Species-specific kinetics and zonation of hepatic DNA synthesis induced by ligands of PPARα

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Abstract

PPARα ligands evoke a profound mitogenic response in rodent liver, and the aim of this study was to characterise the kinetics of induction of DNA synthesis. The CAR ligand, 1,4-bis[2-(3,5-dichoropyridyloxy)]benzene, caused induction of hepatocyte DNA synthesis within 48 hours in 129S4/SvJae mice, but the potent PPARα ligand, ciprofibrate, induced hepatocyte DNA synthesis only after 3 or 4 days dosing; higher or lower doses did not hasten the DNA synthesis response. This contrasted with the rapid induction (24 hours) reported by Styles *et al.* (*Carcinogenesis* **9**:1647-1655). C57BL/6 and DBA/2J mice showed significant induction of DNA synthesis after 4, but not 2, days ciprofibrate treatment. Alderley Park and 129S4/SvJae mice dosed with methylclofenapate induced hepatocyte DNA synthesis at 4, but not 2, days after dosing, and proved that inconsistency with prior work was not due to a difference in mouse strain or PPARα ligand. Ciprofibrate-induced liver DNA synthesis and growth was absent in PPARα-null mice, and are PPARα-dependent. In the Fisher344 rat, hepatocyte DNA synthesis was induced at 24 hours after dosing, with a second peak at 48 hours. Lobular localisation of hepatocyte DNA synthesis showed preferential periportal induction of DNA synthesis in rat, but panlobular zonation of hepatocyte DNA synthesis in mouse. These results characterise a markedly later hepatic induction of panlobular DNA synthesis by PPARα ligands in mouse, compared to rapid induction of periportal DNA synthesis in rat.

Keywords: liver growth, hepatocyte, PPAR, peroxisome proliferation, DNA synthesis

Introduction

The peroxisome proliferators were originally characterised as a class of structurally diverse compounds that caused liver cancer in rodents {Reddy, 1980 #81}, and induced substantial changes in liver ultrastructure, including proliferation of endoplasmic reticulum and peroxisomes. This class of agents is now known to act through activation of the Peroxisome Proliferator-Activated Receptor α (PPARα) {Issemann, 1990 #70}, and it is clear that this class of carcinogen has a non-genotoxic mode of action {Ashby, 1994 #55; Peters, 1997 #40}. The potent carcinogenicity of this class is shown by the fact that Wy-14,643 can induce 100% multifocal liver cancer in rodents after one year {Cattley, 1991 #68}. Ciprofibrate and methylclofenapate are potent PPARα ligands {Mukherjee, 2002 #219} that are also potent hepatocarcinogens {Meyer, 2003 #235; Tucker, 1995 #241}.

The mechanism whereby PPARα ligands cause cancer is still largely unclear {Ashby, 1994 #55; Klaunig, 2003 #242; Peters, 2005 #243}. It is clear that DNA synthesis plays an important role in carcinogenesis, and peroxisome proliferators induce hepatic DNA synthesis which is related to carcinogenesis {Marsman, 1988 #77}. It is therefore desirable to understand the mechanisms controlling the induction of DNA synthesis by ligands of the PPARα, particularly since these ligands augment normal liver size, in contrast to the regenerative growth pathways induced by partial hepatectomy {Mangnall, 2003 #245}. The mechanisms underlying PPARα ligand-induced augmentative liver growth are poorly understood {Menegazzi, 1997 #124; Peters, 2005 #243}, beyond noting that DNA synthesis is dependent upon the PPARα {Peters, 1997 #40}.

The availability of mouse genetic tools {Ledda-Columbano, 2002 #112; Lee, 1995 #53} combined with the potent liver growth effect of PPARα ligands in the mouse {Peters, 1997 #40}, provides a compelling reason for using the mouse to characterise the kinetics and mechanisms of induction of hepatocyte DNA synthesis. It is important to characterise the kinetics of induction of DNA synthesis by PPARα ligands in the mouse, as this information is essential for understanding the relationship with genes that regulate the induction of DNA synthesis. The early kinetics of induction of DNA synthesis by PPARα ligands were characterised using flow cytometry to characterise DNA synthesis in rodent liver {Styles, 1987 #80}. Methylclofenapate (MCP) induced high levels of DNA synthesis by as early as 24 hours after dosing in the Alderley Park (AP) mouse {Styles, 1988 #78}, and the kinetics of induction were confirmed by another study in C57BL/6 mice {Styles, 1990 #73}. However, there is one report using immunohisto-

chemical detection of BromodeoxyUridine (BrdU) incorporation in the CD-1 mouse that shows induction of DNA synthesis only after three/ four days of dosing with the potent PPARα ligand, ciprofibrate, {Ledda-Columbano, 2003 #88}, and so this area is in dispute.

