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Effect of Thioridazine or Chlorpromazine on Increased Hepatic NAD^+ Level in Rats Fed Clofibrate, a Hypolipidaemic Drug

MARIKO SHIN, SUMIYO ASADA, NANAE MIZUMORI, KEIJI SANO AND CHISAE UMEZAWA

School of Pharmacy, Kobe Gakuin University, Nishi-ku, Kobe, Japan

Abstract

The effect of the phenothiazines, thioridazine and chlorpromazine, on the increased hepatic NAD^+ level of rats fed clofibrate, a hypolipidaemic drug, has been investigated.

Short-term (6 days) addition of phenothiazines to the diet negatively affected diet intake and body-weight gain, but increased liver weight and hepatic NAD^+ levels, which was synergistic to clofibrate. The phenothiazines were shown to inhibit hepatic peroxisomal fatty acid oxidation in-vivo, as determined by the increased residual catalase activity. In hepatocytes prepared from clofibrate-fed rats, phenothiazines inhibited not only peroxisomal but also mitochondrial fatty acid oxidation to the same extent. In the hepatocytes, NAD^+ was maintained at the high level until the phenothiazine concentration was increased to 0·2 mM.

The result suggests that the increase of hepatic NAD^+ in rats fed clofibrate is not related to peroxisomal fatty acid oxidation.

The peroxisome, like the mitochondrion, is an important organelle for fatty acid β-oxidation (Lazarow & de Duve 1976). Details of peroxisomal function in lipid metabolism are, however, not yet established. It has been shown that clofibrate, a hypolipidaemic drug and a peroxisome-proliferator, significantly increased the level of hepatic NAD^+ in rats (Loo et al 1995) and that the tryptophan-NAD^+ pathway was specifically activated in hepatocytes prepared from rats fed a clofibrate diet (Shin et al 1996, 1998*a*,*b*). In those animals, the activities of key enzymes in the tryptophan-NAD^+ pathway changed in parallel with the hepatic NAD^+ increase; for example, the activity of quinolinate phosphoribosyltransferase (EC 2.4.2.19) was increased whereas that of α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (EC 4.1.1.45) was drastically reduced. NAD^+ is an essential cofactor in peroxisomal fatty acid oxidation (Lazarow 1978). Increased levels of NAD^+ might promote fatty acid metabolism in peroxisomes without a specific NAD^+ pool. The concentration of CoA, another cofactor of peroxisomal β-oxidation, in the liver is known to increase when clofibrate is administered (Strede & Halvorsen 1979). Actually, the NAD^+/NADH ratio was significantly reduced by increasing the concentration of NAD^+ (Shin et al 1998*a*), possibly because of activated peroxisomal β-fatty acid oxidation.

We have investigated the relationship between hepatic NAD^+ increase and activation of peroxisomal fatty acid oxidation, to determine whether peroxisomal fatty acid oxidation is a prerequisite for the increase in hepatic NAD^+. Phenothiazines such as thioridazine and chlorpromazine are reported to inhibit peroxisomal β-oxidation in-vivo, but not mitochondrial β-oxidation (Leighton et al 1984; Van den Branden & Roels 1985; Van den Branden et al 1987; Skorin et al 1992). We co-administered these drugs to rats fed a clofibrate-diet to obtain further information on the mechanism of the increase in NAD^+.

Correspondence: Mariko Shin, School of Pharmacy, Kobe Gakuin University, Nishi-ku, Kobe, 651-2180, Japan.

Materials and Methods

Materials

Clofibrate (2-(4-chlorophenoxy)2-methylpropanoic acid ethyl ester) was obtained from Wako Pure Chemical Industries, Osaka, Japan. Chlorpromazine-hydrochloride was from Nacalai Tesque, Kyoto, Japan. Thioridazine-hydrochloride and methimazole (2-mercapto-1-methylimidazole) were from Aldrich.

Animals and diets

Male Sprague-Dawley rats (specific pathogen-free, 200–220 g) were obtained from Japan SLC, Shi-

zuoka, Japan. Rats were fed Oriental MF-solid diet as a control for 3 days and MF-powder diet for approximately 4 days for conditioning. Diet and water were freely available.

