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Gene expression changes in rat liver following exposure to liver growth agents: role of Kupffer cells in xenobiotic-mediated liver growth

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Abstract

Many xenobiotics are known to cause liver enlargement and hepatocarcinogenesis in rats, although the molecular mechanisms that underlie this effect remain largely undefined. Human exposure to several of these compounds, including glucocorticoids and peroxisome proliferators may be significant, due to their use in both pharmaceutical and industrial processes. It is therefore important to elucidate the molecular mechanisms underlying this abnormal liver enlargement in rats, as this will enable more accurate extrapolation of the possible outcomes of human exposure. Male Sprague–Dawley rats were dosed with the peroxisome proliferator Wy-14,643 and changes in liver gene expression examined using subtractive suppression hybridisation examined either 12 of 24 hr later. Twenty-five transcripts were identified which showed differential gene expression in liver following exposure toWy-14,643. Biochemical indices of liver growth (DNA synthesis, apoptosis) showed that these changes correlated with the initiation of liver enlargement. Rats were next treated with either Wy-14,643, cyproterone acetate and dexamethasone, chemically and mechanistically-distinct hepatomegalic compounds. Carboxylesterase and Kupffer cell receptor mRNA levels were seen to alter in a qualitatively similar fashion for all three compounds, and in a liver specific fashion. In addition, these changes correlated with a decrease in the density of Kupffer cells within the liver, which are known to release mitogenic cytokines, and have been linked to Wy-14,643-induced cell proliferation. We therefore propose that Kupffer cells play a role in a general mechanism of xenobiotic-mediated liver enlargement.

Keywords: Suppression subtractive hybridisation; Kupffer cell; Hyperplasia; Peroxisome proliferation; Liver enlargement; Cytokine

1. Introduction

Humans are exposed to a variety of xenobiotics that cause liver enlargement (hepatomegaly) in rats and mice, and in some cases may result in rodent hepatocarcinogenesis. Examples of such compounds include a variety of pharmaceutical compounds, herbicides and industrial solvents, demonstrating the likelihood of human exposure and thus an associated risk of hepatocarcinogenesis. Compounds causing liver enlargement may be separated into two groups. Firstly, hepatomegaly may be caused mainly through an increase in the number of hepatocytes (hyperplasia), with examples of such compounds being the peroxisome proliferators (e.g. Wy-14,643) [1] and the anti-androgen and rodent carcinogen CPA [2]. By comparison, hypertrophy, the increase in size of individual hepatocytes, is the major cause of the hepatomegaly induced by the synthetic glucocorticoid DEX, through the intracellular deposition of glycogen [3]. It should be noted however that it is likely that most compounds will produce a mixture of both hyperplasia and hypertrophy, although the precise molecular mechanisms underlying these physiological changes remain undefined, presenting difficulties in risk assessment of these compounds, as well as novel compounds.

While the molecular mechanisms underlying hepatomegaly are unclear, Kupffer cells, the resident hepatic macrophages, have been implicated in hyperplasia due to their ability to release mitogenic cytokines [4]. Rose *et al.* demonstrated that peroxisome proliferators can activate

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Abbreviations: DEX, dexamethasone; CPA, cyproterone acetate; SSH, suppression subtractive hybridisation; BrdU, 5-bromo-2′-deoxyuridine.

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Kupffer cells *in vivo* and *in vitro* [5], and that inactivation of Kupffer cells by either dietary methyl palmitate or glycine prevented peroxisome proliferator-induced hyperplasia in rats [6]. Peroxisome proliferator-activated Kupffer cells have been shown to secrete the cytokine TNFα, and this may in turn activate the transcription factor NF-κB in hepatocytes, thus causing the observed hyperplasia [5]. However, peroxisome proliferator-mediated hepatomegaly has been shown to occur in both TNFα and TNFα-receptor knockout mice, suggesting that TNFα's involvement in this process is not obligatory [7]. It is possible, however, that compensatory pathways involving other cytokines may be up-regulated in these transgenic models, with these alternate pathways mediating the observed changes.

To further examine the molecular mechanisms underlying xenobiotic-mediated liver enlargement we have employed differential gene technology to identify changes in gene expression in rat liver following exposure to a number of classical liver growth agents. By comparison of the transcriptome profiles of these xenobiotics it is possible to identify potential central mechanisms for the liver enlargement effects caused by the structurally diverse chemicals.

2. Materials and methods

2.1. Animals and treatment

Male Sprague–Dawley rats (9–10-week-old) were obtained from Charles River. Upon receipt, animals were randomly assigned into groups of five and housed under controlled conditions. They were maintained on a 12:12 hr light–dark cycle at a room temperature of 21° and food and water were available *ad libitum*. Animals were acclimatised to this environment for 5 days before treatment commenced. Groups of five rats were injected i.p. with one of the following three compounds: Wy-14,643 (50 mg/kg), DEX (100 mg/kg) or CPA (60 mg/kg), in corn oil, daily for up to 3 days. Doses were chosen based on previous literature evidence of doses previously seen to exert hepatomegalic effects on rats ([3,8] and [2], respectively).

Study 1: BrdU (Sigma) was administered via surgically implanted osmotic minipumps (Alzet, Model 2ML1, Charles River) to all animals throughout the study at a concentration of 20 mg/mL in saline, 2 mL per animal. Groups of five animals were killed by cervical dislocation at one of five time-points: 2, 12, 24 hr, respectively after one dose, 24 hr after two consecutive daily doses (48 hr); and 24 hr after three consecutive daily doses (72 hr). Livers were immediately excised, weighed, sliced into approximately 0.5 cm cubes, snap frozen in liquid nitrogen and stored at −80°.

Study 2: Groups of five animals were killed by cervical dislocation at one of three time-points: 24 hr after 1, 2 and 3 consecutive daily doses (24, 48 and 72 hr exposure, respectively). Livers, spleens, testes, kidneys and hearts were immediately excised, weighed, snap frozen in liquid nitrogen and stored at −80°.

2.2. Cell proliferation

Samples of the right lateral liver lobe were fixed in 10% buffered formal saline, processed through to paraffin wax, sectioned, and stained using a monoclonal antibody to BrdU (DAKO Ltd.). For quantitation, at least 2000 hepatocyte nuclei were scored in randomly selected fields at 400× magnification. The percentage of BrdU positive nuclei relative to the total was defined as the labelling index (LI).

