

Short paper

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## Hepatocellular proliferation in response to agonists of peroxisome proliferator-activated receptor $\alpha$ : a role for kupffer cells?

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### Abstract

**Background:** It has been proposed that PPAR $\alpha$  agonists stimulate Kupffer cells in rodents which in turn, release mitogenic factors leading to hepatic hyperplasia, and eventually cancer. However, Kupffer cells do not express PPAR $\alpha$  receptors, and PPAR $\alpha$  agonists stimulate hepatocellular proliferation in both TNF $\alpha$ - and TNF $\alpha$  receptor-null mice, casting doubt on the involvement of Kupffer cells in the mitogenic response to PPAR $\alpha$  agonists. This study was therefore designed to investigate whether the PPAR $\alpha$  agonist PFOA and the Kupffer cell inhibitor methylpalmitate produce opposing effects on hepatocellular proliferation and Kupffer cell activity *in vivo*, in a manner that would implicate these cells in the mitogenic effects of PPAR $\alpha$  agonists.

**Methods:** Male Sprague-Dawley rats were treated intravenously *via* the tail vein with methylpalmitate 24 hrs prior to perfluorooctanoic acid (PFOA), and were sacrificed 24 hrs later, one hr after an intraperitoneal injection of bromodeoxyuridine (BrdU). Sera were analyzed for TNF $\alpha$  and IL-1 $\beta$ . Liver sections were stained immunohistochemically and quantified for BrdU incorporated into DNA.

**Results:** Data show that PFOA remarkably stimulated hepatocellular proliferation in the absence of significant changes in the serum levels of either TNF $\alpha$  or IL-1 $\beta$ . In addition, methylpalmitate did not alter the levels of these mitogens in PFOA-treated animals, despite the fact that it significantly blocked the hepatocellular proliferative effect of PFOA. Correlation between hepatocellular proliferation and serum levels of TNF $\alpha$  or IL-1 $\beta$  was extremely poor.

**Conclusion:** It is unlikely that mechanisms involving Kupffer cells play an eminent role in the hepatic hyperplasia, and consequently hepatocarcinogenicity attributed to PPAR $\alpha$  agonists. This conclusion is based on the above mentioned published data and the current findings showing animals treated with PFOA alone or in combination with methylpalmitate to have similar levels of serum TNF $\alpha$  and IL-1 $\beta$ , which are reliable indicators of Kupffer cell activity, despite a remarkable difference in hepatocellular proliferation.

## Background

Treatment of rodents with agonists of PPAR $\alpha$  results in liver cancer *via* mechanisms that remain unclear. While some studies implicate oxidative stress, caused by an overproduction of H<sub>2</sub>O<sub>2</sub>, as a consequence of peroxisome proliferation [1], others have suggested that these agonists increase rates of hepatocyte DNA synthesis leading eventually to the development of liver tumors [2,3].

In an attempt to delineate how PPAR $\alpha$  agonists may initiate hepatocellular proliferation, investigators have proposed that these agonists stimulate Kupffer cells, the resident liver macrophages, which in turn release mitogenic factors leading to hepatic hyperplasia [2,4,5]. This conclusion is supported by the observation that presumed inactivation of Kupffer cells prevented the mitogenic effect of the PPAR $\alpha$  agonist Wy-14,643 [4]. Furthermore, presence of nonparenchymal cells was required for replicative DNA synthesis in hepatocytes cultured in the presence of Wy-14,643 [6]. In addition, antibodies against tumor necrosis factor *alpha* (TNF $\alpha$ ), presumably released by Kupffer cells upon their activation by agonists of PPAR $\alpha$ , blocked the increase in liver cell replication in response to Wy-14,643. Finally, it was reported that TNF $\alpha$  suppressed apoptosis and induced DNA synthesis, effects which are similar to those produced by PPAR activators [7].

