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# Blunted Induction of Hepatic CYP4A in TNF ($p55^{-/-}/p75^{-/-}$) Double Receptor Knockout Mice following Clofibrate Treatment

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## INTRODUCTION

The cytochrome P450 (CYP) superfamily of monooxygenase enzymes catalyze the oxidation of a diverse array of xenobiotics and endogenous compounds. The CYP4A subfamily is known to perform the ω- and ω-1-hydroxylation of endogenous substrates, including, fatty acids, prostaglandin and arachidonic acid (1–3). This production of hydroxylated fatty acid metabolites produce physiologically relevant effectors, such as 20-hydroxyeicosatetraenoic acid, which is known to constrict microvessels in the periphery and brain (3). In the liver, CYP4A catalyzes the first step (ω-hydroxylation) in the metabolic conversion of fatty monocarboxylic acids to dicarboxylic acids, the preferred substrate for peroxisomal oxidation (2). Therefore, it can be surmised that alterations in CYP4A expression through xenobiotic exposure or disease processes, such as, acute inflammation may influence the metabolic production or activation of important endogenous effectors.

In rodents, CYP4A is significantly induced in the liver by peroxisome proliferating xenobiotics, such as clofibrate (4,5). Induction of CYP4A by peroxisome proliferators is tightly regulated through activation of the peroxisome proliferator activated receptor-alpha (PPARα) [for review see (4)], a member of the nuclear hormone receptor family of transcriptional regulators.

In addition to xenobiotics, initiation of an acute phase response by the administration of bacterial endotoxin (LPS) or particulate irritants is known to induce CYP4A through a PPARα dependent mechanism (6–9). The acute phase response is a complex cascade of events involving a number of inflammatory cytokines including tumor necrosis factor-α (TNFα) (10). To date, the effect of TNFα signaling on peroxisome proliferator mediated induction of CYP4A in the liver has not been evaluated, although several lines of evidence suggest that TNFα may play a prominent modulatory role in this induction process. First, TNFα administration has been shown to depress the expression of PPARα and a number of peroxisomal enzymes in the rat liver (11). Therefore, it would be expected that PPARα dependent induction of CYP4A would also be modulated by TNFα. Second, in rats, administration of TNFα produces similar alterations in hepatic CYP expression as administering LPS (12). These data suggest that TNFα signaling is an important modulator of hepatic CYP expression during an acute phase response, a physiologic condition known to affect CYP4A expression. Therefore, we hypothesize that the induction of CYP4A by the peroxisome proliferator clofibrate is modulated by endogenous TNFα signaling *in vivo*. To test this hypothesis we examined the induction response of hepatic CYP4A in TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice and wild-type mice following clofibrate administration. Additionally, hepatic catalase activity was examined as a marker of peroxisome proliferation.

## METHODS

### Animals and Treatment

Unless otherwise specified, all chemicals were obtained from Sigma Chemicals (St. Louis, Missouri). Twelve to 15-week-old adult male TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice (KO) were generated as previously described (13,14) and maintained on a C57BL/6 X 129 hybrid background (WT). Animals were allowed food and water ad libitum. Clofibrate (400 mg/kg in corn oil) was administered i.p. once daily for three consecutive days to treated animals; control animals received an equivalent volume of corn oil vehicle. Food was removed at midnight on day three and the animals were euthanatized under halothane anesthesia between 7:00 and 9:00 am on day 4. The livers and kidneys were excised, rinsed with ice-cold normal saline, snap frozen in liquid nitrogen and stored at –80°C until analyzed. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

### Microsome Preparation

Microsomes were prepared as previously described (15). In brief, liver tissues were homogenized in 0.154 M KCl, 0.25 M potassium phosphate buffer, pH 7.4 with butylated hydroxy-toluene added as an antioxidant, and centrifuged at 10,000 × g to pellet the membranes. The supernatant was centrifuged at 105,000 × g to separate the microsomal and cytosolic fractions. The microsomal pellet was washed once with 0.154 M KCl. The resulting microsomal pellet was resuspended in 0.25 M sucrose / 0.02 M Tris buffer, pH 7.4 and stored at –80°C until analyzed. Spectral analysis of total CYP content was performed according to the method of Omura

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**Abbreviations:** Cytochrome P450 4A, CYP4A; (TNF ($p55^{-/-}/p75^{-/-}$) Double Receptor Knockout Mice, KO; Interleukin-6, IL-6, Tumor Necrosis Factor-Alpha, TNFα; Wild-Type Mice, WT.



