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Effects of DEHP in the Liver: Modes of Action and Species-Specific Differences

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Abstract

The industrial plasticizer di-(2-ethylhexyl)phthalate (DEHP) is used in manufacturing of a wide variety of polyvinyl chloride (PVC)-containing medical and consumer products. The biological action of DEHP is very similar to chemicals that are collectively known as peroxisome proliferators (PPs). PPs are a structurally diverse group of compounds characterized as nongenotoxic rodent carcinogens. This review focuses on the effect of DEHP in liver, a primary target organ for the pleiotropic effects of DEHP and other PPs. Specifically, liver parenchymal cells, identified herein as hepatocytes, are a major cell type that are responsive to exposure to PPs, including DEHP; however, other cell types in the liver may also play a role. The PP-induced increase in the number and size of peroxisomes in hepatocytes, so called 'peroxisome proliferation' that results in elevation of fatty acid metabolism, is a hallmark response to these compounds in the liver. A link between peroxisome proliferation and tumor formation has been a predominant, albeit questioned, theory to explain the cause of a hepatocarcinogenic effect of PPs. Other molecular events, such as induction of cell proliferation, decreased apoptosis, oxidative DNA damage, and selective clonal expansion of the initiated cells have been also been proposed to be critically involved in PP-induced carcinogenesis in liver. Considerable differences in the metabolism and molecular changes induced by DEHP in the liver, most predominantly the activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)α, have been identified between species. Both sexes of rats and mice develop adenomas and carcinomas after prolonged feeding with DEHP; however, limited DEHP-specific human data are available, even though exposure to DEHP and other phthalates is common in the general population. This likely constitutes the largest gap in our knowledge on the potential for DEHP to cause liver cancer in humans. Overall, it is believed that the sequence of key events that are relevant to DEHP-induced liver carcinogenesis in rodents involves the following events whereby the combination of the molecular signals and multiple pathways, rather than a single hallmark event (such as induction of PPARα and peroxisomal genes, or cell proliferation) contribute to the formation of tumors: (i) rapid metabolism of the parental compound to primary and secondary bioactive metabolites that are readily absorbed and distributed throughout the body; (ii) receptor-independent activation of hepatic macrophages and production of oxidants; (iii) activation of PPARα in hepatocytes and sustained increase in expression of peroxisomal and non-peroxisomal metabolism-related genes; (iv) enlargement of many hepatocellular organelles (peroxisomes, mitochondria, etc.); (v) rapid, but transient increase in cell proliferation, and a decrease in apoptosis; (vi) sustained

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hepatomegaly; (vii) chronic low-level oxidative stress and accumulation of DNA damage; (viii) selective clonal expansion of the initiated cells; (ix) appearance of the pre-neoplastic nodules; (x) development of adenomas and carcinomas.

Absorption, Distribution, Metabolism and Excretion of DEHP and their Relevance to Effects on the Liver

Oral exposure of DEHP, primarily through food and water, is the most likely route of exposure in humans [reviewed in [1]]. It is the only route of exposure known to lead to liver cancers in rodents [2]. Inhalation, dermal, and parenteral routes of exposure, as well as hemodialysis, are also possible in humans but they are not generally considered to be relevant for the potential carcinogenic effect of DEHP in liver or other organs even in susceptible species [reviewed in [1]]. However, these routes of exposures may be significant for some industrial workers and individuals undergoing dialysis.

DEHP is a lipophilic compound and can be absorbed through skin and lungs by both humans and rodents. However, the greatest absorption occurs after oral exposure. Once DEHP enters the gastro-intestinal tract, it is rapidly metabolized to mono(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol via pancreatic lipases (Figure 1). At low concentrations, most of DEHP is absorbed as these two metabolites, but at high doses some unmetabolized DEHP can also be absorbed [3]. It was estimated that human absorption is as high as 25% [4], but rats absorb more than 55% of the oral dose [5]. Furthermore, non-human primates appear to absorb a smaller percentage of the oral dose than rats [6]. Once absorbed, DEHP and its metabolites are distributed throughout the body in the blood where unhydrolyzed DEHP can be metabolized via non-specific esterases. Several studies of DEHP distribution after oral administration in rodents and other species, including monkeys, showed that liver contains the greatest amount of DEHP and its metabolites under the condition of repeated exposure [5].

The primary metabolic conversion of DEHP following oral administration is qualitatively similar in all species studied, including non-human primates and humans, and involves formation of MEHP and 2-ethylhexanol (Figure 1), followed by formation of phthalic acid through the activity of lipases in the gut and many parenchymal organs. Further metabolism can occur via oxidation by cytochrome P450 4A, alcohol and aldehyde dehydrogenases and yields multiple oxidation products of 2-ethylhexanol and the aliphatic side chain of MEHP [Figure 1, reviewed in [1]]. The downstream metabolites of DEHP can also be glucuronated before excretion. More than 100-fold differences in activity of DEHP-metabolizing enzymes were reported to exist in liver, intestine, kidney and lung between rodents and non-human primates [7]. Notably, significantly less activity is found in non-human primates, suggesting that DEHP is metabolized to its active metabolite (MEHP) to a lesser degree in this species as compared to rodents. This is also consistent with previous studies that indicate that lipase activity and absorption of DEHP is significantly less humans and/or non-human primates as compared to rodents [reviewed in [8]].

Excretion of DEHP and its metabolites occurs through urine and feces (from biliary excretion and unmetabolized/unabsorbed matter). In humans, only urinary excretion was studied and showed that 15-25% of the dose is excreted through this route [4]. Animal studies show that urinary excretion is the predominant route of elimination for DEHP metabolites, while unmetabolized parent compound is predominantly eliminated with feces [9]. More than half of the dose is excreted in the first 24 hrs and about 80% is excreted within 5-7 days [6,10,9]. Humans and non-human primates excrete DEHP metabolites predominantly in the form of glucuronidated conjugates, while guinea pigs, hamsters and mice excrete smaller amount of

these conjugates, and none of the metabolites in rat urine were found to be conjugated [11, 12].

People exposed to DEHP in the workplace routinely test positive for DEHP-related metabolites in the urine [13]. Recent studies have found that humans with no known exposures have detectable concentrations of DEHP metabolites in their urine and serum, and these reference levels have been used to estimate human exposure levels. For example, the concentration of urinary MEHP ranged from undetectable levels up to 66.6 ng/mL, with an average concentration of 3.5 ng/mL [14]. However, these values reflected both free and glucuronidated forms of MEHP, which could overestimate the actual biologically active form. Others have estimated that while the total concentration of MEHP (free + glucuronidated) in urine ranges from 1.2 to 26.0 ng/mL, with an average concentration of 3.88 ng/mL, the free concentration of MEHP ranged from undetectable to 10.0 ng/mL [15]. Interestingly, there is good correlation with urinary and serum levels of MEHP [15]. Based on these biomarker levels of DEHP metabolites, it has been estimated that humans are exposed to DEHP at a concentration ranging from 3-52 μg/kg/day [16,17]. While these findings demonstrate environmental exposure to DEHP in the general population, the physiological relevance of this exposure is under debate. This is further complicated by the fact that there could be additive effects from other environmental phthalates that may or may not activate PPARα, and that in some cases (*e.g.*, occupational exposure, dialysis patients, etc.), exposure levels may be significantly greater than those previously reported.

The metabolites of DEHP, primarily MEHP, but not the parental compound *per se*, are considered to be downstream "biologically active" entities that are responsible for its carcinogenic effect in rodent liver [18,19]. In addition, both primary and secondary metabolites of DEHP can activate PPARα and other isoforms of this nuclear receptor family in both rodents and humans [20,21]. Thus, better understanding of formation, distribution, and excretion of DEHP metabolites in different species, especially humans, and across various organs is equally important to studies on species- and tissue-diversity in expression of PPARs (see below).

