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SHORT COMMUNICATION

Pyridine Nucleotide Levels in Liver of Rats Fed Clofibrate- or Pyrazinamide-Containing Diets

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ABSTRACT. Hepatic NAD^+, NADH, and NADPH were increased significantly 3 days after feeding rats with a 0.25% clofibrate diet, increased further after 8 days, and stayed at the same levels 14 days after feeding the diet. The NAD^+/NADH ratio was decreased significantly by feeding the clofibrate diet for 8 days, while the ratio remained unchanged with a 1% pyrazinamide diet. Hepatic quinolinate phosphoribosyltransferase (QAPRTase) (EC 2.4.2.19) activity was increased to 1.8 and 1.3 times that of the control animals in the clofibrate- and the pyrazinamide-fed rats, respectively, while hepatic aminocarboxymuconate-semialdehyde decarboxylase (ACMSDase) (EC 4.1.1.45) activity was decreased to 0 and 19% of that of the control animals. The heat-treated liver homogenate from the pyrazinamide-fed rats contained inhibitory activity toward ACMSDase, while no inhibitory activity was found in the liver homogenate of the clofibrate-fed animals. We conclude that these changes of enzyme activities, which seem due to different mechanisms, may contribute to the increase of pyridine nucleotides in the liver of rats fed clofibrate or pyrazinamide. BIOCHEM PHARMACOL **55**;3:367–371, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. clofibrate- or pyrazinamide-feeding; hepatic pyridine nucleotides; NAD^+/NADH ratio; peroxisomal fatty acid oxidation; quinolinate phosphoribosyltransferase; aminocarboxymuconate-semialdehyde decarboxylase

We previously reported that clofibrate, an anti-hyperlipidemic drug, elevates hepatic NAD^+ in rats [1], and that hepatocytes isolated from clofibrate-fed rats show increased NAD^+ biosynthesis from tryptophan [2]. Nasu *et al.* [3] found that orally or intraperitoneally administered pyrazinamide, an anti-tubercular drug, causes a marked increase in the NAD^+ content in rat liver. This increase in NAD^+ was brought about by a metabolite of pyrazinamide, which happens to be an inhibitor of ACMSDase† [3]. ACMSDase degrades aminocarboxymuconate-semialdehyde, an important intermediate from tryptophan to NAD^+, leading to the glutarate pathway. Inhibition of the metabolic flux of tryptophan via the glutarate pathway by pyrazinamide was confirmed by Cook and Pogson [4] using isolated rat liver cells.

In this paper, to elucidate the mechanism of hepatic NAD^+ increase in rats fed a clofibrate diet, we compared the effect of clofibrate with that of pyrazinamide on pyridine nucleotides in the liver and on the activities of regulatory enzymes in NAD^+ biosynthetic pathways.

MATERIALS AND METHODS

Materials

Clofibrate [2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester] was obtained from Wako Pure Chemical Industries, Ltd. Pyrazinamide was purchased from Nacalai Tesque, Inc. Glutamate dehydrogenase (EC 1.4.1.3) for the oxidation of extracted reduced pyridine nucleotides, alcohol dehydrogenase (EC 1.1.1.1) for NAD^+ assay, and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) for $NADP^+$ assay were obtained from Boehringer–Mannheim.

Animals and Diets

Sprague–Dawley rats (specific pathogen-free; 7-weeks-old) were obtained from Japan SLC, Inc. The 0.25% (w/w) clofibrate diet was prepared as described previously [1]. Pyrazinamide, micropowdered in a mortar, was uniformly mixed with powdered MF diet [1] to make a final concentration of 0.13% or 1% (w/w). Diet and water were given freely.

Assay Methods for Pyridine Nucleotides

Eight groups of male rats, each containing 3 rats, were fed a control diet and a 0.25% clofibrate diet for 3, 8, or 14 days and a 0.13% or 1% pyrazinamide diet for 3 and 8 days. A group of 3 male rats were fed a 0.25% clofibrate diet for 12 days and then were switched to a control diet for 2 days.

