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Review

Chemopreventive Effects of 2-(Allylthio)pyrazine

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A series of organosulfur compounds were synthesized with the aim of developing chemopreventive compounds active against hepatotoxicity and chemical carcinogenesis. 2-(Allylthio) pyrazine (2-AP) was effective in inhibiting cytochrome P450 2E1-mediated catalytic activities and protein expression, and in inducing microsomal epoxide hydrolase and major glutathione S-transferases. 2-AP reduced the hepatotoxicity caused by toxicants and elevated cellular GSH content. Development of skin tumors, pulmonary adenoma and aberrant crypt foci in colon by various chemical carcinogens was inhibited by 2-AP pretreatment. Anticarcinogenic effects of 2-AP at the stage of initiation of tumors were also observed in the aflatoxin B_1 (AFB_1)-induced three-step medium-term hepatocarcinogenesis model. Reduction of AFB_1-DNA adduct by 2-AP appeared to result from the decreased formation of AFB_1-8,9-epoxide via suppression of cytochrome P450, while induction of GST by 2-AP increases the excretion of glutathione-conjugated AFB_1. 2-AP was a radioprotective agent effective against the lethal dose of total body irradiation and reduced radiation-induced injury in association with the elevation of detoxifying gene expression. 2-AP produces reactive oxygen species *in vivo*, which is not mediated with the thiol-dependent production of oxidants and that NF-κB activation is not involved in the induction of the detoxifying enzymes. The mechanism of chemoprotection by 2-AP may involve inhibition of the P450-mediated metabolic activation of chemical carcinogens and enhancement of electrophilic detoxification through induction of phase II detoxification enzymes which would facilitate the clearance of activated metabolites through conjugation reaction.

Key words : 2-(Allylthio)pyrazine, Chemoprevention, Radioprotection, Hepatoprotective Agent, Cytochrome P450, Epoxide hydrolase, Glutathione S-transferase

INTRODUCTION

One of the most promising areas in cancer research is chemoprevention. Chemoprevention is the process of inhibiting, delaying, or reversing the process of carcinogenesis and will ultimately provide benefits to public health by lowering the incidence of human cancers (Morse and Stoner, 1993; Kelloff *et al.*, 1994). Both naturally-occurring and synthetic chemicals have been tested for the possible cancer chemopreventive activities. Organosulfur compounds have been found to possess protective effects against experimental carcinogenesis and mutagenesis. Examples include diallylsulfide present in garlic and onion (Sparmins *et al.*, 1988; Hong *et al.*, 1992; Surh *et al.*, 1995; Wargovich *et al.*, 1992). The compound inhibits expression of cytochrome P450 2E1 (CYP 2E1) (Brady *et al.*, 1991; Haber *et al.*, 1995), and also induces Phase II detoxification enzymes such as glutathione S-transferases and NAD(P)H:quinone oxidoreductase (Sparmins *et al.*, 1988; Reddy *et al.*, 1993; Eaton and Gallagher, 1994). Besides naturally-occuring organosulfur compounds, several synthetic sulfur-containing substances including oltipraz have been shown to exert chemopreventive properties against chemical-induced carcinogenesis in many animal models (Sparmins *et al.*, 1988; Haber *et al.*, 1995; Helzlsouer and Kensler, 1993; Maxuitenko *et al.*, 1993). Studies in this laboratory have shown that organosulfur compounds allylsulfide, allylmercaptan and allylmethylsulfide are effective in suppressing both constitutive and chemical-inducible CYP 2E1 expression (Kwak *et al.*, 1994), and in inducing glutathione S-transferases (GSTs) and microsomal epoxide hydrolase (mEH) and their mRNA levels in rats. (Kim *et al.*, 1994; Kim *et al.*, 1995). A series of compounds were synthesized with the aim of developing potential chemopreventive organosulfur compounds active against hepatotoxicity and chemical carcinogenesis under the assumption that alterations in the activities of Phase I and Phase II enzymes correlate with chemoprevention.

