Effects of Dose Levels of Autoxidized Linoleic Acid on the Drug-Metabolizing System in Rat Liver

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Summary Autoxidized linoleic acid (AL) having 800 meq/kg of peroxide value and 1,700 meq/kg of carbonyl value was given in repeated oral doses at a daily dose of 0 (control)–7.5 ml/kg to male Wistar rats for 5 successive days. The effect of increasing AL dose on the drug-metabolizing system was investigated in rat liver microsomes and S-9 fractions. All the rats of a daily dose of 5.0–7.5 ml/kg died after the third day of consecutive oral doses. The cytochrome P-450 and b5 contents, enzyme activities in electron transfer system, aminopyrin-N-demethylase activity and S-9 activity (metabolic activation of 2-acetylaminofluorene) in the drug-metabolizing system changed essentially in a similar manner, that is, both the contents and the activities were increased by a small dose of AL, and were decreased by a large dose of AL. These results strongly supported the findings in a previous report wherein we observed the periodical effect of AL dose on the drug-metabolizing system.

Key Words autoxidized linoleic acid, microsome, cytochrome P-450, cytochrome b5, electron transfer system, drug-metabolizing activity, aminopyrin-N-demethylase, S-9 activity, membrane disorder

There have been some incompatible findings on the effect of autoxidized oil on drug-metabolizing activity in rat liver microsomes under the respective experimental conditions (1, 2). Therefore, in our previous work (3), AL having 800 meq/kg of peroxide value and 1,700 meq/kg of carbonyl value was administered periodically for 1–15 days at a daily dose of 2.5 ml/kg body weight to male Wistar rats, and the effect of AL on the drug-metabolizing system in rat liver microsomes was studied. The cytochrome P-450 and b5 contents were increased by consecutive oral doses of AL for 3–7 days, then the amount of cytochrome P-450 decreased gradually, but the b5 decreased slightly. Thus, after administration for 11–15 days, the cytochrome P-450 content was significantly lower, but the cytochrome b5 content was rather high.
in comparison with the control group. The aminopyrin-N-demethylase activity was not reduced even after repeated oral dose of AL for 15 days, while the activation of 2-acetylaminofluorene (2-AAF) gradually decreased during administration for more than 3 days, and disappeared after administration for 9 days.

This time we plan to elaborate upon the changes in drug-metabolizing activity caused by increasing dose levels of AL for a relatively short period. Thus, in the present study, changes in cytochrome P-450 and h5 contents, the enzyme activities of electron transfer system, and drug-metabolizing activities are investigated in liver microsomes or S-9 fractions of male Wistar rats administered increasing levels of AL for 5 successive days.

MATERIALS AND METHODS

Chemicals. Linoleic acid was of extra-pure reagent grade from Nakarai Chemical Co. (Kyoto), and its purity as determined by gas chromatography was about 95%. NADPH and NADH were purchased from the Oriental Yeast Co. (Tokyo). Bovine serum albumin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and cytochrome c from horse heart were obtained from Sigma Chemical Co. (USA). Nutrient broth and agar powder were purchased from Difco (USA). The other chemicals were of guaranteed reagent grade from Nakarai Chemical Co. (Kyoto). Pantethine (60% solution) was kindly supplied by Daiichi Seiyaku Co. (Tokyo).

Animals and diet. Male Wistar rats (JCL, Tokyo, Japan), aged 3 weeks, were divided into 6 groups (5 rats/group), and were fed for one month on the same diet (Clea Japan Inc., Tokyo) as described in the previous report (3). The components of the diet consisted of 24.5% vitamin free casein, 45.5% corn starch, 10.0% granulated sugar, 6.0% corn oil, 3.0% avicel, 2.0% KC flock, 1.0% a-starch, 1.0% vitamin mixture without pantothenic acid, and 7.0% mineral mixture. The drinking water contained 0.00625% pantethine, because the diet contained no pantothenic acid and its related compounds. The diet and drinking water were provided ad libitum. No differences in diet and water intakes and growth were observed between the groups before the administration of AL.

Administration of AL. After the initial feeding for one month, AL prepared by the same way as previously described (3) was orally administered to the rats in each group at a daily dose of 0 (control), 1.5, 2.5, 3.5, 5.0, and 7.5 ml/kg body weight.

Preparation of S-9 and microsomes. The S-9 and microsomes were prepared from rat liver as noted previously (3).