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† *Abbreviations:* ACMSDase, aminocarboxymuconate-semialdehyde decarboxylase; and QAPRTase, quinolinate phosphoribosyltransferase.

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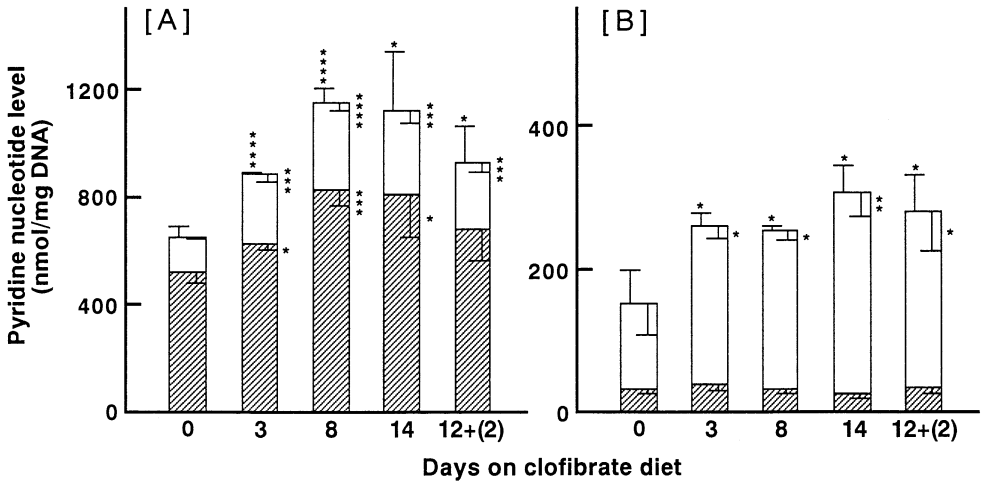


FIG. 1. Pyridine nucleotide levels in the liver of rats fed a 0.25% clofibrate diet. Rats were fed a clofibrate diet as described in "Materials and Methods." Values are means ± SD of three animals. Values marked by an asterisk(s) were significantly different from 0 day values, as determined by Student's *t*-test: (****) $P < 0.001$, (***) $P < 0.005$, (**) $P < 0.01$, and (*) $P < 0.05$. An error bar on the right is for individual pyridine nucleotide (▨, oxidized; □, reduced), while that on the left is for NAD^+(H) in [A] or $NADP^+$(H) in [B]. The number in the parentheses on the horizontal scale indicates the number of days on the control diet.

Four more groups of male or female rats, each containing 3 or 4 rats, were fed a control diet and a 0.25% clofibrate diet for 8 days.

All animals were decapitated; the liver and kidneys were excised immediately and quick-frozen with dry-ice blocks. Pyridine nucleotides were extracted and determined according to Klingenberg [5].

Enzyme Assays

Samples of liver and/or kidneys were homogenized in 3 vol. of ice-cold 0.14 M KCl or 0.25 M sucrose containing 0.1% ethanol in a Potter–Elvehjem-type homogenizer fitted with a Teflon pestle. The homogenate with 0.14 M KCl for assays of QAPRTase, ACMSDase, and ACMSDase inhibitor was centrifuged at 105,000 × *g* for 60 min. For the QAPRTase assay, the supernatant was treated with charcoal. The homogenate with 0.25 M sucrose for the catalase assay was centrifuged at 200 × *g* for 10 min.

QAPRTase and ACMSDase activities were assayed according to Nishizuka and Nakamura [6] and Mehler [7], respectively. ACMSDase inhibitor activity was assayed according to Nasu *et al.* [3]. Catalase activity and DNA content were assayed according to Baudhuin [8] and Burton [9], respectively.

