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Phthalates Rapidly Increase Production of Reactive Oxygen Species in Vivo: Role of Kupffer Cells

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ABSTRACT

The role of oxidants in the mechanism of tumor promotion by peroxisome proliferators remains controversial. The idea that induction of acyl-coenzyme A oxidase leads to increased production of H_2O_2, which damages DNA, seems unlikely; still, free radicals might be important in signaling in specialized cell types such as Kupffer cells, which produce mitogens. Because hard evidence for increased oxidant production in vivo after treatment with peroxisome proliferators is lacking, the spin-trapping technique and electron spin resonance spectroscopy were used. Rats were given di(2-ethylhexyl) phthalate (DEHP) acutely. The spin trapping agent α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone was also given and bile samples were collected for 4 h. Under these conditions, the intensity of the six-line radical adduct signal increased to a maximum value of 2.5-fold 2 h after administration of DEHP, before peroxisomal oxidases were induced. Furthermore, DEHP given with [$^{13}C_2$]dimethyl sulfoxide produced a 12-line electron spin resonance spectrum, providing evidence that DEHP stimulates $^{\cdot}$OH radical formation in vivo. Furthermore, when rats were pretreated with dietary glycine, which inactivates Kupffer cells, DEHP did not increase radical signals. Moreover, similar treatments were performed in knockout mice deficient in NADPH oxidase ($p47^{phox}$ subunit). Importantly, DEHP increased oxidant production in wild-type but not in NADPH oxidase-deficient mice. These data provide evidence for the hypothesis that the molecular source of free radicals induced by peroxisome proliferators is NADPH oxidase in Kupffer cells. On the contrary, radical adduct formation was not affected in peroxisome proliferator-activated receptor α knockout mice. These observations represent the first direct, in vivo evidence that phthalates increase free radicals in liver before peroxisomal oxidases are induced.

Peroxisome proliferators are potentially hazardous chemicals that are widely used and persist in the environment (IARC Working Group on Peroxisome Proliferation, 1995). These agents, administered to rodents, cause hepatomegaly, proliferation of peroxisomes in hepatocytes and marked increases in the enzymes of peroxisomal β-oxidation of fatty acids. Importantly, long-term treatment of rodents with these compounds results in the development of liver tumors (Reddy et al., 1980). Peroxisome proliferators may increase cancer risk in humans, although this idea has been challenged and remains controversial (Dalen and Dalton, 1996; Newman and Hulley, 1996). Whether or not phthalates pose a health risk to humans is controversial (Wilkinson and Lamb, 1999); therefore, determining the mechanisms underlying phthalate-induced effects is important.

Two mechanisms have been proposed for peroxisome proliferator-induced hepatocarcinogenesis: increased cell proliferation/decreased apoptosis and oxidative stress [reviewed in Gonzalez et al. (1998)]. The latter hypothesis is supported by the fact that chemicals of this class up-regulate enzymes that generate H_2O_2 in hepatic peroxisomes. Moreover, increases in 8-hydroxydeoxyguanosine, lipofuscin, and conjugated dienes have been reported in livers of rats treated for long periods with peroxisome proliferators (Goel et al., 1986). On the other hand, some of these points have been challenged. For example, H_2O_2 production increases in vitro with peroxisome proliferators, but not in intact cells, in which fatty acid supply is the limiting factor (Handler et al., 1992). Moreover, several attempts to detect increases in 8-hydroxydeox-

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ABBREVIATIONS: NF-κB, nuclear factor κB; ESR, electron spin resonance; PPARα, peroxisome proliferator-activated receptor α; DMSO, dimethyl sulfoxide; DEHP, Di(2-ethylhexyl) phthalate; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; i.g., intragastric; PKC, protein kinase C; TNFα, tumor necrosis factor α.

yguanosine after treatment with peroxisome proliferators have not been successful (Cattley and Glover, 1993).

Alternatively, it has been hypothesized that peroxisome proliferators first act on Kupffer cells, the resident hepatic macrophages, to increase production of oxidants that act as signaling molecules to activate the transcription factor NF-κB, leading to release of mitogenic cytokines. Indeed, it was recently shown that activation of the transcription factor NF-κB by peroxisome proliferators occurs first in Kupffer cells and is dependent upon reactive oxygen species derived from NADPH oxidase (Rusyn et al., 1998, 2000). Indeed, peroxisome proliferators do this by directly activating Kupffer cells to produce superoxide anion (Rose et al., 1999a).

However, whether or not phthalates increase oxidants in vivo remains unclear. Here, the hypothesis that phthalates increase reactive oxygen species was studied using the spin trapping technique and ESR, a technique that allows direct detection of reactive oxygen species in vivo. The first physical evidence for rapid production of radicals after administration of DEHP in vivo and a role for Kupffer cell NADPH oxidase is presented.

