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CLOFIBRATE-LIKE EFFECTS OF ACETYLSALICYLIC ACID ON PEROXISOMES AND ON HEPATIC AND SERUM TRIGLYCERIDE LEVELS

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Abstract—The effects of acetylsalicylic acid on (1) triglyceride levels of rat liver and rat serum, (2) peroxisomal enzyme activities, and (3) peroxisome-associated polypeptide content were investigated. The rats were maintained on diets containing 1% acetylsalicylic acid for 2 weeks. The triglyceride levels of the livers and sera of rats fed acetylsalicylic acid decreased by 23 and 65 per cent respectively. Cyanide-insensitive palmitoyl-CoA oxidizing activity in the livers of treated rats was 3.7 times greater than in controls, but the activities of catalase and urate oxidase were increased slightly. The observed increase in the hepatic palmitoyl-CoA oxidizing activity was presumably due to an enhancement of the activity in peroxisomes. Furthermore, an increase in the content of a polypeptide associated with peroxisomes in the light mitochondrial fractions from the livers of treated rats was revealed by SDS–polyacrylamide gel electrophoresis. The observed effects of acetylsalicylic acid were very similar to those of clofibrate, a hypolipidemic drug, pointing to a possibility that these effects may be common phenomena in drug-induced proliferation of peroxisomes.

Peroxisomes are cytoplasmic organelles present in various tissues of rodents and several other vertebrates. The organelles are characterized by their content of H_2O_2-producing oxidases (urate oxidase, D-amino acid oxidase, L-α-hydroxy acid oxidase, etc.) and catalase [1].

Clofibrate, a well-known hypolipidemic drug, casues proliferation of hepatic peroxisomes [2,3]. Some other hypolipidemic agents produce similar effects [3], indicating a relationship between their hypolipidemic effects and the proliferation of peroxisomes. Lazarow and de Duve [4] reported that peroxisomes contain a cyanide-insensitive fatty acyl-CoA oxidation system which is markedly increased by treatment with clofibrate. It was further suggested that this enzyme is specific for long-chain fatty acyl-CoA [5].

Electron microscopic studies have demonstrated that the administration of acetylsalicylic acid to rats also induced proliferation of hepatic peroxisomes [6,7]. The number of peroxisomes increased 2- to 3-fold after 10 days of treatment. These effects of acetylsalicylic acid on hepatic peroxisomes were based on morphological observations [6–8] and their biochemical nature was not investigated.

This paper shows that triglyceride levels in liver and serum of rats are reduced by the administration of acetylsalicylic acid, but the peroxisomal fatty acyl-CoA oxidizing activity and the cellular content of a peroxisome-associated polypeptide are increased.

MATERIALS AND METHODS

Materials. CoA and NAD^+ were purchased from Kyowa Hakko Kogyo Co., Tokyo, and palmitoyl-CoA from the Sigma Chemical Co., St. Louis, MO. Acetylsalicylic acid was obtained from the Nippon Chemipha Co., Japan. L-Carnitine was kindly donated by Otsuka Pharmaceutical Factory, Japan.

Administration of acetylsalicylic acid and preparation of liver homogenates. Wistar male rats weighing about 150 g were used. After fasting overnight, rats were fed, *ad lib.* for 2 weeks, a ground Oriental laboratory chow with and without 1% (w/w) acetylsalicylic acid. The animals were weighed and decapitated. The livers were removed, weighed and homogenized in ice-cold 0.25 M sucrose in a Potter–Elvehjem type homogenizer fitted with a Teflon pestle. Serum was pooled for the determination of triglyceride content.

Subcellular fractionation of the liver. A 10% (w/v) liver homogenate was prepared in 0.25 M sucrose and fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and supernatant (S) fractions according to the method of de Duve *et al.* [9]. The pellet from each fractionation step was rehomogenized in 0.25 M sucrose and assayed for fatty acyl-CoA oxidizing activity.

