

CARCINOGEN-METABOLIZING ENZYME ACTIVITY IN THE LIVER: BENEFICIAL EFFECT OF THE PHYTOTHERAPEUTIC COMPOUND YHK

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Short title:

Beneficial effect of YHK on liver carcinogen-metabolizing enzyme activity in the liver

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Summary

In this study we investigated the effect of YHK supplementation on the activities of antioxidant, phase I and phase II metabolizing enzymes involved in detoxification as well on liver antioxidant defense system in rats. YHK was administered for four weeks to Wister rats. At the end of the treatment period, different cytochrome P450 (CYP) isoform and phase II enzyme activities were determined by incubation of the liver microsomes or cytosols with appropriate substrates. Dietary supplementation of YHK (2%, w/v) to male rats for four weeks significantly increased the activities of glutathione peroxidase and catalase to 118% and 87% in liver as compared with corresponding normal diet fed control ($P < 0.05-0.001$). CYP 1A2 activity was markedly increased in all the YHK treatment groups ($P < 0.05$). CYP 1A1 activity was increased significantly in all the groups. Parallel to these changes, YHK feeding to mice also resulted in a considerable enhancement in the activity of phase I and II metabolizing enzymes such as glutathione S-transferase to 1.6 fold and 1.8 fold in liver as compared with corresponding normal diet fed control ($P < 0.05-0.01$). The induction of such detoxifying enzymes by YHK suggest the potential value of this compound as protective agent against chemical carcinogenesis and other forms of electrophilic toxicity. The significance of these results can be implicated in relation to cancer chemopreventive effects of YHK against the induction of tumors in various target organs.

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent cancers worldwide and is most often associated with exposure to environmental factors such as aflatoxin B₁, hepatitis viruses B and C, and alcohol consumption (Bosch 1999). Agents with tumor promoting activity in the liver generally cause several of the following effects: enzyme induction, enlargement of the liver by hypertrophy and/or hyperplasia, an increase in DNA synthesis and/or decrease in apoptotic activity, which is more pronounced in preneoplastic than in unaltered cells, and preferential growth stimulation of precancerous lesions (Shulte 95 and 94). Fruits, vegetables, vitamins and several herbs with diversified pharmacological properties have been shown to be a rich source of cancer chemopreventive agents (Wattenberg 1992). Though these agents can be targeted for intervention at either initiation, promotion or progression stages of multistep processes of carcinogenesis (Digiovanni 1992). Many of these actions have been related to the agents abilities to enhance the activities of carcinogen metabolizing enzymes and by binding with toxicants thus reducing their effective critical concentrations. Hepatic drug metabolizing system consists of mixed-function oxidase or monooxygenase enzymes including phase I enzymes such as cytochrome P450, cytochrome b5 and NADPH-cytochrome P450 reductase and phase II enzymes such as glutathione-S-transferase (GST), sulfatase and UDP-glucuronyl transferase (Sheweita 2000). Alternatively, chemopreventive agents acts as antioxidant and counteract the increased amount of oxidants generated by toxicants (Wattenberg 1985). Among phase I enzymes, CYP 1A1 is primarily involved in the metabolism of polycyclic aromatic hydrocarbons, whereas CYP 1A2 preferentially metabolizes heterocyclic amines and aflatoxin B₁ (Eaton 1995;Williams 1995). These isoforms may play a very important role in activation of these environmental carcinogens. CYP 1A2 is normally expressed in liver. The level of CYP 1A1 expression in the liver is substantially lower than for CYP 1A2 and inducers of CYP 1A may have considerable potential for toxicity}carcinogenicity (Tassaneeyakul et al 1993). GST belongs to a superfamily of multifunctional isoenzymes categorized into three major classes, α , μ and π (Mannervick). Studies have suggested that the GST α possesses high catalytic efficiency towards aflatoxin B₁-8,9-epoxide, the reactive intermediate of the fungus mycotoxin aflatoxin B₁ (Buetler) while the GST μ isoenzyme is most efficient in forming a conjugation of glutathione with carcinogen 4-nitroquinoline-1-oxide (Aceto) and that GST π metabolites preferentially conjugate 7 β , 8 α -dihydroxy-9 α , 10 α -oxy-7,8,10-tetrahydrobenzo(a)pyrene, the ultimate carcinogenic metabolite of benzo(a)pyrene including aflatoxin B₁ or 4-nitroquinoline-1-oxide (Mannervick). Phase I enzymes, which include cytochrome P450, can metabolize not only lipophilic compounds to more polar products but, under some circumstances, can lead to generation of highly reactive electrophiles ((Henderson). Therefore, the balance between phase II and phase I enzymes is likely to be important for determining cellular sensitivity to environmental chemicals. We have previously shown either in vitro and in vivo experimental studies that YHK exerts a potent protective effect on hepatocellular damage and on liver microcirculation in an ischemia-reperfusion model (Marotta, Marotta) as well as exerting potent in vitro protective effect on metal-induced oxidative stress of hepatocytes (manuscript in preparation). Because bioactivation of precarcinogens and detoxification of ultimate carcinogens are mainly carried out by drug metabolizing enzymes in the liver and these may be influenced by specific nutrients, the aim of the present study was to explore the effects of YHK on these enzymes and liver antioxidant.