2-(Allylthio)pyrazine (2-AP), among a series of syn-

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thesized pyrazine derivatives with allylsulfurs, was the most effective in inhibiting CYP 2E1-catalytic activities *in vitro* (Ki, 12 μM) and in suppressing CYP 2E1-catalytic activities and protein expression *in vivo* (Kim *et al.*, 1997). 2-AP suppressed isoniazid-, ethanol-, acetone-, pyridine- or starvation-inducible hepatic CYP 2E1 levels, as shown by both metabolic activities and immunoblot analyses (Kwak *et al.*, 1997). 2-AP was effective in suppressing both constitutive and inducible P450 2E1 expression. Northern blot analysis showed that 2-AP transiently suppressed the hepatic P 450 2E1 mRNA level, suggesting that suppression in P 450 2E1 expression by 2-AP may be mediated in part by transcriptional inactivation (Kim *et al.*, 1997). In addition, oral administration of 2-AP to rats gave rise to marked elevation of GST and mEH levels with increases in their mRNA levels (Kim *et al.*, 1995; Kwak, 1997; Kim *et al.*, 1999) and cellular GSH levels (Kim *et al.*, 1997). In the rat, 2-AP produced significant induction of the major GST subunits including rGSTA 5 (Kwak, 1997; Kim et al., 1999). The mEH level was also increased by 4-fold in the hepatic microsomes derived from 2-AP-treated animals, relative to that in vehicle-treated rats (Kim *et al.*, 1999). The mEH mRNA level was also increased by 18-fold relative to control after 2-AP treatment (Kwak, 1997; Kim et al., 1999).

Hepatoprotective effects

Hepatoprotective effects of 2-AP against toxicants were monitored. 2-AP substantially reduced the hepatic toxicity caused by acetaminophen, carbon tetrachloride or aflatoxin B_1 in animals, as evidenced by reduction in the mortality rate of animals as well as decreases in serum alanine aminotrasferase and sorbitol dehydrogenase activities (Kim *et al.*, 1997; Ha and Kim, 1998). 2-AP also inhibited the hepatotoxicity caused by chloroform, N-nitrosodimethylamine, and N-nitrosodiethylamine (Kwak, 1997). In primary cultured rat hepatocytes, 2-AP inhibited P450 2E1-catalytic activities and protein expression induced by pyridine and increased the expression of mEH and GST proteins (Kwak, 1997). Cytotoxicity induced by acetaminophen or carbon tetrachloride was also inhibited by 2-AP, as supported by decrease in release of lactic dehydrogenase, suggesting that 2-AP protects the hepatocytes against toxicants (Kwak, 1997). 2-AP also reduced acetaminophen-, CCl_4- or AFB_1-induced increases in lipid peroxidation (Kim *et al.*, 1997; Ha and Kim, 1998). Further study was conducted to assess the role of 2-AP in scavenging reactive oxygen species. The protective effect of 2-AP on the DNA strand breakage induced by benzenetriol was assessed by measuring the conversion of supercoiled ϕX-174 DNA to the open relaxed form. 2-AP blocked the benzenetriol-induced conversion of supercoiled DNA to open circular form in a dose-dependent manner. 2-AP completely protected benzenetriol-induced DNA strand breakage with the EC50 of ~220 μM, suggesting that 2-AP effectively scavenges the reactive oxygen species (Kim *et al.*, 1996). 2-AP also inhibited the acetone- or isoniazid-induced conversion of supercoiled DNA to open circular form (Kwak, 1997). The results suggest that antioxidant properties of 2-AP may play a role in protecting against the hepatotoxic effects of the toxicants (Kim *et al.*, 1996; Kwak, 1997).

Cancer chemopreventive effects

Recent studies have shown that the multiplicities of skin tumors formed in female ICR mice treated with vinylcarbamate or vinylcarbamate oxide were significantly inhibited by pretreatment with 2-AP (Surh *et al.*, 1998). 2-AP also inhibited the mutagenecity of vinylcarbamate in Salmonella-microsome assay (Surh *et al.*, 1998). 2-AP has recently been shown to protect against the development of pulmonary adenoma induced by benzo(a)pyrene in NIH mice and to inhibit aberrant crypt foci in colon induced by azoxymethane in rats (unpublished data).