Experimental Procedures

Animals and Treatments. Female Sprague-Dawley rats (200–250 g; Charles River, Raleigh, NC), PPARα knockout [wild-type, SV129 (Lee et al., 1995)] and $p47^{phox}$ knockout [C57BL/6x129 (Jackson et al., 1995)] mice and their correspondent wild-type counterparts (25–30 g) were used in these experiments. Phthalates [di(2-ethylhexyl)phthalate (DEHP), 2-ethylhexanol, ethylhexanoic acid, and phthalic acid] and dimethyl sulfoxide (DMSO) were obtained from Aldrich (Milwaukee, WI), [$^{13}C_2$]DMSO were obtained from Isotech (Miamisburg, OH), and α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (POBN) from Sigma (St. Louis, MO). All other chemicals and reagents were of the highest available purity from standard suppliers.

Animals were given a single intragastric (i.g.) dose (1.2 g/kg) of DEHP, 2-ethylhexanol, ethylhexanoic acid, or phthalic acid. Control animals were given equal amounts of saline i.g. Some rats were given DMSO or [$^{13}C_2$]DMSO (0.5 ml/kg) by gavage at the time of treatment with DEHP. In some experiments, rats were fed glycine containing 5% (w/w) or control AIN-76 diets for 4 days before treatments.

Synthesis of 2-Ethyl[1-^{13}C]hexanol and Di(2-ethyl[1-^{13}C]hexyl)-α,α′-$^{13}C_2$-phthalate. Grignard reagent was prepared by the reaction of 3-bromoheptane (TCI America, OR) and magnesium turnings (Aldrich) in anhydrous diethyl ether (J.T. Baker, Phillipsburg, NJ) solvent. $^{13}CO_2$ (Cambridge Isotope Lab, Inc. MA) was introduced into Grignard reagent with vigorous stirring at 0°C. Subsequently, 30% sulfuric acid was added to decompose the excess Grignard reagent and the magnesium turnings, and to produce 2-ethyl[1-^{13}C]hexanoic acid. The ether phase was extracted with 30% potassium hydroxide, and the solution was washed with *n*-hexane and then acidified with 30% sulfuric acid, pH 1.0. The recovered product was extracted with diethyl ether, dried over anhydrous sodium sulfate, filtered, and concentrated at 40°C under reduced pressure to obtain 2-ethyl[1-^{13}C]hexanoic acid. Freshly prepared ethereal diazomethane (DeBoer and Backer, 1954) was added dropwise to the 2-ethyl[1-^{13}C]hexanoic acid to produce the methyl ester. Excess diazomethane was blown off with nitrogen gas, and the ester was refluxed for 30 min with excess lithium aluminum hydride (Aldrich) to yield 2-ethyl[1-^{13}C]hexanol. Excess lithium aluminum hydride was decomposed with water-saturated diethyl ether. The ether phase was transferred, dried over anhydrous sodium sulfate, filtered, and then concentrated at 40°C under reduced pressure to obtain the 2-ethyl[1-^{13}C]hexanol. Di(2-ethyl[1-^{13}C]hexyl)-α,α′-$^{13}C_2$-phthalate was synthesized as detailed previously with minor modifications (Takeshita and Takizawa, 1977). A solution of 2-ethyl[1-^{13}C]hexanol and pyridine in benzene was added dropwise to phthaloyl-α,α′-$^{13}C_2$ chloride (Isotech) in benzene with vigorous stirring for 30 min at 5 to 10°C. Subsequently, the mixture was refluxed on a boiling water bath for 1 h. After cooling to room temperature, the mixture was filtered to remove the pyridine hydrochloride formed. The solution was washed with water, dried over anhydrous sodium sulfate, and then concentrated at 40°C under reduced pressure. The residue obtained was dissolved in *n*-hexane. The solution was washed with 1% sodium hydrogen carbonate and then with water. The solution was dried and concentrated as detailed above. The identity of final products was confirmed by thin-layer chromatography and high-performance liquid chromatography with commercially available phthalates (Aldrich) as standards (data not shown).

Detection of Free Radicals. Animals were anesthetized with pentobarbital (75 mg/kg), and the gallbladder was cannulated using a 10-cm length of polyethylene 10 tubing. The spin trap POBN (1 g/kg, i.p.) was injected immediately after treatment with phthalates (see above) and bile samples were collected into Eppendorf tubes containing 50 μl of Desferal (3.3 mg/ml; Sigma) for 4 h at 20-min intervals in rats, or as a single 2 h sample in mice. Bile samples were frozen immediately on dry ice and stored at −70°C until analyzed by electron spin resonance (ESR) spectroscopy. ESR spectra were recorded on a Bruker EMX ESR spectrometer with a super high-Q cavity. Instrument settings: microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 0.6 s; time constant, 1.3 s. Spectra were recorded on an IBM-compatible computer interfaced to the spectrometer. Hyperfine coupling constants were determined with a spectral simulation program. ESR analysis of bile from animals treated with xenobiotics has been demonstrated to be useful in monitoring hepatic free radical-adduct formation in vivo (Knecht and Mason, 1988). Furthermore, the radical adducts detected in bile may be derived from both parenchymal and nonparenchymal cells. In addition, POBN is rapidly absorbed and distributed throughout the body after intraperitoneal administration and is relatively stable in vivo (Liu et al., 1999).

