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Trichloroethylene Issue Paper 3:

Role of Peroxisome Proliferator-Activated Receptor Agonism and Cell Signaling in Trichloroethylene Toxicity

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DISCLAIMER

This issue paper does not represent and should not be construed to represent any agency determination or policy. This issue paper has not been externally reviewed. The information is being provided to assist the National Academy of Sciences in their review of the scientific issues surrounding trichloroethylene health risks.

CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

ACO	Acyl-coenzyme A oxidase
CAD	Coronary artery disease
CH	Chloral hydrate
DBP	Dibutyl phthalate
DCA	Dichloroacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
DEHP	Di(2-ethylhexyl) phthalate
DHEA	Dehydroepiandrosterone
EGF	Epidermal growth factor
ES	Esterase
FAH	Foci of altered hepatocytes
FCHL	Familial combined hyperlipidemia
GJIC	Gap junction intercellular communication
GEM	Gemfibrozil
GH	Growth hormone
GW	Gestation week
IGF	Insulin-like growth factor
IGFBP	IGF-binding proteins
IL1	Interleukin 1
LCT	Leydig cell tumor
LPL	Lipoprotein lipase
Ly-6D	Lymphocyte antigen 6 complex locus
MOA	Mode of action
NAS	National Academy of Sciences
NPC	Nonparenchymal cell
PACT	Pancreatic acinar cell tumor
PPAR	Peroxisome proliferator-activated receptor
PPARα	PPARalpha
PPARδ	PPARdelta
PPARλ	PPARgamma
PPRE	Peroxisome proliferator response element
ROS	Reactive oxygen species
SAB	Science Advisory Board

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

SAP	Science Advisory Panel
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TCE	Trichloroethylene
TNFα	Tumor necrosis factor alpha
WY	WY-14,643

PREFACE

Publication of these issue papers is a part of EPA's effort to develop a trichloroethylene (TCE) human health risk assessment. These issue papers were developed to provide scientific and technical information to the National Academy of Sciences (NAS) for use in developing their advice on how to best address the important scientific issues surrounding TCE health risks. As such, these papers discuss a wide range of perspectives and scientific information (current through Fall 2004) on some of these important issues, highlighting areas of continuing uncertainty and data that may be relevant. They are intended to be useful characterizations of the issues, not a presentation of EPA conclusions on these issues. The papers have undergone internal review within EPA, but they have not been externally reviewed. The concepts presented in these papers will eventually be addressed in EPA's revised risk assessment of TCE, after the advice from the NAS, along with comments from the EPA Science Advisory Board and the public, as well as recently published scientific literature, have been incorporated.

AUTHORS AND CONTRIBUTORS

Many individuals contributed to the completion of this set of tichloroethylene (TCE) issue papers. The TCE Risk Assessment Team identified the topics covered by the papers and prepared them for submission to the National Academy of Sciences. The authors wish to thank Dr. Peter Preuss, Dr. John Vandenberg, Mr. David Bussard, Mr. Paul White, Dr. Bob Sonawane, Dr. Hugh Barton, Dr. Aparna Koppikar, Mr. David Bayliss, Dr. William Wood, and Dr. Ila Cote for their input and comments.

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THE TCE ISSUE PAPERS

BACKGROUND

In August 2001, a draft, *Trichloroethylene (TCE) Health Risk Assessment: Synthesis and Characterization,* was released for external review. This draft assessment drew on 16 "state-of-the-science" papers published as a supplemental issue of *Environmental Health Perspectives* (Volume 108, Supplement 2, May 2000). Subsequent to its release, EPA's 2001 draft assessment underwent a peer review by a panel of independent scientists through EPA's Science Advisory Board (SAB), which provided a peer review report in December 2002. In addition, the public submitted more than 800 pages of comments to EPA during a 120-day public comment period.

There are a number of important issues that EPA will need to examine as it moves forward in revising the draft TCE assessment. These include issues raised not only in the SAB peer review and public comments, but also by new scientific literature published since the release of the state-of-the-science papers and EPA's 2001 draft assessment. Some of this research is specific to the study of TCE or its metabolites while some of it describes advances in scientific fields more generally but which have potential relevance to characterizing the human health risks from TCE.

In February 2004, EPA held a symposium so that authors of some of the TCE-specific research that had been published since the release of the draft assessment could present their findings in more detail. This symposium represented only a limited cross section of recently published research, but was reflective of the breadth of new relevant science that EPA will consider in revising the assessment (the presentation slides and a transcript of the meeting are available separately on EPA's website and have already been sent to the NAS).

In 2004, EPA, in cooperation with a number of other federal agencies, initiated a consultation with the National Academy of Sciences (NAS) to provide advice on scientific issues related to the health risk assessment of TCE. It was recognized that a review by an NAS panel of the important scientific issues would be beneficial and informative to clarify the state-of-the-science as EPA moves forward in completing its health risk assessment. A charge was developed for the NAS through an Interagency Workgroup led by the White House Office of Science and Technology Policy.

PURPOSE OF THE TCE ISSUE PAPERS

Although EPA will need to address all of the issues identified in the charge to the NAS panel in updating its assessment, EPA would like to focus the NAS panel's attention on a subset of issues that EPA believes to be most critical in developing a revised risk assessment, as summarized in four issue papers developed by EPA staff:

1. Issues in trichloroethylene pharmacokinetics;
2. Interactions of trichloroethylene, its metabolites, and other chemical exposures;
3. Role of peroxisome proliferator-activated receptor agonism and cell signaling in trichloroethylene toxicity; and
4. Issues in trichloroethylene cancer epidemiology.

Each paper provides an overview of the science issues, a discussion of perspectives on those issues (including the SAB and public comments), and an outline of some of the recently published scientific literature. The pharmacokinetics issue paper also summarizes results from a recent collaboration with the U.S. Air Force on TCE pharmacokinetics, as well as EPA's planned approach for further refinement of the pharmacokinetic modeling of TCE and its metabolites. These scientific areas were selected because they are (a) critical to the hazard and/or dose-response characterization of TCE; (b) scientifically complex and/or controversial; and (c) areas in which substantial important scientific literature has been recently published. The input from the NAS on the topics described in the issue papers, as well as other topics put forth in the charge to the NAS, should help to strengthen EPA's revised TCE assessment.

NEXT STEPS

The advice from the NAS, along with comments already received from the EPA SAB and the public, as well as recently published scientific literature, will be incorporated into a revised EPA risk assessment of TCE, strengthening its scientific basis. Because of the substantial amount of new information and analysis that is expected, the revised draft of the assessment will undergo further peer review and public comment prior to completion.

1. INTRODUCTION

Understanding the mode of action (MOA) by which a chemical or its metabolite induces tumors is key to judgment about the relevance of such tumor data for human risk assessment and is a key feature of the U.S. Environmental Protection Agency's (EPA's) new cancer guidelines. The charge to the National Academy of Sciences (NAS) panel states that advice is being sought regarding the strength and weight of evidence for various MOAs for trichloroethylene (TCE) toxicity and their relevance to humans. Additional issues regarding MOAs delineated in the charge include the identification of key events, adverse effects, potential contributions from multiple MOAs, and MOA differences due to dose-response.

This paper discusses the issues surrounding peroxisome proliferator-activated receptor alpha (PPARα) agonism and TCE toxicity, both in terms of the variety of perspectives that have been put forth on these issues and in light of recently published scientific literature. In particular, an effort is made to highlight recently published research both on PPARα and TCE as well as on PPARα more generally, with an emphasis on areas of scientific uncertainty that have been identified by recent reviews (e.g., SAP 2004, Klaunig et al., 2003; Melnick et al., 2001). Although a large body of information is presented on these topics, this paper is not intended to be a comprehensive, complete review or characterization of PPARα agonism and TCE toxicity, nor is it intended to advocate or critique particular points of view.

1.1. TCE AND PPARα AGONISM

One of the important endpoints associated with TCE exposure is liver cancer. The epidemiological review of Wartenberg et al. (2000) reported that the data suggested an overall excess incidence of liver cancer in humans exposed to TCE. Liver cancer has been reported in mice exposed to TCE but not in rats. Furthermore, TCE metabolites (trichloroacetic acid [TCA], dichloroacetic acid [DCA], and chloral hydrate [CH]) have been shown to cause liver tumors in mice, with DCA also causing liver tumors in rats. DCA and TCA have been the primary focus as the potentially active agents causing hepatocarcinogenicity.

EPA's draft TCE assessment concluded that the MOA for liver cancer for TCE or its metabolites was unknown and considered peroxisome proliferation as one of several possible MOAs for TCE-induced liver tumors. TCE, DCA, and TCA induce peroxisome proliferation in rodents via PPARα activation, although they are considered to be weak peroxisome proliferators (e.g., as compared with the model pharmaceutical drug WY-14,463 [WY]). EPA's draft assessment stated that the role of PPARα activation as a possible MOA was more plausible for TCA than for DCA because TCA induces a more sustained proliferative response than DCA, and

DCA appears to induce effects on cell-signaling processes and on carbohydrate handing at lower concentrations than those associated with peroxisome proliferation or PPARα activation. It was noted that the role of PPARα activation and the sequence of events following activation of this receptor that are important to tumorigenesis are not well defined and, furthermore, that the key events critical to tumor induction and the cross-species relevance of these key events had yet to be identified.

The question of what human health risks may be posed by peroxisome proliferators, both in general and in the case of TCE, remains controversial. The EPA Science Advisory Board (SAB) review of EPA's draft TCE risk assessment recommended that EPA consider giving less weight to peroxisome proliferation as a possible MOA for TCE-induced liver cancer than to some other hypotheses. By contrast, among public comments, some commenters stated that more emphasis should be placed on TCA and a mechanism involving interaction with PPARα and that humans are more generally unresponsive to peroxisome proliferators with regard to the factors that may contribute to liver tumors.

1.2. PERSPECTIVES ON PPARα AGONISM AND TUMOR INDUCTION

Substantial scientific interest exists regarding the role of peroxisome proliferation in rodent hepatocarcinogenesis and its relevance for human carcinogenesis in both the liver and other potential sites. Because of the uncertainty of human cancer risk associated with peroxisome proliferators, delineating the mechanisms of carcinogenesis by these agents is of great interest (Kiss et al., 2001). Despite scientific advances, the mechanism by which PPARα agonists induce liver tumors in rodents is unknown (Melnick et al., 2003; Melnick, 2002, 2001; Miller et al., 2001; Kiss et al., 2001; Bull, 2000; Peters et al., 2000; Zhou and Waxman, 1998; Bojes et al., 1997).

