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Effects of saikosaponin-d on enhanced CCl_4-hepatotoxicity by phenobarbitone

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The effects of saikosaponin-d extracted from the roots of *Bupleurum falcatum* L. on increased toxicity of CCl_4 and increased activities of microsomal enzymes induced by phenobarbitone have been examined. Saikosaponin-d showed protection against the CCl_4-hepatotoxicity enhanced by phenobarbitone. It also inhibited increases in the content of cytochrome P450 and NADPH-cytochrome c reductase activity, which are induced by the phenobarbitone treatment, but the spectral characteristics of P450 were not altered. The rate of microsomal lipid peroxidation by NADPH and CCl_4 was significantly lowered in-vitro in rats pretreated with phenobarbitone and saikosaponin-d compared with those pretreated with phenobarbitone alone.

Phenobarbitone is known to induce microsomal drug-metabolizing enzymes in the rat liver (Staubli et al 1969) and enhance CCl_4 hepatoxicity (Garner & McLean 1969; McLean & McLean 1966; Stenger et al 1970). In contrast, saikosaponin-d, extracted from Bupleuri radix and used for the treatment of chronic hepatitis in oriental medicine, has been reported to decrease the activities of drug metabolizing enzymes (Abe et al 1980) and inhibit experimental hepatic injuries of CCl_4 (Abe et al 1982) and D-galactosamine (Abe et al 1980).

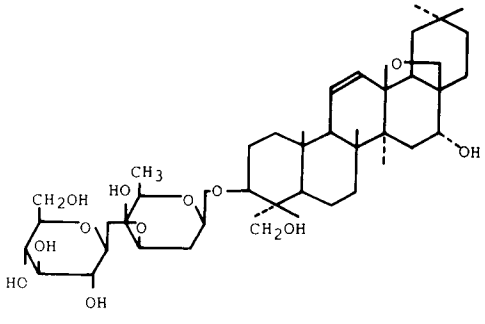
Enzyme induction by phenobarbitone is generally an undesirable effect since it causes a lowering of drug efficacy and drug reliability.

In the present study, the effect of saikosaponin-d on enzyme induction and CCl_4-hepatotoxicity enhanced by phenobarbitone has been investigated in rats.

MATERIALS AND METHODS

Preparation of saikosaponin-d

Saikosaponin-d (I) was extracted from the roots of *Bulpeurum falcatum* L. by Dr Ishii of Shionogi Research Laboratory (Shionogi and Co, Ltd, Osaka, Japan) using the methods of Kubota & Tonami (1967) and Ishii et al (1980).



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Animal procedures

Male, Wistar rats, 180–220g, five to a group, were maintained on a standard diet and had free access to tap water.

In the experiment on acute hepatic injury, the rats were pretreated with 1 mg kg^{-1} of saikosaponin-d, or 50 mg kg^{-1} phenobarbitone or both, once daily (i.m.) for 4 days. The control animals were given an equal amount of 0·9% NaCl (saline). Acute hepatic injury was induced by a single i.p. administration of 0·1 ml of 50% CCl_4 (olive oil) per 100 g weight. Serum assays were conducted 6, 24, 30 and 48 h after the CCl_4 injection. Lipid peroxide levels of liver homogenates were examined 6 h after the CCl_4 injection. Liver tissue was observed by light microscopy 24 h after the CCl_4 injection.

To study the effects on the liver microsomal enzymes, rats given saikosaponin-d, phenobarbitone or both in the above mentioned doses, for 4 days, were used.

Serum assay

Blood specimens taken from the cervical vein were used to obtain serum. Glutamic pyruvic transaminase (GPT) was assayed using a commercial kit (Wako Pure Chemical Industries, Ltd).

Preparation of liver homogenate

The liver was perfused with ice-cold saline, removed and minced in an ice cold 1·15% KCl solution. The liver blocks were homogenized with a Potter-

Elvehjem homogenizer in a 1·15% KCl solution. The homogenate was used for the examination of lipid peroxide in a concentration of 20 mg protein ml^{-1}. The protein content was determined by the method of Lowry et al (1951).

Preparation of microsomes

The liver perfused with ice-cold saline was homogenized with a Potter-Elvehjem homogenizer in 0·25 M sucrose. This homogenate was centrifuged at 1000*g* for 20 min in a refrigerated centrifuge. The resulting supernatant was further centrifuged at 102 000*g* for 90 min and the pellet was washed once in 1·15% KCl. The resuspension contained microsomes of 10 mg protein ml^{-1}.

