Effect of Feeding Clofibrate-Containing Diet on the Hepatic NAD$^+$ Level in Rats

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(Received December 27, 1994)

Summary Feeding rats with a diet containing 0.25% clofibrate for 2 weeks elevated the hepatic NAD$^+$ and total nicotinate levels significantly. Other peroxisome proliferators, such as 2-(4-chlorophenoxy)propionic acid and di(2-ethylhexyl)phthalate, had similar effects. When rats were fed the control diet without clofibrate for 1 week after 2 weeks of the clofibrate diet, the hepatic NAD$^+$ level returned to the control value. Muscular NAD$^+$ content was not affected by the peroxisome proliferators. The results were discussed in relation to induction of peroxisomal β-oxidation enzymes by the peroxisome proliferators.

Key Words: rat, clofibrate, peroxisome proliferators, hepatic NAD$^+$ content, hepatic total nicotinate content, muscular NAD$^+$ content

Clofibrate[2-(4-chlorophenoxy)-2-methylpropionic acid ethyl ester] is a hypolipidemic drug and is also known as a peroxisome proliferator (1). From the findings that peroxisomal acyl-CoA oxidation is strongly stimulated by added NAD$^+$ and that pyridine nucleotides could not be found in highly purified peroxisomes, it is supposed that NAD$^+$ can cross the peroxisomal membrane and that peroxisomes do not possess their own pyridine nucleotide pool (2).

Peroxisome proliferators are known to raise the CoA concentration in the liver and the CoA increase was proved to be due to an increase in CoA synthesis (3). It is, therefore, interesting to see how clofibrate affects the levels of NAD$^+$, another cofactor necessary for peroxisomal fatty acid oxidation, in the liver of rats. As clofibrate was reported to develop myotonia among the treated patients (4, 5), NAD$^+$ and total nicotinate levels were measured not only in the liver but also in the muscle of the rats administered the clofibrate-containing diets.

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Abbreviations: Clofibrate: 2-(4-chlorophenoxy)-2-methylpropionic acid ethyl ester, CPP: DL-2-(4-chlorophenoxy)propionic acid, DEHP: Di(2-ethylhexyl)phthalate
MATERIALS AND METHODS

Chemicals. Clofibrate and DEHP were obtained from Wako Pure Chemical Industries, Ltd., Japan and CPP from Aldrich.

Animals. Male Sprague-Dawley rats (specific pathogen-free; 7 week-old, 210–230 g) were obtained from Japan SLC, Inc. They were housed in individual, suspended, wire-mesh cages at 23°C in a room with a 12-h light-dark cycle. The diets and water were given freely.

Diets. Clofibrate was diluted with ethanol and mixed with the powdered chow pellets (MF diet; Oriental Yeast Co., Japan) to make a final concentration of clofibrate of 0.25% (w/w) and the ethanol was allowed to evaporate. Animals were fed the powdered chow pellets for a couple of days prior to the clofibrate diet in order to allow them to adjust to the powdery diets.

Treatment of animals. Three separate experiments were conducted. In the first experiment, 2 groups of rats (7 rats for the control group and 8 rats for the clofibrate group) were fed the powdered MF diet with or without 0.25% clofibrate for 2 weeks. In the second experiment, 3 groups of rats (6 rats for the control group, 5 rats for the CPP group and 5 rats for the DEHP group) were fed the powdered MF diet or the powdered MF diet containing 0.5% CPP or 2% DEHP for 2 weeks. In the first and second experiments, NAD+ content in the liver and the muscle and total nicotinate content in the liver were determined at the end of the feeding experiments. In the third experiment, 3 groups of rats (4 rats for each group) were used. One group was fed the powdered MF diet and the other 2 groups were fed the powdered MF diet containing 0.25% clofibrate for 2 weeks. One of the clofibrate group of rats were returned to the control MF diet after 2 weeks and maintained on the diet for 1 more week. At the end of the feeding experiment, the NAD+ and DNA contents of the liver were measured.

Sample preparation and assay methods: After decapitation, the liver and the gastrocnemius muscle were quickly excised and frozen. NAD+ and total nicotinate were extracted from the frozen tissue powder with 0.6N HClO4. NAD+ was determined according to the method of Klingenberg (6) and total nicotinate content of the liver was determined microbiologically using Lactobacillus plantarum (ATCC 8014) as a test organism (7). DNA content of the liver was determined after Burton (8).

