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Several nongenotoxic carcinogens uncouple mitochondrial oxidative phosphorylation

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A number of plasticizers and lipid-lowering drugs induce peroxisomes and cause hepatocellular carcinoma in rodents by mechanisms which remain unknown. In this study, seven structurally dissimilar peroxisome proliferating agents were shown to uncouple oxidative phosphorylation in isolated rat liver mitochondria. For example, perfluorooctanoate (0.5 mM) increased succinate-induced (state 4) mitochondrial respiration by over 50% while stimulation of state 3 respiration with ADP was minimal (i.e., uncoupling occurred). Interestingly, compounds which are potent carcinogens in vivo (e.g., Wy-14,643 and perfluorooctanoate) were more powerful uncouplers of oxidative phosphorylation in vitro than weak tumor-causing agents (e.g., valproate). Uncoupling also occurred in vivo. Basal rates of oxygen uptake in perfused livers from chronically treated rats were increased from 137 ± 7 μmol g^{-1}/h in pair-fed controls to 153 ± 5 μmol g^{-1}/h after 2.5 months of feeding Wy-14,643 (0.1% w/v in diet). Concomitantly, rates of urea synthesis from ammonia, a process highly dependent on ATP supply, were reduced almost completely from 104 ± 10 μmol g^{-1}/h to 13 ± 6 μmol g^{-1}/h. Bile flow, another energy-dependent process, was also reduced significantly by treatment with Wy-14,643 in vivo for 24 h. Taken together, these data indicate that energy supply for cellular processes such as urea synthesis and bile flow was disrupted in vivo due to uncoupling of oxidative phosphorylation by Wy-14,643. It is proposed that peroxisomal proliferators accumulate in the liver where they uncouple mitochondrial oxidative phosphorylation and interfere with cellular energetics.

Introduction

A widely publicized theory states that drugs and chemicals which cause proliferation of peroxisomes and are nongenotoxic carcinogens act via mechanisms involving reduced oxygen species [1–4]. This hypothesis maintains that the chemical agents in this class induce the peroxisomal hydrogen peroxide-generating acyl CoA oxidase by interacting with specific intracellular receptors. Hydrogen peroxide formed within the peroxisome is thought to generate other oxidants, such as lipid peroxides, leading to DNA strand breaks. Some experimental evidence supports this hypothesis; however, other results are controversial or have not been substantiated (see Fig. 4, Table II and Discussion).

Lipid-lowering drugs and plasticizers induce peroxisomes and cause a 5- to 20-fold increase in peroxisomal enzymes, which are capable of generating hydrogen peroxide at high rates in vitro [2,5–7]. Further, many of these chemicals, when fed chronically, cause the accumulation of lipofuscin, thought to be an index of oxidative injury [8]. Although these observations are well documented with a variety of agents, their biological relevance remains unclear. However, one important consideration is the relative rate of hydrogen peroxide generation in vitro compared with rates in vivo. Catalase is capable of destroying H_2O_2 at rates ten million times faster than it is produced [9]; therefore, H_2O_2 should never leave the peroxisome. Additionally, treatment of rats in vivo with ciprofibrate and perfluorooctanoate increased hydrogen peroxide production via acyl CoA oxidase in vitro; however, H_2O_2 production was not increased in whole cells, most likely due to rate limitation by fatty acid supply [10,11]. It is difficult, therefore, to envision how hydrogen peroxide of peroxisomal origin could enter the nucleus and alter DNA in vivo.

In 1988, Marsman et al. reported that feeding rats di(2-ethylhexyl)phthalate (DEHP) or Wy-14,643 for up to one year led to similar increases in peroxisomal volume and enzyme activities; however, the incidence

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of hepatic tumors was much greater in the Wy-14,643-treated group [12]. In this study, Wy-14,643 but not DEHP caused sustained cell turnover. These studies demonstrate that tumorigenesis is not quantitatively linked to the induction of peroxisomes.