It was initially proposed that the MOA for liver tumors caused by peroxisome proliferators is due to oxidative damage caused by marked increases in free radical-generating enzymes and peroxisomal β-oxidation might initiate carcinogenesis. Under this hypothesis, it was generally believed that because peroxisome proliferation has not been observed in humans, agents that produced this result in rodents would not present a carcinogenic hazard to humans. For instance, Cattley et al. (1998) state:

> A core set of biochemical and cellular events has been identified in the rodent strains that are susceptible to the hepatocarcinogenic effects of peroxisome proliferators, including peroxisome proliferation, increases in fatty acyl-CoA oxidase levels, microsomal fatty acid oxidation, excess production of hydrogen peroxide, increases in rates of cell proliferation, and expression and activation of the alpha subtype of the peroxisome proliferator-activated receptor (PPAR-α).

> Such effects have not been identified clinically in liver biopsies from humans exposed to peroxisome proliferators or in in vitro studies with human hepatocytes, although PPAR-α is expressed at a very low level in human liver. Consensus was reached regarding the significant intermediary roles of cell proliferation and PPAR-α receptor expression and activation in tumor formation. Information considered necessary for characterizing a compound as a peroxisome proliferating hepatocarcinogen include hepatomegaly, enhanced cell proliferation, and an increase in hepatic acyl-CoA oxidase and/or palmitoyl-CoA oxidation levels. Given the lack of genotoxic potential of most peroxisome proliferating agents, and since humans appear likely to be refractive or insensitive to the tumorigenic response, risk assessments based on tumor data may not be appropriate. However, nontumor data on intermediate endpoints would provide appropriate toxicological endpoints to determine a point of departure such as the LED10 or NOAEL which would be the basis for a margin-of-exposure (MOE) risk assessment approach. Pertinent factors to be considered in the MOE evaluation would include the slope of the dose-response curve at the point of departure, the background exposure levels, and variability in the human response.

A number of public comments on the draft TCE assessment expressed support for this point of view.

However, more recent scientific developments have led some to reevaluate the state of the science concerning the MOA and human relevance of rodent tumors induced by certain peroxisome- proliferating agents. One recent debate on the risk posed to humans by peroxisome proliferators has been focused by the risk assessment of di-(2-ethylhexyl) phthalate (DEHP), a mild peroxisome proliferator in rodent liver (Melnick, 2002, 2001). Melnick et al. (2003) have argued that human cancer risk from PPAR agonists cannot be dismissed. In particular, Melnick (2001) states:

> The literature review presented in this commentary reveals that, although our knowledge of the mechanism of peroxisome proliferation has advanced greatly over the past 10 years, our understanding of the mechanism(s) of carcinogenicity of peroxisome proliferators remains incomplete. Most important is that published studies have not established peroxisome proliferation per se as an obligatory pathway in the carcinogenicity of DEHP. No epidemiologic studies have been reported on the potential carcinogenicity of DEHP, and cancer epidemiologic studies of hypolipidemic fibrate drugs (peroxisome proliferators) are inconclusive. Most of the pleiotropic effects of peroxisome proliferators are mediated by the peroxisome proliferator activated receptor (PPAR), a ligand-activated transcription factor that is expressed at lower levels in humans than in rats and mice. In spite of this species difference in PPAR expression, hypolipidemic fibrates have been shown to induce hypolipidemia in humans and to modulate gene expression (e.g., genes regulating lipid homeostasis) in human hepatocytes by PPAR activation. Thus, humans are responsive to agents that induce

> peroxisome proliferation in rats and mice. Because peroxisome proliferators can affect multiple signaling pathways by transcriptional activation of PPAR-regulated genes, it is likely that alterations in specific regulated pathways (e.g., suppression of apoptosis, protooncogene expression) are involved in tumor induction by peroxisome proliferators. In addition, because DEHP also induces biological effects that occur independently of peroxisome proliferation (e.g., morphologic cell transformation and decreased levels of gap junction intercellular communication), it is possible that some of these responses also contribute to the carcinogenicity of this chemical. Last, species differences in tissue expression of PPARs indicate that it may not be appropriate to expect exact site correspondence for potential PPAR-mediated effects induced by peroxisome proliferators in animals and humans. Because peroxisome proliferation has not been established as an obligatory step in the carcinogenicity of DEHP, the contention that DEHP poses no carcinogenic risk to humans because of species differences in peroxisome proliferation should be viewed as an unvalidated hypothesis.

In addition, the International Life Sciences Institute's Risk Science Institute independently convened a workgroup to update the state of the science regarding PPARα agonist-induced rodent liver tumors and to evaluate the MOA for Leydig cell tumors (LCTs) and pancreatic acinar cell tumors (PACTs), which also are observed frequently in rats with PPARα agonists (Klaunig et al., 2003). They described a proposed mode of action for rodent liver carcinogenesis, including a discussion of key events that were either causal (PPARα activation, perturbation of cell proliferation and/or apoptosis, and selective clonal expansion) or associative (expression of peroxisomal genes; PPARα mediated gene expression of cell cycle, growth, and apoptosis; nonperoxisome lipid gene expression; peroxisome proliferation; inhibition of gap junction intercellular communication [GJIC]; hepatocyte oxidative stress; and Kuppfer cell-mediated events). The workgroup concluded that although the PPARα receptor can be activated in humans, substantial species differences in toxicodynamics make it very unlikely for the downstream key events, and therefore hepatocarcinogenesis, to occur in humans. For instance, in contrast to Melnick et al. (2003), the ILSI workgroup concluded the following with respect to DEHP (Klaunig et al., 2003):

> Using the human relevance framework, the data are sufficient to support the conclusion that a mode of action for the DEHP-induced liver tumor has been established in animals. Comparing the key events of this MOA to humans (and nonhuman primates), the key event of perturbation of cell proliferation and/or apoptosis does not occur in nonhuman primates. In addition, some associative events such as increased peroxisomal enzyme activity and inhibition of GJIC do not occur. This leads to a conclusion that key events of the MOA are not plausible in nonhuman primates (and humans). The strength of this conclusion rests on only one study of a causal key event and several studies of associative

events. However, dynamic and kinetic factors contribute to this decision. The data lead to a conclusion that a carcinogenic response induced via the MOAs for liver tumorogenesis in the rodent is not likely to occur in humans following exposure to DEHP.

In addition, the workgroup concluded that, at present, there is insufficient information to firmly establish an MOA for LCTs and PACTs in rats (Klaunig et al., 2003).

Most recently, on December 9, 2003, the Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel (SAP) met to discuss its review of the set of scientific issues being considered by EPA pertaining to *Proposed Science Policy: Peroxisome Proliferator Activated Receptor-alpha (PPAR-α) Agonist-Mediated Hepatocarcinogenesis in Rodents and Relevance to Human Health Risk Assessment.* The SAP was asked to comment on several issues for which science policy conclusions had been proposed. These issues (and the proposed conclusions) included the following:

1. An MOA for rodent hepatocarcinogenesis (i.e., PPARα agonists activate PPARα leading to an increase in cell proliferation, a decrease in apoptosis, and eventual clonal expansion of preneoplastic cells leading to liver cancer);

2. The relative sensitivity of the fetal, neonatal, and adult rodent (i.e., conclusions about MOA in adults would apply to the young in rodents and humans);

3. The human relevance of PPARα agonist-induced hepatocarcinogenesis (i.e., that humans and nonhuman primates are refractory to the hepatic effects of PPARα agonists);

4. The data requirements sufficient to demonstrate the proposed MOA for rodent liver tumors (i.e., evidence of PPARα agonism, in vivo evidence of increase in size and number of peroxisomes, increases in the activity of acyl-coenzyme A oxidase [ACO] and hepatic cell proliferation, and adequacy of dose-response and temporal sequence between precursor events and liver tumor formation); and

5. The MOAs for other tumors induced by PPARα agonists (i.e., the data are insufficient to support MOAs for other tumors induced by PPARα agonists, such as LCTs and PACTs in rats).

The summary minutes of the meeting became publicly available March 5, 2004 (SAP 2004). In brief, the panel conclusions were as follows:

1. Regarding the MOA for rodent hepatocarcinogenesis, the SAP wrote:

Overall, the majority of the Panel felt the evidence in support of the proposed MOA for PPAR-α agonist induced rodent hepatocarcinogenesis was adequate, though the opinions of individual Panel members ranged from full agreement to complete disagreement. The key event in the MOA is PPAR-α activation. PPAR-α activation triggers multiple events leading to tumorigenesis but the PPAR-α-altered genes in the causal pathway for tumor induction have not been identified. While some of the key events that occur after PPARα activation, such as increased cell proliferation, inhibition of apoptosis, and the clonal expansion of preneoplastic lesions are known, the PPAR-α dependent mechanism for the perturbation of these key events is less well established. Specifically, mechanisms and steps linking key events downstream of PPAR-α activation are not known. The data are sufficient to demonstrate a PPAR-α activation dependence to the MOA, but are inadequate to provide the quantitative linkages associated with a more defined mechanism of action. The Panel members agreed that additional evidence of specific alterations associated with PPAR-α activation would greatly strengthen the proposed MOA.

2. Regarding the relative sensitivity of fetal, neonatal, and adult rodents, the SAP wrote:

   The Panel does not support the OPPTS conclusions. Although fetal and embryonic rats and mice respond to PPAR-α agonists as demonstrated by changes in peroxisomal enzyme activities, strong evidence demonstrating that fetal and neonatal rats do not exhibit an increased sensitivity to PPAR-α agonist-induced hepatocarcinogenesis is lacking. Moreover, conclusions regarding this MOA for human hepatocarcinogenesis should not be applied to developing humans.

3. Regarding the human relevance, the SAP wrote:

   Overall, the majority of the Panel agreed that there are relevant data indicating that humans are less sensitive than rodents to the hepatic effects of PPAR-α agonists. However, the opinions of individual Panel members ranged from full agreement with the proposed OPPTS policy statement, as currently written, to complete disagreement. The majority of the Panel recognized weaknesses in the data that supported the policy noting in particular that the case for lack of human relevance was deficient in the human data.

4. With regard to data requirements, the SAP wrote:

   There was general consensus among the Panel that the proposed data set was adequate and provided a straight forward approach to classifying a chemical as a PPAR-α agonist. The Panel also concurred that the use of PPAR-α knockout mice would be definitive evidence to ascribe a chemical as a PPAR-α agonist, but that the proposed data set would be sufficient in

lieu of the use of this rather costly tool. While the Panel agreed with these data needs, they suggested some clarifications and additional supportive approaches.