Histopathological Changes in Liver Following Exposure to DEHP

Rats and mice are mammalian species that are most susceptible to the characteristic hepatic effects of DEHP and other PPs. Hepatomegaly-marked increase of the liver mass, is the most pronounced early gross pathological change that occurs after continuous oral exposure to DEHP in rodents [22,23]. This effect is robust, sustainable over the time course under conditions of continuous exposure, highly reproducible between laboratories, and appears to be a result of both hypertrophy (increase in the size of each cell) and hyperplasia (increase in the number of cells) of liver parenchymal cells. Microscopically, a midzonal to periportal accumulation of fat, a decline in glycogen deposits in centrilobular areas, structural changes in the bile ducts, and accumulation of lipofuscin granules were reported in rats and mice treated with DEHP for various lengths of time [24,25]. Ultrastructurally, hepatocellular hypertrophy is associated with an increase in both size and number of peroxisomes, mitochondria, and lipid-loaded lysosomes [24,26]. In parallel, a burst of proliferation is observed in DEHP-treated rats and mice. Elevation in the rate of mitosis of hepatocytes is observed as early as 24 hrs after the initial dose [27], and it is gradually increased until about 1 week of exposure, but is not sustained over longer time courses in either rats, or mice [27,28,29]. Furthermore, a decrease in apoptosis was also suggested to be an important additional molecular event that may affect the number of cells in rodent liver following exposure to DEHP, although the apoptosis rates were not investigated past 4 days of exposure and thus the time-course of this event is uncertain [30,31].

The long-term consequence associated with the above mentioned events is the development of hepatocellular adenomas and carcinomas in rodents. A two-year rodent bioassay conducted by the National Toxicology Program indicated that there was a statistically significant positive correlation between DEHP exposure and hepatocellular carcinomas or neoplastic nodules of liver in both sexes of mice (B6C3F1), and rats (F344) [2]. These observations have since been repeated with additional dose- and time-response studies [32] and largely confirmed the original findings. Interestingly, studies have shown that most of the effects of DEHP, such as tumor incidence, liver weight and peroxisomal proliferation are reversible after cessation of treatment which demonstrates a temporal relationship between exposure and liver carcinogenesis [32]. Finally, DEHP is known to promote initiated cell *in vivo* as exemplified by the ability to induce tumors after exposure to a DNA-damaging agent [33,34].

Several studies evaluated the pleiotropic responses to prolonged (from 14 days up to 13 weeks) oral administration of relatively high doses of DEHP (500 to 2500 mg/kg) in several species of ***non-human primates***. In contrast to results found in rodents (see above), no significant increases in liver weight, induction of peroxisomal enzymes, or proliferation of peroxisomes were reported [35,36,37]. Very limited data are available pertaining to hepatic effects in ***humans*** exposed to DEHP. There are two reports suggesting that dialysis patients exhibit hepatic peroxisome proliferation, which presumably could be due to exposure to DEHP or other phthalates in the plastic tubing [38,39] However, no quantified data were provided, so the extent of this putative DEHP-dependent effect is uncertain. Studies that examined patients treated with relatively more potent PPs (e.g. clofibrate, gemfibrazil, or fenofibrate), for prolonged periods of time (*i.e.*, years) are more consistent with the idea that humans do not exhibit peroxisome proliferation in response to exposure to PPs. Similar to findings made in hemodialysis patients, a marginal 50% increase in liver peroxisome number, but not in peroxisome volume is reported in humans treated with clofibrate [40]. In contrast, the majority of studies examining the effect of PP administration in humans have consistently shown no change in hepatic peroxisome proliferation in liver [reviewed in [41]].

There are no known reports of long-term carcinogenesis studies with DEHP or other PPs in non-human primates. Several studies have evaluated the long-term effects of exposure to DEHP in humans. Only one known study evaluated causes of mortality among people exposed to DEHP via inhalation at workplace and found no conclusive evidence of increased death rates from carcinogenesis in this cohort [as reported in [8], original manuscript is not available]. Other known reports investigated workers manufacturing PVC, or patients in long-term contact with PVC-containing medical devices. Several large epidemiological studies that examined the relationship between chronic treatment with lipid-lowering PPs gemfibrozil and clofibrate did not find an association with liver cancer [reviewed in [41]]. Collectively, while an overwhelming number of human epidemiological studies did not show an association between liver cancer and treatment with PPs, they all have important limitations. Thus, it is difficult to draw an unequivocal scientifically-based conclusion for or against a link between long-term DEHP exposure and risk of cancer of liver or other organs in humans.

Pleiotropic Responses to DEHP *in vitro* in Cultured Hepatocytes

Extensive studies on the molecular mechanisms of action of PPs, including DEHP metabolites such as MEHP, have been conducted using hepatocytes from rodent, humans and other species. Such experiments represent the best available direct comparison of responses between rodents and humans. However, it is important to note that the overall magnitude of response to PPs in cultured hepatocytes is typically weaker than that observed *in vivo* in whole liver, which raises a question of whether other cell types (*e.g.*, Kupffer cells, see below) are necessary to achieve the full spectrum of responses [reviewed in [42]].

With respect to the effects of MEHP in cultured isolated ***rat hepatocytes***, several studies reported a significant increase in DNA replication, peroxisomal gene induction, and suppression of apoptosis. While the induction of peroxisomal genes *in vitro* in rodent hepatocytes is robust and comparable to that in whole liver [43,44], the increase in DNA synthesis, a marker of cell proliferation *in vitro* has been reported to be only 120-200%, which is markedly less than that observed *in vivo* [43,44]. Suppression of apoptosis studies in cultured rat hepatocytes were conducted under both "spontaneous" and growth factor (*e.g.*, TGFβ1)-induced conditions [43,44,31]. However, while the qualitative effect of MEHP was similar to that of DEHP in whole rat liver, the magnitude of response was muted even though the rates of apoptosis in culture were much higher than *in vivo* (reference).

One study compared the effect of MEHP and metabolites on the ability of rat, rabbit, guinea pig and ***monkey hepatocytes*** to induce changes in gene expression indicative of peroxisome proliferation [45]. It was concluded that hepatocytes from all species included in the study were responsive to MEHP by inducing peroxisomal palmitoyl-CoA oxidase, but rat hepatocytes were about 30-fold more sensitive based on the dose-response studies. The effects of MEHP on ***human hepatocytes*** have also been studied *in vitro* by several groups [43,44,31]. A general conclusion from these experiments is that human hepatocytes are largely non-responsive to MEHP when DNA synthesis, apoptosis, or peroxisomal gene induction are evaluated. While a trend for activation of β-oxidation capacity of human cells (2- to 4-fold) was shown, these changes were not statistically significant [31,32]. In addition, hepatocytes from one individual exhibited a small, but highly significant increase in DNA replication in response to both MEHP and another phthalate, di-isononyl phthalate (DINP) [32]. It should be noted that a limited number of humans were sampled, which may have affected the significance levels of these studies due to presumably higher genetic variability in human population. In addition, an unknown condition of the livers used for cell isolations, as well as duration and condition of storage of human cells before the experiments may also be considered as confounding variables.

Proposed Modes of Action for DEHP in Hepatocytes

Several major modes of action have been proposed to be responsible for short- and long-term effects of PPs, including DEHP, in the hepatocyte, a target cell for liver carcinogenesis due to these compounds. Below, the molecular events that are thought to be involved in each pathophysiological process are discussed and compared between species.

Activation of PPARα

A discovery of a family of the nuclear receptors, peroxisome proliferator-activated receptors (PPARs) [46], shed light on the mechanism of action of PP compounds, as well as opened new opportunities for therapeutic interventions in many human diseases [47]. These steroid hormone-like receptors act as transcription factors upon ligand binding. PPARs play a number of important roles in normal physiology [reviewed in [47]]. For example, PPARα serves a fundamental role in mammals by acting as a central modulator of signaling molecules that mediate changes in gene expression to maintain lipid homeostasis [48]. PPARs generally form a heterodimer with the retinoid-X receptor (RXR) before translocating to the nucleus and binding to the peroxisome proliferator response elements (PPRE) on DNA, and subsequently activating transcription of specific target genes. PPARs are expressed in all tissues, though the distribution of different isoforms varies between organs and individual cell types. Of the three isoforms that have been cloned to date: α, β/δ, and γ; the former is the primary form responsible for mediating PP-induced effects in the liver and it is ubiquitously expressed in hepatocytes in all species [49]. Still, significant cross-talk between different forms of PPARs and other transcription factors exists [50]; and PPs, including MEHP, are able to activate other PPAR isoforms, particularly PPARγ [21,51].