Given these reservations, the action of several agents, with a range of potencies as tumor-causing agents, was examined on isolated mitochondria in vitro and in vivo. The data support the hypothesis that uncoupling of oxidative phosphorylation is a primary event following exposure to lipid-lowering drugs and phthalate ester plasticizers.

Materials and Methods

Animals and treatment. Male Fisher 344 rats (Charles River Breeding Laboratory, Raleigh, NC) weighing 225 to 350 g were used in these studies. Rats were maintained on NIH-07 chow and purified water ad libitum and housed with a 12-h night/day cycle. Treated rats were fed the same rodent chow blended with 0.1% Wy-14,643 for 2.5 months or given one oral dose of Wy-14,643 (100 mg/kg in olive oil) 24 h prior to determination of bile flow.

Liver perfusion. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and livers were perfused with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with 95% O_2:5% CO_2 in a hemoglobin-free, nonrecirculating system as described previously [13]. Perfusate was pumped into the liver via a cannula placed in the portal vein, and effluent perfusate was collected via a cannula inserted into the inferior vena cava. Oxygen concentration in the effluent perfusate was monitored continuously employing a Clark-type oxygen electrode. Urea synthesis from ammonia (NH_4Cl; 4 mM) was determined colorimetrically [14] and rates of oxygen uptake and urea synthesis were calculated from the influent-effluent concentration differences, the flow rate and the wet weight of the liver.

Measurement of bile flow. Rats were gavaged with Wy-14,643 (100 mg/kg in olive oil) or with the vehicle alone 24 h prior to surgery. While under ether anesthesia, the bile duct was cannulated and bile was collected into tared vessels every 5 min for 30 min. Rates were calculated using the liver wet weight.

Isolation of mitochondria. Mitochondria were isolated in a mannitol-sucrose buffer (pH 7.0) by standard procedures of differential centrifugation [15]. Isolated mitochondria (1 mg/ml) were incubated at room temperature in a 2 ml volume of buffer (pH 7.2) containing 100 mM KCl, 50 mM sucrose, 20 mM Tris-HCl, 5 mM Tris-phosphate, and 10 μM rotenone. Oxygen uptake was measured in a closed vessel with a Clark-type oxygen electrode after addition of succinate (1 μmol) and ADP (0.5 μmol). Protein concentration was determined colorimetrically [16].

Materials. Ciprofibrate was the generous gift of Sterling-Winthop Research Institute, Rensselaer, NY. [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14,643) was purchased from Chemsyn Science Laboratories, Lenexa, KA. Pentadecafluorooctanoate (PFO) and 2-ethylhexanol were acquired from Aldrich, Milwaukee, WI. All other chemicals and reagents were of the highest available purity from standard commercial sources.

Statistics. Data represent means ± SE. Statistical comparisons were performed using Student's *t*-test [17].

Results

Uncoupling of oxidative phosphorylation in isolated mitochondria

Several chemicals, with a range of potencies as nongenotoxic carcinogens, were compared for their ability to uncouple oxidative phosphorylation in isolated mitochondria. An example of the effect of the very potent nongenotoxic carcinogen, perfluorooctanoate (PFO), on oxygen uptake by isolated mitochondria is depicted in Fig. 1. In the absence of ADP, control mitochondria took up oxygen at rates around 25 nmol min^{-1}/mg protein. Values increased over 3-fold when ADP was added, indicating that the mitochondria were well coupled. In contrast, hypometabolic rates of respiration were stimulated to 53 nmol min^{-1}/mg protein in the presence of PFO, illustrative of uncoupling agents.