Results of a previous study in our laboratory do not support the aforementioned conclusions [8], and others [9] showed that activating the retinoid X receptors, the obligatory heterodimer of PPAR, inhibited TNF $\alpha$  production by isolated Kupffer cells. Importantly, it has been shown that Kupffer cells do not express PPAR $\alpha$  receptors [10], and that PPAR $\alpha$  agonists were able to stimulate hepatocellular proliferation in both TNF $\alpha$ - and TNF $\alpha$  receptor-null mice [11,12]. These findings cast doubt on the role of Kupffer cells in the hepatocellular proliferation known to occur in response to PPAR $\alpha$  agonists in rodents.

Serum levels of TNF $\alpha$  and IL-1 $\beta$  are reliable indicators of the Kupffer cell activity status [13]. Thus, according to the hypothesis stipulating that PPAR $\alpha$  agonists activate Kupffer cells as a prerequisite for the induction of hepatocellular proliferation, PFOA is expected to elevate serum levels of these mitogens. Also, it would be expected that hepatocellular proliferation and serum levels of TNF $\alpha$  or IL-1 $\beta$  will be blunted by methylpalmitate which is a known inhibitor of Kupffer cells. Data show that PFOA at a dose which remarkably stimulated hepatocellular proliferation did not produce significant changes in the serum levels of either TNF $\alpha$  or IL-1 $\beta$ . Furthermore, results reveal that Kupffer cell activity and PFOA-induced hepatocellular proliferation are unrelated phenomena, as evidenced by the fact that methylpalmitate did not diminish serum

levels of TNF $\alpha$  or IL-1 $\beta$  when given in combination with PFOA, yet it significantly blocked the hepatocellular proliferative effect of this PPAR $\alpha$  agonist. It is therefore unlikely that mechanisms involving the stimulation of Kupffer cells are responsible for the hyperplasia and the consequent hepatocarcinogenicity attributed to PPAR $\alpha$  agonists in rodents.

## Methods

### **Animal treatment and determination of serum TNF $\alpha$ , IL-1 $\beta$ and triglycerides**

Male Sprague-Dawley rats (150–200 g) were purchased from Sasco (Omaha, NE). Animals received humane care in compliance with the National Research Council's criteria outlined in "Guide for the Care and Use of Laboratory Animals." Rats were treated intravenously *via* the tail vein with methylpalmitate (2 g/kg), or the 20% Tween 80+5% glucose vehicle, 48 hrs prior to sacrifice. Rats also received PFOA (100 mg/kg orally), or the corn oil vehicle, and were sacrificed 24 hrs later, one hr after an intraperitoneal injection of 100 mg/kg bromodeoxyuridine (BrdU) in 0.05 N NaOH. Animal sera were collected and analyzed for TNF $\alpha$ , IL-1 $\beta$  using commercially available kits (R&R Systems, Minneapolis, MN).

### **Measurement of hepatic peroxisomal $\beta$ -oxidation activity and cell proliferation**

$\beta$ -Oxidation assays were performed on liver homogenates as previously reported [14]. Briefly, liver samples were excised and homogenized in 0.25 M sucrose (20% w/v). Cyanide-insensitive palmitoyl-CoA oxidation was used as a measure of activity of peroxisomal  $\beta$ -oxidation enzymes, and was assayed by monitoring the rate of NAD<sup>+</sup> reduction spectrophotometrically at 240 nm using the method of Lazarow and DeDuve [15]. A unit of activity equals 1  $\mu$ mol NAD<sup>+</sup> reduced/min.

A mid-lobe radial section of the right anterior lobe of the liver was flash frozen in liquid nitrogen, embedded in paraffin and serial tissue sections were mounted onto poly-L-lysine coated slides. Following deparaffination and dehydration, one set of slides was stained immunohistochemically for BrdU incorporation, routinely performed in our laboratories [16,17]. Random areas of the slides were chosen for counting stained and unstained hepatocytes (>1000 cells/animal).

### **Statistical Analysis**

Statistics were performed using one-way analysis of variance (ANOVA), or t-test, as appropriate P < .05 was considered significant.