Enzyme assays

NADPH-cytochrome c reductase activity was measured according to Williams & Kamin (1962).

The cytochrome P450 content was determined from CO difference spectra of dithionite-reduced samples with an extinction coefficient of 91 cm^{-1} mM^{-1} (Omura & Sato 1964). The difference spectra were recorded between 370–500 nm.

Assay of lipid peroxide

The lipid peroxide of the liver homogenate was measured according to Ohkawa et al (1979). The values were expressed in nmol malondialdehyde mg $protein^{-1}$ of liver homogenate, which was calculated from the absorbance at 532 nm using 1,1,3,3-tetramethoxypropane (TMP) as an external standard.

Lipid peroxidation in microsomes was measured using NADPH and CCl_4. The reaction mixture contained 2 mg protein of microsomes, 0·2 mg of NADPH and 20 µl of CCl_4 in 0·15 M KCl/10 mM Tris-HCl buffer (pH 7·4). The reaction was started by the addition of CCl_4 and the reaction mixture was incubated at 37 °C. The lipid peroxide level was determined for a definite period of time by the method described above.

Light microscopy

A portion of liver from each animal was fixed in 10% formalin, embedded in paraffin wax and stained with haematoxylin and eosin.

Electron microscopy

A portion of liver was removed, cut into small pieces, and fixed in ice-cold 3% glutaraldehyde solution (0·1 M phosphate buffer, pH 7·4). The specimens were post-fixed in 1% OsO_4 solution, dehydrated in a graded series of ethanol solutions and then embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi HS-9 electron microscope.

Table 1. Effects of saikosaponin-d and phenobarbitone pretreatment on the response to CCl_4.

Treatment	Body wt (g)	Liver wt (g)	Serum GPT level after CCl_4 administration (U $litre^{-1}$) 6 h	24 h	30 h	48 h	Lipid peroxide level of liver homogenate* (nmol MDA mg^{-1} $protein^{-1}$ of liver homogenate)
Control	249·0 ± 9·0	10·08 ± 1·06	22·2 ± 5·2	22·7 ± 4·3	21·0 ± 2·6	17·0 ± 1·7	1·80 ± 0·24
CCl_4	221·0 ± 6·0	8·35 ± 0·20	59·1 ± 6·1	4256·3 ± 521·3	3523·4 ± 340·8	947·6 ± 134·1	2·47 ± 0·19
Pheno. + CCl_4	207·6 ± 2·9	9·27 ± 0·38	171·1 ± 42·7	5741·9 ± 625·0	5882·6[a] ± 714·3	4725·0[b] ± 351·4	3·29 ± 0·37
s.s.d. + CCl_4	207·7 ± 5·6	7·34 ± 0·21[b]	34·5 ± 8·8	228·0[b] ± 73·8	72·4[b] ± 16·1	43·4[b] ± 11·1	1·96 ± 0·19
Pheno. + s.s.d. + CCl_4	194·5 ± 4·6[b]	8·13 ± 0·22	87·4 ± 20·3	4219·1[1] ± 343·4	2600·1[1] ± 418·1	810·6[1] ± 142·1	2·09 ± 0·11[2]

[a] $P < 0·01$, [b] $P < 0·001$: Statistically significant difference from CCl_4-treatment.
[1] $P < 0·05$, [2] $P < 0·01$: Statistically significant difference between groups with phenobarbitone and with phenobarbitone and saikosaponin-d.
* 6 h After CCl_4 administration.
Values are the mean ± s.e.

