Biochemical Pharmacology, Vol. 42, No. 2, pp. 239–246, 1991
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EFFECT OF SORBIC ACID FEEDING ON PEROXISOMES AND SORBOYL-CoA METABOLIZING ENZYMES IN MOUSE LIVER

SELECTIVE INDUCTION OF 2,4-DIENOYL-CoA HYDRATASE

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(*Received* 20 *December* 1990; *accepted* 5 *March* 1991)

Abstract—On the basis of the finding that sorbic acid (SA)-induced hepatoma was correlated with the depletion of reduced glutathione (GSH) in mouse liver (Tsuchiya *et al.*, *Mutation Res* **130**: 267–262, 1984), the possible conversion of SA to a metabolite which is reactive with SH-compounds was studied. Sorboyl-CoA was hydrated and then reduced to 3-keto-4-hexenoyl-CoA by the combined actions of mitochondrial hydratase (crotonase) and L-3-hydroxyacyl-CoA dehydrogenase. Upon the addition of GSH or coenzyme A, 3-keto-4-hexenoyl-CoA was nonenzymatically converted to another 3-ketoacyl-CoA derivative, possibly a Michael type adduct, in a time- and concentration-dependent manner. Alternatively, sorboyl-CoA can be reduced by 2,4-dienoyl-CoA reductase and completely β-oxidized without the generation of 3-keto-4-hexenoyl-CoA. Two-week feeding of mice of 15% SA caused a 2.0-fold induction of peroxisome β-oxidation in the liver. SA caused a marked induction (3.6-fold) of hydratase toward sorboyl-CoA but a less pronounced induction (1.3-fold) of 2,4-dienoyl-CoA reductase, leading to about a 3-fold elevation in the hydratase : reductase ratio. The elevated ratio was sustained throughout the period of SA feeding up to 12 weeeks. Thus, a large amount of SA could be converted to 3-keto-4-hexenoyl-CoA during this period. Oxidative stress caused by a depleted cellular SH-pool together with the induction of peroxisome proliferation by SA-feeding may implicate the mechanism by which non-mutagenic SA caused hepatoma.

Sorbic acid (SA,† 2,4-hexadienoic acid) is an antimicrobial preservative for food with very low acute and chronic toxicity [1]. Although no mutagenicity has been observed [2], it has been reported that 88-week feeding on a 15% SA-containing diet caused hepatoma in mice [3]. Since the intestinal contents of mice fed on SA showed mutagenicity, it has been proposed that orally given SA was converted to unidentified mutagenic metabolites in the intestine which, after transportation to the liver, caused hepatoma [4, 5].

Induction of peroxisome proliferation has been implicated in the mechanism by which non-mutagenic compounds, such as clofibrate and di(2-ethylhexyl)phthalate (DEHP), cause hepatoma [6, 7]. A number of structurally dissimilar compounds can induce peroxisome proliferation. Several hypolipidemic drugs, certain phthalate ester plasticizers, and high fat diets constitute major categories of peroxisomal proliferators [6]. Since all peroxisome proliferators are, or can be, converted to the corresponding carboxylic acid by hydrolysis or oxidation, it seems likely that SA induces peroxisome proliferation.

Another possible mechanism could be the conversion of SA to a metabolite which is reactive with SH-compounds in liver. We were interested in the striking similarities in structure and possible metabolic fate between SA and 4-pentenoic acid (4-PA), a known inhibitor of mitochondrial fatty acid β-oxidation [8] (Scheme 1). The precise mechanism by which 4-PA inhibits mitochondrial β-oxidation has been demonstrated by Schulz [9]. According to this mechanism [9], the direct β-oxidation of 4-PA via 2,4-pentadienoyl-CoA leads to the formation of 3-keto-4-pentenoyl-CoA. This can inhibit 3-ketoacyl-CoA thiolase irreversibly, possibly owing to the formation of a covalent bond between the SH-group of the enzyme and the acryloyl residue of 3-keto-4-pentenoyl-CoA. Highly reactive 3-keto-4-pentenoyl-CoA spontaneously forms an adduct in the presence of free coenzyme A (CoA) [9]. Similarly, the direct β-oxidation of SA can yield 3-keto-4-hexenoyl-CoA, which is analogous to 3-keto-4-pentenoyl-CoA but has an extra methyl-group (Scheme 1). Although the reactivity for Michael addition of the methylacryloyl (crotonoyl)-residue in 3-keto-4-hexenoyl-CoA seems lower than that of the acryloyl-residue in 3-keto-4-pentenoyl-CoA because of its steric hindrance, it is still possible that 3-keto-4-hexenoyl-CoA reacts to sequester free CoA and thereby reduces the cellular

