

ACCELERATED PAPER

Expression of base excision repair enzymes in rat and mouse liver is induced by peroxisome proliferators and is dependent upon carcinogenic potency

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Elevated and sustained cell replication, together with a decrease in apoptosis, is considered to be the main mechanism of hepatic tumor promotion due to peroxisome proliferators. In contrast, the role of oxidative stress and DNA damage in the carcinogenic mechanism is less well understood. In view of possible induction of DNA damage by peroxisome proliferators, DNA repair mechanisms may be an important factor to consider in the mechanism of action of these compounds. Here, the ability of peroxisome proliferators to induce expression of base excision repair enzymes was examined. WY-14,643, a potent carcinogen, increased expression of several base excision DNA repair enzymes in a dose- and time-dependent manner. Importantly, expression of enzymes that do not repair oxidative DNA damage was not changed. Moreover, less potent members of the peroxisome proliferator group had much weaker or no effects on expression of DNA repair enzymes when compared with WY-14,643. Collectively, these data suggest that DNA base excision repair may be an important factor in peroxisome proliferator-induced carcinogenesis and that induction of DNA repair might provide further evidence supporting a role of oxidative DNA damage by peroxisome proliferators.

Introduction

Peroxisome proliferators are a diverse group of chemicals and therapeutic agents. In rodents these compounds cause hepatomegaly, proliferation of peroxisomes in hepatic parenchymal cells and marked increases in the activity of enzymes required for peroxisomal β -oxidation of fatty acids (1). These changes persist at steady-state levels as long as peroxisome proliferators are administered. Long-term exposure results in the development of liver tumors in rodents (2). Rodents are much more sensitive to the effects of peroxisome proliferators than dogs, non-human primates or humans. This difference in sensitivity appears to be due to differences in the PPAR α receptor and its response elements (reviewed in ref. 3). Most reviews of peroxisome proliferators conclude that humans are at little or no risk for cancer from exposure to these agents (4).

The major mechanisms that have been proposed for peroxisome proliferator-induced hepatocarcinogenesis are: (i) increased cell proliferation leading to promotion of spontaneously initiated lesions; (ii) oxidative stress, resulting from disproportionate increases in the levels of oxidants. However, recent observations that oxidant-dependent activation of the transcription factor NF- κ B plays a critical role in increased hepatocyte proliferation caused by WY-14,643 (5) and ciprofibrate (6) suggest that these mechanisms may not be mutually exclusive and oxidants may be involved in signaling increased cell proliferation.

It has been suggested that reactive oxygen species play a role in the initiation and promotion steps of carcinogenesis induced by peroxisome proliferators. Recently, direct evidence of rapid peroxisome proliferator-induced generation of hydroxyl radicals *in vivo* has been presented (7). Indeed, production of reactive oxygen species may lead to DNA damage via hydroxyl radicals and products of lipid peroxidation. Oxidative stress is hypothesized to be a common pathway for many non-genotoxic chemical carcinogens (8). However, the role of oxidative stress has been questioned. Indeed, when compared with direct DNA damaging agents, the magnitude of response following carcinogenic exposures to chemicals thought to work through oxidants has been small. Several attempts to assess oxidative DNA damage by peroxisome proliferators using direct measurement of adducts produced equivocal results (9,10). Moreover, the artifactual formation of oxidized base damage due to artifactual auto-oxidation reactions in assays requiring extraction of DNA has plagued this experimental approach (11).

On the other hand, it is known that DNA repair enzymes are induced both *in vivo* and *in vitro* by oxidative stress (12). Several DNA repair genes involved in oxidative damage have been identified and it is believed that the predominant pathway used for removal of oxidized bases is the base excision repair (BER) pathway. Several proteins are involved in this multi-step repair process (12). For instance, the primary pathway for removal of 8-hydroxydeoxyguanosine (8-OH-dG) appears to be OGG1, a glycosylase/lyase, which excises this adduct from DNA and cleaves 3' to AP (abasic) sites, leaving a 3'-cleaved AP site (13). The DNA is then cleaved 5' to the AP site by AP endonuclease (APE), the gap is filled by polymerase β (Pol β) and the newly synthesized DNA is sealed by ligase (14). While BER is considered the main pathway for oxidative DNA damage, nucleotide excision repair and long patch repair have also been shown to remove oxidative damage from DNA (15). Importantly, expression of enzymes that participate in these processes may be induced following increased production of reactive oxygen species (16) or chemical exposure (17).