2.3. Apoptosis

Samples of the right lateral liver lobe were fixed and sectioned as for the proliferation studies. Apoptotic bodies (AB) were identified by the TUNEL method (TdT-mediated dUTP digoxigenin nick end labelling), using the Apoptag staining kit (Appligene Oncor). An estimate of the total number of hepatocytes in each section was made by measuring the area of the liver section and counting the number of hepatocytes in 15 high power fields. The apoptotic index (AI) was defined as the number of ABs per 100 normal hepatocytes.

2.4. Suppression subtractive hybridisation

This procedure was performed as described elsewhere [9]. Briefly, messenger RNA (mRNA) was extracted from approximately 80 mg of liver using the PolyATract® System 1000 kit (Promega). Following phenol/chloroform extraction and ethanol precipitation, 2.5 μg mRNA derived from single animals was subjected to SSH using the PCR-Select™ cDNA Subtraction kit (Clontech) according to the manufacturer's instructions. Secondary PCR reactions from the cDNA subtraction procedure were run on a 2% MetaPhor agarose gel (Flowgen) and each differentially expressed band extracted from the gel and reamplified using the original nested PCR primers supplied with the PCR-Select™ cDNA Subtraction kit. PCR products were then immediately ligated into a TOPO TA Cloning® vector (Invitrogen) and transformed into *E. coli* TOP10F′ One Shot™ cells (Invitrogen). DNA from colonies containing inserts was then prepared using DNA minipreps (Promega).

In order to eliminate false positives, duplicate dot blots of purified plasmid DNA were prepared, and probed with either the final differential display reaction or the 'reverse-subtracted' differential display reaction using the ECL direct nucleic acid labelling and detection system (Amersham).

Clones exhibiting true differential expression were sequenced on an ABI3700 capillary autosequencer, using the M13 Reverse Primer, and sequences matched against

the EMBASE database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/).

2.5. Quantitative-PCR analysis (Q-PCR)

Total RNA was prepared using the Bio/RNA-Cell™ kit from Bio/Gene Ltd. Total RNA was quantified using the RiboGreen RNA Quantitation kit (Molecular Probes). Three micrograms of total RNA and 150 ng of random hexamers as primer were used to synthesise cDNA (RT+) using the Superscript™ Preamplification System for First Strand cDNA Synthesis (Gibco-BRL). In order to identify any genomic DNA contamination of RNA samples, a control cDNA synthesis reaction for each sample was carried out in the absence of enzyme (RT−).

Q-PCR reactions were set up using FAM reporter dye/TAMRA quencher dye labelled probes in conjunction with appropriate primer sets, all designed using the Primer Express Software and purchased from Applied Biosystems. TaqMan Universal PCR Mastermix was purchased from Applied Biosystems, and reactions set up according to the manufacturer's instructions, with the exception that all volumes were halved to give a final reaction volume of 25 μL. Q-PCR reactions were carried out using an ABI7000 SDS instrument and quantitation carried out using the ABI proprietary software against a standard curve generated from rat genomic DNA. To eliminate any genomic DNA contamination of RNA samples, values obtained for RT− reactions were subtracted from those obtained for RT+ reactions. Inter animal variation within each treatment group was routinely less than 10%, and the compound variation observed for fold induction measurements was routinely less than 15%. Values outside these levels were re-measured.

2.6. Cell quantitation

Samples of the left lateral liver lobe were fixed in 10% buffered formal saline, processed through to paraffin wax, sectioned, and stained with haematoxylin and eosin. Quantitation of hepatocytes and Kupffer cells was carried out in 10 random fields per slide at 400× magnification. Mean numbers of Kupffer cells and hepatocytes and the mean ratio of hepatocyte number to Kupffer cell number per field for each animal were calculated.

3. Results

3.1. Liver weight, cell proliferation and apoptosis

Administration of Wy-14,643, DEX and CPA to male Sprague–Dawley rats caused the characteristic, time-dependent increases in liver weight previously reported [2,3,8] (Table 1). Changes in compound-induced liver weight were similar between the two studies (data not shown).

Table 1
Effect of xenobiotic exposure on rat liver weight

Exposure (hr)	Wy-14,643 (50 mg/kg per day)	Dexamethasone (100 mg/kg per day)	Cyproterone acetate (60 mg/kg per day)
2	−4% ± 2	+3% ± 4	+2% ± 5
12	−5% ± 2	+26% ± 6**	+22% ± 4***
24	+2% ± 5	+53% ± 6**	+29% ± 8***
48	+17% ± 3**	+70% ± 8***	+62% ± 10***
72	+37% ± 9**	+70% ± 11***	+49% ± 9***

Data represents mean percentage change in liver weights as compared to appropriate vehicle control, derived from five animals per treatment group. Statistical analysis was performed using ANOVA with Bonferroni *post-hoc* analysis, and asterisks represent *P*-values: (*), $P < 0.05$; (**), $P < 0.01$; (***), $P < 0.001$.

The effect of compound exposure on liver DNA synthesis was determined by immunohistochemical staining for BrdU incorporation. Figure 1 demonstrates that, in accordance with the literature, treatment of rats with either Wy-14,643 [10] or CPA [11] resulted in increased hepatocyte proliferation, as demonstrated by increased BrdU LI, whereas DEX treatment produced no significant effect on LI [12] (Fig. 1a). For Wy-14,643 and CPA, the increase of hepatocyte proliferation observed showed a degree of overlap with the increases in liver weights observed (Table 1).

Next, the effect of Wy-14,643, CPA and DEX on hepatocyte apoptosis was examined by measurement of percentage apoptotic indices (Fig. 1b). Although all 3 compounds have been recorded in the literature to suppress apoptosis [13–15], statistically significant apoptosis suppression was only recorded following DEX treatment in this study. The timing and magnitude of this inhibition of apoptosis appeared to correlate with the observed DEX-induced increases in liver weights (Table 1).

3.2. Suppression subtractive hybridisation

Gene-hunting experiments were carried out to identify differentially expressed genes in rat liver 12 and 24 hr after one dose of Wy-14,643. Twenty differentially expressed sequences were identified following confirmation of regulation via dot blot analysis (false-positive rate was 40% at 24 hr and 5% at 12 hr). Subsequent BLAST interrogation of the NCBI database identified high identity rat matches for 11 of the transcripts (Table 2).

