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EFFECTS OF ACETYLSALICYLIC ACID ON PARAMETERS RELATED TO PEROXISOME PROLIFERATION IN MOUSE LIVER

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Abstract—Male C57 BL/6 mice were exposed to 1.0% (w/w) acetylsalicylic acid (ASA) in their diet for 10 days and effects related to peroxisome proliferation were subsequently examined. A 2.2-fold increase in mitochondrial protein content was obtained. The activities of the peroxisomal enzymes, lauroyl-CoA oxidase, palmitoyl-CoA oxidation and catalase, were enhanced 4.5-, 4.0- and 2.1-fold, respectively. There was a dramatic increase (9.1-fold) in microsomal cytochrome P450 IVA-catalysed activity, a 1.6-fold induction of total microsomal P450 content and a 2-fold induction of microsomal cytochrome P450 reductase activity (measured as NADPH-cytochrome *c* reductase). Catalase activity in the cytosol was induced 5.2-fold and DT-diaphorase activity was increased 3.5- and 3.2-fold in the cytosol and mitochondria, respectively. There was a significant increase in the susceptibility of microsomes to lipid peroxidation. Smaller increases in superoxide dismutase, glutathione transferase and glutathione peroxidase activities were also observed. The possible relevance of these effects to the pharmacology of ASA is discussed.

Key words: acetylsalicylic acid; peroxisome proliferation; fatty acid β-oxidation; cytochrome P450 IVA; catalase; DT-diaphorase; liver; mouse

Peroxisomes are cellular organelles which are found in almost every eukaryotic cell [1–4]. One of the most characteristic features of peroxisomes is that this organelle is remarkably proliferated in response to structurally diverse xenobiotic agents, so-called peroxisome proliferators (e.g. clofibrate, nafenopin, phtalates, perfluoro fatty acids, etc.) in rodents. Some of these agents are components of drugs and others are widely used in industry.

Peroxisome proliferation increases the number and size of hepatic peroxisomes, greatly induces peroxisomal acyl-CoA† oxidase (which is generally rate-limiting for peroxisomal fatty acid β-oxidation) and causes a lesser induction of peroxisomal catalase, which degrades H_2O_2. The catalase activity of hepatic cytosol is much increased and liver mitochondrial protein enhanced [5]. Many xenobiotic-metabolizing enzymes are also affected by peroxisome proliferators, for example, DT-diaphorase, cytosolic epoxide hydrolase, etc. There is invariably induction of microsomal cytochrome P450 IVA, which is specialized for the ω- and ω-1-hydroxylation of fatty acids, when peroxisome proliferation occurs [31–33].

Long-term treatment of rodents with peroxisome proliferators has been found to give rise to liver tumours [6]. Investigation of the mechanism for hepatomegaly and hepatocarcinogenesis caused by several representatives of this class of substances has revealed that they are apparently not directly genotoxic. Because peroxisome proliferators increase acyl-CoA oxidase activity much more than catalase, Reddy and Lalwani [7] hypothesized that the main cause for carcinogenesis may be a leakage of excess oxidase-produced H_2O_2 from peroxisomes. However, a definitive correlation between H_2O_2 leakage and hepatocarcinogenesis has not been established.

ASA (aspirin) is a widely used clinical drug, which makes it especially interesting in studies concerning peroxisome proliferation. The aim of this work has been to investigate the effects of acetylsalicylic acid on parameters related to peroxisome proliferation in mouse liver. In the future the mechanism underlying these effects and their possible relevance to the clinical properties of this drug will be studied further.

MATERIALS AND METHODS

Chemicals

Lauric acid, 1-chloro-2,4-dinitrobenzene, H_2O_2 (30%), NAD^+, KCN, NaN_3 (E. Merck, Darmstadt, Germany), palmitoyl-CoA, lauroyl-CoA, NADPH, CoA, cytochrome *c*, ASA, menadione and dicoumarol (Sigma Chemical Co., St Louis, MO, U.S.A.) and horseradish peroxidase (Boehringer-Mannheim, Bromma, Sweden) were all purchased from the sources indicated.