No previous reports, however, have described changes in DNA repair enzymes associated with peroxisome proliferator-induced carcinogenesis. Since BER is a major mechanism for removal of oxidative lesions from DNA (18), we investigated the expression of several DNA glycosylases, APE, DNA

polymerases and ligases in livers of rats (Fisher 344) and mice (C57Bl/6) treated with peroxisome proliferators. To assemble a comprehensive set of liver tissues, a combination of samples from several different studies performed at the University of North Carolina at Chapel Hill, Chemical Industry Institute of Toxicology, or by the National Toxicology Program has been used.

WY-14,643 causes a dose-dependent increase in expression of DNA BER enzymes in rodent liver

Mammalian *N*-methylpurine-DNA glycosylase (MPG) has broad substrate specificity and primarily is capable of hydrolysis of 3-methyladenine. It has also been shown to hydrolyze 1,*N*⁶-ethenoadenine *in vitro* and weak activity towards 8-OH-dG has been reported (19). A quantitative reverse transcriptase PCR assay (19) was used to test the hypothesis that peroxisome proliferators induce expression of MPG in rat liver. The use of *in vitro* synthesized reference RNA allowed quantitation of the PCR products (MPG and standard) after gel electrophoresis and autoradiography (Figure 1A). The effect of a 21 day treatment with WY-14,643 (1000 p.p.m.) on MPG mRNA in whole rat liver is shown in Figure 1B. In livers of animals fed a regular chow diet, expression of MPG mRNA was low (89 ± 16 fg/ μ g total RNA), however, it was elevated 2-fold after treatment with WY-14,643 (164 ± 21 fg/ μ g total RNA). Moreover, a dose-dependent increase in expression of MPG was observed in rats treated with WY-14,643 for 90 days (Figure 1C). The amount of MPG mRNA was 2- or 6.5-fold higher in livers of rats treated with 100 or 500 p.p.m. WY-14,643, respectively, than in control animals. Collectively, these results support the hypothesis that potent peroxisome proliferators (i.e. WY-14,643) may inflict DNA damage and that levels of DNA repair gene expression could be used to establish a gene response profile after exposure to these chemicals.

Next, we used a recently developed multi-probe RNase protection assay for BER enzymes. This approach distinguishes the presence of multiple expressed DNA repair genes simultaneously from a single sample, which allows comparative analysis of different mRNA products both within and between samples. This is a highly sensitive and specific approach for detection and quantitation of gene expression at the mRNA level. It should be noted, however, that the RNase protection assay template sets (a kind gift of BD PharMingen, San Diego, CA) used here for rat and mouse tissues were under development and differed slightly in the composition of DNA repair genes evaluated. Total mRNA was isolated from livers of rats fed control or WY-14,643 (1000 p.p.m.)-containing diet for up to 22 weeks. A time-dependent 3- to 12-fold increase in mRNA for OGG1, APE, MPG and Pol β was observed (Figure 2). Importantly, expression of several enzymes that are not related to oxidative DNA damage (e.g. *O*⁶-methylguanine-DNA methyltransferase and polymerase δ) was not changed. It should be noted that our findings of an increased expression of mRNA for BER proteins were corroborated recently when it was reported that Pol β and APE protein levels were increased markedly after treatment with WY-14,643 for 6 days (20).