RESULTS

No significant difference in diet intake (g/day) was observed among the groups [control: 21 ± 0.6 (3 days), 20 ± 1.3 (8 days); clofibrate: 21 ± 3.2 (3 days), 20 ± 2.3 (8 days)] except for the pyrazinamide group [0.13%: 24 ± 1.5 ($P < 0.05$, by Student's *t*-test) (3 days), 22 ± 1.2 (8 days); 1%: 19 ± 0.6 ($P < 0.05$) (3 days), 20 ± 1.5 (8 days)], while body weight gain (g/day) in a group of rats fed either the clofibrate diet [3.8 ± 1.3 ($P < 0.005$) (3 days), 6.4 ± 0.9 (8 days)] or the pyrazinamide diet [0.13%: 6.3 ± 0.9 ($P < 0.05$) (3 days), 7.0 ± 0.8 (8 days); 1%: 4.2 ± 0.4 ($P < 0.001$) (3 days), 5.6 ± 0.4 ($P < 0.005$) (8 days)] was less than that of the control group [8.1 ± 0.5 (3 days), 7.0 ± 0.1 (8 days)], especially when the feeding period was 3 days.

Pyridine nucleotide levels in the liver of rats fed the clofibrate diet are shown in Fig. 1. NAD^+, NADH, and the sum of NAD^+ and NADH increased significantly after feeding the clofibrate diet for 3 days, increased further after 8 days, and stayed at the same levels until day 14. Although switching to the control diet for 2 days after 12 days of feeding the clofibrate diet tended to decrease pyridine nucleotides, NADH, the sum of NAD^+ and NADH, NADPH, and the sum of $NADP^+$ and NADPH were still significantly higher compared with the control levels. These changes of pyridine nucleotides were parallel to that of the activity of catalase, a marker enzyme of peroxisomes (data not shown).

Pyridine nucleotides in the kidneys of the same animals were lower than those in the livers and only NADH and the sum of NAD^+ and NADH were increased significantly after feeding a clofibrate-containing diet for 8 days (data not shown).

On day 8, when pyridine nucleotides increased to their maximum levels, the effects of clofibrate were compared with those of pyrazinamide. The effect of pyrazinamide on the level of pyridine nucleotides appeared slightly earlier than that of clofibrate and reached a plateau (data not shown). Although pyrazinamide increased the sum of NAD^+ and NADH, its effect was less than the effect of clofibrate even at the 1% level (7.6 times of clofibrate in molar ratio) (Table 1). The NAD^+/NADH ratio was decreased in the liver of the rats fed a 0.25% clofibrate diet

TABLE 1. Pyridine nucleotide levels in the liver of rats fed a clofibrate or pyrazinamide diet

Pyridine nucleotides	Pyridine nucleotide levels (nmol/mg DNA)		
	Control diet	Clofibrate diet	Pyrazinamide diet
NAD^+	517 ± 44	812 ± 48*	704 ± 42†
NADH	126 ± 3	327 ± 19‡	176 ± 17†
NAD^+ + NADH	643 ± 43	1139 ± 63‡	880 ± 33*
NAD^+/NADH	4.1 ± 0.4	2.5 ± 0.1*	4.0 ± 0.6
$NADP^+$	28.9 ± 3.4	34.0 ± 5.6	37.5 ± 4.5
NADPH	123 ± 43	218 ± 12§	158 ± 13
$NADP^+$ + NADPH	152 ± 47	252 ± 8§	195 ± 12
$NADP^+$/NADPH	0.25 ± 0.05	0.16 ± 0.03	0.24 ± 0.04

Rats were fed a 0.25% clofibrate or a 1.0% pyrazinamide diet for 8 days. Values are means ± SD of 3 animals.

*–§ Significantly different from control values, as determined by Student's *t* test: *$P < 0.005$, †$P < 0.01$, ‡$P < 0.001$, and §$P < 0.05$.

compared with that of the control rats, while no significant difference was observed when the rats were fed pyrazinamide (Table 1).

The effect of clofibrate on pyridine nucleotides was less pronounced in females than in males, but a similar effect was observed (data not shown). The effects of clofibrate were also recognized by another peroxisomal proliferator, di-2(ethylhexyl)phthalate (data not shown).