Of the remaining sequences, eight showed high identity to sequences within EST or genome sequence databases, presumably representing rat transcripts for which the entire mRNA is not present in the main database. In silico walking through contiguous EST fragments, or cross genome comparisons allowed identification of the respective coding region, and interrogation of the NCBI database with these sequences produced high identity matches (Table 2). Finally, one sequence showed no high identity matches to either the main, EST or genome databases and hence has

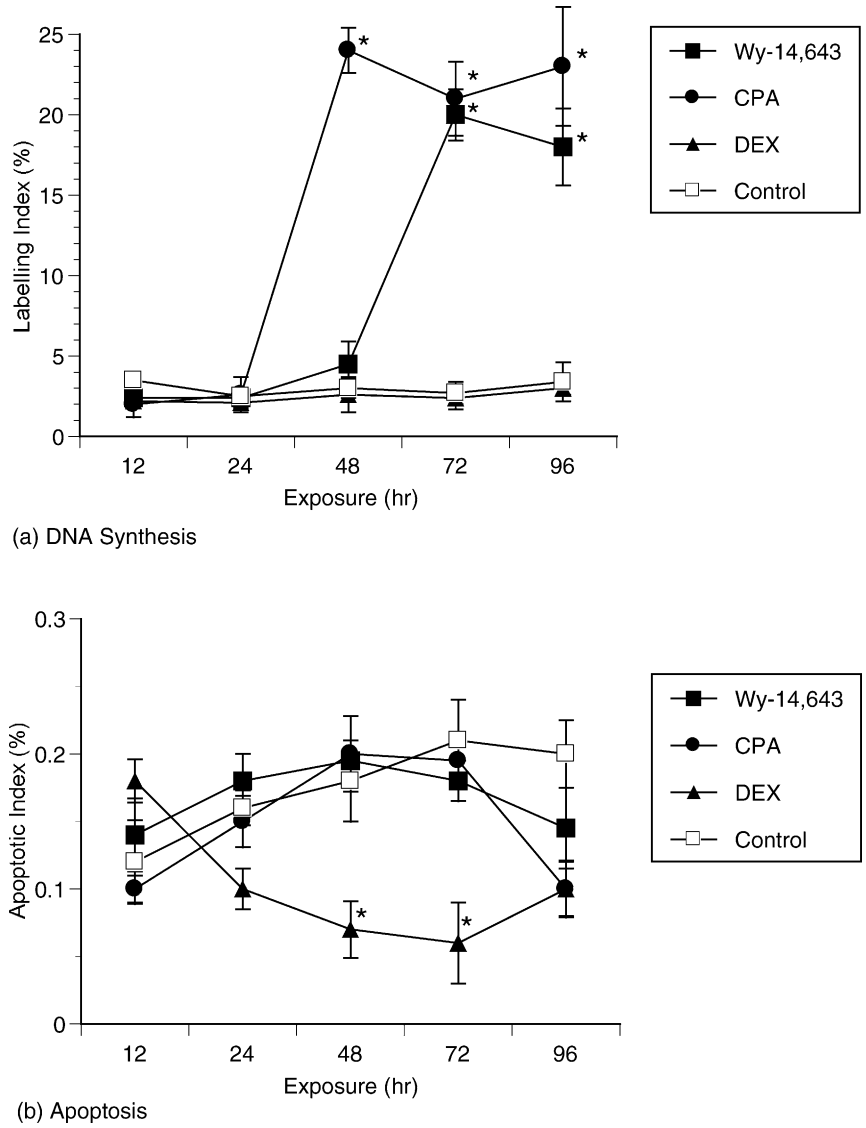

Fig. 1. Effect of xenobiotics exposure on liver DNA synthesis and apoptosis. Rats were exposed to either 50 mg/kg per day Wy-14,643, 100 mg/kg per day DEX or 60 mg/kg per day CPA for 3 days in corn oil vehicle. Liver sections were taken and the percentage of BrdU positive nuclei (labelling index; a) or apoptotic bodies (b) identified in each liver section as described in materials and methods. Each data point represents the mean labelling index derived from five animals; 15 fields per animal (5 each from centrilobular, midzonal and periportal regions) were counted representing approximately 6000 hepatocytes or apoptotic bodies. Error bars represent SEM and statistical analysis, comparing control to treated animals at each time point, was performed using a one-way ANOVA, with Bonferroni *post-hoc* analysis. $^{*}P < 0.05$.

been classed as 'novel'. The identified genes were documented to be involved in a variety of processes including cell proliferation, protein degradation, and oxidative stress, indicating pathways that may be involved in peroxisome proliferator mechanisms of action.

3.3. Q-PCR analysis of gene expression changes

If any of the gene expression changes identified via SSH form are exclusively involved in a common pathway for xenobiotic-mediated liver enlargement then these changes would need to fulfil three criteria. Firstly, the changes in gene expression must be shared by chemically and mechanistically dissimilar liver growth agents. Secondly, they would occur only in liver, as other organs do not undergo trophic responses to these compounds. Finally, they would not occur in the absence of liver enlargement. To examine the first two of these we have studied the effect of DEX, CPA and Wy-14,643 on the gene expression of four of the genes shown to be differentially expressed in liver in other organs, namely spleen, heart, testis and kidney. The transcripts chosen for further study represent different biochemical functions within the cell. Carboxylesterase is involved in the metabolism of many xenobiotics (although not Wy-14,643, DEX or CPA), catalysing the hydrolysis of aromatic and aliphatic esters. Cathepsin H and Ubiquitin activating enzyme (E1) are both involved in protein degradation, which may play a role in the response

Table 2
Identification of genes differentially expressed in male rat liver following exposure to 50 mg/kg dose of Wy-14,643