## Results

### Effect of methylpalmitate on PFOA-induced

#### hepatocellular proliferation and peroxisomal $\beta$ -oxidation

PFOA significantly increased liver/body weight ratios from control values of  $4.2 \pm 0.2\%$  to  $5.5 \pm 0.2\%$ , an effect which was not altered by prior administration of methylpalmitate (Fig 1A). Since the increase in liver weight in response to PPAR agonists has a hyperplastic as well as a hypertrophic component, we investigated the effect of methylpalmitate on the hyperplastic component which is blamed for the hepatocarcinogenic effect of these chemicals. Methylpalmitate significantly reduced PFOA-induced levels of BrdU incorporation into hepatocyte DNA (Fig 1B). Hepatocellular BrdU labeling index was  $0.66 \pm 0.21\%$  in control rats (Fig 1B). PFOA increased labeling indices to  $5.8 \pm 0.9\%$  (Fig 1B), and pretreatment with methylpalmitate diminished PFOA-induced labeling indices by 57% (Fig 1B).

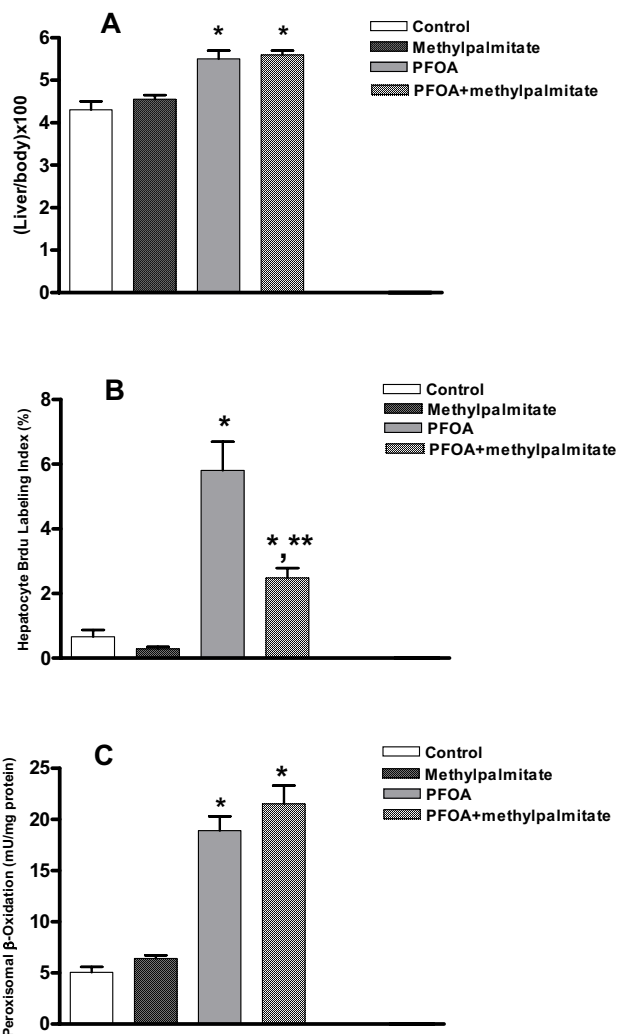
In contrast to the significant inhibitory effect exerted by methylpalmitate on PFOA-induced hepatocellular proliferation, methylpalmitate did not influence the ability of this PPAR $\alpha$  agonist to induce liver peroxisomal  $\beta$ -oxidation activity. PFOA caused a significant increase in peroxisomal  $\beta$ -oxidation activity from a basal value of  $5.1 \pm 0.53$  mU/mg protein to  $19.1 \pm 1.4$  mU/mg protein (Fig 1C). Activity remained at  $21.5 \pm 1.8$  mU/mg protein when methylpalmitate was given simultaneously with PFOA (Fig 1C).

### Serum TNF $\alpha$ and IL-1 $\beta$ Levels in Treated Rats

In PFOA-treated animals, serum levels of TNF $\alpha$  were  $67 \pm 9$  pg/ml (Fig 2). Treatment with methylpalmitate prior to PFOA did not exert detectable effects on these levels reaching  $71 \pm 5$  pg/ml (Fig 2A). Similarly, serum levels of IL-1 $\beta$  in PFOA-treated rats of  $62 \pm 8$  pg/ml were not significantly altered by prior administration of methylpalmitate, as levels were  $54 \pm 6$  pg/ml (Fig 2B). In examining whether hepatocellular labeling indices correlated with serum levels of either TNF $\alpha$  or IL-1 $\beta$ , it was found that such a correlation did not exist, as  $r^2$  values ranged from 0.021 to 0.0016, respectively (Fig 3).

