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Effects of Naturally Occurring Furanocoumarins on Lipid Peroxidation and Carbon Tetrachloride Induced Hepatotoxicity in Mice

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Several naturally occurring furanocoumarins significantly inhibited microsomal lipid peroxidation not only mediated by endogeneous iron and NADPH but also initiated by CCl_4 metabolites. Phellopterin, a potent inhibitor of cytochrome P-450, exhibited an almost complete inhibition of CCl_4-induced hepatotoxicity as measured by sGPT activity 24 hr after CCl_4 intoxication, whereas other furanocoumarins such as imperatorin, byakangelicin and oxypeucedanin methanolate exerted no protective effect. When compared with other cytochrome P-450 inhibitors (SKF-525A, AIA) and silymarin given at the same dose level (ED_{50}), phellopterin still showed a significant inhibition of hepatotoxicity which was even stronger than that of AIA, known as a typical suicide inhibitor. Phellopterin was partially effective when given 30 min after CCl_4 treatment. Repeated administrations of phellopterin, however, resulted in a complete loss of the protection against CCl_4-induced hepatotoxicity.

Key words: Naturally occurring furanocoumarins, Phellopterin, Lipid peroxidation, CCl_4-induced hepatotoxicity, sGPT

INTRODUCTION

Activation of xenobiotics to highly reactive intermediates occurs to such an extent that many of these chemicals are transformed to hepatotoxins, and as such are involved in the etiology and pathogenesis of liver disorders, both carcinogenic and noncarcinogenic. Typical hepatotoxicants such as carbon tetrachloride are biologically inert, but they are converted to highly reactive hepatotoxic products primarily by the liver microsomal mixed function oxidase (MFO) system (Slater, 1982). CCl_4-induced infliction of hepatocellular damage, therefore, involves the bioactivation of CCl_4 to free radicals which resulted from reductive dehalogenation by cytochrome P-450 and consequent lipid peroxidation (Recknagel, 1967; McCay *et al.*, 1984; Stater, 1987).

We have recently reported that several furanocoumarins isolated from Angelicae plants most freqently prescribed in Chinese medicines caused a profound inhibitory activity of the hepatic microsomal cytochrome P-450 dependent MFO system, accompanied by the loss of cytochrome P-450 itself (Shin and Woo, 1986, 1990). Methoxalen, one of psoralen derivatives to be used for the treatment of psoriasis, has been reported to have protective effects on CCl_4-induced hepatotoxicity and its inhibition is suggested to be due to the blocking of activation of CCl_4 mediated by cytochrome P-450 (Labbe *et al.*, 1987). At present, however, little information is available concerning the relationship between CCl_4-induced hepatic injury as well as lipid peroxidation and regulation of microsomal cytochrome P-450 enzyme system.

The present study aims at the evaluation of time dependent effects of some structurally related naturally occurring furanocoumarins on CCl_4 metabolism with respect to NADPH, CCl_4-dependent lipid peroxidation as well as CCl_4-induced hepatotoxicity.

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MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats of CD strain weighing 150-200g and male albino mice of dd strain weighing 20-25g were used and maintained in central animal facility at constant temperature and humidity throughout the experiments. Water and lab chows were allowed *ad. lib.* Furanocoumarins such as isoimperatorin, imperatorin, phellopterin, oxypeucedanin methanolate and bya-

kangelicin were isolated from Angelicae dahuricae Radix (Shin *et al.*, 1988). The identity of these compounds were evaluated by spectral analysis by comparison with authentic samples. SKF-525A was a gift from Smith, Kline and French Lab. Philadelphia, PA, and AIA was a gift from Hoffman-LaRoche, N.J. Nutley, U.S.A.. Other chemicals such as CCl_4, NADPH, EDTA and silymarin were reagent grade commercially available.

Preparation of microsomal fraction

Rats were fasted overnight and killed by cervical dislocation. Blood was collected and the livers were removed, washed with 1.5% KCl soln and homogenized in 0.1 mM Na^+/K^+ phosphate buffer (pH 7.4, containing 0.05 mM EDTA). Washed microsomes were isolated by differential ultracentrifugation and the pellets thus obtained were stored at −50℃ until required.