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† Abbreviations: DT, DPNH/TPNH (i.e. NADH/NADPH); TLC, thin-layer chromatography; ASA, acetylsalicylic acid; CoA, coenzyme A; GST, glutathione transferase; GTP, glutathione peroxidase; SOD, superoxide dismutase.

Animals and treatment

Male C57 Bl/6 mice (ALAB, Sollentuna, Sweden) weighing about 18–22 g were used throughout this study. The animals were housed in steel cages in groups of three or four with a 12 hr light/dark cycle at 25°. They were given commercial food pellets R_3 and tap water. ASA was administered in the diet, which was prepared according to Lundgren *et al.* [8]. A control diet was prepared in the same manner, but without addition of ASA. The food was stored at −20° prior to use. All dosages are given as weight percentage in the diet. A 1% dose corresponds to ingestion of approximately 1.5 g/kg daily. There were no signs of toxicity at any of the doses or with any of the periods of administration employed.

Preparation of liver subcellular fractions was performed as described previously [9]. Peroxisomes were recovered primarily in the mitochondrial fraction.

Assays

Lauroyl-CoA oxidase. Activity was monitored in the mitochondrial fraction by quantitation of H_2O_2 production with a fluorometric method according to Pooch and Yamazaki [10].

Palmitoyl-CoA oxidation. The mitochondrial fraction was measured as the reduction of NAD^+ at 340 nm in the presence of KCN as an inhibitor of mitochondrial β-oxidation [11, 12].

Catalase activity. This was assayed in the mitochondrial and cytosolic fractions by monitoring the disappearance of H_2O_2 at 240 nm [13].

DT-diaphorase. Activity was determined by the method of Ernster [14].

SOD. Activity was assayed using a fluorometric method, according to Segura-Aguilar [15].

GST. Activity was measured spectrophotometrically at 340 nm using 1-chloro-2,4-di-nitrobenzene as a second substrate [16, 17].

GTP. Activity was quantitated spectrophotometrically by the method of Gunzler *et al.* [18].

Microsomal cytochrome P450. This was quantitated according to Omura and Sato [19].

NADPH-cytochrome P450 reductase. Activity was measured spectrophotometrically as NADPH-cytochrome *c* reductase [20].

Cytochrome P450 IVA. This was assayed using [^{14}C]lauric acid as diagnostic substrate. The incubation medium was loaded onto a Merck Silica gel TLC plate, the plate developed and the radioactivity quantified using a Rita radioactivity TLC scanner [21].

Lipid peroxidation. This was determined by measuring the uptake of O_2 in the presence of ADP, Fe^{3+} and NADPH using an oxygen electrode [22]. Thiobarbituric acid-reactive compounds, which are products of lipid peroxidation, were assayed using the method of Ernster and Nordenbrand [23].

Protein. This was determined according to Lowry *et al.* [24] with bovine serum albumin as the standard.

Statistical analysis. Each experimental group contained three or four animals. Data are given as means ± SD and the results of the Student's *t*-test are given where appropriate.

RESULTS

Effects on the protein contents of subcellular fractions

Feeding mice with ASA at different doses for 10 days had an effect on the protein contents of the subcellular fractions (Table 1). Protein contents increased significantly in the three subcellular fractions at doses of 0.5 and 1.0% (w/w). There was a 2.2-fold increase in the protein content of the mitochondrial fraction at the 1.0% dose. Although the protein contents increased significantly in microsomal and cytosolic fractions at these two doses, the increase was much greater in the mitochondrial fraction. There were no effects on the protein contents at a dose of 0.1%.

Effects on peroxisomal enzymes

When the mice were treated with 1.0% ASA for 10 days, the liver peroxisomal lauroyl-CoA oxidase and palmitoyl-CoA oxidation were increased by 4.5- and 4.0-fold, respectively (Fig. 1). The former catalyses the first step of peroxisomal β-oxidation of fatty acids, producing H_2O_2, while the latter measures the whole process of peroxisomal β-oxidation. The peroxisomal catalase was slightly induced (2-fold).

Effects on microsomal enzymes

Peroxisome proliferation is always accompanied by induction of IVA isozymes of microsomal cytochrome P450. Using 1.0% ASA in the diet for 10 days, a slight induction of total microsomal P450 content was obtained. Further assays revealed some induction of NADPH-cytochrome P450 reductase and a relatively large induction (9.1-fold) of cytochrome P450 IVA (Fig. 2).