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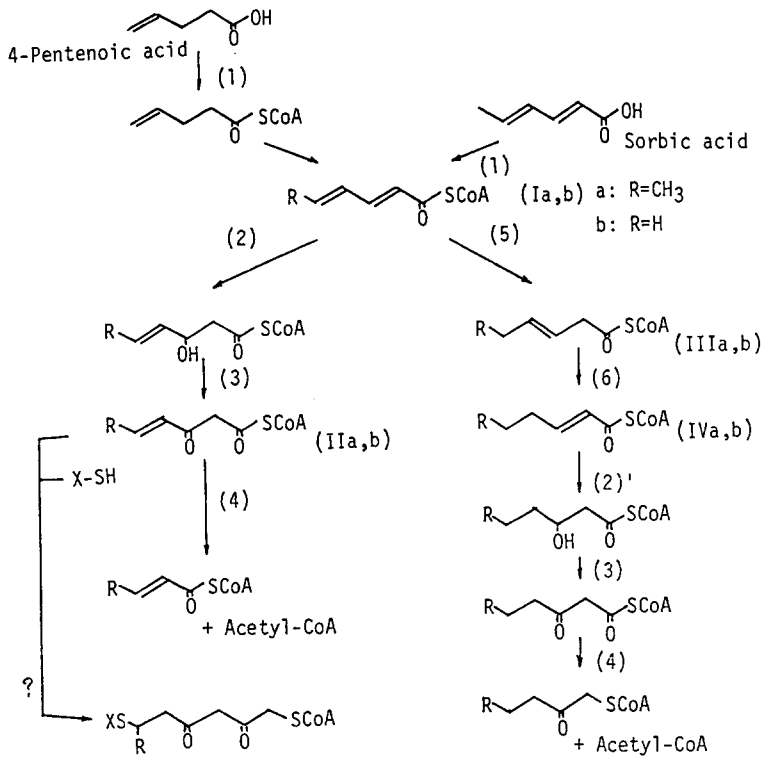
† Abbreviations: SA, sorbic acid; SH, sulfhydryl; GSH, reduced glutathione; DEHP, di(2-ethylhexyl)phthalate; 4-PA, 4-pentenoic acid.



Scheme 1. β-Oxidation pathway of sorbic acid and 4-pentenoic acid. (Ia) sorboyl-CoA; (Ib) 2,4-pentadienoyl-CoA; (IIa) 3-keto-4-hexenoyl-CoA; (IIb) 3-keto-4-pentenoyl-CoA; (IIIa) 3-hexenoyl-CoA; (IIIb) 3-pentenoyl-CoA; (IVa) 2-hexenoyl-CoA; (IVb) 2-pentenoyl-CoA. (1) acyl-CoA synthetase; (2) 2,4-dienoyl-CoA hydratase; (2)′ crotonase; (3) L-3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase, (5) 2,4-dienoyl-CoA reductase, (6) *cis*-3,*trans*-2-enoyl-CoA isomerase.

SH pool. Indeed, depletion of cellular reduced glutathione (GSH) in the liver has been observed after three-month feeding of 15% SA in mice [4]. An alternative pathway of β-oxidation by which 4-PA can be degraded has been established [10]. According to this pathway, 2,4-pentenoyl-CoA can be reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to 3-pentenoyl-CoA which can be finally degraded to acetyl-CoA and propionyl-CoA without the generation of the active intermediate, 3-keto-4-pentenoyl-CoA [9, 11, 12]. 2,4-Dienoyl-CoA reductase is located mainly in rat liver mitochondria [13, 14] and can be markedly induced by peroxisome proliferators [12–15]. The finding that clofibrate feeding abolishes the inhibition of mitochondrial β-oxidation by 4-PA in rat hepatocytes [12] strongly supports this mechanism by which 4-PA is metabolized to a suicidal substrate [9]. In addition, this finding indicates that the balance of the two enzymes 2,4-dienoyl-CoA hydratase and reductase is crucial. Hence it is very interesting to examine the effect of SA feeding on the activities of the two enzymes, which determine the rate of the conversion from SA to a potential active metabolite, 3-keto-4-hexenoyl-CoA.

The following results are outlined in this paper: first, an induction of hepatic peroxisome proliferation was caused by SA feeding in mice. Second, SA caused a marked induction of 2,4-dienoyl-CoA hydratase but a less pronounced induction of 2,4-dienoyl-CoA reductase. Third, it was found that SA could be converted to 3-keto-4-hexenoyl-CoA by mitochondrial enzymes and that this compound was able to react with SH-compounds spontaneously.

MATERIALS AND METHODS

Materials. Sorbic acid and crotonic anhydride were obtained from the Tokyo Kasei Kogyo Co. (Tokyo, Japan); NAD, NADPH and coenzyme A were obtained from the Kyowa Hakko Co. (Tokyo, Japan); ATP, crotonase from bovine liver, 3-hydroxyacyl-CoA dehydrogenase from pig heart, and lactate dehydrogenase were from the Sigma Chemical Co. (St Louis, MO, U.S.A.); *trans*-2-, *trans*-4-decadienoic acid and *trans*-2-, *trans*-4-hexadecadienoic acid were gifts from Dr M. Mizugaki (Tohoku University, Sendai). All other chemicals were of analytical grade. CoA esters were synthesized by mixed-anhydride methods [16]. Crotonyl-CoA was synthesized from crotonic anhydride [17].