The single nucleotide BER pathway is a favorable mechanism for removal of oxidized bases and is dependent upon the interaction between DNA Pol β and DNA ligase I (21). Indeed, only ligase I, but not ligase III or other enzymes of the 'long

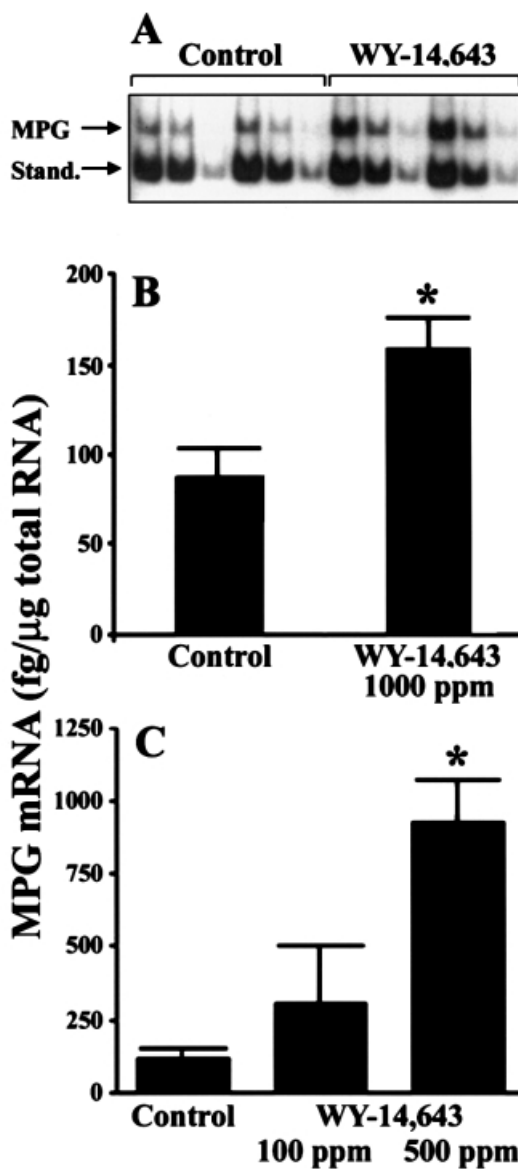


Fig. 1. MPG mRNA is induced in rat liver by dietary feeding of WY-14,643. (A) An autoradiogram of PCR products of native mRNA and synthetic MPG RNA (a kind gift of Dr S. Mitra, University of Texas, Galveston, TX) after *Eco*RI digestion is shown. Fisher 344 rats were fed either control diet (NIH-07) or diet containing WY-14,643 (1000 p.p.m. for 21 days). Total cellular RNA was isolated using a QuickPrep extraction kit (Amersham Pharmacia Biotech) and RT-PCR was performed as described in Roy *et al.* (19). Three 1:2 serial dilutions of each sample mixture containing 2 μ g total cellular RNA and 540 fg synthetic RNA standard were made, amplified by PCR and resolved on 6% polyacrylamide gels. The 471 bp product of cellular MPG amplification and 366 bp product of reference RNA amplification after enzymatic cleavage of the 105 bp non-radioactive fragment are indicated. Representative autoradiogram. (B) Data shown are results of densitometry analysis of images from the experiment detailed in (A). (C) As above but rats were given 0 (Control), 100 or 500 p.p.m. WY-14,643 for 90 days. Data reported as means \pm SEM from four separate experiments. Asterisks (*) denote statistical differences ($P < 0.05$) from the control group by one way ANOVA and Student–Newman–Keuls *post hoc* test.

patch' repair pathway, was induced in mouse liver after dietary treatment with WY-14,643 (500 p.p.m.) for 7 days (Figure 3). Similar to what was observed in rats, WY-14,643 (500 p.p.m.) caused an ~3-fold increase in mRNA for OGG1, TDG, APE, MPG and Nth1 in mouse liver (data not shown). These findings