Considering Table 1, and Figs 1 and 2, it can be concluded that ASA caused significant peroxisome proliferation in mouse liver.

Effects on enzymes which detoxify reactive oxygen and decompose H_2O_2

Peroxisome proliferation produces H_2O_2, which can then give rise to even more reactive oxygen species. To investigate the effect of ASA on enzymes detoxifying H_2O_2 and reactive oxygen, hepatic cytosolic catalase and DT-diaphorase were measured, and found to be dramatically increased (Fig. 3), whereas only small effects on hepatic GST, GTP and SOD were obtained.

Effects on microsomal and mitochondrial susceptibility to lipid peroxidation

At a dose of 1.0% (w/w) for 10 days, some increase in susceptibility to lipid peroxidation in the microsomal fraction compared with the control group was obtained. This lipid peroxidation was initiated by ADP–Fe^{3+}–NADPH, it was determined by measuring the uptake of O_2. The reaction was stopped by adding 3.5% trichloroacetic acid. Formation of products of lipid peroxidation, so-called thiobarbituric acid-reactive material, increased (Table 2).

Effects of dose and period of administration on peroxisomal and related enzymes

Mice were fed ASA at three different doses, i.e.

Table 1. Effects of dietary exposure to ASA (w/w) for 10 days on the protein contents of the mitochondrial, microsomal and cytosolic fractions from mouse liver

Treatment	Mitochondrial protein*	Microsomal protein*	Cytosolic protein*
None (control)	9.95 ± 1.20	12.4 ± 1.82	30.3 ± 1.28
0.1% ASA	9.46 ± 1.80	15.1 ± 1.65	35.1 ± 2.27†
0.5% ASA	14.4 ± 2.20†	20.2 ± 0.93‡	37.9 ± 0.92‡
None (control)	12.2 ± 1.10	11.3 ± 1.74	33.8 ± 2.40
1.0% ASA	26.4 ± 4.35‡	16.0 ± 1.83†	47.7 ± 1.60‡

* mg/g liver.
Significantly different from control †$P < 0.05$, ‡$P < 0.001$.

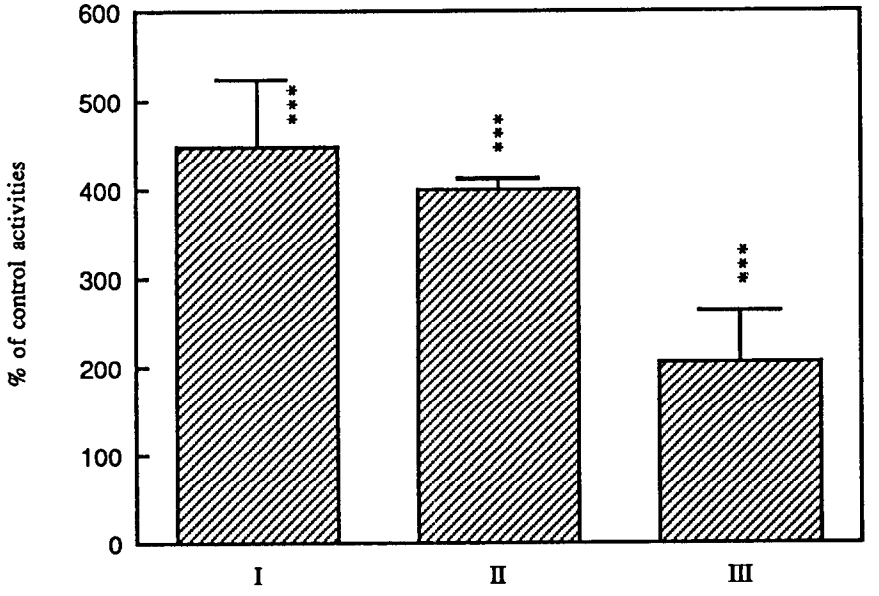

Fig. 1. Effects of ASA (1.0%, w/w, 10 days) on hepatic peroxisomal lauroyl-CoA oxidase(I), palmitoyl-CoA oxidation(II) and catalase(III) activities. The control activities (i.e. 100%) were 0.574 ± 0.066 μmol/min, 0.229 ± 0.029 μmol/min and 16.9 ± 2.53 mmol/min per gram of liver, respectively. Significantly different from control: ***$P < 0.01$.