In response to a clarification by EPA that data demonstrating PPAR-α agonist activity could be submitted in the absence of testing in long-term carcinogenesis studies, the SAP wrote that:

> [A] Panel member observed that in the absence of testing in standard long-term rodent carcinogenicity studies, it is not possible to determine whether the chemical would operate through a PPAR-α agonist MOA producing rodent liver tumors. A chemical with PPAR-α agonist activity may either: 1) not cause cancer in rodents, 2) cause liver cancer in rodents by the proposed PPAR-α agonist MOA, 3) cause liver cancer by a MOA other than the proposed PPAR-α agonist MOA (e.g., cytotoxicity), or 4) cause cancer at sites other than the liver (with or without liver cancer). The Panel concurred that an overriding requirement is that other MOAs have been excluded. For example, rigorous tests must be performed to exclude mutagenicity, other forms of DNA damage (clastogenicity), or overt cytotoxicity directly produced by the test compound, or its metabolic products.

5. Finally, with regard to other tumors, the SAP wrote:

> In addition to the hepatic tumors that appear to be a general occurrence in rats and mice, nine PPAR-α agonists have been reported to induce Leydig cell tumors (LCTs) and pancreatic acinar cell tumors (PACTs) in rats. Together with the hepatic tumors, this is referred to as the tumor triad. The Panel was in agreement with the OPPTS conclusion that chemicals that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

1.3. ISSUES FOR NAS TO CONSIDER REGARDING PPARα AGONISM AND TCE

NAS input regarding the state of the science on PPARα agonism as it relates to the toxicity of TCE and its metabolites would be particularly important for several reasons as EPA revises its risk assessment of TCE. First, as noted above, a variety of divergent perspectives exist regarding the role of PPARα in TCE toxicity and, more generally, the actions of and human health risks from PPARα agonists. Second, scientific interest in PPARα has greatly increased in recent years owing to possible pharmaceutical applications, and research on the effects resulting from activation of this receptor is expanding at an extremely rapid pace.

Because of the quantity and quality of recent scientific research on PPARα, EPA needs to assess both the possible role(s) of PPARα in TCE toxicity and the potential human health hazard from TCE exposure in the light of this new literature. In particular, some of this new information may be important in addressing areas of scientific uncertainty that have been identified in the recent reviews cited above. Therefore, EPA would like to seek advice from NAS on the following issues/questions:

1. What effects from PPARα agonism, either peroxisomal or extra-peroxisomal, may be plausibly involved in TCE-induced rodent liver tumors?

2. Can changes in gene expression or methylation status be adequately characterized to determine whether the changes induced by peroxisome proliferators and the changes induced by TCE are consistent with each other?

3. Have PPARα agonism and related downstream effects been demonstrated to be both *necessary* and *sufficient* to account for TCE-induced liver tumors in rodents?

    a. How adequate are studies in PPARα knockout mice for demonstrating the dependency of TCE-induced hepatocarcinogenesis on PPARα agonism?

    b. Is it possible the TCE-induced biological effects related to PPARα agonism that occur independently of peroxisome proliferation contribute to carcinogenicity?

    c. What cell-signaling or other mechanisms *independent* of those resulting from PPARα agonism may be plausibly involved in TCE-induced liver carcinogenesis?

4. What does the available evidence tell us about the relative sensitivity of humans to possible MOAs of TCE or its metabolites to induce liver tumors, particularly as related to PPARα agonism?

    a. What can be concluded about the actions of PPARα agonism in humans and its relationship to MOAs of cancer?

    b. What can be concluded about human sensitivity to the adverse effects from PPARα agonism by TCE and its metabolites, given species differences in response to PPARα agonism and the state of knowledge of effects in various species?

    c. What other factors (such as baseline actions and responses related to PPARα, genetic polymorphisms, gender, and life stage) modulate human sensitivity to PPARα agonism by TCE or its metabolites?

5. Are there other cancer or noncancer effects of TCE for which PPARα agonism may be plausibly involved?

This issue paper summarizes and outlines some of the recently published scientific literature identified by EPA that may be informative as to TCE toxicity. Section 2 discusses the limited recent information on TCE or its metabolites and PPAR, and Section 3 focuses on recent information on PPARα agonists in general that may be relevant to TCE toxicity. In addition, throughout Section 3, we have drawn on the SAP discussion of areas of scientific uncertainty potentially relevant to the addressing the TCE-related questions above, focusing in particular on their discussion of the data they reviewed and what additional data they thought could help to clarify the science. Although there may not be definitive answers to the above questions given the current state of the science, NAS input regarding the interpretation and characterization of existing evidence would help to strengthen EPA's revised assessment.

2. CONSIDERATIONS FOR PEROXISOME PROLIFERATION AND OTHER EFFECTS OF TCE AND METABOLITES

EPA will need to integrate information specific to TCE with the broad database of information on PPARα agonists (presented in Section 3) to characterize the potential role of PPARα agonism in TCE-induced effects. Several TCE-specific issues related to this integration are summarized in this section. Section 2.1 discusses recent evidence regarding which TCE metabolite(s) may be involved in TCE-induced liver tumors and to what extent PPARα agonism may be involved for different TCE metabolites. Section 2.2 discusses independent information on liver tumor phenotypes that may inform cross-species extrapolation and thus bear on the plausibility of various mechanisms related to PPARα agonism. Finally, Section 2.3 discusses recent data on TCE's effects on transcriptional regulation and their potential relationship to gene expression effects observed from PPARα agonists more generally.

2.1. CONTRIBUTION OF TCE METABOLITES TO TUMOR INDUCTION AND EXPOSURE-LEVEL CONSIDERATIONS FOR THEIR EFFECTS

TCE and its metabolites TCA and DCA have all been shown to activate PPARs to varying degrees. Maloney and Waxman (1999) reported TCA and DCA at high concentrations in vitro activated PPARα in humans. There did not appear to be a difference between species in activation of PPARα, but there was a difference in activation of PPAR gamma (PPARγ). TCA and DCA showed no PPARγ activity in humans, but TCA activated PPARγ in mice (Maloney and Waxman 1999). Recent data have helped to elucidate the potential roles of each of these compounds in TCE liver tumor induction and the extent to which PPARα agonism may play a

role, although definitive conclusions do not appear to have been reached. As described in a separate issue paper, the work of Bull et al. (2004) and Bull et al. (2002) suggests that neither TCA nor DCA can be assigned individually as the agent causing TCE-induced liver tumors in mice, that both TCA and DCA may be involved, and that TCA and DCA may affect each other's toxicity.

With regard to PPARα, both TCA and DCA can elicit peroxisome proliferation in rodents at high enough exposure levels. However, is peroxisome proliferation a key step in TCE-induced carcinogenesis? Consideration needs to be given to the other effects that have been reported in conjunction with liver tumor induction by TCE and its metabolites, and the exposure levels at which they occur, to examine the plausibility of these effects as key events.

A study by Pereira et al. (2004a) investigated a number of effects of DCA related to liver tumor induction. They reported DCA-induced hypomethylation in mouse liver at exposure levels that also induced glycogen accumulation and peroxisome proliferation. To test the involvement of DNA hypomethylation in the carcinogenic activity of DCA, the effect of methionine on both activities was examined. Following 8 weeks of exposure, methionine altered DCA-induced DNA hypomethylation and marginally reduced glycogen accumulation; however, it did not alter the increased liver/body weight ratio or the proliferation of peroxisomes. After 44 weeks of exposure, DCA induced foci of altered hepatocytes and hepatocellular adenomas with methionine having varying effects on the multiplicity of foci that were dependent on dose. Other studies concerning the hypomethylation of DCA and TCA (Tao et al., 2004; Tao et al., 2000) are discussed in a separate issue paper. Tao et al. (2004) reported no difference in hypomethylation between DCA and TCA.

A recent study by Laughter et al. (2004) has used the PPARα knockout mouse to try to establish the role of PPARα in responses to TCE and its metabolites. Overall, the study authors concluded that "[t]hese data support the hypothesis that PPARα plays a dominant role in mediating the effects associated with hepatocarcinogenesis upon TCE exposure." Male wild-type and knockout mice received either TCE for either 3 days (1,500 mg/kg) or 3 weeks (0 - 1,500 mg/kg). TCE treatment increased liver weight in wild-type mice. Knockout mice did not show a statistically significant effect from TCE treatment but had greater liver-to-body weight ratios than wild-type mice at all levels of exposure, including controls. This study also examined liver to body weight ratios after 1 week of either TCA or DCA exposure in wild-type or PPARα knockout male mice. There was no difference in liver weight ratios between the two types of mice after TCA exposure and only a small difference at the highest dose of DCA. Liver weight changes were suggested as a surrogate for peroxisomal proliferative activity, although neither peroxisome proliferation nor changes in glycogen content of the liver was directly measured. Differences in experimental protocol and relatively high baseline measures of liver-to-body

weight ratios in control PPARα knockout mice made comparisons between TCE effects and those of its metabolites difficult in this study. Additional discussion of this study is in Sections 2.3 and 3.5, with Section 3.5 also discussing general issues related to interpreting results using PPARα knockout mice.

Based on relevancy of exposure levels to reported effects, Bull (2004) and Bull et al. (2004) have recently suggested that peroxisome proliferation is not a key step in liver tumor induction. They report that a direct comparison in the no-effect level or low-effect level for induction of liver tumors in the mouse and several other endpoints shows that, for TCA, liver tumors occur at lower concentrations than peroxisome proliferation in vivo but that PPARα activation occurs at a lower dose than either tumor formation or peroxisome proliferation. A similar comparison for DCA shows that liver tumor formation occurs at a much lower exposure level than peroxisome proliferation, PPARα activation, or hypomethylation. However, apoptosis is suppressed at levels that also induce liver tumors as well as decreases in insulin and increases in glycogen. In addition, they report that these chemicals are effective as carcinogens at doses that do not produce cytotoxicity. Thus, the authors suggest that TCA and DCA encourage clonal expansion of initiated cells through subtle, selective mechanisms.

2.2. IMPLICATIONS OF PHENOTYPE FOR TCE-INDUCED TUMORS AND HUMAN RISK

Tumor phenotype has been used to investigate the MOA of TCE through inferences of the assignment of metabolite(s) as the active agent in rodent liver tumor production. It has also been used to examine the differences in tumors produced by peroxisome proliferators and other agents. Tumor phenotype is discussed in Section 2.2 of *Interactions of Trichloroethylene, Its Metabolites, and Other Chemical Exposures* with regard to the potential active agents of TCE toxicity and to the effects of potential co-exposures on the toxicity pattern of TCE. Consistent with SAP advice to investigate other MOAs besides PPARα agonism, phenotype may help in determining the relevancy of TCE-induced rodent tumors to human risk and may be informative regarding the plausibility of contributions from PPARα agonism. Among the different types of liver tumor, hepatocellular neoplasms predominate by far in both animals and humans. Foci of altered hepatocytes (FAH) precede both hepatocellular adenomas and carcinomas in rodents and, in humans with chronic liver diseases that predispose them, hepatocellular carcinomas. Striking similarities in specific changes of the cellular phenotype of preneoplastic FAH are emerging in experimental and human hepatocarcinogenesis, irrespective of whether this was elicited by chemicals, hormones, radiation, viruses, or, in animal models, by transgenic oncogenes or *Helicobacter hepaticus*. Several authors have noted that the detection of phenotypically similar FAH in various animal models and in humans prone to developing or bearing hepatocellular

carcinomas favors the extrapolation from data obtained in animals to humans (Bannasch et al., 2003; Su and Bannasch, 2003; Bannasch et al., 2001).