Characterisation of the kinetics of induction of DNA synthesis is crucial for understanding the relationship with induced genes that might regulate this response, and so we have examined the time course of induction of hepatocyte DNA synthesis by PPARα ligands in several mouse strains, and in the rat. In contrast to previous reports, we show that there are species-specific kinetics of induction of DNA synthesis, and that the zonation of PPARα ligand-induced DNA synthesis is different between mouse and rat.

Materials And Methods

All the chemicals were of the highest quality available. Ciprofibrate was a kind gift of Sanofi-Aventis, and methylclofenapate (MCP), a kind gift of Dr C R Elcombe (CXR Biosciences, Dundee) was synthesised by Lancaster synthesis Ltd, Morecambe, Lancs, UK. TCPOBOP was obtained from Sigma (Poole, UK). 129S4/SvJae mice, and their PPARα null congenic strain (129S4/Jae-Ppara$^{tm1Gonz/tm1Gonz}$) {Lee, 1995 #53} were a kind gift of Frank Gonzalez (NIH, MD, USA), and were maintained as a colony in house. C57BL/6J:CRL and DBA/2J mice were obtained from Charles River Laboratories, Alderley Park (AP) mice from AstraZeneca Pharmaceuticals, and male Fischer 344 rats (F344/NHsd) were purchased from Harlan UK Ltd (Bicester, UK).

Chronic treatment protocol. Mice (8-9 weeks old) or rats (14-15 weeks old) were randomised to treatment groups (normally n=6), then acclimatised to 10% orange juice in tap water as their sole source of drinking water for one week. Bromodeoxyuridine (BrdU) was then added to the 10% orange juice (0.8 mg ml^{-1} final concentration), and after one day of exposure to the BrdU, animals were dosed by gavage with xenobiotic in corn oil (20 ml kg^{-1} bodyweight), then dosed as indicated until killed by pentobarbital overdose. Body weight was determined daily throughout the procedure.

Acute rat treatment protocol. Rats (14-15 weeks old) were randomised to treatment group, then dosed with peroxisome proliferator (in corn oil, 20 ml kg^{-1} bodyweight) by gavage. At the indicated time after treatment, animals were dosed *i.p.* with 100 mg kg^{-1} bodyweight of BrdU in sterile phosphate-buffered saline. Animals were killed by *i.p.* pentobarbital overdose 2 hours after BrdU administration.

Immunohistochemistry. A blood sample was taken at necropsy, and serum prepared, followed by storage at -80°C until determination of alanine aminotransferase activity (ALT). The liver was weighed, and a section of left lobe and small intestine were fixed in 10% neutral buffered formalin overnight at room temperature. Fixed tissues were used to prepare blocks, then heat induced epitope retrieval used with the Amersham mouse anti-BrdU labelling system to visualise BrdU; the specific nuclear staining required both primary and secondary antibodies, and administration of BrdU (data not shown). Slides were counterstained with Harris' haemotoxylin, then mounted with distyrene, plasticiser and xylene. For each animal, 2000 hepatocyte nuclei were scored in random fields, and the labelling index is (BrdU labelled hepatocyte nuclei/ total hepatocyte nuclei)*100. Hepatocytes were identified morphologically. A sample of small intes-

tine from each animal was tested as a positive control. For the determination of zonal distribution of labelled hepatocytes, the method was essentially as described {Barrass, 1993 #92}. Briefly, a field was defined as a radius of five to seven cells around either the portal space (the periportal region) or the central vein (the perivenous region), using small vessels of similar size. Five fields were counted for each of the periportal and centrilobular zones for each animal, and the total number of labelled nuclei was recorded.

Statistics. Students t-test was used for comparison of two groups, and a paired t-test for examining body weight loss. ANOVA followed by a post-hoc test (Dunnett's, Newman-Keuls) was used for multiple comparisons. For time-course studies, the control group timepoints were tested to determine if they were significantly different from each other. If not, the control group values were pooled, and the pooled values used for comparisons against the treated groups (Fig. 2A, C, Fig. 3, Fig. 4, Fig. 5A).