Animals and treatment. Four-week-old male JcI/

ICR mice (16–20 g) were obtained from the Nihon Kurea Co. The mice were fed *ad lib.* on a CE-2 diet (Nihon Kurea Co.) or a diet containing 15% (w/w) SA, prepared according to the composition described by Tsuchiya *et al.* [5], for 2–12 weeks.

Subcellular fractionations. The mice livers were isolated and perfused with ice-cold 0.9% NaCl. Part of the liver was frozen at −70° until the enzymes were assayed. The other part of the liver was homogenized with cold 0.25 M sucrose and fractionated into nuclear, heavy mitochondrial, light mitochondrial, microsomal and cytosolic fractions by the method of de Duve *et al.* [18]. Each fraction was homogenized in 50 mM potassium phosphate (KP_i, pH 7.0) containing 0.1% (w/v) Triton X-100 prior to enzyme assay. For the separation of mitochondria and peroxisomes, part of the light mitochondrial fraction (*ca.* 70 mg) was suspended in 0.25 M sucrose (2 mL), and layered on discontinuous sucrose density gradients (total volume 33 mL) and centrifuged according to the method of Osumi and Hashimoto [19]. Fractions (2.3 mL) were collected from the tops of tubes, and diluted with an equal volume of 50 mM KP_i (pH 7.4) containing 0.2% (w/v) Triton X-100 prior to enzyme assay. The frozen liver was thawed, homogenized in nine volumes of 50 mM KP_i (pH 7.4), and centrifuged at 600 *g* for 10 min. The resulting supernatant was diluted with an equal volume of 50 mM KP_i (pH 7.4) containing 0.2% (w/v) Triton X-100, and the enzymes were assayed.

Enzyme assays. Glutamate dehydrogenase [20], urate oxidase [18], and catalase [21] were assayed according to the known methods. Peroxisomal β-oxidation and mitochondrial β-oxidation activities were determined at 37° as described by Osumi and Hashimoto [19] and Shindo and Hashimoto [22], respectively. 2,4-Dienoyl-CoA reductase was assayed as described previously [15] in the presence of 0.1 mg/mL bovine serum albumin (BSA) for 40 μM sorboyl-CoA or 0.15 mg/mL for 30 μM 2,4-decenoyl-CoA. 2,4-Dienoyl-CoA hydratase was assayed by monitoring NADH formation at 340 nm. A typical assay mixture of 0.8 mL contained 0.2 M KP_i (pH 8.0), 125 μM NAD, 0.1 mg/mL BSA, 40 μM sorboyl-CoA, 3-hydroxyacyl-CoA dehydrogenase (1.6 units), 1 mM KCN, 0.0125% (w/v) Triton X-100 and enzyme solution. For the assay with 2,4-decenoyl-CoA (40 μM), 0.2 mg/mL BSA was added. Crotonase was assayed by monitoring a decrease in absorbance at 280 nm. A typical assay mixture of 0.8 mL contained 0.2 M KP_i (pH 8.0), 5 mM EDTA, 20 μM crotonyl-CoA, BSA (0.1 mg/mL) and enzyme solution. Alternatively, crotonase was assayed by following the formation of NADH in the presence of 3-hydroxyacyl-CoA dehydrogenase (0.8 units), 125 μM NAD and 1 mM KCN. All enzyme assays except for β-oxidation assays were carried out at 25°.

Enzymatic conversion of sorboyl-CoA to 3-keto-4-hexenoyl-CoA and reaction with SH-compounds. The reaction was carried out according to the methods of Schulz [9] with minor modifications. A typical reaction mixture of 1.0 mL contained 0.1 M Tris (pH 8.5), 25 mM $MgCl_2$, 125 μM NAD, 10 mM sodium pyruvate, lactate dehydrogenase (1 unit), L-3-hydroxyacyl-CoA dehydrogenase (0.8 unit), crotonase (5 units) and 60 μM sorboyl-CoA. The reaction was started by the addition of crotonase to the sample cuvette. After the absorbance at 337 nm reached a maximum, GSH or CoA was added as indicated. In some cases, the reaction mixture was filtered with Centricon 10 (Amicon, Danvers, MA, U.S.A.) at 4° prior to the addition of SH-compounds. The extinction coefficient of the Mg^{2+}-enolate complex of 3-keto-4-hexenoyl-CoA, an approximate value of $18.4 \times 10^3\ M^{-1}\ cm^{-1}$, was calculated on the basis of the increase in absorbance at 337 nm and, after addition of EDTA, the decrease in absorbance

