RESEARCH COMMUNICATION

Dietary Administration of Inositol and/or Inositol-6-phosphate Prevents Chemically-induced Rat Hepatocarcinogenesis

Hae-Jeung Lee, Sang-Ah Lee, Haymie Choi*

Abstract

Chemoprevention is considered a rational strategy for dietary approaches to prevention of cancer. Multiple lines of evidence suggest that many of our dietary principles are able to intervene in the multistage carcinogenesis process and phytic acid (inositol hexaphosphate, IP6), a phytochemical present in a variety of plant species, has been shown to prevent various cancers, including those of the mammary gland, colon and liver. However, the mechanism of chemoprevention by IP6 has not been fully elucidated. In the present study, we examined the effects of inositol and/or IP6 supplementation on rat hepatocarcinogenesis initiated by diethylnitrosamine (DEN) and promoted by partial hepatectomy (PH). Supplementation with either inositol or IP6, or their combination, starting one week prior to administration of DEN, resulted in a significant decrease in both the area and the number of placental glutathione S-transferase positive (GST-P+) foci, a preneoplastic marker for DEN-initiated hepatocarcinogenesis. The administration of inositol and/or IP6 in drinking water caused marked enhancement in the glutathione S-transferase (GST) activity. In addition, the production of thiobarbituric acid reactive substances and the catalase activity were significantly reduced in rats supplemented with inositol and /or IP6. Based on these findings, it is likely that the chemopreventive effects of inositol and/or IP6 on rat hepatocarcinogenesis initiated by DEN and promoted by PH are associated with induction of GST activity and suppression of lipid peroxidation.

Key Words : Inositol - inositol hexaphosphate (IP6) - hepatocarcinogenesis - placental glutathione S - transferase positive foci - glutathione S-transferase

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Introduction

Phytic acid (inositol hexaphosphate, IP6) exists in plants normally as a salt, most commonly in association with sodium or potassium. Inositol and its phosphated derivatives are found not only in plants (Clements and Darnell, 1982), but also in mammalian tissues (Sakamoto et al., 1993), where they participate in the regulation of vital cellular functions, such as signal transduction, proliferation and differentiation (Shamsuddin and Vucenik, 1999). Interestingly, several recent studies have shown that IP6 modulates prostate (Singh et al., 2004), colon (Shamsuddin et al., 1989; Shamsuddin and Ullah, 1998), mammary (Shamsuddin et al., 1989; 1995; 1996), and liver (Hirose et al., 1991; 1999) carcinogenesis. The plausible mechanisms for anticancer effects of IP6, although not fully elucidated yet, include antioxidant function, control of cell signaling, inhibition of cell proliferation, induction of cell differentiation, and enhancement of immune responses (Baten et al., 1989; Fox and Eberl, 2002; Vucenik and Shamsuddin, 2003).

Modulation of the intracellular phosphate pool and signal transduction cascades is considered to be associated with anticarcinogenic effects of a wide spectrum of naturally occurring substances. It has been reported that administration of IP6 alters the intracellular inositol phosphate pool (Shamsuddin, 1997). Huang et al (1997) have documented evidence that IP6 inhibits the activation of activator protein 1 (AP-1) and cellular transformation by targeting phosphatidylinositol-3 kinase (PI3K), a key enzyme in the intracellular signal transduction cascade regulating cell proliferation. IP6 also reduces the rate of cellular proliferation by controlling cell division (Saied and Shamsuddin, 1998) through induction of $p21^{WAF1}$ and p53-mediated cell cycle arrest. It has also been reported that, IP6 is capable of inducing differentiation of K-562 human erythroleukemia (Shamsuddin et al., 1992), HT-29 human

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colon carcinoma (Yang and Shamsuddin, 1995), and prostate cancer (Shamsuddin and Yang, 1995) cells. Moreover, the transformation of $HepG_2$ human liver cancer cells was reversed to the normal phenotype by supplemention with IP6 (Vucenik et al., 1998).