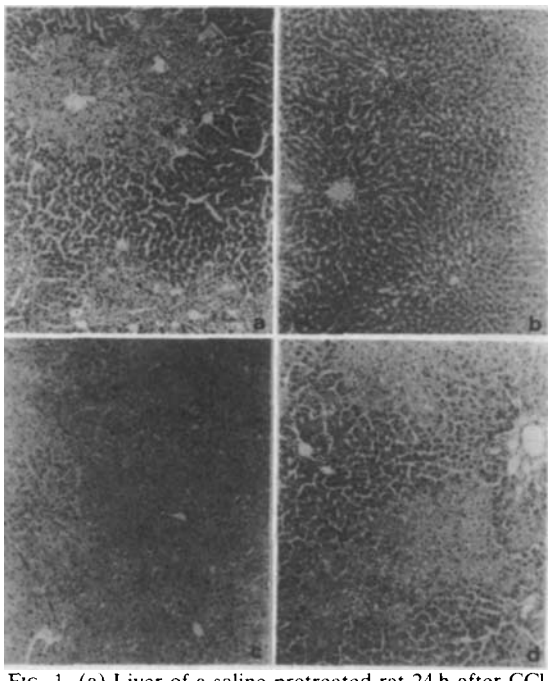

FIG. 1. (a) Liver of a saline-pretreated rat 24 h after CCl_4 administration (×150). Centrilobular necrosis and inflammatory infiltration are noted. (b) Liver of a saikosaponin-d pretreated rat 24 h after CCl_4 administration (×150). Less extensive necrosis is present. (c) Liver of a phenobarbitone-pretreated rat 24 h after CCl_4 administration (×150). Coagulative necrosis is extensively noted. (d) Liver of a rat pretreated with phenobarbitone and saikosaponin-d 24 h after CCl_4 administration (×150). The centrilobular necrosis is less extensive and the periportal zones are well preserved.

RESULTS

Effects of saikosaponin-d on hepatotoxicity

Serum GPT level. The administration of CCl_4 to saline-pretreated rats produced a maximal increase in the serum GPT level after 24 h and the level began to decrease after 30 h. Phenobarbitone pretreatment showed a significant increase in serum GPT level 30 h after CCl_4 injection. In contrast, saikosaponin-d pretreatment did not produce an increase in serum GPT after the administration of CCl_4. When the rats were pretreated with both phenobarbitone and saikosaponin-d, the serum GPT level 24 h after the CCl_4-injection was significantly lower than that produced in the rats pretreated with phenobarbitone alone (Table 1).

Lipid peroxide level in liver. The lipid peroxide level of the homogenate obtained 6 h after the CCl_4 injection showed that the rats given phenobarbitone pretreatment had a much higher level than the controls. The concomitant administration of saikosaponin-d and phenobarbitone inhibited this enhancement of lipid peroxide formation (Table 1).

Light microscopic observation. The administration of CCl_4 to rats pretreted with saline produced intense centrilobular necrosis at 24 h. The hepatocytes in centrilobular zones were enlarged and contained lipids. The hepatocytes in the periportal zones were also enlarged and the normal architectural pattern was destroyed (Fig. 1a). Pretreatment with saikosaponin-d resulted in remarkable protection against the liver cell damage caused by CCl_4 (Fig. 1b) whereas phenobarbitone pretreatment resulted in widespread coagulative necrosis (Fig. 1c). Saikosaponin-d inhibited the enhancement of CCl_4-hepatotoxicity by phenobarbitone-pretreatment. This was evident in the rats pretreated with phenobarbitone and saikosaponin-d, as no coagulative change was seen and areas with a normal architectural pattern were more extensive than in the animals pretreated with phenobarbitone alone, although necrosis was still present (Fig. 1d).

Effects of phenobarbitone and saikosaponin-d on microsomal enzyme activity in liver

Administration of phenobarbitone caused an increase in liver weight but no effect on the serum GPT level and lipid peroxide level in liver. Saikosaponin-d did not affect the liver weight, the serum GPT level and the lipid peroxide level as shown in Table 2.

Microsomal fractions were isolated from rat livers of each group and the activity of NADPH-cytochrome c reductase and the P450 content were compared.

Comparison of the rats pretreated with saline to those pretreated with phenobarbitone only, showed the volume of microsomes fractionated from 1 g of liver in the latter group to be significantly increased; those pretreated with saikosaponin-d only showed a diminished microsomal volume. The rats pretreated with phenobarbitone and saikosaponin-d showed an increased volume in the microsomal fraction compared with the saline-pretreated rats, but compared with the phenobarbitone-pretreated rats an inhibitory effect was observed.

NADPH-cytochrome c reductase activity in the rats treated with phenobarbitone and saikosaponin-d was lowered in comparison with the rats treated with phenobarbitone alone, and its level was identical to that of the saline-pretreated rats. The combination of phenobarbitone and saikosaponin-d inhibited the increase in the P450 content induced by the phenobarbitone, but no shift in the wavelength peak was observed in microsomes isolated from rats treated

Table 2. Effects of phenobarbitone and saikosaponin-d on serum GPT level, liver weight, lipid peroxide level of liver, microsomal protein, and microsomal enzymes.