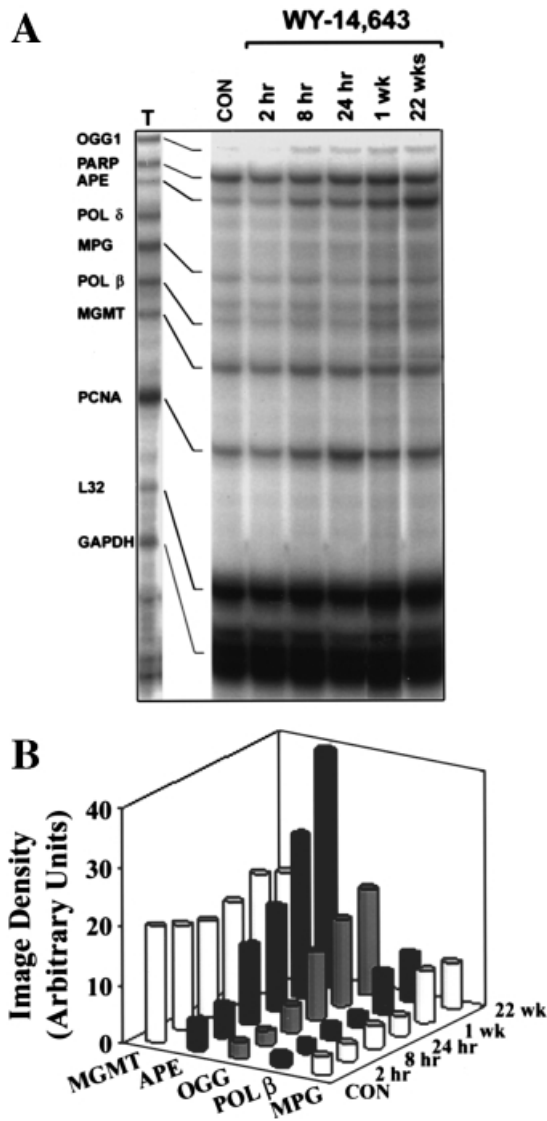


Fig. 2. WY-14,643 causes a time-dependent increase in expression of base excision DNA repair enzymes in rat liver. (A) Total RNA was isolated from livers of rats treated with WY-14,643 (1000 p.p.m.) for up to 22 weeks using a QuickPrep extraction kit (Amersham Pharmacia Biotech) and dissolved in RNase-free water. Expression of base excision DNA repair enzymes was analyzed with an RNase protection assay using rodent multi-nucleotide RNA probe template sets (a generous gift of BD PharMingen). Riboprobes were synthesized in the presence of [³²P]dUTP to yield labeled antisense RNA probes. The RNase protection assay was performed on 40 µg of individual RNA samples using a RiboQuant multi-probe RNase protection assay kit (BD PharMingen). Protected fragments were separated on QuickPoint nucleic acid separation gels (Novex, San Diego, CA), dried and exposed to X-ray film. Representative autoradiogram from three separate experiments. (B) Data shown are results of densitometry analysis of images from the experiment detailed in (A). The intensity of protected bands was quantified using an image analyzer and normalized to the intensity of housekeeping genes L32 and GAPDH.

are important because they show that WY-14,643 induces expression of the whole pathway responsible for the repair of oxidative DNA damage, DNA glycosylases, APE, Pol β and ligase I.

Potent carcinogens are more potent inducers of BER enzymes gene expression

WY-14,643 is one of the most potent carcinogens among the peroxisome proliferators. To test whether less potent

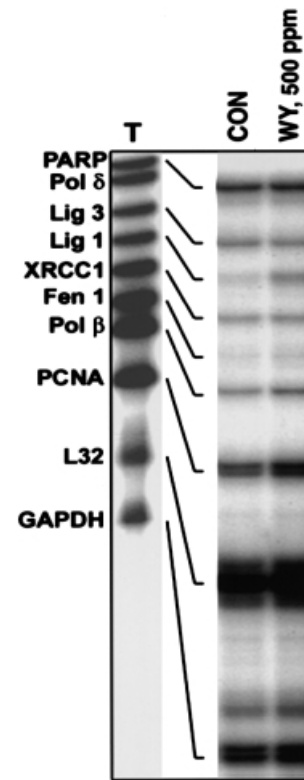


Fig. 3. Expression of ligase I is induced in mouse liver by WY-14,643. Mice (C57Bl/6) were fed a diet containing the potent carcinogen WY-14,643 (WY, 500 p.p.m.) for 7 days. Total RNA was isolated from liver samples and used in the RNase protection assay as detailed in Figure 2. Representative autoradiograms from three separate experiments.