Gene identity	Accession number identified by BLAST		Location within gene[a]	Time-point (hr)
	Rat	Other		
Up-regulated				
D-Dopachrome tautomerase	Z36980		cds	12
Copper/zinc superoxide dismutase	NM017050		cds + 3′ utr	12
RN protein	U93197		3′ utr	12
Mitochondrial ribosomal L1 protein	BM386958[b]	Rat: XM214008	3′ utr	12
ATP synthetase subunit f	BM986304[b]	Rat: XM221890	cds + 3′ utr	12
Transthyretin-like protein	BM282409[b]	Mouse: AK013177	3′ utr	12
Cathepsin H	Y00708		cds + 3′ utr	24
Preproalbumin	V01222		cds + 3′ utr	24
Selenoprotein P	D25221		3′ utr	24
TATA binding protein	BU760138[b]	Mouse: D01034	3′ utr	24
Ubiquitin activating enzyme E1	CA508764[b]	Mouse: D10576	3′ utr	24
Metallopanstimulin	BQ078913[b]	Human: L19739	?	24
Pyruvate dehydrogenase	AI1409946[b]	Mouse: AK049272	3′ utr	24
Down-regulated				
Carboxylesterase	M20629		cds	12
Fetuin (pp63)	X63446		cds + 3′ utr	12
α2u globulin	M26837		cds + 3′ utr	12
Kupffer cell receptor	J03734		cds + 3′ utr	24
Apolipoprotein E	J02582		cds + 3′ utr	24
V1a arginine vasopressin receptor (mouse)	NM053019	D49729	?	24

[a] 3′ utr: 3′ untranslated region; cds: coding sequence.
[b] The only available rat sequence were unidentified ESTs, gene identity was obtained by re-interrogation of the NCBI database using this EST sequence. In the case of pyruvate dehydrogenase, an EST contig was constructed due to the poor conservation between the long mouse and rat 3′ utr sequences.

to toxic insult. Finally, Kupffer cells have previously been linked to peroxisome proliferator-mediated liver growth [5] and so the Kupffer cell receptor, a type II C-lectin containing cell surface receptor involved in cell–cell recognition and adhesion was chosen for further examination.

Figure 2 shows the time-dependent expression of these four transcripts in rat liver following exposure to Wy-14,643, CPA, DEX or vehicle control. It can be seen that Cathepsin H transcript levels alter differently for the three test compounds (Fig. 2a). Both Wy-14,643 and DEX treatment result in little change in Cathepsin H transcript levels, with small, but statistically significant increases observed between 12 and 48 hr exposure, before levels decrease below control level after 72 hr of exposure. In comparison, whereas CPA treatment produces a statistically down-regulation of Cathepsin H levels, compared to control between 12 and 72 hr. A similar pattern of expression is seen for the other transcript whose product is involved in protein degradation, Ubiquitin activating enzymes (E1; Fig. 2b). As with Cathepsin H, both Wy-14,643 and DEX cause a transient increase in E1 levels between 12 and 48 hr exposure before returning to, or below, control levels. However, E1 levels are unaltered by CPA treatment throughout the exposure period.

In contrast to the expression profiles of Cathepsin H and E1, transcript levels of both the Kupffer cell receptor (Fig. 2c) and liver carboxylesterase (Fig. 2d) showed similar responses to all three compounds. In both cases, exposure to Wy-14,643, CPA or DEX resulted in a time-dependent decrease in the levels of expression. This decrease occurred earlier for Kupffer cell receptor, with the first statistically significant decreases seen after 12 hr exposure, and reached a higher magnitude, with a maximal 8-fold decrease in levels observed after 72 hr DEX treatment.

Therefore, of the four transcripts studied only liver carboxylesterase and Kupffer cell receptor fulfilled the first criteria for components of a central mechanism, i.e. they demonstrated qualitatively similar changes in transcript levels in response to three structurally distinct xenobiotics that all cause liver enlargement. To examine the second criteria, that changes were exclusive to the liver, transcript profiling was carried out in the spleen, heart, testis and kidney or animals exposed to Wy-14,643, CPA or DEX. Kupffer cells are unique to the liver, and hence it is logical to presume that changes seen in Kupffer cell receptor levels are also unique to the liver. However, as macrophages in other parts of the body also contain type II C-lectin containing cell surface receptors it is possible that a closely related species would show similar regulation. However, no other receptors sufficiently similar to be detected by the Q-PCR probe set were detected in any other organs (data not shown), and thus it was concluded that changes in Kupffer cell receptor transcript levels were unique to the liver. The carboxylesterase isoenzyme identified as down-regulated in rat liver following Wy-14,643 exposure is also described as 'liver specific' [16], and QRT-PCR analysis failed to detect significant transcript levels in any other organs (data not shown). Figure 3 shows the