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and Sato (16). Total protein content was determined by the method of Lowry (17).

### Microsomal and Cytosolic Enzyme Activities

The CYP4A subfamily is known to catalyze the ω- and ω-1-hydroxylation of lauric acid in mouse liver and kidney (1,18). As verification, two independent methods were utilized to determine lauric acid hydroxylase activity in these microsomes. The combined ω- and ω-1-lauric acid hydroxylase activity (total-lauric acid hydroxylase activity) was measured according to the radiometric method of Giera and van Lier (19). The individual ω- and ω-1-lauric acid hydroxylase activities were determined using the $^{14}$C-lauric acid reverse phase HPLC method of Romano *et al.* (20). Catalase activity was determined according to the spectrophotometric method of Beers and Sizer (21).

### Western Blot Analysis

Microsomal protein (7.5 ug) and 1170 fmol clofibrate induced rat CYP4A1 standard (Gentest, Woburn, MA) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5% polyacrylamide) and blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California). The nitrocellulose membranes were blocked with 5% non-fat milk in phosphate buffered saline (PBS), probed with polyclonal goat anti-rat CYP4A1 antibody (Gentest, Woburn, Massachusetts) in PBS 1% non-fat milk, extensively washed in PBS 0.1% Tween 20 and incubated with alkaline phosphatase-conjugated monoclonal anti-goat/sheep IgG antibody. The membranes were washed and developed by soaking in CDP-Star/Nitro-Block II (Tropix, Bedford, Massachusetts) chemiluminescent substrate and exposed to X-ray film according to the manufacturers directions. Band densities were quantitated utilizing NIH image version 1.59 software and a Power Macintosh based image analysis system. The standard curve demonstrated linearity from 0 to 3512 fmol CYP4A1 per lane.

### Statistical Analysis

Multiple comparisons were performed utilizing SPSS software (SPSS, Chicago IL). All comparisons were made via a two factor ANOVA with Fisher's LSD post-hoc determination. Statistical significance was set at $P < 0.05$ *a priori.*

## RESULTS

### Effect of TNF p55/p75 Double Receptor Knockout on Clofibrate Induction of Hepatic CYP4A Activities and Protein

No significant differences were detected in constitutive CYP4A activity or protein content between KO and WT mice (Fig. 1A–C and Fig. 2A,B). Clofibrate treatment significantly induced CYP4A activity and protein in both KO and WT mice ($P < 0.05$, Fig. 1A–C and Fig. 2). A significant interaction effect ($P < 0.05$) of TNF double receptor knockout on clofibrate induction occurred for all three CYP4A associated activities analyzed (total-, ω- and ω-1-lauric acid hydroxylase activity), demonstrating a blunting of induction in KO mice (Fig. 1A–C, respectively). The induction of total-, ω- and ω-1-lauric acid hydroxylase activities observed in the KO mice