Table 1. Effect of sorbic acid feeding for 2 weeks on peroxisomal and mitochondrial enzymes in mouse liver homogenates

	Control	Sorbic acid
	(nmol/min/mg protein)	
Catalase	0.80 ± 0.15	1.37 ± 0.32†
Urate oxidase	5.52 ± 1.50	10.90 ± 1.56‡
Glutamate dehydrogenase	5.47 ± 0.65	5.17 ± 0.77
Peroxisomal β-oxidation	7.92 ± 1.06	15.41 ± 2.91‡
Mitochondrial β-oxidation	0.94 ± 0.04	0.86 ± 0.12
2,4-Dienoyl-CoA reductase		
Sorboyl-CoA	6.17 ± 0.48	8.28 ± 1.54*
2,4-Decenoyl-CoA	15.1 ± 2.0	18.9 ± 3.1
2,4-Dienoyl-CoA hydratase		
Sorboyl-CoA	1.38 ± 0.21	4.99 ± 0.65‡
2,4-Decenoyl-CoA	0.93 ± 0.17	3.33 ± 0.38†
Crotonase	225 ± 18	1027 ± 192‡

Results are means ± SD for five mice. Mitochondrial β-oxidation was measured with the heavy mitochondrial fraction. The recovery of the fraction was estimated from the recovered activity of glutamate dehydrogenase.

Statistically significant effects are indicated by *$P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$.

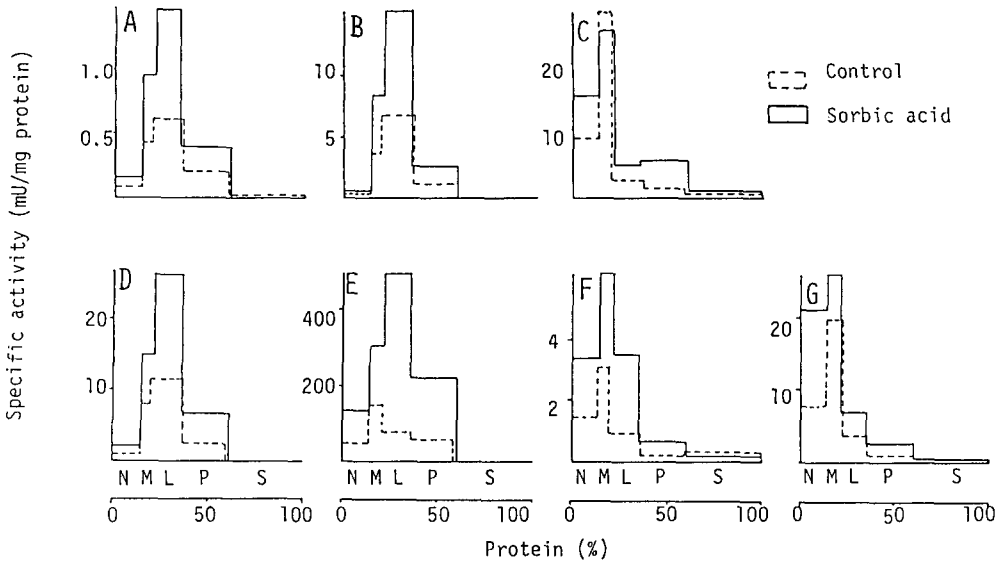


Fig. 1. Subcellular distribution of 2,4-dienoyl-CoA reductase, 2,4-dienoyl-CoA hydratase, crotonase and some marker enzymes in the control and SA-fed mouse livers. The livers from control mice (N = 5) or SA-fed mice (N = 5) were homogenized and fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and cytosolic (S) fractions, and enzyme activities were determined as described in the text. The activity of crotonase was expressed in μmol/mg. (A) Catalase; (B) urate oxidase; (C) glutamate dehydrogenase; (D) peroxisomal β-oxidation; (E) crotonase; (F) 2,4-dienoyl-CoA hydratase; (G) 2,4-dienoyl-CoA reductase.

at 300 nm using the value of $19.1 \times 10^3\ M^{-1}\ cm^{-1}$ for sorboyl-CoA [23].

RESULTS

Effects of sorbic acid feeding on peroxisomes and sorboyl-CoA metabolizing enzymes

The feeding of SA (15%) for 2 weeks of mice caused inductions in peroxisomal enzymes, peroxisomal β-oxidation (2.0-fold), urate oxidase (1.9-fold) and catalase (1.7-fold) in the liver homogenates (Table 1). In contrast, no change was observed in mitochondrial β-oxidation and in the activity of the mitochondrial marker enzyme glutamate dehydrogenase. 2,4-Dienoyl-CoA hydratase activity toward sorboyl-CoA increased markedly (3.6-fold), whereas 2,4-dienoyl-CoA reductase activity toward sorboyl-CoA increased only 1.3-fold. In agreement with the report that a purified 2,4-dienoyl-CoA reductase from rat liver shows the maximum activity toward 2,4-decenoyl-CoA [15], 2,4-dienoyl-CoA reductase activity in mouse liver showed higher activity toward 2,4-decenoyl-CoA. However, the increase of reductase activity toward this substrate was not significant, whereas a marked increase (3.6-fold) in 2,4-dienoyl-CoA hydratase for 2,4-decenoyl-CoA was observed.