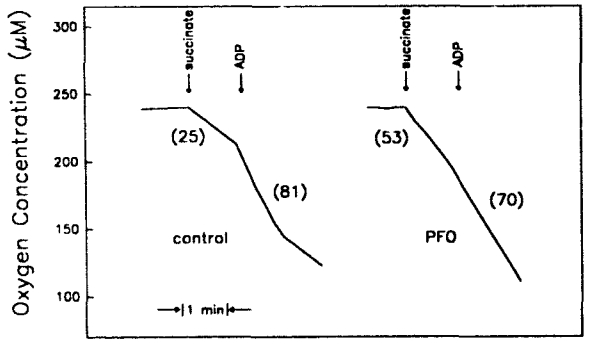

Fig. 1. Effect of perfluorooctanoate (PFO) on oxygen uptake by isolated mitochondria. Mitochondria were isolated from rat liver by standard procedures of differential centrifugation [15]. Mitochondria were incubated at room temperature in 2 ml of buffer (pH 7.2) containing 100 mM KCl, 50 mM sucrose, 20 mM Tris-HCl, 5 mM Tris-phosphate, and 10 μM rotenone. Oxygen concentration was measured in a closed vessel (2 ml) with a Clark-type oxygen electrode after addition of succinate (1.0 μmol) and ADP (0.5 μmol) in the presence or absence of PFO (1.0 μmol). Rates of oxygen uptake, shown in parentheses (nmol min^{-1}/mg protein; average data, $n = 6$), were calculated from the change in oxygen concentration per unit time. Protein was determined by the method of Lowry et al. [16].

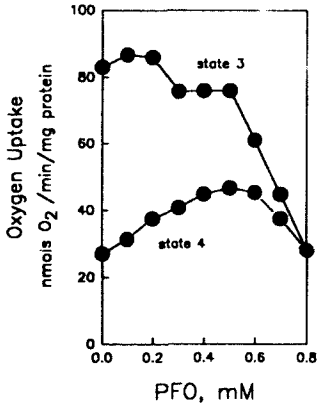

Fig. 2. Uncoupling of mitochondrial respiration by perfluorooctanoate (PFO). Conditions as described in Fig. 1. Mitochondria were incubated with concentrations of PFO indicated on the abscissa. Rates of oxygen uptake were calculated as described in Fig. 1. Mean ± SE ($n = 6$).

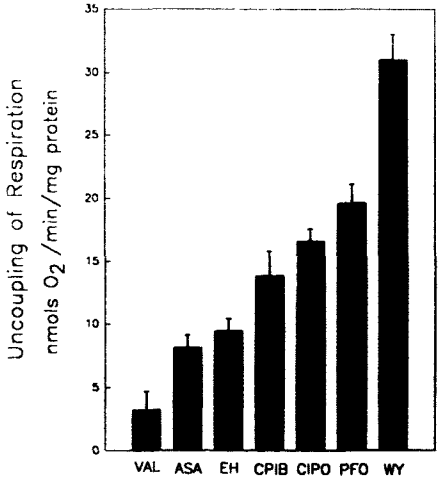

Fig. 3. Uncoupling of mitochondria by seven structurally dissimilar peroxisomal proliferating agents. Mitochondria were isolated from rat liver as described in the legend to Fig. 1 and were incubated at room temperature with valproate (VAL, 3 mM), acetylsalicylic acid (ASA, 2.5 mM), 2-ethylhexanol (EH, 2.5 mM), clofibrate (CPIB, 2.5 mM), ciprofibrate (CIPO, 0.9 mM), perfluorooctanoate (PFO, 0.5 mM) or Wy-14,643 (WY, 0.125 mM). In many cases these concentrations were much lower than the concentration required to inhibit state 3 respiration. Oxygen uptake was measured with a Clark-type oxygen electrode following addition of succinate (1 μmol). Data are presented as the increase in oxygen uptake from basal levels (25 ± 3 nmol O_2 min^{-1}/mg protein) using succinate as substrate. Bars represent average data from 4 rats per group ± SE.

Fig. 2 depicts the dose-dependent stimulation of state 4 rates of respiration (i.e., uncoupling) due to perfluorooctanoate. In the concentration range from 0.6 to 0.8 mM, perfluorooctanoate inhibited both state 3 and state 4 rates of oxygen uptake. Thus, it is clear that PFO first uncouples and then inhibits oxidative phosphorylation as the concentration is elevated. In fact, all seven structurally dissimilar peroxisomal proliferators were shown to uncouple oxidative phosphorylation (Fig. 3). Interestingly, weak tumor-causing agents such as valproate were mild uncouplers, whereas more potent carcinogens, such as PFO and Wy-14,643, uncoupled oxidative phosphorylation to a greater degree. A comparison of the ED_{50} estimated for each compound supports this relationship (Table III). As is observed with many uncouplers, higher concentrations of compound led to inhibition of active state 3 rates of oxygen uptake in isolated mitochondria [18].