Acyl CoA Oxidase Activity. Acyl CoA oxidase is localized in peroxisomes, and its activity, measured as formaldehyde formed from hydrogen peroxide generated by peroxisomal β-oxidation, is a measure of induction of peroxisomes (Inestrosa et al., 1979). Liver samples (~100 μg) were homogenized in 10 volumes of 0.25 M sucrose buffer. A reaction mixture [1.4 ml; for details see Rose et al. (1997)] was warmed to 37°C and mixed with 200 μl of homogenate and the reaction was terminated after 10 min with 40% trichloracetic acid, which was added before homogenate to the blanks. The solution was centrifuged to pellet protein, and 1.0 ml of supernatant was added to 0.2 ml of Nash Reagent to measure formaldehyde after 30 min of incubation at 37°C. Protein concentration was determined by the method of Bradford (1976).

Statistics. Data presented are representative ESR spectra from three to five separate experiments per group. For statistical comparisons, one-way analysis of variance with Tukey's multiple comparison test was used. A p value less than 0.05 was selected before the study to determine statistical differences between groups.

Results

Effect of DEHP and Ethyl Hexanol on Radical Adduct Formation. Here, the effects of DEHP and its metabolites on the production of reactive oxygen species in rodent liver in vivo were studied. Weak radical adduct ESR signals were detected in bile from rats injected intraperitoneally with POBN (Fig. 1A); however, a significant 2- to 3-fold increase in the free radical adduct signals was detected in the bile of rats 2 h after intragastric administration of DEHP (Fig. 1B). Spectral simulation revealed the presence of two

free radical adduct spectra with hyperfine coupling constants: (I) a^N = 15.6 ± 0.2 G, a_β^H = 2.6 ± 0.2 G; and (II) a^N = 15.6 ± 0.2 G, a_β^H = 3.5 ± 0.1 G (Fig. 1C). Spectrum I is most likely to derive from a family of carbon-centered radical adducts (POBN/˙L, about 90% of the combined radical adduct) based on the hyperfine coupling constants and comparatively broad ESR linewidth of 0.8 G. In contrast, the narrow linewidth (0.35 G) and large β hydrogen coupling of adduct II (about 10%) match the properties of POBN/˙CO_2^- possibly formed by the abstraction of a hydrogen atom from endogenous formate (Burkitt et al., 1993).

Furthermore, 2-ethylhexanol, a nongenotoxic carcinogen, peroxisome proliferator, and metabolite of DEHP, produced a similar ESR spectrum, indicating that metabolites of DEHP may account for the radical species (Figs. 1, E and F). The intensity of the radical adduct increased maximally about 2.5-fold 2 h after administration of DEHP and 2-ethylhexanol (data not shown). However, when other metabolites of DEHP, such as 2-ethylhexanoic acid and phthalic acid were tested in this system, no changes in free radical adduct levels relative to control samples were detected (data not shown). To determine whether the radical adduct detected here was derived from DEHP and/or 2-ethylhexanol, analogs containing ^{13}C labels at the most likely points of radical adduct formation were synthesized (i.e., di(2-ethyl[1-^{13}C]hexyl)-α,α′-$^{13}C_2$-phthalate and 2-ethyl[1-^{13}C]hexanol). The ^{13}C-labeled carboxyl group was suspected to be the source of the POBN/˙CO_2^-, whereas the labeled alcohol could have formed the lipid-like radical adduct. Treatments using each of the compounds gave no spectra with double the number of ESR lines because of the presence of ^{13}C (Fig. 1D for DEHP, data not shown for 2-ethylhexanol), indicating that neither DEHP nor 2-ethylhexanol molecules were involved directly in the free radical adduct formation. This result supports the conclusion that DEHP or its metabolites do not form reactive species per se; rather, they activate production of reactive species by oxidant-generating enzymes in the liver.