Enzyme assays. Cyanide-insensitive fatty acyl-CoA oxidizing activity was determined by measuring the palmitoyl-CoA-dependent reduction of NAD^+ spectrophotometrically at 340 nm as described by Lazarow and de Duve [4]. The incubation mixture contained, in a total volume of 1.0 ml, the following constituents: 30 mM potassium phosphate buffer (pH 7.4), 0.2 mM NAD^+, 50 μM CoA, 6 mM dithiothreitol, 1 mM KCN, 0.015% bovine serum albumin, 14 μM palmitoyl-CoA, 0.01% Triton X-100 and varying amounts of enzyme. Mitochondrial β-oxidation could not be detected because of the presence of KCN [4]. One unit of activity was defined as the amount of enzyme that reduced 1 μmole of NAD^+/min.

Catalase activity was determined spectrophotometrically at 240 nm [10]. One unit of activity is defined as the amount of enzyme that brought the k value to 1, where k is the decrease in extinction at 240 nm per sec at 25°.

Table 1. Changes in liver weight and triglyceride levels in liver and serum of rats fed diets containing acetylsalicylic acid*

	Liver weight		Triglyceride levels	
	(g)	(% of body wt)	Liver (mg/g liver)	Serum (mg/dl)
Control	7.9 ± 1.0	4.0 ± 0.1	7.1 ± 0.5	88.2 ± 9.2
Acetylsalicylic acid-treated	7.0 ± 0.7	5.0 ± 0.2†	5.5 ± 0.3‡	39.4 ± 3.1†

* Rats were fed for 2 weeks on a diet with or without 1% acetylsalicylic acid. Results are expressed as the means ± S.D. of five rats.

† Changes were statistically significant when compared with the group maintained on the standard diet, $P < 0.01$.

‡ The decrease was statistically significant when compared with the group maintained on the standard diet, $P < 0.05$.

Urate oxidase activity was determined by the procedure of Hayashi *et al.* [11]. One unit of the activity was defined as the amount of enzyme equivalent to a k value of 1, where k is the decrease in extinction of uric acid at 290 nm per min at 37°. These latter two enzymes were used as marker enzymes for peroxisomes.

Monoamine oxidase activity was determined according to the method of Turuski *et al.* [12] with modifications, using *m*-nitrobenzylamine as a substrate. The reaction mixture contained 67 mM phosphate buffer (pH 7.2), 3.3 mM *m*-nitrobenzylamine and 0.5 ml of the enzyme preparation, in a final volume of 1.5 ml. The reaction was started by the addition of the enzyme at 37°; after 30 min, the reaction was terminated by the addition of 1 ml of 1 mM 2,4-dinitrophenylhydrazine. Hydrazone formed in the mixture was converted to the quinoid species by adding 1 ml of 1.5 N KOH. Isopropyl acetic acid (4 ml) was added to the mixture and, after mixing, the quinoid species was extracted into the isopropyl acetic acid by centrifugation. Two ml of the extract was transferred to another tube and a scarlet color was developed by the addition of 1 ml isoamyl alcohol. The resulting *m*-nitrobenzaldehyde was measured in terms of the absorbance at 505 nm. One unit of the activity was defined as the amount of enzyme that produced 1 nmole of *m*-nitrobenzaldehyde from *m*-nitrobenzylamine per min under the assay condition. The enzyme served as a marker enzyme for mitochondria.

The activities of carnitine acetyltransferase and carnitine palmitoyl transferase were determined spectrophotometrically by measuring the amount of CoA-SH released from acetyl-CoA or palmitoyl-CoA, respectively, with 5,5′-dithiobis-(2-nitrobenzoate), at 412 nm [13]. For both enzymes, one unit of the activity was defined as the amount of enzyme that produced 1 nmole of CoA–SH from acetyl-CoA or palmitoyl-CoA per min.

Determination of triglyceride levels of the liver and serum. Triglyceride levels of the livers and the sera were determined by the method of Van Handel-Kawade with modification as described previously [14].

Determination of protein content. Protein concentration was determined by the method of Lowry *et al.* [15], with bovine serum albumin as a standard.

Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis. The SDS–polyacrylamide gel electrophoresis was performed according to the method of Reddy and Kumar [16]. Gels of 7.5% acrylamide were prepared from stock solutions of 30% acrylamide and 0.8% *N,N′*-bis-methylene acrylamide. The separation gel was 10 cm. The light mitochondrial pellets were suspended in 3% SDS and a sample containing 150 μg of protein was loaded onto a stacking gel. Electrophoresis was carried out at 3 mA/gel until the marker dye reached the bottom of the gel (about 8 hr). The proteins were stained for 6 hr with 0.1% Coomassie brilliant blue in 20% trichloroacetic acid.