Measurements of the enzyme activities in electron transfer system. The NADPH-cytochrome c reductase activity was determined by the method of Omura and Takesue (4). NADPH and cytochrome c from horse heart were added to the liver microsomal suspension, and an increase in absorbance at 550 nm (AE550) was determined. The specific activity was calculated according to the following equa-
tion: specific activity (µmol/min/mg protein) = ΔE<sub>550</sub>/min × volume (ml) of reaction mixture/21.1 × mg protein in reaction mixture.

The NADH-ferricyanide reductase activity was determined by the method of Takesue and Omura (5). The NADH, potassium ferricyanide, and liver microsomal suspension were used as electron donor, electron acceptor, and enzyme, respectively. The specific activity was calculated according to the following equation: specific activity (µmol/min/mg protein) = ΔE<sub>420</sub>/min × volume (ml) of reaction mixture/1.02 × mg protein in the mixture, where ΔE<sub>420</sub> is a decrease in absorbance at 420 nm in the reaction system.

The NADH-cytochrome c reductase activity was determined by the method of Takesue and Omura (6). NADH and cytochrome c from horse heart were added to the liver microsomal suspension, and an increase in absorbance at 550 nm (ΔE<sub>550</sub>) was determined. The specific activity was calculated according to the following equation: specific activity (µmol/min/mg protein) = ΔE/min × volume (ml) of reaction mixture/21.1 × mg protein in the mixture.

Other measurement methods. Cytochrome P-450 and cytochrome b<sub>5</sub> contents were determined by the method of Omura and Sato (7) as previously described (3). The metabolic activation of 2-AAF, which we termed S-9 activity, and aminopyrine-N-demethylase activity were determined according to the methods of Yahagi (8) and Kato et al. (9), respectively. Protein was determined by the procedure of Lowry et al. (10) with bovine serum albumin as the standard.

Statistical analysis. Student's t-test was used to determine statistical significance. The variability of the data is presented as mean ± SD.

RESULTS

Lethal dose level

All the rats of the 7.5 ml/kg group had died by the fourth day of consecutive oral doses. Two rats of the 5.0 ml/kg group died on the fourth day of consecutive oral doses, and the other rats died on the fifth day. Two rats of the 3.5 ml/kg group died on the fifth day, and the other rats lived until the morning after the fifth day of consecutive oral doses. On the fourth day of the doses, the groups of a daily dose of 5.0 ml/kg and 7.5 ml/kg were respectively supplemented with 5 rats. Thus, the effects of AL dose on the drug-metabolizing system were investigated for the groups of a daily dose of 0–3.5 ml/kg for 5 successive days and for the groups of a daily dose of 5.0–7.5 ml/kg for 2 successive days.

Changes in body weight

No difference in growth between the six groups of rats was observed during the initial feeding period. Even after AL administration, body weights in the 1.5 ml/kg and 2.5 ml/kg groups were not significantly different from that of the control group, though a retardation of body weight gain occurred on the first day at a daily dose of 2.5 ml/kg (Fig. 1). The body weights in the 3.5–7.5 ml/kg groups kept on decreasing
Fig. 1. Changes in body weight by increasing AL dose. —○—, control (without AL dose); ---○---, 1.5 ml AL/kg body weight; —■—, 2.5 ml AL/kg body weight; ---■---, 3.5 ml AL/kg body weight; —△—, 5.0 ml AL/kg body weight; ---△---, 7.5 ml AL/kg body weight.

Fig. 2. Changes in cytochrome P-450 content in rat liver microsomes by increasing AL dose. ◦, AL doses for 5 successive days; □, AL doses for 2 successive days.

Fig. 3. Changes in cytochrome b5 content in rat liver microsomes by increasing AL dose. ◦, AL doses for 5 successive days; □, AL doses for 2 successive days.

Changes in cytochrome P-450 and cytochrome b5 contents

The cytochrome P-450 and b5 contents were measured after AL administration.
Fig. 4. Changes in NADPH-cytochrome c reductase of electron transfer system by increasing AL dose. ●, AL doses for 5 successive days; ○, AL doses for 2 successive days.

Fig. 5. Changes in NADH-cytochrome c reductase of electron transfer system by increasing AL dose. ●, AL doses for 5 successive days; ○, AL doses for 2 successive days.