carcinogens from this group of compounds have similar effects on DNA repair enzymes, rats were fed WY-14,643 or the weaker carcinogens di(2-ethylhexyl)phthalate (DEHP) and clofibric acid for 22 weeks. Both DEHP (12000 p.p.m.) and clofibric acid (5000 p.p.m.) increased expression of mRNA for OGG1, APE, MPG and Pol β by ~2- to 3-fold, however, these effects fell short of the profound changes caused by WY-14,643 (1000 p.p.m.) (Figure 4). It should be noted that since WY-14,643, DEHP and clofibric acid cause a similar initial increase in cell proliferation, the differences in expression of DNA repair enzymes observed here are not due to a rapid growth of liver mass. Furthermore, when WY-14,643 (1000 p.p.m.) and DEHP (12000 p.p.m.) were administered to rats for 7 days, induction of OGG1 APE and Nth1 was observed only in WY-14,643-treated animals (data not shown). Similar effects were observed with gemfibrozil, another potent rodent carcinogen, but not with the weak carcinogen dibutyl phthalate (Figure 5). Specifically, both WY-14,643 (500 p.p.m.) and gemfibrozil (16000 p.p.m.) administered to rats for 90 days increased expression of DNA BER enzymes, while dibutyl phthalate (10000 p.p.m.) had no effect.

It has been hypothesized that the hepatocarcinogenicity of peroxisome proliferators is due to oxidative stress. At least two possible sources of oxidants following administration of peroxisome proliferators have been proposed: (i) peroxisomal acyl CoA oxidase in parenchymal cells; (ii) NADPH oxidase in Kupffer cells (reviewed in ref. 22). The data presented here are important for mechanistically based risk assessment of peroxisome proliferators for several reasons. First, further evidence supporting the role of oxidative DNA damage in the

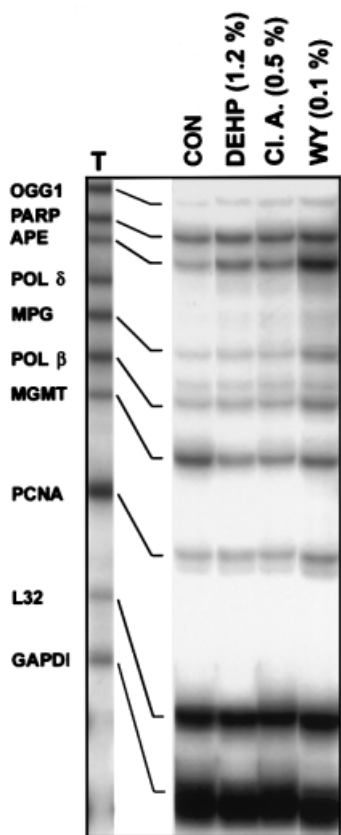


Fig. 4. Chronic treatment with peroxisome proliferators that are potent carcinogens induces increases in DNA BER enzymes expression. Total RNA was isolated from livers of rats treated with DEHP (1.2% w/w), clofibrac acid (Cl. A., 0.5% w/w) or WY-14,643 (WY, 0.1% w/w) for 22 weeks and used in the RNase protection assay as detailed in Figure 2. Representative autoradiogram from three separate experiments.