Materials & Methods

Animals were housed in stainless steel wire-mesh cages and kept in an environmentally-controlled vivarium (temperature, ventilation, humidity and light-dark cycle) and with free access to food (commercial rodent diet). The animals were allowed to acclimatize for five days before the study.

Experimental protocol.

For studying, the effect of dietary supplementation of YHK on antioxidant, phase I and phase II metabolizing enzymes, the rats were randomly divided into control and experimental groups consisting of twenty animals in each group. These animals were fed with either normal diet (control group) or 2% YHK diet (experimental group) which was prepared by mixing normal diet and YHK, with a final concentration of YHK fixed at 2%. This defined feeding regimen was kept for four weeks. The selection of dose of YHK was based on previous studies where significant cancer chemopreventive effects were observed when added to either adriamycin and/or cis-platinum (Marotta et al. personal data presented at Natural Health Product Conference, Montreal, 2004). After four weeks of treatment, the animals were sacrificed by cervical dislocation, and whole liver was quickly removed, rinsed in cold 0.9% NaCl and perfused immediately with ice cold saline (0.85% sodium chloride) and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17%) using a Potter-type Teflon glass homogenizer. Part of the homogenate was centrifuged at 800 g for 15 min at 4°C using Hitachi cold centrifuge model CR15B to separate nuclear debris. The aliquot so obtained was centrifuged at 12,000 rpm for 30 min at 4°C to obtain postmitochondrial supernatant which was used as a source of enzymes. The rest of the sample was used for liver microsomes and cytosols extraction which was carried out by a differential centrifugation method. Briefly, homogenates were subjected to centrifugation at 0 000×g for 15 min at 4 °C in a refrigerated centrifuge (OM 3593 IEC Co. Ltd.USA). The supernatant was then centrifuged 105 000×g for 60 min at 4 °C in a preparative ultracentrifuge (20PR-52D; Hitachi, Tokyo). The pellet of microsomes was suspended in the homogenization solution in the homogenizer and centrifuged again. The supernatant (cytosol fraction) after discarding any floating lipid layer and appropriate dilution, was used for enzyme assays. as described above (Robson et al 1987) and stored in 20 mM phosphate buffer (pH 7.4) containing 20%wv glycerol at -80°C until analysis. The microsomal protein content was determined by the method of Lowry et al (1951). The P450 content was determined by the method described by Omura & Sato (1964).

Liver antioxidant assay

Glutathione peroxidase activity was measured according to the procedure of Mohandas et al. (1984). The reaction mixture consisted of 1.44 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (0.5 mM), 0.1ml sodium azide (1.0 mM), 0.05 ml glutathione reductase (1.0 EU/ml), 0.1 ml GSH (1.0 mM), 0.1 ml NADPH (0.1 mM), 0.1 ml hydrogen peroxide (0.019 M), 0.025 ml and 0.05 ml of renal and hepatic PMS (10% w/v) in a total volume of 2.0 ml. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of 6.22×10^3 M/cm. Catalase activity was determined by the method of Claiborne (1985) and further modifications by Ansar et al. (1999). Briefly, the assay mixture consisted of 1.0 ml phosphate buffer (0.05 M, pH 7.0), 0.975 ml hydrogen peroxide (0.019 M), 0.025 ml of renal and hepatic PMS (10% w/v) in a total volume of 2.0 ml. Catalase activity was calculated following decomposition of hydrogen peroxide measured as a decrease in absorbance at 240 nm.