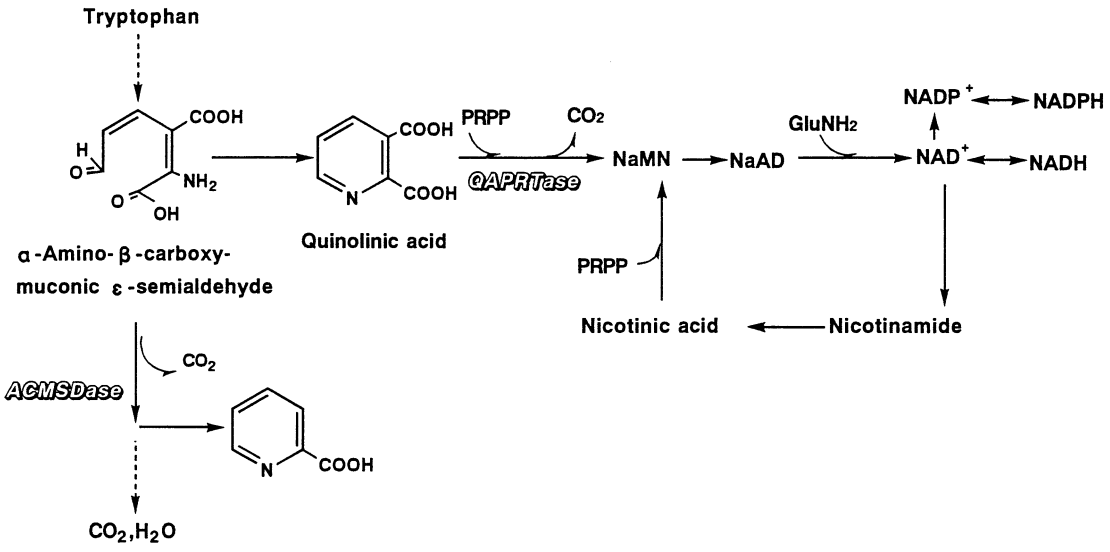
To clarify the mechanism of action of these drugs on tryptophan-NAD^+ metabolism, activities of two key enzymes, namely ACMSDase, which regulates the breakdown of the carbon skeleton of tryptophan through the glutarate pathway, and QAPRTase, which regulates the biosynthesis of NAD^+ from quinolinate (Scheme 1), were determined. As shown in Table 2, both clofibrate and pyrazinamide increased QAPRTase activity in the liver. Pyrazinamide was less effective than clofibrate at the equimolar level (0.13%) and increasing its content in the diet had no further effect. ACMSDase activity in the liver could not be detected in the clofibrate-fed rats. Pyrazinamide at the 1% level in the diet also reduced the hepatic ACMSDase level to less than 20% of the control value. In the kidney, however, only the higher level of pyrazinamide was inhibitory to the enzyme (Table 2).

Activity inhibitory toward ACMSDase was found in the liver of rats fed a high pyrazinamide diet, while no such inhibitor existed in the liver of the clofibrate-fed rats.

DISCUSSION

Clofibrate is an anti-hyperlipidemic drug, known as a peroxisome proliferator [10]. We recently found that clofibrate increases the hepatic NAD^+ in rats [1]. We also found that in hepatocytes isolated from clofibrate-fed rats NAD^+ biosynthesis from tryptophan is stimulated by decreasing the flux of tryptophan via the glutarate pathway [2]. The conversion ratio of tryptophan to niacin obtained by measuring urinary metabolites of niacin is elevated significantly in clofibrate-fed rats compared with control rats [11]. In this report, we showed that not only NAD^+ but also other types of pyridine nucleotides were increased in the liver of rats fed a clofibrate diet.

As can be seen from Table 1, the NAD^+/NADH ratio decreased significantly in the clofibrate-fed rats. The differences in the NAD^+/NADH ratios found between the rats fed the clofibrate diet and those fed the pyrazinamide diet could be due to peroxisomal fatty acid oxidation (clofibrate is a known peroxisome proliferator, while no such effect was reported on pyrazinamide). Peroxisomal palmitoyl-CoA oxidase in the liver of rats fed the clofibrate



SCHEME 1. Biosynthesis of pyridine nucleotides in rat liver. An outline of NAD^+ biosynthesis and key enzymes in rat liver is shown. Abbreviations: QAPRTase, quinolinate phosphoribosyltransferase; and ACMSDase, aminocarboxymuconate-semialdehyde decarboxylase.