Treatment	Body wt (g)	Serum GPT (U litre^{-1})	Liver wt (g)	Lipid peroxide of liver[1]	Microsomal protein[2]	NADPH-cyt.C reductase[3]	Cyt.P450[4]
Control	196·5 ± 0·5	22·7 ± 1·0	8·70 ± 0·14	1·05 ± 0·05	3·96 ± 0·03	161·9 ± 7·1	0·66 ± 0·03
Pheno.	193·0 ± 0·5	17·5 ± 1·4	10·34[a] ± 0·11	1·09 ± 0·05	7·53[c] ± 0·22	247·5[c] ± 2·6	1·64[c] ± 0·14
s.s.d.	192·0 ± 4·0	21·3 ± 1·7	8·72 ± 0·44	0·93 ± 0·03	3·15 ± 0·14	83·8[c] ± 4·4	0·45[a] ± 0·07
Pheno. + s.s.d.	195·0 ± 1·0	20·3 ± 1·4	10·16 ± 0·22	1·25 ± 0·05	4·73[c]*** ± 0·12	182·9*** ± 6·3	1·37[c]* ± 0·07

[1] nmol of MDA mg protein^{-1} of liver homogenate.
[2] mg of protein g^{-1} of weight of liver.
[3] nmol of cytochrome c reduced min^{-1} mg protein^{-1} of microsome.
[4] nmol mg protein^{-1} of microsome.
[a] $P < 0·025$, [b]$P < 0·01$, [c]$P < 0·001$ (statistically significant difference from control).
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ (statistically significant differences between groups with phenobarbitone and with phenobarbitone and s.s.d.).
Values are the mean ± s.e.

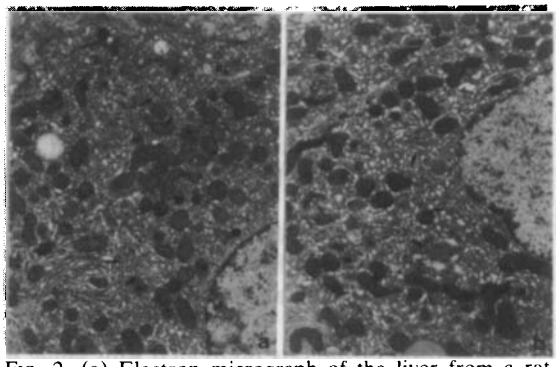

FIG. 2. (a) Electron micrograph of the liver from a rat treated with phenobarbitone (50 mg kg^{-1}) for 4 days (×3000). Numerous smooth-surfaced tubules and vesicles (arrow) are seen in the cytoplasm. (b) Electron micrograph of the liver from a rat treated with phenobarbitone (50 mg kg^{-1}) and saikosaponin-d (1 mg kg^{-1}) for 4 days (×3000). The increase in smooth-surfaced tubules and vesicles is inhibited and mild dilatation of the rough endoplasmic reticulum (arrow) is observed.

with saikosaponin-d alone or with phenobarbitone and saikosaponin-d.

Electron microscopical observation. Rat liver pretreated by phenobarbitone resulted in a proliferation of the vesiculated form of smooth endoplasmic reticulum (Fig. 2a). The administration of saikosaponin-d with phenobarbitone prevented this proliferation and showed a slight dilatation in the rough endoplasmic reticulum (Fig. 2b).

Effects on lipid peroxidation in liver microsomes

The rate of lipid peroxidation in liver microsomal fractions prepared from rats of each group were examined in-vitro using NADPH and CCl_4 (Fig. 3).

