in *Pparα*-null mice. Biliary bile acid/phospholipid ratios were higher in

31   *Pparα*-null mice than wild-type mice. Results from these studies suggest that PPARα is

32   protective against PFOAand have a potential role for drug induced hepatobiliary injury.

33   **Key words**

34   Peroxisome proliferator-activated receptor-α, Perfluorooctanoic acid, hepatobiliary

35   injury, bile acid transporter, histopathology

36

Introduction

36

37   Peroxisome proliferator-activated receptor-α (PPARα) is a ligand-activated receptor

38   that mediates critical transcriptional regulation of genes associated with lipid

39   homeostasis. PPARα is also suggested to have important roles in inflammation, immune

40   response and hepatocarcinogenesis, however the mechanism has not been clarified.

41   Perfluorooctanoic acid (PFOA) is a fluorinated eight-carbon member of the

42   perfluoroalkyl acid family that is amphiphilic and is used in the preparation of

43   surfactants and fabricants [1]. The potential health risk for PFOA arises from its

44   ubiquitous distribution and persistence in the environment, and its presence in humans

45   and wildlife[2,3]. PFOA is assumed to be a weak PPARα ligand because of its low degree

46   PPAR-α transcriptional activations among PPAR- α ligands [4] and is carcinogenic to

47   rodents [5,6].

48   The pathophysiological roles of PPARα in toxicity caused by PFOA is well delineated

49   by PPAR α null mice [7,8].   Rosen et al (2008) demonstrated that ablation of PPAR-α

50   changes profiles of transcripts related to fatty acid metabolisms, inflammation,

51   xenobiotic metabolism and cell cycle regulation [7].   Qualitative changes in transcripts

52 modified hepatortoxicity significantly in PPARα null mice, leading a conclusion that

53 PPARα is required for PFOA-induced cellular alterations in mouse hepatocytes.

54 Recently, Hays et al. demonstrated that a weak PPARα ligand, bezafibrate, induces

55 cholestasis without neoplastic changes in *Pparα*-null mice, and have concluded that

56 PPARα protects against potential cholestasis, while it facilitates tumor promotion [9].

57 They have also demonstrated that a very specific PPARαligends, Wy-14,643, does not

58 induce cholestastis [9]. Thus, the toxicity profile of a chemical that up- or down-regulates

59 via PPARα-dependent and independent pathways may be modified depending on its

60 affinity to PPARα and its dose.

61 A reasonable conjecture would be that PFOA , which is known as a PPARα weak

62 ligand, might also induce cholestatic disease in PPARα null mice. No study on PFOA

63 has ever investigated so far biliary duct toxicity. This study examined whether PFOA

64 has the potential for inducing cholestatic disease and the role of PPARα has in

65 protecting against chemical induced choestasis.We investigated whether PFOA induces

66 cholestasis in *Pparα*-null mice and the dose-response relationship between PFOA and

67 toxicological responses in PPARα wild and null mice. It is well known that cholestasis

68 is not a common response in mice, although it is a very common response to

69   xenobiotics including therapeutic drugs in human [10]. Thus, the null genotype of PPARα

70   might reveal bile duct toxicity of many PPARα inducers otherwise overlooked,

71   although they may be recognized only at very high doses[11]. .

72   **Subjects and methods**

73   *Animals and treatment*

74   Wild[k1]-type mice (129S4/SvlmJ) and *Pparα*-null mice (129S4/SvJae-Pparα[tm1Gonz]/J)

75   were originally provided from Dr. Frank J. Gonzalez (National Cancer Institute,

76   Bethesda, MD) and housed in Kyoto University Institute of Laboratory Animals. All

77   experiments were performed with male mice aged 8–10 weeks (22–25 g). 39 wild-type

78   mice and 40 *Pparα*-null mice were randomly assigned to four groups in accordance

79   with the administered doses of PFOA (0, 12.5, 25, 50 µmol/kg/day). PFOA ammonium

80   salt (>98% purity) was purchased from Fluka Chemical (Steinheim, Switzerland) and

81   dissolved in deionized water. Mice were treated by oral gavage (8 ml/kg) daily for 4

82   weeks and killed by euthanasia at the end of 4 weeks, at which time, blood, liver and

83   bile werecollected.

84   Livers were weighed, and the tissue was fixed in 10% neutral-buffered

85   formalin for light microscopic examination or 1% glutaraldehyde/1.44%

86   paraformaldehyde solution for transmission electron microscopy for ultrastructural

87 examination. The remaining portion was flash-frozen in liquid nitrogen and stored at

88 −80°C.

89 *Biochemical measurements*

90   Biochemical analyses were performed on plasma samples. These analyses were

91 examined by Nagahama Life Science Laboratory, Oriental Yeast Co. Ltd. (Shiga, Japan)

92 included aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as

93 hepatocellular damage markers, total bilirubin (T-Bil) and total bile acid (TBA) as

94 cholestatic markers, and total cholesterol (T-Cho) and triglyceride (TG) as fat

95 metabolism markers.

96 *Histology*

97   For light microscopy, livers were processed by routine paraffin sectioning and

98 staining with hematoxylin and eosin (HE). For the determination of apoptosis, a

99 terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling

100 TUNEL test was performed. An Apop Tag kit (Oncor, Gaithersburg, MD) was used

101 according to the manufacturer's recommendations.

102 For ultrastructural studies, livers were post-fixed in 1% osmium tetroxide in 0.2 M

103 phosphate buffer, routinely dehydrated through a graded ethanol series, and embedded

104 in Epon using the Luft method [12]. Sections were cut in 80 μm on a Leica EM UC6

105   ultramicritome (Hitachi, Tokyo, Japan) with a diamond knife, and stained by the

106   Reynolds method [13]. The grids were examined under a Hitachi 7650 transmission

107   electron microscope (Hitachi, Tokyo, Japan).