Subcellular distribution of enzyme activities was studied with differential centrifugation (Fig. 1). The distribution of 2,4-dienoyl-CoA reductase was similar to that of glutamate dehydrogenase, being located mainly in the mitochondria, in agreement with previous findings with rat liver [13, 14]. SA feeding did not change the distribution. 2,4-Dienoyl-CoA hydratase was found mainly in the N (nuclear), M (heavy mitochondrial) and L (light mitochondrial) fractions, suggesting that 2,4-dienoyl-CoA hydratase was located both in mitochondria and peroxisomes. SA feeding markedly increased crotonase (hydratase toward crotonyl-CoA) activity in the N, M, L and P (microsomal) fractions. The increase in the L and P fractions was apparently greater than in the N and M fractions, suggesting that induction of the peroxisomal bifunctional enzyme was more marked than that of mitochondrial crotonase. In contrast, increased activity of 2,4-dienoyl-CoA hydratase by SA was observed mainly in the N, M and L fractions, suggesting that mitochondrial activity was mainly increased. Separation of mitochondria and peroxisomes in the L fraction by sucrose density centrifugation also indicated that an induction of mitochondrial was more marked than that of peroxisomal 2,4-dienoyl-CoA hydratase (Fig. 2).

Prolonged feeding of sorbic acid

The prolonged feeding of SA for up to 12 weeks did not change the peroxisomal enzyme activities; elevated activities of peroxisomal β-oxidation and catalase compared to the control group were observed throughout the period of SA treatment. Relative liver weight gradually increased compared to the control and became significant with time. Both the marked elevation (3.6–5.0-fold of control) of 2.4-dienoyl-CoA hydratase and the slight elevation (1.3–1.5-fold) of 2,4-dienoyl-CoA reductase were unchanged by prolonged sorbic acid feeding.

Enzymatic conversion of sorboyl-CoA to 3-keto-4-hexenoyl-CoA and reaction with SH-compounds

The conversion of sorboyl-CoA to a potential active

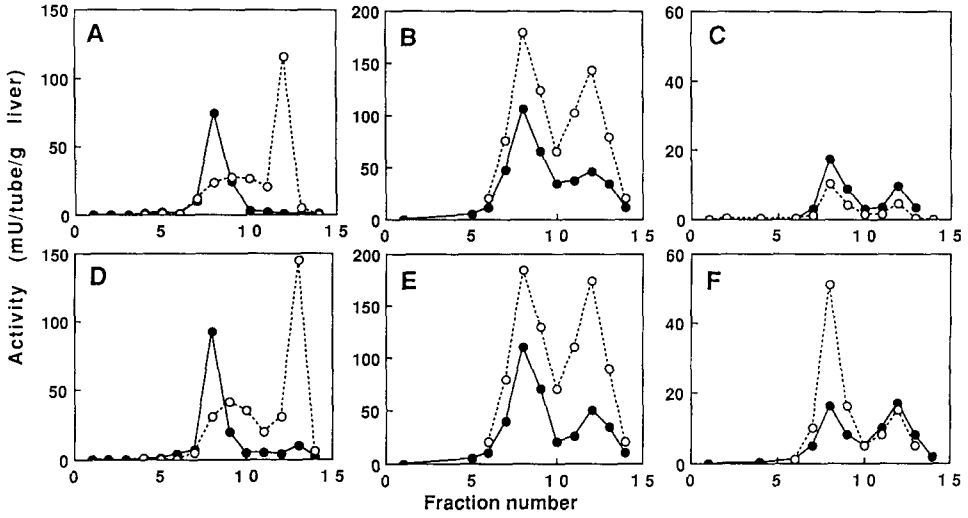


Fig. 2. Sucrose density gradient centrifugation of the light mitochondrial fraction. Sucrose solutions were layered discontinuously in 35 mL tubes, as described in the text. Light mitochondrial fractions (70 mg/2 mL, 2.5 g liver) were layered above the gradient, and then centrifuged. Enzymes were assayed as described in the text. Crotonase activity was expressed in μmol/tube/g liver. (A) and (D) Glutamate dehydrogenase (—●—) and urate oxidase (- - -○- - -); (B) and (E) 2,4-dienoyl-CoA reductase for sorboyl-CoA (—●—) and *trans*-2-, *trans*-4-decadienoyl-CoA (- - -○- - -); (C) and (F) crotonase (—●—) and 2,4-dienoyl-CoA hydratase for sorboyl-CoA (- - -○- - -). (A–C) control mice; (D–F) SA fed mice.