Uncoupling of oxidative phosphorylation by Wy-14,643 in vivo

This class of compounds is very lipophilic and would be expected to accumulate in vivo. Therefore, uncoupling of oxidative phosphorylation in isolated mitochondria with relatively high concentrations of test chemical may not reflect events under physiological conditions. Therefore, oxidative phosphorylation was assessed in vivo in rats fed the potent nongenotoxic carcinogen, Wy-14,643 (0.1% in the diet) under conditions which produce tumors by one year [12]. After 2.5 months of treatment, Wy-14,643 tended to increase oxygen uptake per g of tissue in the perfused liver, suggesting uncoupling of oxidative phosphorylation (Table I). Because of a compound-related increase in liver size (hyperplasia) and mitochondrial content, oxygen uptake by the organ was actually increased 3-fold when the data were expressed on the basis of body weight (Table I).

TABLE I

Effect of treatment with Wy-14,643 in vivo on oxygen uptake and urea synthesis in the perfused liver

Rats were fed 0.1% Wy-14,643 in lab chow for 2.5 months. Livers were perfused and oxygen uptake was monitored with a Clark-type oxygen electrode. Rates of urea synthesis from ammonia (4 mM) were calculated from the influent-effluent concentration differences, the flow rate and the wet weight of the liver. [a], tendency for P between 0.05–1.0; *, $P < 0.001$ as compared to control ($n = 6$).

	Oxygen uptake μmol/g liver per h	Oxygen uptake μmol/100 g body weight per h	Liver weight / 100 g body weight	Urea synthesis μmol/g per h
Control	137 ± 7	465 ± 26	3.4 ± 0.1	104 ± 10
Wy-14,643	153 ± 5 [a]	1330 ± 45 *	8.7 ± 0.2 *	13 ± 6 *

To test the hypothesis that the increase in oxygen uptake following Wy-14,643 treatment in vivo was due to uncoupling of oxidative phosphorylation, urea synthesis was studied. Urea synthesis from ammonia is a process highly dependent on ATP supply and is disrupted by uncoupling. Treatment with Wy-14,643 for