All synthetic PPs, including some of the downstream metabolites of DEHP, function as PPARα ligands. For example, MEHP and 2-ethylhexanoic acid can both trans-activate PPARα reporter constructs in transiently transfected cells [20], but there is a large species difference as rodent PPARα is transactivated by MEHP with lower concentrations and with greater efficacy as compared to human PPARα [20,21,51]. Treatment of transfected cells with DEHP caused no change in reporter activity demonstrating that DEHP must be metabolized in order to transactivate PPARα [20]. The ability of DEHP metabolites to transactivate PPARα also correlates well with the ability to increase PPARα-dependent target gene expression. Increased activity of PPARα-regulated genes by MEHP, but not un-metabolized parent compound DEHP, was first reported to occur in COS1 monkey kidney epithelial cells [46]. A number of other studies have also shown good correlation between transactivation of PPARα in vitro with increased expression of PPARα target gene expression. Additionally, these changes also confirm the reported species differences as changes in transactivation and target gene expression occur with lower concentrations and result in changes in target gene expression with greater efficacy with rodent PPARα as compared to human PPARα [20,52, 21,31,53,51]. Interestingly, MEHP, but not 2-ethylhexanoic acid, can also transactivate PPARγ and this also correlates well with the ability to induce functional changes in adipocyte differentiation indicative of PPARγ activity [51,21,20,52,21,31,53]. Lastly, MEHP is also reported to transactivate PPARβ/δ [51]. However, the induction of many target genes induced by DEHP is mediated by PPARα as these changes in gene expression do not occur in *PPAR*α-null mice [25]. Thus, while there is good reason to examine the PPARα-mediated effects induced by DEHP and DEHP metabolites, it remains possible that DEHP and DEHP metabolites could alter cellular functions by interacting with other PPARs and/or through other "non-PPAR"-mediated events (discussed below). It is also worth noting that MEHP is a relatively weak PPARα agonist as compared to fibrates or more highly specific ligands such as GW7647 [reviewed in [8]]. Additionally, the concentration required to transactivate human PPARα *in vitro* [30 μM; [51]] is considerably greater than the average concentration found in human serum [~18 nM; [15]].

It has been hypothesized that there are at least three molecular levels of possible ***species-specific differences*** in pleiotropic responses to PPs that concern PPARs, including the relative expression levels of the receptor, differences in the ability of PPARα to activate target gene expression, and differences in the ability of the receptor to interact with other transcriptional proteins. While it appears that human hepatocytes contain fully functional PPARα [54,55], the relative expression levels of this receptor in human liver is about 10-fold lower than that in the rat or mouse [54,56]. However, another report suggests that human liver may express comparable levels of PPARα as compared to rodents [57]. In addition, truncated and polymorphic forms of the protein have been found in human population [56,58,59,60,61,62]. In one case, the truncated form of PPARα was shown to act as a dominant negative [62]. Still, MEHP can bind to the human PPARs, but there are clear differences in the ability of MEHP to transactivate PPARα between rodents and humans [53]. Thus, while there is evidence suggesting that humans may express less PPARα as compared to rodents, in addition to polymorphic alleles, the functional significance of these variants is uncertain [reviewed in [41]].

The ability of the activated receptor to bind to PPREs on the genes responsible for the pleiotropic response of PPs has also been postulated to underlie the reported species difference in PPARα activity. When activated, both rodent and human PPARs are capable to *trans*-activate reporter genes driven by rodent-specific PP-responsive promoters [63,20,21]. Conversely, human PPARα rescues the PP-induced peroxisomal proliferation response in liver of *PPARα*-null mice when it is over-expressed transiently (with adenovirus injection), or permanently (in transgenic mice) [52,64]. However, several human-specific PPREs in peroxisomal genes studied to date were found to be largely non-responsive to *trans*-activation by either mouse or

rodent PPARα [65,31]. Thus, there is some uncertainty regarding the role of polymorphisms in functional PPREs in the reported species differences observed in response to PPs. Importantly, genes responsible for lipid lowering effects of several PP compounds contain PPREs that are highly responsive to activation in humans, but not rats [66,67]. Clearly, this discordance underscores the importance of this level of regulation of PPARα-mediated events in different species. This is also consistent with working showing that expression of acyl-CoA oxidase mRNA is not increased in human liver following exposure to fibrate therapy [68], while it is well documented that this mRNA increases significantly in fibrate-treated rodents.

It has also been hypothesized that other proteins that interact with PPARα could alter the specificity of gene activation and this may differ between cell types and species [69]. It was shown that some proteins, such as heat shock protein (Hsp)90 and the hepatitis virus B X-associated protein 2 (XAP2), complex with PPARα in the cytoplasm and may serve to suppress ligand activation [70,71]; however, no data for potential species differences in this mechanism are available at this time.

On a more functional level, PPARα activation is intertwined with other modes of action for pharmacological and unwanted (*e.g.*, liver carcinogenesis) effects of DEHP and other PPs. The development of the *PPARα*-null mouse opened new research opportunities that helped to define the causal links that exist in rodent liver between PPs, induction of lipid and xenobiotic metabolism genes, proliferation of peroxisomes and other organelles in hepatocytes, increases in cell proliferation, and decreases in apoptosis. Most notably, the *PPARα*-null mouse was instrumental for demonstrating that the receptor is required for liver tumorigenesis induced by a model PP and potent rodent hepatocarcinogen WY-14,643. Specifically, a long-term study in *PPARα* homozygous and nullizygous mice showed that feeding WY-14,643 for up to 11 months resulted in all wild-type mice developing hepatocellular adenomas or carcinomas [72]. *PPARα*-null mice administered the same treatment did not develop tumors, nor were any of the other pleiotropic responses associated with PP-treatment observed. Similar negative results were obtained recently for another PP, bezafibrate [73]. While no long-term carcinogenesis study was performed with DEHP in *PPARα*-null mice, it was shown that for up to 24 weeks of feeding a carcinogenic dose of DEHP (12,000 ppm) failed to induce proliferation of peroxisomes or liver enlargement in *PPARα*-null mice [25].

Most recently, the examination of the mechanisms determining species differences in peroxisome proliferator response between mice and humans became possible with a *PPARα*-humanized mouse line [64]. Human *PPARα* was expressed in liver of *PPARα*-null mouse strain, thus providing an *in vivo* model to examine the reported species difference. In early studies where these mice were fed WY-14,643 or another PP, fenofibrate, the *PPARα*-humanized and wild-type mice responded to treatment with PPARα ligands as revealed by induction of genes encoding peroxisomal and mitochondrial fatty acid metabolizing enzymes and resultant decrease of serum triglycerides. However, only the wild-type mice and not the *PPARα*-humanized mice exhibited hepatocellular proliferation and hepatomegaly [64]. More recently, long-term feeding studies demonstrate that the humanized PPARα mice are also resistant to Wy-14,643-induced liver cancer [74]. This demonstrates that it is highly likely that there are large species differences in the receptor function, and establish that following ligand activation, the PPARα-mediated pathways controlling lipid metabolism are independent from those controlling the cell proliferation pathways that lead to hepatocellular carcinogenesis (see below).

Proliferation of Peroxisomes and Induction of Peroxisomal Proteins

Peroxisome proliferators, as the name implies, cause an increase in the number and size of peroxisomes. These ubiquitous organelles are present in essentially all cell types and are largely responsible for fatty acid β-oxidation of very long chain fatty acids, oxidation of cholesterol

derivatives, D- and L-amino acids oxidation, and purine catabolism. In livers of rodents, the peroxisomes house an array of H_2O_2-generating oxidases, including acyl-coA oxidase, along with H_2O_2-degrading enzyme catalase [75]. PPARα acts as a transcription factor involved in regulating expression of several target genes in peroxisomes, most of which contain a PPRE in their promoter region [76,77]. Many of the genes identified as PPARα target genes are involved in fatty acid metabolism [78]. For example, mitochondrial and peroxisomal fatty acid enzyme expression is effected by PPARα.

Activity of enzymes involved in lipid β-oxidation (*i.e.*, acyl-coA oxidases) is dramatically increased in livers of rats and mice following even a single-dose treatment with PP compounds [79]. By far, this hallmark feature associated with administration of PPARα ligands in rats and mice is the best and well-accepted phenotype that serves as a biomarker that determines the classification of chemical compounds into the class of peroxisome proliferators. A number of investigators over past 30 years demonstrated that DEHP exhibits similar effects on peroxisomal oxidases and other enzymes, as well as density and number of peroxisomes in hepatocytes, in both ***rats and mice*** [23,24,80,32]. Multiple strains of rats and mice and both sexes in these species are responsive, and both time- and dose-response have been reported.

