Role of Oxidative Stress in Age Dependent Hepatocarcinogenesis by the Peroxisome Proliferator Nafenopin in the Rat

Wolfgang Huber, Bettina Kraupp-Grasl, Hermann Esterbauer, and Rolf Schulte-Hermann

Institut für Tumorbiologie und Krebsforschung der Universität Wien, Borschkegasse 84, A-1090 Vienna [W. H.], and Institut für Biochemie der Universität Graz, Schubertstrasse 1, A-8010 Graz [H. E.], Austria

ABSTRACT

Recently old rats were found to be much more susceptible than young rats to the hepatocarcinogenic effect of a 55-59-week treatment with the peroxisome proliferator nafenopin (NAF) (B. Kraupp-Grasl, W. Huber, H. Taper, and R. Schulte-Hermann, Cancer Res., 51: 666–671, 1991). In the present study indicators of oxidative stress were measured in the livers of the same animals (male Wistar). NAF enhanced peroxisomal β-oxidation 10-12-fold and reduced glutathione peroxidase activity by 40–50%. Indicators of lipid peroxidation like thiobarbituric acid reactive substances and malondialdehyde were both decreased by one-third and two-thirds, respectively. Of the oxidation-sensitive polysaturated fatty acids linoleic acid and docosahexaenoic acid were decreased by 40% and two-thirds, respectively, but the particularly sensitive arachidonic acid remained unchanged. Taken together these data suggest that NAF did not significantly enhance lipid peroxidation in the present experiment. All NAF effects were of the same magnitude in the old and young animals. Therefore, the considerably stronger induction of hepatocarcinoma by NAF in the old animals was not associated with evidence of enhanced oxidative stress. These findings are consistent with the hypothesis that NAF acts hepatocarcinogenically by promotion of tumor development from preneoplastic lesions occurring spontaneously with age.

INTRODUCTION

A number of peroxisome proliferators have been found to induce liver cell carcinoma in lifetime animal bioassays (1–6). These chemically heterogeneous compounds include environmental pollutants (2, 5–8), hypolipidemic (1–3, 5, 9), uricosuric (10), and analgesic drugs (2, 5). Thus far, no convincing evidence for genotoxicity or tumor initiating capacity has been presented from enhanced generation and/or reduced inactivation of active oxygen species in the old animals. Therefore, in the present study we tested for oxidative stress in livers from the same old and young animals used in the previous carcinogenicity study (23). The following parameters were selected: peroxisomal β-oxidation as possible initial event; GSH peroxidase as important antioxidant; and TBARS and MDA as indicators of lipid peroxidation. Moreover, fatty acid patterns were determined because of reports that lipid peroxidation reduced the content of PUFA's in vitro (24–27) and in vivo (28–33).

The results obtained suggest that the enhanced susceptibility of old rats to the hepatocarcinogenic action of nafenopin is not associated with evidence of enhanced oxidative damage in these animals.

MATERIALS AND METHODS

Materials and Treatment. Male Wistar rats of different age were obtained from Kleintierfarm Madörin, Fullündorf, Switzerland. They were partly treated with nafenopin at the dose of 100 mg/kg body weight in their diets for 55–59 weeks. The young animals were 13 weeks of age at the start of treatment, the old ones were 57 weeks. Treatment was equal for both ages. A more detailed description is given by Kraupp-Grasl et al. (23).

There were four treatment groups: group 1, old nafenopin-treated; group 2, old controls; group 3, young nafenopin-treated; group 4, young controls. At the end of the experiment the animals were killed by carbon dioxide inhalation and subsequent decapitation. Livers were examined morphologically for the presence of neoplastic lesions or tumors (results presented in Ref. 23). Samples taken from these livers were frozen at −30°C before being analyzed biochemically.

Chemicals. NAF was a kind gift of Ciba-Geigy (Basel, Switzerland); CoA, palmitoyl-CoA, FAD and NADPH were purchased from Boehringer Mannheim (Mannheim, Germany). The dye reagent for the protein determination was a standard mixture from Bio-Rad (München, Germany). Essentially fatty acid free bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO). All the other chemicals used were of the highest purity available, mostly from Merck (Darmstadt, Germany).

