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Effects of Ginseng Saponins on Morphine 6-Dehydrogenase

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Abstract—The possible mechanisms of ginseng saponins on the inhibition of the development of morphine tolerance and physical dependence were investigated in the aspects of morphine metabolism by morphine 6-dehydrogenase. The administration of morphine causes a reduction of non-protein sulfhydryl contents in the liver, because morphinone metabolized from morphine by morphine 6-dehydrogenase conjugates with sulfhydryl compounds. However, ginseng saponins inhibited the activity of morphine 6-dehydrogenase which catalyzed the production of morphinone from morphine. In addition, ginseng saponins inhibited the reduction of non-protein sulfhydryl levels by increasing the level of hepatic glutathione. These results suggest that the dual action of the above plays an important role in the inhibition of the development of morphine tolerance and physical dependence. On the other hand, it was observed that less polar components of ginseng saponins with parent structures were more active components *in vitro*.

Keywords—Morphine 6-dehydrogenase • ginseng saponins • morphine • morphinone

The metabolism of morphine has been studied extensively in many animal species. Morphine is metabolized mainly via glucuronide conjugation and N-demethylation[1~3]. Dihydromorphinone is identified in the acid hydrolyzates of urine from various animal species given morphine[4,5]. Morphine 6-dehydrogenase which catalyzes the production of morphinone from morphine and codeinone from codeine has been recently purified from guinea pig liver[6].

Morphinone is about nine times more toxic than morphine in mice, and it binds irreversibly to tissue proteins and opiate receptors. This binding relates the toxicity including lethality of, tolerance to and physical dependence on morphine[7,8]. On the other hand, the administration of morphine causes a reduction of non-protein sulfhydryl contents in the liver. A part of morphinone produced conjugates with sulfhydryl compounds such as cysteine and glutathione reducing tissue protein bindings with morphinone, and this conjugation plays a role on detoxication of this metabolite[7].

Schole has reported that ginseng extracts increase the hepatic glutathione levels in rats[9]. It has been demonstrated that ginseng saponins inhibit the development of morphine tolerance and physical dependence[10]. However, this role of ginseng saponins on morphine detoxication related to the inhibition of the development of morphine tolerance and physical dependence is not clear.

The effects of ginseng saponins on morphine 6-dehydrogenase activity and on the formation of morphinone were investigated in this experiment to define possible mechanisms of ginseng saponins on the development of morphine tolerance and physical dependence. In addition, active components of ginseng saponins which inhibit morphine 6-dehydrogenase were also studied in this experiment.

Experimental Methods

Drugs—Morphine hydrochloride (Dae-won Pharm. Co., Ltd.), ginseng total saponins (GTS), prtopanaxadiol saponins (PD), protopanaxatriol saponins (PT), ginsenosides (Rb_1, Rb_2, Re, Rg_1) [extracted and purified by Namba *et al.*'s methods[11], supplied by Korean Ginseng and Tobacco Research Institute] and aglycones [20 (*S*) protopanaxadiol and 20(*S*) protopanaxatriol were prepared by Shibata *et al.*'s methods[12]] were dissolved in saline or buffer solution with 0.5% DMSO just before the test.

Measurement of liver morphine 6-dehydrogenase activity from guinea pig and rabbit *in vitro*—These tests were made into two steps. The first test was the effects of GTS, PD and PT on morphine 6-dehydrogenase to screen the most active fraction with NADP or NAD at optimum pH or 7.4, and the second, saponin fractions, single components and aglycones to screen the active components with NADP at optimum pH referring to the first result.

All the procedures for the purification of the enzyme from guinea pig and rabbit livers were carried out by the method of Yamano *et al.*[6,13]. Briefly, the liver was homogenized in 100 mM sodium phosphate buffer (pH 7.4) containing 100 mM 2-mercaptoethanol. The enzyme was purified from the 9,000×g supernatant fraction of the guinea pig and rabbit liver by saturated ammonium sulfate (pH 7.4) precipitation followed by Matrex green A column chromatography, Sephadex G-100 gel filtration and DE 32 column chromatography.