Non-human primates and ***humans*** are considered to be largely non-responsive species when proliferation of peroxisomes is considered as an endpoint. While some reports of modest, often non-significant, induction of peroxisomal oxidases in cultured human hepatocytes and *in vivo* in patients on chronic hemodialysis have been brought forward (see above), it is well accepted that proliferation of peroxisomes is a minor and not reliable phenotype caused by DEHP, or other PPs in people. It should be noted, however, that some PPs, clofibrate and fenofibrate, when given to cynomolgus monkeys for 15 days in doses higher than therapeutic (9.4 and 4 times, respectively), caused a treatment-related increase in liver weight, periportal hepatocellular hypertrophy, increase in the number of peroxisomes (~3 times) and mitochondria (~2.5 times), and increase in activity of peroxisomal enzymes [81].

While peroxisome proliferation and induction of fatty acid metabolism are hallmark responses to PPs, a causal link between this response and liver tumor formation, even in rodents, is debated. One of the original hypotheses that linked proliferation of peroxisomes with liver carcinogenesis was the fact that H_2O_2-generating oxidases are induced disproportionately compared to H_2O_2-degrading enzyme catalase thus causing a state of chronic oxidative stress in the liver that contributes to carcinogenesis via oxidative DNA damage [see below and [79]]. Although it is clear that peroxisome proliferation occurs in response to treatment with PPs, including DEHP, the role of this organelle in the hepatocarcinogenic response does not appear to be a sole causative event. This is based on the finding that the degree of peroxisome proliferation observed in rats fed peroxisome proliferators does not correlate well with tumorigenicity [28]. It is plausible, however, that there exists a threshold above which peroxisome proliferation could be of sufficient magnitude to lead to oxidative damage to cellular macromolecules. While this hypothesis has not been tested, peroxisomal induction continues to be regarded as an important associative endpoint [8].

Induction of Non-Peroxisomal Metabolism Proteins

In addition to peroxisomal fatty acid catabolism, PPs are known to also affect fatty acid β-oxidation in mitochondria, and ω-hydroxylation in microsomes. These effects, together with the recently established role of PPARα in fatty acid uptake and transport, ketogenesis, and lipogenesis in liver [reviewed in [82]], clearly point to the overall importance of PPARα-ligands for physiological regulation of lipid and energy metabolism. Specifically, it was shown that PPARα regulates mitochondrial fatty acid oxidation by controlling expression of a key oxidative enzyme, medium-chain acyl-CoA dehydrogenase [83]. The ω-hydroxylation is governed by a subfamily of cytochrome P450 4A enzymes that are known to be regulated by

PPARα [84,85]. A number of electron transport chain proteins in the mitochondria, as well as CYP4A isoforms are known to be "leaky" enzymes that can generate secondary reactive oxygen species and thus potentially contribute to oxidative DNA damage and carcinogenesis due to PPs (see below); thus, these two non-peroxisomal pathways are important to consider in the evaluation of the mechanisms of DEHP and related compounds.

Indeed, DEHP and its metabolites MEHP and 2-ethylhexanol were shown to cause a significant dose-related induction of CYP4A1 and lauric acid ω-hydroxylase activity in ***rat*** liver *in vivo* even at about ¼ of a dose used for the 2-year NTP carcinogenesis study [86,87]. Similar effects were observed in cultured rat hepatocytes [45], and in the mouse [25,88]. Induction of CYP4A genes by DEHP is dependent on the presence of functional PPARα [25]. Interestingly, MEHP fails to induce ω-hydroxylase activity in cultured hepatocytes from ***other species***, such as guinea pig, rabbit and monkey [45]. No experiments have been performed on human hepatocytes to address the effects of DEHP or its metabolites on these endpoints.

The effects of DEHP on the mitochondria appear to be more complex than just regulation of expression of β-oxidation proteins. Despite the fact that expression of some genes encoding mitochondrial fatty acid β-oxidation enzyme is regulated by the PPARα [83,89], numerous studies showed that phthalates and other PPs inhibit mitochondrial β-oxidation in ***rodent hepatocytes*** as a result of diminished mitochondrial respiration at the level of cytochrome c reductase [90,91,92]. While this effect on the mitochondria may explain the accumulation of lipids in the rodent liver observed following administration of phthalates [93], it is not known what effect this may have on the carcinogenesis. Furthermore, it is not presently known whether similar effects are also observed in human cells. It remains possible that inhibition of mitochondria function by PPs including DEHP could occur through PPARα-independent mechanism, which will be discussed later.

It is likely that other genes that modulate metabolism in hepatocytes are also inducible by DEHP in a PPARα-dependent manner. For example, a recent report showed that CYP1A1, a known Ah receptor target gene, is highly inducible by PPARα ligands, including MEHP, in ***human cell lines*** CaCo-2 (derived from colon adenocarcinoma), HepG2 (hepatoma), A549 (lung adenocarcinoma), as well as primary human keratinocytes [94]. This induction specifically involved PPARα and required 2 PPRE sites that were located within the CYP 1A1 promoter. Whether this also occurs in normal human hepatocytes is yet to be established. CYP 1A1 is a major P450 isoform that is responsible for bioactivation of a number of environmental procarcinogens, and it was reported that DEHP and other PPs enhance DNA adduct formation in rat liver when co-administered with benzo[*a*]pyrene [95]. Thus, the fact that MEHP and other PPs are capable of regulating CYP 1A1 in PPARα-mediated manner in human cells may have significant implications for the assessment of potential carcinogenic risk by environmental compounds that are PPARα ligands. However, others have shown that clofibrate inhibits expression of CYP1A1 and CYP1A2 in liver cells [96]. This would suggest that treatment with PPs such as DEHP would reduce bioactivation of chemical carcinogens. Thus, it is currently unclear how PPs regulate other important CYPs in the liver, and whether these changes result in physiological significant changes.

Induction of Cell Proliferation

Several classes of rodent non-genotoxic liver carcinogens, such as PPs and phenobarbital-like compounds, exhibit a characteristic pattern of changes in hepatocyte proliferation rates. This process is characterized by a rapid, within hours of a single dose of a compound, and remarkable surge in the number of proliferating cells. The increase reaches a peak of several- to more then ten-fold over control levels within a week after initiation of continuous exposure, yet it subsides gradually to nearly control levels despite the prolongation of the treatment [28]. The proliferative response appears to be mitogenic in nature as there is no evidence of cell necrosis

in the first weeks of treatment with PPs and other compounds. It is well accepted that induction of cell proliferation is an important step in the tumorigenesis by genotoxic and non-genotoxic carcinogens. Increased cell replication facilitates the fixation of DNA damage (either from direct interaction with DNA, or indirect oxidative damage) and silencing of tumor suppressor genes. These conditions promote clonal expansion of mutated cells and development of focal lesions in the liver that progress to adenomas and carcinomas.

Induction of cell proliferation is a phenotype characteristic of exposure to PPs in ***rat and mouse liver*** [97,28,98]. Extensive time- and dose-response studies of proliferative effects of DEHP are available in the *rat* [24,28,27]. A rapid burst of cell proliferation (peak at 3 days) occurs in a dose-dependent manner and subsides by 7-14 days of treatment [24]. Sustained increase in cell proliferation is not observed, however there are some reports of an increased proliferation at 52 weeks of treatment [28]. In the *mouse*, several reports provide data showing that DEHP causes an increase in cell proliferation early after initiation of treatment [30,32], and that such an increase may also be present at 40 weeks of exposure [29]. However, no conclusive dose- and/or time-response study was conducted with DEHP in mice to elucidate the shape of the dose-/time-response curve of DEHP on cell proliferation in the liver.

There is no *in vivo* evidence for cell proliferation or hyperplasia (*i.e.*, liver enlargement) in response to DEHP or other PPs in ***human***, or ***non-human primate liver***. The only available studies that evaluated cell proliferation (number of proliferating cell, or liver size), and/or markers of increased cell cycle in the liver were performed with other PPs. One report showed that clinical hepatomegaly did not occur in 12 human patients treated with fenofibrate for 4-86 months [99]. In studies on non-human primates, administration of clofibrate, ciprofibrate, or fenofibrate for either 2 or 13 weeks had no significant effect on relative liver weight with doses at therapeutic levels [81]. Dose of these drugs that exceeded therapeutic by 4- to 9-fold did cause a significant increase in liver weight. This was, however, attributed to hypertrophy of hepatocytes due to induction of peroxisomes and mitochondria, but not increased proliferation since no evidence of increased mitotic figures, Ki-67-positive cells, or mRNA markers of enhanced cell proliferation (e.g., proliferating cell nuclear antigen, cyclin D, c-myc, etc.) was detected in liver [81]. In another study where the effects of di-isononyl phthalate (DINP) and DEHP were evaluated in young adult male cynomolgus monkeys after 14 days of treatment, there were no statistically significant changes in total hepatic DNA synthesis, or liver weights [37].