Lipid peroxidation assay

Microsomal lipid peroxidation was measured by the method of Pessarye *et al.* (1982). In brief, to measure the peroxidation triggered by the reduction of endogenous iron, reaction mixtures contained, in a final volume of 3.5 ml, rat liver microsomes (5-6 mg, protein/ml), 0.2 mM NADPH and furanocoumarins dissolved in 25% dimethylsulfoxide. To measure the peroxidation initiated by CCl_4 metabolites, 0.2 mM NADPH, 1.5 mM EDTA and 1 mM CCl_4 were additionally added. The reaction mixtures were incubated under air at 37℃ for 20 min and the reaction was terminated by adding 2 ml of 20% TCA. After centrifugation, 3 ml of supernatant was added to 1 ml of 0.67% 2-thiobarbituric acid and heated at 90-100℃ for 10 min. After cooling, the absorbance was measured at 532 nm and the amount of malonic aldehyde (MDA)-reactive products formed was calculated using a molar extinction coefficient of $1.56\times10^{-5}M^{-1}cm^{-1}$.

Measurement of CCl_4-induced hepatotoxicity

CCl_4-induced hepatotoxicity was evaluated by measuring serum glutamic pyruvic transaminase (sGPT) activity in mice. SGPT activity was measured by the method of Reitman and Frankel (1957), in blood samples drawn by ocular puncture, 24 hr after the administration of CCl_4 dissolved in corn oil.

RESULTS AND DISCUSSION

Effect of furanocoumarins on microsomal lipid peroxidation

Table I was shown of the effects of 5 naturally occurring furanocoumarins on TBA reactant formation by rat liver microsomes. Lipid peroxidation was initiated either by NADPH or CCl_4 and the reaction mixture was incubated for 20 min. The results indicate that at 1 mM concentration, furanocoumarins such as phellopterin, isoimperatorin, imperatorin which had a nonpolar prenyl side chain moiety showed a profound inhibition of the NADPH-dependent lipid peroxidation by 95, 85 and 88%, respectively, but oxypeucedanin methanolate and byakangelicin possessed more polar side chain were less effective inhibitors.

Corresponding to these results, furanocoumarins exhibited similar tendencies in affecting the CCl_4-dependent lipid peroxidation. Incubation of microsomes in the presence of phellopterin, isoimperatorin, imperatorin at the same concentration *in vitro* resulted in a profound inhibition of CCl_4-induced lipid peroxidation.

And furthermore, the three compounds also showed a strong inhibition of TBA-reactant formation when incubated hepatic microsomes from rats killed 90 min

Table I. Effects of furanocoumarins on lipid peroxidation in rat liver microsomes *in vitro*

	TBA-reactants (nmoles/mg prot./20 min)	
	With NADPH	With EDTA and CCl_4
Control	0.751± 0.081	0.248± 0.017
Isoimperatorin	0.113± 0.027** (85)[a]	0.058± 0.015** (77)
Imperatorin	0.091± 0.032** (88)	0.028± 0.009** (89)
Oxypeucedanin methanolate	0.425± 0.032* (43)	0.108± 0.060* (56)
Phellopterin	0.040± 0.009** (95)	0.039± 0.002** (84)
Byakangelicin	0.406± 0.028* (46)	0.110± 0.025* (56)

The peroxidation of microsomal lipids was measured in two different systems: microsomes (5-6 mg protein) were incubated with (a) NADPH (0.2 mM) and furanocoumarins (1 mM), or (b) in the presence of NADPH (0.2 mM), EDTA (1.5 mM), CCl_4 (1 mM) and furanocoumarins (1 mM).
Significantly different from the control: *$p<0.05$, **$p<0.01$.
[a]% inhibition

Table II. Effects of pretreatment with furanocoumarins on lipid peroxidation induced by CCl_4

Treatments	TBA-reactants (nmoles/mg prot./20 min)
Control	0.474± 0.029
Isoimperatorin	0.246± 0.077* (48)[a]
Imperatorin	0.117± 0.024* (75)
Oxypeucedanin methanolate	0.423± 0.048 (11)
Phellopterin	0.057± 0.003** (88)
Byakangelicin	0.269± 0.018* (43)

Rats were pretreated with furanocoumarins (100 mg/kg, i.p.) 90 min prior to the determination of microsomal lipid peroxidation. Control rats received vehicle (0.5% CMC) only. The rat liver microsomes (5-6 mg protein) isolated were incubated with NADPH (0.2 mM), EDTA (0.05 mM), CCl_4 (1 mM). Significantly different from control: *$p<0.05$, **$p<0.01$.
[a]% inhibition