Results

Experiments were undertaken to determine whether PPARα ligands induce hepatic DNA synthesis in mice at 24 hours after dosing {Styles, 1990 #73; Styles, 1988 #78}. Male 129S4/SvJae mice were administered the potent PPARα ligand, ciprofibrate, and hepatocytes labelled with BrdU, essentially as described for the rat acute dosing protocol (Materials and Methods), except with *i.p.* administration of ciprofibrate. This protocol yielded satisfactory staining of intestinal nuclei for BrdU as a positive control for labelling, but showed no significant induction of hepatocyte DNA synthesis over 18-42 hours, over a dose range of 25-100 mg ciprofibrate kg^{-1} bodyweight (unpublished data). Administration of BrdU at 2 hours before death limits the window for detection of DNA synthesis to two hours, and so BrdU was administered continuously in the drinking water to ensure that there was continuous exposure to BrdU. Continuous exposure to BrdU is known to be more sensitive for detecting low levels of DNA synthesis, or where DNA synthesis occurs over a period of days {Eldridge, 1990 #226}. Although BrdU has been administered in the drinking water at doses of 1 mg ml^{-1}, *e.g.* {Ledda-Columbano, 2003 #88}, administration of BrdU at 0.8 mg ml^{-1} in the drinking water led to significant body weight loss in Balb/c or 129S4/SvJae mice (data not shown); several studies show body weight loss with BrdU in the drinking water {Reome, 2000 #238; Jecker, 1997 #240}, attributed to the bitter taste of the BrdU. Therefore, 129S4/SvJae mice were acclimatised to 10% orange juice as the sole source of drinking water for one week, before adding the BrdU (0.8 mg ml^{-1}) to the 10% orange juice; the hypothesis was that the orange juice would mask the taste of the BrdU. This protocol yields stable mouse body weights (Fig. 1A), and effective labelling of a positive control tissue, the small intestine (Supplementary Figure 1): note that the nuclear labelling of intestinal cells is uniform throughout the length of villus stained, showing that there is consistent BrdU-staining, and suggesting consistent bioavailability of BrdU, throughout the period of dosing with BrdU. A single dose of 100-400 mg kg^{-1} ciprofibrate did not increase serum ALT levels, demonstrating that these doses of ciprofibrate did not cause hepatotoxicity, and regenerative regrowth in the liver (Fig. 1A). These doses did increase liver weight at 48 hours after dosing, which was statistically significant (Fig. 1A), but failed to cause a statistically significant increase in labelling of hepatocyte labelling index (Fig. 1A).

In order to verify that the chronic BrdU labelling protocol leads to efficient incorporation of BrdU label in liver, as well as intestine, animals were treated with the CAR agonist, 1,4-bis[2-(3,5-dichoropyridyloxy)]benzene (TCPOBOP), at 3 mg kg^{-1}, which is characterised to cause

rapid induction of DNA synthesis in CD-1 mice {Ledda-Columbano, 2003 #88}. There was a statistically significant three-fold increase in ALT in male, but not female, mice (data not shown), showing a small effect on liver cell damage. The liver to body weight ratio was significantly increased in both male and female mice (Fig. 1B), and the hepatocyte labelling index was induced by TCPOBOP treatment to 25-50% (Fig. 1B,C,D). Thus the BrdU dosing methodology leads to effective labelling of hepatocyte DNA synthesis that has been induced within 48 hours of xenobiotic (TCPOBOP) treatment in 129S4/SvJae mice; consequently an artefact of dosing methodology can be excluded as a reason for the failure to detect induction of hepatic DNA synthesis in mouse by PPARα ligands (Fig. 1).

The time course of the hepatic response to ciprofibrate was examined, to determine if the DNA synthesis response occurs at a later stage than in the first two days after dosing. Fig. 2 shows that liver weight is significantly increased as early as 2 days after dosing, and that liver weight has increased by ~100% at 6 days after dosing commenced. The DNA synthesis response was later than the liver growth, with significant induction after three days of administration of ciprofibrate (although with large variation) and subsequently (Fig. 2A). In order to ensure that the kinetics of induction of DNA synthesis was not an artefact of too high or low a dose, the hepatocyte DNA synthesis response was examined after three or four days of dosing with ciprofibrate (Fig. 2B). As little as 10 mg kg^{-1} day^{-1} of ciprofibrate caused a significant increase in liver to body weight ratio, and there was a tendency for the increase in weight to be larger after four days, compared to three days (Fig. 2B). In this experiment, the induction of DNA synthesis was lower at three days than in the previous experiment (Fig. 2A,B), but the induction of DNA synthesis was statistically signficant. At four days after dosing, there was a robust induction of hepatocyte DNA synthesis at 30, or 100 mg kg^{-1} day^{-1} of ciprofibrate (Fig. 2B), and this latter dose was demonstrated to give the highest induction of DNA synthesis. The hepatic effects of ciprofibrate were examined in female mice to determine if there was a sex difference in response. The liver to body weight ratio was significantly induced after three or four days of dosing (Fig. 2C), but hepatocyte DNA synthesis was only significantly increased after four (but not three) days of dosing (Fig. 2C), comparable with the male, showing that there is no marked sex difference in induction of hepatocyte DNA synthesis by PPARα ligands. Thus ciprofibrate induced hepatocyte DNA synthesis only after three/ four days of dosing, and the dose giving the highest induction of DNA synthesis has been defined.