Biochemical Studies. All the assays described below were carried out in whole liver homogenate prepared with a motor driven Ultra-Turrax (Janke and Kunkel, Germany) at full speed. The samples were kept on ice or were refrigerated during preparation.

Protein Determination. Protein concentration was measured according to the method of Bradford and Bio-Rad with bovine serum albumin as the standard (34). The protein concentration was always determined in the final dilution from which the material for the corresponding assay was also taken.

Peroxisomal β-Oxidation. Peroxisomal β-oxidation was determined spectrophotometrically according to the method described by Lazarow (35) with minor modifications such as the addition of nicotinamide (2 mm). Tissue samples were homogenized and further diluted to the appropriate concentrations in 0.25 M sucrose. Three concentrations of every homogenate were assayed, each of them twice. The percentage of liver in the assay was between 0.123 and 0.029% in the untreated and between 0.029 and 0.005% in the treated animals. A Hamilton syringe was used for the application of palmitoyl-CoA. The results are expressed as nmol NADH formed/min/mg of protein.
expressed in nmol NADPH consumed/min/mg protein.

concentrations of every homogenate were assayed twice. The percentage
Germany). Measurement was done at 254 nm. Acetonitrile (70%) was
acetonitrile (1:1, v/v) was carried out as described. In addition the
chromatography according to the method described by Esterbauer et
al. (37), using an amino phase column (Lichrocart 250-4; Merck,
Germany). Malondialdehyde was determined by high performance liquid
chromatography showed effects of age and NAF treatment
similar to those of the thiobarbituric acid reaction. In both age
groups values declined by approximately two-thirds after NAF
administration was increased approximately 10-12-fold by NAF treat
Dixon (40) was carried out. Six of 132 values (4.6%) were eliminated
with a confidence limit of 0.95.

RESULTS

Peroxisomal β-Oxidation. The activity of peroxisomal β-oxi
dation was increased approximately 10-12-fold by NAF treat
ment. This increase was observed in both age groups and was
slightly more pronounced in the young. In NAF treated animals no
age difference in activity was found. Detailed data are given in
Table 1.

GSH Peroxidase. The activity of GSH peroxidase decreased
by approximately 40-50% after NAF treatment. The decrease
was somewhat more pronounced in the old than in the young
animals (decrease, 51 versus 39%). No difference was detected
between the activities in old and young animals. Detailed data are
given in Table 2.

TBARS. A decrease in TBARS by approximately one-third
as compared to controls was found after treatment with NAF.
These findings are statistically significant in both age groups
and the decrease was somewhat more pronounced in the young
animals than in the old (decrease, 47 versus 28%). In NAF
treated animals there was no age difference. Detailed data are
given in Table 3.

MDA. Determination of MDA by high performance liquid
chromatography showed effects of age and NAF treatment similar
to those of the thiobarbituric acid reaction. In both age
groups values declined by approximately two-thirds after NAF
treatment. Again, age differences were absent in the treated rats
(Table 4).

Generally, only a relatively small part of the TBARS was
present as MDA. In the untreated animals this fraction was
17% (old) and 14% (young); after NAF treatment it was 9% in
both age groups. This finding does not support the opinion
present as MDA. In the untreated animals this fraction was
similar to those of the thiobarbituric acid reaction. In both age
groups values declined by approximately two-thirds after NAF
treatment. Again, age differences were absent in the treated rats
(Table 4).

TABLE 1

<table>
<thead>
<tr>
<th>Old</th>
<th>Untreated</th>
<th>NAF</th>
<th>Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>6.80 ± 0.89</td>
<td>71.65 ± 18.86</td>
<td>10.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N*</td>
<td>8</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>5.79 ± 0.62</td>
<td>74.33 ± 12.44</td>
<td>12.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N*</td>
<td>7</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio (old/young)</td>
<td>1.17</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N, number of animals; NS, not significant.