0.1, 0.5 and 1.0% (w/w), for 10 days and some peroxisomal and related enzymes were measured. The results showed that the effect of ASA on peroxisome proliferation increased with increasing doses (Fig. 4). On the other hand, mice were also treated with ASA at a dose of 1.0% (w/w) for different periods (Fig. 5). From Fig. 5 it can be seen that the activities of enzymes which produce H_2O_2 (lauroyl-CoA oxidase, palmitoyl-CoA oxidation) were first induced to their peak levels, but the activities of enzymes which were responsible for decomposing H_2O_2 were induced later and continued to increase during the period of treatment. In order to obtain information on the effect of long-term, low-dose treatment with ASA in mouse liver, mice were treated with ASA at a dose of 0.1% (w/w) for 2 months. Under these conditions there were no significant changes in hepatic peroxisomal lauroyl-CoA oxidase, cytosolic catalase or microsomal ω- and ω-1-hydroxylation (data not shown).

DISCUSSION

ASA has been studied as a peroxisome proliferator primarily in rat [25, 26] and mouse [8, 27] livers. To date there have been no reports on the effects of ASA on lauroyl-CoA oxidase in peroxisomes, catalase in cytosol, cytochrome P450 IVA in microsomes, lipid peroxidation in microsomes and mitochondria, GTP, SOD in mitochondria and cytosol in mouse liver. This is the first report regarding these parameters and provides a detailed picture of peroxisome proliferation and related effects of ASA in mouse liver. Like other peroxisome proliferators, ASA increased the hepatic mitochondrial protein content (Table 1), enhanced

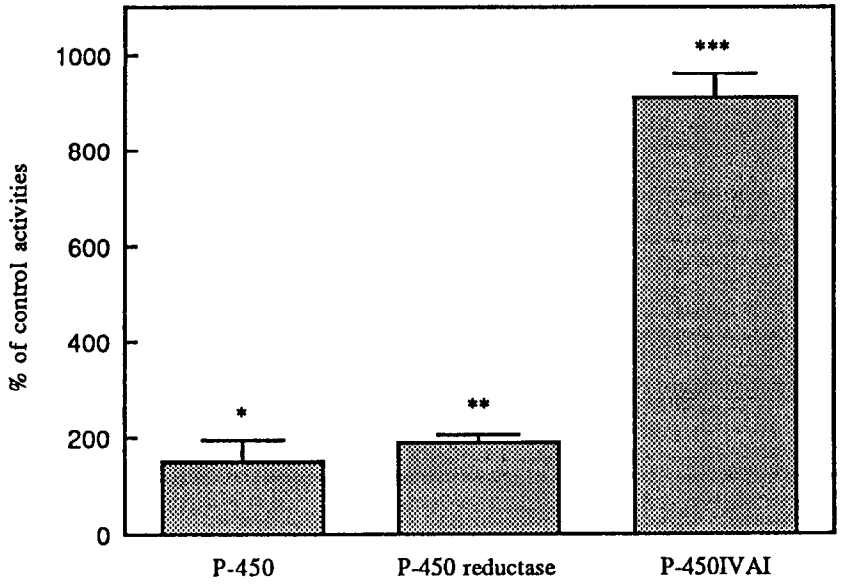

Fig. 2. Effects of ASA (1.0%, w/w, 10 days) on hepatic cytochrome microsomal P450, P450 reductase and P450 IVA. The control values (i.e. 100%) were 11.6 ± 1.30 nmol/g liver, 3.04 ± 0.140 nmol/min per gram of liver and 30.0 ± 7.42 nmol/min per gram of liver, respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group.