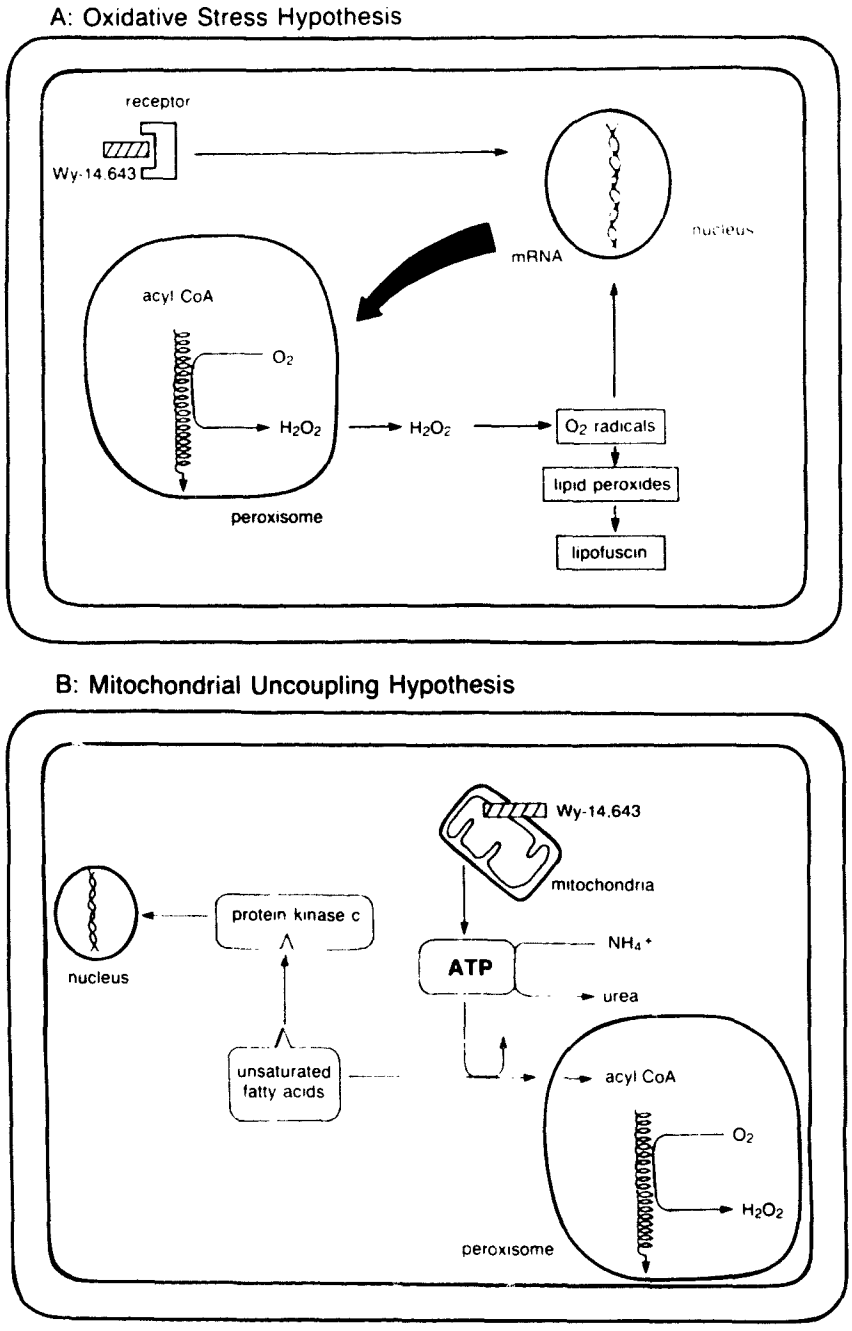

Fig. 4. Schematic representations of the oxidative stress and the mitochondrial uncoupling hypotheses. (A) The oxidative stress hypothesis states that there are specific cytosolic receptors for this class of chemicals (e.g., Wy-14,643) leading to induction of peroxisomes via increases in mRNA coded for peroxisomal protein. H_2O_2 produced from peroxisomal β-oxidation then diffuses into the cytosol forming radical species which damage DNA. (B) The uncoupling hypothesis states that impaired mitochondrial function leads to a decline in ATP, thereby elevating intracellular unsaturated fatty acids which activate protein kinase c leading to increased cell turnover.

2.5 months diminished urea synthesis dramatically from 104 ± 10 to 13 ± 6 μmol/g per h (Table I). Bile flow, another energy-dependent process, was assessed as well in animals treated with Wy-14,643 for 24 h. Exposure to Wy-14,643 for this short period of time decreased bile flow significantly from 121 ± 10 to 79 ± 5 * μl/g per h (* $P < 0.01$; $n = 4$), indicating that uncoupling occurs rapidly.

Discussion

Mitochondria as a primary target of nongenotoxic carcinogens

We hypothesize that peroxisome proliferators, which are highly lipophilic, accumulate in mitochondrial membranes where they uncouple oxidative phosphorylation and interfere with cellular energetics and ion gradients. The fact that the lipid-lowering drug clofibrate, which causes hepatic tumors in rodents, and many of its structural analogs uncouple mitochondrial respiration in vitro has been known for some time [19–22]. In this study, a comparison was made of the effects of seven different peroxisome proliferators on isolated mitochondria. All seven compounds uncoupled oxidative phosphorylation to varying degrees (Fig. 3; Table III). Interestingly, agents which are more potent carcinogens in vivo (PFO and Wy-14,643) appeared to be more powerful uncouplers of oxidative phosphorylation in vitro. Therefore, there may be a correlation