The standard incubation mixture contained 1.5 μM morphine, 1.5 μM NAD or NADP, 0.1 ml of enzyme solution and 100 mM tricine-NaOH buffer (pH 9.1 or pH 7.4) in a total volume of 1.5 ml. The incubation time was 120 min under nitrogen. The activities of morphine 6-dehydrogenase were measured by the change in absorbance at 340 nm employing a 1cm light path in Shimadzu UV 150 spectrophotometer at 25°C. Protein concentration was determined by the method of Lowery *et al.*[14]

Determination of morphinone from morphine by morphine 6-dehydrogenase of guinea pig liver *in vitro*—PT shown as the most active fraction in the previous test was only used for the further experiments. Morphine 6-dehydrogenase of guinea pig liver prepared in the previous step was also used in this test[6]. GTS and their aglycones were dissolved in 100 mM tricine-NaOH buffer or with 0.5% DMSO, respectively. The incubation time was 120 min at 37°C. After incubation, one ml filtrate passed through a 0.45 μm membrane filter and was poured onto Sep-Pak C_{18} cartridge, washed with 20 ml of water and eluted with 1.5 ml of methanol. The methanol eluate was used for estimating the background in HPLC. Morphinone contents were determined by high performance liquid chromatography (HPLC)[15]. The chromatographic equipment consisted of a model 510 pump, a Model U6K injector and a Model 440 absorbance detector. The column used was a 6×150 mm YMC AL-312 Octadecyl-saline (ODS). The flow rate was 2 ml/min and the peaks were monitored at 214 nm. Retention time was 18 min. The peak area was determined by a Shimadzu Chromatopac C-R1B integrator. Triplicate samples were determined by the peak

area compared to that obtained from control.

Fractionation of PT saponin components by silica gel column chromatography—The components of PT were fractionated by silica gel column chromatography to identify the principal inhibitory components. The elution was carried out first with 80 ml of chloroform applied on a Sephadex G-10 column (1×30 cm) and then the residue was passed through the same column with 100 ml of chloroform/methanol(9 : 1), 28 ml of chloroform/methanol(8 : 2) and 4 ml of choloroform/methanol(6 : 4), in turn.

Determination of hepatic non-protein sulfhydryl contents in mice—This test was made on GTS, PD and PT to examine the correlation between the contents of liver non-protein and the detoxication degrees of morphinone. Male mice(23~28 g) were given 10 mg/kg of morphine(s.c.) once a day for a period of 6 days and ginseng saponins were administered orally 30 min prior to morphine injection daily. The mice were killed by decapitation 24 hours after the final morphine injection. The liver was removed and the non-protein sulfhydryl concentration was determined by a modification of the method of Ellman[16] as follows: Liver homogenates(20%) in 5% trichloroacetic acid containing 1 mM Na-EDTA were centrifuged at 2,000×g for 5 min. The clear supernatant(0.4 ml) was transferred to a tube containing 4.55 ml of 0.1 M NaH_2PO_4-Na_2HPO_4 pH 8.0, and 50 μl of 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) was added. After mixing, absorbance at 410 nm was recorded against a reagent blank to determine non-protein sulfhydryl concentration.

Results

Inhibitory effects of ginseng saponins on morphine 6-dehydrogenase—Ginseng saponins had the inhibitory effects on liver morphine 6-dehydrogenase from the guinea pig by using NAD and NADP as co-factors at the optimum pH (pH 9.1) and at pH 7.4 respectively(Table I). Especially, PT was more effective than PD. About 50% of morphine 6-dehydrogenase was

Table I. Effects of GTS, PD and PT saponins on guinea pig liver morphine

Saponin fraction	Conc. (mg/ml)	Inhibition(%)			
		with NADP		with NAD	
		Optimum pH	pH 7.4	Optimum pH	pH 7.4
GTS	10.0	36.6	—	—	—
	1.0	7.9	18.1	9.5	9.1
	0.1	0	3.6	0	0
	0.01	0	0	0	—
PD	10.0	44.9	—	—	—
	1.0	7.3	15.5	6.9	9.1
	0.1	0	0	0	0
	0.01	0	0	0	0
PT	10.0	75.1	—	—	—
	1.0	53.1	67.8	44.4	67.3
	0.1	34.5	51.1	28.6	45.4
	0.01	4.0	11.9	4.8	—
	0.001	0	3.9	0	—

—, not determined.

Table II. Effects of ginseng saponins and their aglycones on guinea pig liver and rabbit liver morphine 6-dehydrogenase

Compound	Concentration(mg/ml)	Inhibition(%)	
		Guinea pig	Rabbit
GTS	1.0	4.0	7.3
Ginseng saponins from leaf	1.0	7.5	6.6
PD	1.0	5.1	5.7
PT	1.0	49.4	48.4
	0.1	24.0	12.1
Ginsenoside Rb_1	1.0	0.0	3.1
Ginsenoside Rb_2	1.0	1.8	9.3
Ginsenoside Re	1.0	4.0	4.5
Ginsenoside Rg_1	1.0	7.7	1.7
20(*S*)-Protopanaxadiol	0.025	—	28.9
	0.01	39.0	17.2
	0.005	18.8	10.8
	0.001	5.7	—
20(*S*)-Protopanaxatriol	0.05	38.8	12.5
	0.025	11.8	5.3
	0.01	3.9	3.1

NADP was used as a cofactor at optimum pH.
—, not determined.