Inositol has been shown to elicit anti-cancer effect on pulmonary (Estensen and Wattenberg, 1993; Wattenberg, 1999), colon (Ullah and Shamsuddin, 1990), and mammary carcinogenesis to the same extent as IP6 (Shamsuddin et al., 1995; 1998). Oral administration of inositol suppresses hepatic carcinogenesis in mice (Nishino et al., 1996) and the combination of IP6 and inositol synergistically enhanced the antineoplastic effect in Sprague-Dawley rats treated with a classical tumor initiator, 7,12-dimethylbenz(a)anthracene (Shamsuddin et al., 1995).

The purpose of the present study was to evaluate inhibitory effects of inositol and/or IP6 on rat hepatocarcinogenesis and to determine whether their combination can synergistically potentiate any chemopreventive activity.

Materials and Methods

Animals

Male Sprague-Dawley rats (4 weeks) were supplied by the Animal Care Facility (Chemical Institute of Daeduk, Korea) and were acclimatized for 2 weeks before launching the study. Animals were kept for 8 weeks in plastic cages under standard conditions (room temperature 20 ± 1˚C, relative humidity 55 ± 1%, 12 hr light/dark cycle), given food and liquid *ad libitum*, recorded daily and weighed weekly. Rats were divided into five groups, each consisting of 12 animals. The basal diet was prepared as described previously (Kim et al., 2000) with slight modification and contained: 20 g casein, 54.7 g corn starch, 15 g corn oil, 1.0 g vitamins and mixed without inositol, 5g α-cellulose, 4.0 g minerals, and 0.3 g DL-methionine per 100 g diet. All groups were given basal diet, which was composed of 15% (g/100g) corn oil (31% of total calories).

Chemicals

Diethylnitrosamine (DEN), myo-inositol, IP6 (as dodecasodium salt from corn), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), and avidin-biotin-peroxidase complex (Vectastain Elite ABC kit) used for immunohistochemical assay were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-rat GST-P antibody (Vector Co., USA) was kindly provided by Prof Yeong-Soon Lee, Veterinary Public Health Lab, College of Veterinary Medicine, Seoul National University.

Treatment of Animals

Hepatocarcinogenesis was initiated by DEN and was promoted by PH using a modified medium-term bioassay protocol (Ito et al., 1980; 1988; 1989). Each animal received a single intraperitoneal injection of DEN (200 mg/kg body weight) dissolved in saline. After 3 weeks of DEN administration, rats were subjected to a 2/3 PH and were sacrificed at 8 weeks. Inositol (2% w/v), or IP6 (2% w/v), or a combination of inositol and IP6 (1% w/v each) in drinking water (pH 7.4 adjusted) were given from week 2 after initiation with DEN as shown in Figure 1. The actual phytic acid content in the 2% IP6 liquid was 1.4%, the pH being adjusted to pH 7.4.

Sample Collection

Animals were killed by decapitation at 8 weeks. Before sacrifice, animals were kept under fasting for 12 h (Figure 1). Livers were promptly excised, weighed, finely minced in cold 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM of EDTA and homogenized. Each homogenate was centrifuged at 1000 ×g for 10 min, and the supernatant was further centrifuged at 10,000 ×g. Pellets were homogenized in potassium phosphate buffer (pH 7.4) for the catalase assay. The remaining supernatant was centrifuged at 105,000 ×g. Peroxisomal (including mitochondria, lysosomes, and peroxisomes), microsomal, and cytosolic fractions were prepared by differential centrifugation (Wolfe, 1993) and stored at -70˚C until used.