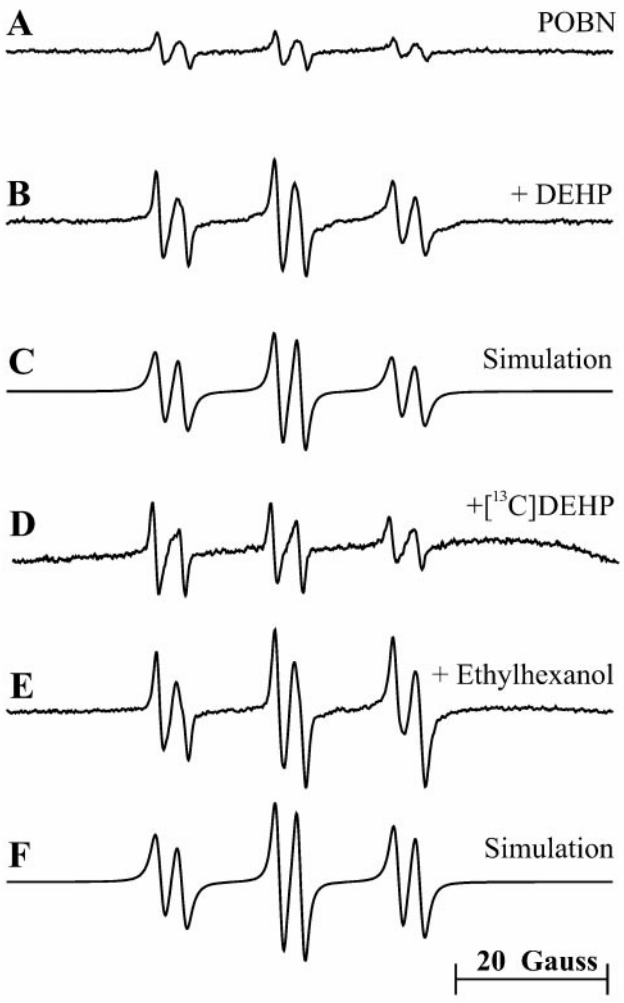

Fig. 1. Effects of di(2-ethylhexyl)phthalate and 2-ethylhexanol on ESR spectra of POBN/free radical adducts in rat liver. A, the ESR spectrum of radical adducts detected in bile of rats 2 h after administration of POBN (1 g/kg, i.p.). B, same as in A, but rats were also given DEHP (1.2 g/kg, i.g.). C, computer simulation of spectra shown in B. D, same as in A but rats were also administered di(2-ethyl[1-^{13}C]hexyl)-α,α′-$^{13}C_2$-phthalate ([^{13}C]DEHP; 1.2 g/kg, i.g.). E, same as in A, but rats were also given 2-ethyhexanol (1.2 g/kg, i.g.). F, computer simulation of spectra shown in E.

Because peroxisome proliferators can activate isolated Kupffer cells directly to produce superoxide anion (Rose et al., 1999a), it was hypothesized that a superoxide anion-derived radical adduct was involved. Furthermore, it is possible that the toxicity of superoxide anion in vivo is potentiated by iron-catalyzed generation of hydroxyl radical (˙OH), which could abstract hydrogen from endogenous formate (Minotti and Aust, 1989). Because of high reactivity and low stability of ˙OH-derived adducts of POBN, direct spin-trapping of this radical in biological systems is difficult (Halliwell and Grootveld, 1987). This experimental difficulty is remedied with the introduction of DMSO into the systems studied. Hydroxyl radical reacts with DMSO to yield methanesulfonic acid and methyl radical (˙CH_3) (Klein et al., 1981). The latter reacts rapidly with POBN to form relatively stable ESR-detectable nitroxides; this approach has been used in vivo to detect ˙OH (Kadiiska et al., 1995). To determine whether DEHP-induced radical adducts were caused by ˙OH, DMSO (0.5 ml/kg, i.g.) was given at the time of DEHP treatment (Fig. 2). Indeed, direct evidence for formation of a ˙CH_3 mediated adduct of POBN in the presence of DMSO (Fig. 2B) was obtained when [$^{13}C_2$]DMSO was given with DEHP and a characteristic 12-line ESR signal of POBN/˙$^{13}CH_3$ was observed (Fig. 2C; hyperfine coupling constants: species I (POBN/˙CO_2^-): a^N = 15.8 G, and a_β^H = 3.4 G; species II (POBN/˙$^{13}CH_3$): a^N = 16.0 G, a_β^H = 2.8 G, and a_β^{13C} = 4.9 G; and species III (POBN/˙L): a^N = 15.6 G and a_β^H = 2.7 G). Collectively, these results provide direct evidence in support of the hypothesis that DEHP rapidly causes formation of ˙OH in vivo.

Kupffer Cell NADPH Oxidase Is the Source of Oxidants Due to Phthalates in Vivo. To test the hypothesis that Kupffer cells are causally involved in increased radical production because of the phthalates observed here, glycine, an agent that inactivates Kupffer cells (Wheeler et al., 1999), was used. DEHP increased production of POBN/radical adducts in rats fed control diet for 4 days (Fig. 3B). However, when animals were fed dietary glycine (5% w/w), no increase in POBN/radical signal caused by DEHP was observed (Fig. 3D). It is concluded that rapid activation of Kupffer cells by phthalates leads to formation of reactive oxygen species in vivo.