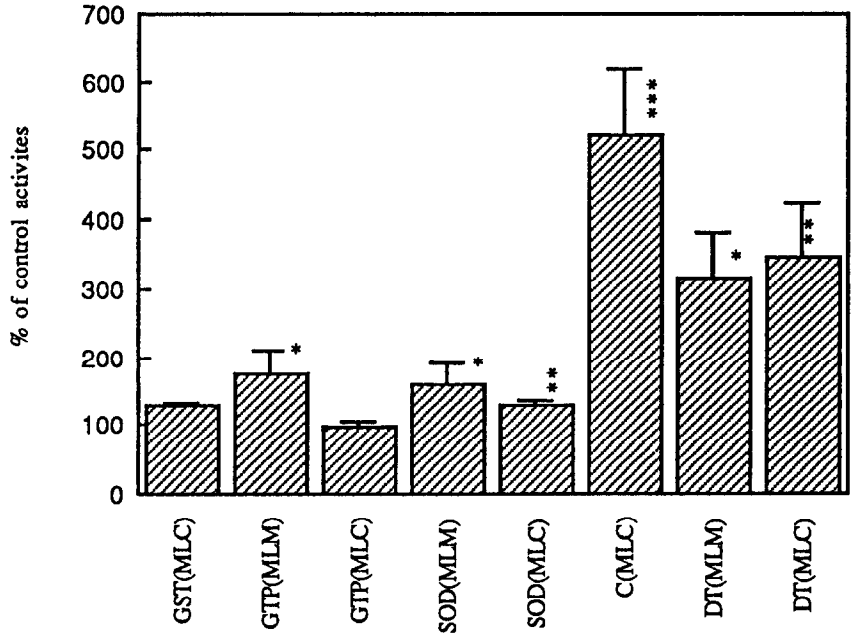

Fig. 3. Effects of ASA (1.0%, w/w, 10 days) on enzymes which detoxify reactive oxygen and decompose hydrogen peroxide. The control activities (i.e. 100%) were as follows: GST, 0.262 ± 0.031 mmol/min per gram of liver; GTP, 0.820 ± 0.150 and 28.6 ± 4.02 nmol/min per gram of liver in mitochondria and cytosol, respectively; SOD, 4.07 ± 0.79 and 95.8 ± 8.87 nmol/min per gram of liver in mitochondria and cytosol, respectively; C, 2.54 ± 0.936 mmol/min per gram of liver; DT, 11.11 ± 2.42 nmol/min per gram of liver and 3.76 ± 1.57 mmol/min per gram of liver in mitochondria and cytosol, respectively. MLC, mouse liver cytosol; MLM, mouse liver mitochondria. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group.

Table 2. Effects of dietary treatment with 1% ASA (w/w) for 10 days on lipid peroxidation in microsomal and mitochondrial fractions from mouse liver

Subfraction	Group	O_2-consumption*	Thiobarbituric acid reactive material†
Microsome	Control	24.9 ± 4.49	5.97 ± 0.29
	Treated	61.8 ± 22.0‡	11.5 ± 2.98‡
Mitochondria	Control	18.7 ± 5.36	4.32 ± 1.12
	Treated	18.1 ± 3.86	3.13 ± 1.22

* nmol O_2/min/mg protein.
† nmol malondiadehyde/min/mg protein.
‡ Significantly different from control: $P < 0.05$.