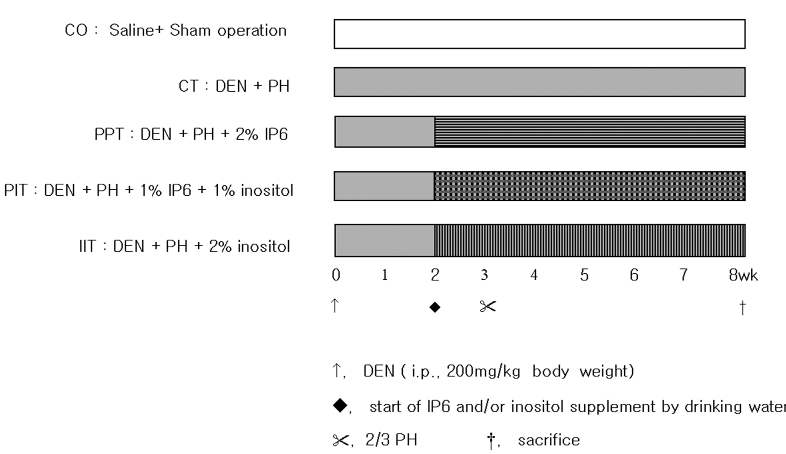

Figure 1. Experimental Protocol

Experimental liquids were administered for 6wks : PPT (IP6 2%), PIT (1% IP6 + 1% inositol), and IIT (2% inositol). All except CO group rats received a single intraperitoneal injection of diethylnitrosamine (DEN), subjected to 2/3 partial hepatectomy (PH) at week 3, and sacrificed at week 8.

Analysis of Placental Glutathione S-transferase Positive (GST-P+) Foci

At autopsy, liver sections of 2-3 mm were cut with a razor blade and fixed in ice-cold acetone for immunohistochemical examination of GST-P+ foci. The avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab. Inc., Burlingame CA; GST-P antibody, MBL Co., Japan; Hsu et al., 1981) was used to visualize GST-P+ foci that are commonly regarded as putative preneoplastic lesions. The areas and numbers of GST-P+ foci (> 0.2 mm in diameter) in liver sections were measured using an image analyzer (Quantinet 520, Cambridge Instruments, USA)

Biochemical Assays

GST activities in the hepatic cytosolic fraction were determined according to the method of Habig et al. (1974). The conjugation of GSH with CDNB was monitored by reading absorbance changes at 340 nm using a dual beam spectrophotometer (Beckman DU650). The concentration of thiobarbituric acid reactive substances (TBARS) was determined according to the method of Buege and Aust (1978). Malondialdehyde, the product of lipid peroxidation reacts with TBA to form a chromophore that is detectable at 535 nm. The catalase activity was determined by measuring the enzymatic decomposition of H_2O_2 as reported by Abei (1984). Protein was quantified using the modified Lowry method (1951), with bovine serum albumin as the standard.

Statistical Analysis

SAS 8.1 statistical software was used to analyze the data. Differences among groups were determined by analysis of variance (ANOVA) with the Duncan's multiple range test at $p < 0.05$.

Results

Gross Observation

Animals of all groups were kept under close observation for intake of diet and liquid throughout the study period. There was no significant difference in liquid or basal diet intake among different treatment groups. However, liquid intake was apparently lower in animals given IP6 (2%) compared with those receiving either inositol (2%) or a combination of inositol and IP6. Final body and liver weights in carcinogen treated groups did not significantly vary. Relative liver weights (%) in animals given inositol and/or IP6 were decreased, but were not significantly different between these supplemented groups.

Suppression of Carcinogen-induced GST-P+ Foci Formation by Inositol and/or IP6

In all groups treated with DEN, placental GST-P+ foci were developed. These foci are known to be the most effective biochemical phenotypic markers for DEN-initiated preneoplastic lesions (Sato, 1989). The areas and the numbers of the GST-P+ foci (mean diameter >0.2 mm) in inositol and/or the IP6 supplemented groups were significantly lower than those of the carcinogen treated animals (Figure 2). All three groups supplemented with inositol, IP6 plus inositol, and IP6 exhibited reduced hepatic GST-P+ foci development. The frequency of GST-P+ foci in the group supplemented with a combination of inositol and IP6 was not significantly different from that observed in groups supplemented with either inositol or IP6. The present study, thus revealed a lack of synergistic effect of IP6 and inositol in terms of suppressing DEN-induced hepatocarcinogenesis in rats.