Table III. Effects of PT on the formation of morphinone from morphine by guinea pig liver morphine 6-dehydrogenase

Fraction	Concentration (mg/ml)	Inhibition(%) Morphinone formed with	
		NAD	NADP
PT	0.1	14	11
	1.0	47	38

inhibited by 1 mg/ml concentration of PT. The enzymes from the guinea pig (49.4% by 1 mg/ml) and rabbit (48.4% by 1 mg/ml) were similarly inhibited by PT.

Inhibitory effects of ginsenosides and their aglycones on morphine 6-dehydrogenase—Morphine 6-dehydrogenase under NAD was inhibited by ginsenosides and their aglycones at optimum pH (Table II). 20(*S*)-Protopanaxadiol (39% of inhibition by 0.1 mg/ml) had 10 times more inhibitory effects than 20(*S*)-protopanaxatriol (3.9% of inhibition by 0.01 mg/ml).

Inhibitory effects of PT on the morphinone formation from morphine—Further researches were used for PT, which had the most inhibitory effects on morphine 6-dehydrogenase. The enzyme preparations, 9,000×g supernatant from the guinea pig liver, were used to measure the inhibitory effects of PT under NAD and NADP as cofactors respectively (Table III). Morphinone production by morphine 6-dehydrogenase under NAD was inhibited upto almost 47% of the control level by 1 mg/ml concentration of PT.

Inhibitory effect of PT components fractionated by column chromatography on guinea pig liver morphine 6-dehydrogenase—It is necessary to fractionate, isolate and identify the principal inhibitory components of PT components in steps. The inhibitory effects of each fraction was tested on morphine 6-dehydrogenase for the first step (Table IV). Fraction

Table IV. Effect of PT components fractionated by silica gel column chromatography on guinea pig liver morphine 6-dehydrogenase

Fraction	Effluent	Weight (mg)	Inhibition(%) Concentration(mg/ml)				
			0.2	0.1	0.05	0.02	0.01
1	$CHCl_3$	0.5	—	—	—	—	16.6
2~4	$CHCl_3$	6.0	—	—	83.9	58.4	45.0
5~8	$CHCl_3$	3.1	83.2	70.0	—	27.5	21.5
9~14	$CHCl_3$/MeOH(9:1)	5.2	32.2	21.5	—	—	6.0
15~18	$CHCl_3$/MeOH(9:1)	32.6	—	14.1	—	—	—
19	$CHCl_3$/MeOH(9:1)	9.7	—	9.4	—	—	—
20~22	$CHCl_3$/MeOH(8:2)	18.8	—	6.0	—	—	—
23~24	$CHCl_3$/MeOH(8:2)	12.2	—	1.3	—	—	—
25~26	$CHCl_3$/MeOH(8:2)	9.9	—	3.4	—	—	—
27~31	$CHCl_3$/MeOH(8:2)	8.0	—	6.7	—	—	—
32~33	$CHCl_3$/MeOH(8:2)	1.0	—	5.4	—	—	—
34~38	$CHCl_3$/MeOH(8:2)	3.3	—	3.4	—	—	—
39~43	$CHCl_3$/MeOH(6:4)	6.2	—	5.4	—	—	—

with NADP at optimum pH.
—, not determined.

2~4 was the most inhibitory, 83% of inhibition by 0.05 mg/ml concentration, 58% by 0.02 mg/ml concentration. It is presumed that one or two components of PT which possess a low polarity play a role of an essential part in inhibition. Further experiment is required for the isolation of an active single component.

Increase of the hepatic non-protein sulfhydryl contents by ginseng saponins— The non-protein sulfhydryl contents in mice treated with GTS, PD and PT respectively are shown on the upper panel of Fig. 1. Those values are generally a little bit higher than that of the saline group. The lower panel shows the values of ginseng saponins and morphine concomitant treated groups, but the values of the non-protein sulfhydryl contents in ginseng treated groups show higher than that of the morphine control group. Ginseng inhibited the reduction of hepatic non-protein sulfhydryl contents caused by continuous injection of morphine for 6 days (Fig. 2).