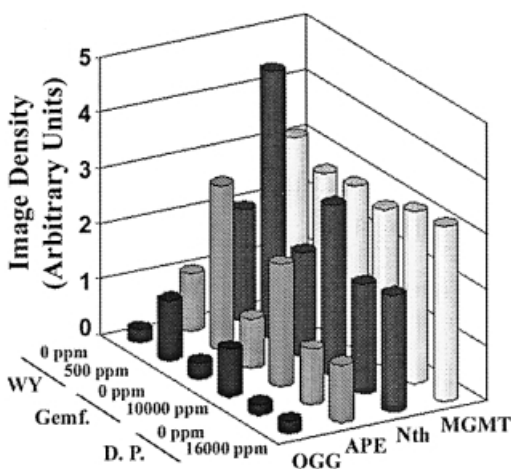


Fig. 5. Sub-chronic treatment with the potent carcinogens WY-14,643 and gemfibrozil, but not with the weak carcinogen dibutyl phthalate, increases expression of DNA excision repair enzymes. Total RNA was isolated from livers of rats treated with WY-14,643 (WY, 500 p.p.m.), dibutyl phthalate (D.P., 10000 p.p.m.) or gemfibrozil (Gemf., 16000 p.p.m.) for 90 days and used in the RNase protection assay as detailed in Figure 2. Data shown are results of densitometry analysis and the intensity of protected bands was quantified using an image analyzer and normalized to the intensity of housekeeping genes L32 and GAPDH.

mechanism of action of peroxisome proliferators is provided. Specifically, we suggest that DNA BER may be an important factor in peroxisome proliferator-induced carcinogenesis and that induction of DNA repair reflects an increase in oxidized bases following treatment with these compounds. The small and inconsistent increases in oxidative DNA damage observed in previous studies may be due to an inability to observe real increases due to the artifactual formation of oxidative DNA damage during DNA isolation. Most of the studies have reported 8-OH-dG values in control rat livers of ~ 2 per 10^{-5} bp. These values can be dramatically reduced through the use of free radical trapping reagents and antioxidants (23, 24). Reductions in artifactual 8-OH-dG are likely to increase the level of detectable oxidative DNA damage resulting from exposure to peroxisome proliferators.

Second, overexpression of DNA repair enzymes *per se* may play an important role in the mechanism of action of peroxisome proliferators. For instance, Pol β is normally expressed at low levels and has high infidelity in replicating DNA (25). Recently it was shown that overexpression of Pol β may result in increased spontaneous mutagenesis (26). Moreover, it was hypothesized that an excess of Pol β may disrupt functions of other DNA polymerases by introducing illegitimate deoxyribonucleotides or mutagenic base analogs like those produced by oxidative stress (27).

In addition, APE (Ref-1) is a multifunctional protein that stimulates DNA binding by a number of transcription factors, such as NF- κ B and AP-1 (28). Interestingly, peroxisome proliferators increase the activity of NF- κ B in rodent liver and it was hypothesized that NF- κ B activation plays a role in increased cell proliferation induced by these compounds (reviewed in ref. 22). Therefore, the marked increase in APE expression observed here might be important for the promotional activity of peroxisome proliferators. On the other hand, APE is known to regulate transactivation of p53 (29). This transactivation only requires the C-terminus and has been suggested to delay the G₁/S transition and enhance BER (29). Given that p53 has been shown to enhance BER (30), it is possible that up-regulation of APE expression by peroxisome proliferators leads to enhanced p53-dependent BER. The significance of APE transactivation of the pro-apoptotic functions of p53 is unclear, since peroxisome proliferators are known to decrease apoptosis in liver (31).

An additional concern is that DNA repair enzymes could be up-regulated unevenly, so that a state of imbalanced DNA repair may exist. Indeed, imbalanced DNA repair may lead to formation of both mutagenic and clastogenic lesions. In the case of oxidative DNA damage, if glycosylase and APE are overabundant (relative to DNA polymerase or DNA ligase) then DNA strand breaks might accumulate that could consequently influence cell viability and induce chromosomal damage (32). Whether peroxisome proliferators cause imbalanced repair has yet to be determined.

Finally, since current risk assessment of peroxisome proliferators is based on important differences between humans and rodents in expression of PPAR α (reviewed in ref. 3), the approach of analyzing expression of DNA repair enzymes can be used in further studies to assess whether or not BER enzymes, as a biomarker of oxidative stress, are induced by WY-14,643 in PPAR α knockout mice.

In summary, the results of this study provide new information that supports a role of oxidative stress as a mechanism of carcinogenesis for peroxisome proliferators. It demonstrates a

clear induction of DNA repair pathways associated with oxidative DNA damage that is related to the dose and length of exposure, as well as the potency of the peroxisome proliferator for inducing hepatic carcinogenesis.

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