Table 1. Effect of Inositol and/or IP6 on Final Body Weights, Liver Weights, and Relative Liver Weights

Group	Final body weight (g)	Liver weight (g)	Relative liver weight (%)[1]
CO	465.1 $\pm$ 8.90^{a}	11.91 $\pm$ 0.34^{a}	2.56 $\pm$ 0.04^{a}
CT	438.3 $\pm$ 10.8^{b}	10.77 $\pm$ 0.54^{ab}	2.49 $\pm$ 0.07^{ab}
PPT	427.3 $\pm$ 6.01^{b}	9.91 $\pm$ 0.36^{b}	2.27 $\pm$ 0.09^{c}
PIT	439.9 $\pm$ 9.20^{ab}	10.53 $\pm$ 0.39^{b}	2.39 $\pm$ 0.05^{abc}
IIT	437.4 $\pm$ 9.10^{b}	9.92 $\pm$ 0.39^{b}	2.32 $\pm$ 0.07^{bc}

[1] Relative liver weight = {Liver weight (g) / Body weight (g)} - 100
Hepatocarcinogenesis was induced by DEN injection and partial hepatectomy. CO, control with saline and shame operation; CT, carcinogen treated; PPT, carcinogen treated + 2% IP6; PIT, carcinogen treated + 1% IP6 + 1% inositol; IIT, carcinogen treated + 2% inositol. Values are mean $\pm$ SD. Values with the different superscripts (a, b, c) are significantly different at p<0.05 by Duncan's multiple range test.

Inositol and/or IP6 Induced GST Activity in Carcinogen-treated Rat Liver

Since inositol and IP6 were found to inhibit DEN- and PH-induced GST-P+ foci formation, effects of inositol and IP6 on the activity of GST, a phase 2 detoxifying enzyme (Kensler, 1997), were examined. The GST activity in carcinogen-treated rat liver was increased by inositol and

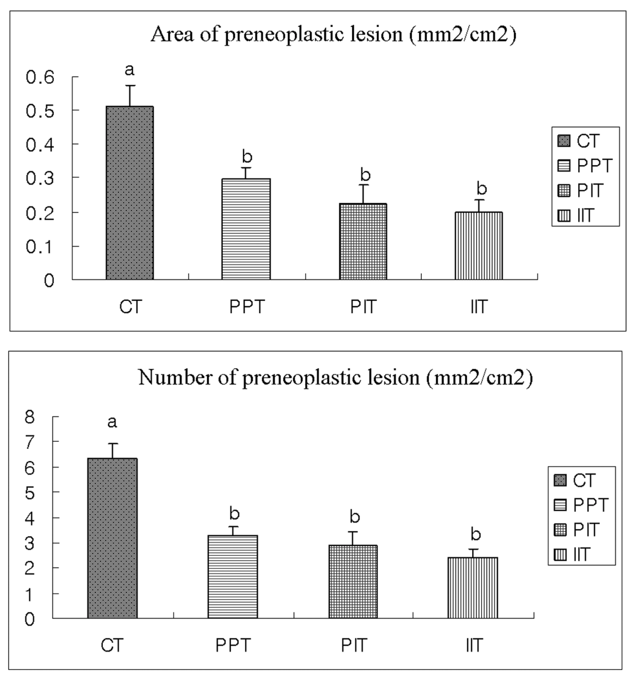

Figure 2. The Effect of Additional Inositol and/or IP6 on the Area and Number of GST-P+ Foci in Rats Treated with DEN and Subjected to Partial Hepatectomy. CT, carcinogen treated; PPT, carcinogen treated + 2% IP6; PIT, carcinogen treated + 1% IP6 + 1% inositol; IIT, carcinogen treated + 2% inositol. Values are means $\pm$S.E. Means with different superscripts (a, b) are significantly different (by analysis of variance, $p < 0.05$)