These studies had used the 129S4/SvJae mouse, and strain differences were one explanation for the fact that the response seen in (Fig. 2) is much later than that reported by Styles {Styles, 1988 #78; Styles, 1990 #73}. Therefore, we investigated the induction of hepatocyte DNA synthesis in C57BL/6J mice; however the results are essentially similar to those seen in 129S4/SvJae mice, with significant induction of hepatocyte DNA synthesis after four (but not two) days of dosing with ciprofibrate (Fig. 3A). DBA/2J mice were also dosed using a similar protocol (Fig. 3B); liver: body weight ratio is significantly induced at two days after dosing, but the hepatic labelling index is not significantly increased at two days after dosing, but is significantly increased at four and six days after dosing commenced. It is therefore less likely that the discrepancy between this study, and the work of Styles {Styles, 1988 #78; Styles, 1990 #73}, is simply due to an idiosyncracy of the 129S4/SvJae mouse strain, since these workers had shown that methylclofenapate induces hepatocyte DNA synthesis by 24 hours in C57BL/6 mice {Styles, 1990 #73}. In order to exclude the possibility that ciprofibrate may have some effect that is not mediated by the PPARα, the effects of ciprofibrate were tested in congenic 129S4/SvJae mice which are nullizygous for the PPARα (unpublished data). These data showed that ciprofibrate at 100 mg kg^{-1} day^{-1} had no significant effect on liver to body weight ratio, nor on the hepatocyte labelling index, or ALT (data not shown), consistent with the work of Peters et al. {Peters, 1997 #40}. This proves that the induction of liver growth and hepatic DNA synthesis by ciprofibrate requires PPARα, and given that ciprofibrate is a known PPARα ligand and peroxisome proliferator{Meyer, 2003 #235; Mukherjee, 2002 #219}, this constitutes proof that these effects are caused by a direct action of ciprofibrate on the PPARα.

Styles showed rapid induction of hepatic DNA synthesis in Alderley Park (AP) mice, using MCP, and given the importance of this report, we undertook a direct comparison between 129S4/SvJae and AP mice using the same dose of the same peroxisome proliferator (MCP) used by Styles {Styles, 1988 #78}. MCP caused a significant induction of liver growth at days two to four after dosing commenced (Fig. 4A) in 129S4/SvJae mice, and showed significant induction of hepatocyte DNA synthesis after three or four (but not two) days of dosing (Fig. 4A); this is consistent with previous data using the PPARα ligand, ciprofibrate (Fig. 1-Fig. 3). When this experiment was repeated using AP mice, similar results were obtained (Fig. 4B): hepatic growth was statistically significant after 2-4 days of dosing, but labelling index was not significantly increased after one, two or three days dosing, and was only statistically significantly increased after four days of dosing with MCP. These data exclude mouse strain or peroxisome proliferator as a cause of the delayed hepatic DNA synthesis response seen with our data, as compared to

the results of Styles {Styles, 1988 #78; Styles, 1990 #73}, and provide strong evidence that the induction of hepatic DNA synthesis by PPARα ligands is delayed until after three/ four days in the mouse.

A direct comparison was made with the induction of hepatic DNA synthesis by ciprofibrate in the Fisher 344 rat. Hepatocyte DNA synthesis was significantly increased at 24, 48 and 96 hours after dosing when using the chronic BrdU dosing protocol, attaining a 38% labelling index (Fig. 5A). The labelling index at the 24 hour time point was significantly different from control (control $1.33 \pm 0.37\%$ (mean and SD) versus ciprofibrate $4.4 \pm 2.3\%$) on a t-test. Chronic dosing with BrdU involves exposing animals to the labelling agent for a period of days, thereby leading to a high background value for DNA synthesis in the control group as a result of DNA synthesis over the whole of this period. Therefore, the experiment was repeated using an acute *i.p.* dose of BrdU at two hours before killing the animals, to characterise the DNA synthesis response within the shorter time frame of the first 48 hours after dosing. The liver weight was slightly, but significantly, increased at 30 and 36 hours after dosing, with a larger increase at 48 hours after dosing with ciprofibrate (Fig. 5A). In contrast to the mouse (Fig. 1-Fig. 4), ciprofibrate significantly induced hepatocyte DNA synthesis as early as 24 hours after dosing, with levels falling back to background before a second wave of DNA synthesis at 48 hours after the first dose (Fig. 5B). The induction of hepatocyte DNA synthesis at 24 hours was examined by varying the dose of ciprofibrate, confirming an early induction of hepatocyte DNA synthesis at 24 hours, and further showing that 50-200 mg ciprofibrate kg^{-1} bodyweight are optimal doses for inducing hepatocyte DNA synthesis (Fig. 5C). At 300 mg ciprofibrate kg^{-1} bodyweight, there is no significant induction of liver to bodyweight ratio, and no induction of hepatocyte DNA synthesis; the diminished nature of these responses, compared with lower doses, suggests that 300 mg kg^{-1} bodyweight is overtly toxic and is suppressing the liver growth response. These results demonstrate that there is a species difference between mouse and rat in the kinetics of induction of hepatocyte DNA synthesis by PPARα ligands.