108   *Analysis of PFOA*

109   Determination of PFOA in whole blood, bile and liver was performed using a

110   modification of a method originally developed by Yline et al [14]. Diluted blood, bile or

111   homogenized liver was combined with 10 μl of a 1 μg/ml solution of $^{13}C_2$-PFOA as an

112   internal standard. One milliliter of tetrabutylammonium (TBA) hydrogen sulfate and 2

113   ml 0.5 M sodium carbonate buffer solution (pH adjusted to 10) were combined and

114   vortexed, then 2 ml methyl *tert*-butyl ether (MTBE) was added and vortexed. The tube

115   was centrifuged to separate the aqueous and organic phases, and 1 ml of the MTBE

116   layer was extracted, transferred to a glass tube, and evaporated to dryness at 38°C

117   under a gentle stream of dry nitrogen. The residue was then re-dissolved in 100 μl of

118   100 mM benzyl bromide acetone for 1 hour at 80°C and transferred to an autosampler

119   vial. Extracts were analyzed using gas chromatography-mass spectrometry (Agilent

120   6890GC/5973MSD, Agilent Technologies Japan, Ltd., Tokyo, Japan) in electron impact

121   ionization mode. PFOA was separated on an HP-5MS column (30 m length, 0.25 mm i.d.,

122   0.25 μm film thickness) with a helium carrier gas. Splitless injections (2 μl) were

123   performed with the injector set at 220 °C, and the split was opened after 1.5 min. The

124   initial oven temperature was 60 °C for 1.5 min, ramped at 15 °C min^{-1} to 100 C°, and

125   then at 40 °C min^{-1} to 240 °C. Recoveries of $^{13}C_2$ PFOA from biological samples (n=3)

126   were 94 ± 2.6 % for blood, 97 ± 4.4 % for bile and 94.7 ± 4.9 % for liver, respectively.

127   *Measurement of 8-hydroxydeoxyguanosine (8-OHdG) in the liver*

128   8-OHdG/dG levels were measured as an indicator of oxidative DNA damage. The

129   frozen livers were minced and gently homogenized in a homogenizer by 5 strokes in

130   lysis solution (Qiagen, Tokyo, Japan), DNA was extracted from mice frozen liver using a

131   DNA Extractor WB kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) [15]. DNA

132   was digested completely to nucleotides by combined treatment with Nuclease P1 (Wako

133   Pure Chemical Industries, Ltd.) and alkaline phosphatase (Sigma Chemical Co., St.

134   Louis, MO). Then the resulting deoxynucleoside mixture was injected into a high

135   performance liquid chromatography apparatus (LC-10ADvp, Shimadzu, Kyoto, Japan)

136   equipped with both a UV detector (SPD-10AVvp, Shimadzu) and an electrochemical

137   detector (Coulochem model-5200-2, ESA, MA) [16]. Each liver was examined in duplicate

138   and the means were reported.

139   *Quantitative RT-PCR analysis for multidrug resistance protein 2 (Mdr2) and tumor*

140   *necrosis factor α (TNF-α)*

141   Quantitative Real time (RT)-PCR was used to study *Mdr2* and *TNF-α* mRNA

142   expression in the liver. Total RNA was extracted from the liver using RNeasy Lipid

143   Tissue Mini Kit (Qiagen, Tokyo, Japan). Aliquots (10 ng) were amplified using

144   QuantiTect® SYBR® Green RT-PCR (Qiagen,Tokyo, Japan). Quantification of the

145   amplified products was performed on an ABI PRISM 7700 Sequence Detection System

146   (Applied Biosystems Japan, Tokyo, Japan). All expression data were normalized to

147   glyceraldehyde 3-phophate dehydrogenase (*GAPDH*) mRNA from the same individual

148   sample, to correct for differences in efficiency of RNA extraction and quality.

149   The following primers were used for RT-PCR: GAPDH: forward,

150   5'-ATGGTGAAGGTCGGTGTGAA-3';   reverse,

151   5'-GAGTGGAGTCATACTGGAAC-3', [17] corresponding to GenBank accession

152   number M32599; Mdr2: forward, 5'-ATCCTATGCACTGGCCTTCTGGT-3'; reverse,

153   5'-GAAAGCATCAATACAGGGGGCAG-3', [18] corresponding to GenBank accession

154   number NM_008830; TNF-α. forward, 5'-TCTTCTCAAAATTCGAGTGACAAG-3';

155   reverse, 5' –GAGAACCTGGGAGTAGACAAGGTA-3', (note: designed in our lab)

156   corresponding to GenBank accession number NM_013693.

157   *Determination of bile acid/ phospholipid ratio (BA/PL) in bile*

158   Commercially available kit was used for determination of bile acid and

159   phospholipid contents in bile (Wako Pure Chemical Industries). For bile acid

160   determination, 0.1 μl bile was diluted in 200 μl double distilled H_2O, that was added to

161   500 μl 3-α-hydroxysteroid dehydrogenase, incubated at 37°C for 10 minutes, added to

162   500 μl response fixing solution, and absorbance was read at 560 nm using a Hitachi

163   U-2000A spectrophotometer (Hitachi, Tokyo, Japan). For phospholipid determination,

164   0.4 μl bile was diluted in 20 μl double distilled H_2O, that was added to 3.0 ml color

165   reagent (Phospholipid-C Test Wako, Wako Pure Chemical Industries), incubated at

166   37°C for 5 min, and absorbance was read at 600 nm against a color reagent blank.