RESULTS

Liver weights and triglyceride levels in liver and serum. The effects of acetylsalicylic acid on liver weight and triglyceride levels in liver and serum are shown in Table 1. An increase in liver weight was found in the

Table 2. Effects of acetylsalicylic acid on enzyme activities of liver*

Parameter	Enzyme activities (units/g liver)	
	Control	Acetylsalicylic acid
Cyanide-insensitive palmitoyl-CoA oxidation	0.38 ± 0.11	1.44 ± 0.43†
Catalase	48.1 ± 2.7	56.6 ± 5.9
Urate oxidase	3.04 ± 0.15	3.68 ± 0.27
Carnitine acetyltransferase	784 ± 125	7251 ± 1349†
Carnitine palmitoyltransferase	699 ± 74	3284 ± 769†
Protein (mg/g liver)	251 ± 5	254 ± 5

* Rats were fed a chow containing 1% acetylsalicylic acid for 2 weeks. Results are expressed as the means ± S.D. from five rats.

† The increase was statistically significant when compared with the group fed with the standard diet, $P < 0.01$.

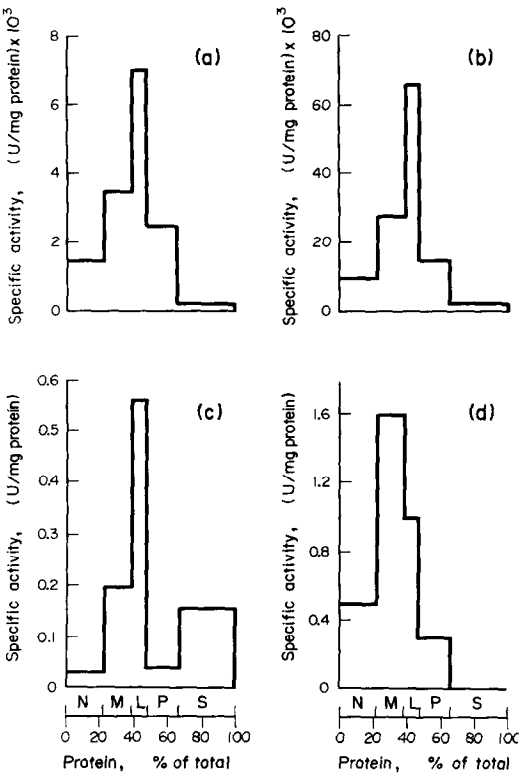

Fig. 1. Subcellular fractionation of rat liver. The liver was homogenized in 9 vol. of ice-cold 0.25 M sucrose, and was fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and supernatant (S) fractions according to the method of de Duve *et al.* [9]. The enzyme activities were determined as described in Materials and Methods. Distribution of the activities of: (A) cyanide-insensitive palmitoyl-CoA oxidation; (B) urate oxidase; (C) catalase; and (D) monoamine oxidase.

rats treated with this compound. The increase was higher than that reported by Hruban *et al.* [7]. The responses to acetylsalicylic acid were remarkably different in liver and in serum. Although the drug reduced or lowered the triglyceride levels in both cases, the hepatic level was only slightly lower than the untreated control, while the serum triglyceride level dropped to 44% that of normal rats.

Enhancement of various enzyme activities in the liver by acetylsalicylic acid. Table 2 shows the effects of acetylsalicylic acid in the diet on peroxisomal enzymes and on the enzymes related to fatty acid metabolism in rat liver. Cyanide-insensitive palmitoyl-CoA oxidizing activity was increased by about 3.7-fold. The activities of catalase and urate oxidase, well-known peroxisomal enzymes, seemed to be slightly elevated, though not significantly, by the drug. The activities of carnitine acetyltransferase and carnitine palmitoyltransferase were 9.3 and 4.7 times those of the control respectively. Total protein content was not affected by the treatment.