In ***cultured rat hepatocytes***, MEHP shows a modest (up to 2-fold) yet significant effect on replicative DNA synthesis [43,44,100]. It should be noted, however, that the magnitude of response is much lower than that seen in rat liver *in vivo* and it has been hypothesized that other cells in liver (e.g., Kupffer cells) may play an important role by potentiating the proliferative response of the hepatocytes by producing mitogenic cytokines (see below). Studies with ***cultured human hepatocytes*** failed to produce any evidence of increased proliferation even though they were generally conducted in parallel with rat hepatocyte assays and at comparable doses [43,44,100]. It should be noted that a limited number of humans were sampled which may have affected the significance levels of these studies due to presumably higher genetic variability in human population. In addition, an unknown condition of the livers used for cell isolations, as well as duration and condition of storage of human cells before the experiments may also be considered as confounding variables.

Induction of cell proliferation by PPs, including DEHP, is absolutely dependent on the presence of the functional PPARα in hepatocytes. Feeding of the carcinogenic doses of DEHP or WY-14,643 failed to induce cell proliferation or liver enlargement in *PPARα*-null mice [25, 72]. A similar loss of proliferative response is seen in cultured hepatocytes from *PPARα*-null mice [101]. Interestingly, a recent study that used hepatocyte transplantation to generate

chimeric livers composed of *PPARα*-null and-positive hepatocytes in *PPARα*-null or -positive mice examined a relationship between PPARα status and hepatocyte's ability to proliferate in response to PPs *in vivo* [102]. When treated with WY-14,643 for 7 days, both *PPARα*-null and -positive hepatocytes in chimeric livers displayed elevated DNA synthesis regardless of host receptor status, as long as at least some hepatocytes contained the receptor. These findings suggest that the mitogenic response to peroxisome proliferators does not require the presence of active *PPARα* in all hepatocytes. Most recently, it was shown that *PPARα*-humanized mice exhibited neither hepatocellular proliferation, nor hepatomegaly when fed WY-14,643 or another PP, fenofibrate [64].

Collectively, cell proliferation appears to be the PP-induced phenotype that best correlates with the carcinogenic potential of PPs in rats and mice. Induction of cell cycle appears to be linked, at least partially, to PPARα-mediated signaling in the hepatocyte; however, the exact mechanism of such interaction is not presently known. Several hypothesis suggesting a link via mitogen activated kinases, such as p38 [103], and/or Ras-mediated signaling [104] have been recently brought forward. These leads, while intriguing, require further validation and it is not clear whether any of these mechanisms are relevant for the mode of action of DEHP. Furthermore, it is plausible that other, non-PPARα-mediated events in hepatocytes and/or other cell types in liver may be important for induction of cell proliferation in rodent liver (see below).

Suppression of apoptosis

Apoptosis is a physiological process that is highly conserved due to its importance in maintaining correct cell number in various tissues, and removal of damaged or unwanted cells. In adult animals, apoptosis acts as a safeguard to prevent cells with damaged DNA from progressing to a tumor. Several studies have shown that agonists of PPARα, including DEHP, suppress rates of apoptosis in ***rat and mouse liver in vivo*** [105,106,31]. Thus, it was hypothesized that the cells that would normally be removed by apoptosis may then persist for further mitogenic stimulation by non-genotoxic carcinogens, giving rise to tumors. Interestingly, one study that quantified the age-dependent effect of PPARα agonists on liver apoptosis revealed that only livers of middle age and senescent rats were sensitive, while no effect was observed in young animals [107]. There are no known reports on the anti-apoptotic effects of DEHP or other PPs in ***livers of humans or non-human primates***.

In ***cultured rodent hepatocytes***, MEHP and other PPs suppress both spontaneous hepatocyte apoptosis, and that induced by the physiological negative regulator of liver growth, transforming growth factor (TGF)β1, DNA damaging drugs etoposide or hydroxyurea, or anti-Fas anibody [43,44,108]. Furthermore, viability assays from isolated cultured hepatocyte have shown that cells from PP-treated rats are viable in standard culture for at least 4 weeks, as compared to ~8 days for cells from control animals, and signs of apoptosis (*i.e.*, condensed or fragmented nuclei) were less frequently observed in PP-treated cultures [109]. It appears that PPARα is required to mediate inhibition of apoptosis since hepatocytes from *PPARα*-null mice are refractory to the effects of PPs (nafenopin) on spontaneous, or TGFβ1-induced apoptosis [101,110]. The inhibition of apoptosis by PPARα-mediated signaling can be eliminated by introducing a negative effector regulator of this nuclear receptor [111]. Conversely, the anti-apoptotic effects of PPs are also dependent on non-parenchymal cells (NPCs, most likely Kupffer cells, see below). Highly purified hepatocytes show no response, but the phenotype is rescued by co-culturing with NPCs [110,112]. Interestingly, the PPARα status of NPCs is irrelevant for this effect since *PPARα*-wild type and -null NPCs produced a similar result [110], and Kupffer cells do not express PPARα [113]. Collectively, whereas it is clear that DEHP and other PPARα agonists lead to inhibition of apoptosis, the specific target genes mediating these events remain unidentified.

Several published reports provide conclusive evidence that ***cultured human hepatocytes*** are non-responsive to anti-apoptotic effects of MEHP [44], and other PPs [43]. The lack of an effect is true for both spontaneous and cytokine-induced (TGFβ1, or TNFα/amanitine) apoptosis. It should be noted, however, that human hepatocyte cultures used in these experiments may have contained up to 5% of non-parenchymal cells [43].

Production of Reactive Oxygen Species

Reactive oxygen species are thought to be intimately associated with the mechanism of tumorigenesis by PPs, including DEHP. This assumption is based to a large degree on a fact that various proteins that are induced by these chemicals in liver parenchymal cells (peroxisomes, mitochondria and microsomes, see above) are prone to formation of hydrogen peroxide and other oxidants. Indeed, it was hypothesized that such overproduction of oxidants might cause DNA damage and lead to mutations and cancer [114,114,115]. In addition, recent discoveries show that reactive oxygen species play an important signaling role in a rapid increase of parenchymal cell proliferation caused by peroxisome proliferators [116,116]. Collectively, it appears that oxidant-related molecular events could interact with other pathways activated by peroxisome proliferators *in vivo* in rodent liver, and thus it is critically important to understand their precise mechanism of action.

In ***rat and mouse liver in vivo*** and in ***isolated cultured hepatocytes***, peroxisome proliferators induce disproportionate increases in the activity of enzymes that generate and degrade hydrogen peroxide, oxidize DNA, lipids and other molecules. Reddy and co-workers originally proposed that fatty acyl-CoA oxidase in the peroxisome is the enzyme responsible for oxidative stress by peroxisome proliferators [117,118]; however, mice lacking this protein, instead of being protected from chemically induced liver cancer, develop liver tumors spontaneously, possibly as a result of a hyper-activation of PPARα by unmetabolized lipids [119]. A number of indirect confirmations for DEHP-initiated increases in oxidants have been collected over past two decades [120,121,122,123,124,125]. It should be noted, however, that causative relevance of some of such evidence to the carcinogenic effect of PP in general, and DEHP in particular, has been questioned and contrasting views have been presented [126,127,128,129, 130]. It has been shown that PPARα is required for generation of oxidants in mouse liver after prolonged [weeks, [131]], but not acute [hours, [132]] exposure to PPs. In addition, Kupffer cells have been suggested to be a potential source of oxidants in rat and mouse liver after treatment with DEHP or other PPs [see below, [133,132]]. Collectively, despite the fact that few question a role of oxidative stress in the mechanism of action of these compounds, precise molecular source(s) of reactive oxygen species that are activated by peroxisome proliferators are still a subject of intense debate.

There are no known reports on the ability of DEHP or other PPs to induce production of reactive oxygen species in ***livers of humans or non-human primates***, or ***in cultured liver cells*** from these species.