metabolite, 3-keto-4-hexenoyl-CoA was studied with a similar approach to that for 2,4-pentadienoyl-CoA reported by Schulz [9]. Incubation of sorboyl-CoA with mitochondrial crotonase and 3-hydroxyacyl-CoA dehydrogenase in the presence of NAD^+, NAD^+-regenerating system and Mg^{2+} led to a decrease in the characteristic absorbance of sorboyl-CoA at 290 nm and a concomitant increase in the absorbance at 337 nm in a time-dependent manner (Fig. 3A). The absorbance at 337 nm, comparable to that of the Mg^{2+}-enolate complex of 3-keto-4-pentenoyl-CoA at 334 nm [9], appears to be due to the Mg^{2+}-enolate complex of 3-keto-4-hexenoyl-CoA, because the addition of excess EDTA caused the disappearance of the peak at 337 nm. Similarly, the peak at 337 nm did not appear when Mg^{2+} was omitted from the incubation mixture, whereas a new absorbance at 339 nm, presumably due to the enol form of 3-keto-4-hexenoyl-CoA, appeared when the pH of the incubation mixture was brought to 11.

The reaction of 3-keto-4-hexenoyl-CoA with SH-compounds was studied. The addition of 2.0 mM GSH to 3-keto-4-hexenoyl-CoA (Fig. 3A, the curve at 24 min) caused the disappearance of its chromophore at 337 nm and concomitant appearance of a new peak at 315 nm (Fig. 3B). The appearance of a new chromophore at 315 nm, which was also observed with the addition of CoA (data not shown), was dependent on time (Fig. 3B) and the concentration of SH-compounds (Fig. 4). The separation of enzymes by ultrafilitration did not affect the spectrum change caused by SH-compounds, indicating that this event is non-enzymatic. The incubation of crotonyl-CoA in the same manner yielded a chromophore at 303 nm due to the Mg^{2+}-complex of 3-ketobutanoyl-CoA. However, the addition of 2.0 mM GSH did not cause any change in the spectrum. This result clearly indicates that a double bond at 4-position of 3-keto-4-hexenoyl-CoA is essential for the formation of a chromophore at 315 nm, and excludes the possibility that the change in the spectrum was simply due to the destruction of the Mg^{2+}-enolate complex by SH-compounds. Therefore, it is very likely that the noncatalytic reaction of 3-keto-4-hexenoyl-CoA with an SH-compound yielded a new compound; possibly a Michael adduct in which the SH-group was attached to carbon 5 position of 3-keto-4-hexenoyl-CoA, as reported for 3-keto-4-pentenoyl-CoA [9].

DISCUSSION

In mice, SA can be degraded completely to CO_2 [24]. There are two pathways of β-oxidation by which SA is degraded. According to a classic pathway of β-oxidation, SA, after conversion to sorboyl-CoA, is hydrated and then dehydrated to 3-keto-4-hexenoyl-CoA, which is cleaved into 2-butenoyl-CoA and acetyl-CoA.

Importantly, 3-keto-4-hexenoyl-CoA, an intermediate of this pathway, can react with free CoA as shown in the present study. According to the newly discovered pathway [10], sorboyl-CoA is reduced directly by 2,4-dienoyl-CoA reductase to 3-hexenoyl-CoA, which after isomerization to 2-hexenoyl-CoA, is degraded to three molecules of acetyl-CoA by regular β-oxidation. The two enzymes reductase and hydratase for 2,4-dienoyl-CoA thus constitute the branch point of the SA degradation pathway. In agreement with the previous reports (reviewed in Ref. 12), the measurement of the activities of the