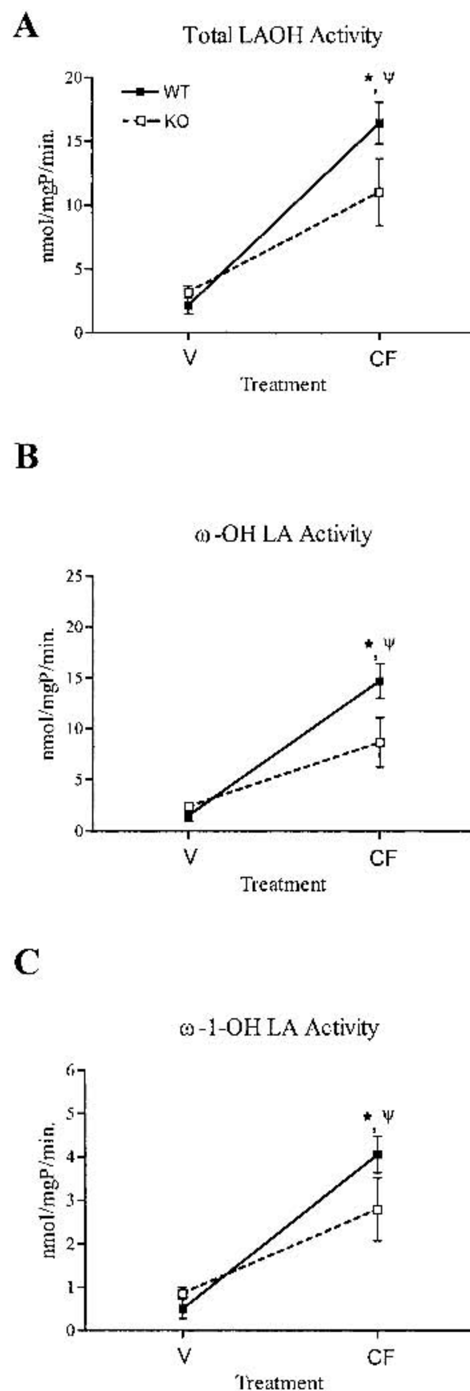


**Fig. 1.** Blunted induction of hepatic CYP4A associated lauric acid hydroxylase activities in TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice following clofibrate administration. TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice (KO) and wild-type mice (WT) were administered clofibrate (400 mg/kg in corn oil) or corn oil once daily on three consecutive days. Microsomal CYP4A associated lauric acid hydroxylase activities were determined as described in the Methods. Abbreviation: LAOH = lauric acid hydroxylase. Asterisk denote significant difference from WT for a given treatment [clofibrate (CF) or corn oil vehicle (V)], psi indicates a significant induction of lauric acid hydroxylase activity following clofibrate treatment in WT and KO mice. All interactions of TNF double receptor knockout on clofibrate induction of lauric acid hydroxylase activities were significant, with significance set at $P < 0.05$, n = 7.