167   *Western blot analysis of Bsep and Mrp2*

168   Western blot analysis was carried out for quantification of the protein levels

169   of the canalicular bile salt export pump (Bsep) and the canalicular multidrug

170   resistance-associated protein 2 (Mrp2). Membrane protein samples mixed with sample

171   loading buffer (15 μg protein/lane) were loaded after heating for 10 minutes at 70°C

172   onto a 3–8% Tris-Acetate gel. Following electrophoresis, proteins in the gel were

173   electrotransferred to PVDF-plus membranes (Immobilon-P Transfer Membrane;

174   Millipore) for 1 hour at 30 V at 4°C. Membranes were blocked for 1 hour at room

175   temperature with 5% non-fat dry milk in Tris-buffered saline that contained 0.05%

176   Tween-20 (TBS-T). Blots were then incubated for 1 hour at room temperature with

177   the primary polyclonal antibody of rabbit Bsep, which was kindly provided by Rexue

178   Wang, (British Columbia Cancer Research Center, Vancouver, BC, Canada) and rat

179   Mrp2, which was kindly provided by Bruno Stieger (University Hospital, Zurich,

180   Switzerland). GAPDH antibody was used as a loading control. Each primary antibody

181   was diluted in blocking buffer (1:5000 for Bsep, 1:4000 for Mrp2, 1:1000 for

182   GAPDH). After thorough washing, blots were incubated with donkey anti-rabbit IgG

183   horseradish peroxidase-linked secondary antibody (1:4000 dilution with 5% non-fat

184   milk in TBS-T) for 1 hour. Immunoreactive bands were detected with an enhanced

185   chemical luminescence (ECL) kit (Immobilon Western; Millipore). Bsep and Mrp2

186   proteins were visualized by exposure to Fuji Medical X-Ray film (FUJIFILM Medical

187   Co., Ltd, Tokyo, Japan).

188   *Statistical analysis*

189   Nine or 10 animals were studied in each group. All results were expressed as

190   mean ± SD. Comparisons between two groups were performed using an unpaired

191   Student's *t* test, and Dunnett's test for dose–response experiments. Levene's test was

192   used to assess the equality of variance. Trend test was performed using Jonckheere's

193   test. *P* < 0.05 was considered to be statistically significant.   Statistical analyses were

194   done on SAS software (ver.8.2).

195   **Results and discussion**

196   *Body and liver weights (Table1)*

197   Body and liver weight changes after exposure to PFOA in both groups of mice are

198   shown in Table 1. Absolute and relative liver weights (% body weight) were increased

199   approximately three fold in wild-type or *Pparα*-null mice, and induction of

200   hepatomegaly reached plateau levels in both genetic backgrounds at doses higher or

201   equal to 12.5 μmol/kg. These results demonstrated that PFOA induced hepatomegaly

202   through non-PPARα-mediated pathways as previously reported [19].

203

204   *Biochemical analysis (Table2)*

205   In wild-type mice, judging from the plasma AST and plasma ALT values, 12.5

206   or 25 μmol/kg PFOA caused hepatocellular damages with slight changes in T-Bil and

207   TBA. The hepatocellular damages seemed to   increase with increase in dose. Mild

208   cholestasis was apparent at 50 μmol/kg, at which dose, mild increases in T-Bil and TBA

209   were observed. T-Cho was decreased after treatment with PFOA at 25 and 50 μmol/kg.

210   TG was increased after treatment with PFOA at 12.5 and 25 μmol/kg, but was the same

211   level at 50μmol/kg.

212   In *Pparα*-null mice, 12.5 or 25 μmol/kg PFOA treatment induced mild

213   hepatocellular damages indicated by ALT but those changes were not accompanied by

214   elevation of T-Bil or TBA. At 50 μmol/kg, PFOA, however, induced extensive

215   hepatocellular damages and cholestasis simultaneously with a sharp contrast with wild

216   cholestasis in wild mice. TG metabolism was significantly disturbed, even at 12.5

217   μmol/kg, while cholesterol metabolism was disturbed only at the highest dose of 50

218   μmol/kg.

219   Biochemical analysis suggested a significant modification of liver toxicity

220   of PFOA by PPARα. Hepatocytes were more vulnerable than bile duct cells to

221   PFOA in wild-type mice. In contrast, ablation of PPARα rendered the

222   hepatocytes tolerable to PFOA-induced damage, at doses lower than 50

223   μmol/kg, while extensive hepatic and bile duct injuries occurred at 50

224   μmol/kg as shown in next section. In addition, metabolism of both T-Cho and

225   TG was impaired more extensively in *Pparα* null than wild-type mice.

226   *Histology*

227   In PFOA wild-type mice, PFOA induced hepatocellular hypertrophy. The

228   liver parenchyma showed dose-dependent eosinophilic cytoplasmic changes that were

229 morphologically consistent with peroxisome proliferation (Fig1 A-D). However, no fat

230 droplets or focal necrosis were observed in control or treated mice at any doses. Bile

231 duct epithelium showed a slight increase in thickness, which suggested that slight

232 cholangiopathy occurred at 25 and 50 µmol/kg (Fig. 1C, D).

233   The histological appearance in control *Pparα*-null mice showed greater

234 occurrence of microvesicular steatosis than in wild control mice (Fig. 1E). In

235 PFOA-treated *Pparα*-null mice, the hepatocytes showed not only hepatocellular

236 hypertrophy, but also cytoplasmic vacuolation and a increase in microvesicular steatosis

237 (Fig. 1F-H). Focal necrosis was detectable at 50 µmol/kg (Fig. 1I). The most

238 characteristic change was cholangiopathy. Although it was found in both wild and

239 PPARa null mice treated with PFOA at 25 (Fig. 1 C and G) and 50 µmol/kg (Fig.1 D

240 and H), it was more intensive in the latter than in former(Fig. 1C, D, G, H). In particular,

241 it was shown in *Pparα*-null mice that bile ducts were surrounded by a few inflammatory

242 cells and areas of fibrosis andbile plaque (Fig. 1H).

243   TUNEL staining demonstrated increased apoptosis in hepatic cells, hepatic

244 arterial walls and bile-duct epithelium in wild-type mice treated with PFOA at 25 and

245 50 µmol/kg (Fig. 2A, B, E, F). On the other hand, in *Pparα*-null mice, positive staining

246 was observed mainly in bile duct epithelium at 25 and 50 µmol/kg (Fig. 2C, D, G, H).