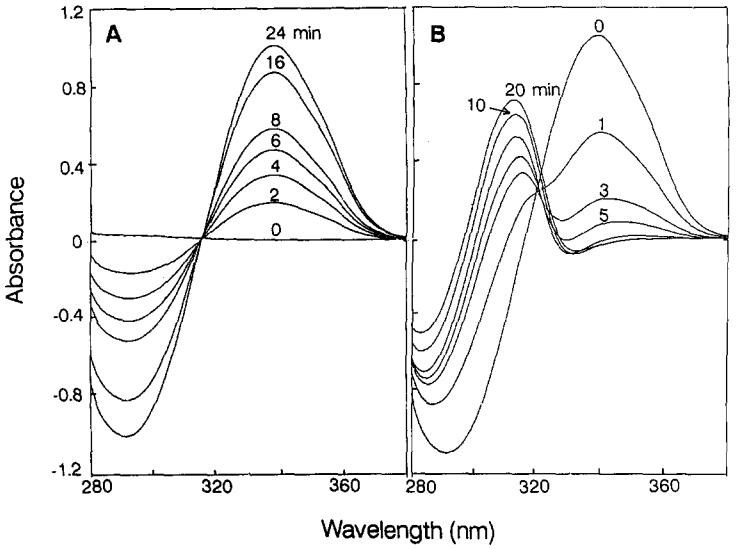


Fig. 3. (A) Conversion of sorboyl-CoA to 3-keto-4-hexenoyl-CoA (Mg^{2+}-enolate complex) catalysed by crotonase and L-3-hydroxyacyl-CoA dehydrogenase. The reference cuvette contained all compounds except for crotonase, as described in the text, and the reaction was started by adding crotonase to the sample cuvette. Spectral changes after 0, 2, 4, 6, 8, 16 and 24 min were recorded. (B) Spectral changes of 3-keto-4-hexenoyl-CoA (Mg^{2+}-enolate complex) by the addition of 2 mM GSH after 0, 1, 3, 5, 10 and 20 min. GSH was added both to the sample and reference cuvette, after 24 min in (A).

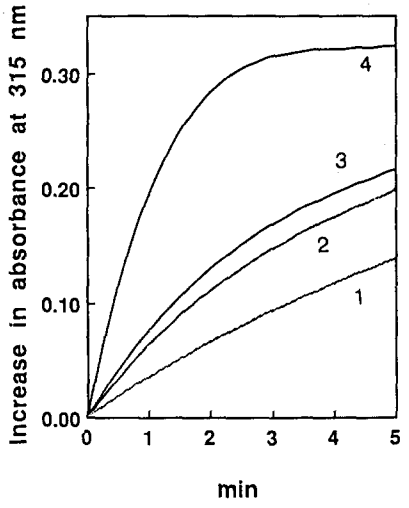


Fig. 4. New chromophore at 315 nm, dependent on the concentration of SH-compounds. Mg^{2+}-enolate complex of 3-keto-4-hexenoyl-CoA (20 μM) was prepared as described in Fig. 3A and reacted with the following concentrations of SH-compounds: (1) 0.5 mM CoA; (2) 1.0 mM CoA; (3) 2.0 mM CoA; (4) 2.0 mM GSH.

two enzymes in this study indicated that the 2,4-dienoyl-CoA reductase-dependent pathway is the major pathway. When sorboyl-CoA was used as a substrate, the ratio of the activities of 2,4-dienoyl-CoA hydratase to reductase was 0.22 in the control liver homogenate. However, SA feeding for 2 weeks elevated the ratio to 0.60 because of a selective induction of 2,4-dienoyl-CoA hydratase. In rat liver, 2,4-dienoyl-CoA reductase was markedly induced by clofibrate and DEHP treatment [12–15]. However, even after the prolonged feeding of SA for 12 weeks, the induction of 2,4-dienoyl-CoA reductase was still less pronounced than that of 2,4-dienoyl-CoA hydratase, and the elevated ratio of hydratase to reductase was thereby sustained during the period. Consequently, in the livers of mice fed with SA, approximately 28–38% of the administered SA can be degraded via the 2,4-dienoyl-CoA hydratase-dependent pathway, which leads to the formation of 3-keto-4-hexenoyl-CoA.

The present study shows that 3-keto-4-hexenoyl-CoA can react with CoA or glutathione to form an unidentified compound. The structural similarity of 3-keto-4-hexenoyl-CoA to 3-keto-4-pentenoyl-CoA, derived from 4-PA [9], suggests the formation of a Michael-type adduct. The reactivity of 3-keto-4-hexenoyl-CoA appears to be much lower than that of 3-keto-4-pentenoyl-CoA. Indeed, the concentration of free CoA that we used in this study was approximately 10-fold higher than that used for the 4-PA experiment by Schulz [9]. This may explain why SA, unlike 4-PA [10], does not show acute toxicity [1] and does not affect mitochondrial β-oxidation, as shown in this study. However, long term SA feeding at a high concentration (15%) can yield a large amount of 3-keto-4-hexenoyl-CoA by the selective induction of 2,4-dienoyl-CoA hydratase. Possibly, the amount of this compound was large enough to sequester free CoA and thereby deplete the cellular SH pool, as indeed seen in the depletion of cellular GSH levels (40% of control) after 3-month feeding on 15% SA [4].