Table 2 GSH peroxidase activity (μmol NADPH/min/mg protein)

<table>
<thead>
<tr>
<th>Old</th>
<th>Untreated</th>
<th>NAF</th>
<th>Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>0.757 ± 0.234</td>
<td>0.369 ± 0.030</td>
<td>0.49</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N*</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>0.617 ± 0.186</td>
<td>0.377 ± 0.081</td>
<td>0.61</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>N*</td>
<td>7</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio (old/young)</td>
<td>1.23</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N, number of animals; NS, not significant.

Table 3 Content of TBARS (nmol/mg protein)

<table>
<thead>
<tr>
<th>Old</th>
<th>Untreated</th>
<th>NAF</th>
<th>Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>0.883 ± 0.073</td>
<td>0.633 ± 0.127</td>
<td>0.72</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N*</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>1.189 ± 0.263</td>
<td>0.634 ± 0.099</td>
<td>0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N*</td>
<td>9</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio (old/young)</td>
<td>0.74</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N, number of animals; NS, not significant.

Table 4 Content of malondialdehyde (pmol/mg protein)

<table>
<thead>
<tr>
<th>Old</th>
<th>Untreated</th>
<th>NAF</th>
<th>Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>152.1 ± 24.1</td>
<td>58.1 ± 25.1</td>
<td>0.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N*</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>168.1 ± 19.1</td>
<td>60.1 ± 33.1</td>
<td>0.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N*</td>
<td>8</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio (old/young)</td>
<td>0.91</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N, number of animals; NS, not significant.
OXIDATIVE STRESS, AGE, AND HEPATOCARCINOGENESIS BY NAFENOPIN

Table 5 Fatty acid content (molar percentage of total fatty acids identified)

<table>
<thead>
<tr>
<th>N*</th>
<th>C16</th>
<th>C18</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C20:4</th>
<th>C22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old NAF-treated</td>
<td>6</td>
<td>29.60 ± 1.05*</td>
<td>20.77 ± 1.62</td>
<td>9.51 ± 1.31</td>
<td>21.40 ± 1.39</td>
<td>1.52 ± 0.55</td>
</tr>
<tr>
<td>Untreated</td>
<td>5</td>
<td>29.69 ± 2.06</td>
<td>11.49 ± 2.13</td>
<td>15.61 ± 0.45</td>
<td>20.93 ± 0.56</td>
<td>4.99 ± 0.45</td>
</tr>
<tr>
<td>Young NAF-treated</td>
<td>5</td>
<td>29.30 ± 1.14</td>
<td>19.41 ± 1.69</td>
<td>8.96 ± 0.45</td>
<td>22.87 ± 1.78</td>
<td>1.55 ± 0.60</td>
</tr>
<tr>
<td>Untreated</td>
<td>3</td>
<td>34.52 ± 5.56</td>
<td>7.20 ± 2.56</td>
<td>15.67 ± 0.63</td>
<td>22.42 ± 2.98</td>
<td>4.12 ± 2.43</td>
</tr>
</tbody>
</table>

* N, number of animals. ** Mean ± SD.

Table 6 Fatty acid content: ratios and statistical evaluation

<table>
<thead>
<tr>
<th>Ratios</th>
<th>C16</th>
<th>C18</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C20:4</th>
<th>C22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAF/untreated Old animals</td>
<td>1.00</td>
<td>1.00</td>
<td>1.81</td>
<td>0.61</td>
<td>1.02</td>
<td>0.30</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Young animals</td>
<td>0.85</td>
<td>1.12</td>
<td>2.70</td>
<td>0.57</td>
<td>1.02</td>
<td>0.38</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Old/young</td>
<td>NAF-treated</td>
<td>1.01</td>
<td>0.96</td>
<td>1.07</td>
<td>1.06</td>
<td>0.94</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.86</td>
<td>1.08</td>
<td>1.59</td>
<td>1.00</td>
<td>0.93</td>
<td>1.21</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS, not significant.