Cytosol phase I and phase II enzymes

Determination of CYP 1A1/CYP 1A2 activity. The activity of CYP 1A1 and CYP 1A2 was determined using phenacetin as a specific substrate probe as described by Tassaneeyakul et al (1993). The activity of the high affinity component (CYP 1A2) of phenacetin- *O*-deethylase was determined by incubating 5 μ m phenacetin with liver microsomes (0.5 mg mL⁻¹) for 30 min. The

reaction was terminated by addition of 1 m NaOH. The formation of the metabolite, paracetamol, was measured by a specific HPLC method (Tassaneeyakul et al 1993). The activity of the low affinity isozyme CYP 1A1 was determined by using phenacetin at a concentration of 300 μ M, approximately the K_m of CYP 1A1 reported in rat liver microsomes (Boobis et al 1981). The procedures for incubation and HPLC assay were the same for CYP 1A2.

Hepatic cytosolic glutathione-S-transferase activity was determined using a spectrophotometric (340nm) method (Habig et al 1974) and further modifications (Iqbal 1996). This procedure was based on the enzyme catalysed condensation of glutathione with the model substrate 1-chloro-2,4-dinitrobenzene (CDNB). Briefly, the reaction mixture consisted of 1.825 ml phosphate buffer (0.1 M, pH 6.5), 0.1 ml reduced glutathione (1.0 mM), 0.05 ml CDNB (1.0 mM), 0.025 ml and 0.01 ml of renal and hepatic PMS (10% w/v), respectively, in a total volume of 2.0 ml. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6×10^3 M/cm.

Statistical analysis

Significance was established by analysis of variance and the level of significance was determined by employing a Duncan's multiple-range test. Data were expressed as means (SD) and a probability value of <0.05 was regarded as indicating that a significant difference existed between experimental groups.

Results

The dose of YHK used in the present study did not produce any apparent sign of toxicity such as weight loss or reduced diet and water consumption, throughout the feeding regimen (data not shown).

The effect of dietary supplementation of YHK to rats on the activities of antioxidant enzymes in liver tissues was evaluated and results are shown in table 1. Addition of 2% YHK to the diets of 4 weeks old rats for 30 days resulted in a normal weight gain and was well tolerated. The dietary supplementation of YHK resulted in a significant elevations in the activities of glutathione peroxidase and catalase to 118% and 87% as compared with normal diet fed control ($P<0.05-0.001$). The increase occurred in glutathione peroxidase activity was comparably higher than what observed in catalase activity (table 1).

The effect of dietary supplementation of YHK on phase II metabolizing enzymes such as glutathione S-transferase are shown in table 2. The dietary supplementation of YHK enhances the activities of glutathione S-transferase to about 1.6 fold fold as compared to animals fed with normal diet ($p<0.05-0.001$).

Glutathione-S-transferase activity

A significant increase in the activity of cytosolic glutathione- S-transferase was observed in the rats treated with YHK ($p<0.05$) In YHK-fed group, the contents of P450 were significantly increased in male rats (2.66 ± 0.55 nmol.mg MS pro⁻¹) compared with those in the control group (1.08 ± 1.04 nmol.mg MS pro⁻¹; $P<0.01$). In particular the CYP 1A was significantly increased by YHK treatment ($p<0.05$).

The results indicated that there was a difference of hepatic microsomal drug-metabolizing enzymes under normal conditions in different sex rats. However, the effect of YHK was comparably effective in either sex. (Table 1).

Discussion

Dietary antioxidants protect laboratory animals against the induction of tumors by a variety of chemical carcinogens. Among possible mechanism of protection against chemical carcinogenesis could be mediated via-antioxidant dependent induction of detoxifying enzymes.

There is increasing evidence that nutrition plays an important causative role in the initiation, promotion and progression stages of several types of human cancer (Katiyar & Mukhtar 1996a). The diet may contain many chemicals that can antagonize the effects of chemical carcinogens. One of the mechanisms could be by modulation of the enzyme systems involved in the activation and deactivation of chemical carcinogens (Parke & Ioannides 1981; Guengerich 1984). A large number of the potentially genotoxic environmental chemicals and natural products, to which man is exposed, require metabolic activation to exhibit their mutagenic and carcinogenic effects (Eaton et al 1995). This bioactivation is mainly carried out by some of the phase I enzymes including cytochrome P450 (CYP), which give rise to reactive intermediates that attack DNA and other cellular macromolecules (Smith et al 1995). Inhibition of bioactivating enzymes and/or induction of detoxication enzymes by either naturally occurring substances or synthetic agents continues to be a promising chemopreventive strategy.