## Discussion

Increased hepatocellular replication has been advanced as an important factor in liver cancer induced by PPAR $\alpha$  agonists in rodents [18]. In an attempt to elucidate mechanisms involved in the hepatocellular proliferation caused by these agonists, studies have produced conflicting evidence with regards to the role of Kupffer cells in this process [19-23]. This study was therefore undertaken to test the hypothesis that hyperplasia induced by PPAR $\alpha$  agonists correlates with Kupffer cell activity. The executed experiments examined the effect of the selective PPAR $\alpha$  agonist, PFOA [24] on serum levels of TNF $\alpha$  and IL-1 $\beta$ ,

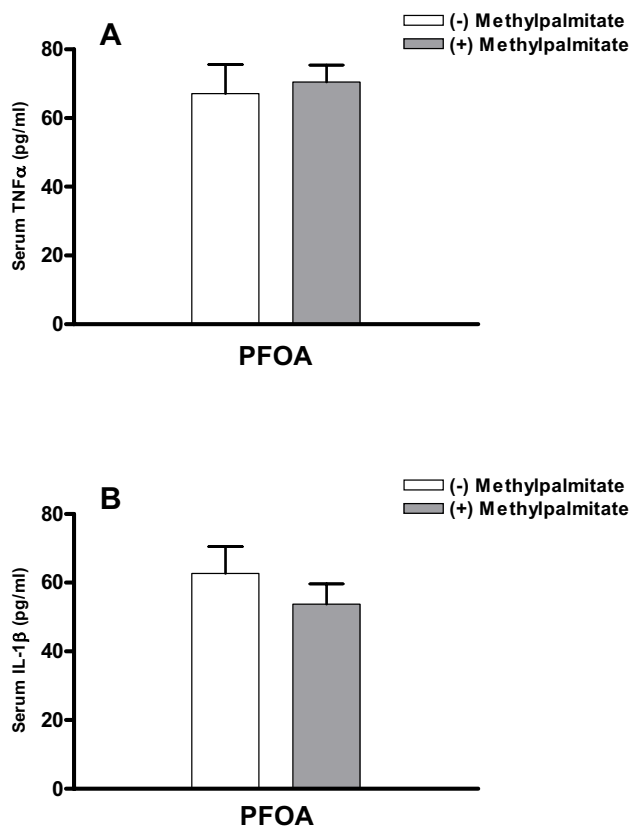


**Figure 1**

**Effect of methylpalmitate on liver/body weight ratios, hepatocellular proliferation and peroxisomal  $\beta$ -oxidation in response to the PPAR $\alpha$  agonist PFOA.** Animals were treated, livers were excised, weighed, and liver/body weight ratios were calculated (A). Hepatocyte BrdU labeling indices (B), and peroxisomal  $\beta$ -oxidation activity (C) were determined as described under "Methods". Data are means  $\pm$  SEM of 5 animals per group. \* $p < 0.05$  compared to the control group. \*\* $p < .01$  compared to the PFOA group.

reliable indicators of Kupffer cell activity [13], in the absence and presence of methylpalmitate, known inhibitor of Kupffer cell activity [8].