Kupffer cells, like other macrophages, have a strong capacity to generate reactive oxygen species via several enzymatic pathways (Decker, 1990). It was recently demonstrated that

in mice deficient in the p47^{phox} subunit of NADPH oxidase, both the activation of NF-κB and the increase in cell proliferation caused by WY-14,643 were prevented nearly completely (Rusyn et al., 2000). Therefore, here we tested the hypothesis, using knockout mice deficient in NADPH oxidase, that NADPH oxidase is causally responsible for increased oxidant production caused by peroxisome proliferators. Similar to what was observed in the rat, ESR spectra composed of free radical adducts I and II were detected in bile from p47^{phox} wild-type and knockout mice injected intraperitoneally with POBN (Figs. 4, A and C, respectively). However, intragastric administration of DEHP resulted in a 2- to 3-fold increase in formation of POBN/radical adducts in wild-type mice (Fig. 4B). Although this spectrum seems to be primarily composed of POBN/$^{\cdot}CO_2^-$, simulation reveals that POBN/$^{\cdot}CO_2^-$ contributes only about 26% to signal intensity. Its presence is accentuated because of the inverse square relationship between line width and peak height. Importantly, DEHP failed to increase production of oxidants in NADPH oxidase-deficient mice (Fig. 4D), thus providing evidence for the hypothesis that NADPH oxidase is the source of radicals activated by phthalates in vivo.

It was hypothesized initially that phthalates increase oxidants in rodent liver via acyl-CoA oxidase in peroxisomes (Goel et al., 1986). Indeed, peroxisomes are markedly increased in both size and number in liver parenchymal cells of rats and mice treated with phthalates (Ganning et al., 1984). Increases in hepatic protein levels of acyl CoA oxidase and other peroxisomal enzymes are evident as soon as ~4 h after administration of peroxisome proliferators (Reddy et al., 1986). In this study, activity of acyl CoA oxidase was measured in liver homogenates from wild-type and p47^{phox} knockout mice treated with DEHP. An increase of ~2.5-fold in activity of this marker peroxisomal enzyme was detected 5 h after a single dose of DEHP in both wild-type (control, 1.6 ± 0.3 nmol/min/mg protein; 5 h DEHP, 4.2 ± 0.2) and knockout mice (1.7 ± 0.1 and 4.2 ± 0.2, respectively). However, no increase was evident after 2 h (wild-type, 2.0 ± 0.3 nmol/min/mg protein; knockout: 2.2 ± 0.4), when radical adducts were detected in wild-type but not NADPH oxidase-deficient mice (see above).

Peroxisome proliferation involves activation of an intracellular receptor (i.e., PPARα) that acts as a transcription factor to up-regulate synthesis of many lipid-metabolizing enzymes [reviewed in Gonzalez et al. (1998)]. Experiments with PPARα knockout mice showed unequivocally that hepatocellular proliferation and tumors caused by peroxisome proliferators require this receptor (Peters et al., 1997). Because no direct evidence has been presented to address the role of peroxisomes in increased production of oxidants caused by phthalates, PPARα knockout mice were treated with DEHP and POBN and bile was collected for ESR analysis. Importantly, ESR spectra of similar intensity (2- to 3-times control

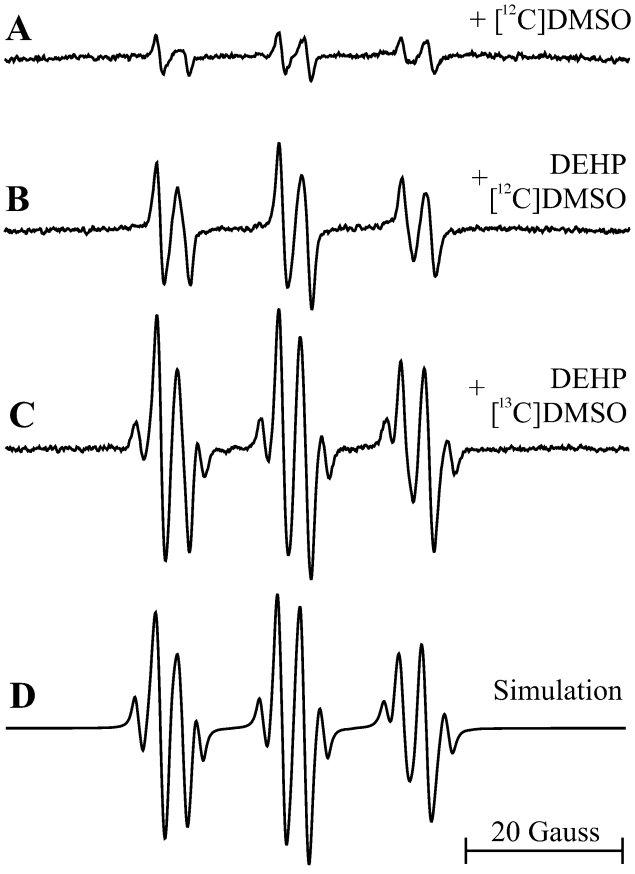

Fig. 2. DEHP-induced radical adducts involve hydroxyl radicals. A, the ESR spectrum of radical adducts detected in bile of rats 2 h after administration of POBN (1 g/kg, i.p.) and DMSO ([^{12}C]DMSO, 0.5 ml/kg, i.g.). B, same as in A, but rats were also given DEHP (1.2 g/kg, i.g.). C, the ESR spectrum of radical adducts detected in bile of rats 2 h after administration of POBN, DEHP, and [$^{13}C_2$]DMSO (0.5 ml/kg, i.g.). D, computer simulation of spectra shown in C.