Given these distinct results in mouse and rat, the lobular zonation of induction of hepatocyte DNA synthesis in liver was examined, as previously described {Barrass, 1993 #92}. Fig. 6A shows that there is marked periportal distribution of labelled hepatocyte nuclei in the rat, whereas the mouse shows a pan-lobular distribution of labelled cells. Quantification of these data (Fig. 6B) shows that there is a difference in the zonation of induced hepatocyte DNA synthesis between the rat and mouse, with statistically significant preferential periportal induction of

DNA synthesis by PPARα ligands in the Fisher344 rat, and no significant difference between periportal and centrilobular induction of DNA synthesis in the 129S4/SvJae mouse. The data in Fig. 6B are typical of data from 129S4/SvJae mice treated with 30-100 mg ciprofibrate kg^{-1} day^{-1} for three or four days, 100 mg ciprofibrate kg^{-1} day^{-1} for three to 6 days, 25 mg kg^{-1} day^{-1} methylclofenapate for three or four days, or C57BL/6 mice treated with 100 mg ciprofibrate kg^{-1} day^{-1} for four days (Data not shown). Likewise, the preferential periportal distribution of induced DNA synthesis in rat hepatocytes is consistent over a dose range of 50-200 mg ciprofibrate kg^{-1}, and when using a chronic BrdU administration regime (data not shown).

Discussion

The mouse and rat show differential kinetics of induction of hepatocyte DNA synthesis after exposure to PPARα ligands, with the mouse response being delayed to three days after dosing. Given that these results differ from previous work {Styles, 1988 #78; Styles, 1990 #73}, it was necessary to to undertake extensive controls. Immunohistochemical detection of incorporated BrdU has been extensively validated for detection of replicating hepatocytes (*e.g.* {Eldridge, 1990 #226; Ledda-Columbano, 2003 #88}), and positive controls include labelling of intestine, labelling in liver from mice treated with the CAR ligand, TCPOBOP and that results obtained using acute and chronic BrdU-dosing protocols were comparable. Hence the labelling regime and immunohistochemical detection system were robustly validated for detecting early induction of DNA synthesis. BrdU treatment can decrease body weight (unpublished data), and so mouse body weight was measured, although this elementary control is not often described in the literature; mouse body weight was found to be unaffected by BrdU in all experiments shown. PPARα ligands can cause (focal) necrosis (*e.g.* {Woods, 2007 #212}), thereby leading to regenerative growth; therefore serum ALT (a marker of liver cell damage) was shown to be unaffected by the doses of PPARα ligands used in this study, and there was no evidence of necrosis detected by examination of histological sections (not shown). These control experiments prove unambiguously that ciprofibrate fails to induce hepatocyte DNA synthesis within two days after dosing.

Styles found rapid induction of hepatocyte DNA synthesis by PPARα ligands in Alderley Park (AP) {Styles, 1988 #78} and heterozygous Snell dwarf mice on C57BL/6 {Styles, 1990 #73}, whereas our results were obtained in 129S4/SvJae mice, suggesting a strain difference in response. Mouse strain can affect liver function {Akiyama, 2001 #221; Manenti, 1994 #223}, but published evidence that strain differences affect peroxisome proliferation is inconclusive {Budroe, 1992 #63; Dwivedi, 1989 #250; Jones, 1995 #50}. C57BL/6 and DBA/2J mice were treated with ciprofibrate, showing that the kinetics of induction of liver growth and DNA synthesis were similar to that in 129S4/SvJae mice. AP mice are an outbred stock of Swiss origin {Beck, 2000 #224; Chia, 2005 #225}, and so we directly tested in AP mice using the same dose of the same PPARα ligand described in {Styles, 1988 #78}. The induction of hepatocyte DNA synthesis response was delayed to three/ four days after administration of MCP in 129S4/SvJae and in AP mice, thus showing remarkably little effect of strain difference in the response of the mouse to PPARα ligands in three different mouse strains. Thus, we have been unable to repli-

cate the results of Styles in both mouse strains; while it is possible that the AP mice, being out-bred, may have undergone strain drift, the C57BL/6 mice are inbred, and are unlikely to show significant strain drift. Moreover, the concordance between the results obtained in 129S4/SvJae, AP, C57BL/6J and DBA/2J mice in our hands excludes the possibility that the discordance between our data, and those of Styles {Styles, 1988 #78; Styles, 1990 #73}, arises from mouse strain differences.

The possibility that ciprofibrate had off-target (*i.e.* non-PPARα mediated) effects that confounded its liver growth function was also considered. Ciprofibrate is a potent PPARα ligand {Mukherjee, 2002 #219}, has similar effects to another potent {Bell, 1991 #67; Bell, 1991 #65} PPARα ligand, MCP, and the use of PPARα-nullizygous mice provides compelling evidence that the liver growth and DNA synthesis effects of ciprofibrate are mediated by the PPARα.