Discussion

Ginseng saponins have inhibitory effects on the development of morphine tolerance and physical dependence, and these effects are supposed to be associated with a newly equilibrated state of neurologic function in the central nervous system[10]. In this experiment, the possible mechanism of ginseng saponins on the inhibition of development of morphine tolerance and physical dependence was investigated in the aspects of metabolism and excretion.

Recently, Toki *et al.* have reported that morphinone also binds irreversibly to -SH of opioid receptors or -SH of tissue protein and plays a part in the toxicity of morphine, and morphinone has a role in the development of tolerance and physical dependence[7]. Therefore, administration of morphine reduces the hepatic non-protein sulfhydryl concentration and induces hepatocellular damage[18]. On the other hand, a

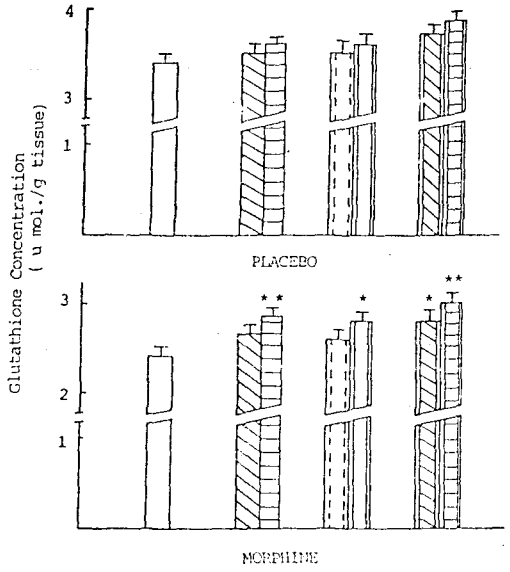

Fig. 1. Effects of GTS, PD and PT on the inhibition of hepatic glutathione level decrease in mice by daily injection of morphine for 6 days

Morphine 10 mg/kg s.c. was injected into the mice every 24 hours for 6 days. GTS, PD and PT were administered orally to the respectively group once a day 30 minutes prior to morphine injection for 6 days. The placebo group was treated with saline or each dose of ginseng saponins without morphine. Upper panel, placebo group; lower panel. morphine group.

*$p<0.05$,

**$p<0.01$, compared with that of morphine control.

GTS 50 mg/kg
GTS 200 mg/kg
PD 50 mg/kg
PD 200 mg/kg
PT 50 mg/kg
PT 200 mg/kg
Saline

part of morphinone binds covalently to the sulfhydryl compounds such as glutathione and cysteine, and these conjugations involve detoxication of this metabolite. So, pretreatment with glutathione or cysteine significantly increased the survival rate of mice at a lethal dose of morphinone[17]. In this experiment, ginseng saponins inhibit the decrease of the hepatic non-protein level in mice treated with morphine, in agreement with Schole's finding that ginseng increased the hepatic glutathione level[9]. A possible explanation for these observation is proposed that ginseng saponins inhibit the development of morphine tolerance and physical dependence providing more hepatic non-protein sulfhydryl contents.

GTS and their aglycones inhibit morphine 6-dehydrogenase. Consequently, the inhibition of this enzyme activity by ginseng saponins reduces morphinone production from morphine and this results in the reduction of morphine toxicity. PT was more active than PD saponin in the inhibition of morphine 6-dehydrogenase. Morphine 6-dehydrogenase under NAD was inhibited upto almost 50% of control by 1 mg/ml PT. 20(*S*)-Protopanaxadiol was more effective than 20(*S*)-protopanaxatriol. In these results, the lower polar components, PT and 20(*S*)-protopanaxadiol, are active components in the inhibition of morphine 6-dehydrogenase. PT was the most active component in GTS, PD and PT.

The components of PT were fractionated by silica gel column chromatography and inhibitory effects of each fraction on morphine 6-dehydrogenase were tested, because PT was the most inhibitory in this experiment. Even though this test was made *in vitro*, it was demonstrated that ginseng saponins which possess a low polariy were active in the inhibition of morphine 6-dehydrogenase activity.

Conclusion

Ginseng saponins increased the hepatic non-protein SH level, inhibited the decrease of the hepatic non-protein level in mice treated with morphine and the formation of morphinone by the inhibition of morphine 6-dehydrogenase. It was proposed that these dual actions of ginseng saponins may partially involve the mechanism of inhibitory effects on the development of morphine tolerance and physical dependence.

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