247   The ultrastructure of livers from control wild-type mice (Fig. 3A) exhibited

248   numerous glycogen granules, normal lamellar arrangement of the rough endoplasmic

249   reticulum (RER), a few normal dense peroxisomes and mitochondria. In contrast to

250   control livers, treated wild-type mice (Fig. 3B-D) displayed, dose-dependent,

251   hepatocyte hypertrophy, reduction or disappearance of glycogen granules, degranulation

252   and disruption of the RER, nuclear vacuoles, extensive peroxisome proliferation, and

253   slight proliferation of mitochondria. There were larger numbers and sizes of

254   dark-staining peroxisomes and increased small, round-shaped mitochondria (Fig. 3B-D,

255   I).

256   In control *Pparα*-null mice (Fig. 3E) there were discernible amounts of small fat

257   deposits in the cytoplasm. In treated *Pparα*-null mice (Fig. 3F-H) there were

258   dose-dependent hepatocyte hypertrophy, decreased amounts of glycogen granules,

259   degranulation and disruption of the RER, and increased numbers of mitochondria. There

260   is increased cytoplasmic lipid accumulation to varying extents, extensive mitochondrial

261   changes that consisted of slight swelling, decreased matrix density and inconspicuous

262   criste, but no peroxisome proliferation (Fig. 3F-H, J). In addition, bile duct epithelium

263   showed degradation of cytoplasmic structure, vacuolization, and disintegration of nuclei

264   and organelles. Severe bile duct epithelium injury was observed, with periductal

265   infiltration of fibroblasts and macrophages, and fibrosis (Fig. 3K).

266   *Pharmacokinetics of PFOA in whole blood, bile and liver (Table3)*

267   In order to investigate whether the absence of PPARα changed the

268   pharmacokinetics of PFOA, the concentration of PFOA was determined in whole blood,

269   bile and liver after dosing for 4 weeks. The concentrations of PFOA in whole blood

270   increased in proportion to dose, in both wild-type and *Pparα*-null mice (Table 3). On

271   the other hand, the concentrations in liver reached similar saturation levels at 12.5

272   μmol/kg in wild-type and *Pparα*-null mice.

273   The concentrations of PFOA in bile increased with dose; it increased by 13.8

274   times from 56.8 μg/ml at a dose of 12.5 μmol/kg to 784 μg/ml at 25 μmol/kg, and 38

275   times to 2170 μg/ml at a dose of 50 μmol/kg in wild-type mice. Enhanced PFOA

276   excretion indicate that the liver has a PFOA transport capacity from hepatocytes to

277   bile duct that can be mediated, at least partly by PPARα. In contrast, much lower

278   increases were observed in *Pparα*-null mice. PFOA concentrations increased by 3.2

279   times from 19.6 μg/ml at a dose of 12.5 μmol/kg to 62.9 μg/ml at 25 μmol/kg, and by

280   19.5 times to 383.0 μg/ml at a dose of 50 μmol/kg, demonstrating existence of capacity-

281   limited and PPARα-independent PFOA transport.

282   *8-OHdG levels in liver and quantitative RT-PCR of TNF-α mRNA*

283   In wild-type mice, PFOA did not elevate the levels of 8-OHdG in liver

284   significantly at any dose (Fig. 4A). In contrast, in *Pparα*-null mice, the levels of

285   8-OHdG tended to increase dose-dependently ($P < 0.05$), which was significantly

286   increased at 50 μmol/kg ($P < 0.05$) (Fig. 4A). PFOA did not alter the levels of *TNF-α*

287   mRNA in wild-type mice (Fig. 4B). However, PFOA upregulated *TNF-α* mRNA

288   significantly at doses of 25 and 50 μmol/kg in *Pparα*-null mice ($P < 0.01$ and $P < 0.05$,

289   respectively) (Fig. 4B).

290   These data demonstrated that ablation of PPARα exacerbated oxidative

291   damage and enhanced production of inflammatory cytokines after PFOA administration.

292   *Quantitative RT-PCR for Mdr2, bile acids/ phospholipids ratio in bile*

293   We investigated three prototypical hepatobiliary transporters. Mdr2 transports

294   biliary phospholipids from hepatocytes to bile via the canalicular phospholipid flippase,

295   which alleviates bile acid toxicity in cholangiocytes [20]. Bsep transports bile acid from

296   hepatocytes to bile via the canaliculi to keep bile acid concentrations constant in bile

297   [21,22], and is resistant to canalicular damage in humans [23]. Mrp2 is a transporter of bile

298   acid and is a sensitive indicator of canalicular damage [24]. Recently, PFOA has been

299   reported to regulate liver transporters, organic anion transporting polypeptides (Oatps)

300   and multidrug resistance–associated proteins responsible for uptake of bile acids (BAs)

301 and other organic compounds into liver, primarily via activation of PPARα [25,26]. For

302 investigating expression of Mdr2, we performed quantitative RT-PCR instead of

303 Western blotting because the Mdr2 antibody was not specific for mouse Mdr2 (data not

304 shown). In wild-type mice, the expression of *Mdr2* mRNA was significantly

305 upregulated by PFOA at 12.5, 25 and 50 μmol/kg ($P < 0.05$, $P < 0.01$ and $P < 0.01$,

306 respectively) (Fig. 4C). In *Pparα*-null mice, *Mdr2* mRNA was not induced by PFOA at

307 12.5 μmol/kg, however, it was induced significantly at 25 and 50 μmol/kg ($P < 0.05$ and

308 $P < 0.01$, respectively) (Fig. 4C). This non-PPARα-mediated increase in Mdr2 may

309 likely be attributable to the increase in bile acid at high dose[27] or other nuclear

310 receptors [28]. To confirm adaptive phospholipid transport, we examined the biliary bile

311 acid to phospholipid (BA/PL) ratio (Fig. 4D). As expected, BA/PL ratio decreased

312 significantly in a dose-dependent manner in PFOA-treated wild-type mice ($P < 0.01$).