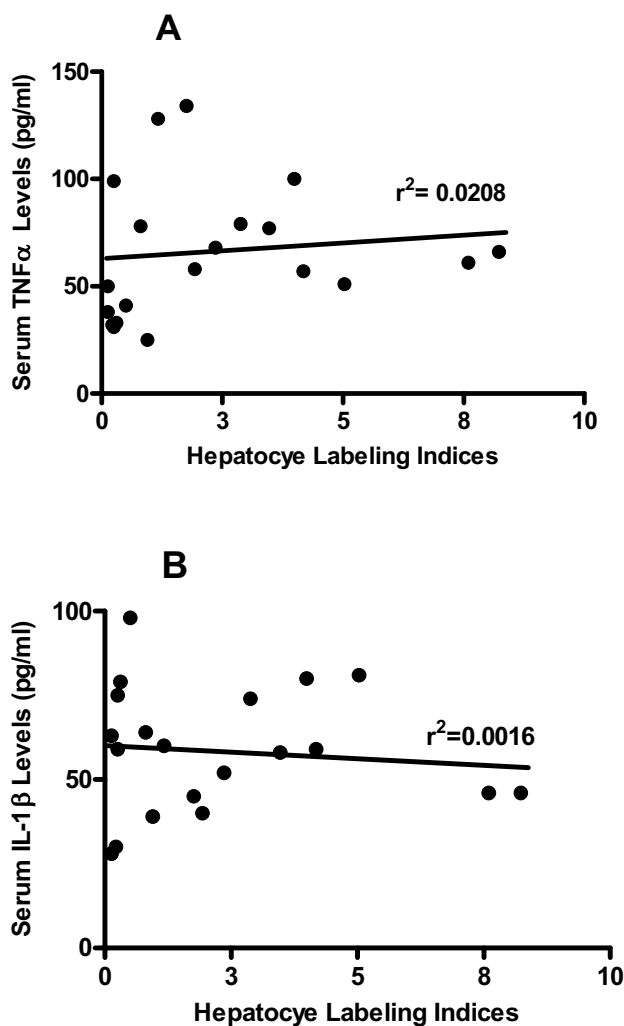
Consistent with previous studies [25], PFOA caused significant hepatomegaly and hyperplasia in treated animals (Fig 1A & 1B). This effect was observed in the absence of a



**Figure 2**  
**Effect of methylpalmitate on serum levels of TNF $\alpha$  and IL-1 $\beta$ .** Animals were treated, and sera were collected and analyzed for TNF $\alpha$  (A) and IL-1 $\beta$  (B) as described under "Methods". Data are means  $\pm$  SEM of 5 animals per group.

concomitant effect on serum levels of TNF $\alpha$  and IL-1 $\beta$  (Fig 2). Furthermore, methylpalmitate which is a known inhibitor of Kupffer cell activity failed to modulate the effect of PFOA on TNF $\alpha$  and IL-1 $\beta$  serum levels (Fig 2), while significantly diminishing its hepatoproliferative effect (Fig 1B), dissociating these two phenomena. Indeed, examining the relationship between BrdU hepatocyte labeling indices and serum TNF $\alpha$  and IL-1 $\beta$  levels in all treated animals revealed a very poor correlation (Fig 3), making the assertion that activation of Kupffer cells is a prerequisite for the hepatocellular proliferation caused by PPAR $\alpha$  agonists a strenuous one.

It is noteworthy that results observed in this study with methylpalmitate mimic very closely those we obtained earlier with the mitochondrial inhibitor, rotenone [26]. In a previous study, we showed that rotenone inhibited hepatocellular proliferation in response to the PPAR $\alpha$  agonist Wy-14,643 without interfering with the ability of



**Figure 3**  
**Lack of correlation between BrdU labeling indices and serum mitogen levels.** Linear regression analyses were performed on data generated from animal groups depicted in Fig 1, for TNF $\alpha$  (A) and IL-1 $\beta$  (B).

this agonist to induce peroxisomal  $\beta$ -oxidation [26], an effect observed in this study for methylpalmitate with PFOA as the PPAR $\alpha$  agonist (Fig 1B & 1C). In addition, preliminary results (not shown) indicate that methylpalmitate produces effects on serum lipid metabolites and hepatocellular BrdU indices in the opposite direction from those caused by the natural congener, palmitic acid. This finding suggests that methylpalmitate may interfere with intermediary metabolism in a manner that diminishes the cellular ability to produce energy necessary for fueling cell proliferation. However, the exact mechanism by which this fatty acid derivative selectively inhibits hepatocyte, but not peroxisomal, proliferation in

response to the PPAR $\alpha$  agonists remains unclear, and requires further investigation.

### Authors' contributions

IA and WB treated animals, and collected tissues and sera, as well as performed peroxisomal  $\beta$ -oxidation assays. MLC measured serum mitogen levels, and performed BrdU labeling experiments. MB conceived, designed and coordinated the study.

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