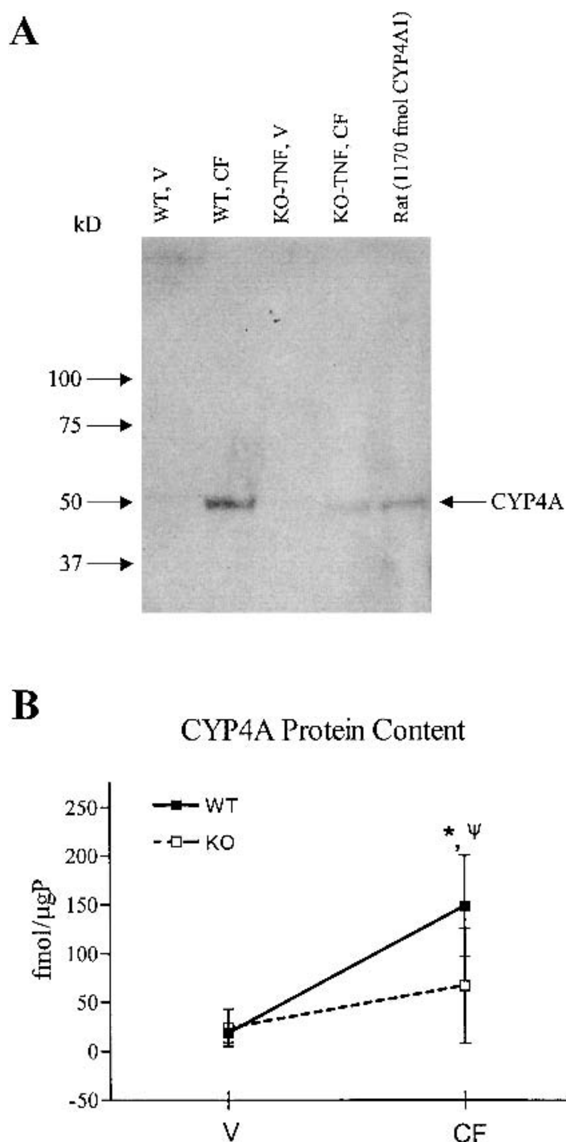


**Fig. 2.** Western blot analysis demonstrates the blunted induction of hepatic CYP4A protein in TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice following clofibrate administration. TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice (KO) and wild-type mice (WT) were administered clofibrate (CF; 400 mg/kg in corn oil) or corn oil once daily on three consecutive days. (A) 7.5 μg of liver microsomal protein from individual animals and CYP4A1 standard rat microsome were separated by SDS polyacrylamide electrophoresis, blotted onto nitrocellulose and probed with anti-rat CYP4A1 polyclonal antibody as described in the Methods. (B) Densitometric quantification of the CYP4A band for individual animals on Western blot is presented (n = 6). Abbreviation: μgP = μg of microsomal protein. Asterisk denote significant difference from WT for a given treatment [clofibrate (CF) or corn oil vehicle (V)], psi indicates a significant induction of CYP4A protein content following clofibrate treatment in WT and KO mice. All interactions of TNF double receptor knockout on clofibrate induction of CYP4A protein content were significant, with significance set at $P < 0.05$.

were 67, 59% and 69% of WT mice, respectively. Analysis of the individual ω- and ω-1-lauric acid hydroxylase activities demonstrates a similar pattern of induction as total-lauric acid hydroxylase activity with ω-lauric acid hydroxylase activity making up the majority of lauric acid hydroxylation. In parallel with CYP4A associated activities, the induction of CYP4A protein was significantly blunted in KO compared with WT mice (45% of WT, $P < 0.05$ for the interaction, Fig. 2). Clofibrate treatment significantly induced hepatic catalase activity in both KO mice and WT mice ($P < 0.05$, Table I). A significant interaction effect was not observed for TNF double receptor knockout on clofibrate induction of catalase activity.

## DISCUSSION

This investigation provides the first evidence that an intact TNFα response is necessary for the maximal induction of hepatic CYP4A by clofibrate. Induction of hepatic CYP4A activities and protein was significantly blunted in KO mice compared with WT mice, indicating that endogenous TNFα signaling may play a positive modulatory role in the induction of CYP4A by clofibrate i*n vivo.*

In contrast to these findings, published hepatocyte culture studies have consistently demonstrated a negative effect of inflammatory cytokines on clofibrate induction of CYP4A expression. In rat hepatocytes, Parmentier *et al.* demonstrated that two cytokines released during an acute phase response (IL-1 and IL-6) could repress the induction response of CYP4A and PPARα activation by clofibrate treatment (22,23). The hepatocyte culture systems used in these studies by design are virtually devoid of other cell types and related cytokine signaling pathways. Therefore, the lack of cytokine "cross talk" between cell types in hepatocyte culture systems may explain the discordance between this *in vivo* study and those findings in hepatocyte culture systems.