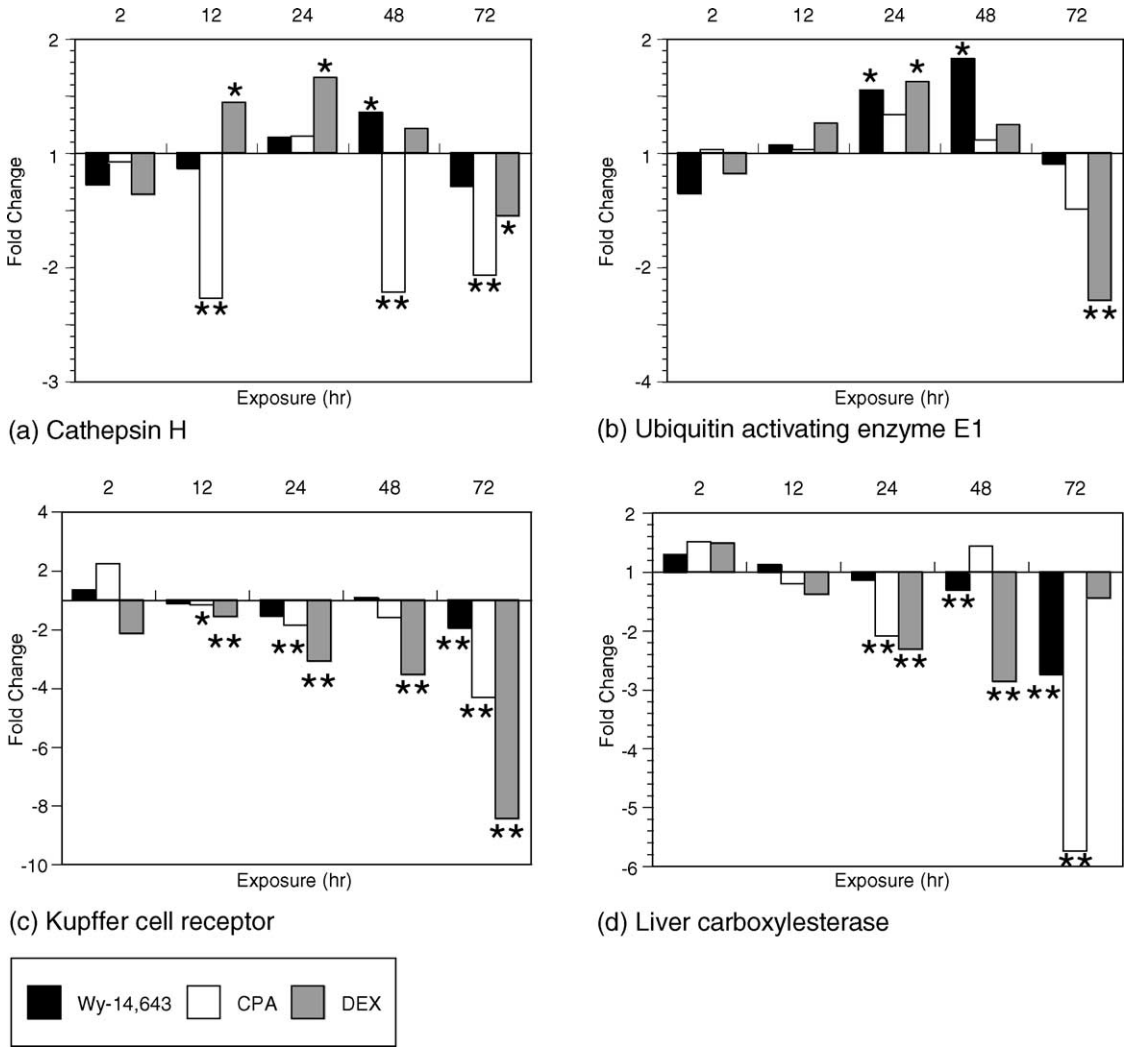

Fig. 2. Xenobiotic-mediated changes in transcript levels in rat liver. Rats were exposed to either 50 mg/kg per day Wy-14,643, 100 mg/kg per day DEX or 60 mg/kg per day CPA in corn oil vehicle. Total RNA was prepared from livers at the indicated time points and Q-PCR carried out as described in materials and methods. Each data point represents the fold change in transcript level derived from the mean change in five treated animals as compared to the mean change of five control animals at each time point. Statistical analysis was performed using a one-way ANOVA, with Bonferroni *post-hoc* analysis. $^{*}P < 0.05$, $^{**}P < 0.01$.

levels of expression of Cathepsin H and ubiquitin activating enzyme E1 transcripts in spleen, heart, testis and kidney in response to exposure to Wy-14,643, CPA or DEX. Both Cathepsin H and E1 show alterations in expression in response to the xenobiotics in at least one other organ (Fig. 3a and b).

Based on these analyses, it can be seen that Cathepsin H and ubiquitin activating enzyme E1 fail both criteria to be considered exclusive components of a central mechanism of liver enlargement in response to xenobiotics. In comparison, both the Kupffer cell receptor and carboxylesterase fulfil both criteria in that their expression levels are altered in a qualitatively similar fashion by all the xenobiotics, and that this change occurs only in the liver.

3.4. Kupffer cell quantitation

The absolute changes in Kupffer cell receptor mRNA levels observed following compound treatment could potentially occur through either a decrease in the number of receptors per Kupffer cell, a decrease in the absolute number of Kupffer cells or a combination of these two effects.

Livers from animals treated with 1, 2 and 3 doses (24, 48 and 72 hr, respectively) of Wy-14,643, DEX or CPA as well as control livers were analysed to address this question. Numbers of Kupffer cells and hepatocytes were counted in randomly selected fields, and mean hepatocyte:Kupffer cell ratios calculated significant vs. control values at the same time-point (Fig. 4). All three compounds caused a decrease in the number of Kupffer cells per field, and DEX also caused a decrease in the number of hepatocytes per field (Fig. 4a and b). Calculation of the hepatocyte:Kupffer cell ratio revealed a significant increase in ratio following 72 hr of Wy-14,643 or CPA treatment, which may clearly be attributed to the prominent reduction in Kupffer cell number as hepatocyte number did not significantly change. In contrast, DEX treatment resulted

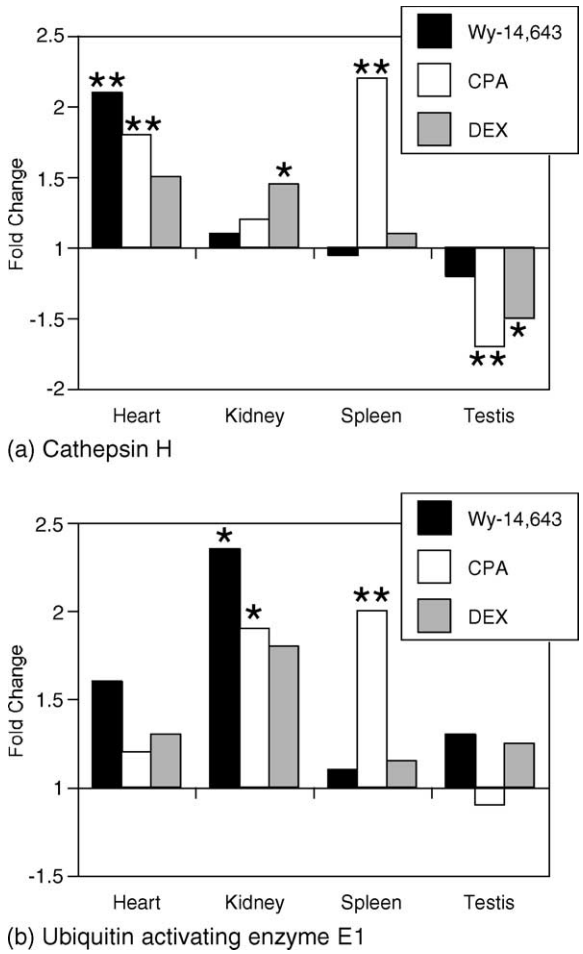

Fig. 3. Xenobiotic-mediated changes in transcript levels in rat spleen, heart, testis and kidney. Rats were exposed to either 50 mg/kg per day Wy-14,643, 100 mg/kg per day DEX or 60 mg/kg per day CPA in corn oil vehicle. Total RNA was prepared from spleen, heart, testis and kidney following 48 hr exposure and Q-PCR carried out as described in materials and methods. Part (a) depicts changes in Cathepsin H transcript levels and Part (b) Ubiquitin activating enzyme E1 transcript levels. Each data point represents the fold change in transcript level derived from the mean change in five treated animals as compared to the mean change of five control animals at each time point. Statistical analysis was performed using a one-way ANOVA, with Bonferroni *post-hoc* analysis. $^{*}P < 0.05$, $^{**}P < 0.01$.

in a small, but significant, increase in the hepatocyte:Kupffer cell ratio (Fig. 4c), presumably due to the simultaneous decrease in both the number of Kupffer cells and hepatocytes per field.