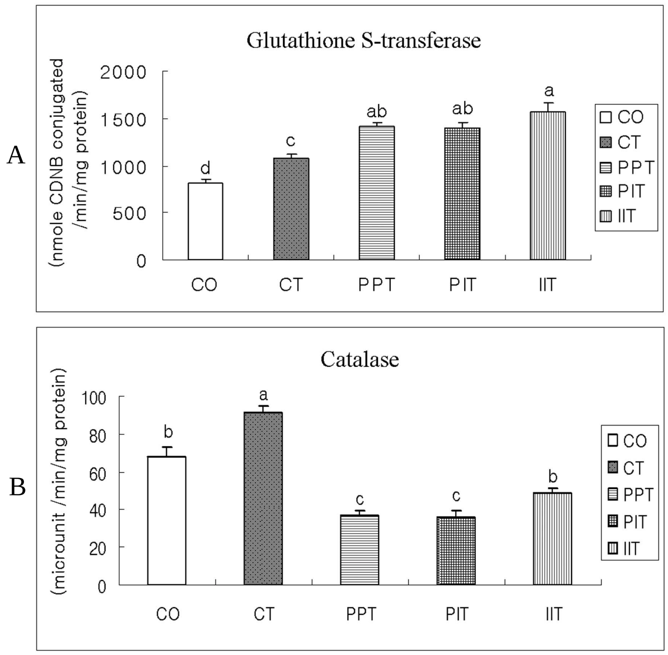

Figure 3. Efects of Inositol and/or IP6 on Glutathione S-trnasferase (A) and Catalase (B) Activity in DEN-induced and PH-promoted Rat Liver. CO, control treated with saline and shame operation; CT, carcinogen treated; PPT, carcinogen treated + 2% IP6; PIT, carcinogen treated + 1% IP6 + 1% inositol; IIT, carcinogen treated + 2% inositol. Values are means ±S.E. Means with different superscripts (a, b, c, d) are significantly different (by analysis of variance, $p < 0.05$).

IP6 supplementation (Figure 3A). Rats supplemented with inositol alone had a relatively high hepatic GST activity compared to other supplemented groups. The GST activity also inversely correlated with the area and the number of GST-P+ foci (r = 0.76, r = 0.71, p<0.01, respectively).

Inhibition of Catalase Activity by Inositol and/or IP6 in Rat Liver Treated with Carcignogen

It has been reported that reactive oxygen species (ROS) are generated in hepatocytes after treatment with DEN (Roomi et al., 1997) resulting in lipid peroxidation, and partial hepatectomy further stimulates the release of ROS by up-modulating TNF-alpha (Diehl, 2000). Hepatocytes from rats treated with DEN followed by PH are vulnerable to oxidative damage that may lead to hepatocarcinogenesis (Sanchez-Perez et al., 2005). One of the antioxidant enzymes is catalase that converts carcinogen-induced H_2O_2 into H_2O and protects cells from oxidative damage. Therefore, an attempt was made to examine the effects of inositol and/or IP6 on catalase activity in DEN- and PH-treated rat liver. The catalase activity was significantly elevated in animals treated with carcinogen alone and was significantly decreased in groups supplemented with either inositol and/ or IP6 (Figure 3B).

Supplementation with Inositol and/or IP6 Reduced the Level of Carcinogen-induced TBARS in Rat Liver

Oxidative stress is reported to be closely linked to cancer and mutation (Horton, 1987), and lipid peroxidation often leads to cell damage and even stimulates tumor promotion (Cerutti, 1985). The antioxidant property of IP6 is reported to contribute to inhibition of hydroxyl radical generation by the Fenton reaction, subsequent lipid peroxidation and DNA damage (Shamsuddin, 1995). To investigate whether the antioxidant effects of inositol and IP6 contributes to the chemopreventive effects of these phytochemicals in chemically-induced rat hepatocarcinogenesis, effects of inositol and/or IP6 on TBARS were studied. TBARS content, which is used as a lipid peroxidation index, was significantly elevated after treatment of DEN followed by PH in a positive correlation with the area and number of GST-P+ foci (r = 0.67, r= 0.62, p<0.01 respectively). Animals supplemented with inositol and/or IP6 showed a lower level of TBARS than the group treated with carcinogen alone (Table 2). TBARS content was decreased in the order of IP6 < IP6 plus inositol < inositol < control < carcinogen alone treated groups. Therefore, the antioxidant function of IP6 could partly contribute to the inhibition of carcinogenesis by these phytochemicals.