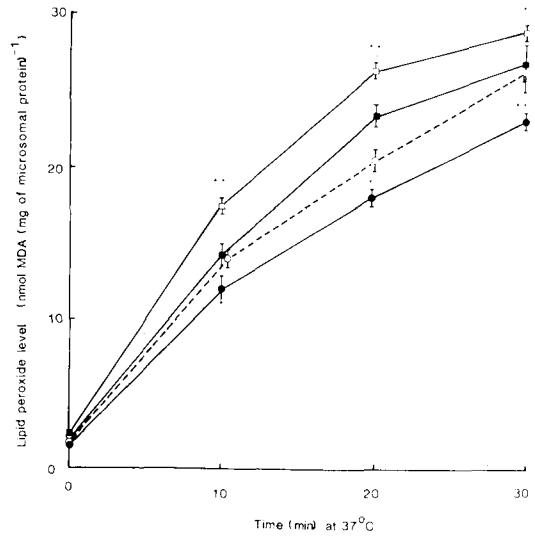

FIG. 3. Time course of CCl_4-induced microsomal lipid peroxidation in-vitro. Liver microsomes were prepared from the rats treated with saline (control) (○), phenobarbitone alone (□), Saikosaponin-d alone (●) and with phenobarbitone and saikosaponin-d (■). The reaction conditions were described under materials and methods. *$P < 0·025$, **$P < 0·001$ (statistically significant differences from control). [a]$P < 0·025$, [b]$P < 0·01$, [c]$P < 0·001$ (statistically significant differences between groups treated with phenobarbitone alone and with phenobarbitone and saikosaponin-d).

Microsomal lipid peroxide formation was markedly increased in rats pretreated with phenobarbitone and reduced in rats pretreated with saikosaponin-d when compared to the saline-pretreated rats. The amount of lipid peroxide formed in the liver microsomes of rats pretreated with phenobarbitone and saikosaponin-d was significantly lower than that of the rats pretreated with phenobarbitone alone.

DISCUSSION

Phenobarbitone pretreatment has been known to alter the response of the liver to a CCl_4 challenge. This altered hepatotoxic response is thought to be related to an increase of smooth endoplasmic reticulum in the liver (Garner & McLean 1969; Slater 1966; Slater & Sawyer 1969). The mechanism of CCl_4-induced liver cell necrosis is considered to be due to the enzymatic activation of CCl_4 to a CCl_3 free radical within the membranes of the endoplasmic reticulum (Farber 1979) and a liver cell with an augmented SER would be more vulnerable to CCl_4 than one with a normal complement. In contrast, Krishnan & Stenger (1966) have reported that CCl_4 hepatoxicity was increased in fasted rats, in which a reduction of hepatocellular SER was observed. Smuckler & Hultin (1966) also doubted the relation between the drug-metabolizing enzyme system and the development of hepatotoxic necrosis by phenobarbitone.

In the present study we found that saikosaponin-d reduces the phenobarbitone enhanced CCl_4-hepatotoxicity. The histological observations also demonstrated that, in the animals treated with phenobarbitone alone, CCl_4 consistently produced massive coagulative necrosis all over a hepatic lobule; whereas, in the animals treated with saikosaponin-d and phenobarbitone, the change of parenchymal cell was not coagulative and necrotic change did not extend to whole lobule. This inhibitory effect of saikosaponin-d on phenobarbitone-enhanced CCl_4 hepatotoxicity may be due to a reduction in the drug-metabolizing enzyme system, since saikosaponin-d inhibits an increase of the P450 content and NADPH-cytochrome c reductase activity caused by phenobarbitone treatment. In fact, the production of lipid peroxide by CCl_4 was lowered both in-vivo and in-vitro in the liver of a rat treated with phenobarbitone and saikosaponin-d compared with that in a rat treated with phenobarbitone alone. However, the protective effect of saikosaponin-d on the liver is not limited to CCl_4-induced liver cell necrosis. We have reported a protective effect of saikosaponin-d on D-galactosamine-induced liver cell necrosis in rats (Abe et al 1980). Since a cell membrane defect is a terminal feature in various kinds of liver cell necrosis, the protective action of saikosaponin-d may be due to the stabilization or strengthening of cellular cytomembranes. A stabilizing action of saikosaponin-d on rat red cell membranes has been demonstrated (Abe et al 1981). Accordingly, the protective action of saikosaponin-d against phenobarbitone enhanced CCl_4-hepatotoxicity may be partly due to stabilization of cell membrane of hepatocyte.

In spite of the unknown mechanism of action, administration of saikosaponin-d with phenobarbitone may have clinical application. Especially, the inhibitory action of saikosaponin-d against the induction of drug metabolizing enzyme systems could be useful for the prevention of a diminished drug efficacy and metabolic tolerance produced by the long-term administration of phenobarbitone.

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