4. Discussion

Many xenobiotics cause liver growth in rats by mechanisms that remain largely undefined. Several of these compounds have significant human exposure, but as their molecular mechanisms of action are not fully understood accurate human risk assessment is complicated. Examples of such compounds include Wy-14,643, DEX and CPA, each of which, in agreement with the published literature, significantly increased rat liver weight in this study [2,3,8]. In the case of Wy-14,643 and CPA this was at least partly attributable to hyperplasia resulting from increased rates of DNA synthesis. Increases in hepatocyte size may result through the deposition of glycogen within the cells following DEX treatment. All three compounds have previously been shown to cause inhibition of apoptosis, which could lead to liver enlargement through the prevention of normal hepatocyte death [13–15]. In the current study inhibition of apoptosis was only statistically significant for DEX; a possible explanation for this is that the low basal rate of apoptosis (approximately 0.2%) combined with higher inter animal variation (approximately 15%) has masked effects with the other two compounds. It is interesting to note that both treatments cause a decrease, albeit a non-significant one, after 96 hr of treatment.

Differential gene expression analysis identified 25 transcripts whose expression was altered in rat liver following treatment with Wy-14,643. None of the classically expected peroxisome proliferator-induced genes (e.g. CYP4A1, ACOX) were shown to alter upon treatment, but this is perhaps unsurprising as SSH utilises a suppression step to bias equalise differences between rare and abundant transcripts. In doing so the regulated gene set identified will be different to those previously seen with less sensitive methods, and may not include highly expressed transcripts such as CYP4A1. Of these, four were selected for further analysis, based upon their biochemical roles and literature evidence. Cathepsin H is a member of the lysosomal cysteine proteinases, probably the most active proteinases in the body. As well as a role in protein degradation it is involved in regulating other cellular functions including cell growth, and tumour metastasis [17]. Ubiquitin activating enzyme E1 is involved in the selective degradation of many proteins, including the tumour suppressor p53 [18]. Through the targeted degradation of this and other cell cycle proteins the ubiquitin cycle may play an important role in cell cycle control and potentially hepatocarcinogenesis [19]. The expression of Cathepsin H and ubiquitin activating enzyme (E1), whose products are involved in protein degradation, were seen to change in several organs in response to compound treatment. While of interest in terms of the overall body response to these compounds, such a change suggests they are not exclusively involved in abnormal liver growth. Carboxylesterases are a family of enzymes involved in the lipid and xenobiotic metabolism, and expression levels of these enzymes have previously been shown to be altered by several classes of xenobiotics [20]. Consequently, further study of this transcripts regulation was undertaken due to its established role in the body's response to xenobiotic exposure. Carboxylesterase expression, however, was shown to change only in rat liver in response to compound treatment, where all three compounds caused a significant repression of gene expression. Liver carboxylesterase belongs to the Type B carboxylesterase/lipase