After 7 days, animals were divided into six groups (four animals per group). Animals in groups 1 and 2 (the 'A groups') were given clofibrate plus either 0·25% thioridazine or 0·1% chlorpromazine, respectively. Animals in groups 3, 4 and 5 (the 'B groups') were given thioridazine, chlorpromazine or clofibrate, respectively. Animals in group 6 (the 'C group') were control animals. In the 1st week, a control diet was freely available to groups 3, 4 and 6 and a clofibrate-diet to groups 1, 2 and 5. In the 2nd week, phenothiazine-containing diets were started for groups 1, 2, 3 and 4. Each diet was fed in the manner of pair-feeding against A groups, with water freely available. The clofibrate content of the diets was 0·25 or 0·5% in experiments A and B, respectively. Clofibrate was added as described elsewhere (Loo et al 1995) and phenothiazine was added to the control diet (MF powder), at the designated final concentration (w/w), after grinding with a pestle and mortar.

Animal experiments

Rats were killed by decapitation 2 h after intraperitoneal injection of 3-amino-1,2,4-triazole (1 g kg^{-1} in 0·9% NaCl (saline))-methanol(3·5 mmol kg^{-1}), an inhibitor of catalase-hydrogen peroxide complex (Oshino et al 1973; Inestrosa et al 1979; Mannaerts et al 1979; Leighton et al 1984; Van den Branden et al 1984; Van den Branden & Roels 1985; Small et al 1985; Van den Branden et al 1987; Skorin et al 1992). The liver was quickly removed and weighed and a portion was quickly frozen with blocks of dry ice and finely powdered. NAD^+ and total niacin content were determined according to Klingenberg (1985) or by microbioassay (Snell & Wright 1941). Total liver homogenate was prepared with 3 vol 0·25 M sucrose-0·1% ethanol and total or residual catalase activity was assayed at 0°C, by the titanium oxysulphate method (Baudhuin 1974), the supernatant was obtained by centrifugation of the homogenate at 250 *g* for 10 min. One unit is the amount of catalase that breaks down 90% substrate (H_2O_2 1.5 mM) in 50 mL at 0°C in 1 min; the maximum reaction time is 10 min. Residual catalase activity is the catalase activity that remains after inhibition by aminotriazole-methanol (Van den Branden et al 1984, 1987; Van den Branden & Roels 1985). When residual catalase activity is low, H_2O_2 production is high, i.e. higher peroxisomal β-oxidation. The DNA content of the liver (mg) was determined as described by Burton (1968).

Preparation of hepatocytes and conditions of incubation

Hepatocytes were prepared from rats fed a 0·5% clofibrate-containing diet for 2 weeks, using collagenase as described by Seglen (1976). The cells (more than 85% viability when checked by the trypan-blue exclusion test) were suspended in Krebs-Ringer bicarbonate buffer supplemented with 5 mM glucose, 0·5 mM glutamine, 50 mM methanol, 10 mM semicarbazide hydrochloride and 0·18 mM de-fatted bovine serum albumin (Chen 1967) and 0·54 mM fatty acid (palmitate or laurate) in an Erlenmeyer flask, so that each milliliter of cell suspension contained approximately 6 mg wet weight of cells (Leighton et al 1984). Incubations were performed by shaking the flasks in a water bath at 37°C for 60 min at 75 oscillations min^{-1} in an atmosphere of 95% O_2–5% CO_2 (v/v). Samples were extracted from the incubation mixture with 0·5 M $HClO_4$.

Assays

The supernatant after centrifugation was used for assay of formaldehyde, as a marker of peroxisomal fatty acid oxidation. The formaldehyde, generated by peroxidation of methanol by the hydrogen peroxide released during peroxisomal fatty acid oxidation, was trapped as its semicarbazone. For assay of β-hydroxybutyrate as mitochondrial fatty acid oxidation marker and the NAD^+ content of the hepatocytes, the supernatant was adjusted to neutral pH and used after centrifugation. Formaldehyde, β-hydroxybutyrate and NAD^+ were determined according to Nash (1953), Williamson & Mellanby (1974) and Klingenberg (1985), respectively.

Statistics

Data are expressed as means ± s.d. of results from three animals or from hepatocytes from three animals. Significant differences from control values were determined by Student's *t*-test. Statistical significance was indicated by $P < 0.05$.

Results

Diet intake and body-weight gains in experiment 1 are shown in Table 1. In the 1st week, diet intake and body-weight gain were comparable with those in the conditioning period (data not shown). However, in the 2nd week of pair-feeding with diet containing thioridazine or chlorpromazine, diet intake dropped to a half of that of the 1st week and body weight decreased to below the starting weight. Animals given thioridazine showed eye-mucus symptom, which was alleviated slightly by co-administration of clofibrate. Thioridazine- and

Table 1. Diet intake and body-weight gain.