TABLE 2. Activities of key enzymes and inhibitor in the Trp-NAD pathway

Diet	QAPRTase (μmol/hr/g)	ACMSDase (μmol/hr/g)		ACMSDase inhibitor (inhibitory units/g)*
	Liver	Liver	Kidney	Liver
Control	0.054 ± 0.003	1.28 ± 0.20	8.56 ± 1.60	0
Clofibrate (0.25%)	0.101 ± 0.014†	0‡	9.33 ± 1.81	0
Pyrazinamide (0.13%)	0.071 ± 0.006§	1.34 ± 0.46	7.10 ± 2.25	50.7 ± 33.6
(1%)	0.072 ± 0.005†	0.24 ± 0.13‖	2.42 ± 1.15†	184 ± 22.8†

Rats were fed each diet for 8 days. Values are means ± SD of 3 animals.

* One unit is expressed as the amounts of inhibitor exhibiting a half-maximum inhibition of the enzyme activity assayed under standard assay conditions, as described in "Materials and Methods."

†–‖ Significantly different from control values, as determined by Student's *t*-test: †$P < 0.01$, ‡$P < 0.001$, §$P < 0.05$, and ‖$P < 0.005$.

diet was increased remarkably compared with that of the control rats, when the hydrogen peroxide production by peroxidase-coupled oxidation of scopoletin [12] was measured (data not shown). Hepatocytes from the clofibrate-fed rats produced significantly more hydrogen peroxide than the pyrazinamide-fed and the control rats. These results indicate that fatty acid oxidation in peroxisomes is stimulated in the liver of clofibrate-fed rats, which is reflected by a decrease in the NAD^+/NADH ratio. As a sex-specific difference in the effects of clofibrate-induced peroxisomal enzyme induction has been reported in rats [13, 14], female rats were also tested. The influence of clofibrate on pyridine nucleotides was also shown in female rats but it was a little less effective (data not shown).

As clofibrate stimulated NAD^+ synthesis from tryptophan, inhibiting the metabolic flux of tryptophan via the glutarate pathway, but not from nicotinate [2], we assayed the activities of two enzymes catalyzing steps leading to the glutarate pathway (ACMSDase) and to NAD^+ synthesis (QAPRTase) from tryptophan. Both clofibrate and pyrazinamide increased QAPRTase activity by 85 and 30%, respectively. Clofibrate, on the other hand, decreased hepatic ACMSDase activity to a non-detectable level, while renal enzyme was not affected. Pyrazinamide was inhibitory to both hepatic and renal ACMSDase activities.

According to Whitehouse *et al.* [15], the main metabolite of pyrazinamide is pyrazinoic acid, and 5-hydroxypyrazinoic acid and 5-hydroxypyrazinamide follow. Rat liver aldehyde oxidase was responsible for the oxidation of pyrazinamide to 5-hydroxypyrazinamide, while pyrazinoic acid was the microsomal deamination product of pyrazinamide [16]. One of those metabolites of pyrazinamide seems to cause inhibition of ACMSDase [3].

The possibility of inhibitory substances was investigated, using heat-treated kidney and liver homogenates of the clofibrate-fed rats. As shown in Table 2, activity inhibitory toward ACMSDase was found in the liver of the rats fed high and low pyrazinamide-containing diets. In the liver of rats fed the clofibrate diet, however, no inhibitory activity was found. Therefore, the enzyme activity below the limit of detection in the liver of the clofibrate-fed rats was not due to the existence of inhibitory substances.

Both elevated QAPRTase activity and ACMSDase activity at non-detectable levels contributed to the high hepatic NAD^+ level in the clofibrate-fed rats. These effects of clofibrate on the pyridine nucleotides or key enzyme activities were also shown by another peroxisome proliferator. The acceleration of NAD^+ biosynthesis from tryptophan together with the increased biosynthesis of CoA in the liver of clofibrate-fed rats [17] will favor fatty acid oxidation. Studies on the mechanism of regulation of the two enzymes and its relation to the peroxisome proliferative effect of clofibrate are underway.

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