Aflatoxin B_1 (AFB_1), a mycotoxin produced by the fungus *Aspergillus flavus*, is known to induce the hepatocellular carcinoma. In oriental countries, food contamination with AFB_1 with high incidence of hepatitis B virus infection has been a serious problem (Eaton and Gallagher, 1994). The anticarcinogenic effects of 2-AP at the stages of initiation of tumors were evaluated in the aflatoxin B_1 (AFB_1)-induced three-step medium-term hepatocarcinogenesis model (Haber-Mignard *et al.*, 1996). The studies were conducted to define the dose-response characteristics for 2-AP inhibition of AFB_1-induced tumorigenesis using a marker of presumptive liver preneoplastic foci, GST-P. Male Sprague-Dawley rats were treated with 2-AP at the daily oral doses of 10, 25, and 50 mg/kg for 16 consecutive days, during which four repeated doses of AFB_1 (1.0 mg/kg, i.p.) were given to the animals. Rats were then subjected to two-thirds of hepatectomy, followed by administration of phenobarbital. Focal areas of hepatocellular alteration were identified after 44 days and preneoplastic foci expressing the placental form of glutathione S-transferase (GST-P) were quantified by immunostaining of liver sections. Whereas AFB_1 (1.0 mg/kg) administration significantly reduced the increase in body weight, 2-AP returned the body weights to normal. Rats treated with AFB_1 showed 100% incidence of hepatic foci formation, a mean number being 20.9 ± 3.1 foci/cm^2 with the mean size (areas) of 32.1 ± 11.4 (10^{-2} mm^2). Percentage of the section area occupied by GST-P positive foci was 5.6 ± 1.5%. Treatment with 2-AP significantly reduced the parameters associated with GST-P positive foci.

Mean sizes (diameter) of the foci were significantly smaller in the animals treated with 2-AP than those of control. The percentage of section areas occupied by GST-P-positive foci was reduced 65, 85 and 96% at the doses of 10, 25 and 50 mg/kg of 2-AP, respectively (Ha *et al.*, 1998). Ornithine decarboxylase (ODC) serves as the rate-limiting enzyme in the polyamine biosynthesis (Pegg and McCann, 1982). Polyamines play an essential role in cell proliferation, which is essential for tumor promotion. Thus, the induction of ODC has been implicated in carcinogenesis (Russel, 1985: OBrien, 1976; Kingsnorth *et al.*, 1983) and used as an intermediate biomarker in chemopreventive studies (Rao *et al.*, 1993; Kojima *et al.*, 1993; Rozhin, *et al.*, 1984). ODC activity in AFB_1-exposed rats was determined by the three-step medium-term hepatocarcinogenesis assay. Animals treated with AFB_1 (1 mg/kg, 4 times) showed increases in ODC activity as compared with vehicle-treated, from 2.5 ± 0.3 to 5.6 ± 0.6 pmol/mg protein. ODC activity in the liver was significantly decreased by 40% to 66% in rats treated with 2-AP at the dose of 10, 25 and 50 mg/kg, as determined at the 44 days post-treatment (Ha *et al.*, 1999).

It has been shown that the major detoxification pathways for AFB_1 in rodents involve GSH conjugation catalyzed by GST (Eaton and Gallagher, 1994). Glutathione has a variety of cellular function in protecting cellular macromolecules against reactive intermediates (Molders and Jernestron, 1983). Because glutathione is a natural antioxidant, it can protect cells during the initiation phase of carcinogenesis (Boyland and Chasseud, 1979). The effects of 2-AP on liver GST enzymes and GSH contents were evaluated to determine whether 2-AP altered the early phase of AFB_1-induced carcinogenesis. GST activity and total GSH content in rat liver treated with 2-AP (50 mg/kg, once a day for 14 days) and AFB_1 (1.0 mg/kg, 4 times) were monitored. Treatment of rats with 2-AP produced 40~150% increases in GST activity relative to control during the treatment. Administration of AFB_1 alone resulted in fluctuation in GST activity, which declined to the basal level in two days. Coadministration of AFB_1 and 2-AP resulted in a 2.5-fold increase in the GST activity at 14 days post-treatment. (Ha *et al.*, 1999). The time course of the hepatic GSH contents following treatment of rats with AFB_1 or AFB_1 plus 2-AP was studied. Hepatic GSH contents increased by 60% in rats treated with 2-AP (50 mg/kg). AFB_1 administration decreased the GSH content in the liver. Treatment of rats with both 2-AP and AFB_1 resulted in an increase in the GSH content, as compared with AFB_1 treatment alone. The next study was designed to determine whether 2-AP could increase the biliary excretion of AFB_1-conjugate in rats exposed to AFB_1. Rats were pretreated with 2-AP (p.o.) at the daily dose of 50 mg/kg for 5 consecutive days. AFB_1 (5 mg/kg) was administered intraperitoneally 2 h after the last dose of 2-AP. Amounts of principal AFB_1 metabolites, AFB_1-glutathione (AFB_1-GSH), and AFP_1-glucuronide secreted in bile juice was increased by 56% and 50%, respectively after 2-AP treatment, which was in agreement with the observation after oltipraz treatment by Holeski (Holeski *et al.*, 1987). Thus, it is likely that induction of GST by 2-AP increases the excretion of glutathione-conjugated AFB_1, which may contribute to its chemopreventive effects against hepatocarcinogenesis induced by this carcinogen. Pharmacokinetic studies have shown that excretion of acetaminophen-glucuronide in bile and urine was significantly increased in 2-AP-pretreated rats, showing that 2-AP might induce the UDP glucuronyl transferases (Kwak *et al.*, 1998).