Group	Diet intake		Body-weight gain		
	1st Week (g/7 days)	2nd Week (g/6 days)	Start (g)	1st Week (g/7 days)	2nd Week (g/6 days)
Control	140 ± 0	62 ± 0	285 ± 18	+(21 ± 7)	−(26 ± 2)
Thioridazine	137 ± 2	50 ± 11	291 ± 5	+(19 ± 4)	−(24 ± 6)
Chlorpromazine	137 ± 3	58 ± 2	278 ± 6	+(23 ± 4)	−(30 ± 3)
Clofibrate	138 ± 2	51 ± 0	292 ± 7	+(23 ± 6)	−(35 ± 4)
Clofibrate + thioridazine	137 ± 9	69 ± 8	279 ± 7	+(29 ± 5)	−(16 ± 4)
Clofibrate + chlorpromazine	142 ± 9	63 ± 8	272 ± 12	+(31 ± 6)	−(24 ± 2)

Rats were fed control or 0·25% clofibrate-diet during the first week (7 days) and then thioridazine or chlorpromazine was added to this diet for the second week (6 days). Values are means ± s.d. of results from four animals.

Table 2. Effects of phenothiazines on liver-weight, DNA, NAD and total niacin level in the liver of rats fed 0·25% clofibrate diet (experiment A).

Group	Liver weight (g)	DNA (mg g^{-1} liver)	NAD (nmol mg^{-1} DNA)	Total niacin (nmol mg^{-1} DNA)
Control	7·9 ± 1·6	2·07 ± 0·09	293 ± 40	441 ± 36
Thioridazine	10·1 ± 1·9*	1·30 ± 0·15‡	398 ± 73*	569 ± 42‡
Chlorpromazine	8·8 ± 1·1	1·53 ± 0·11‡	325 ± 40	464 ± 18
Clofibrate	10·7 ± 1·2**	1·64 ± 0·06¶	471 ± 33**	618 ± 44**
Clofibrate + thioridazine	13·6 ± 3·2†	1·14 ± 0·09§	630 ± 49‡	839 ± 39§
Clofibrate + chlorpromazine	13·6 ± 2·3‡	1·13 ± 0·05§	593 ± 39‡	799 ± 25§

Values are means ± s.d. of results from four animals. §$P < 0·001$, ‡$P < 0·005$, †$P < 0·01$, *$P < 0·05$, significantly different from results for control or clofibrate diet, respectively, as determined by Student's t-test. **$P < 0·001$, ¶$P < 0·005$, significantly different from results for control diet.

chlorpromazine-treated animals developed hepatic hypertrophy (liver-weight increase and DNA decrease; Table 2), although the effect of chlorpromazine was less than that of thioridazine at the dose used. When the phenothiazines were co-administered with clofibrate, more severe hepatic hypertrophy was observed (Table 2).

The effect of phenothiazines on liver residual catalase activity is shown in Table 3. Administration of phenothiazines significantly increased residual catalase activity, which indicates inhibition of peroxisomal fatty acid oxidation by these drugs.

Results from determination of hepatic NAD^+ and total niacin are shown in Table 2. Clofibrate increased hepatic NAD^+ significantly. The ratio of NAD^+ to total niacin was approximately 70% in each group. Total niacin includes microbiologically active niacin derivatives, namely NAD^+, $NADP^+$, nicotinic acid and nicotinamide. Chlorpromazine alone had no effect, but when co-administered with clofibrate, NAD^+ was increased beyond the level induced by clofibrate alone. On administration of 0·5% clofibrate (experiment B), body-weight gain, diet intake and liver weight were the same as in experiment A except for hepatic NAD^+ level. As shown in Table 3, the effect of clofibrate on the level of NAD^+ and total niacin in experiment B was far larger than that in the experiment A. However, co-administered phenothiazines had no additional effect on NAD^+ level. On administration of 0·25 or 0·5% clofibrate (experiments A and B), hepatic NAD^+ levels were significantly increased. When phenothiazines, reported inhibitors of peroxisomal fatty acid oxidation, were administered, the hepatic NAD^+ level was increased, often synergistically, by the administration of clofibrate.