Table 2. The Effect of Inositol and/or IP6 on Lipid Peroxidation

Group	Treatment	No. of rat	TBARS (nmole/mg protein)
CO	Saline	9	0.500 ± 0.024[b]
CT	DEN+PH	9	0.633 ± 0.042[a]
PPT	DEN+PH+2% IP6	9	0.244 ± 0.009[c]
PIT	DEN+PH+1% IP6+1% inositol	9	0.256 ± 0.016[c]
IIT	DEN+PH+2% inositol	9	0.281 ± 0.010[c]

CO, control treated with saline and sham operation; CT, carcinogen treated; PPT, carcinogen treated + 2% IP6; PIT, carcinogen treated + 1% IP6 + 1% inositol; IIT, carcinogen treated + 2% inositol. Values are mean ± SE. Values with the different superscripts (a, b, c) are significantly different at p<0.05 by Duncan's multiple range test.

Discussion

Accumulating evidence from laboratory-based and population studies suggest that many of our dietary phytochemicals may exploit as potential chemopreventive agents. There is growing interest in identifying new chemopreventive agents from dietary sources. Phytate (IP6) has previously been reported to inhibit the development of neoplastic lesions in the promotional stage of rat liver carcinogenesis (Hirose et al., 1999). The present study was, therefore, conducted to investigate the possible chemopreventive effects of inositol and IP6 on hepatocarcinogenesis in rats treated with DEN and subjected to PH. Our study revealed that the development of GST-P+ foci, one of the biomarkers of carcinogen-induced hepatic preneoplastic leisons, was significantly inhibited by inositol and/or IP6 supplements. However, the inhibitory effects of inositol and IP6 on DEN- and PH-induced GST-P+ foci formation was not significantly different from that observed by using a combination of inositol and IP6.

IP6 has been reported to cause a statistically significant increase in the hepatic level of GST in normal rat (Singh et al., 1997), but the role of IP6 in modulating GST activity during DEN-induced hepatocarcinogenesis has not been studied yet. Since supplementation with inositol and/or IP6 reduced the formation of GST-P+ foci, attempt was made to examine the effect of these phytochemicals on hepatic GST activity in rat liver exposed to carcinogen and PH. Our study revealed that the elevated GST activity in rat liver treated with DEN- and PH, was more elevated with supplementing IP6 and/or inositol in the drinking water. Inositol induced the GST activity to a greater extent than IP6 or IP6 plus inositol did. The number and the area of the preneoplastic lesions in the liver were negatively correlated with the GST activity.

On the other hand, the catalase activity was elevated in carcinogen-treated liver, which may reflect cellular endeavour to eliminate the oxidative stress induced by DEN and 2/3 PH. The catalase activities of the rats-supplemented with IP6 and/or inositol groups were significantly decreased compared with animals treated with carcinogen alone. Especially in IP6 supplemented group, the catalase activity was the lowest, suggesting no need to increase catalase activity to eliminate hydrogen peroxide because phytate already scarvenged the origin of free radicals. Catalase activity by inositol supplement was significantly decreased compared with carcinogen treated alone. Inositol phosphates, including IP6 were reported to have the capacity to scavenge ROS (Graf et al., 1987). Inositol phosphates could be synthesis from inositol and those could eliminate the origin of free radicals (Shamsuddin, 1991). In support of this assumption, inositol supplement was reported to decrease the lipid peroxidation (Raj et al., 1995).

Many of the important biological activities of IP6 may be attributed to its antioxidant activity. The 1,2,3-triphosphate functional group of IP6 retains a conformation that allows specific interaction with transition metal ions such as iron, thereby inhibiting their ability to catalyze hydroxyl radical formation by Fenton reaction (Graf et al., 1987; Graf and Eaton, 1990). IP6 has been reported to decrease the content of lipid peroxides in normal rat and pig, respectively (Singh et al., 1997; Porres et al., 1999). Muraoka and Miura (2004) have suggested that lipid peroxidation of phytic acid is due to xanthine oxidase inhibition and blocking the formation of the initiator of lipid peroxidation. In our model, lipid peroxidation was dramatically decreased by IP6 supplementation, suggesting that IP6 exerts an anticarcinogenic effect through an antioxidant mechanism.