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Parke, D. V., Ioannides, C. (1981) The role of nutrition in toxicology. *Annu. Rev. Nutr.* **1**: 207±234

Smith, T., Hong, J., Wang, Z., Yang, C. (1995) How can carcinogenesis be inhibited ? *Ann. N.Y. Acad. Sci.* **768**: 82±90

Cancer prevention may occur by various different mechanisms. These include reduced metabolic toxification and/or enhanced detoxification, which lower the amount of the ultimate initiating carcinogen. Furthermore, in the post-initiation phase reduced growth of initiated/preneoplastic cells may impair the process of tumor promotion.

The activities of hepatic drug-metabolizing enzymes, especially cytochrome P450 and sulfotransferase, were regulated through the sex-related secretion pattern of growth hormone^[37]. Some studies reported the sex-related effect on drug -metabolizing enzymes^[38,39]. However, in our study, no marked sex difference in the effects of long-term treatment with YHK on hepatic drug-metabolizing enzymes in rats was observed.

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[38](#)Finnen MJ, Hassall KA. Effects of hypophysectomy on sex differences in the induction and depression of hepatic drug-metabolizing enzymes in the rat. *J Pharmacol Exp Ther* 1984; 229: 250-254

[39](#)Kobayashi Y, Ohshiro N, Suzuki M, Sasaki T, Tokuyama S, Yoshida T, Yamamoto T. Sex-related effect of hemin on cytochrome P450 and drug-metabolizing enzymes in rat liver. *J Toxicol Sci* 2000; 25: 213-222

Glutathione conjugation is probably the most effective means of detoxification of reactive intermediates (Wilkinson & Clapper 1997).

Wilkinson, J., Clapper, M. L. (1997) Detoxification enzymes and chemoprevention. *Proc. Soc. Exp. Biol. Med.* **216**: 192±200

ROS are widely generated in biological system either by normal metabolic pathways or as a consequence of exposure of chemical carcinogen is extensively studied and may results in membrane dysfunction, protein inactivation, DNA damage and ultimately contribute to the multisteps process of carcinogenesis (Sun 1990; Perchellet & Perchellet 1989). The collective action of both antioxidants and phase II enzymes such as glutathione S-transferase and quinone reductase, besides small nonenzymatic water soluble biomolecules, is to afford protection against the adverse effects of oxidants or reactive metabolites of precarcinogens (Sun 1990 ; Perchellet & Perchellet 1989). Reiners et al. (1991), have shown the depleted levels of antioxidant enzymes in 7,12-dimethylbenz(a)anthracene-12-O-tetradecanoylphorbol-13-acetate-treated skin and in skin tumors induced chemically. Depletion of these enzymes following exposure to carcinogens and /or tumor promoter is also known (Sun 1990 ; Perchellet & Perchellet 1989). On the contrary, cancer chemoprevention studies have shown that following the administration of chemopreventive agents, the levels of antioxidant enzymes are elevated in various organs of test animals (Wattenberg 1990).The significant enhancement in the activity of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, catalase and phase II enzymes like glutathione S-transferase and quinone reductase in the various organs of mice fed with YHK suggest that it may contribute to the cancer chemopreventive effects observed with curcumin (Satoskar et al. 1986; Huang et al. 1992 ; Sharma 1976). These results showed that YHK feeding to rats resulted in the induction of glutathione linked enzymes which are known to be involved in detoxification of electrophilic product of lipid peroxidation may contribute to its anti-inflammatory and anti-cancer activities.

The primary antioxidant enzyme catalase possess a slow catalytic activity at low intracellular levels of its substrates H_2O_2 , under this condition, glutathione peroxidase plays the predominant role in the detoxification of peroxides from the cells and /or tissues (Raes et al. 1987). Several reports suggest the pronounced effects of peroxides as compared to O_2 in producing cytotoxicity/genotoxicity in the cellular systems (Sun 1990 ; Perchellet & Perchellet 1989). Besides , the highly reactive OH, generated from H_2O_2 via the Haber-Weiss-like–Fenton reaction (Perchellet & Perchellet 1989), is known to damage macromolecules, specifically DNA, to produce pathological alterations (Sun 1990; Perchellet & Perchellet 1989). In view of these facts, the enhancement in the activity of glutathione peroxidase and catalase in the liver of YHK-fed animals suggest that such a treatment could protect the cells/tissues against the cytotoxic/genotoxic effects of peroxides and OH.

