Effect of Peroxisome Proliferators on Rat’s Liver Peroxisomal Enzymes

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KEY WORDS: peroxisome proliferators, nafenopin (NAF), perfluorooctanoic acid, 2,4-dichlorophenoxyacetic acid, 2,4,5 trichlorophenoxyacetic acid, acyl CoA oxidase, amino acid oxidase, malondialdehyde

ABSTRACT
Treatment of rodents with peroxisome proliferators such as nafenopin (NAF), perfluorooctanoic acid (PFOA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T) is suggested to be associated with a remarkable increase in peroxisomal \[\text{oxyhemeperoxidase}\] activity. Lipid peroxidation was assessed by measuring malondialdehyde liver tissue level. This study was conducted on 80 male Wistar rats classified into five groups, with a minimum of 15 rats per group. Group I animals received only basal diet (BD) (laboratory rodent diet, a constant nutrition formula from LabDiet Dealer, Tripoli, Lebanon). Groups II to V received NAF 0.1%, PFOA 0.02%, 2,4-D 0.05%, and 2,4,5-T 0.05%, respectively, which were admixed to BD. Fourteen weeks later, the rats were killed and their livers were used for biochemical analysis and measurement of enzyme activity.

The activity of acyl CoA oxidase was significantly increased by about 50%, 20%, 3%, and 2-fold in groups II, III, V, and IV, respectively. The activity of D-amino acid oxidase showed a significant increase \((P<0.01)\) in only group V, while the activity of glycolate oxidase was not significantly changed. Malondialdehyde was measured as a predictor of lipid peroxidation, and it showed a significant increase \((P<0.05)\) in groups II and III. Results of this study confirmed the remarkable increase in peroxisomal \[\text{oxyhemeperoxidase}\] activity. Lipid peroxidation was assessed by measuring malondialdehyde liver tissue level. This study was conducted on 80 male Wistar rats classified into five groups, with a minimum of 15 rats per group. Group I animals received only basal diet (BD) (laboratory rodent diet, a constant nutrition formula from LabDiet Dealer, Tripoli, Lebanon). Groups II to V received NAF 0.1%, PFOA 0.02%, 2,4-D 0.05%, and 2,4,5-T 0.05%, respectively, which were admixed to BD. Fourteen weeks later, the rats were killed and their livers were used for biochemical analysis and measurement of enzyme activity.

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INTRODUCTION
Peroxisomes are membrane-bound cytoplasmic organelles present in all mammalian cells except red blood cells. However, their number, size, and enzyme profile varies among different tissues. Biochemical analysis reveals a content of several oxidative enzymes. The feature common to all these enzymes is that they generate hydrogen peroxide ($H_2O_2$) as a product of their reaction. One feature of peroxisomes is that they are inducible and, therefore, an administration of the hypolipidemic drug clofibrate to rats leads to a 10-fold increase in number of peroxisomes per liver cell. Peroxisomes are involved in lipid degradation and biosynthesis. A $\beta$-oxidation pathway similar in function to that of mitochondria is present in peroxisomes.

Peroxisome proliferators, nafenopin (NAF), perfluorooctanoic acid (PFOA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T), are known nongenotoxic carcinogens in rodents. They induce predictable pleiotropic responses in the liver characterized as early adaptive changes and late carcinogenic effects. Adaptive changes include hepatomegaly, peroxisome proliferation, and induction of peroxisomal and microsomal enzymes. In addition to the early adaptive changes, peroxisome proliferators also induce liver tumors on long-term administration in rodents. Now there is considerable evidence indicating that peroxisome proliferator-induced hepatocarcinogenesis is dependent on oxidative stress that causes DNA damage and cell proliferation. In rodents treated with potent peroxisome proliferators, the activity in $H_2O_2$ generating enzymes like acyl CoA oxidase (the first and rate-limiting enzyme of the peroxisomal $\beta$-oxidation system) is increased by 10- to 30-fold. Acyl CoA oxidase converts acyl CoA to enoyl CoA, a step in which $H_2O_2$ is generated. Increase in $H_2O_2$ generating enzymes is associated with minimal increase or considerable decrease in $H_2O_2$ degrading enzymes further accentuating oxidative stress. If this persists, the oxidative stress coupled with enhanced lipid peroxidation may participate in extensive DNA damage and may create conditions favorable for neoplastic development.

Other peroxisomal enzymes, e.g., amino acid oxidase and glycolate oxidase, may be involved in this oxidative stress in the same way, as they are known to increase $H_2O_2$ production.

MATERIALS AND METHODS
Animals
Male Wistar rats, initially weighing 180 to 200 g, were used throughout. The animals were housed under standard conditions and were allowed free access to food and tap water. Minimum of 15 rats per group were tested.

Chemicals
NAF and chemicals for enzyme activities and malondialdehyde were obtained from Sigma Chemical Co. (St. Louis, MO). PFOA, 2,4 dichlorophenoxyacetic acid (2,4-D), and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T) were supplied by Aldrich (Beers, Belgium).