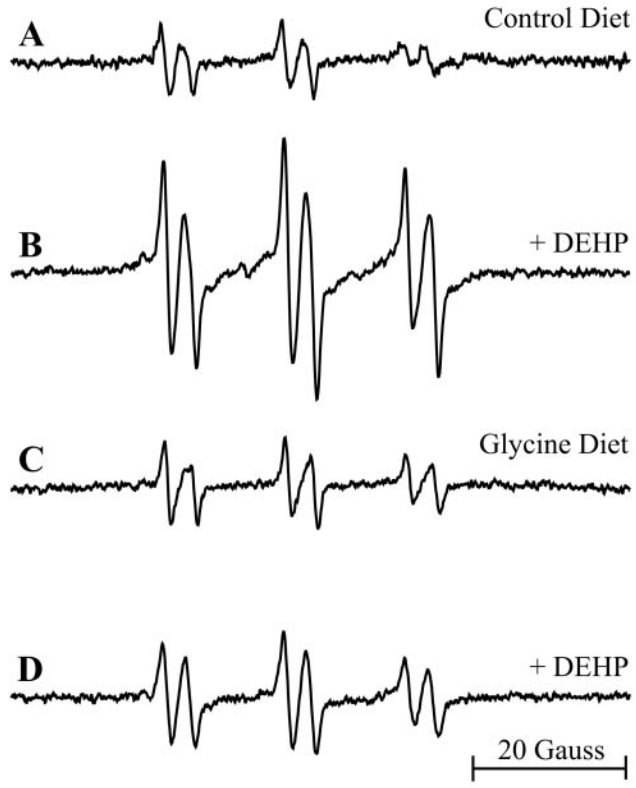

Fig. 3. Glycine prevents DEHP-induced radical production. A, the ESR spectrum of radical adducts detected in bile of rats fed control (AIN-76) diet for 4 days 2 h after administration of POBN (1 g/kg, i.p.). B, same as in A, but rats were also given DEHP (1.2 g/kg, i.g.). C, the ESR spectrum of radical adducts detected in bile of rats fed a glycine-containing (5% w/w) diet for 4 days 2 h after administration of POBN. D, same as in C, but rats were also given DEHP.

levels) were detected in both wild-type and PPARα knockout mice treated with DEHP (Figs. 5B and D). ESR spectra were similar to free radical adduct species I and II detected in bile from p47^{phox} wild-type and knockout mice (see above). These data support the hypothesis that PPARα is not involved in the rapid increase in oxidants caused by phthalates detected here. Moreover, because Kupffer cells do not express PPARα (Peters et al., 2000), this result further supports the idea that Kupffer cells but not hepatocytes are responsible for early increases in oxidants in response to phthalates.

Discussion

Direct Evidence for Production of Hydroxyl Radical after Treatment with Phthalates. The role of oxidants in the mechanism of action of peroxisome proliferators remains controversial despite numerous efforts to link these compounds to oxidized DNA [reviewed in Rose et al. (1999b)]. No direct evidence of increased oxidant production in vivo after treatment with these chemicals has yet been presented. On the other hand, a plethora of indirect evidence both in support and in opposition to the hypothesis that peroxisome proliferators increase oxidants has been presented. Recently, the in vivo formation of free radicals after exposure to several toxic chemicals has been demonstrated using the spin trapping technique and ESR spectroscopy (Kadiiska et al., 1998). Indeed, in vivo spin trapping is a useful tool because it allows direct detection and characterization of oxidants in tissues and body fluids (Liu et al., 1999).

Data presented here are important because they represent the first observation of rapid phthalate-induced free radical production in vivo (Fig. 1). The radical adducts detected in this study were not caused by a direct reaction of phthalates with the spin trapping agent because treatment with [^{13}C]-labeled analogs of DEHP and 2-ethylhexanol had no effect on ESR spectra (Fig. 1C). Because phthalates per se were not the source of oxidants, the hypothesis that these compounds produce radicals in vivo by activation of Kupffer cells was tested. Indeed, the evidence that the Kupffer cell NADPH oxidase is the source of oxidants at least immediately after treatment with these chemicals is presented (Figs. 3 and 4).