The change in cytochrome P-450 content is shown in Fig. 2. The maximal content was obtained by the dose of 2.5 ml/kg, but the content decreased sharply and significantly (0.025 < p < 0.05) in the 3.5 ml/kg group in comparison with control, and the greater decrease was observed in the dose of 5.0–7.5 ml/kg. The change in cytochrome b<sub>5</sub> content is shown in Fig. 3. The cytochrome b<sub>5</sub> content increased significantly in the 1.5 (0.01 < p < 0.025)–3.5 (0.05 < p < 0.1) ml/kg groups. The maximal content was observed in the 2.5 ml/kg group. Unlike cytochrome P-450, the cytochrome b<sub>5</sub> content did not significantly decrease in the 5.0–7.5 ml/kg group in comparison with control, although it decreased significantly (0.001 < p < 0.005) in comparison with the 2.5 ml/kg group.

Changes in enzyme activities of electron transfer system

As shown in Fig. 4, the NADPH-cyt. c reductase activity increased significantly (0.001 < p < 0.005) at a daily dose of 2.5 ml/kg in comparison with the control, and the maximal activity was obtained at this dose level. However, the enzyme activity decreased significantly in the 3.5 (0.005 < p < 0.01) ml/kg group and the 5.0 (p < 0.001)–7.5 (p < 0.001) ml/kg group in comparison with the group of maximal activity, although no differences were observed compared with control.

The mean of NADH-cyt. c reductase activity showed the maximal activity in
Fig. 6. Changes in NADH-ferri (CN) reductase of electron transfer system by increasing AL dose. ●, AL doses for 5 successive days; ○, AL doses for 2 successive days.

Fig. 7. Changes in aminopyrin-N-demethylase activity in rat liver S-9 fraction by increasing AL dose. ●, AL doses for 5 successive days; ○, AL doses for 2 successive days.

the 2.5 ml/kg group, but the value was not significantly different from both control and 1.5 ml/kg groups (Fig. 5). However, the enzyme activity decreased significantly (0.01 < p < 0.025) in the 3.5 ml/kg group in comparison with the 2.5 ml/kg group, and also decreased significantly in the 5.0–7.5 ml/kg group in comparison with the groups of a daily dose of 0 (p < 0.001)–3.5 (0.001 < p < 0.005) ml/kg.

The NADH-ferri (CN) reductase activity decreased gradually by a daily dose of 0–3.5 ml/kg for 5 days, but the enzyme activity decreased suddenly and significantly by a daily dose of 5.0 ml/kg for 2 days, compared with those of the groups of 0 (p < 0.001)–3.5 (0.01 < p < 0.025) ml/kg (Fig. 6).

Changes in aminopyrin-N-demethylase activity

Aminopyrin-N-demethylase activity responded to AL orally dosed, as shown in Fig. 7. The maximal mean value of aminopyrin-N-demethylase activity was obtained by the dose of the 1.5 ml/kg, but it was not significantly different from the values of the 0 and 2.5 ml/kg groups. The enzyme activity of the 3.5 ml/kg group was significantly lower than those of the 1.5 (0.025 < p < 0.05) and 2.5 (0.01 < p < 0.025) ml/kg groups. At a daily dose of 5.0–7.5 ml/kg for 2 days, the activities decreased significantly in comparison with those of the 0 (0.001 < p < 0.005)–2.5 (p < 0.001) ml/kg groups, though no differences were observed between the above groups and the dose group of 3.5 ml/kg for 5 days.

Changes in S-9 activity

As shown in Fig. 8, the S-9 activity rose significantly at a daily dose of 1.5
Fig. 8. Changes in S-9 activity in rat liver S-9 fraction by increasing AL dose. The metabolic activation of 2-AAF with the S-9 fraction was measured by Ames’ test. The number of revertants of *Salmonella typhimurium* TA 1538 was used as a measure of the total drug-metabolizing activities. I, control (without AL dose); II, 1.5 ml AL/kg body weight for 5 days; III, 2.5 ml AL/kg body weight for 5 days; IV, 3.5 ml AL/kg body weight for 5 days; V, 5.0 ml AL/kg body weight for 2 days; VI, 7.5 ml AL/kg body weight for 2 days.