2.3. TRANSCRIPTIONAL REGULATION IN RESPONSE TO TCE EXPOSURE: ADEQUACY FOR MOA

Information has recently emerged on the effects both of TCE and its metabolites and of PPARα agonists more generally on gene expression. Gene expression studies have been expected to hold great promise for the elucidation of MOAs in general and, in particular, with relationship to the potential contributions from PPARα agonism. As mentioned in Section 1.3, one of the key issues for these studies is whether gene expression changes can be adequately characterized for TCE and its metabolites specifically as well as for peroxisome proliferators in general, so as to provide profiles which can be compared. Discussion of these issues in relation to peroxisome proliferators in general follows in Sections 3.3 and 3.5.

With regard to TCE specifically, although there is some information on changes in DNA methylation from TCE metabolites, as discussed above, TCE-specific data using DNA arrays remain limited. Studies attempt to examine changes in transcriptional regulation following TCE exposure to establish a pattern of response that can be related to an MOA. However, such changes must be considered within the context of the experimental paradigm used for the test condition and may be difficult to interpret. Furthermore, these data may be not only limited for examination of changes induced by TCE exposure but also inadequate for comparison to a standard set of changes expected by PPARα agonism—if such a profile exists (see Section 3.3).

In a screening analysis of 148 genes for xenobiotic-metabolizing enzymes, DNA repair enzymes, heat shock proteins, cytokines, and housekeeping genes, Bartosiewicz et al. (2001) report gene expression patterns in the liver in response TCE. TCE-induced gene induction was highly selective; only Hsp 25 and 86 and Cyp2a were up-regulated at the highest dose tested. Collier et al. (2003) report differentially expressed mRNA transcripts in embryonic hearts from Sprague-Dawley rats exposed to TCE and show that sequences down-regulated with TCE exposure appear to be those associated with cellular housekeeping, cell adhesion, and developmental processes, while TCE exposure up-regulated expression of numerous stress-response and homeostatic genes.

Laughter et al. (2004) recently examined transcription profile using macroarrays containing approximately 1,200 genes in response to TCE exposure. Forty three genes were significantly altered in the TCE-treated wild-type mice and 67 genes significantly altered in the TCE-treated PPARα knockout mice. Out of the 43 genes expressed in wild-type mice upon TCE exposure, 40 genes were dependent on PPARα. These genes included CYP4a12, epidermal growth factor receptor and additional genes involved in cell growth. The interpretation of this

information is difficult because PPARα knockout mice are more sensitive to a number of hepatotoxins partly because of defects in the ability to effectively repair tissue damage in the liver (Shankar et al., 2003a; Mehendale, 2000). Further, a comparison of gene expression profiles between controls (wild-type and PPARα knockout) were not reported. A more general discussion of the heterogeneity of PPARα effects in knockout mice follows in Section 3.5.

3. ISSUES RELATED TO PPAR AGONISM AND MOAs

Chronic diseases (such as diabetes, obesity, atherosclerosis, and cancer) are responsible for most deaths in developed societies. The evidence that PPAR activity may be involved in these ailments and can be modulated by such drugs as thiazolidinediones and fibrates has generated a huge research effort into PPARs (Kersten et al., 2000). The generation of data relevant to characterization of the human response to PPARα has been extensive since release of the draft EPA assessment of TCE.

A wide range of chemicals induce tumors when tested in laboratory strains of rats and mice of both genders. The classes of chemicals with such activity include DEHP and other phthalates; chlorinated paraffins; chlorinated solvents (such as TCE and perchloroethylene); and certain pesticides, hypolipidemic pharmaceuticals, and endogenous hormones and fatty acids (Klaunig et al., 2003; Gonzalez, 2002). Several different MOAs for tumor induction have been postulated, some beginning with PPARα activation as a causal first step (Klaunig et al., 2003).

An examination of the full spectrum of PPARα activity is necessary to make a comprehensive comparison with TCE-induced effects and thereby assess the potential role of PPARα agonism in TCE toxicity and its relevance to human risk. For instance, although humans are responsive to PPARα agonism, as evidenced by the efficacy of hypolipidemic fibrate drugs, studies of TCA and DCA in human hepatocyte cultures seem to indicate that the human liver is refractory to markers of peroxisome proliferation (Walgren et al., 2000a, b). However, as discussed previously, other studies have noted that extra-peroxisomal actions consistent with PPARα agonism by TCE metabolites occur at concentrations much lower than those producing peroxisome proliferation and tumor induction (Bull, 2004).

Some of the issues and perspectives surrounding this spectrum of activity are outlined in the following sections, including the variety of observed responses and potential species, strain, and inter-individual differences in those responses. Section 3.1 discusses the pleiotropic nature of responses to PPARα agonism beyond peroxisome proliferation alone and presents data on how these varied effects may be related to disease endpoints. Section 3.2 discusses data related to effects of PPARα agonists on nonparenchymal cells that may be PPARα-independent and their

potential role in carcinogenesis. Section 3.3 discusses information on PPARα agonism and changes in gene expression, with particular emphasis on changes in DNA methylation and their potential role in carcinogenic transformation. Section 3.4 presents recent information and perspectives on species differences in response to PPARα agonists, including a detailed discussion of the SAP's perspectives on the question of human sensitivity. Section 3.5 discusses the adequacy of data from PPARα knockout mice on the MOA for PPARα agonists. Finally, Section 3.6 describes recent data on intrinsic factors, including genetic polymorphisms, gender, and life stages, that may modulate responses to PPARα agonism. NAS input regarding interpretation of these data and perspectives will be valuable as EPA revises its draft TCE risk assessment.

3.1. PPAR RECEPTOR ACTIVITIES, INTERACTIONS, AND PLEIOTROPIC NATURE

This section discusses the pleitropic nature of responses to PPARα agonism beyond peroxisome proliferation alone and presents data on how these varied effects may be related to disease endpoints. Although early hypotheses regarding the MOA for PPARα agonists had focused on induction of peroxisomes, more recent data suggest a broader spectrum of effects that may be related to adverse outcomes. A large body of scientific evidence has been recently published to describe the functions and actions of PPARα and its relationship to other receptors.

Although chemicals that are agonists for the PPARα receptor have been traditionally identified in rodents by their induction of peroxisomes, many actions other than peroxisome proliferation are associated with PPARα activation. As noted by the ILSI workgroup (Klaunig et al., 2003), "While the term 'peroxisome proliferator' has been very useful in providing a shorthand term for a group of chemically diverse agents that induce a common pleiotropic response, the term is also somewhat misleading since it acknowledges only a very limited component of the responses caused by this group of agents." Scatena et al. (2003) further suggest that PPARα agonists produce myriad extraperoxisomal effects that are not necessarily dependent on their interaction with PPARα and that, therefore, the biochemical profile and a therapeutic role of this class of PPAR ligands is more complex than those previously proposed. Peroxisome proliferators are heterogeneous in action and the receptors extremely pleiotrophic. This pleotropic effect has been shown by the phenotypes of PPARα knockout mice.

Some agonists have been shown to display activity toward more than one receptor, which complicates data interpretation across chemicals. Shimizu et al. (2004) reported that expression of these genes (PPARα or PPARγ2) is induced through the same peroxisome proliferator response element in the liver and adipose tissue, where the two PPAR subtypes are specifically expressed. Blanquart et al. (2003) report that transcriptional activity of the PPARs is regulated

by post-translational modifications, such as phosphorylation and ubiquitination. Phosphorylation of PPARs is controlled by environmental factors activating different kinase pathways leading to the modulation of their activities. PPAR degradation by the ubiquitin-proteasome system modulates the intensity of the ligand response by controlling the level of PPAR proteins in the cells.

Thus, although the SAP concluded that the concordance of the hypothesized MOA of PPARα activation, increased cell proliferation, decreased apoptosis, and clonal expansion of preneoplastic cells is supported with data for several PPARα agonists, they also wrote:

> One Panel member noted several inconsistencies in the supporting data however. These include observations from long-term carcinogenicity studies of the PPAR-α agonist gemfibrozil, where a dose-related increase in liver tumors was observed in male rats, while in females, a dose-dependent decrease in liver tumors was seen (IARC, 1996). In another example, studies in rats with two PPAR-α agonists, WY-14,463 and DEHP, demonstrated that doses that produced equivalent levels of hepatic peroxisome proliferation, measured as peroxisome number and peroxisomal enzyme activity, produced markedly different liver tumor incidences (Marsman et al., 1988). Another Panel member noted that these differences may be due to sex, species, and strain differences in pharmacokinetics.

The heterogeneity effects from PPARα agonism have been supported by recent studies, which suggest chemical, gender, and dose-specific effects on gene regulation. For example, Fan et al. (2003) investigated peroxisome proliferator effects on other components of the P450-metabolizing system that are often a rate-limiting component in P450-dependent reactions. The down-regulation of the P450R protein was gender- and tissue-specific, in that exposure to peroxisome proliferators led to increases in P450R protein in female rat livers (di-n-butyl phthalate [DBP] only) and male rat kidneys (WY, gemfibrozil [GEM], and DBP). Poole et al. (2001) report that chronic exposure to WY and GEM, but not to DBP, led to decreases in carboxyesterase ES-4 in male rat livers, but only GEM increased it in females. WY exposure led to decreased ES-10 in male and female rat livers, while DBP increased ES-10 in females. In the kidney, chronic exposure to WY or DEHP in wild-type mice had down-regulation of ES-4 and ES-10. These decreases in kidney esterase (ES) expression were not observed in PPARα knockout mice. The authors concluded that ES protein expression is under complex sex- and compound-dependent control by peroxisome proliferators. Recent information has been reported for gender differences in TCE-induced peroxisome proliferation in mice (Nakajima et al., 2000). No remarkable sex difference was observed in induction of peroxisome proliferation, as measured morphologically, but a markedly higher induction of several enzymes and PPARα protein and mRNA was found in males.

Other extraperoxisomal effects of PPARα agonists have been reported relating to changes in cell bioenergetics. Zhou and Wallace (1999) report that GEM and WY induced the mitochondrial permeability transition as characterized by calcium-induced swelling and depolarization of membrane potential, both of which were inhibited by cyclosporine A. Fenofibrate, clofibrate, ciprofibrate, and DEHP, on the other hand, caused a direct dose-dependent depolarization of mitochondrial membrane potential. However, the mechanism of membrane depolarization varied among the test chemicals. Bezafibrate and TCE elicited no effect on succinate-supported mitochondrial bioenergetics. The authors concluded that most, but not all, peroxisome proliferators they studied interfered with mitochondrial bioenergetics, and the specific biomolecular mechanism differed among the individual compounds.