Protein concentrations were determined by the method of Lowry et al (9) with bovine serum albumin as a standard.

Statistical analysis: The statistical significance of differences between mean values was assessed by one-way analysis of variance (one-way ANOVA) with a subsequent two-tailed unpaired t-test. Differences were considered significant when p < 0.05.
RESULTS

The amount of clofibrate and the period of its administration are the same as those recommended for peroxisome proliferation and induction of enzymes and proteins involved in the metabolism of fatty acids (I). The results of the first experiment are shown in Table 1. No differences in body weight gain and diet intake were observed between the control and the clofibrate-fed groups during the experimental period of 2 weeks. Hepatic NAD$^+$ and total nicotinate contents per gram of wet tissue were increased significantly, while muscular NAD$^+$ content was not different in the two groups. Though only NAD$^+$ was measured among pyridine nucleotides, the fact that total nicotinate was significantly increased in the liver of the clofibrate-fed animals suggests the possibility of increases in other types of pyridine nucleotides. This is because L. plantarum used as a test organism can grow not only on nicotinate and NAD$^+$ but also on nicotinamide and other pyridine nucleotides as well.

Clofibrate is a known peroxisome proliferator. To find out whether the increase in hepatic NAD$^+$ found in the rats treated with clofibrate was specific for this drug, the second experiment was performed with other peroxisome proliferating chemicals such as dl-2-(4-chlorophenoxy)propionic acid (CPP), the chemical structure of which is analogous to that of clofibrate and di(2-ethylhexyl)phthalate (DEHP), a plasticizer for the production of plastics. The results of the second experiment are shown in Table 2. Hepatic NAD$^+$ and total nicotinate contents per gram of wet tissue increased significantly in the rats fed the CPP- and DEHP-containing diets but to a lesser extent compared with the values in the rats fed the clofibrate-containing diet (Table 1). Muscular NAD$^+$ was not affected by CPP or DEHP. CPP is reported to lower serum cholesterol but to have no effect on serum triglycerides (I0). DEHP is known to increase both peroxisomal and mitochondrial $\beta$-oxidation due to the increase of de novo synthesis of the individual enzymes of these pathways (I1). NAD$^+$ content in rat liver is known to remain unchanged

| Table 1. Effects of 0.25% clofibrate in diet on tissue NAD$^+$ and total nicotinate levels. |
|---------------------------------------------------------------|-------------|-------------|
| Body weight gain (g/2 weeks) | 44.9±9.4    | 50.0±8.2    |
| Diet consumed (g/2 weeks) | 255±9.7     | 259±16.7    |
| NAD$^+$ (nmol/g wet tissue) |
| liver | 571±22 | 1,090±87*  |
| muscle | 421±46 | 458±30     |
| Total nicotinate in liver (nmol/g wet tissue) | 871±61 | 1,610±220* |

Numbers in parentheses show the number of animals. Values are expressed as M±SD. *Significantly different from the control value at the 0.1% level by Student's t-test.
Table 2. Effects of other peroxisome-proliferating agents on tissue NAD\(^+\) and total nicotinate levels.

<table>
<thead>
<tr>
<th></th>
<th>Control (6)</th>
<th>0.5% CPP (5)</th>
<th>2% DEHP (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g/2 weeks)</td>
<td>62.7±15.1</td>
<td>64.0±17.9</td>
<td>70.0±8.4</td>
</tr>
<tr>
<td>Diet consumed (g/2 weeks)</td>
<td>271±6.2</td>
<td>275±10.4</td>
<td>276±5.8</td>
</tr>
<tr>
<td>NAD(^+) (nmol/g wet tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td>553±14</td>
<td>900±43*</td>
<td>929±39*</td>
</tr>
<tr>
<td>muscle</td>
<td>506±26</td>
<td>539±37</td>
<td>513±18</td>
</tr>
<tr>
<td>Total nicotinate in liver (nmol/g wet tissue)</td>
<td>782±53</td>
<td>1,020±43*</td>
<td>990±40*</td>
</tr>
</tbody>
</table>

Numbers in parentheses show the number of animals. Values are expressed as M±SD. *Significantly different from the control values at the 0.1% level by Student's t-test.