Oxidative DNA Damage

Oxidative stress to DNA has been hypothesized to be a common pathway for many nongenotoxic chemical carcinogens [134]. An overwhelming number of studies draw a connection between chemical exposure, DNA damage, and cancer based on detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a highly mutagenic lesion, in DNA isolated from organs of *in vivo* treated animals [reviewed in [135]]. Kasai *et al.* [136] showed that treatment with ciprofibrate, a potent peroxisome proliferator, leads to a two-fold increase in the number of 8-OHdG adducts ***in vivo in rat liver*** genomic DNA. Similar observations were made for DEHP [122,137]. In addition to 8-OHdG, increases in other DNA alterations that were detectable by ^{32}P-postlabeling assay have been reported after chronic administration of PPs

clofibrate and WY-14,643 [138]. Despite the ever growing number of published reports that describe high levels of oxidative stress-induced DNA adducts in livers of rats and mice treated with peroxisome proliferators, a concern exists as to whether increases in 8-OHdG represent damage to genomic DNA, a confounding contamination with mitochondrial DNA, or an experimental artifact [139,127,129,131,129]. Recent reports indicate that earlier estimates of ubiquitous persistence of 8-OHdG in genomic DNA were greatly (up to 1000-fold) exaggerated, most likely due to significant artifactual oxidation of guanine bases during isolation of DNA [140]]. These recent developments cause a serious concern as to whether previous studies on oxidative DNA adducts, especially 8-OHdG, should be reconsidered.

One possible explanation to an apparent lack of convincing increases in oxidant-modified DNA bases despite the fact that conclusive evidence of increased oxidant production after treatment with PPs has been obtained in a variety of studies including direct *in vivo* measurements [132], is that oxidative stress to DNA in a living animal is a chronic event that is much weaker in its magnitude than treatment of cells *in vitro* with a concentrated solution of hydrogen peroxide. Indeed, it was shown that chronic treatment with PPs, including DEHP, elicits an adaptive response such as increased DNA repair in both rat and mouse liver [141]. The degree of induction of DNA repair gene expression correlated with a dose and a carcinogenic potency of the PP compounds. Furthermore, it was shown that oxidative DNA damage is a PPARα-dependent event [131].

In view of possible induction of DNA damage by PPs, compensatory induction of DNA repair may be an important factor to consider in the mechanism of action of these compounds. First, increased repair may mask actual levels of oxidative DNA damage by removing the bulk of the lesions that are introduced by a chemical treatment. Second, in the process of DNA repair by a base-excision mechanism, additional mutagenic lesions, such as abasic sites and single strand breaks, are introduced and if the induction of enzymes that participate at different steps of the repair is uneven, there is potential for accumulation of mutagenic intermediates [142, 142]. Third, if DNA polymerase β, a repair-specific enzyme that has low fidelity for DNA replication, is present in significant amounts in the nucleus of a proliferating cell, mutations can be introduced by erroneous base pairing by this error prone protein [143]. Collectively, oxidative stress to DNA is most likely involved in the mechanism of carcinogenesis by PPs. It appears that gene expression for oxidative stress-related DNA repair proteins, but not levels of DNA adducts, is a reliable biomarker that can be used as proof of a carcinogenic potential of PPs.

There are no published reports on the effects of PPs on oxidative stress to DNA in ***cultured rodent, human, or non-human primate hepatocytes***.

Inhibition of Gap Junctional Intercellular Communication

Gap junctional intercellular communication can be modulated during the cancer process and it decreases progressively during multi-stage rat hepatocarcinogenesis [reviewed in [144]]. Blockage of cell-to-cell communication has particularly been associated with the tumor promotion stage of chemical carcinogenesis and has been suggested as a mechanism of action for the tumor promotion process [145]. Additionally, the inhibition of gap junctional intercellular communication observed following treatment with tumor promoting compounds correlates with species and strain sensitivity of the chemical [145].

All of the available data on this mode of action of PPs was generated by J.E. Klaunig and co-workers. DEHP, DINP and other PPs were shown to inhibit gap junctional intercellular communication in the ***liver of rats or mice*** [146]. This inhibition persists while DEHP treatment continues, but reverses when treatment is stopped [147]. It also correlates with the dose- and species-specific tumorigenicity of DEHP and may be used as a predictive indicator of the

nongenotoxic carcinogenic potential of PPs [148]. MEHP has also been shown to inhibit gap junctional intercellular communication in ***cultured rat and mouse hepatocytes*** in a concentration-dependent manner [149,150]. Inhibition of gap junctional intercellular communication in rodent cells was substantially reversed within 24 hrs of chemical removal [150]. In contrast, cell-to-cell communication is not inhibited by MEHP in ***cultured hamster, cynomolgus, or human hepatocytes*** [150], or ***in vivo in non-human primate*** studies [37].

Clonal Expansion of Previously Initiated Cells

Induction of cell proliferation and reduction of apoptosis that are caused by PPs in rodent liver (see above) likely play a central role in further genetic alterations in hepatocytes that are initiated either by PP-induced oxidative stress, or due to other endogenous factors (*e.g*., replication errors, normal oxidative metabolism, etc). DEHP and other PPs are commonly classified as tumor promoters based on their ability to promote clonal expansion of the initiated cells both *in vivo* and *in vitro* in rodent liver. PPs selectively stimulate growth of cells within PP-induced basophilic foci and adenomas in ***rat liver*** [151,34]. DEHP and other PPs promote hepatocellular neoplastic growth induced by genotoxic compounds in mouse and rat liver [33,152,18]. Furthermore, progression from initiated cell to hepatic carcinomas is dependent on the continued presence of the PPs [153]. Since initiation is considered to occur spontaneously and irreversibly, old animals may have a greater accumulation of spontaneously initiated hepatocytes than young ones. Indeed, when the carcinogenicity of the PP compound WY-14,643 was compared in young (starting age 2 months) and old (starting age 15 months) rats, old rats had a 5- to 7-fold higher yield of grossly visible hepatic tumors following 22 weeks of dietary WY-14,643 when compared to young rats [154].

Potent PPs, such as nafenopin and WY-14,643, can promote the clonal expansion of anchorage-independent ***rat and mouse hepatocytes*** [155,156], and other cells [18] *in vitro* which suggests that these compounds perturb the balance of mitosis and apoptosis, leading to the net outgrowth of initiated clones. In contrast, hamster, guinea pig, and ***human hepatocytes*** do not respond to mitogenic effects of nafenopin [155]. No studies have been performed with MEHP or other metabolites of DEHP *in vitro* in animal or human hepatocytes.

It is not clear whether this mode of action is PPARα-dependent as there are no published reports that examined the relationship between receptor status and promotional activity of PPARα agonists.

Proposed Modes of Action for DEHP in Kupffer Cells

DEHP and other PPs increase proliferation of rodent liver parenchymal cells both *in vivo* and *in vitro*; however, the effect on isolated hepatocytes from rats and mice is much less robust and persistent (*i.e*., 8- to 10-fold increases *in vivo* versus only up to 2-fold increases *in vitro*), regardless of the dose of the compound used [28,43,44]. Most interestingly, in highly purified rodent hepatocytes, PPs fail to further increase DNA synthesis [112,110,157]. The possible explanation of this difference is the involvement of nonparenchymal cell(s) in whole liver that is lost in isolated cultured hepatocytes. It was hypothesized that such cell-cell interaction occurs via PP-induced activation of resident hepatic macrophages, Kupffer cells that are the predominant source of mitogens and co-mitogens in liver [reviewed in [42]]. Several laboratories in the past decade have demonstrated that Kupffer cells are involved in a number of acute PP-mediated pleiotropic responses in rodent liver. It was shown that Kupffer cell activation by PPs (i) is independent of PPARα, (ii) involves generation of reactive oxygen species, and (iii) leads to production of mitogenic cytokines [reviewed in [116]]. It is not presently known whether Kupffer cell-specific events play a role in long-term effects of DEHP and other PPs, and if these mechanisms are operational in species other than rats and mice. Finally, it should be noted that apart from one *in vitro* study that used MEHP [158], and two

in vivo studies that used DEHP [132,88], all other reports cited below in support of this mode of action have used other PPs.

Activation of Kupffer Cells is Independent of PPARα

Tumor necrosis factor (TNF)α is a cytokine that is one of the master regulators of liver growth and regeneration and it is produced by Kupffer cells in liver [159]. The first evidence of activation of Kupffer cells by PPs was shown in isolated perfused rat liver. It was demonstrated that nafenopin and WY-14,643 doubled uptake of particulate colloidal carbon, reflecting activation of Kupffer cells [160]. Then, it was reported that neutralizing antibody to TNFα blocked WY-14,643-induced cell proliferation in the ***rat liver in vivo***, and that TNFα was detected immunohistochemically exclusively in Kupffer cells following the treatment [161]. It was also shown that TNFα stimulates proliferation and decreases apoptosis in cultured rat hepatocytes, mimicking the effects of PPs with the exception of induction of peroxisomes [162]. Indeed, studies in the rat demonstrated increases in whole-liver TNFα mRNA [163], and serum TNFα protein [164] levels following treatment with PPs. It should be noted, however, that one study that reports an extensive time-course study with WY-14,643 in mice (from 2 to 72 hrs) found no induction of TNFα, or other cytokine mRNAs in mouse liver [165].