A related effect of peroxisome proliferators that has been reported is pronounced mitochondrial proliferation and increased activity of mitochondrial enzymes in liver tumors (Bannasche et al., 2001; Zhou and Wallace, 1999). Bannasch (1996) reports that the hypothesized DNA damage caused by marked increases in free radical-generating enzymes of the peroxisomal β-oxidation through H_2O_2 (Reddy and Rao, 1989) is not supported by the findings in rats treated with the peroxisome proliferator dehydroepiandrosterone (DHEA). The adrenocortical hormone DHEA is a potential natural regulator of the peroxisomal compartment (Depreter et al., 2002). Amphiphillic cell foci preceding the appearance of hepatocellular neoplasms do not develop from the perivenular zones, in which the most pronounced peroxisome proliferation occurs, but from the periportal areas in which the prevailing cellular alteration is proliferation of mitochondria. Polyak et al. (1998) have examined mitochondria with regard to neoplasia, largely because of their role in apoptosis and other aspects of tumor biology. The mitochondrial genome is particularly susceptible to mutations because of the high level of reactive oxygen species (ROS) generation in this organelle, coupled with a low level of DNA repair. They report mutations in the mitochondrial genome in the majority of human colorectal cancer examined. Possible effects of peroxisome proliferators on mitochondrial genomics have not been investigated.

PPARα agonism is also thought to be involved in several different diseases, effects, and receptor pathways. The table presented as an appendix to this document cites some of the recent literature investigating such relationships and demonstrates the pleiotropic nature of the receptor. Along with the liver, other target organs and systems affected include the muscle, cardiovascular system, small intestine, testes, ovary, thyroid, adrenal axis, and immune system. A large variety of cells have also been noted to be involved with PPARα responses that not only include the parenchymal cell of the liver (hepatocytes) but also macrophages. Processes affected include lipid and glucose metabolism; inflammatory cytokine production and recruitment to inflammatory sites; and control of glucocorticoids, growth hormones (GHs), P450 genes

(including CYP2B, CYP2C, and CYP4a family members), fasting, bile acid synthesis, macrophage cholesterol homeostasis, key proteins involved in all stages of atherosclerosis, liver fatty-acid binding protein, thyroid hormone and estrogen action, male rat-specific alpha 2u globulin, a mouse homologue of alpha 2u, glutathione S-transferase, and glutathione reductase. Effects on the vulnerability of the liver to other insults such as acetominophen have also been reported.

3.2. PPARα EFFECTS: RELATIONSHIP TO NONPARENCHYMAL LIVER CELLS

Another issue raised recently with respect to the role of PPARα agonism in liver carcinogenesis has been whether such agonists also independently (of PPARs) activate nonparenchymal liver cells (such as Kupffer cells), and whether such activation may be necessary for tumor induction. For instance, the SAP wrote that "[one] Panel member observed that there is a large body of data demonstrating that PPAR-α agonists activate Kupffer cells through a PPAR-α independent mechanism, resulting in the release of cytokines capable of stimulating parenchymal cell mitosis and suppressing apoptosis (Rolfe et al., 1997; Rusyn et al., 2001; Parzefall et al., 2001; Hasmall et al., 2001)." The ILSI workgroup (Klaunig et al., 2003) also noted some of the issues raised by these same studies, stating that "[i]n light of these in vitro results, one should be mindful of the potential meaning of results with respect to the responsiveness (or lack thereof) in human hepatocyte assay systems that would have the Kupffer cells removed during preparation."

The role of oxidants in the mechanism of tumor promotion by peroxisome proliferators remains controversial, but recent data suggest a possible relationship between oxidants and Kupffer cell activities. Although the early idea that induction of ACO leads to increased production of H_2O_2, which damages DNA, seems unlikely now, free radicals might be important in signaling in specialized cell types such as Kupffer cells, which produce mitogens (Rusyn et al., 2001). Current data support a role for cytokines such as the mitogenic cytokine tumor necrosis factor alpha (TNFα) and interleukin 1 (IL1) in hepatocarcinogenesis. Specifically, Rusyn et al. (2000) state the following:

> Oxidative DNA damage caused by leakage of H_2O_2 from peroxisomes was hypothesized initially as the mechanism by which these compounds cause liver tumors. It seems unlikely that oxidants of peroxisomal origin explain the mechanism of action of peroxisome proliferators because treatment with these compounds in vivo does not lead to increased H_2O_2 production. On the other hand, Kupffer cell-derived oxidants, such as superoxide, may play a role in initiating TNFα production that leads to hepatocyte proliferation.

The authors also report that peroxisome proliferators activate the transcription factor NF-kappaB, one of the major regulators of TNFα expression, in Kupffer cells. Rusyn et al. (2001, 2000) report a body of work that suggests that peroxisome proliferator-induced cell proliferation and tumors require the PPARα, but that PPARα is not involved in TNFα production by Kupffer cells because it is not expressed in this cell type. They suggest that both Kupffer cell TNFα and parenchymal cell PPARα are required and suggest that phthalate peroxisome proliferators increase free radicals in the liver before peroxisomal oxidases are induced.

Hasmall et al. (2001) report results consistent with nonparenchymal cells (NPCs) being required for peroxisome proliferator-induced growth but not for peroxisome proliferation. These data support a role for NPCs in facilitating a response of hepatocytes to peroxisome proliferators that is ultimately dependent on the presence of PPARα in the hepatocyte. Holden et al. (2000) report that TNFα acts downstream or independently of PPARα to mediate the suppression of apoptosis and induction of DNA synthesis by peroxisome proliferators. In their in vitro model, the peroxisome proliferator nafenopin does not appear to mediate de novo TNFα gene expression, suggesting that the response to nafenopin may be mediated by bioactivation or release of pre-existing TNFα protein from Kupffer cells. Bojes et al. (1997) reported that neutralizing antibodies to TNFα blocks increases in protein kinase C and cell proliferation due to WY, and Peters et al. (2000) have suggested that Kupffer cells play a central role in peroxisome proliferator-induced carcinogenesis, most likely via mechanisms involving increases in superoxide, activation of nuclear factor kappaB, and production of TNFα. Recent evidence suggests that responses of hepatocytes to peroxisome proliferators is not only dependent on PPARα but also on the trophic environment provided by nonparenchymal cells and by cytokines such as TNFα (Roberts et al., 2002; Parzefall et al., 2001). Additionally, the ability of peroxisome proliferators to suppress apoptosis and induce proliferation depends on survival signaling mediated by p38 mitogen-activated protein kinase.

The gene expression of TNFα has been compared with that of nafenopin and epidermal growth factor (EGF) to study the potential relationship between both. Chevalier et al. (2000) report that proteins showing an altered expression pattern in response to nafenopin differed from those showing altered expression in response to EGF. However, many proteins showing altered expression following stimulation with TNFα were common to both the EGF and nafenopin responses. They report 32 proteins with altered expression following stimulation with nafenopin, including muscarinic acetylcholine receptor 3, intermediate filament vimentin, and the beta subunit of the ATP synthase, and suggest that these nonperoxisomal protein targets offer insights into the mechanisms of peroxisome proliferator-induced carcinogenesis in rodents.

The SAP noted that "the mechanism for the induction of cell proliferation and apoptosis suppression induced by PPAR-α agonists is not known. One significant factor to consider is the role of nonparenchymal hepatic cells in these process." They also wrote more generally:

> The Panel agreed that there were considerable uncertainties as to the significance of associated key events, such as hepatic acyl CoA oxidase induction, with regard to the tumor forming potential of PPAR-α agonists in rats and mice. PPAR-α agonists can bind directly to PPAR-α, but may also perturb interactions with the RXR [retinoid-X receptor] binding partner, the binding of co-activators and co-repressors to the receptor, or the availability and action of endogenous ligands or inhibitors.

3.3. PPAR AGONISM AND EPIGENETICS

Recent data have also suggested that PPARα agonism affects gene expression, particularly with respect to DNA methylation. These effects are potentially important because of the role played in carcinogenesis by gene expression changes. As discussed in Section 2.3, attempts have also been made to explore the effects of TCE on transcriptional regulation. Pereira et al. (2004a) have reported that DCA induces DNA demethylation at concentrations that also induce peroxisomes. Questions naturally arise, then, as to whether changes in gene expression or methylation status induced by peroxisome proliferators can be adequately characterized and whether they are consistent with effects from TCE. This section discusses recent literature both on the potential role that DNA methylation plays in carcinogenesis and on reported effects by PPARα agonists on DNA methylation and gene expression.

The role of chromatin in mediating the transformation of a normal cell into a malignant state is particularly interesting. On one hand, there is the discovery that aberrant methylation patterns occur in an increasing number of tumor suppressor and DNA repair genes that determine carcinogenic transformation; on the other hand, there is the existence of a series of methyl-DNA binding activities that recruit co-repressor complexes and modify the structure of the chromatin to produce a transcriptionally silenced state. Many nuclear receptor genes can be silenced through aberrant methylation in tumors; epigenetic silencing, therefore, represents an additional mechanism that modifies expression of key genes during carcinogenesis (Berger and Daxenbichler, 2002; Ballestar and Esteller, 2002). Inactivation of tumor suppressor genes is central to the development of all common forms of human cancer. This inactivation often results from epigenetic silencing associated with hypermethylation rather than intragenic mutations. In human cells, the mechanisms underlying locus-specific or global methylation patterns remain unclear (Rhee et al., 2002).

Regarding epigenetic mechanisms, the SAP wrote:

> One member of the Panel expressed a concern, which was shared by some other Panel members, that the MOA and evaluation of human relevance was lacking in its assessment of altered gene expression that could be associated with altered methylation of DNA. There is evidence that DNA methylation is modified in rodents following exposure to PPAR-α agonists (Ge et al., 2001, Ge et al., 2002, and Pereira, et al., 2004[a]).
>
> Given the accepted role for DNA methylation in gene imprinting and the loss of imprinting in cancer etiology (see for example McClachlan et al., 2001), such a role for PPAR-α agonists in causing similar alterations in humans should be explored before human relevance can be appropriately evaluated, particularly for exposure during early life stages and for questions regarding site concordance.

In addition, the SAP also noted that

> PPAR-α agonists not only modulate the expression of genes with PPREs [peroxisome proliferator-response elements] but they may also regulate gene expression by altering levels of gene methylation (Ge, et al., 2001). Such DNA methylation is known to be involved in imprinting and alterations or loss of imprinting can directly or indirectly impact disease risk at later life stages (Cui, H. et al., 2003).