313 However, no such significant adaptation was observed in PFOA-treated *Pparα*-null

314 mice, suggesting that bile duct protective mechanism characterized by increasing

315 phospholipid transport into bile did not work in the null mice.

316 *Western blotting for Bsep and Mrp2*

317   Protein levels of Bsep were downregulated in treated wild-type mice

318 significantly at 50 μmol/kg ($P < 0.01$). In contrast, in *Pparα*-null mice, protein level of

319   Bsep was increased significantly at 12.5 μmol/kg ($P < 0.01$), however decreased

320   significantly at 50 μmol/kg ($P < 0.05$) (Fig. 5). The decreased levels of Bsep in

321   *Pparα*-null mice were very likely induced by severe injury of the hepatobiliary system

322   and inflammation [29,30]. Protein levels of Mrp2 decreased in both wild-type mice and

323   *Pparα*-null mice at 50 μmol/kg ($P < 0.05$ for both types) (Fig. 5).

324   Bile duct transporters demonstrated that there were several PPARα-mediated

325   adaptive responses in wild-type mice to alleviate toxicity of PFOA, such as

326   up-regulation of Mdr2 and down-regulation of Bsep. In contrast, these responses were

327   not mobilized in concert in PFOA-treated *Pparα*-null mice. Ablation of PPARα made

328   mice highly susceptible to bile duct injury. Mrp2 protein levels decreased in both

329   wild-type and *Pparα*-null mice, which might be independent to PPARα.

330

331   To embark this study, we have hypothesized that PFOA is a potential toxicity for

332   bile duct as Bezafibrate does [9]. As expected, PFOA was shown to induce cholestatic

333   lesions more intensively in PPARa null mice than in wild mice as demonstrated by

334   clinical and pathological investigation. Simultaneously, we could demonstrate clear

335   differences in dose dependent mobilization of transporters, Mdr2 and Bsep, between

336   wild and null mice. Furthermore, there were differences in inducing 8-OHdG, TNF-a

337   induction and BA/PL ratios in bile between wild and null mice. This is the first study to

338   demonstrate a potential toxicity of PFOA for cholestatic disease and PPARa dependent

339   and independent responses.

340   Although hepatomegaly and increases in AST and ALT were observed in both

341   wild-type and PPARa null mice, microscopic appearance and ultrastructure of liver

342   indicated different modes of action, including biomarkers investigated in this study.

343

344   In terms of the mechanism of bile duct injury, we focused on the changes in bile

345   compositions and expression levels of hepatobilliary transporters. BA/PL ratio was

346   decreased immediately in PFOA-treated wild-type mice. On the contrary it was

347   increased at 12.5μmol/kg and decreased gradually at higher PFOA dose in *Ppara*-null

348   mice. In wild-type mice, the decreased BA/PL ratio may protect against bile duct-injury

349   induced by the effects of the toxic bile. Expression of *Mdr2* mRNA was clearly

350   upregulated in all treated wild-type mice, whereas it was less upregulated in *Ppara*-null

351   mice, which was consistent with BA/PL ratio in both groups of mice. The bile acid

352   transporter, Bsep, also showed different responses between wild and null mice.

353   Decreased Bsep levels were observed in both genetic background mice at higher doses,

354   while *Ppara*-null mice showed a transient increase in Bsep protein levels at lowest dose,

355   12.5 μmol/kg. Although the entire signal transduction for eliciting responses remains

356   entirely unknown, several other factors such as farnesoid X receptor-α (FXRα), which is

357   known to downregulate Bsep [31,32,33] and CAR [28] may also be involved.

358   In conclusion, this study revealed the new insights that PPARα is protective against

359   cholestastis induced by the weak PPARα ligand PFOA in using mouse model. PFOA

360   mobilized adaptive processes regulated by PPARα- fat metabolism by mitochondria and

361   peroxisomes, oxidative stress, TNF-α and hepatobiliary transport systems. So we

362   propose that PPARα activators may induce either hepatocellular or bile duct injury,

363   depending on their affinity to PPARα and dose level. If so, cholestasis and its associated

364   morbidities may also be taken into account for risk assessment of PFOA in humans

365   since species differences is well characterized in PPARα-associated signal transduction

366   [34] . Further studies are needed to clarify this hypothesis.

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481

482

Figure titles and legends

483 **Figure 1. Effects of PFOA on the mouse liver by oral gavage for 4weeks.**

484 Hematoxylin-eosin stained sections of liver from control wild-type mice (A), wild-type

485 mice treated with PFOA at 12.5 μmol/kg (B), 25 μmol/kg (C), 50 μmol/kg (D), control

486 *Pparα*-null mice (E), *Pparα*-null mice treated with PFOA at 12.5 μmol/kg (F), 25

487 μmol/kg (G), 50 μmol/kg (H, I). Original magnification, ×200, (A-H) ×40 (I). Wild-type

488 mice treated with PFOA (B-D) have diffuse hepatocyte hypertrophy with numerous

489 eosinophilic cytoplasmic granules. Control *Pparα*-null mice (E) has scattered small fat

490 vacuoles. Centrilobular fat accumulations were increased dose-independently in

491 *Pparα*-null mice treated with PFOA at 12.5 μmol/kg (F), 25 μmol/kg (G), 50 μmol/kg

492 (H, I). Focal necrosises are scattered with fat accumulation and proliferation of bile

493 ductules is prominent in the portal tracts in *Pparα*-null mice treated with PFOA at 50

494 μmol/kg (I). Diffuse hepatocyte hypertrophy was observed in both mouse lines treated

495 (B-D, F-H). Bile duct epithelial thickness (arrow) was observed in both mouse lines

496 treated at 25 μmol/kg (C, G) and 50 μmol/kg (D, H). Diffusely distributed, fine, fatty

497 droplets and ground-glass appearance is showed at 12.5 μmol/kg (F) and 25 μmol/kg

498 (G) in *Pparα*-null mice. Note hyperplastic changes in the biliary duct epithelium with

499 bile plaque (arrow head) and fibrosis (open circle) as evidenced by proliferation of bile

500 ductules (arrow) in *Pparα*-null mice treated with PFOA at 50 μmol/kg (H). cv, central

501 vein; pv, portal vein; ha, hepatic artery; bd, bile duct; f, fat droplet; ne, necrosis.