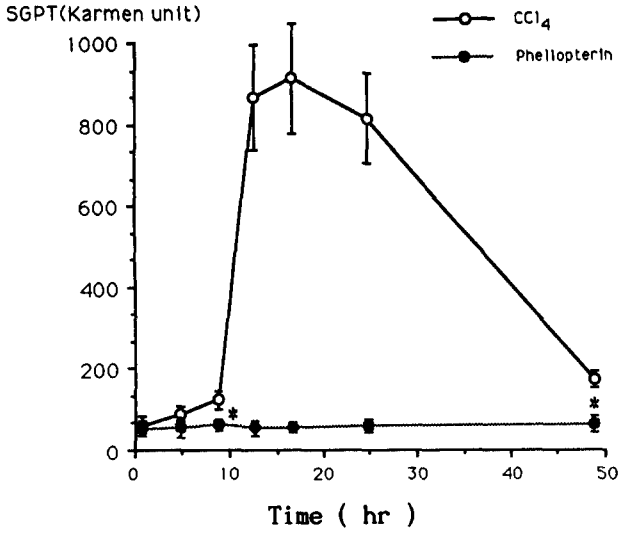

Fig. 1. Effect of pretreatment of phellopterin on the time course of sGPT activity after administration of CCl_4. Mice were administered with phellopterin (100 mg/kg i.p.) 30 min prior to CCl_4 (0.02 ml/kg, i.p. in corn oil). sGPT activity was determined at 4 hr intervals upto 12 hr, 24 hr and 48 hr after CCl_4 treatment. Data are means± S.E. for 8 mice. The asterisks indicate significant differences from values in control mice: *p<0.01.

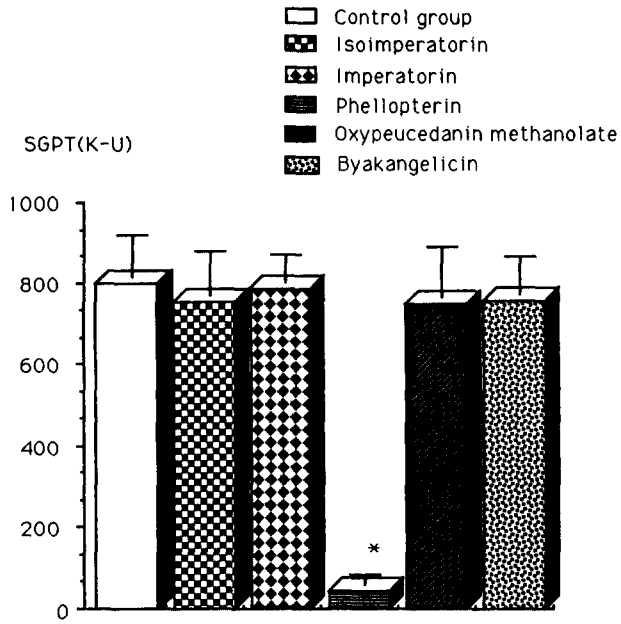

Fig. 2. Effect of pretreatment of furanocoumarins on sGPT. Mice were administered with various furanocoumarins (100 mg/kg, i.p.) 30 min prior to CCl_4 (0.02 ml/kg, i.p. in corn oil). sGPT activity was determined 24 hr after CCl_4 treatment. Results are means± S.E. for 8 mice. Significantly different from the CCl_4-treated control: *p<0.001.

after their administration (100 mg/kg, i.p.), whereas oxypeucedanin methanolate and byakangelicin were shown to have far weaker inhibitory activities (Table