Activated NADPH oxidase complex generates superoxide anion [reviewed in Jones (1994)]; however, based on ^{13}C experiments and coupling constants, the radical adduct detected in this study was assigned as originating from hydroxyl radical (Fig. 2). It is most likely that in liver, where both superoxide dismutase and transition metals are present, superoxide anion was rapidly transformed to H_2O_2 and than to hydroxyl radical via a metal-catalyzed Haber-Weiss reaction [reviewed in Dunford (1987)]. Another possible mechanism for hydroxyl radical generation in biological systems is a reaction of superoxide with nitric oxide to form peroxynitrite, which leads to "hydroxyl-like" radical formation (Halliwell et al., 1999).

It is not clear how phthalates activate NADPH oxidase in

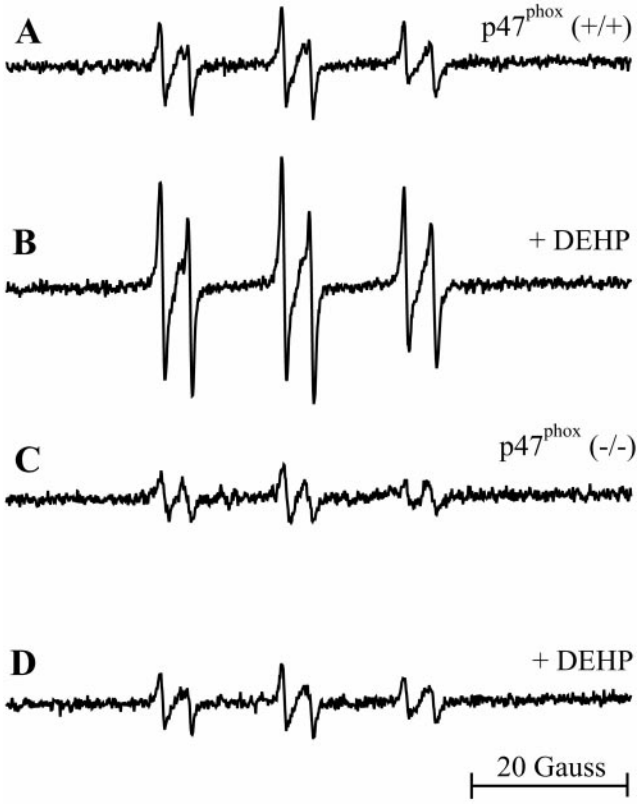

Fig. 4. DEHP does not increase formation of radical adducts in NADPH oxidase-deficient (p47^{phox} knockout) mice. A, the ESR spectrum of radical adducts detected in bile of p47^{phox} wild-type (+/+) mice 2 h after administration of POBN (1 g/kg, i.p.). B, same as in A, but mice were also given DEHP (1.2 g/kg, i.g.). C, the ESR spectrum of radical adducts detected in bile of p47^{phox} knockout (−/−) mice 2 h after administration of POBN. D, same as in C, but mice were also given DEHP.

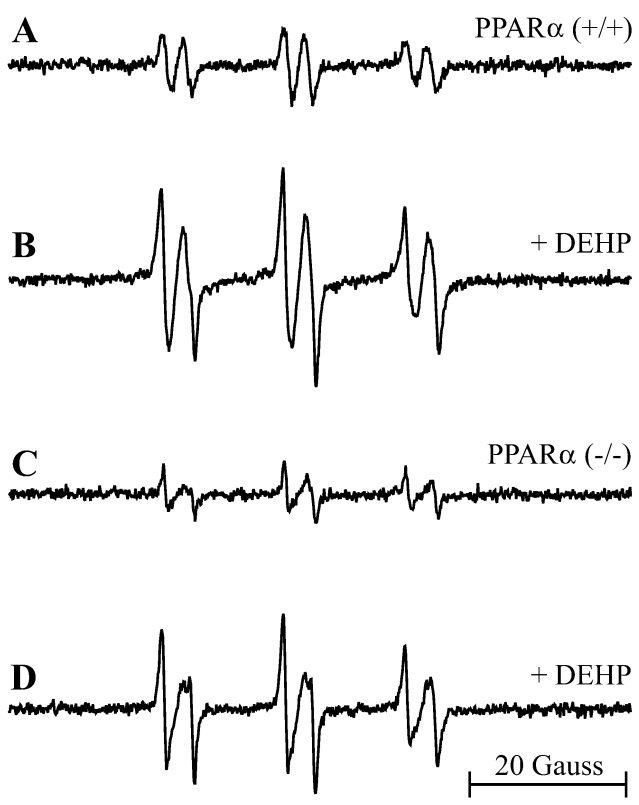

Fig. 5. DEHP-induced radical adducts are elevated in both wild-type and PPARα knockout mice. A, the ESR spectrum of radical adducts detected in bile of PPARα wild-type (+/+) mice 2 h after administration of POBN (1 g/kg, i.p.). B, same as in A, but mice were also given DEHP (1.2 g/kg, i.g.). C, the ESR spectrum of radical adducts detected in bile of PPARα knockout (−/−) mice 2 h after administration of POBN. D, same as in C, but mice were also given DEHP.