502 **Figure 2. Distribution of apoptotic cell in liver PFOA treated by oral gavage for**

503 **4weeks by immunohistochemistry for TUNEL.**

504 Wild-type mice treated with PFOA at 25 μmol/kg (A, E) and 50 μmol/kg (B, F),

505 *Pparα*-null mice treated with PFOA at 25 μmol/kg (C, G) and 50 μmol/kg (D, H).

506   Original magnification, ×100 (A-D), ×400 (E-H) the extended a part surrounded with a

507   square in A-D, respectively. Wild-type mice treated with PFOA at 25 μmol/kg (A, E)

508   and 50 μmol/kg (B, F) show diffuse positive stains in hepatocyte, vessel wall, and bile

509   duct epithelium (arrow). *Pparα*-null mice treated with PFOA at 25 μmol/kg (C, G) and

510   50 μmol/kg (D, H) show positive stains mainly in bile duct epithelium (arrow head).

511   cv, central vein; pv, portal vein; ha, hepatic artery; bd, bile duct.

512   **Figure 3. Ultrastructure of hepatocyte and bile duct epithelium cells in control and**

513   **after treatments of wild-type mice and *Pparα*-null mice with PFOA by oral gavage**

514   **for 4weeks.**

515   Hepatocytes from control wild-type mice (A), wild-type mice treated with PFOA at 12.5

516   μmol/kg (B), 25 μmol/kg (C), 50 μmol/ kg (D, I), Control *Pparα*-null mice (E),

517   *Pparα*-null mice treated with PFOA at 12.5 μmol/kg (F), 25 μmol/kg (G), 50 μmol/kg

518   (H, J), Bile duct epithelial cell (BEC) of *Pparα*-null mice treated with PFOA at 50

519   μmol/kg (K).

520   Numerous glycogen granules (circle) are observed in control wild-type mice (A). The

521   increased number and size of dark staining peroxisomes were shown in treated

522   wild-type mice (B-D, I). Hepatocytes from control *Pparα*-null mice (E) are similar to

523   control wild-type mice with fewer fat droplets (f) in cytoplasm. In contrast to controls,

524   treated *Pparα*-null mice (F-H, J) also display hepatocyte hypertrophy, decreased

525   glycogen granules, degranulation and disruption of the rough endoplasmic reticulum,

526   and increased mitochondria in dose-dependently. The marked different points contrasts

527   to wild-type mice treated with PFOA are increased fat droplets in cytoplasm, a few

528   peroxisomes, and a variable size and shape of mitochondria (F-H, J). Note that

529   peroxisomes are markedly increased and slightly enlarged in size in wild-type mice

530   treated with PFOA at 50 μmol/kg (I), and mitochondria are pleomorphic, enlarged (*),

531   and disorganization of cristae (arrowhead) in *Pparα*-null mice treated with PFOA at 50

532   μmol/kg (J). BECs (K) are showed degradation of cytoplasmic structure, vacuolization,

533   disintegration of nuclei and organelles and surrounded with fibroblasts and collagen. p,

534   peroxisome; f, fat droplet; v, vacuole. (A-H) Bar = 4 μm, (I, J) Bar = 1 μm, (K) Bar =

535   10 μm.

536   **Figure 4. (A) Effects of PFOA on 8-hydroxydeoxyguanosine from unfractionated**

537   **livers of wild-type and *Pparα*-null mice.** This figure reveals the levels of 8-OHdG tend

538   to increase dose-dependently in *Pparα*-null mice (Jonckheere`s test, $P < 0.05$), in which

539   the levels are increased significantly at 50 μmol/kg ($P < 0.05$).

540   **Effects of PFOA on hepatic expressions of (B) *TNF-α* mRNA and (C) *Mdr2***

541   **mRNA in wild-type mice and *Pparα*-null mice.** (B) The expressions of *TNF-α* mRNA

542   are significantly increased in *Pparα*-null mice treated with PFOA at 25 ($P < 0.01$) and

543   50 μmol/kg ($P < 0.05$). (C) The expressions of *Mdr2* mRNA are significantly

544   up-regulated in wild-type mice treated with PFOA at all doses (at 12.5 μmol/kg, 25

545   μmol/kg and 50 μmol/kg, $P < 0.05$, $P < 0.01$, respectively). In *Pparα*-null mice treated

546   with PFOA, the expressions of *Mdr2* mRNA are not induced at 12.5 μmol/kg, however

547   induced at 25 μmol/kg ($P < 0.05$) and 50 μmol/kg ($P < 0.01$) significantly.

548   **(D) Effects of PFOA on biliary total bile acid/phospholipid (BA/PL) ratio.**

549   Biliary BA/PL ratios show significant decrease in wild-type mice treated with PFOA

550   dose-dependently ($P < 0.05$). However, no such significant adaptation is observed in

551   *Pparα*-null mice treated with PFOA. Data are presented as mean ± SD from 9 to 10

552   animals in each group. Trend test is Jonckheere`s test. *$P < 0.05$, **$P < 0.01$ versus

553   control controls in each group. Log-transformation was performed for expressions of

554   *Mdr2* mRNA levels due to heteroscedusticity.