The effect of phenothiazines on fatty acid oxidation in hepatocytes prepared from rats fed clofibrate diet (0·5%, 2 weeks) is shown in Table 4. In the reaction system, glutamine was added for the synthesis of NAD^+ (Shin et al 1996) and methanol and semicarbazide were for trapping hydrogen peroxide, generated by the peroxisomal oxidation of fatty acids (Leighton et al 1984), as formaldehyde. More laurate than palmitate was oxidized peroxisomally (data not shown). Thioridazine was more inhibitory than chlorpromazine toward fatty acid oxidation, as reported by Leighton et al (1984). In our experiment phenothiazines inhibited mitochondrial and peroxisomal fatty acid oxidation to the same extent. This result is different from that reported by Leighton's group (Leighton et al 1984).

Table 3. Effects of phenothiazines on NAD and total niacin content and fatty acid oxidation in the liver of rats fed 0·5% clofibrate diet (experiment B).

Group	NAD (nmol mg^{-1} DNA)	Total niacin (nmol mg^{-1} DNA)	Residual catalase activity (units mg^{-1} DNA)
Control	349 ± 43	514 ± 15	6·3 ± 0·2
Thioridazine	397 ± 21	683 ± 52†	11·1 ± 0·9§
Chlorpromazine	314 ± 29	569 ± 60	10·0 ± 1·5‡
Clofibrate	761 ± 116**	1035 ± 196¶	9·9 ± 0·7**
Clofibrate + thioridazine	930 ± 63*	1192 ± 87	15·0 ± 3·2*
Clofibrate + chlorpromazine	842 ± 64	1067 ± 70	17·7 ± 4·0†

Values are means ± s.d. of results from four animals. §$P < 0·001$, ‡$P < 0·005$, †$P < 0·01$, *$P < 0·05$, significantly different from results for control or clofibrate diet, respectively, as determined by Student's t-test. ¶$P < 0·001$, **$P < 0·05$, significantly different from results for control diet.

Table 4. Effects of phenothiazines on mitochondrial or peroxisomal laurate oxidation in hepatocytes prepared from clofibrate-fed rats.

Drug	Amount added (mM)	Relative activities or levels (%)		
		NAD level	β-Hydroxybutyrate production	H_2O_2 production
None		100 ± 7·4	100 ± 14·3	100 ± 1·5
Thioridazine	0·1	107 ± 5·4	14·4 ± 4·3	40·2 ± 2·9
	0·2	104 ± 5·5	2·7 ± 0·5	6·3 ± 2·3
	0·5	28·1 ± 5·2	1·4 ± 0·1	1·8 ± 0·8
Chlorpromazine	0·1	102 ± 13·5	60·1 ± 2·0	65·5 ± 2·2
	0·2	101 ± 6·0	54·6 ± 4·2	37·2 ± 2·2
	0·5	54·9 ± 8·0	9·4 ± 0·7	2·2 ± 0·3

Each result is a value relative to that obtained for the 'no addition' groups. Each control value (no drug added) was: NAD 0·46 ± 0·03 nmol mg^{-1} cells, n = 3, after 60 min incubation; β-hydroxybutyrate production 10·1 ± 1·5 nmol h^{-1} mg^{-1} cells, n = 3; H_2O_2 production 29·1 ± 0·4 nmol h^{-1} mg^{-1} cells, n = 3. Values are expressed as means ± s.d. of results from three animals.

However, it is noteworthy that even when fatty acid oxidation was inhibited, NAD^+ level was maintained at the high level until the concentration of phenothiazines reached 0·2 mM. These results in-vitro (Table 4) were consistent with those obtained in-vivo (Tables 2 and 3).

Discussion

In this work, phenothiazines (thioridazine or chlorpromazine) were used to investigate the mechanism of hepatic NAD^+ increase in rats fed a clofibrate diet. In the hepatocytes isolated from the clofibrate-fed rats, NAD^+ biosynthesis from tryptophan was stimulated by reducing the flux of tryptophan via the glutarate pathway (Shin et al 1996). Moreover, the NAD^+/NADH ratio was significantly reduced in the liver of clofibrate-fed rats (Shin et al 1998). These results suggest that administration of clofibrate generated conditions conducive to peroxisomal fatty acid oxidation. In rodents, administration of phenothiazines (thioridazine or chlorpromazine) is reported to inhibit hepatic peroxisomal fatty acid oxidation (Leighton et al 1984; Van den Branden & Roels 1985; Van den Branden et al 1987; Skorin et al 1992). To detect in-vivo changes in hepatic H_2O_2 production originating from peroxisomal fatty acid oxidation, a combination of the catalase inhibitor, 3-amino-1,2,4-triazole and methanol was used. Lower residual catalase activity reflects a higher level of H_2O_2, i.e. greater activity of peroxisomal β-oxidation. Table 3 shows that administration of phenothiazines for 1 week significantly increased residual catalase activity, which indicates inhibition of peroxisomal fatty acid oxidation by these drugs. The increase of residual catalase activity as a result of the clofibrate diet might be because of the increase in total catalase activity. In another experiment without administration of methimazole-methanol, total catalase activity (units $(mg\,DNA)^{-1}$) in the liver of rats fed control or 0·25% clofibrate diet for 2 weeks was 12·5 ± 2·3 (n = 6) and 34·7 ± 4·6 (n = 3). When rats were fed 0·25 or 0·5 % clofibrate diet for 2 weeks the hepatic NAD^+ level was significantly increased (Tables 2 and 3). Clofibrate induced a significant increase in levels of hepatic NAD^+ and total niacin, although