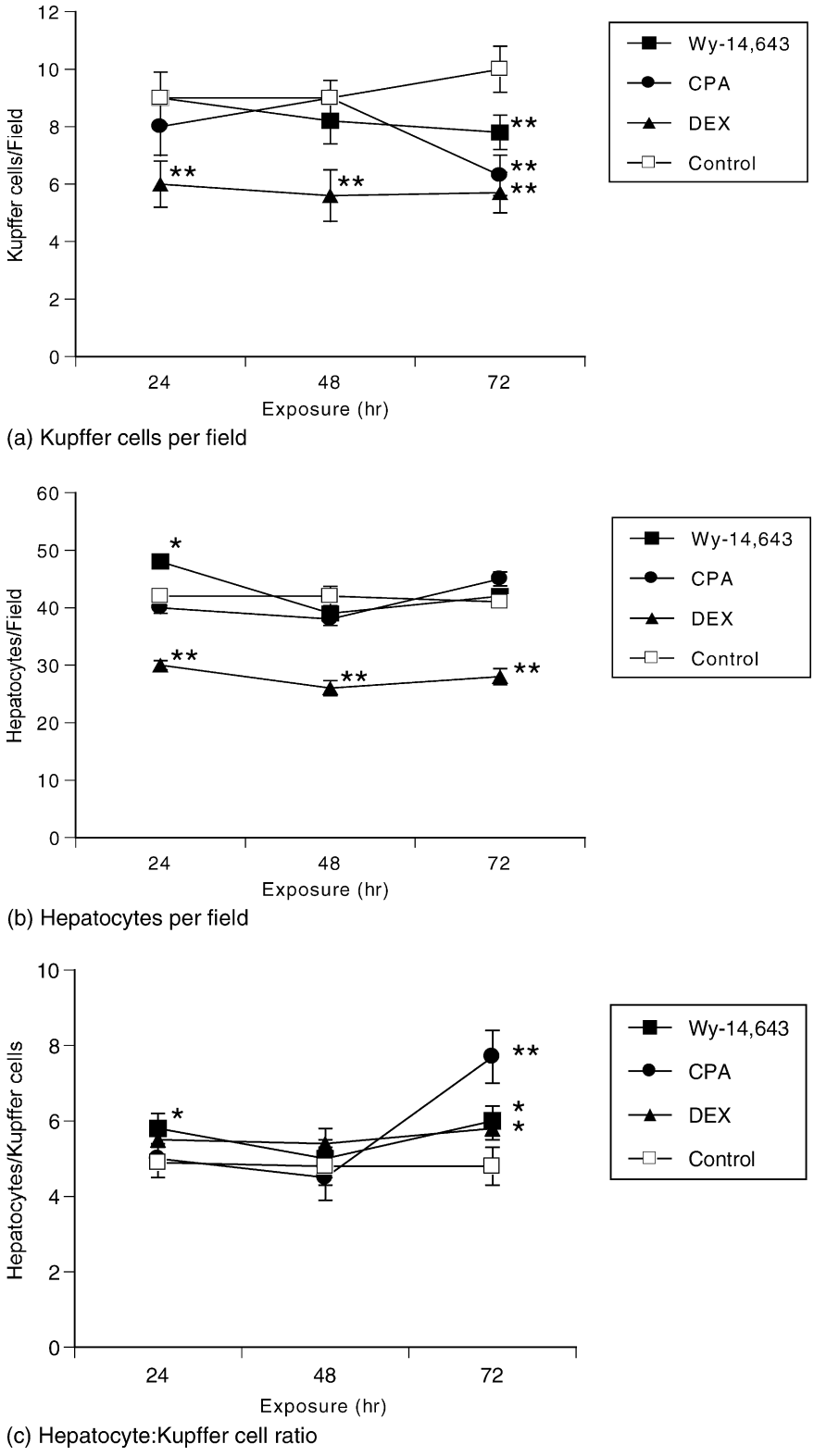

Fig. 4. Xenobiotic exposure causes a change in the hepatocyte:Kupffer cell ratio. Rats were exposed to either 50 mg/kg per day Wy-14,643, 100 mg/kg per day DEX or 60 mg/kg per day CPA in corn oil vehicle. Liver sections were taken and hepatocyte and Kupffer cell numbers determined as detailed in materials and methods. Part (a) represents mean Kupffer cells per field from 10 random fields and Part (b) the mean hepatocyte number from 10 random fields. Part (c) shows the resulting hepatocyte:Kupffer cell ratio. Error bars represent SEM and statistical analysis, comparing control to treated animals at each time point, was performed using a one-way ANOVA, with Bonferroni *post-hoc* analysis. $^{*}P < 0.05$, $^{**}P < 0.01$.

family, with specificity towards aromatic and aliphatic esters. Carboxylesterase levels have previously been shown to be altered by several xenobiotics, including both glucocorticoids and peroxisome proliferators. DEX causes a significant decrease in carboxylesterase protein levels and activity [21], whereas the evidence for peroxisome proliferators is more equivocal, with both increases and decreases in carboxylesterase levels being reported [22,23]. However, carboxylesterase levels have also been shown to alter in response to a number of xenobiotics which do not promoter liver growth; it is hence unlikely that regulation of the expression of this gene plays a key role in any central pathway of xenobiotic-mediated liver growth.

The identification of a surface-receptor specific for Kupffer cells was of particular interest with regard to a potential mechanism of xenobiotic-induced liver growth as Kupffer cells are capable of secreting various cytokines and growth factors on activation [24]. They are also known to interact with hepatocytes through factor-mediated communication, receptor-mediated endocytosis and gap-junction communication to perform roles in liver surveillance [25]. The Kupffer cell receptor is a type II C-lectin containing membrane receptor, with specificity towards fucose and galactose. Its specific role has yet to be fully elucidated, although such carbohydrate receptors have been hypothesised to be involved in regulation of cell numbers within the liver [26]. Specifically, Kupffer cells have been shown to be activated by peroxisome proliferators [27], and evidence is accumulating for their role in peroxisome proliferator-mediated liver growth [5,28].

Significant down-regulation in mRNA levels of the Kupffer cell-surface receptor in rat liver was observed for all three compounds, suggesting a common effect. Moreover, no expression was observed with any of the other organs tested, demonstrating that the gene changes observed were specific to the Kupffer cell receptor, and also Kupffer cells specifically and not macrophages in general. This indicates that the differential expression was liver- and therefore possibly xenobiotic-mediated liver growth-specific. Histochemical analysis suggests that the cause of this decrease in Kupffer cell receptor mRNA levels was an effect of Kupffer cells density within the liver, which significantly decreased with all three treatments. One possible explanation for this would be an anti-inflammatory action of the compounds, potentially suppressing Kupffer cells as part of this action. Both DEX and peroxisome proliferators have been shown to possess anti-inflammatory properties [29,30] although the evidence for any anti-inflammatory action of CPA is equivocal. Further work is required to fully define the potential role of any anti-inflammatory effects of these compounds on Kupffer cell number. The cause of this decrease in Kupffer cell density is however unlikely to be exactly the same for all three compounds however. The increase in the hepatocytes:Kupffer cell ratio caused by Wy-14,643 and CPA could result from two alternative scenarios. Firstly, increased numbers of hepatocytes due to compound-mediated hyperplasia would effectively dilute the Kupffer cells in a 'larger' liver. Alternatively, there could be a decrease in the overall level of Kupffer cells in the liver, caused by a specific toxicity towards this cell type within the liver. In comparison, DEX causes substantial hypertrophy of hepatocytes, leading to a decrease in the number of Kupffer cells per field, probably through a dilution effect. There is also a small, but significant, change in the ratio of hepatocytes:Kupffer cells caused by DEX and this may be caused by either of the scenarios discussed for Wy-14,643 and CPA.

These observations regarding the effects of a variety of hepatomegalic compounds on Kupffer cell number and distribution has defined a putative novel role for Kupffer cells in xenobiotic-induced liver growth (Fig. 5). As previously indicated, Wy-14,643 is thought to interact with Kupffer cells resulting in the release of cytokines and growth factors, including TNFα [31]. This release of cytokines and growth factors is probably responsible for the rapid burst of hepatocyte DNA synthesis and proliferation following administration of peroxisome proliferators to rats. We hypothesise that following this initial Kupffer cell activation and resultant stimulation of hepatocyte DNA synthesis, a reduction or dilution of the Kupffer cells secreting these growth-inducing cytokines and growth factors occurs. This may account for the pattern of hepatocyte DNA synthesis and cell proliferation recorded following treatment of rats with certain liver growth inducing compounds—following the rapid, initial wave of hepatocyte DNA synthesis and cell proliferation, replication rates slow and may even approach control rates, even if the compound is continually administered [10]. In fact this increased hepatocyte:Kupffer cell ratio may explain the overall growth response of the liver to these compounds, in that a peak liver weight is attained within just a few days of administration. In the case of CPA, although studies indicate that Kupffer cells are unlikely to be essential for the initiation of the hyperplasic response, they may still be involved in the maintenance of cell proliferation [32]. The observed increase in hepatocyte:Kupffer cell ratio and the subsequent decrease/dilution of mitogenic cytokines may explain the similarities in the pattern of growth of the liver following CPA treatment to that recorded following treatment with peroxisome proliferators.

It is also possible that other non-parenchymal cells may be involved in the maintenance of increased hepatocyte DNA synthesis. Indeed, Ito cells are known to produce a number of hepatotrophic and homeostatic cytokines including TGFα, EGF, and HGF [33,34].