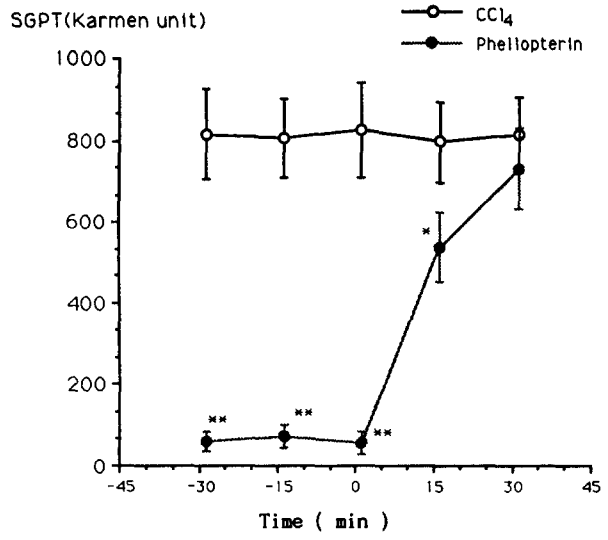

Fig. 3. Influence of time interval between the administration of CCl_4 (0.02 ml/kg, i.p.) and that of phellopterin (100 mg/kg, i.p.) on sGPT activity 24 hr after administration of CCl_4. Results are means± S.E. for 7-10 mice. Significantly different from the CCl_4-treated control: *p<0.05, **p<0.01.

II). From the fact that furanocoumarins with a more nonpolar side chain moiety exhibited a far stronger inhibition of lipid peroxidation than those with more polar side chains, it can be readily postulated that the inhibitory potency is associated with the lipophilicity of the inhibitors.

Effect of furanocoumarins on CCl_4-induced hepatotoxicity

Time course studies on CCl_4-induced hepatotoxicity as measured by sGPT activity in control mice were revealed that a rapid and a maximum rise in sGPT values was attained at around 12 hr after CCl_4 treatment retaining its level upto 24 hr and then returning to the normal level at 48 hr. These phenomena indicate that the rise in sGPT is due to its formation of free radicals which occurs as an initial events in the hepatotoxicity caused by CCl_4 (Fig. 1).

To see whether the preventive effects on rise in sGPT induced by CCl_4 is common in naturally occurring psoralen derivatives, mice were pretreated with various furanocoumarins at the same dose level (100 mg/kg, i.p.) 30 min prior to the CCl_4 treatment and their effects on sGPT were compared measuring 24 hr after CCl_4 intoxication. As indicated in Fig. 2, phellopterin at 100 mg/kg, i.p., produced an almost complete prevention of sGPT elevation, but other furanocoumarins such as imperatorin, isoimperatorin, oxypeucedanin methanolate and byakangelicin failed to affect sGPT activity at the same dose level. And furthermore, the complete prevention of sGPT elevation by phellopterin persisted throughout the experimental periods upto 48 hr after CCl_4 treatment (Fig. 1).

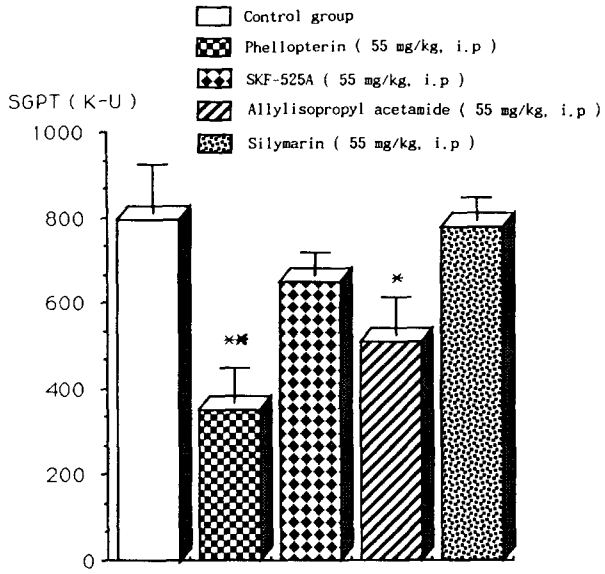

Fig. 4. Comparison of the effects of pretreatment with different drug-metabolizing enzyme inhibitors and silymarin on sGPT. Mice were administered with test compounds 30 min prior to CCl_4. sGPT activity was determined 24 hr after CCl_4 treatment. Results are means ± S.E. for 8 mice. Significantly different from the CCl_4-treated control: *$p<0.05$, **$p<0.01$.