the effect was less than that observed when the diet was freely available (Loo et al 1995). This difference must be because of drastically reduced clofibrate uptake during pair-feeding compared with the group of phenothiazines disliked by animals (Table 1). When co-administered with phenothiazines, reported as inhibitors of peroxisomal fatty acid oxidation, clofibrate raised hepatic NAD^+ levels significantly, often synergistically (Tables 2 and 3).

Phenothiazines, especially thioridazine, resulted in increased liver weight and reduced DNA level similarly to clofibrate after administration for 6 days (Table 2). However, after long-term administration of 0.25% thioridazine diet there is no significant induction of enzymes related to peroxisomal β-oxidation, and light microscopy of liver sections revealed no changes of peroxisomes in rat liver (Van den Branden et al 1987). Chlorpromazine had less effect than thioridazine.

To gain more insight into peroxisomal function, specific inhibitors of peroxisomal β-oxidation, especially effective in-vivo, would be of great value. Leighton et al (1984) reported the selective inhibition of peroxisomal β-oxidation by three phenothiazines (chlorpromazine, fluphenazine and thioridazine), of which thioridazine proved to be the most effective in in-vitro systems (for example isolated rat hepatocytes). The activity of peroxisomal β-oxidation, which produces H_2O_2 in the first step (Lazarow & de Duve 1976), was determined by measuring H_2O_2 generation. Van den Branden & Roels (1985) showed, by estimation of residual catalase activity, that in-vivo administration of thioridazine inhibited hepatic peroxisomal β-oxidation in mice; they also showed that 3-hydroxybutyrate concentration was not reduced by thioridazine treatment (50–250 μmol kg^{-1}, in saline, given intraperitoneally) in fed or 24-h-starved mice. They subsequently discussed the short- and long-term influence of phenothiazines on liver peroxisomal fatty acid oxidation in rodents (Van den Branden et al 1987). Phenothiazines such as thioridazine (0·1 to 0·5%, w/w) and chlorpromazine (0·1%, w/w) inhibited peroxisomal β-oxidation most effectively when administered to rats for 1 week after administration of the diet containing the drugs. The inhibitory effects could not be restored for long-term administration. This effect was only transitory in rats or not detectable in mice. The inhibition of peroxisomal fatty acid oxidation by chlorpromazine in hepatocytes was attributed to inhibition of carnitine octanoyltransferase (Van den Branden et al 1987). Carnitine octanoyltransferase in the peroxisomes is believed to participate in the transport of the products of fatty acid oxidation (Skorin et al 1992). However, Skorin et al reported that in whole cells chlorpromazine acts as an uncoupler of mitochondrial oxidative phosphorylation and reduces the concentration of ATP in the cell (Skorin et al 1992). In our experiments using hepatocytes prepared from rats fed clofibrate, thioridazine and chlorpromazine inhibited not only peroxisomal β-oxidation activity but also mitochondrial activity (Table 4). Causes of this discrepancy are not clear but it might be a result of the different condition of hepatocytes prepared from clofibrate- or nafenopin-treated rats (Leighton et al 1984).

These results suggest that the increase in hepatic NAD^+ in the rat fed clofibrate (Tables 2 and 3) might not be related to the practical metabolism of fatty acids in peroxisomes. The NAD^+ increase might be caused by transcription of genes coding key enzymes of Trp-NAD^+ biosynthesis in relation to the peroxisome-proliferator-activated receptors expressed by the administration of clofibrate. Further investigation of this possibility are in progress.

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