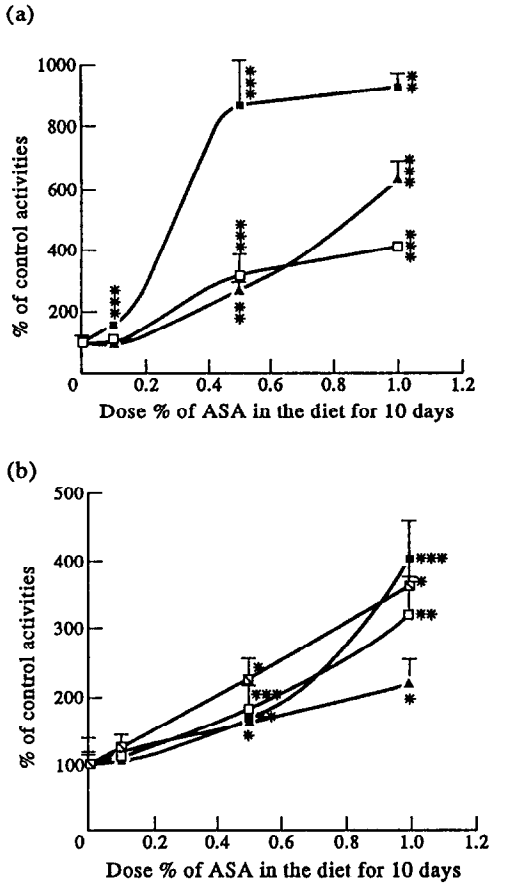

Fig. 4. Effects of dose (0.1%, 0.5% and 1.0%, w/w, 10 days) of ASA on peroxisomal and related enzymes. (a) Microsomal P450IVAI (■), peroxisomal lauroyl-CoA oxidase (▲) and peroxisomal palmitoyl-CoA oxidation (□). The control activities (i.e. 100%) were 27.1 ± 3.74 nmol/min per gram of liver, 0.376 ± 0.075 mmol/min per gram of liver and 0.312 ± 0.062 mmol/min per gram of liver. (b) Catalase in cytosol (■) and in mitochondria (▲). DT-diaphorase in cytosol (□) and DT-diaphorase in mitochondria (□). The control activities (i.e. 100%) were 2.54 ± 0.936 mmol/min, 16.9 ± 2.53 mmol/min, 3.76 ± mmol/min and 11.11 ± 2.42 nmol/min per gram of liver, respectively.

certain hepatic peroxisomal enzymes (Fig. 1), induced IVA isoenzymes of hepatic microsomal cytochrome P450 (Fig. 2) and certain other enzymes (Fig. 3). ASA is a moderate peroxisome proliferator compared with perfluoro-octanoic acid (a very potent peroxisome proliferator) [28–30], although ASA caused a potent induction of hepatic microsomal cytochrome P450 IVA activity.

Peroxisome proliferation is invariably accompanied by induction of microsomal cytochrome P450 IVA and, indeed, there may be a mechanistic relationship between these two process [31–33]. The effect of ASA on this enzyme was, in fact, more potent than its effect on peroxisomal fatty acid β-oxidation, since as much as a 6-fold induction of IVA on the second day and a 10-fold increase on the fifth day when mice were fed ASA at a dose of 1.0% (Fig. 5) were obtained. Most other peroxisome proliferators exert at least as great an effect on peroxisomal fatty acid β-oxidation as on cytochrome P450 IVA [28, 29].

It is well known that ASA (aspirin) inhibits the synthesis of prostaglandins from arachidonic acid by blocking cyclo-oxygenase activity [34, 35]. Cytochrome P450 IVA is capable of metabolizing arachidonic acid to ω- and ω-1-hydroxy-arachidonic acids [36, 37]. Therefore, the possibility arises that ASA also inhibits the synthesis of prostaglandins by another mechanism as well, i.e. by inducing cytochrome P450 IVA, which lowers the substrate (arachidonic acid) concentration.

Another finding was that peroxisomal lauroyl-CoA oxidase activity was strongly induced, while, in contrast, peroxisomal catalase activity was less induced (Fig. 1), as is usually the case with peroxisome proliferators [8, 38, 39]. These imbalanced inductions might result in elevated H_2O_2 and reactive oxygen levels in the cell. Measurements of hepatic enzymes which detoxify H_2O_2 and reactive oxygen showed DT-diaphorase (cytosol and mitochondria) and catalase (cytosol) to be greatly increased, with only small induction of GST in cytosol, GTP and SOD in mitochondria and cytosol (Fig. 3).

To determine whether these inductions of detoxifying enzymes were enough to protect cells from oxidative stress, the effects of ASA on the susceptibility of microsomal and mitochondrial fractions from mouse liver to lipid peroxidation were tested; increase in O_2-consumption and thiobarbituric acid-reactive material in the microsomal fraction