TABLE II

Comparison of elements of oxidative stress and mitochondrial uncoupling hypotheses

Hypothesis	Evidence for or against	Reference
Oxidative stress hypothesis		
Specific receptor	+	Lalwani et al., 1983
	+	Issemann and Green, 1990
	–	Milton et al., 1988
Induce peroxisomes	+	Moody and Reddy, 1978
		Gray et al., 1983
		Fahl et al., 1984
		Feller et al., 1987
in vitro H_2O_2 production	+	Moody and Reddy, 1978
		Gray et al., 1983
		Fahl et al., 1984
		Feller et al., 1987
in vivo H_2O_2 production	–	Foerster et al., 1981
		Handler and Thurman, 1988
		Tamura et al., 1990
		Handler et al., 1992
Lipofuscin accumulation	+	Conway et al., 1989
8-Hydroxydeoxyguanosine adducts	+	Kasai et al., 1989
	–	Hegi et al., 1990
Mutagenic	–	Von Daniken et al., 1981
		Goel et al., 1985
		Gupta et al., 1985
Tumor production	+	Svoboda and Azarnoff, 1979
		Reddy and Lalwani, 1983
Mitochondrial uncoupling hypothesis		
Lipophilic properties	+	Lake et al., 1987
Uncoupler of oxidative phosphorylation in vitro	+	Katyal et al., 1972
		Mackerer et al., 1973
		Goudonnet et al., 1978
		This paper: fig. 3
Uncoupler of oxidative phosphorylation in vivo	+	This paper: Table I
Weight loss	+	Svoboda and Azarnoff, 1979
		Rhodes et al., 1986
		Marsman et al., 1988
Elevated unsaturated free fatty acids	+	Lock et al., 1989
Activation of protein kinase C	+	Bojes et al., 1992
Stimulated cell turnover	+	Marsman et al., 1988
Tumor production	+	Svoboda and Azarnoff, 1979
		Reddy and Lalwani, 1983

between mitochondrial uncoupling and the ability of these chemicals to cause tumors.

Recently, it was demonstrated that 2-ethylhexanol, a metabolite of the common plasticizer di(ethylhexyl) phthalate, uncoupled oxidative phosphorylation in isolated mitochondria, dissipated the mitochondrial membrane potential, and prevented the uptake of radiolabeled Ca^{2+} [23,24]. Therefore, as a consequence of mitochondrial uncoupling in vivo, ATP supply for ion pumps would be decreased which could indirectly increase levels of intracellular calcium. It is possible that calcium is involved in the stimulation of cell turnover. Indeed, norepinephrine, which increases intracellular free calcium pools [25], potentiates the effect of growth factors on cell replication [26].

One argument against this hypothesis is that there is little indication that established mitochondrial uncouplers (e.g., dinitrophenol (DNP) and long-chain fatty acids) are nonmutagenic carcinogens. On the other hand, fatty acids, like the chemicals being considered here, have long been known to be tumor promoters [27–29] and mild peroxisome proliferators [30]. Furthermore, pentachlorophenol has been shown to be a hepatic carcinogen in rodents [31]. Further, the fact that DNP is not known to cause tumors may have a trivial explanation. The feeding studies performed with this agent, largely in the 1930's, most likely led to death long before tumors could appear [32].

Comparison of oxidative stress and mitochondrial uncoupling hypotheses

Oxidative stress. The oxidative stress and mitochondrial uncoupling hypotheses are compared in Fig. 4. The oxidative stress hypothesis states that specific cytosolic receptors for these compounds exist and lead to an increase in mRNA coded for peroxisomal proteins (Fig. 4A). Following induction, hydrogen peroxide produced from β-oxidation of fatty acyl CoA within the peroxisome diffuses from the organelle into the cytosol forming highly reactive radical species which damage DNA. These activated oxygen species also lead to formation of lipofuscin, indicative of increased lipid peroxidation. In contrast, the uncoupling hypothesis states that impaired mitochondrial function leads to a decline in cellular ATP, thereby elevating free fatty acids which activate protein kinase *c*, leading to replicative DNA synthesis (Fig. 4B).