AFB_1 requires P450-mediated bioactivation to produce carcinogenic and mutagenic metabolites *in vivo* (Miller, 1978). The principal metabolites implicated in AFB_1-carcinogenesis and mutagenesis include reactive AFB_1-8,9-oxide (AFBO), which has been shown to bind to nucleic acids and proteins (Croy *et al.*, 1978; Essigmann *et al.*, 1970). The effect of 2-AP (10, 25 and 50 mg/kg) on AFB_1 adduct formation was examined in rats. Levels of radiolabelled AFB_1 covalently bound to hepatic DNA, RNA and protein were reduced by 73, 60 and 49%, respectively, in animals treated with 2-AP for 5 days prior to a single exposure to AFB_1 (0.6 mg/kg) (Ha *et al.*, 1999). Exposure of animals to AFB_1 results in the formation of AFB_1-N^7-guanine as a predominant DNA adducts in the liver (Kensler *et al.*, 1992). Studies have also shown that the excretion of AFB_1-N^7-guanine adduct into the urine occurred in a dose-dependent manner following a single exposure of rats to AFB_1 and that the amount of adduct excreted was proportional to the amount of AFB_1-DNA adduct formed in the liver (Goopman *et al.*, 1993; Bennet *et al.*, 1981). 2-AP pretreatment also caused a 45% reduction in the urinary elimination of AFB_1-N^7-guanine adduct over the 24-h postdosing period (Ha *et al.*, 1999). Levels of radiolabelled AFB_1 covalently bound to calf thymus DNA catalyzed by microsomes obtained from 2-AP-treated rats (10 and 50 mg/kg, for 5 days) were reduced 47~66% *ex vivo*. The effect of 2-AP on AFB_1-DNA adduct formation was also determined *in vitro*. When [^{3}H]-AFB_1 and calf thymus DNA were incubated with 2-AP (0.07~1.3 mM), AFB_1 binding to DNA in the presence of S9 fraction was inhibited by 2-AP. Studies were extended to determine whether 2-AP was active in inhibiting the oxidation of AFB_1, which is primarily catalyzed by P450 2B and P450 3A in the rat liver to the reactive AFBO (Burke *et al.*, 1994: Langouët *et al.*, 1997). Hepatic microsomal pentoxyresorufin-*O*-dependentylase (PROD) and ethoxyresorufin-*O*-deethylase (EROD) activities were measured in phenobarbital (PB)-, dexamethasone (DEXA)- and 3-methylcholanthrene (3-MC)-induced microsomes. Microsomal PROD activity was inhibited in PB- or

DEXA-induced microsomes by 2-AP (IC_{50} was 2.5 and 1.5 μM, respectively). However, the IC50 value of 2-AP inhibition on the EROD activity was 15 mM (Ha and Kim, 1998). Thus, the reduction of AFB_1-DNA adduct by 2-AP is likely to result from the decreased formation of AFB_1-8,9-epoxide via suppression of cytochrome P450. These results support the conclusion that both induction of phase II enzymes and inhibition of AFB_1 activation by 2-AP might be related to the chemoprotective effect of 2-AP against liver toxicants.