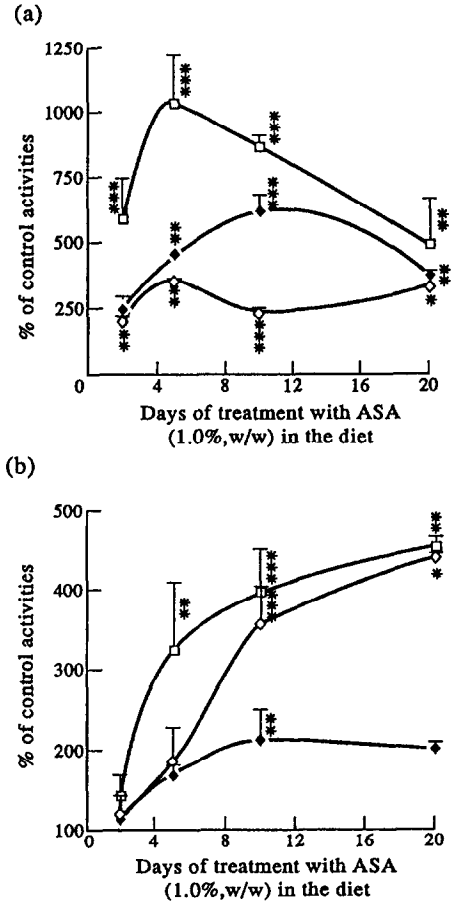

Fig. 5. Effects of period of ASA administration (1.0%, w/w for 2, 5, 10 or 20 days) on peroxisomal and related enzymes. (a) Microsomal P450 IVA (□): the control activities (i.e. 100%) were 19.0 ± 0.48, 19.0 ± 5.53, 20.4 ± 4.44 and 27.9 ± 10.4 nmol/min per gram of liver for 2, 5, 10 and 20 days, respectively. Peroxisomal lauroyl-CoA oxidase (◆): the control activities were 409 ± 122, 415 ± 54.4, 224 ± 32.9 and 195 ± 39.3 nmol/min per gram of liver for 2, 5, 10 and 20 days, respectively. Peroxisomal palmitoyl-CoA oxidation (◇): the control activities were 372 ± 27.0, 285 ± 12.3, 243 ± 68.9 and 222 ± 30.7 nmol/min per gram of liver for 2, 5, 10 and 20 days, respectively. (b) Catalase in cytosol (□): the control activities were 2.27 ± 0.52, 2.19 ± 0.18, 2.96 ± 0.61 and 2.41 ± 0.42 mmol/min per gram of liver for 2, 5, 10 and 20 days, respectively. Catalase in mitochondria (◆): the control activities were 33.5 ± 9.14, 22.6 ± 7.88, 16.2 ± 2.00 and 12.7 ± 2.15 mmol/min per gram of liver. DT-diaphorase in cytosol (◇): the control activities were 10.7 ± 4.53, 10.5 ± 4.26, 9.99 ± 3.15 and 8.67 ± 2.81 μmol/min per gram of liver for 2, 5, 10 and 20 days, respectively.

were found (Table 2). The results suggest that the treated cells were more susceptible to oxidative stress. The time curve (Fig. 5) revealed that the DT-diaphorase and catalase activities in hepatic cytosol were induced later and continued to increase, while lauroyl-CoA oxidase and palmitoyl-CoA oxidation were first induced to their peak levels and became somewhat lower after 10 days of treatment. Lauroyl-CoA oxidase and palmitoyl-CoA oxidation produce H_2O_2, which can be transformed into even more reactive species of oxygen, whereas catalase and DT-diaphorase detoxify the H_2O_2 and reactive quinones. It seems that long-term treatment may stimulate cells to produce more enzymes protecting against H_2O_2 or reactive oxygen.

It should be emphasized that lipid peroxidation may be closely related to the severity of atherosclerosis. There is evidence that oxidative modification of low-density lipoprotein (LDL) may induce vascular inflammation and even give rise to autoimmune reactions in the vascular wall, finally resulting in atherosclerosis and myocardial infarction [40]. Since ASA significantly increased microsomal susceptibility to lipid peroxidation in mouse liver, chronic high doses of ASA might possibly play a role in development of atherosclerosis.

Some patients receiving long-term treatment with ASA for atherosclerosis and heart-attack prevention receive a very low dose (about 75 mg ASA/day) [41]. Whether or not there are significant effects of such long-term, low-dose administration of ASA in humans on the parameters investigated here needs to be further investigated. It will also be interesting to elucidate the mechanism for the induction of cytochrome P450 IVA and its biological function in peroxisome proliferation.

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