555   **Figure 5. Effects of PFOA on Hepatic Bsep and Mrp2 protein levels.**

556   Each panel represents an individual experiment. There is a significant decrease in Bsep

557   protein level in wild-type mice treated with PFOA at 50 μmol/kg ($P < 0.01$). In

558   *Pparα*-null mice treated with PFOA, the levels are increased significantly at

559   12.5 μmol/kg ($P < 0.01$), however decreased significantly at 50 μmol/kg ($P <$

560   0.05). There is a significant decrease in Mrp2 protein levels in both wild-type

561   and *Pparα*-null mice treated with PFOA at 50 μmol/kg ($P < 0.05$). Control

562   wild-type mice, w0; wild-type mice PFOA treated with 12.5 μmol/ kg, w12.5;

563   25 μmol/kg, w25, 50 μmol/kg, w50; control *Pparα*-null mice, n0; *Pparα*-null

564   mice treated with PFOA at 12.5 μmol/kg, n12.5; 25 μmol/kg, n25, 50 μmol/kg,

565   n50. Black bars, wild-type mice; white bars, *Pparα*-null mice. Densitometric

566   values are presented as mean ± SD of 3 animals in each group. *$P < 0.05$, **P

567   <0.01 versus control in each group. Trend test is Jonckheere`s test.

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569

Table 1 Body weight and liver weight changes after exposure to PFOA in wild-type and *Ppara*-null mice

PFOA dose	At the start of the experiment		At sacrifice after 4 week dosing				
Levels (μmol/kg)	Gross Body weight (g)	Body weight - Liver weight (g)[a]	Gross Body weight (g)	Liver weight (g)	Relative liver weight (%)	Body weight - Liver weight (g)	Body weight gain excluded liver
Wild-type							
0 (n=9)	23.9 ± 1.97	23.0 ± 1.89	26.6 ± 2.13	1.0 ± 0.08	3.7 ± 0.4	25.7 ± 2.22	2.7 ± 1.36
12.5 (n=10)	23.8 ± 0.79	22.9 ± 0.76	27.5 ± 2.07	3.2 ± 0.20 ***	11.3 ± 0.6***	24.7 ± 1.98	1.8 ± 1.95
25 (n=10)	24.2 ± 1.98	23.3 ± 1.90	25.5 ± 1.94	3.3 ± 0.30 ***	12.9 ± 0.8***	22.5 ± 1.71	-0.9 ± 1.64 ***
50 (n=10)	24.5 ± 1.67	23.6 ± 1.61	23.0 ± 2.90 **	3.3 ± 0.45 ***	13.1 ± 0.9***	20.5 ± 2.50	-3.1 ± 2.09 ***
PPARα[(-/-)]							
0 (n=10)	22.7 ± 1.53	21.6 ± 1.46	25.0 ± 1.56	1.0 ± 0.12	4.7 ± 2.1	24.1 ± 1.37	2.5 ± 0.58
12.5 (n=10)	23.2 ± 1.87	22.1 ± 1.78	27.9 ± 1.99 **	3.3 ± 0.45 ***	11.6 ± 1.7 ***	25.1 ± 1.78	3.0 ± 1.44
25 (n=10)	23.5 ± 1.54	22.4 ± 1.47	27.4 ± 0.93 *	3.4 ± 0.23 ***	11.9 ± 1.2 ***	24.5 ± 1.16	2.1 ± 1.37
50 (n=10)	23.4 ± 1.88	22.3 ± 1.80	26.4 ± 2.07	3.4 ± 0.51 ***	13.0 ± 1.6 ***	23.7 ± 2.64	1.4 ± 1.98

Note: Values are expressed as mean ± SD

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Dunnett's test compared with 0 μmol/kg

[a] Liver weight was assumed as 3.7% in wild-type and 4.7% in *Ppara*-null mice of before body weight.

Table 2 Plasma analysis of PFOA-treated wild-type and *Ppara*-null mice

PFOA dose levels (μmol/kg)	AST (IU/L)		ALT (IU/L)	
	Wild-type	PPARα$^{(-/-)}$	Wild-type	PPARα$^{(-/-)}$
0 (n=9, 10)	145± 71.1	137 ± 25.9	26 ± 7.1	23 ± 7.2
12.5 (n=10)	175 ± 29.2	145 ± 31.5†	176± 62.4^{+++}	136 ± 45.3^{+++}
25 (n=10)	265 ± 146.2*	152 ± 20.2†	284± 158.9^{+++}	176 ± 42.8^{+++}
50 (n=10)	365 ± 106.0***	870± 523.5***††	328± 128.9^{+++}	1356 ± 744$^{+++††}$

PFOA dose levels (μmol/kg)	T-Bil (mg/dl)		TBA (mmol/L)	
	Wild-type	PPARα$^{(-/-)}$	Wild-type	PPARα$^{(-/-)}$
0 (n=9, 10)	0.09 ± 0.05	0.06 ± 0.02	4.5 ± 7.2	2.4 ± 2.6
12.5 (n=10)	0.05 ± 0.01*	0.02 ± 0.01†††	4.5 ± 1.6	1.0 ± 0†††
25 (n=10)	0.09 ± 0.03	0.03 ± 0.01†††	9.0 ± 4.6	1.4 ± 0.6††
50 (n=10)	0.15 ± 0.04**	0.47 ± 0.39****†	12.5 ± 9.9	34.8 ± 9.1***††

PFOA dose levels (μmol/kg)	T-Cho (mg/dl)		TG (mg/dl)	
	Wild-type	PPARα$^{(-/-)}$	Wild-type	PPARα$^{(-/-)}$
0 (n=9, 10)	115 ± 9.7	136 ± 26.8†††	59 ± 17.6	45 ± 17.3
12.5 (n=10)	109 ± 17.7	84 ± 21.9***†	87 ± 15.7**	91 ± 35.5**
25 (n=10)	95 ± 15.4**	87 ± 13.7***	89 ± 28.4**	105 ± 23.8***