Radioprotective Effects

Studies in our laboratories revealed that γ-ray ionizing radiation causes alterations in the hepatic mEH and GST gene expression with induction of the proteins and that the gene expression is enhanced by oltipraz (Nam *et al.*, 1997; Kim *et al.*, 1997a). Cellular radioprotective mechanisms can provide protection against a subsequent exposure to radiation. We have shown that oltipraz reduced the number of degenerated hepatocytes against ionizing irradiation and improved the liver function. Oltipraz was a *bona fide* radioprotective agent against the lethal dose of total body irradiation (TBI), as supported by the increase in the 30-day survival rate of animals (Kim *et al.*, 1998a). The increase in the survival rate was related with the improvement in the liver function. Thus, oltipraz was effective in reducing radiation-induced injury in association with the elevation of mEH and GST gene expression. The role of mEH and GST expression in protecting animals against radiation-induced injury was further supported by substantial increases in the mortality rate in irradiated animals by dexamethasone treatment (Nam *et al.*, 1998). The enhancer region of the mEH and certain GST genes include a glucocorticoid responsive element, which negatively regulates the expression of the genes (Hayes and Pulford, 1995). We previously found that dexamethasone decreased the constitutive and inducible expression of hepatic mEH and GST mRNA (Nam *et al.*, 1998).

The effects of a subtoxic dose of ionizing radiation on the expression of mEH and GST proteins and mRNA were assessed to establish whether radiation-induced changes in the gene expression was also increased by 2-AP. The levels of mEH and major GST mRNA were elevated as a function of time after 2-AP, and the expression of mEH and rGSTA2 mRNAs was additively enhanced after exposure of animals to both TBI and 2-AP. Rats exposed to a single 0.5 Gy γ-ray radiation in combination with 2-AP exhibited greater enhancement in the expression of the mRNA levels at day 1 than those after 3 to 5 consecutive daily treatments (Kim *et al.*, 1998). The study clearly demonstrated that 2-AP in conjunction with ionizing radiation altered the expression of the genes, which might be associated with the adaptive responses after γ-ray irradiation. This was fairly consistent with the response caused by oltipraz. 2-AP increased the 30-day survival rate in the irradiated mice. The effect of 2-AP was also similar to that of oltipraz in terms of reducing the mortality rate of mice against a lethal dose of ionizing radiation. The study demonstrated that 2-AP failed to modulate radiation-inducible changes in hematology. The ratio of myeloid to erythroid bone marrow cells in mice exposed to TBI + 2-AP failed to differ from that of TBI alone. Hence, both 2-AP and oltipraz failed to improve the radiation-inducible suppression in bone marrow. The increase in the survival rate of γ-irradiated mice with improvement in liver morphology rather supported the possibility that enhanced expression of the antioxidant enzymes by 2-AP might be associated with the radioprotective response.

The effect of 2-AP on the expression of hepatic GST was comparable to that of oltipraz, although oltipraz was more effective in enhancing radiation-inducible increases in rGSTA3 and rGSTA5 (Kim *et al.*, 1997a). mEH and rGSTA2 appeared to be more sensitive than others to the oxidative stress caused by γ-irradiation in combination with 2-AP. Nonetheless, both 2-AP and oltipraz induce the mRNA levels of other major GST subunits and have the moiety of pyrazine in their chemical structures. Pyrazine binds to cytochrome P450 2E1 with high affinity and competitively inhibits the metabolic activity of P450 2E1. Pyrazine moiety was also highly associated with the expression of P450 2E1 (Kim and Novak., 1993). The role of P450 2E1 expression on the radioprotective efficacy of xenobiotics should be further studied in conjunction with their modulation of intracellular redox potential. A preliminary study revealed that pyrazine alone failed to exert radioprotective effects. Allylthiobenzimidazole, which has the allylthio moiety, was not active in improving the radiation-induced mortality rate (Kim *et al.*, 1998b). Thus, neither pyrazine nor allylthio moiety alone seems to be responsible for scavenging radiation-induced free radicals. Our studies demonstrated that 2-AP was efficacious in inducing mEH and major GSTs in the liver, that ionizing radiation in conjunction with 2-AP stimulated the expression of hepatic mEH and GST genes, and that the enhanced expression of the genes by 2-AP might be associated with the protective and adaptive responses against ionizing radiation.