Subcellular distribution of cyanide-insensitive palmitoyl-CoA oxidizing activity in rat liver and the effect of acetylsalicylic acid on the distribution of peroxisomal enzymes. The results of subcellular fractionation of the liver homogenate are summarized in Fig. 1. The highest specific activities of catalase and urate oxidase, the marker enzymes for peroxisomes, were found in the light mitochondrial (L) fraction. The highest specific activity of monoamine oxidase, as a marker enzyme for mitochondria, was found in the heavy mitochondrial (M) fraction. The subcellular distribution patterns of these enzyme activities agree with previous results [17]. The specific activity of cyanide-insensitive palmitoyl-CoA oxidation, as well as the activities of catalase and urate oxidase, was highest in the light mitochondrial fraction. However, the distribution of the palmitoyl-CoA oxidizing activity in the supernatant (S) fraction was similar to that of urate oxidase rather than to that of catalase since little activity was detected in this fraction. Table 3 shows the effects of acetylsalicylic acid on subcellular distributions of activities of the peroxisomal enzymes in the liver. The subcellular distribution patterns of the activities of catalase and urate oxidase indicated that peroxisomes are mainly recovered in the heavy (M) and light (L) mitochondrial fractions. Subsequently, the activities of these peroxisomal enzymes were observed in the large granule (ML) fraction. The specific activity of the palmitoyl-CoA oxidation in the ML fraction was quadrupled by the administration of acetylsalicylic acid, but the activity in the S fraction was not affected. The activity of urate oxidase was not affected in both the ML and the S fractions by the treatment. However, the catalase activity was enhanced in both the ML and the S fractions.

SDS–polyacrylamide gel electrophoresis of the light mitochondrial fractions. Electrophoretic profiles of the light mitochondrial pellets from the livers of rats fed clofibrate and acetylsalicylic acid are shown in Fig. 2. A marked increase in the content of a polypeptide, with

Table 3. Effects of acetylsalicylic acid on the subcellular distribution of peroxisomal enzymes in rat liver*

	Palmitoyl-CoA oxidation (units/mg protein) × 10^3		Catalase (units/mg protein)		Urate oxidase (units/mg protein) × 10^3	
	Control	Acetylsalicylic acid	Control	Acetylsalicylic acid	Control	Acetylsalicylic acid
ML fraction	5.34 ± 1.11	21.25 ± 4.14†	0.300 ± 0.020	0.350 ± 0.028	48.5 ± 3.9	51.3 ± 5.1
S fraction	0.21 ± 0.05	0.22 ± 0.11	0.136 ± 0.020	0.201 ± 0.040	1.8 ± 0.1	1.9 ± 0.1

* The ML fraction, which contained both the heavy mitochondrial (M) and the light mitochondrial (L) fractions, was prepared from pellets obtained by centrifugation (from 600 *g* for 10 min to 12,500 *g* for 20 min). The S fraction was prepared from supernatant fractions obtained by centrifugation at 105,000 *g* for 60 min. The results are expressed as the means ± S.D. from five rats.

† The increase was statistically significant when compared with the group given the standard diet, $P < 0.01$.

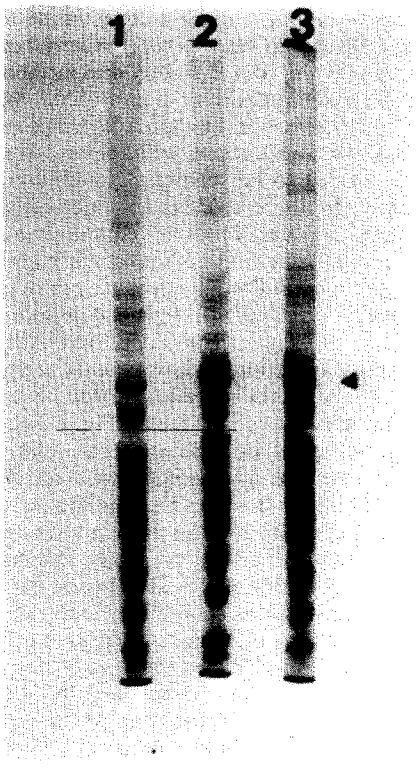

Fig. 2. SDS–polyacrylamide gel electrophoresis of the light mitochondrial pellets from the liver of normal, clofibrate- or acetylsalicylic acid-treated rats. Rats were fed *ad lib.* for 2 weeks on a standard diet containing 0.25% clofibrate or 1% acetylsalicylic acid. Approximately 150 μg of proteins of each fraction were analyzed. Key: (1) normal; (2) clofibrate-treated rats and (3) acetylsalicylic acid-treated rats.

an apparent mol. wt of 80,000 (arrow head), was observed in the SDS-polyacrylamide gel for both clofibrate-treated and acetylsalicylic acid-treated rats. Reddy and Kumar [16] reported that the marked increase in the polypeptide band caused by clofibrate treatment was due to induction of a specific peroxisomal protein.