The two-electron reduction of the metabolic products of polycyclic aromatic hydrocarbons such as quinones, catalyzed by quinone reductase also known as DT-diaphorase, has been considered to be a detoxification pathway, since the resulting hydroquinones may be conjugated and excreted through mercapturic acid pathways (Monks et al. 1992). These quinones in addition to electrophilic characteristics, are well known oxidants (Monks et al. 1992) covalently bind to DNA forming depurinating adducts and play a definitive role in cancer induction (Cavalieri et al. 1997). The semiquinone, the product of one electron reduction of quinines via microsomal NADPH-cytochrome P-450, may be toxic perse or react with molecular oxygen, forming O_2 and regenerating the parent quinines, which is then available for rereduction and thereby undergoes a futile redox cycling (Monks et al. 1992). The net result of such a redox cycling is an oxidative stress resulting from disproportionate consumption of cellular reducing equivalent and generation of reactive oxygen species such as O_2 , H_2O_2 and OH (Sun 1990; Perchellet & Perchellet 1989). A phase II enzyme such as glutathione S-transferase not only catalyzes the conjugation of both hydroquinones and epoxides of polycyclic aromatic hydrocarbon with reduced glutathione for their excretion, but also shows low activity towards organic hydroperoxides for their detoxification from cells/tissues (Ketterer et al. 1987). It is therefore reasonable to assume that increased activities of glutathione S-

transferase and quinone reductase in liver YHK-fed rats play an important role in relation to the cancer chemopreventive effects of this compound (Marotta et al., personal communication, Natural Health Product Conference, Montreal, 2004).

In conclusion, YHK may owe its anticarcinogenic potential to its ability to modulate the initiation stage of chemical carcinogenesis by affecting the enzyme systems that catalyse the activation and detoxification processes. It could be envisaged that the mutagenic and carcinogenic process, and the ultimate risk of developing a chemically-induced cancer, lie in the delicate balance between phase I carcinogen activating enzymes and phase II detoxifying enzymes.

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There is a considerable emphasis on identifying potential chemopreventive agents present in food consumed by human population. A substantial body of data from epidemiological studies indicate that diet has profound impact on the multistep processes of chemical carcinogenesis (Doll & Peto 1981). Thus the frequent consumptions of fruits and vegetables have been linked to reduced risk for carcinogenesis (Wattenberg & Coccia 1990). Prevention studies for cancer control involving complete reduction or elimination of human exposure to these environmental factor may not always be possible; however, as an alternative approach, the agents for alleviating the carcinogenic effect of several of these substances have been identified and tested for their chemopreventive action (Wattenberg 1992). Wattenberg (1992), Boone et al. (1990), and Kelloff et al. (1992) reviewed the results of many chemoprevention studies in laboratory animal models and in human setting. Although the use of medicinal plants or their active principles in the preventions and/ or treatment of chronic diseases is based on the experience of traditional systems of medicine from different ethnic societies, their use in modern medicine suffer from lack of scientific evidence. Attention has been focused on identifying dietary phytochemical, which possesses ability to block the process of carcinogenesis and thereby suppress toxicity .

Table 1 Effects of long-term YHK consumption on microsomal enzymes

	Group	P450 nmol/mg MS pro
♂	YHK (n=10)	2.66±0.55 ^a
	Control (n=10)	1.08±1.04
♀	YHK (n=10)	0.66±0.42
	Control (n=10)	0.36±0.18

^aP<0.01 vs ♂ control, ^bP<0.05 vs ♀ control, ^cP<0.05 vs ♀ control.

Table 2: Effect of 2% dietary supplementation of YHK to rats on antioxidant enzyme activities in liver

Enzyme Activities	Treatment Groups	Liver
Glutathione peroxidase activity (nmol NADPH oxidized/min/mg protein)	Control group	203.4 ± 9.4
	Experimental group % of Control	387.1 ± 7.2a 173
Catalase activity (nmol H ₂ O ₂ consumed/min/mg protein)	Control group	419.9 ± 82.3
	Experimental group % of Control	658.4 ± 56.3a 164

Data represent mean \pm S.E. of twenty animals. For statistical significance, student's t- test was used between normal diet-fed control and YHK- fed experimental groups.

A $p < 0.001$.

B $p < 0.05$.

Table 3 : Effect of 2% dietary supplementation of YHK to rats on phase II enzyme activities in the liver

Enzyme Activities	Treatment Groups	Liver
Glutathione S-transferase activity (nmol CDNB formed/ min/mg protein)	Control group	1121.6 \pm 119.0
	Experimental group	1692.4 \pm 78.2a
	% of Control	162

Data represent mean \pm S.E. of twenty animals. For statistical significance, student 's t-test was used between normal diet-fed control and YHK diet- fed experimental groups.

A $p < 0.001$.

B $p < 0.05$.