Molecular Basis for Detoxifying Enzyme Induction

We have shown that 2-AP suppresses the constitutive and inducible cytochrome P450 2E1 expression and was effective in blocking the toxicant-induced liver injury (Kim *et al.*, 1997b). 2-AP was effective in increasing mEH and major GST subunits in the liver

and exhibited radioprotective effect against γ-ionizing radiation in animal models. 2-AP also increased the intracellular GSH level in the liver, as was observed after oltipraz treatment (Kim *et al.*, 1997b). The GSH level was increased even in the animals treated with hepatotoxicants including carbon tetrachloride and acetaminophen. Thus, both oltipraz and 2-AP were comparably effective in enhancing the intracellular GSH level as well as in inducing phase II enzymes. Thus, the anticarcinogenic effects of oltipraz and 2-AP, chemoprotective agents, have been attributable to their electrophilic character and the induction of phase II detoxifying enzymes.

We were motivated to compare the *in vitro* conversion of molecular oxygen to reactive oxygens by the agents and the mechanistic basis of their detoxifying enzyme induction *in vivo* and determined the differential DNA protective effects by the agents against autooxidation of benzenetriol in the absence or presence of ferrous iron. Oltipraz caused a single strand DNA breakage in the presence of β-mercaptoethanol, as shown by complete conversion of supercoiled ϕX-174 DNA to the open circular form, whereas 2-AP did not alter the DNA topology. Although Oltipraz failed to prevent a benzenetriol (BT)-induced single strand breakage of DNA, 2-AP effectively reduced the conversion of supercoiled DNA to the open circular form induced by BT. 2-AP also completely prevented BT- and ferrous iron-catalyzed ϕX-174 DNA degradation. In contrast, oltipraz further stimulated the DNA damage. Thus, these agents differed each other in thiol-dependent conversion of DNA topology with only 2-AP being active in preventing DNA damage caused by autooxidation of BT in the absence or presence of iron. The difference in iron-catalyzed DNA degradation by oltipraz and 2-AP may result from their differential iron-binding property. Our studies revealed for the first time that oltipraz failed to protect ϕX-174 DNA against BT- and iron-catalyzed oxidation, whereas 2-AP prevented degradation of the DNA. Thus, only 2-AP was effective without further metabolism in inactivating ferrous iron *in vitro,* which may be due to iron-chelating effect. The iron-binding capability may provide part of the mechanistic basis for the chemoprotective effects caused by 2-AP. Oltipraz and other dithiolethiones have been found to increase ferritin, a major iron-storage protein in the liver. Treatment of animals with 1,2-dithiole-3-thione resulted in diminished levels of intracellular free iron, suggesting that increased ferritin content sequesters intracellular iron (Primiano *et al.*, 1996). It is possible that metabolite(s) produced from oltipraz may interact with iron, as observed with 2-AP, which remains to be established. Despite the opposite effects of oltipraz and 2-AP on the DNA topology, both agents effectively induce phase II enzymes, showing the possibility that the *in vitro* capability of conversion of molecular oxygen might not be related to the induction of the enzymes *in vivo.*