Table 3. Effect of changing feeding schedules on hepatic NAD\(^+\) level

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control (4)</th>
<th>Clofibrate (4)</th>
<th>Clofibrate-control (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver NAD(^+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/g liver)</td>
<td>614±59</td>
<td>895±70*</td>
<td>602±55</td>
</tr>
<tr>
<td>(nmol/mg DNA)</td>
<td>621±126</td>
<td>1,393±120*</td>
<td>532±69</td>
</tr>
</tbody>
</table>

Numbers in parentheses show the number of animals. In the control or clofibrate diet group, rats were fed MF diet or MF diet containing 0.25% clofibrate for 2 weeks. In the clofibrate-control diet group, rats were fed MF diet containing 0.25% clofibrate for 2 weeks and then MF diet for 1 more week. Values are expressed as M±SD. *Significantly different from the control values at the 0.1% level, by Student's t-test.

even in the physiological conditions such as fasting and diabetes (12), which suggests the existence of a mechanism to maintain the NAD\(^+\) level within fairly narrow range. It is, therefore, interesting that the three hypolipidemic and peroxisome-proliferating agents tested showed a similar hepatic NAD\(^+\)-increasing effect, though CPP and DEHP were somewhat less effective than clofibrate.

In the third experiment, whether an increase in the hepatic NAD\(^+\) observed after feeding the clofibrate-diet could be reversed by eliminating clofibrate from the diet was investigated. Clofibrate causes an increase in the liver weight because of hyperplasia and hypertrophy (13). The results of the third experiment were, therefore, expressed per wet weight and per mg DNA. As shown in Table 3, the hepatic NAD\(^+\) of the rats which were fed the MF diet containing 0.25% clofibrate for 2 weeks and then the MF diet for 1 more week was not significantly different from that of the control rats when expressed either per gram of tissue or per milligram of DNA.

DISCUSSION

In this report, we showed that administration of clofibrate and other peroxi-
some proliferators to rats increased the concentration of the hepatic NAD$^+$. The liver plays an important role in the so-called pyridine nucleotide cycle, which provides other organs with a constant supply with nicotinamide as a precursor for NAD$^+$ synthesis ($14,15$).

The NAD$^+$ level in the liver is maintained within a fairly narrow range unless an unphysiological amount of nicotinic acid or nicotinamide is given ($16$). Therefore, it is interesting that clofibrate and other peroxisome proliferators induced an increase in the concentration of liver NAD$^+$.

Peroxisome proliferators are the structurally diverse chemicals which cause a marked increase in the number of peroxisomes in the liver and increase the levels of mRNAs encoding enzymes of peroxisomal $\beta$-oxidation and microsomal $\omega$-oxidation ($13$). Peroxisome proliferators exert their effects on lipid metabolism ($13$). The elevation of NAD$^+$ content in the liver as a whole after feeding a clofibrate-containing diet is favorable for lipid metabolism, especially $\beta$-oxidation of long-chain fatty acids in hepatic peroxisomes since they do not have their own NAD$^+$ pool ($2$).

Hashimoto showed that the quantities of peroxisomal $\beta$-oxidation enzymes increased about 25- to 50-fold by feeding a diet with 2% DEHP for 2 weeks but rapidly returned to the levels of the control animals 1 week after withdrawal of DEHP from the diet ($17$). In this study, we indicated that the concentration of hepatic NAD$^+$, which is closely related to lipid metabolism, was raised by feeding the rats peroxisome proliferators (Tables 1 and 2), and returned to the control level 1 week after removing clofibrate from the diet (Table 3). Shindo et al found that the CoA content increased 5-fold in the liver of rats which received a diet containing 2% DEHP for 2 weeks but the CoA content of other tissues was not changed by DEHP treatment ($18$). A mechanism for clofibrate activation of gene expression of acyl-CoA oxidase, a rate-limiting enzyme in peroxisomal fatty acid metabolism, has been demonstrated ($19$). Both the elevation of hepatic NAD$^+$ content found in this study and that of CoA found by Shindo et al. ($18$) are supposed to contribute to acceleration of long-chain fatty acid oxidation in the liver after treatment with the peroxisome proliferators.

The concentration of the muscular NAD$^+$ was not decreased by the peroxisome proliferators (Tables 1 and 2) and there seems to be no relationship between myotonia caused by clofibrate administration and the muscular NAD$^+$ level.

Considering the diverse biochemical functions of NAD$^+$ such as a substrate for protein ADP-ribosylation ($20$) and a precursor of an intracellular signal substance ($21$), how increased hepatic NAD$^+$ is related to the pharmacological effects of this drug or its side effects is interesting and worth investigating.

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