It is difficult to explain the difference in kinetics of induction of DNA synthesis reported by Styles {Styles, 1988 #78; Styles, 1990 #73}, and in this study. Table 1 compares the methodolgy used by Styles et al and in this paper, and the principal remaining variable is that this study used immunohistochemical detection of incorporated BrdU, whereas Styles detected incorporated BrdU by isolation of hepatocytes, and flow cytometry of the isolated hepatocytes {Styles, 1987 #80}. PPARα ligands cause an increase in liver size, and the consequent increase in hepatocyte size (unpublished data) could cause differential recovery of liver cells during the hepatocyte isolation procedure between control and treated animals, or alternatively may differentially affect the propidium iodide staining. There are no controls to determine whether this is so; and both of these issues could introduce artefactual error into the determination of the proportion of labelled hepatocytes by flow cytometry. Miller *et al.* were unable to replicate the results of Styles on rat hepatocyte ploidy {Miller, 1996 #46; Styles, 1987 #80}, which shows difficulty in reproducing results obtained by the flow cytometry methodology. The reason for the difference in results remains obscure, but we have excluded the choice of PPARα ligand, dose, sex and strain differences as possible variables. Further, our results are consistent with other reports {Ledda-Columbano, 2003 #88}, and so we conclude that the induction of hepatocyte DNA synthesis is delayed until three days after administration of PPARα ligand, and we propose that the results obtained by Styles are an artefact arising from the cell isolation and flow cytometric analysis of liver cells.

The delayed induction of hepatocyte DNA synthesis in the mouse is distinct from the rapid induction of DNA synthesis in the rat. Our results in the rat are consistent with our previous demonstration that PPARα ligands cause rapid (*ca*. 24 hours) induction of DNA synthesis in rat hepatocytes *in vitro* {Plant, 1998 #35; Plant, 1998 #39}, and the finding that the PPARα ligands, nafenopin and Wyeth-14,643, causes induction of hepatic DNA synthesis at 24 hours after dosing in Wistar and Fisher344 rats, respectively {Menegazzi, 1997 #124; Miller, 1996 #46}. However, it is essential to use the same methodology in the same laboratory to obtain a reliable comparison of mouse and rat. This delayed response of the mouse to induction of hepatocyte DNA synthesis is reminiscent of the response to partial hepatectomy, where mouse hepatocyte DNA synthesis commences 12-16 hours later than in the rat; after partial hepatectomy, it has been shown that the faster response of the rat hepatocyte is cell autonomous {Weglarz, 2000 #229}. However, the delayed induction of DNA synthesis in mouse does not reflect an intrinsic lack of capability in the mouse hepatocyte, as TCPOBOP triggers a fast DNA synthesis response, signalling via the CAR receptor. Thus the kinetics of induction of hepatocyte DNA synthesis are specific to the signalling pathway initiating DNA synthesis. The delayed induction of DNA synthesis response in mouse by PPARα ligands is unlikely to be due to a species difference in the amount of the PPARα, since the receptor is present at high levels in mouse, compared to other rodents {Bell, 1998 #36; Choudhury, 2000 #31; Choudhury, 2004 #12}.

The distinct species-specific kinetics of induction of hepatocyte DNA synthesis is associated with altered zonation of induced DNA synthesis, where the rat shows preferential induction of DNA synthesis in the periportal region, and the mouse shows pan-lobular induction of DNA synthesis. The zonation of induction of enzymes by various xenobiotics has been extensively demonstrated {Bars, 1992 #64; Oinonen, 1994 #232}, but the zonal induction of DNA synthesis is much less well characterised, although reliable methods have been established {Barrass, 1993 #92}. Our results confirm and extend the findings of Barrass *et al.*, by showing that a distinct PPARα ligand, ciprofibrate, also induces zonal induction of hepatocyte DNA synthesis in rat, and by showing that DNA synthesis does not show zonal distribution in several mouse strains. The periportal distribution of induced hepatocyte DNA synthesis in rat is distinct from the preferentially centrilobular induction of cytochrome P450 and peroxisomal enzymes induced by PPARα-ligands {Bars, 1993 #60; Bell, 1991 #67}, yet the induction of both DNA synthesis and enzymes are PPARα-dependent. This suggests that PPARα-independent mechanism must be responsible for the distinct zonation of DNA synthesis and peroxisomal enzymes. Characterisation of the zonal distribution of the PPARα- associated coactivators, or microRNAs that are

known to be required for induction of DNA synthesis {Matsumoto, 2007 #3} {Shah, 2007 #2}, are obvious candidates for mediating these PPARα-independent effects. While it is known that Apc is the "zonation-keeper" in mouse liver {Benhamouche, 2006 #251}, it is not clear that exactly the same pathway applies in rat liver. Given the complex web of interactions between Apc and β-catenin, and that β-catenin signalling regulates liver growth pathways {Tan, 2006 #252}, it can be hypothesised that species differences in regulation of the β-catenin signalling pathway mediate PPARα-ligand induced hyperplasia and lobular localisation. Given the availability of liver-specific knockouts of β-catenin {Tan, 2006 #252}, this hypothesis is partly experimentally tractable.