Many of the effects of peroxisome proliferators have been shown to be mediated via the action of the steroid hormone receptor PPARα, and transgenic mice lacking this receptor are refractory to peroxisome proliferator-induced hepatocarcinogenesis [35]. This may appear to be in conflict with

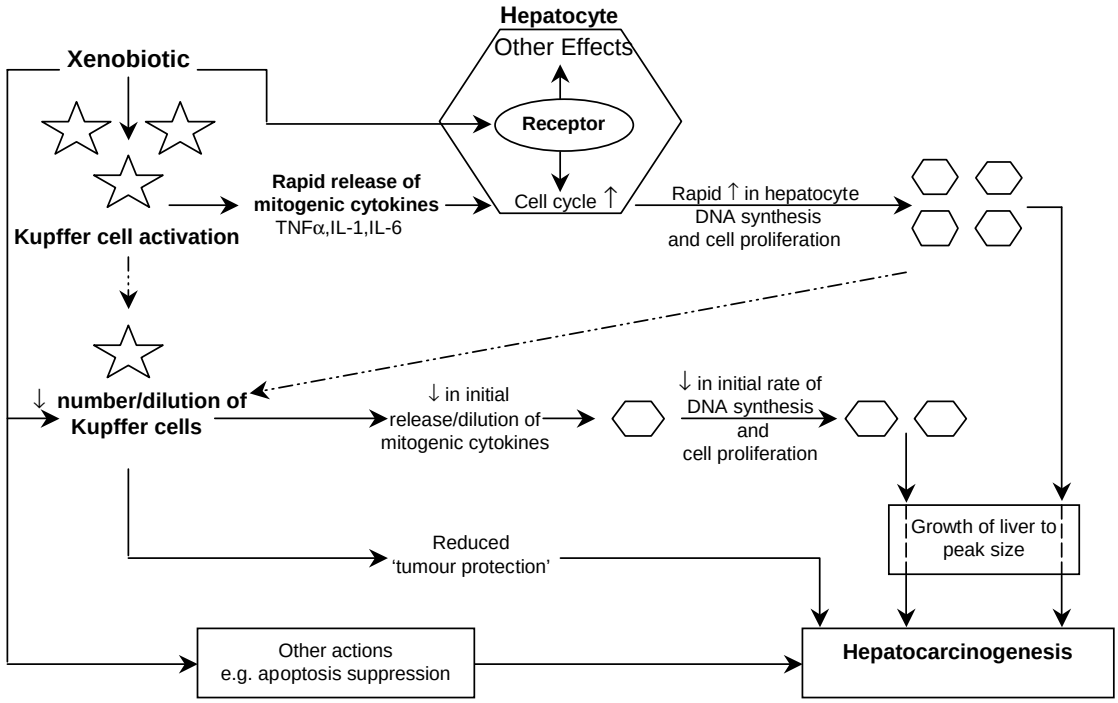

Fig. 5. Proposed model for the role of Kupffer cells in controlling xenobiotic-mediated liver growth. Activated Kupffer cells release mitogenic cytokines which rapidly stimulate a high rate of hepatocyte DNA synthesis and cell proliferation. Following initial xenobiotic-induced Kupffer cell activation, a reduction in Kupffer cell number and/or dilution of Kupffer cells due to hepatocyte proliferation occurs. This results in a reduction/dilution in the release of mitogenic cytokines, reduction in the rate of DNA synthesis and cell proliferation, and hence the rate of growth of the liver slows and the liver reaches a maximum size.

the role of Kupffer cells in peroxisome proliferator-mediated liver growth as these cells lack this receptor [36]. However, recent work by Pauley *et al.* has shown that peroxisome proliferator-mediated growth regulation may be independent of PPARα activation [37]. In this study, primary rat hepatocytes were employed, and while such cells may undergo peroxisome proliferator-mediated DNA synthesis [38], changes in gene expression profiles between *in vivo* and *in vitro* may exist that mask the PPARα input. In addition, it is possible that the mechanisms that regulate DNA synthesis *in vitro* are different from those that regulate proliferation, and the resultant increase in liver size, *in vivo*. One solution to this apparently contradictory data is that PPARα effects on the hepatocyte cell cycle replace the role of Kupffer cells secreted cytokines once Kupffer cell density has been reduced, potentially causing the long-term effects of these chemicals.

This change in hepatocyte:Kupffer cell ratio provides an intriguing hypothesis on possible roles of Kupffer cells in non-genotoxic hepatocarcinogenesis. It is thought that the close apposition of hepatocytes and Kupffer cells in the normal liver may afford some protection against hepatocellular carcinogenesis. Indeed, Kupffer cells have been demonstrated to exert a number of antitumour actions and have been suggested as capable of destroying cells that exhibit abnormal growth properties [32]. A number of factors produced by Kupffer cells have been identified as being involved in macrophage-mediated tumour-cell killing, including arginase, hydrogen peroxide, superoxide, IFNα/β, and the C3a component of complement [39]. Interestingly, depletion of Kupffer cells in genotoxin-mediated liver cancer has become a recognised phenomenon [40]. Therefore, it is possible that the observed loss or change in distribution of Kupffer cells following administration of Wy-14,643 and CPA may partly explain the mechanisms of carcinogenesis following chronic administration of these non-genotoxic compounds to rats, although further experimentation is required to assess such a hypothesis. Such observations may also explain the lack of hepatocarcinogenic action of DEX, where no change in the hepatocyte:Kupffer cell ratio was observed. These observations raise interesting questions regarding a possible role of Kupffer cells as a safeguard against the process of carcinogenesis in the liver. Further analysis of changes in Kupffer cell numbers and distribution following exposure to other compounds with liver growth and/or hepatocarcinogenic properties will be necessary to confirm this hypothesis and any potential link to carcinogenicity; it will also allow the assessment of whether the liver enlargement or hepatocarcinogenic profile of novel compounds can be estimated by measuring this effect.

The step-wise, selective experiments performed herein have resulted in the identification of candidate genes that may play a central role in xenobiotic-mediated liver enlargement. In conjunction with evidence provided by previous investigations, a working hypothesis incorporating a

novel role of Kupffer cells in liver growth mediated by Wy-14,643, and possibly other liver growth agents, is proposed (Fig. 5).

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