In the absence of peroxisome proliferators, acute inflammation and associated inflammatory cytokines are known to induce CYP4A activity. In rats, Sewer *et al.* demonstrated that CYP4A associated ω- and ω-1- lauric acid hydroxylase activities were induced in a strain specific manner following LPS administration (7). In Fischer 344 rats ω-lauric acid hydroxylase activity was induced by LPS administration, while in Sprague Dawley rats no change in ω-lauric acid hydroxylase activity was observed following LPS administration (7). In addition, ω-1-lauric acid hydroxylase activity decreased in both Fischer and Sprague Dawley rats following LPS administration (7). Sewer *et al.* went on to show that several inflammatory particulates ($BaSO_4$, celite and kaolin) could induce ω-1-lauric acid hydroxylase activity while only celite induced both ω- and ω-1-lauric acid hydroxylase activities (6). In light of the differential induction of CYP4A associated ω- and ω-1-lauric acid hydroxylase activities by different inflammatory stimuli, we examined the clofibrate induction profile of these activities in KO and WT mice following clofibrate treatment. In addition, a non-HPLC radiometric method developed by Giera and van Lier for determining total-lauric acid hydroxylase activity in microsomes was used for verification. In KO and WT mice the clofibrate induction pattern of ω-, ω-1- lauric acid hydroxylase and total-lauric acid hydroxylase activities were identical. Both ω- and ω-1- lauric acid hydroxylase activities were blunted in KO mice compared with WT mice ($P < 0.05$ for the interaction, Fig. 1B,C). Therefore, clofibrate induction of total-, ω- and ω-1- lauric acid hydroxylase activities was affected similarly by the absence of an intact TNFα response in KO mice.

Activation of the nuclear hormone receptor PPARα is

**Table I.** Effect of TNF ($p55^{-/-}/p75^{-/-}$) Double Receptor Knockout on Clofibrate Induction of Hepatic Catalase Activity

| Group | Catalase activity (μmol/min/mgP) | % |
|---|---|---|
| (1) WT, V | 349.0 ± 87.9 | 100 |
| (2) WT, CF | 884.7 ± 50.59[1,3,4] | 253 |
| (3) KO, V | 370.3 ± 76.8[2,4] | 100 |
| (4) KO, CF | 1039.0 ± 134.9[1,2,3] | 281 |

*Note:* Data (n = 7) are given as mean ± S.D. TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice (KO) and wild-type mice (WT) were administered either clofibrate (CF) [400 mg/kg in corn oil] or corn oil vehicle (V) on three consecutive days and catalase activity determined spectrophotometrically as described in the Methods. Catalase activity = μmol $H_2O_2$ decomposed per minute per mg microsomal protein (mgP). Superscript numbers denote a statistical difference between respective groups as numbered in the table, with significance set at $P < 0.05$.

known to play a central role in hepatic CYP4A induction and peroxisome proliferation following clofibrate treatment. Therefore, we sought to determine the effect of endogenous TNFα signaling on peroxisome proliferation and PPARα activation following clofibrate treatment. In the present study, the animals were euthanatized 24 h following the last dose of clofibrate, long after PPARα activation would be expected to peak in the nucleus (2–3 h) (24). Therefore, we chose to use catalase activity as an indirect indicator of PPARα activation and peroxisome proliferation. Catalase performs the detoxification of peroxide to water in cell peroxisomes, and has been shown to be inducible by peroxisome proliferators such as clofibrate (25). In all mouse strains tested herein, hepatic catalase activity was significantly induced (2.5–3.5-fold, Table I) by clofibrate. No interaction effect of TNF double receptor knockout on the induction of catalase activity by clofibrate was detected. These data suggest that the mechanism by which TNFα signaling influence clofibrate induction of hepatic CYP4A *in vivo* is independent from mechanisms impacting peroxisome proliferation. These findings are consistent with those of Hasmall *et al.*, where TNFα was shown to be permissive for the PPARα-dependent hepatic growth response following exposure to the peroxisome proliferator nafenopin, without affecting peroxisome proliferation as indicated by peroxisomal beta-oxidation activity (26).

In conclusion, the data presented herein suggest that an intact TNFα response is necessary for the maximal induction of hepatic CYP4A activity and protein by clofibrate. The blunting of hepatic CYP4A induction by clofibrate in the absence of TNF p55/p75 receptors in KO mice did not parallel peroxisome proliferation as demonstrated by catalase activity, suggesting that TNFα signaling modulates clofibrate induction of hepatic CYP4A by mechanisms not impacting peroxisome proliferation.

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