The definition of the rapid induction of hepatocyte DNA synthesis in rat by PPARα ligands opens up opportunities for investigating the mechanism of induction of the growth response, by relating the early gene induction events to subsequent hyperplasia. There is evidence that the liver growth programme induced by PPARα ligands is quite distinct from that seen during regeneration or after treatment with growth factors *e.g.* {Menegazzi, 1997 #124; Plant, 1998 #35; Plant, 1998 #39}: the PPARα-ligand induced growth defined in this paper offers a tractable system for investigating how augmentative liver growth is regulated.

Acknowledgements

TABLE 1: Comparison Of Studies Of Induction Of Liver Hyperplasia

Study	Styles A	This study	This study	Styles A	Styles B	This study	This study	This study	This study	This study
Species	Rat			Mouse						
Strain	AP	F344/NHsd	F344/NHsd	AP	B6.DW-Pou1f1^{dw}/J heterozygotes	AP	C57BL/6J	129S4/SvJae	129S4/SvJae	DBA/2J
PPARα agonist	MCP	Cip	Cip	MCP	MCP	MCP	Cip	MCP	Cip	Cip
Dose	5, 12, 25 mg kg^{-1} day^{-1}	50, 100,200, 300 mg kg^{-1} day^{-1}	50 mg kg^{-1} day^{-1}	5, 12, 25 mg kg^{-1} day^{-1}	25 mg kg^{-1} day^{-1}	25 mg kg^{-1} day^{-1}	100 mg kg^{-1} day^{-1}	25 mg kg^{-1} day^{-1}	10,30, 100, 200, 300 mg kg^{-1} day^{-1}	100 mg kg^{-1} day^{-1}
Route of administration	gavage in corn oil	gavage in corn oil	gavage in corn oil	gavage in corn oil	gavage in corn oil	gavage in corn oil	gavage in corn oil	gavage in corn oil	gavage in corn oil	gavage in corn oil
Dose of BrdU	25 μMol	326 μMol kg^{-1}	2.61 mM	25 μMol	25 μMol	2.61 mM	2.61 mM	2.61 mM	2.61 mM	2.61 mM
Route of administration	i.p. injection 6 hours before kill	i.p. injection 2 hours before kill	In drinking water from one day before Cip	i.p. injection 6 hours before kill	i.p. injection 6 hours before kill	In drinking water from one day before MCP	In drinking water from one day before Cip	In drinking water from one day before MCP	In drinking water from one day before Cip	In drinking water from one day before Cip
Assessment of labelling index	Flow Cytometry	IHC	IHC	Flow Cytometry	Flow Cytometry	IHC	IHC	IHC	IHC	IHC

A comparison of the methods used to measure hepatic DNA synthesis by Styles *et al* in publications A {Styles, 1988 #78} or B {Styles, 1990 #73}, and this study. MCP is methylclofenapate, Cip is ciprofibrate, i.p. is intraperitoneal, and IHC is immunohistochemistry. In this study, BrdU was present continuously in the drinking water, beginning one day before administration of PPARα agonist.

Figure Legends

FIG. 1. Ciprofibrate does not induce hepatic DNA synthesis within 48 hours in mice. A, Groups of six male 129S4/SvJae mice (8-9 weeks old) were acclimatised to 10% orange juice as sole source of drinking water for one week, then BrdU added to the orange juice at a final concentration of 0.08%(w/v). After one day on BrdU, animals were dosed by gavage with corn oil vehicle (Control), or the indicated dose of ciprofibrate; animals were killed two days after dosing with ciprofibrate. The top left panel shows relative bodyweight of animals in each group (each animal is normalised to day 0, which is set at 1). Results are shown as mean ± Standard Deviation. The period where BrdU was administered is shown by a horizontal line, labelled "BrdU", and the time of administration of ciprofibrate by a vertical arrow labelled "Ciprofibrate". Body weight was tested with a paired t-test, and there were no significant differences from the time of initial treatment with BrdU. The top right panel shows serum ALT in this experiment; there are no significant differences. Bottom left panel shows the liver-to-bodyweight ratio is shown as a percentage; values marked with a * are significantly different at $P<0.05$ (Dunnett's multiple comparison test). The bottom right panel shows labelling indices determined for liver sections; all animals were shown to have labelling in intestinal samples (data not shown). There were no significant differences from control at $P<0.05$. B, groups of male or female mice were treated (as in A), but with a single gavage dose of 3 mg of TCPOBOP kg^{-1} of bodyweight, dissolved in corn oil. The left panel shows body weight, and the right panel shows hepatocyte labelling index. An asterisk denotes $P<0.05$ (t-test versus control). There was no effect on body weight in this experiment, but TCPOBOP caused a statistically-significant three-fold increase in serum ALT in male (but not female) mice (not shown). C and D, show typical liver sections labelled with an anti-BrdU antibody (black nuclei) and counterstained with haemotoxylin, for male control and TCPOBOP-treated animals, respectively. The scale bar is 50 μm.