Gene imprinting is an epigenetic mechanism for accomplishing persistent change in gene expression (McLachlan et al., 2001). Pereira et al. (2004b) report that the ability of nongenotoxic colon carcinogens to cause DNA hypomethylation is correlated with their carcinogenic activity in the colon of the mouse and rat. In humans, a striking correlation between genetic instability and methylation capacity suggested that methylation abnormalities may play a role in chromosome segregation processes in cancer cells (Lengauer et al., 1997). Herman et al. (1998) suggest that genes involved in DNA mismatch repair are associated with microsatellite instability in colorectal cancer and with instability resulting from epigenetic inactivation in association with DNA methylation.

Effects of genetic disruption of PPARdelta (PPARδ) have also been implicated as an effector of the tumorigenicity of human colon cancer cell with suppression of PPARδ contributing to growth-inhibitory effects of the adenomatous polypsis coli/beta-catenin pathway (Park et al., 2001). PPARδ expression has been reported to be elevated in human colorectal cancers (He et al., 1999).

Evidence suggests that the peroxisome proliferators 2,4-dichlorophenoxyacetic acid (2,4-D), dibutyl phthalate (DBP), GEM, and WY have the ability to alter the methylation and expression of the c-myc protooncogene. All four peroxisome proliferators caused

hypomethylation of the c-myc gene in the liver, which supports the hypothesis that the peroxisome proliferators prevent methylation of hemimethylated sites (Ge et al., 2002, 2001).

Genes that may be altered in response to PPARα agonism leading to a tumorigenic response have yet to be identified. The same limitations stated for this approach in Section 2.3 are applicable here. Hasmall et al. (2002) report gene expression profiling of mRNA from wild-type versus PPARα-null mice to show a three- to sevenfold down-regulation of hepatic lactoferrin in response to DEHP. The authors suggest that the regulation of iron-binding proteins by PPARα ligands plays a role in peroxisome proliferator-mediated liver growth but not in peroxisome proliferation.

Depreter et al. (2002) have reported modulation of the peroxisomal gene expression pattern by DHEA and vitamin D. After 1 and 6 days of treatment with DHEA and 25-hydroxycholecalciferol, relative transcription levels of 39 selected genes were evaluated in female rats. Expression levels of 16 (of which 11 were peroxisomal) genes were altered. Pex 11, ACO, multifunctional enzyme type 1, thiolase 1, phytanoyl-CoA hydroxylase, 70 kDa peroxisomal membrane protein, and very long chain ACO synthetase were up-regulated; three others were down-regulated. Vitamin D caused down-regulation of six genes.

Meyer et al. (2003) used cDNA microarrays to study the expression profiles of 26 hepatocellular carcinomas developing spontaneously in peroxisomal fatty ACO-null mice. The development of liver tumors in these null mice is due to sustained activation of PPARα by the unmetabolized substrates of ACO, which serve as natural PPARα ligands. Comparisons of the null mouse liver tumor expression profiles with those induced by ciprofibrate or diethylnitrosamine showed that these mice share a number of deregulated (up- or down-regulated) genes with ciprofibrate-induced liver tumors. Some genes identified previously as PPARα regulated were CD36, lymphocyte antigen 6 complex locus (Ly-6D), and C3f. For all 3 classes of liver tumors, 12 genes were up-regulated and included an uncharacterized RIKEN cDNA, lipocalin 2, insulin-like growth factor-binding protein 1, Ly-6D, and CD63 among others.

3.4. PPAR ACTIVITY AND SPECIES DIFFERENCES

This section presents recent information and perspectives on species differences in response to PPARα agonists, including a detailed discussion of the SAP's perspectives on the question of human sensitivity. Marked species and tissue differences in the expression of PPARs and responses to PPAR agonists complicate the extrapolation of preclinical data to humans. For example, despite the observation that these compounds are rodent carcinogens, PPARα ligands such as the hypolipidemic fibrates have been used extensively in the clinic over the past 20 years to treat cardiovascular disease and side effects of clinical fibrate use have not been widely reported. Graham et al. (2004), however, recently reported significantly increased incidence of

hospitalized rhabdomyolysis in patients treated with fibrates both alone and in combination with statins. Adverse clinical responses have also been seen with PPARγ ligands that were not predicted by preclinical models (Boitier et al., 2003).

Concerns have been raised about the adequacy of human data to determine the carcinogenic potential in humans for drugs that cause peroxisome proliferation in rodents (Melnick, 2001; Newman and Hulley, 1996). Newman and Hulley (1996) provided a review of methods and interpretation of carcinogenicity studies in rodents and results of clinical trials in humans and concluded that all members of the two most popular classes of lipid-lowering drugs (the fibrates and the statins) cause cancer in rodents, in some cases at levels of animal exposure close to those prescribed to humans. They also concluded that evidence of carcinogenicity of lipid-lowering drugs from clinical trials in humans is inconclusive because of inconsistent results and insufficient duration of followup. While the ISLI Workgroup (Klaunig et al., 2003) also stated that "[t]he available epidemiological and clinical studies are inconclusive," the group concluded that these studies "nonetheless, do not provide evidence that peroxisome proliferators cause liver cancer in humans."

3.4.1. Recent Data on Species Differences in PPAR Expression and Response

Several different hypotheses regarding the mechanisms behind species differences have been investigated. Roberts et al. (2000) suggest that these species differences between humans and rats and mice can be attributed to a reduced quantity of full-length functional PPARα in human liver. Hasmall et al. (2000) have suggested that the human ACO gene promoter differs from the rat ACO promoter at three bases within the PPRE and appears to be refractory to peroxisome proliferators from studies of DEHP.

Pugh et al. (2000) report that short-term studies of peroxisome proliferators di-isononyl phthalate and DEHP at high exposure levels in young adult male cynomolgus monkeys after 14 days of treatment showed no distinctive treatment-related effects in the liver, kidney, or testes upon histological examination. No changes were noted in any of the hepatic markers for peroxisomal proliferation.

Both the hamster and the guinea pig have PPARα, and the guinea pig receptor has been characterized to be fully functional, as demonstrated in reporter gene expression assays. However, the guinea pig PPARα is expressed at low levels in the liver, and the currently favored hypothesis to explain species differences in hepatic peroxisome proliferation invokes the low level of PPARα as the principal determinant of species responsiveness. On the other hand, the demonstration that guinea pigs and humans undergo hypolipidemia induced by PPARα agonists calls into question the MOA of PPARα agonists in "nonresponsive" species (Choudhury et al., 2000).

O'Brien et al. (2001) treated rats and hamsters with WY and observed decreases in alpha-tocopherol content and total superoxide dismutase (SOD). DT-diaphorase was decreased in activity following WY treatment in rats but only sporadically affected in hamsters. Rats and hamsters treated with DBP demonstrated increased SOD activity at 6 days; however, in the rat, DBP decreased SOD activity at 90 days and alpha-tocopherol content was decreased throughout. In GEM-treated rats and hamsters, a decrease in alpha-tocopherol content and an increase in DT-diaphorase activity were observed.

3.4.2. SAP Perspectives on Species Differences and Human Sensitivity

This section outlines SAP perspectives on the issues of species differences and human sensitivity, specifically noting the discussion of the data they reviewed, and of what data they believed may help to clarify these issues. As previously noted in Section 1.2, overall, the majority of the SAP members agreed that relevant data indicate humans being less sensitive than rodents to the hepatic effects of PPARα agonists, but opinions of the individual panel members on the proposed OPPTS policy statement as written ranged from full agreement to complete disagreement. The SAP further elaborated:

> In addition, the Panel members agreed that the MOA and its application to addressing human relevance would be greatly strengthened by additional evidence of the specific alterations associated with PPAR-α activation that lead to the more general steps of hepatocellular proliferation, clonal expansion of initiated hepatocytes and tumor development.
>
> ...
>
> Considering the proposed MOA, there was agreement that PPAR-α is present in humans and that the receptor is activated in human liver following exposure to known agonists. Accordingly, the proposed MOA for PPAR-α agonist-induced hepatocellular carcinogenesis in rodents is plausible for humans. There was also agreement that the nature of gene expression associated with hepatocellular PPAR-α activation is qualitatively different between humans and rodents. This difference may result from species differences in PPREs, but there are few data available that identify these potentially important differences, particularly in humans. Humans are at least as sensitive to activation end-points that lead to hypolipidemia but are much less sensitive to other end-points normally associated with peroxisome proliferation.
>
> ...
>
> Whereas PPAR-α activation is a very specific component of the MOA, the other steps deemed to be causally-related, namely increased hepatocellular proliferation and clonal expansion of initiated hepatocytes leading to tumor development were very general and non-specific. Overall, the Panel members agreed that additional evidence of specific alterations associated with PPAR-α activation in primates and especially humans would greatly strengthen the proposed MOA.

On this last subject, the linkage between PPARα activation and cell proliferation, the SAP further noted:

> There was a general consensus that the data linking PPAR-α activation to increased cell proliferation in all species was relatively weak. The strongest evidence in support of the importance of this step in subsequent tumor development is derived from the PPAR-α knockout mouse studies in which no increase in hepatic cell proliferation and no tumors are observed after 11 months of treatment (Peters et al., 1997).

Discussion of the limitations of the knockout model, including SAP perspectives and discussion of some new studies, follows in Section 3.5.

With regard to data from other animals besides rats, mice, and humans, the SAP wrote:

> The available data from other animals includes guinea pigs, hamsters, dogs and non-human primates. In all cases, these animals demonstrate reduced liver sensitivities to PPAR-α agonists....Collectively, the Panel was split on the applicability of data from other animals to contribute to a weight of evidence regarding the hepatocarcinogenic effects of PPAR-α agonists in humans.

The SAP discussed human data in some detail, writing:

> Although much of the data cumulatively support the hypothesis that agonist-induced human PPAR-α (hPPAR-α) activation fails to follow the MOA seen in rodent livers, namely, increased liver cell proliferation, decreased apoptosis, formation of preneoplastic foci and clonal expansion of these foci into liver tumors, the weight of evidence for this MOA and consequences of agonist-induced PPAR-α activation events in humans is less well defined than in rodents. Human liver biopsy data, while limited, indicate that clinical administration of PPAR-α agonists results in increases in the number and volume density of hepatic peroxisomes. The Panel agreed that the available cancer epidemiological data on pharmacologic PPAR-α agonists are too limited in study size and duration to provide any relevant information to evaluate human relevance. As such, data from other animals, including non-human primates, along with in vitro studies in human hepatocytes, or cell lines, provide the basis for evaluating the relevance of the proposed MOA in humans.
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> ...
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> Although the human data are limited, the existing data do provide some important information for consideration. Human liver contains functional PPAR-α receptors and the fibrate class of drugs is able to activate this receptor to alter the expression of genes involved in lipid metabolism that induce hypolipidemia. Chronic exposure data reported in humans for two different PPAR-α agonists suggest that humans do not respond to PPAR-α agonists by an increase of the associated key events (such as cell proliferation, suppressed apoptosis, and clonal expansion of

preneoplastic hepatic lesions) observed during PPAR-α activation in rats and mice exposed to these agonists. In addition to the short duration of exposure and the use of therapeutic doses (lower than the doses used in studies with rats and mice), the limitations of these studies include the use of weak agonists. The human epidemiology data from short duration follow up (5 year time period) indicated an early increase in GI tract tumors, although liver cancer was not reported independently. However, no differences were noted after 8 years of follow up. Evidence for peroxisome proliferation and increased cell proliferation was lacking in human liver biopsies. Problems with these observations include the high variability in assessing peroxisome increases in biopsy material that are not representative of all zones of the liver, and whether the timing of biopsy sample acquisition was appropriate for detecting an increase in cell proliferation.