Inositol supplemented group also showed a decreased lipid peroxidation level. It remains unclear how inositol suppresses lipid peroxidation. One plausible mechanism is that inositol could facilitate the synthesis of inositol phosphates (IP6, IP5, IP4, etc.), and these compounds then exert antioxidant effects. In a recent study, Miyamoto et al. (2000) have demonstrated that the hydrolysis products of IP6 possess a chelating ability. Once inside cells, inositol might undergo re-phosphorylation to form inositol phosphates, which in turn, may chelate crucial cations (Shamsuddin, 1999) involved in the generation of ROS. If this is the case, inositol could reduce the TBARS content by lowering inositol phosphates (IP3, IP4, IP5). Raj and Devis (1995) demonstrated that addition of myo-inositol decreased the peroxidant effect of hydrogen peroxide. Thus, we assume that the antioxidant effect of inositol might be mediated by lower inositol phosphates than IP6.

It has been reported that inositol and IP6 decrease the hepatic concentrations of lipids and the hepatic activities of lipogenic enzymes in rat liver (Tetsuyuki, 1997), which may contribute to their ability to lower lipid peroxidation. Until now, inhibition of lipid peroxidation by IP6 and lower inositol phosphates is considered to be mediated by a chelating mechanism. Besides an antioxidant mechanism, other mechanisms, such as the suppression of p53 or the modulation of other cancer associated genes, may account for chemopreventive effects of IP6. Recently, G0/G1 arrest and S phase inhibition of human cancer cell lines by IP6 were reported (Sharma et al., 2003; El-Sherbiny et al., 2001). In conclusion, inositol and/or IP6 exerts chemopreventive effects in chemically-induced rat hepatocarcinogenesis, at

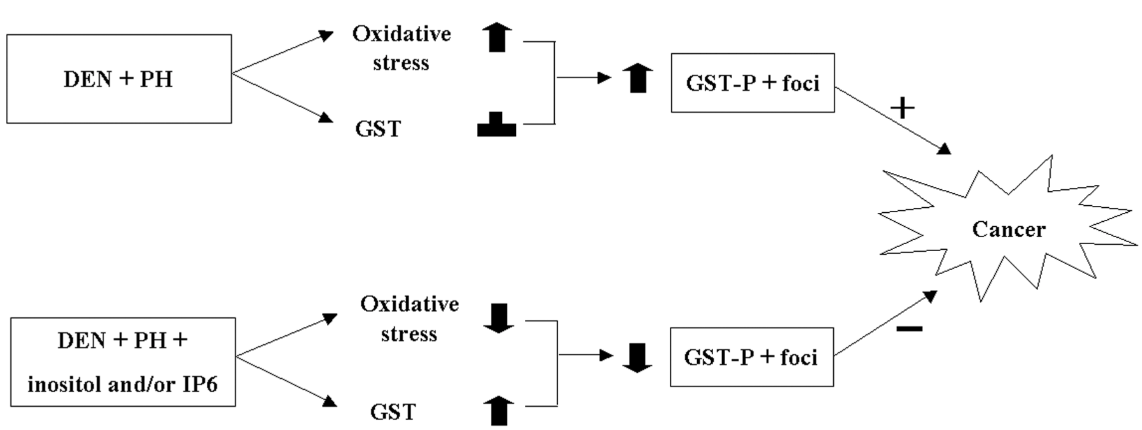

Figure 4. Proposed Mechanisms Underlying Chemopreventive Effects of Inositol and IP6 Against DEN-induced and PH-promoted Rat Hepatocarcinogenesis

least in part, by inducing carcinogen detoxifying enzyme such as GST, and/or scavenging of ROS (Figure 4). Considering the impact of dietary prevention of cancer, further investigation should be conducted to clarify underlying mechanisms of chemoprevention by inositol and IP6.

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