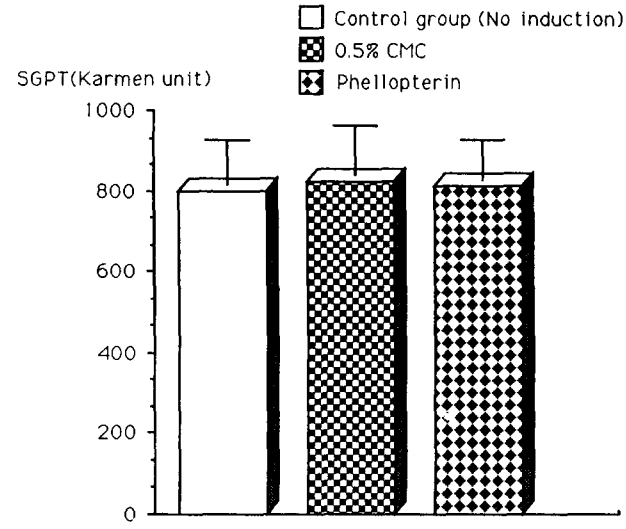

Fig. 5. Effect of repeated treatments of phellopterin on sGPT activity in mice. Mice were pretreated with phellopterin (70 mg/kg, i.p. for three days), while other mice received 0.5% CMC.

In previous communications, various naturally occurring furanocoumarins including phellopterin have been demonstrated to cause a significant decrease not only in hepatic MFO but microsomal cytochrome P-450 (Shin and Woo, 1990). Phellopterin has been shown to have the most potent inhibitory activity of MFO *in vitro* as well as *in vivo* among various furanocoumarins tested and moreover, it has been demonstrated that phellopterin caused a remarkable loss of rat liver cytochrome P-450 *in vivo*. Therefore, the prevention of CCl_4-induced hepatotoxicity by phellopterin is explained by its potent inhibitory action of microsomal cytochrome P-450, and it acted only at the early activation step of cytochrome P-450 catalized free radical formation of CCl_4. This assumption was turned out to be valid in estimation of the alteration in sGPT activity in terms of the time intervals between the administration of CCl_4 and that of phellopterin. As shown in Fig. 3, sGPT activity was not significantly decreased when phellopterin was treated 30 min after CCl_4 (0.02 ml/kg, i.p.), although the enzyme activity was partially but still significantly inhibited when phellopterin was treated 15 min after CCl_4. The ED_{50} value, the dose of phellopterin which caused 50% protection of CCl_4-induced hepatotoxicity was estimated by sGPT after graded doses of phellopterin and calculated to be 54.6 mg/kg, i.p.

Interestingly, SKF-525A, one of prototype MFO inhibitor, AIA, known as a typical suicide inhibitor of P-450 and silymarin, one of plant-derived hepatoprotective principle, given at the same doses, were almost or completely without effect on CCl_4-induced hepatotoxicity in mice as shown in Fig. 4. These results indicate that phellopterin is a far more stronger suicide substrate for cytochrome P-450 than SKF-525A and even suicide inhibitor itself (AIA), and consequently, caused a more profound decrease in metabolic activation of CCl_4.

Indeed, in a previous experiment, we have shown that MFO inhibitory potency of phellopterin was somewhat greater than that of SKF-525A. So far as the present results concern, the mode of the hepatoprotective effect of silymarin might be essentially different from that of phellopterin.

It has been previously demonstrated that furanocoumarins possessed biphasic responses i.e. both inhibitory and inducing effects on microsomal MFO system (Woo *et al.*, 1983). To see whether the induction of microsomal cytochrome P-450 with repeated treatments of phellopterin affect CCl_4-induced hepatotoxicity or not, mice were pretreated with phellopterin (70 mg/kg, i.p.) for three consecutive days and its effect on sGPT activity was estimated injecting CCl_4 (0.02 ml/kg, i.p.) 48 hr after the last dose of phellopterin. As expected, sGPT activity of the phellopterin-treated group measured 24 hr after CCl_4 intoxication was found to be not significanly different from those of the control (Fig. 5).

In conclusion, naturally occurring furanocoumarins tested prevent not only the development of lipid peroxidation which essentially initiated by the reduction of endogenous iron, but also its initiation by decreasing the microsomal cytochrome P-450 mediated formation of the trichloromethyl free radical and the consequent abstraction of hydrogen atoms from lipids. Phellopterin, one of the potent suicide inhibitor of liver cytochrome P-450, decreases the metabolic activation of CCl_4 and

almost completely prevents its hepatotoxicity in mice. Post-treatment with phellopterin was only partially effective when given early. Phellopterin may be preferred to other inhibitors as a possible tool to decrease metabolic activation and determine its role in hepatotoxicity of drugs and chemicals.

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