Experimental procedures
Rats were classified into five groups:

Group I: animals received only basal diet (BD) (laboratory rodent diet, a constant nutrition formulation from LabDiet Dealer, Tripoli, Lebanon)

Group II: NAF 0.1% was admixed to BD.

Group III: PFOA 0.02% was admixed to BD.

Group IV: 2,4-D 0.05% was admixed to BD.

Group V: 2,4,5-T 0.05% was admixed to BD.
Fourteen weeks later, the rats were killed and their livers were used for biochemical analysis.

Biochemical analysis

Measurement of enzyme activity

Liver samples homogenized in ice-cold 0.2 M sucrose buffered with 3 μM imidazole. The pH of the medium was adjusted to 7.4. The homogenates were used for estimation of enzyme activity. The activity of peroxisomal acyl CoA oxidase, amino acid oxidase, and glycolate oxidase was determined by the methods previously described.19

The measurement was based on the fluorometric detection of H₂O₂ in a total volume of 0.5 mL, the assay mixture containing 0.1 mL of diluted liver homogenate, 0.02 μM of flavin adenine dinucleotide, 80 μM glycyglycine buffer (pH 8.3), 10 μM homovanillic acid, and 4 units of horseradish peroxidase.

The reaction was started for:

- Glycolate oxidase by the addition of 200 μM glycolate,
- D-amino acid oxidase by the addition of 500 mM D-proline, and
- Acyl CoA oxidase by the addition of 0.1 mM palmitoyl CoA.

Fluorescence was measured spectrophotometrically at excitation and emission wavelengths of 325 and 426 nm, respectively.

Protein determination

Using bovine serum albumin as a reference standard, protein concentration was measured by the method of Lowry et al.20

Table 1. Enzyme Activity (μU/mg protein) ± SEM

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Acyl CoA Oxidase</th>
<th>D-amino Acid Oxidase</th>
<th>Glycolate Oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Basal diet</td>
<td>2.0 ± 0.45</td>
<td>17.0 ± 0.9</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td>II</td>
<td>NAF (0.1%)</td>
<td>136.0 ±11*</td>
<td>3.0 ± 0.4</td>
<td>4.0 ± 0.16</td>
</tr>
<tr>
<td>III</td>
<td>PFCA (0.02%)</td>
<td>57.0 ± 5.0*</td>
<td>10.0 ± 1.7</td>
<td>12.0 ± 2.5</td>
</tr>
<tr>
<td>IV</td>
<td>2,4-D (0.05%)</td>
<td>6.0 ± 0.9*</td>
<td>17.0 ± 1.6</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>V</td>
<td>2,4,5-T (0.05%)</td>
<td>6.0 ± 0.7*</td>
<td>35.0 ± 2.6*</td>
<td>4.0 ± 0.2</td>
</tr>
</tbody>
</table>

*Significant difference from basal diet.

Malondialdehyde assay of liver tissue

Lipid peroxidation of liver tissue was assessed by measuring malondialdehyde using the thiobarbituric acid assay technique.21 Liver samples were homogenized in 0.02 M sodium phosphate buffer pH 7.4, 1 mL of 17.5 trichloroacetic acid (TCA) and 1 mL of 0.6% thiobarbituric acid, pH 2, were added to 1 mL of homogenate containing 1.0 to 1.7 mg of liver tissue protein. This mixture was placed in boiling water bath for 15 minutes and then allowed to cool. One mL of 70% TCA was added and the mixture was allowed to incubate. Samples were centrifuged for 15 minutes at 2000 rpm and the absorbance was read spectrophotometrically at wavelength of 534 nm against reagent blank. The amount of malondialdehyde was expressed in nm and was calculated using a molar extinction coefficient of 1.56 X 10⁵ M⁻¹ cm⁻¹. Malondialdehyde is expressed as nM/mg protein of liver tissue.

RESULTS

Effect of tested peroxisomes proliferators on acyl CoA oxidase activity

As shown in Tables 1 and 2: NAF-treated group (group II) shows a more than 50-fold increase in the activity of acyl CoA oxidase as compared with control group (group I). A significant increase (P<0.05) in lipid peroxidation, as predicted by the malondialdehyde level, is shown in this group.

The increase in the activity of acyl
CoA oxidase in the PFOA 0.02%–treated rats (group III) reaches more than 20 times its activity in the control group (group I). Also, a significant increase \((P<0.05)\) in lipid peroxidation is shown in this group.

Group IV, which was treated with 2,4-D, also shows a highly significant increase in the activity of acyl CoA oxidase as compared with control group (group I).

A highly significant increase in the activity of acyl CoA oxidase, nearly three times that of the control group is also shown in the 2,4,5-T–treated group (group V). This group also shows a highly significant increase \((P<0.01)\) in D-amino acid oxidase activity as compared with control group.