Finally, regarding in vitro data with human hepatocytes, the SAP wrote:

The strength of the hypothesis that humans are less sensitive to agonist-induced PPAR-α-mediated hepatocarcinogenesis lies in the human primary hepatocyte data. The Panel was again divided on the interpretation and utility of these data. First, there was a difference of opinion on the applicability of the in vitro studies used to assess the ability of human hepatocytes to proliferate in response to treatment with a PPAR-α agonist. Although limited in total sample size, these studies have shown that in vitro cultured human hepatocytes respond differently to PPAR-α agonists when compared to in vitro cultured rodent hepatocytes. As discussed in more detail below, whether these differences are attributable to true interspecies differences or reflect differences in human and rodent hepatocyte culture preparations remains an open question. In parallel experiments with in vitro cultured rodent hepatocytes, in vitro cultured human hepatocytes fail to display several of the key responses deemed essential for the MOA in agonist-induced PPAR-α-mediated rodent hepatocarcinogenesis, those being increased cell proliferation and decreased apoptosis. Furthermore, in vitro cultured human hepatocytes appear to be less responsive to upregulation of peroxisomal genes and proliferation of peroxisomes, two key associative events of agonist-induced PPAR-α-mediated rodent hepatocarcinogenesis. Several Panel members suggested that further experiments in human primary hepatocytes (co-cultured with and without Kupffer cells; see comments below) would be useful if they provide additional biochemical data that demonstrate reduced levels of PPAR-α expression in human liver and an inability for agonist-induced PPAR-α to modulate the gene expression for several key peroxisomal enzymes.

Evidence for Kupffer cell involvement in PPARα agonist liver effects was discussed previously in Section 3.2, and touched on again in Section 3.5. The importance of this issue with regard to human relevance was noted by the SAP:

> For example, the growth permissive factors released from activated Kupffer cells following PPAR-α agonist exposure are absent and may explain the lack of induction of DNA synthesis seen in cultured human hepatocytes. Support for this possibility has been demonstrated in rodent cultures in vitro (Rose, et al., 1999). In these studies, PPAR-α agonists were unable to induce DNA synthesis in purified preparations of rodent hepatocytes (devoid of nonparenchymal cells), while PPAR-α agonist-induced DNA synthesis was restored upon the addition of nonparenchymal cells, or medium derived from activated Kupffer cells, to the purified hepatocyte cultures.

3.5. HETEROGENEITY OF PPARα EFFECTS IN PPARα KNOCKOUT MICE

Although studies using PPARα knockout mice have been used to support the dependency of PPARα agonism on liver tumor induction, several concerns have been raised regarding the adequacy of this model. These are related to both existing study designs and to whether the intrinsic characteristics of these knockout mice mean that they exhibit differing responses from those of wild-type mice independent of effects related to PPARα agonism. PPARα knockout mice can be useful in describing effects that are associated only with activation of the receptor but not necessarily the effects associated only with peroxisome proliferation. Animal studies in PPARα-null mice also may be inadequate to fully describe effects of lack of the receptor on cancers because they show an absence of nonspecific effects only at the high doses associated with peroxisome proliferation.

Some SAP members expressed concerns over the adequacy of the "knockout" or "null" mouse model to demonstrate the dependency on PPARα agonism to induce hepatocarcinogenesis in mice. The SAP wrote:

> There was agreement among most, but not all, of the Panel that data from the PPAR-α -/- mouse indicate the requirement for the activation of PPAR-α in the MOA of the hepatocarcinogenic effect of these agents. A few Panel members expressed concern over the short duration of the studies in the PPAR-α -/- mouse (i.e., 11 months vs. 24 months in standard cancer bioassays), which rendered the studies incapable of assessing the lifetime liver cancer risk of PPAR-α agonists in this knockout mouse model, and thus, inadequate to conclusively demonstrate that PPAR-α activation is required for hepatocarcinogenesis.

Regarding the interplay between Kupffer cells and knockout mice, the SAP wrote:

> It was noted that arguments against the involvement of the Kupffer cells comes from studies in the PPAR-α null mice. In these mice, agonists failed to elicit a DNA synthetic response. Since this model is replete with Kupffer cells, the lack of DNA synthesis has been interpreted as indicating that the Kupffer cell is not required. On the other hand, some members of the Panel felt that the

communication and/or interplay between PPAR-α agonism and Kupffer cells has not been fully characterized and as such, the null mouse, lacking PPAR-α, is not directly applicable to the human situation in which PPAR-α is present and can be activated.

The recent study by Laughter et al. (2004), which as discussed previously used PPARα knockout mice to try to investigate the role of PPARα in response to TCE and its metabolites, also illustrates the potential difficulties in interpreting studies which use knockout mice. For instance, as mentioned previously, knockout mice did not show a statistically significant effect from TCE treatment but had greater liver-to-body weight ratios than wild-type mice at all levels of exposure, including controls. Moreover, measures of induction of hepatocyte proliferation (BrdU incorporation) showed that baseline levels were also elevated in PPARα knockout mice compared with wild-type mice. At the second highest exposure level (1,000 mg/kg), both wild-type and PPARα knockout mice had elevated levels of hepatocyte proliferation with high variability in response. These increases did not appear to be statistically different from each other, but such an analysis was not made by the authors. In the 3 week study, TCE toxicity was observed at the highest dose in the knockout mice that was not observed in the wild-type mice — all knockout mice were moribund and had to be removed from the study. Inspections of livers and kidneys from the group did not reveal overt signs of toxicity that would lead to morbidity. At the same dose, wild-type mice exhibited mild granuloma formation with calcification or mild hepatocyte degeneration. Kidney-to-body weight ratios were increased by TCE in wild-type but not in knockout mice, with WY having no effect on the kidney in either strain. There results suggest that the knockout mouse may exhibit differences in response with the wild-type mouse that may be independent of the peroxisomal effects of PPARα agonism.

Many phthalates are considered to be relatively weak peroxisome proliferators and have been studied in knockout mice. These studies help illustrate that elimination of PPARα activity has effects on expression of other genes. Valles et al. (2003) report that exposure of di-isononyl phthalate in SV129 wild-type, SV129 PPARα-null, and B6C3F1 mice shows a varied pattern of gene expression that was dependent on gender and age, with some changes in gene expression dependent on PPARα activity and others not. An additional gene was shown to be down-regulated in wild-type mice but up-regulated in PPARα-null mice, indicating more complex regulation by PPARα and additional factors. Macdonald et al. (2001) carried out quantitative proteomic analyses of DEHP-treated wild-type or PPARα-null mouse livers. Fifty-nine proteins were identified where altered expression was both PPARα- and peroxisome proliferation-dependent. In addition, six proteins regulated by the deletion of PPARα were identified, possibly indicating an adaptive change in response to the loss of this receptor. Proteins identified as being regulated by PPARα are known to be involved not only in lipid metabolism pathways but also in

amino acid and carbohydrate metabolism, mitochondrial bioenergetics, and stress responses, including several genes not previously reported to be regulated by PPARα.

The report of Anderson et al. (2002) on liver regeneration and hepatic gene expression following partial hepatectomy in wild-type and PPARα-null mice showed that PPARα-null mice had a 12- to 24-hour delay in liver regeneration associated with a delayed onset and lower peak magnitude of hepatocellular DNA synthesis. Furthermore, these mice had a 24-hour lag in the hepatic expression of the G(1)/S checkpoint regulator genes Ccnd1 and cMyc and increased expression of the IL-1beta cytokine gene. Hepatic expression of Ccnd1, cMyc, IL-1r1, and IL-6r was induced in wild-type mice, but not in PPARα-null mice, following acute exposure to the potent PPARα agonist WY, indicating a role for PPARα in regulating the expression of these genes. The authors suggest that liver regeneration in PPARα-null mice is transiently impaired and is associated with altered expression of genes involved in cell cycle control, cytokine signaling, and fat metabolism.

Jia et al. (2003) report disruption of the inducible beta-oxidation pathway in mice at the level of fatty ACO, the first and rate-limiting enzyme, resulting in spontaneous peroxisome proliferation and sustained activation of PPARα. Mice with complete inactivation of peroxisomal beta-oxidation at the level of the second enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-PBE) of the inducible pathway and D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase (D-PBE) of the noninducible pathway (L-PBE-/-D-PBE-/-), exhibit severe growth retardation and postnatal mortality with none surviving beyond weaning. L-PBE-/-D-PBE-/- mice that survived exceptionally beyond the age of 3 weeks exhibited overexpression of PPARα-regulated genes in the liver, despite the absence of morphological evidence of hepatic peroxisome proliferation.

There is a gender difference in expression of PPARα in rodents that has been explored using knockout mice. Lewitt et al. (2001) report mice lacking PPARα knockout have a sexually dimorphic phenotype with PPARα influencing the insulin-like growth factor (IGF)/IGF-binding proteins (IGFBP) response to feeding, particularly in males. Following fasting and refeeding, IGFBP-1 and insulin concentrations were higher in males than in females and were further increased in PPARα knockout, suggesting significant hepatic insulin resistance. The authors suggest that gender differences in the IGF system contribute to the PPARα knockout phenotype. It has been suggested that elevated serum levels of IGF-I and leptin are associated with increased risk of developing cancer (Hursting et al., 2003; Sandhu et al., 2002; Liu et al., 2001; Thompson et al., 1999).

3.6. INTRINSIC FACTORS PPAR-RELATED AFFECTING RISK

Another key element to evaluating of risk that PPAR agonists may pose to humans is how intrinsic factors may modulate that risk. Among the key factors for which some recent data exist regarding differential responses are genetic polymorphisms, gender, and life stages. Intrinsic susceptibility is also a factor for epidemiologic investigations of fibrate drugs because patients who are being studied are generally taking the drug for a disturbance that has been already manifested in lipid metabolism. In general with such studies, an awareness of what human samples are being studied, and under what conditions, is needed to understand the possibility of a false negative signal.