FIG. 2. Kinetics of hepatic response to ciprofibrate in male and female mice. A, Groups of male 129S4/SvJae mice were treated with BrdU, as described in A, and ciprofibrate at 100 mg kg^{-1} day^{-1}, with the exception that animals were killed at 2-6 days after starting dosing with ciprofibrate. There was no significant effect on relative bodyweight, or serum ALT (Data not shown). The left panel shows the liver to bodyweight ratio, and the right panel shows the hepatocyte labelling index; graphs show the number of days after dosing with corn oil/ ciprofibrate on the X-axis. B, Groups of male 129S4/SvJae mice were dosed with the indicated daily dose of ciprofibrate or vehicle control, and killed after three or four days (the three and four day ex-

periments were not contemporaneous), essentially as described in A. There was no significant effect on relative bodyweight, or serum ALT (Data not shown). The left panel shows the liver to bodyweight ratio, and the right panel shows the hepatocyte labelling index. C, groups of female 129S4/SvJae mice were treated daily with 100 mg kg^{-1} ciprofibrate or vehicle, as in panel B. There was no significant effect on relative bodyweight, or serum ALT (Data not shown).

FIG. 3. Effect of strain and PPARα on hepatic response to ciprofibrate. A, Groups of six C57BL/6J mice were dosed with 100 mg kg^{-1} day^{-1} ciprofibrate, or vehicle control, essentially as described for Fig. 2, and killed on days 2 and 4. The left panel shows liver to body weight ratio, and the right panel shows hepatocyte labelling index. B, Groups of six DBA/2J mice were dosed with with ciprofibrate as for A above, and killed on days 2, 4 and 6. The left panel shows liver to body weight ratio, and the right panel shows hepatocyte labelling index. Statistically-significant difference from the control group is indicated by an asterisk (P<0.05).

FIG. 4. Hepatic response to methylclofenapate in 129 and AP mice. A, Groups of six 129S4/SvJae mice were dosed with 25 mg kg-1 day-1 methylclofenapate essentially as described for Fig. 2. The left panel shows liver to body weight ratio, and the right panel shows hepatocyte labelling index, where * =P<0.05 (Dunnett's multiple comparison test). B, As for A, but using Alderley Park (AP) mice.

FIG. 5. Ciprofibrate induces hepatic DNA synthesis in F344 rats. A, Groups of six Fisher 344 rats were acclimatised to 10% orange juice, and then 0.08% BrdU in the 10% orange juice, essentially as described for Figure 1. Animals were then dosed with corn oil vehicle or 50 mg kg^{-1} day^{-1} ciprofibrate, and killed on the indicated day for determination of liver weight and labelling index. B, Groups of six Fisher 344 rats were dosed by gavage with 50 mg kg^{-1} day^{-1} ciprofibrate, or corn oil vehicle, and injected with 100 mg kg^{-1} BrdU at 2 hours before termination, as described in the acute labelling protocol in materials and methods. The left panel shows liver to bodyweight ratio at the indicated time, and the right panel shows hepatocyte labelling index. *=P<0.05 (Dunnett's multiple comparison test). C, essentially as described in B, rats were dosed with the indicated dose of vehicle or ciprofibrate, dosed with BrdU *i.p.* at 22 hours, and killed after 24 hours. Liver to bodyweight ratio is shown in the left panel, and hepatocyte labelling index in the right panel.

FIG. 6. Zonation of ciprofibrate-induced hepatocyte DNA synthesis. A, representative photomicrographs of ciprofibrate induced mouse (left) or rat (right) liver sections, after staining for

BrdU incorporation. B, the left panel shows quantification of zonal distribution of periportal (PS) and centrilobular (CV) hepatocyte DNA synthesis in 129S4/SvJae mice treated with 100 mg kg^{-1} day^{-1} ciprofibrate for four or six days. Individual animal values are shown, and the mean and Standard Deviation are superimposed as a cross with error bar. There was no significant difference between periportal and centrilobular DNA synthesis. B, as for A, but with Fisher 344 rat after two or four days administration. An asterisk indicates that the periportal values are significantly different from the centrilobular values at $P<0.05$ (t-test).

References

Figure 1

Figure 2

Figure 3

A

B

Figure 4

Figure 5

Figure 6