**DISCUSSION**

Rodent bioassays establish that some of the common drugs and persistent environmental pollutants are hepatocarcinogens, and with chronic exposure, they lead to hepatocellular carcinoma (HCC) development. Their hepatotoxicity and carcinogenicity appear to be related directly to the extent of their oxidative metabolism, which is primarily catalyzed by liver cytochrome P450 enzymes and yields multiple reactive and toxic metabolites. At least some of these active metabolites may achieve their deleterious effects via a mechanism that involves peroxisome proliferation.\(^2\-6\)

Peroxisome proliferation is a trophic phenomenon in the liver, originally described after administration of the hypolipidemic drug clofibrate to rodents.\(^2\) This proliferative response is characterized in the short term by a dramatic increase in both the size and number of peroxisomes. Peroxisomes are the principal sites of metabolism of long-chain and very long-chain fatty acids so their proliferation is associated with up-regulation of peroxisomal fatty acid \(\beta\)-oxidation enzymes and microsomal P4504A fatty acid hydroxylase enzymes, as well as increased cell differentiation and liver weight gain. Long-term exposure to peroxisome proliferators leads to HCC.\(^16,23\)

A broad spectrum of structurally diverse compounds, including certain hypolipidemic drugs, herbicides, industrial solvents, and the adrenal steroid dehydroepiandrosterone (DHEA), has been shown to induce peroxisome proliferation.\(^2,4,14,24\) These peroxisome proliferators stimulate liver growth and tumor formation by a nongenotoxic mechanism, i.e., one that does not involve DNA damage caused by the peroxisome proliferators or their metabolites.\(^2,4\)

In this study, data are presented on the levels of peroxisomal enzymes and a fatty acid oxidation predictor, malondialdehyde, in the liver tissue of rats that were exposed to well-known peroxisome proliferators, the hypolipidemic drug, NAF, the perfluorinated hydrocarbon, PFOA, and the herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).

Acyl CoA oxidase, the first enzyme

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**Table 2. Malondialdehyde µM/mg Protein ± SEM**

<table>
<thead>
<tr>
<th>Group</th>
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<tr>
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<td>PFOA (0.02%)</td>
<td>4.2 ± 0.33*</td>
</tr>
<tr>
<td>IV</td>
<td>2,4-D (0.05%)</td>
<td>2.8 ± 0.19</td>
</tr>
<tr>
<td>V</td>
<td>2,4,5-T(0.05%)</td>
<td>3.4 ± 0.21</td>
</tr>
</tbody>
</table>

*Significant difference from basal diet.
of peroxisomal oxidation system, together with the malondialdehyde levels in rats’ liver tissues were shown to be significantly elevated by the chemicals used in this study. This is indicative of proliferation of peroxisomes with upregulation of peroxisomal [·] -oxidation of fatty acids. Many investigators have reported the same findings. With respect to the agent that produced maximum effects, we found that NAF followed by PFOA are the most potent among the tested chemicals.

Peroxisome proliferator-activated receptors (PPAR), a ligand-activated transcription factor and a member of the steroid receptor superfamily, has been shown to be activated by diverse peroxisome proliferators and can thus mediate their peroxisome proliferative effects. So stimulation of PPARs by our chemicals may be the cause of this marked increase of acyl CoA oxidase activity and lipid peroxidation. In addition, Zhou and Waxman found that activation of PPAR induces a dramatic proliferation of peroxisomes in rodent hepatocytes and ultimately leads to HCC.

The other measured enzymes, D-amino acid oxidase and glycolate oxidase, did not show the same trend except for D-amino acid oxidase in the 2,4,5-T-treated group. These enzymes may need a more prolonged exposure for their behavior to be manifested. So the marked effects of the tested chemicals are the induction of peroxisomes proliferation and up regulation of peroxisomal β-oxidation of fatty acids. This may result in production of large amount of H2O2 which may in turn initiate carcinogenesis via oxidative DNA damage. Although some caveats have been raised, the general consensus is the significant role of oxidative stress in peroxisome proliferators-induced hepatocarcinogenesis. The significance and contribution of high levels of H2O2 generating fatty acid oxidation enzymes in HCC and growth of these tumors is not clear. It is unlikely that these enzymes cause further increases in oxidative stress because HCC contains high levels of the antioxidant enzymes superoxide dismutase and catalase that degrade free radicals. The increase in antioxidant enzymes in HCC indicates adaptive changes in the tumor to increasing levels of free radicals. Fibroblasts exposed to high concentrations of H2O2 showed amplification of the catalase gene, an adaptive process to prevent oxidative stress.

At the same time, peroxisome proliferators may act as tumor promoters by stimulating DNA replication. In addition to the previous possibilities, it has proven that peroxisome proliferators may act to inhibit the normal process of apoptosis in liver.

Although it is not yet clear whether peroxisome proliferators are also carcinogenic in humans, some epidemiological studies have reported the development of cancer in some patients who received long-term treatment with fibrates. Such studies may become quite important in light of the fact that certain groups in the population are exposed to relatively high levels of peroxisome proliferators, including farmers using certain herbicides and pesticides, workers in the chemical industry who are exposed to PFOA and phthalates, and patients with heart problems who receive hypolipidemic drugs. Therefore, peroxisome proliferators need to be investigated with regard to their mechanism of carcinogenesis in rodents and their effects on humans.

REFERENCES