Inactivation of Kupffer cells in rat liver *in vivo* by methyl palmitate or glycine prevented the increases in both TNFα mRNA and protein, and cell proliferation due to the peroxisome proliferator WY-14,643 [166,163]. It appears that activation of Kupffer cells in whole liver occurs very rapidly. In rat liver, activity of the transcription factor NF-κB, a major regulator of TNFα transcription, is transiently increased in 2-8 hrs followed by a steady decline to near control levels at 36 hrs following treatment with WY-14,643 [167]. As early as 2 hrs after WY-14,643 treatment, the active form of NF-κB was localized predominantly in Kupffer cells with values 20- to 25-times greater than in hepatocytes where a small increase in NF-κB binding was observed, but only 8 hrs later. A study that evaluated the ability of DEHP to activate production of reactive oxygen species in rat and mouse liver also concluded that DEHP activates Kupffer cells within hours of treatment and before induction of peroxisomal enzymes [132]. Finally, recent gene expression profiling study examined transcriptional changes induced by DEHP in mouse liver [88]. In addition to many genes that have been traditionally associated with hepatocyte-specific responses to PPs, a number of known components of the TNF/IL-1 signaling pathways, including *Irak2, Myd88, Ikbkg* and others were induced very early (2 hrs) and declined at later times (24 hrs) after acute treatment with DEHP, consistent with other studies showing a time-course of Kupffer cell activation.

Studies with cultured rodent hepatocytes, Kupffer cells, or mixed cell populations strengthened the evidence for the role of Kupffer cells. Both WY-14,643 and MEHP directly activate ***rat Kupffer cells in vitro*** to produce oxidants [158]. Furthermore, it was shown that LY-171883, a peroxisome proliferator, was taken up by Kupffer cells via phagocytosis leading to their activation [as reported in [42]]. Addition of non-parenchymal cells, or conditioned medium from non-parenchymal cell cultures, increased DNA synthesis 2-3 fold and suppressed TGFβ1-induced apoptosis by 50-70% in highly purified rat hepatocytes [112]. Furthermore, in both rats and mice, removal of non-parenchymal cells from normal hepatocyte cultures prevented both the nafenopin- and TNFα-induced increases in DNA synthesis and suppression of hepatocyte apoptosis; this response was restored by returning non-parenchymal cells to the purified hepatocytes [112,110].

It appears that Kupffer cell activation by PPs in PPARα-independent. In rat Kupffer cells, from naïve or WY-14,643-treated rats, neither PPARα mRNA, nor protein could be detected [113]. Moreover, when Kupffer cells from wild-type or *PPARα*-null mice were treated with

WY-14,643 *in vitro*, superoxide production was similar [113]. In addition, non-parenchymal cells isolated from *PPARα*-null mice, like those isolated from the wild-type mice, restored the proliferative hepatocyte response to nafenopin that is lost in highly purified liver parenchymal cells [110]. However, as expected, *PPARα*-null hepatocytes remained non-responsive to PPs, irrespective of the genotype of the added non-parenchymal cells. Finally, one study examined the potentiation of the effect of TNFα and a peroxisome proliferator WY-14,643 on cell proliferation in purified cultured rat hepatocytes [157]. It was concluded that an increase in mitogenic cytokine production by Kupffer cells is necessary for stimulation of DNA synthesis in purified rat parenchymal cells since the presence of both the PP and TNFα is required for a maximal proliferative response similar to that in whole liver.

Kupffer Cell-Mediated Production of Reactive Oxygen Species

Stimulation of Kupffer cells results in activation of NADPH oxidase and production of superoxide anion [168]. Indeed, both WY-14,643 and MEHP, are able to increase superoxide production by isolated rat Kupffer cells in a dose-dependent manner, indicating that they can affect Kupffer cells directly [158]. This effect of WY-14,643 was from activation of NADPH oxidase, since Kupffer cells isolated from $p47^{phox}$-null mice, that lack the ability to activate this oxidant-generating protein, show no increase in superoxide production [169]. In addition, direct evidence for increased oxidant production *in vivo* after treatment with DEHP was obtained using a spin-trapping technique and electron spin resonance (ESR) spectroscopy [132]. Specifically, when rats were given DEHP acutely for 2 hrs, an ESR-detectable radical adduct signal was detected. No increase in the radical signal due to DEHP was observed when Kupffer cells were inactivated *in vivo* with glycine pre-treatment, or in $p47^{phox}$-null mice. The rapid DEHP-induced free radical production *in vivo* occurred long before H_2O_2-generating enzymes in peroxisomes were induced, and it was not dependent on PPARα status.

It was suggested that Kupffer cell-derived oxidants may play a role in signaling increases in cell proliferation caused by PPs via a mechanism involving TNFα and NF-κB [133]. Indeed, both activation of NF-κB and increases in cell proliferation due to a single dose of WY-14,643 were prevented completely when rats were pretreated with diphenyleneiodonium, an inhibitor of NADPH oxidase [169]. Furthermore, WY-14,643-induced activation of NF-κB, increase in TNFα mRNA, and acute increases in liver weight and cell proliferation did not occur in $p47^{phox}$-null mice. Combined, these results provide strong evidence for a role of NADPH oxidase in Kupffer cells as a source of oxidants increased by treatment with PPs.

Since PPARα is not involved in activation of Kupffer cells by PPs (see above), it is not clear how exactly such activation occurs upstream of the NADPH oxidase. Furthermore, several published reports questioned the role for TNFα in PP-induced cell proliferation in rodent liver, and the possibility of involvement of the Kupffer cell-specific events in PP-induced carcinogenesis is uncertain (see below).

Lack of Data on a Sustained Role of the Kupffer Cell-Derived Cytokines in PP-Induced Rodent Liver Carcinogenesis

There remains uncertainty in establishing a causal link between Kupffer cell-mediated production of cytokines and long-term effects of PPs as there is no published report that examined such possible connection. The only published report that suggests that Kupffer cell activation may persist for longer than a few days compared the effects of WY-14,643 and DEHP that both elevate hepatocyte replication during the first few days of treatment; however, only WY-14,643 sustains rates of proliferation with long-term treatment [as reported in [28]]. It was found that Kupffer cells isolated from rats fed WY-14,643 generated superoxide at rates significantly greater than cells from controls for up to 3 weeks of treatment; however,

superoxide production was not stimulated by feeding DEHP for the same period of time [158].

Two separate studies attempted to define the role of TNFα signaling in hepatocellular growth induced by PPs by administering the potent peroxisome proliferator, WY-14 643 (for up to 4 days), to mice nullizygous for TNF-receptor 1 (TNFR1), TNFR2, both receptors, or TNFα protein [165,170]. Neither study found any evidence of abrogated PP-induced proliferative response in mouse liver as a result of a null genotype. It should be noted, however, that since cytokine-induced signaling is highly redundant, the knockout models used in these studies may have compensated for the lack of a particular signaling molecule. When cytokine expression was examined in non-tumorous liver tissue or adenomas in wild type mice fed with a carcinogenic dose of WY-14,643 for 52 weeks, no difference in expression of TNFα, IL-6, or TNFR1&2 was found [165]. However, IL-1β mRNA was significantly elevated and it was postulated that cytokines other than TNFα may be important liver co-mitogens in PP-treated rodents.

The potential for the Kupffer cell-derived oxidants to contribute to oxidative DNA damage following exposure to PPs has also been questioned. The analysis of expression of base excision DNA repair genes was used to assess whether this sensitive *in vivo* biomarker of oxidative stress to DNA can be used to determine the source of DNA-damaging oxidants following treatment with PPs. Using *PPARα*- and $p47^{phox}$-null mice treated with WY-14,643 for 4 weeks, the report concluded that DNA-damaging oxidants are generated by enzymes that are induced after activation of PPARα, such as those involved in lipid metabolism in peroxisomes, and are not the result of activation of NADPH oxidase in Kupffer cells [131]. Furthermore, no increase in hepatic BrDU labeling index was detected in *PPARα*-null mice fed the Wy-14,643 diet for 1 or 5 weeks which suggests that the effects of peroxisome proliferators on replicative DNA synthesis in rodent liver are mediated by PPARα, but not Kupffer cells [72].