50 (n=10)	86 ± 11.6***	226 ± 23.0***†††	51 ± 18.4	114 ± 32.1***†††

AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin

TBA, total bile acid; T-Cho, total cholesterol; TG, triglyceride

Data are expressed as mean ± SD

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Dunnett's test compared with 0 μmol/kg

+$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$ by Dunnett's test after log-transformation due to heteroscedasticity (Levene's test $P < 0.05$)

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ by t-test compared with wild-type and *Ppara*-null mice at same PFOA-dose level

Table 3 Whole blood, bile and liver concentrations of PFOA in wild-type and *Ppara*-null mice

	PFOA concentration (µg/ml)					
PFOA dose levels (µmol/kg)	Whole blood		Bile		Liver	
	Wild-type	PPARα$^{(-/-)}$	Wild-type	PPARα$^{(-/-)}$	Wild-type	PPARα$^{(-/-)}$
0 (n=9, 10)	nd	nd	nd	nd	nd	nd
12.5 (n=10)	20.6 ± 2.4	19.3 ± 2.2	56.8 ± 26.9	19.6 ± 2.2	181.2 ± 6.3	172.3 ± 8.9
25 (n=10)	46.9 ± 3.2	36.4 ± 2.7[*]	784.0 ± 137.6	62.9 ± 16.7[**]	198.8 ± 15.4	218.3 ± 14.5
50 (n=10)	64.2 ± 6.5	71.2 ± 8.0	2174.0 ± 322.4	383.0 ± 109.9[**]	211.6 ± 13.3	239.7 ± 25.0

Data are expressed as mean ± SD.

*P < 0.05, **P < 0.01, ***P < 0.001 by *t*-test compared between wild-type and *Ppara*-null mice.
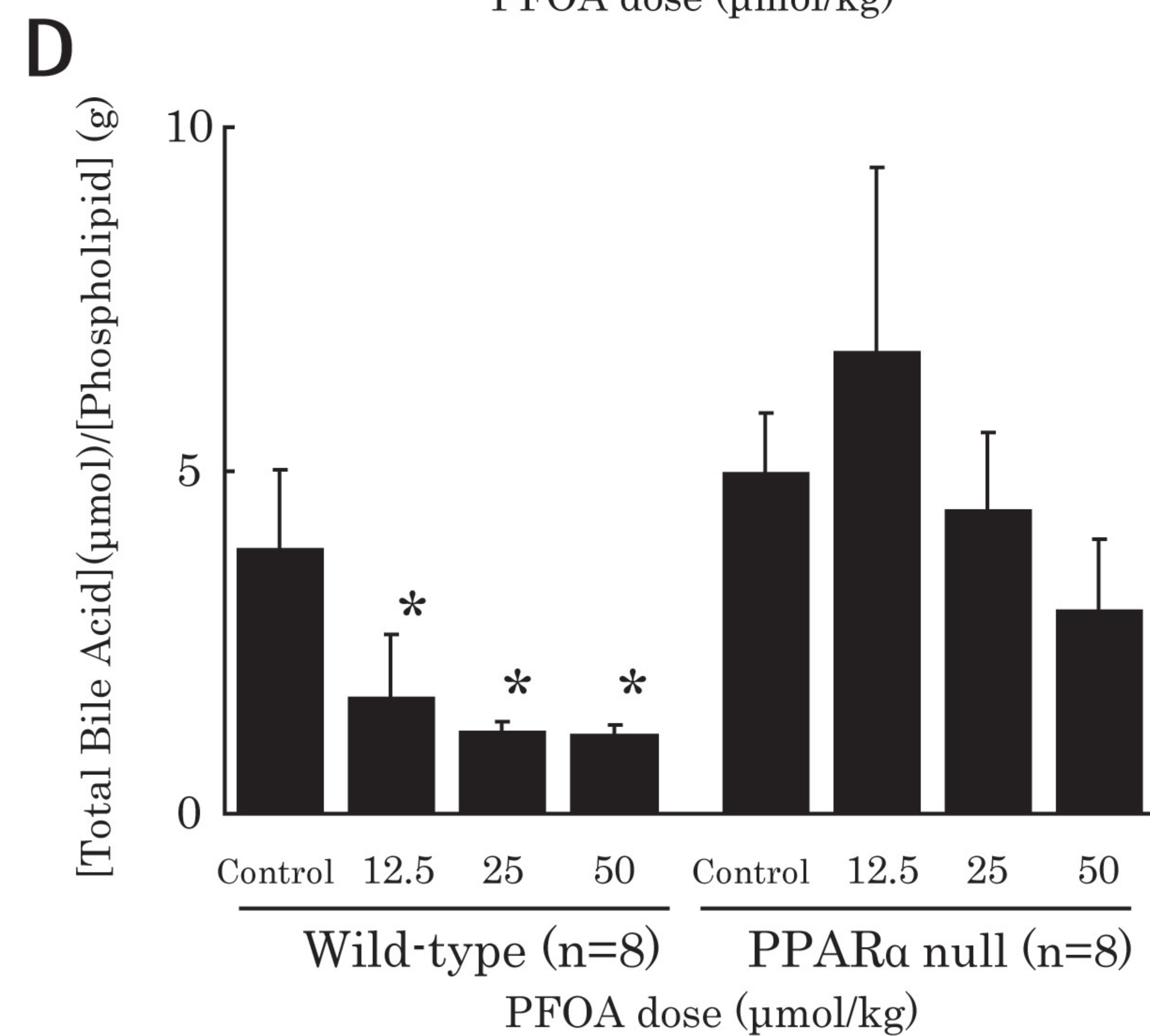
nd; not detected (less than 0.001µg/ml)

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5