DISCUSSION

Hepatic peroxisomes are proliferated by treatment with clofibrate, a hypolipidemic drug [2,3]. Hruban *et al.* [6,7] reported that the administration of acetylsalicylic acid to rats caused a proliferation of hepatic peroxisomes. However, little is known about the biochemical events taking place in peroxisomes after the treatment of animals with acetylsalicylic acid.

To date, hypolipidemic drugs [2,3] have been used mostly to study drug-induced proliferation of peroxisomes. A decrease in serum triglyceride levels has been observed concurrently with the proliferation of the particles. We have shown in this paper (Table 1) that acetylsalicylic acid also causes a marked drop of serum triglyceride levels despite the fact that this compound is structually unrelated to other commonly used hypolipidemic agents. These results strongly suggest that the decrease in triglyceride levels in the liver and serum is related to the proliferation of hepatic peroxisomes.

Recently, Lazarow and de Duve [4,5] reported that cyanide-insensitive fatty acyl-CoA oxidation occurs in peroxisomes. This observation is supported by our subcellular fractionation experiments with liver. The highest specific activity of the fatty acyl-CoA oxidation was found in the light mitochondrial fraction which also contained the highest activities of marker enzymes for the peroxisomes, i.e. catalase and urate oxidase. The fatty acyl-CoA oxidation activity increased after administration of acetylsalicylic acid (Table 2), and the increase was observed in the ML fraction but not in the S fraction (Table 3). This finding indicates that the increase in the activity was due to an increase in activity in peroxisomes. It is known that catalase in the liver is localized in the matrix of peroxisomes and is readily released from the particles into the cytoplasm (S fraction) by rupture of the membrane. However, urate oxidase is not readily released even when the membrane is broken, because the enzyme is tightly bound to the peroxisomal core [11]. Our results of subcellular distribution of catalase and urate oxidase (Fig. 1) are consistent with such a location. The ratio of the total oxidizing activity in peroxisomes compared to the supernatant fraction was 19.5. This was similar to the value obtained for urate oxidase (20.5) rather than that for catalase (1.7). Furthermore, little cyanide-insensitive palmitoyl-CoA oxidizing activity was detected in the S fraction of the liver of both the control and the acetylsalicylic acid-treated rats (Fig. 1 and Table 3). This low activity may be an indication that the oxidation system, like urate oxidase, is tightly bound to peroxisomal components.

It is known that carnitine palmitoyltransferase occurs in mitochondria, and that carnitine acetyltransferase occurs in peroxisomes and mitochondria [13]. In our experiments, carnitine acetyltransferase activity in liver increased more than the activity of carnitine palmitoyltransferase. Carnitine acetyltransferase activity may be required not only for the mitochondrial function but also for the transport of acetyl-CoA produced by peroxisomal β-oxidation to other subcellular organelles, i.e. mitochondria.

Reddy and Kumar [16] reported that a marked increase in the content of a polypeptide with a mol. wt of 80,000, identifiable as a peroxisomal protein, was observed by SDS–polyacrylamide gel electrophoresis of the large granule fraction of the livers of rats treated with several hypolipidemic drugs, including clofibrate. They suggested that the increase of polypeptide may be associated with hypolipidemic effects. We have obtained an analogous result using acetylsalicylic acid. The effects of acetylsalicylic acid and clofibrate on the content of the polypeptide were essentially the same.

In this paper, we have shown a decrease in triglyceride levels of the liver and serum, an enhancement of peroxisomal fatty acyl-CoA oxidizing activity and an increase in peroxisomal polypeptide with a mol. wt of 80,000 from livers of rat fed acetylsalicylic acid. These phenomena are similar to those caused by treatment with clofibrate. This suggests the existence of a common mechanism directly relating these events to the proliferation of peroxisomes which may be playing an important role in fatty acid metabolism.

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