Kupffer cells; however, one possible mechanism is via increases in calcium and activation of PKC, which leads to phosphorylation and assembly of NADPH oxidase subunits. Indeed, supporting evidence for this hypothesis has been reported. Specifically, it was shown that peroxisome proliferators increase intracellular free calcium in cultured Kupffer cells (Hijioka et al., 1992). Furthermore, dietary glycine, an agent that disrupts calcium signaling via hyperpolarization of cell membrane, blocked hepatocellular proliferation because of peroxisome proliferators (Rose et al., 1997). Finally, treatment of Kupffer cells in vitro with peroxisome proliferators increases activity of PKC 3-fold; both glycine and staurosporine, an inhibitor of PKC, inhibited superoxide production and increased PKC activity completely (Rose et al., 1999a). Furthermore, because oxidants here are produced before H_2O_2-generating enzymes are induced by phthalates in hepatocytes and are independent of PPARα, an intracellular receptor that mediates pleiotropic responses to phthalates in parenchymal cells (Fig. 5), it is concluded that induction of peroxisomes is not responsible for increases in reactive oxygen species detected here.

Role of Oxidants in the Mechanism of Action of Phthalates: Harmful Species or Signaling Molecules? Oxidative stress may regulate cell proliferation [reviewed in Nakamura et al. (1997)]. Specifically, oxidants may play a role in tumor promotion by modulating the expression of a family of prooxidant genes that are related to cell growth and differentiation. It was recently suggested that low levels of oxidants might play a role in signaling increases in proliferation of liver parenchymal cells caused by peroxisome proliferators via a Kupffer cell-mediated mechanism involving TNFα and NF-κB (Rose et al., 1999b). Indeed, taking oxidant production in specific liver cell types (i.e., Kupffer cells) into consideration may provide important insights into the mechanisms by which these chemicals elicit proliferative responses in rodent liver. Importantly, evidence that supports the concept that oxidants play a significant role in the peroxisome proliferator-induced proliferative response by participating in signaling in Kupffer cells has been presented. For example, Kupffer cells are known to be activated by peroxisome proliferators in vivo (Bojes and Thurman, 1996). Moreover, peroxisome proliferators rapidly activate the redox-sensitive transcription factor NF-κB in Kupffer cells in an oxidant-dependent manner that results in production of mitogenic cytokines, such as TNFα, and increases in cell proliferation [reviewed in Rose et al. (1999b)]. Furthermore, monoethylhexylphthalate, a key lipophilic metabolite of DEHP, increased superoxide anion production in isolated Kupffer cells in a dose-dependent manner, indicating that phthalates can activate Kupffer cells directly (Rose et al., 1999a).

TNFα stimulates DNA synthesis in primary hepatocytes (Beyer and Theologides, 1993) and it was shown that TNFα from Kupffer cells is involved in the action of phthalates (Rose et al., 1999b). We have shown recently that in mice lacking active NADPH oxidase, peroxisome proliferators fail to increase early cell proliferation in the liver (Rusyn et al., 2000). This phenomenon is caused by a lack of activation of transcription factor NF-κB and production of mitogenic cytokines by these chemicals in the absence of active NADPH oxidase. However, PPARα is required for the increased cell proliferation and tumors caused by peroxisome proliferators (Peters et al., 1997). How can both TNFα and PPARα be required? One idea is that both factors are required for maximal stimulation of cell proliferation. In fact, it is known that peroxisome proliferators increase proliferation of liver parenchymal cells both in vivo and in vitro; however, the in vitro effect is much less robust and persistent (i.e., 8- to 10-fold increases in vivo versus only about 2-fold increases in vitro) regardless of the dose of the compound used (Marsman et al., 1988). Moreover, in highly purified hepatocytes, it was shown recently that peroxisome proliferators alone fail to increase proliferation of parenchymal cells in vitro; however, when peroxisome proliferator is added with TNFα, cell proliferation is potentiated significantly. This data supports the hypothesis that both peroxisome proliferator and TNFα are required for maximal stimulation of cell proliferation in liver parenchymal cells (Parzefall et al., 2001).

Collectively, this work provides the first direct evidence that phthalates induce formation of radicals in vivo. This finding establishes a role for oxidants in the mechanism of hepatocellular proliferation by this important class of chemicals via the following sequence of events: phthalates rapidly activate Kupffer cells, resulting in production of oxidants from NADPH oxidase, activation of NF-κB, and release of mitogenic cytokines such as TNFα. This leads to proliferation of parenchymal cells. Concomitantly, phthalates activate PPARα in hepatocytes, leading to induction of peroxisomes, a phenomenon necessary for induction of tumors months later.

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