Table 2. Prolonged effect of 15% SA feeding on relative liver weight, catalase, peroxisomal β-oxidation, 2,4-dienoyl-CoA reductase and 2,4-dienoyl-CoA hydratase in mouse liver

Feeding (week)	SA treatment	Relative liver weight (%)	Catalase	Peroxisomal β-oxidation	2,4-Dienoyl-CoA reductase	2,4-Dienoyl-CoA hydratase
				(nmol/min/mg)		
4	−	6.21 ± 0.76	0.76 ± 0.12	6.80 ± 0.97	5.81 ± 0.61	0.73 ± 0.12
	+	6.80 ± 0.39*	0.90 ± 0.17	12.16 ± 1.96‡	8.98 ± 1.04‡	3.64 ± 0.33‡
8	−	5.59 ± 0.47	0.74 ± 0.12	6.58 ± 1.36	6.28 ± 0.73	0.87 ± 0.13
	+	7.72 ± 1.09†	1.21 ± 0.16‡	14.07 ± 2.73‡	8.13 ± 1.22*	3.16 ± 0.28‡
12	−	6.26 ± 0.21	0.69 ± 0.16	6.97 ± 1.15	5.71 ± 0.70	1.18 ± 0.20
	+	8.44 ± 0.05‡	1.09 ± 0.14*	14.97 ± 2.68‡	7.95 ± 0.95†	4.46 ± 0.51‡

2,4-Dienoyl-CoA reductase and 2,4-dienoyl-CoA hydratase were assayed with sorboyl-CoA as a substrate. Results are mean ± SD for five mice.

Statistically significant effects are indicated by *$P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$.

While SA induces some peroxisome proliferation, studies with fatty acids of various chain lengths [25], *S*-alkylthioacetic acids [26] and dicarboxylic acids [26] show that it is only induced by the compounds having long-acyl chains. In contrast, certain short-chain fatty acids, such as perfluoro-octanoic acid [27] and valproic acid [28], can induce peroxisome proliferation. Since they cannot be β-oxidized, blocking of β-oxidation seems to be an essential factor for peroxisome proliferation. Although it is not clear whether SA can be β-oxidized at a rate comparable to that of hexanoic acid, it appears that a requirement of β-oxidation must increase in the liver of mice fed on SA at a high concentration. Thus, it is likely that the induction of peroxisome β-oxidation may be an adaptive response to satisfy this requirement.

In the present study, the subcellular distribution of 2,4-dienoyl-CoA reductase showed that the degradation of SA via the 2,4-dienoyl-CoA reductase-dependent pathway took place mainly in mitochondria, in agreement with previous findings for rat liver [13, 14]. 2,4-Dienoyl-CoA hydratase activity was found in both mitochondria and peroxisomes. However, since it is generally accepted that fatty acids shorter than hexanoic acid are not β-oxidized in peroxisomes [29], it is not clear whether SA can be metabolized in peroxisomes. In contrast to the remarkable induction of peroxisomal bifunctional enzyme, a marked induction of 2,4-dienoyl-CoA hydratase in mitochondria, but not in peroxisomes, was observed in the present study. Thus, it is likely that mitochondria but not peroxisomes plays an important role in SA degradation and conversion to 3-keto-4-hexenoyl-CoA. Partially hydrogenated marine oil containing trans-unsaturated fatty acids which are poorly β-oxidized in mitochondria induced peroxisomal β-oxidation [30]. Valproic acid, a peroxisome proliferator, inhibits β-oxidation in mitochondria [31] but not in peroxisomes [32]. Thus, occupation of mitochondrial enzymes by SA and its metabolites could be a trigger of peroxisome proliferation.

Long term administration of peroxisome proliferators to rodents resulted in the development of hepatocellular carcinoma [6, 7]. Unlike many carcinogens, peroxisome proliferators are not DNA reactive or mutagenic [7]. Since they cause a marked induction of H_2O_2-generating peroxisomal β-oxidation enzymes, it has been hypothesized that the carcinogenicity may be mediated by oxidative DNA damage resulting from persistent peroxisome proliferation [6, 7]. Increased lipid peroxidation and increases in hepatic lipofuscin by peroxisome proliferators [7, 33] support this hypothesis. Recent reports demonstrated that administration of peroxisome proliferators caused a significant increase in the formation of 8-hydroxydeoxyguanosine in the liver DNA of rats [34–36]. For SA, the significant induction of peroxisomal β-oxidation implies that the carcinogenic mechanism of SA may involve oxidative stress. Indeed, depletion of GSH level was observed in SA-fed mice [4]. However, since the induction of peroxisomal β-oxidation by SA was far less potent than that seen in carcinogenic peroxisome proliferators [6, 7], it is conceivable that the elevation of H_2O_2 level by peroxisome proliferation is not large enough to cause tumors. However, it should be noted that SA feeding also caused a selective induction of 2,4-dienoyl-CoA hydratase which could dramatically increase the conversion of SA to 3-keto-4-hexenoyl-CoA. Thus, it is plausible that the depletion of cellular SH, possibly due to this reaction, enhances the oxidative stress resulting from the induction of peroxisomal H_2O_2-generating enzymes, to cause DNA damage in the liver of SA-fed mice.

Acknowledgement—The authors thank Prof. Michinao Mizugaki for the gift of 2,4-dienoic acids.

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