PPARα-independent mechanisms

It is clear from the previous discussion that many of the biological effects resulting from exposure to DEHP and its metabolites are due to PPARα-mediated events. However, it is essential to point that there are other possible mechanisms of action that may also contribute to DEHP associated toxicity. Since prolonged exposure to DEHP can cause testicular toxicity in the absence of PPARα expression [33], this demonstrates that this compound can cause toxicity independent of receptor activation. Indeed, PPAR ligands can cause mitochondrial dysfunction, activate intracellular kinases signaling pathway, inhibit enzymes, and possibly interact with other receptors or unidentified intracellular targets, and there is good evidence that these effects are mediated independent of receptor activation [171]. For example, DEHP and other PPARα ligands causes direct depolarization of mitochondrial membrane potential leading to inhibition of ATP synthesis [172]. While the potential for DEHP to activate kinase signaling, inhibit enzymes or interact with other unidentified molecular targets has not been examined to date, since other PPARα ligands have been shown to have similar effects [171], it remains possible that these pathways contribute to DEHP-dependent toxicity. However, it is also worth noting that the biological significance of many of these changes with respect to modulating toxicity is largely unknown.

Summary

DEHP is clearly carcinogenic in liver in both sexes of rats and mice. It is evident that a number of molecular signals and multiple pathways in several cell types in the liver, rather than a single molecular event such as activation of PPARα and peroxisomal genes, or cell proliferation, contribute to the formation of liver tumors in susceptible rodent species. While exposure to DEHP and other phthalates is common in general population in humans, only limited DEHP-

specific human data is available. The majority of experimental human data that is available in the peer reviewed literature comes from *in vitro* studies in cultured human liver cells and these studies suggest that key pathways that are responsible for liver carcinogenesis in rats and mice either do not occur in human cells, or the response is considerably weaker. The lack of adequate epidemiological studies on long-term effects of DEHP and other PPs constitutes an important gap in our knowledge on the potential for these agents to cause human liver tumors. Activation of PPARα and the subsequent downstream events mediated by this transcription factor may be one central mechanism of action (Figure 2), but the average concentration of DEHP and DEHP metabolites found in humans is considerably lower than that required to transactivate PPARα in vitro. Coupled with the observations that humans appears to be relatively refractory to many of the effects induced by exposure to DEHP, due to a variety of species differences, suggests that the average human population may not be at risk for PPARα-mediated liver cancer. However, certain exposures (*e.g.*, occupational or dialysis exposures) could still represent significant concern. More importantly, given the potential for DEHP and other PPARα ligands to induce alterations independent of PPARα activation, it remains possible that these pathways contribute significantly to toxicity (Figure 2). This is an area of research that deserves evaluation.

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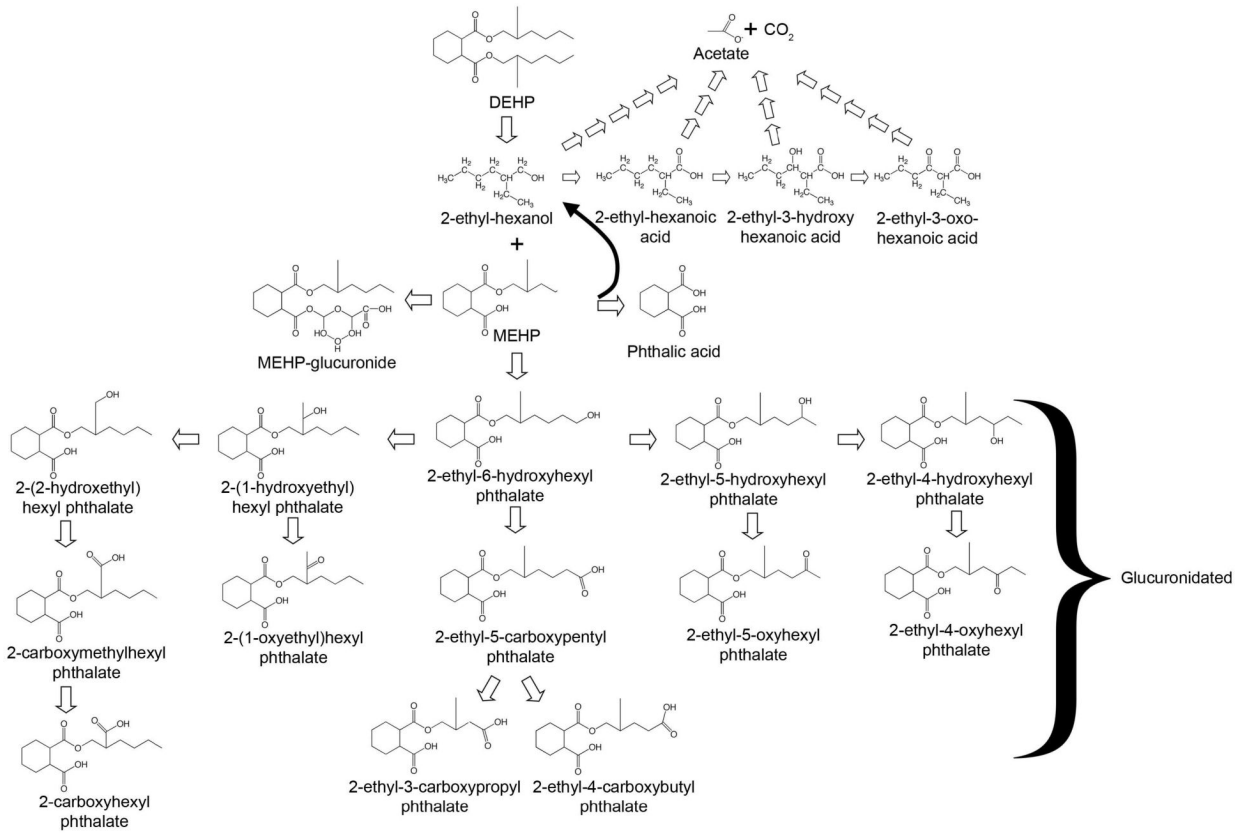

Figure 1.

Metabolic fate of DEHP. Lypolysis of DEHP by esterases in different tissues results in the formation of MEHP and 2-ethylhexanol. 2-ethylhexanol can be further metabolized with its ultimate fate being acetate and carbon dioxide. MEHP can be further metabolized by esterases to form another molecule of 2-ethylhexanol and phthalic acid. Alternatively, the sidechain of MEHP can be further oxidized to a variety of metabolites. MEHP and any of its downstream metabolites can be glucuronidated and then excreted.

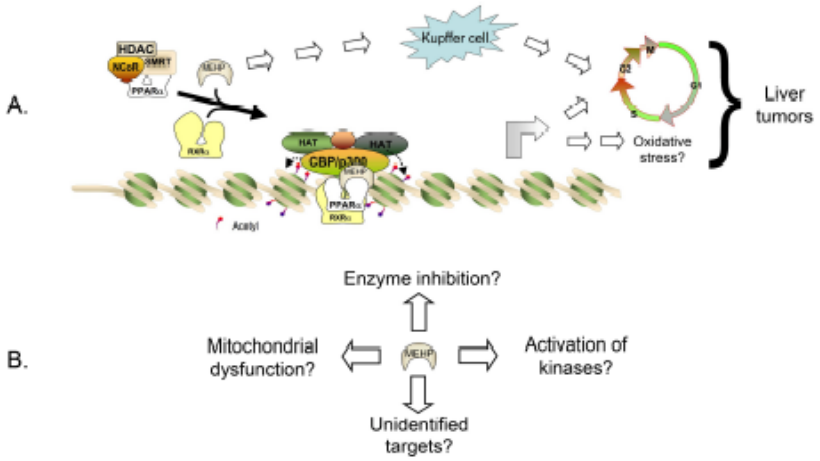

Figure 2.

Postulated mechanisms of DEHP-mediated toxicity in liver. (A) DEHP causes liver cancer in rodent models via activation of the nuclear receptor, PPARα, which leads to changes in gene expression that cause increased cell proliferation/inhibition of apoptosis and/or oxidative stress. DNA mutations that may or may not be due to the increased oxidative stress are “fixed” in cells due to the increased cell proliferation leading to liver tumors. DEHP and other PPARα ligands can also activate Kupffer cells and influence cell proliferation, independent of PPARα. (B) In addition to this well-documented mechanism of action, DEHP could also cause PPARα-independent changes including activation of intracellular kinases, enzyme inhibition or interactions with other molecular targets that could potentially influence DEHP-dependent toxicity. However, the role, if any, of these changes is still uncertain.