Evidence that some chemicals in this class react with specific receptors in cytosol from rat liver has been presented [33]. Recently, a cloned member of the steroid hormone receptor family was shown to be activated by Wy-14,643, nafenopin and clofibrate [34]; however, whether it plays a role in tumor formation has not been established. Also, it is well established that this class of chemicals does not interact directly with DNA [35–38] (Table II). One report describes

TABLE III

Effect of seven structurally dissimilar peroxisome proliferators on mitochondrial oxidative phosphorylation

Rat liver mitochondria were isolated as described in Materials and Methods and incubated with various concentrations of the above compounds in experiments typified by Fig. 2. State 4 respiration was measured in a closed chamber after addition of succinate (1.0 μmol). The ED_{50} was estimated from the dose-dependent stimulation of state 4 respiration by each agent.

Compound	ED_{50} (mM)
Wy-14,643	0.08
Perfluorooctanoate	0.18
Ciprofibrate	0.35
Acetylsalicylic acid	1.00
2-Ethylhexanol	1.20
Clofibric acid	1.40
Valproic acid	1.90

formation of 8-hydroxydeoxyguanosine following treatment with ciprofibrate [39], suggesting interactions of oxygen radicals with DNA; however, background levels in that study were very high, making interpretation difficult. Furthermore, Hegi et al. reported that 8-hydroxyguanosine did not increase following treatment with nafenopin [40].

The observation that lipofuscin appears following exposure of animals to plasticizers and lipid-lowering drugs is consistent with the oxidative stress hypothesis. However, one must interpret this result cautiously, since lipofuscin may not be a specific marker for radical production. Proof that peroxisomal proliferators cause the formation of oxygen radicals could be obtained with techniques such as spintrapping of lipid radicals [41] or measurement of lipid hydroperoxides in vivo [42]. Unfortunately, such experiments have not yet been performed in vivo. Since evidence for the role of specific receptors and alterations in DNA and the basis for lipofuscin in cancer causation is not compelling, the oxidative stress hypothesis must be questioned.

Mitochondrial uncoupling hypothesis. The consequence of uncoupling in vivo is a decline in intracellular ATP synthesis. In this study it was shown that urea synthesis, a process highly dependent on ATP supply, was almost totally abolished in the perfused liver after chronic treatment with Wy-14,643 (Table I), indicating that Wy-14,643 diminished ATP supply. This reduction in urea synthesis determined in vitro by addition of ammonia to the perfused liver most likely does not occur to the same extent in vivo since the rats appeared healthy throughout the duration of Wy-14,643 treatment and did not go into a hepatic coma. Bile flow, which is also energy dependent [43,44], was also diminished significantly 24 h after treatment with Wy-14,643 (see Results). It is possible that uncoupling of mitochondrial oxidative phosphorylation diminishes the

synthesis of acyl CoA compounds due to lack of energy and, thereby, elevates intracellular free fatty acids (Fig. 4B). Fatty acids activate protein kinase *c* [45], which is known to stimulate cell proliferation [27–29]. Indeed, phorbol esters, which are classical tumor promoters, stimulate protein kinase C [46]. This sequence of events may or may not be linked to the increase in peroxisomes after exposure to this class of chemicals.

In support of the involvement of cell turnover, a strong (Wy-14,643) but not a weak (DEHP) tumorigenic agent caused sustained cell turnover during many months of chronic feeding [12]. Moreover, a positive correlation was made recently between increased cell proliferation and hepatocarcinogenesis [47].

While support for the mitochondrial uncoupling hypothesis exists (Table II), it is by no means firmly established. Only after more information on protein kinase *c* [56] and fatty acids is obtained in vivo after chronic exposure to Wy-14,643 and related compounds can this sequence of events be assessed in its entirety. Then it will be clear if mitochondrial uncoupling by lipid-lowering drugs and plasticizers is involved in the mechanism of action of peroxisome proliferating carcinogens in the liver.

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