We have previously reported (3) that both cytochrome P-450 and \( b_s \) contents increased by repeated AL administration for 3–7 days at a daily dose of 2.5 ml/kg, and that thereafter cytochrome \( b_s \) content scarcely decreased in contrast with cytochrome P-450 content. Patzelt-Wenczler (11) has reported that the cytochrome \( b_s \) content increased in liver microsomes of vitamin E-deficient rats, and that it was lowered by applications of vitamin E to the control level. We concluded that a rise of *in vivo* lipid peroxidation induces cytochrome \( b_s \), and supposed that this increase of cytochrome \( b_s \) leads to the acceleration of desaturation of fatty acids and
provides the unsaturated fatty acids in microsomal membrane in order to repair the
membrane injured by dosed AL and in vivo lipid peroxidation. In the present study,
responses of the drug-metabolizing system to dosed AL were investigated in liver
microsomes or S-9 fractions from rats administered increasing levels of AL. The
maximal contents of cytochrome P-450 and b5 were observed at a daily dose of
2.5 ml/kg for 5 days, but the cytochrome P-450 content decreased significantly at a
daily dose of 3.5 ml/kg for 5 days and at a daily dose of 5.0–7.5 ml/kg for 2 days
(Fig. 2). On the other hand, the content of cytochrome b5 was scarcely decreased by
the dose of 3.5 ml/kg for 5 days and 5.0 ml/kg for 2 days (Fig. 3). These results
obtained in the present experiments are evidence supporting our previous con-
clusion and supposition described above.

We have also previously reported that cytochrome P-450 is easier to break
down than cytochrome b5 during successive oral doses of AL at a daily dose of
2.5 ml/kg for relatively long periods. Jeffry et al. (12) have reported that linoleic acid
hydroperoxide destroys cytochrome P-450 in microsomes without destroying
cytochrome b5 in vitro. We have previously supposed from these facts that the
decrease of cytochrome P-450 content might be caused by the endogenous lipid
hydroperoxide from the in vivo lipid peroxidation accelerated by a large amount of
exogenous AL that arose from the elongation of the dose period (3). The data
shown in Figs. 2 and 3 obtained in the present experiments also basically support
our supposition described above.

The results shown in Figs. 4, 5, and 6 suggest that each of the enzymes in
electron transfer system is also impaired by a large amount of AL dose. However,
NADPH-cytochrome c reductase is more difficult to break down compared with the
other enzymes in electron transfer system, because no differences were observed
between the 5.0–7.5 ml/kg groups and control group, though the reason has not
been clear.

The changes in aminopyrin-N-demethylase and S-9 activities by increasing
dose levels of AL were essentially the same as those observed for cytochrome P-450
content, i.e., the drug-metabolizing activities increased in a small amount of AL
dose, and decreased in a large amount of AL dose (Figs. 7 and 8). We inferred from
these facts that the decrease of drug-metabolizing enzyme activities after a large
amount of AL dose resulted from a disorder of the microsomal membrane and the
lowering in content of cytochrome P-450 which plays a central role in the
hydroxylation of 2-AAF at the membrane, or from the inactivation of acetyl-
transferase which forms the final mutagen, though the possibility exists that the
lowering of cytochrome P-450 content and in drug-metabolizing activities by a large
amount of AL dose might arise indirectly from the reduction in digestion and
absorption abilities owing to the injury of digestive organs.

In the administration period of 5 consecutive days, cytochrome P-450 content
and drug-metabolizing enzyme activity were significantly induced in a daily dose of
2.5 ml/kg, compared with control. A small part of the ingested AL is accumulated in
the rat liver (13), and the AL contains some kinds of xenobiotics such as aldehydes
including malonaldehydes, hydroperoxy alkenals, hydroperoxy epoxides, and polymers. It is well known that cytochrome P-450 content and drug-metabolizing activity are induced by ingesting lyophilic xenobiotics, and also there have been some reports (14–18) that both cytochrome P-450 and \( b_5 \) act as peroxidase on lipid hydroperoxide. Therefore, it seems that these phenomena are reasonable responses against xenobiotics which are metabolized by the drug-metabolizing system in rat liver. Within this dosage range (i.e. 2.5 ml/kg), ingested AL and in vivo lipid peroxidation caused by the AL may be able to be disposed by in vivo antioxidation mechanisms, such as catalase (19), glutathione peroxidase (20), superoxide dismutase (19), cytochrome P-450 (14–16) and \( b_5 \) (17, 18), or antioxidants, such as vitamin E and C (21). However, the cytochrome P-450 contents and drug-metabolizing functions were lowered even with this dose level by the elongation of dose period, as previously described (3). In the present experiments, the cytochrome P-450 and drug-metabolizing activities were also decreased by large amounts of AL dose. These findings show that the damages by both given AL and in vivo lipid peroxide caused by the AL may exceed the peroxide disposition resulting from in vivo antioxidants or antioxidation mechanisms, when dose period was elongated, or a large amount of AL was given. Further investigations are necessary to clarify the effect of antioxidant on the drug-metabolizing system in rat liver given AL.

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