Fatty Acid Content. NAF did not change the total content of fatty acids in liver homogenate. However, changes were observed in the content of individual fatty acids. C18:1 was increased by 80–170%, and C18:2 was reduced by approximately 40%, both changes being slightly more pronounced in the young. C22:6 was decreased by approximately two-thirds in both age groups. The latter reduction was significant only in the old group because of high standard deviations and low mean values (between 1.5 and 5 mol%). No NAF related changes were observed in the content of C16, C18 and C20:4. The comparison between fatty acid contents in young and old animals revealed no statistically significant differences after NAF treatment. The same is true for the untreated animals with the exception of C18:1 where the content is approximately 60% higher in the old. Detailed data are given in Table 5; the statistical evaluation is given in Table 6.

DISCUSSION

NAF increased peroxisomal β-oxidation and decreased GSH peroxidase activity which is consistent with results on various peroxisome proliferators obtained by other authors (15–20). TBARS and MDA both were reduced after NAF treatment. Whether or not this reduction is a specific effect of NAF or results from the experimental conditions remains to be elucidated; in any event these findings do not support the working hypothesis that liver carcinogenesis in our experiment was associated with enhanced lipid peroxidation. Likewise, previous studies on oxidative injury after various peroxisome proliferators have not shown uniform increases. TBARS or MDA were only slightly increased or even unchanged (14, 20), lipofuscin contents were increased (16, 19, 45), and the in vitro formation of OH radicals was stimulated (46), but decreases were observed in conjugated dienes after short treatment periods (15, 22, 47). These decreases were reversed to normal or even to increases after approximately 50 days of treatment (16, 48).

As a further potential marker of oxidative stress we studied PUFA levels. Pronounced PUFA decreases were found in previous studies in several in vivo models where lipid peroxidation occurs. These models involve, e.g., liver (28–31), kidney (32), and adrenal gland (33). After NAF treatment we found a decrease in C18:2 and C22:6 but no change in C20:4. Since C20:4 has been shown to be particularly sensitive to peroxidation in vivo (28–33) it seems possible that the decreases in C18:2 and C22:6 are due not to peroxidation but to other NAF effects. Indeed, C18:2 and C22:6 were found to be much better substrates for peroxisomal β-oxidation than C20:4 (49, 50). Moreover, it has been suggested that an induction of desaturases and elongases by peroxisome proliferators might lead not only to the observed increase in C18:1 but also to enhanced metabolism of C18:2 (51–53). Therefore, the PUFA data do not support the hypothesis of increased lipid peroxidation under the conditions of our study. It may be added that the present work does not rule out the possibility of more subtle peroxidation-related NAF effects such as altered permeability of nuclear membranes to radicals, oxidative damage of DNA, superoxide dismutase levels, or others.

Most remarkably no relevant differences between NAF effects in old and young animals were observed. This finding strongly contrasts with the high tumor yield in old but not in young rats following identical treatments with NAF. In fact none of the biochemical parameters studied exerts a response correlating with the divergent tumorigenic effects in the two age groups.

In addition we have studied rates of cell proliferation in the same livers from young and old rats because it has been argued that enhanced lipid peroxidation would increase cell death and thereby the rates of cell replication. The results showed that cell proliferation rates were not enhanced after prolonged NAF treatment, and no age difference was observed in either normal tissue or preneoplastic foci.5

It might be argued that the enhanced tumor response in old rats might have been caused by a higher accumulation of NAF in old age. Although hepatic levels of the agent were not determined, the NAF effects on the biochemical parameters shown in this paper and on liver size and DNA5 were all of a similar extent in both age groups which suggests an absence of major concentration differences.

In summary our findings do not support the hypothesis that enhanced oxidative stress was responsible for the high susceptibility of old rats to the hepatocarcinogenic effects of NAF. However, a major surplus in old rats has been observed in the same livers from young and old rats because it has been argued that enhanced lipid peroxidation would increase cell death and thereby the rates of cell replication. The results showed that cell proliferation rates were not enhanced after prolonged NAF treatment, and no age difference was observed in either normal tissue or preneoplastic foci.

5 B. Kraupp-Grasl et al., manuscript in preparation.
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REFERENCES

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