The possibility of genetic polymorphism and the association of PPARα polymorphism with metabolic diseases is the subject of a number of recent studies. Familial combined hyperlipidemia (FCHL) is a common genetic lipid disorder present in 10% of patients with premature coronary artery disease (CAD). Eurlings et al. (2002) report that the PPARα gene is a modifier of the FCHL phenotype. Lacquemant et al. (2000) screened the PPARα gene for mutations to test the genetic contribution of the PPARα in diabetes and its vascular complications. They concluded that it is unlikely that PPARα gene has a major role in diabetes and CAD in their populations, although they cannot exclude a minor contribution of the PPARα gene to the risk of coronary heart disease associated with Type 2 diabetes through a modulation of atherogenic plasma lipids.

Regarding gender differences, some of the effects on gender dependencies related to gene regulation were discussed in Section 3.1. In addition, male rats have been reported to be more responsive to fibrates than female rats. Jalouli et al. (2003) report that male rats had higher levels of hepatic PPARα mRNA and protein than female rats. Fasting increased hepatic PPARα mRNA levels to a similar degree in both sexes. Hypophysectomy increased hepatic PPARα mRNA and protein levels and was more pronounced in females than in males but was not mediated by GH. The authors suggest that sex hormones regulate the sex difference in hepatic PPARα levels but not via the sexually dimorphic GH secretory pattern.

Regarding questions about life stages, Michalik et al. (2002, 2001) and Wahli (2002) report that in rodents, PPARα, PPARδ, and PPARγ show specific time- and tissue-dependent patterns of expression during fetal development and in adult animals. In addition to citing evidence that suggested differences in β-oxidation capabilities in developing rodents and humans, the SAP wrote

> It was also considered that differences in cell proliferation, xenobiotic metabolism, and other factors in the developing rodent (or human) could affect sensitivity to PPAR-α hepatocarcinogenesis. Therefore, information on the

expression of the PPAR-α during ontogeny as well as responses of embryonic and fetal human hepatocytes to PPAR-α agonists should be evaluated before concluding that the developing human conceptus is unresponsive to PPAR-α agonist exposures.

More specifically, the SAP summarized a number of results relating to early life stages as follows:

> Published reports have shown that both the expression of PPAR-α and the assembly of peroxisomes occur late in the development of rats and mice. Furthermore, it has been shown that, as in adult livers, embryonic, fetal and neonatal livers of rats and mice respond to PPAR-α agonists by increasing peroxisome number, peroxisome volume density, liver weight, and the expression of the peroxisomal enzyme palmitoyl CoA oxidase. This suggests that at least some of the cellular macromolecules involved in the proposed PPAR-α agonist MOA are functional and responsive to PPAR-α agonists in rat and mouse embryonic, fetal, and neonatal livers. However, data on the hepatocarcinogenic response of rat and mouse embryonic, fetal, and neonatal livers to PPAR-α agonists are lacking and, therefore, no conclusions can be made at this time as to the relative sensitivity of these early life stages to PPAR-α agonist induced hepatocarcinogenicity.
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> Although the exposure of pregnant rats and mice led to increases in peroxisomal enzyme activities and increases in liver weight in embryonic, fetal, and neonatal liver tissues, other parameters involved in the proposed MOA, such as cell proliferation, inhibition of apoptosis and clonal expansion of preneoplastic cells, were not examined in these studies. In addition, responses to PPAR-α agonists in the fetal and neonatal rat and mouse, as measured by the peroxisomal enzyme expression levels, suggest that there are differences in young animals relative to adults. It is unclear how these differences in enzyme expression levels might translate into differences in sensitivity to hepatocarcinogenesis. Regarding the comparison of changes in liver weights across early and later life stages, it is inappropriate to assume that a given proliferative response seen at one stage of life is equivalent to a similar proliferative response at another stage of life. For example, an increase in liver weight during the neonatal period might result in a much greater lifetime risk of cancer than an equivalent increase occurring during adulthood, because a larger number of cells in the neonatal liver will undergo multiple cell divisions than in the adult. Finally, none of the studies examining the response of the rodent in utero or during early life stages were carried out with the late onset of tumors as a specific endpoint.
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> Conclusions regarding the relevance of the PPAR-α agonist MOA for human hepatocarcinogenesis applied to adults may not apply to the young. In contrast to adult human liver, there are no data establishing PPAR-α expression levels in embryonic, fetal and neonatal human liver.
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> ...

In contrast to embryonic and fetal rodent liver in which cytochrome P450 enzymes are expressed near, during and after birth (Ring et al. 1999), embryonic and fetal human livers possess metabolic activation capabilities resulting from the early developmental expression of cytochrome P450 enzymes. Moreover, the expression profiles of xenobiotic metabolizing enzymes and isozymes are different in embryonic, fetal, neonatal and adult human livers. Like the gene expression profile of xenobiotic metabolizing enzymes, it is difficult to disregard the possibility that there could be differences between the expression of PPAR-α and its transcriptional co-factors in the human conceptus and adult human liver. In addition, metabolic differences in rats and mice play an important role in determining the degree of response to some PPAR-α agonists (Lake, 1995) and that could also apply to the human conceptus.

Differences in peroxisome biogenesis have been reported during the ontogenic development of rodents and humans. While the assembly of peroxisomes in rats and mice, including the insertion of β-oxidation enzymes into the peroxisomes, occurs near birth, the assembly of human peroxisomes has been observed as early as 8 weeks of gestation (Espeel, et al, 1997). The number and density of peroxisomes plateau by 17 weeks of gestation in humans. Moreover, acyl-CoA oxidase and 3-ketoacyl CoA thiolase are immunodetectable in the peroxisomes by 10 and 9 weeks of gestation, respectively. These observations suggest differences in β-oxidation capabilities in developing rodents and humans and therefore information on the expression of the PPAR-α during ontogeny, as well as responses to PPAR-α agonists in embryonic and fetal human hepatocytes should be evaluated before concluding that the developing human conceptus is unresponsive to PPAR-α agonist exposures.

Finally, there is also evidence that peroxisome proliferators are much more potent in producing tumors in older rats than in younger ones, even though effects on peroxisome proliferation and cell proliferation were the same (Youssef et al., 2003; Chao et al., 2002; Youssef and Badr, 2002). Promotional effects of PPAR agonists for tumors induced by other MOAs are described in the issue paper on interactions. However, a promotion effect in older animals with already initiated foci could be the MOA for increased sensitivity of older rats to PPARα effects.

4. SUMMARY

As scientific information that can aid in the hazard characterization of TCE has increased since the last assessment, so has the field of PPARα activation and its nonperoxisomal effects in humans. The receptor appears to be pleiotropic in its actions, and those actions also seem to be chemical, gender, age, and concentration dependent. There is additional insight as to what types of cell signals and relationships exist with PPARα activation. Recent work on the role of the

Kupffer cell in effects resulting from PPARα appears to be important to understanding hepatocarcinogenesis. However, the way in which peroxisome proliferators induce tumors in rodents is still unknown, and the relevancy of those tumors to human risk is controversial. The task of NAS is as follows: Given the substantial amount of new information on PPARα agonism in general and more limited information regarding its relationship to TCE, advise EPA on the interpretation of new data and on whether conclusions can be made about the role of PPARα agonism in TCE toxicity and its relevance to human health risks.

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APPENDIX

Recent Literature on Effects Associated With PPAR Agonism or Related to Its Mechanisms of Action

Effects	Reference
Obesity and atherosclerosis	Kersten et al. (2000)
Diabetes	Vohl et al. (2000); Shankar et al. (2003a, b, c); Holden et al. (1999); Lacquemant et al. (2000)
Cardiomyopathies	Nohammer et al. (2003)
Familial combined hyperlipidemia	Eurlings et al. (2002)
Increased susceptibility from aging	Chao et al. (2002); Youssef and Badr (2002); Youssef et al. (2003)
Atherosclerosis, inflammation, cancer, infertility, and demyelination	Berger and Moller (2002); Berger and Wagner (2002), Barbier et al. (2002)
Acetaminophen hepatotoxicity	Chen et al. (2000)
Cardiac cell metabolism	Jiang et al. (2004)
Lipid and glucose metabolism	Brisson et al. (2002)
Muscle lipid homeostasis	Muoio et al. (2002)
Lipoprotein lipase (LPL)	Nohammer et al. (2003)
Fatty acid oxidation	Kersten et al. (2001); Watanabe et al. (2000)
Liver fatty-acid-binding protein (liver and small intestine)	Poirer et al. (2001)
Triglyceride and fatty acid metabolism	Barbier et al. (2002)
Macrophage cholesterol homeostasis	Barbier et al. (2002)
Hypoglycemia, role for the development of insulin resistence in response to a Western-type high-fat diet	Guerre-Millo et al. (2001)
Regulation during fasting	Escher et al. (2001); Poirer et al. (2001)
Genes implicated in the inflammatory response (NFkappaB, AP-1, C/EBP beta, STAT-1 and NFAT)	Blanquart et al. (2003)
Tumor necrosis factor-alpha	Bojes et al. (1997); Rusyn et al. (2000); Holden et al. (2000); Chevalier et al. (2000); Peters et al. (2000); Roberts et al. (2002)

Effects	Reference
Early inflammation phase of the healing	Michalik et al. (2001)
Control by glucocorticoids (corticosterone)	Lemberger et al. (1996); Plant et al. (1998)
Connexin32 (major gap junction forming protein in liver)	Moennikes et al. (2003)
Fetal or neonatal CYP4A mRNA expression	Simpson et al. (1996); Simpson et al. (1995)
P450 genes, including CYP2B, CYP2C, and CYP4A family members	Fan et al. (2003)
Glutathione S-transferase glutathione peroxidase and glutathione reductase	O'Brien et al. (2001a)
Growth hormone and STAT5b	Zhou and Waxman (1999); Zhou et al. (2002)
Carboxylesterases in the liver	Poole et al. (2001)
L-pyruvate kinase (glycolytic enzyme)	Pan et al. (2000)
Bile acid synthesis and catabolism in the liver (UDP-glucuronosyltransferase)	Barbier et al. (2003a, b); Sinal et al. (2001)
Constitutive myocardial beta-oxidation of the medium and long chain fatty acids, octanoic acid, and palmitic acid	Watanabe et al. (2000)
Proteins not involved in lipid metabolism but are implicated in the pathogenesis of heart disease	Vosper et al. (2002)
Ovarian function	Komar et al. (2001)
Estrogen action	Klotz et al. (2000); Zhu et al. (1999); Xu et al. (2001)
Testicular degeneration	Dufour et al. (2003); Gazouli et al. (2002)
Thyroid hormone action	Miller et al. (2001)
Regulation of CYP1A1	Seree et al. (2004)
Rat male-specific alpha 2u-globulin	Corton et al. (1997, 1998)