The molecular basis for detoxifying enzyme induction was comparatively evaluated under the assumption that production of reactive oxygens by the chemoprotective agents would lead to the antioxidant responsive element (ARE)-mediated transcriptional activation of the genes. Northern blot analysis revealed that rats exposed to buthionine sulfoximine, a GSH depleting agent, prior to treatment with either oltipraz or 2-AP exhibited greater increases in the hepatic mEH and rGSTA2 mRNA levels at 12 h than those treated with each chemoprotective agent alone. Furthermore, oltipraz- or 2-AP-inducible mEH and rGSTA2 mRNA levels were completely reduced following concomitant treatment of animals with cysteine, indicating that oxygen free radicals produced by the chemoprotective agents could be reduced by cysteine supplementation. The activated oxygens are potentially responsible for the transcriptional induction of anticarcinogenic enzymes (Kensler *et al.*, 1992; Hayes and Pulford, 1995). BSO depletes the intracellular GSH content, whereas cysteine contributes to elevation of the GSH level (Stipanuk *et al.,* 1992). BSO inhibits the heavy subunit of γ-glutamylcysteine synthase, which possesses all of the catalytic activity for GSH feedback (Mulcahy *et al.*, 1995). Oguro *et al.* showed that stilbene oxide induced heme oxygenase-1 mRNA with concomitant decreases in the hepatic GSH level and that BSO, an inhibitor of GSH biosynthesis, enhanced GSH depletion and augumented the increase in heme oxygenase-1 mRNA (Oguro *et al.*, 1997). Oltipraz as well as hypoxia have been also shown to induce heme oxygenase-1 in rat tissues (Primiano *et al.*, 1996; Lee *et al.*, 1997). The favorable formation of thiol-protein mixed disulfides after BSO pretreatment may shift the balance between the formation of reactive oxygen species and the transcriptional activation of the detoxifying genes. The hypothesis that production of activated oxygens is responsible for mEH and GST enzyme induction was also confirmed in the GSH depleting state caused by BSO. The present study revealed that both oltipraz and 2-AP further elevated the mEH and rGSTA2 mRNA levels in the liver in the GSH-depleting animals, while the mRNA levels inducible by the chemoprotective agents were decreased by cysteine supplementation. Thus, the increased intracellular GSH level did not appear to contribute to the enhanced production of reactive oxygen species by oltipraz or 2-AP. The antioxidant enzymes were rather induced to greater extents by these agents in the decreased intracellular GSH level. The greater elevation in the hepatic mEH and rGSTA2 mRNA levels in the GSH-depleting animals provided evidence that production of reactive oxygen species, which is correlated with the transactivation of ARE, appeared not to be mediated by non-enzymatic breakdown of the agents in the presence of thiol, but presumably by bioactivation of the agents *in vivo.*

The intracellular thiol level has been implicated in the regulation of transcriptional expression of a variety of genes (Rushmore *et al.*, 1991; Pinkus *et al.*, 1996). It has been proposed that the gene of NAD(P)H: quinone oxidoreductase, a detoxifying antioxidant enzyme, was transcriptionally activated in association with NF-κB activation by oltipraz or hypoxia (Yao and O'dwyer, 1995). NF-κB activation by oxidative stress has been correlated with the cellular oxidation state, which may translate a redox sensor into a chemical signal that leads to transcriptional activation of the appropriate genes (Primiano *et al.*, 1997; Schreck *et al.*, 1991). The NF-κB factor level was monitored in rats to determine the possible role of NF-κB activation in the expression of mEH and rGSTA2 by these chemoprotective agents. Neither oltipraz nor 2-AP stimulated the nuclear factor-κB (NF-κB) activation in the liver at early times, as assessed by gel shift analysis, and LPS-induced activation of NF-κB failed to be enhanced or blocked by concomitant treatment of rats with the agents.

The results provided evidence that: oltipraz differs with 2-AP in preventing DNA damage *in vitro*; both agents produce reactive oxygen species *in vivo*, which is not mediated with the thiol-dependent production of oxidants; and NF-κB activation is not involved in the induction of the detoxifying enzymes by these agents *in vivo*. Metabolic conversion of oltipraz and/or 2-AP is likely to be coupled with the steady state consumption of reduced GSH content in the cells. Intracellular GSH level appeared to be inversely correlated with the increases of mEH and GST mRNAs, as supported by the observation that increases of the mRNA levels were enhanced by concomitant treatment of animals with the chemoprotective agents and BSO. A number of hepatotoxicants increase intracellular GSH levels as part of adaptive responses unless cellular viability is decreased (McMillan and Jollow, 1992; Simplicio *et al.*, 1998). The oxidative stress from the chemopreventive agents may stimulate the synthesis of glutathione to compensate for the altered cellular oxidation state in conjunction with the transcriptional activation of phase II antioxidant enzymes. This compensatory increase in the intracellular GSH level may be associated with smaller increases in mRNA levels at later times after multiple treatment with oltipraz than at early times. Hence, the elevation of the intracellular GSH content by either oltipraz or 2-AP is unlikely to result in the transcriptional activation of phase II enzymes. Rather, the reduced GSH level caused by oxidative stress would subsequently feedback-stimulate the production of GSH as well as the induction of phase II enzymes.

In summary, 2-AP was effective in protecting the liver against carcinogen-induced toxicity and hepatocarcinogenesis. The mechanism of chemoprotection by 2-AP might involve inhibition of the P450-mediated metabolic activation of chemical carcinogen and enhancement of electrophilic detoxification through induction of phase II detoxification enzymes which would facilitate the clearance of activated metabolites through conjugation reaction.

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