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Inhibition by Phenyl *N-tert*-Butyl Nitrone of Early Phase Carcinogenesis in the Livers of Rats Fed a Choline-deficient, L-Amino Acid-defined Diet[1]

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Abstract

Male Wistar rats were fed a choline-deficient, L-amino acid-defined (CDAA) diet alone or in combination with a nitrone-based free radical trapping agent, phenyl *N-tert*-butyl nitrone (PBN) in the drinking water at the concentrations of 0.013, 0.065, and 0.130% for 12 weeks. PBN inhibited the changes that are normally induced in the livers of rats by the CDAA diet feeding, *i.e.*, development of putative preneoplastic lesions, proliferation of connective tissue, reduction of glutathione *S*-transferase activity, formation of 8-hydroxyguanine in DNA, and an increase in inducible cyclo-oxygenase (COX2) activity. PBN, however, did not prevent the increases in the COX2 mRNA or protein levels brought on by the CDAA diet. These results indicate that the loss of glutathione *S*-transferase activity and COX2 induction may play significant roles in rat liver carcinogenesis by the CDAA diet and that PBN prevents neoplasia not only by its radical scavenging activity but also by inhibiting COX2 activity at the catalytic level.

Introduction

A nitrone-based free radical trapping agent, PBN,[3] has been shown to protect animals from various oxidative disorders such as endotoxin shock (1). Although these have been usually attributed to the compound's scavenging and thereby detoxifying ability for reactive oxygen species, we have demonstrated that PBN also inhibited the induction of *iNOS* gene during its protective action against lipopolysaccharide-induced endotoxin shock in mice (1). The enzyme, iNOS, produces a nitric oxide radical that interacts with signal transduction systems and mediates a variety of physiological and toxic events including carcinogenesis (2). The gene expression of *iNOS* is induced and regulated by various factors such as NF-κB, interleukin-1β, and tumor necrosis factor α (1–3). Signal transduction systems and their influence on arachidonic acid metabolism and prostaglandin synthesis have attracted attention in relation to their involvement in the etiology of carcinogenesis (4). COX2 is considered particularly important in carcinogenesis (4); its induction is also regulated by factors such as NF-κB (5), and a communication between iNOS and COX2 through their metabolites has been shown (6). There exist, therefore, comprehensive mechanisms underlying many disease processes, and the preventive effects of PBN may arise from the multidirectional inhibition of processes involving reactive oxygen and nitrogen species. It is thus of interest to explore the exact effects of PBN on carcinogenesis. We have recently established a model for rat liver carcinogenesis induced by the chronic feeding of a CDAA diet (7–9). Because hepatocellular carcinomas arise in the absence of any known carcinogens and in the presence of continuous liver injuries resulting in frank cirrhosis (7–9), this model has an advantage in investigating the mechanisms underlying hepatocarcinogenesis under circumstances relative to endogenous origins of cancers. Thus far, we have shown that, in its underlying mechanisms, oxidative injury to subcellular components play crucial roles along with the liver injuries (7, 9) and that augmented cyclo-oxygenase-mediated arachidonic acid metabolism may also participate (8). In addition, we have quite recently found that 1′-acetoxychavicol acetate, an inhibitor of NF-κB activation and *iNOS* expression *in vitro* (10), significantly inhibits putative preneoplastic lesion development in the livers of rats fed the CDAA diet by reducing the magnitude of the initiation (9). Furthermore, Rushmore *et al.* (11) demonstrated that PBN prevents diene conjugate formation in rat hepatocyte nuclei brought about by the feeding of a semipurified choline-deficient diet. Therefore, this model is of interest to examine the effects of PBN on carcinogenesis. The results we have obtained are presented here.

Materials and Methods

Animal Experiment. A total of 30 male Wistar rats, 5 weeks old, (Charles River Japan, Inc., Atsugi, Kanagawa, Japan), were acclimatized for 1 week and then divided into 6 groups of 5 animals each. The CDAA diet and the control CSAA diet were obtained from Dyets Inc., (Bethlehem, PA). PBN was synthesized and purified to 99.997% purity according to the method of Janzen and Haire (12) in our laboratories. It was dissolved in double-distilled water. Rats in groups 1–4 and 5–6 were allowed free access to the CDAA and CSAA diets, respectively, for a experimental period of 12 weeks. At the same time, group 2 (low-dose group), group 3 (middle-dose group) and groups 4 and 6 (high-dose groups) were given drinking water *ad libitum* containing 0.013, 0.065, and 0.130% of PBN, respectively. The PBN doses were used based on the previous report (1). At the end of week 12, all of the rats were killed under light ether anesthesia by exsanguination, and livers were excised. Body weight and the average intake of food, water, and PBN were monitored weekly.

Liver Sample Preparation, Assays, and Statistics. Three serial 4-μm-thick, acetone-fixed, paraffin-embedded liver sections were prepared. Two of them were used for histological examination after H&E and Azan-Mallory stainings, and the remaining one was for immunohistochemical quantification of putative preneoplastic, GST-P-positive focal lesions performed as previously described (7–9). Liver portions (~2 g) were homogenized in 15 mM Tris-HCl buffer (pH 7.9), containing 250 mM sucrose, 15 mM NaCl, 30 mM KCl, 5 mM disodium ethylenediaminetetraacetate, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 0.15 mM spermine, 0.5 mM spermidine, 1 mM DTT, 0.77 nM aprotinin, 4.32 nM leupeptin, and 0.10 μM phenylmethyl sulfonyl fluoride (all these reagents from Sigma Chemical Co., St. Louis, MO), and centrifuged at 4500 × *g* and 4°C for 10 min. The resultant supernatants were stored at −20°C and used for the assays: spectrophotometry for GST activity with chlorodinitro benzene and glutathione (both from Sigma) by the method of Habig *et al.* (13); Western blotting for COX2 protein using anti-COX2 monoclonal antibody (Transduction Laboratories, Lexington, KY);

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[3] The abbreviations used are: PBN, phenyl *N-tert*-butyl nitrone; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor κB; COX2, inducible cyclo-oxygenase; CDAA diet, choline-deficient, L-amino acid-defined diet; CSAA diet, choline-supplemented, L-amino acid-defined diet; GST, glutathione *S*-transferase; GST-P, GST placental form; 8-OHG, 8-hydroxyguanine; PGE_2, prostaglandin E_2.

Table 1 *General findings and the data for the GST-P-positive lesions*

Group[a]	Treatment(s)	Final body wt (g)	Relative liver wt (g/100 g body wt)	Av. food intake[b] (g/kg body wt/day)	Av. water intake[b] (ml/kg body wt/day)	Av. PBN exposure[b] (mg/kg body wt/day)	GST-P-positive lesions	
							no./cm^3	Size (mm^3)
1	CDAA	274 ± 16[c,d]	4.44 ± 0.27[d]	51 ± 11	63 ± 14	0	190 ± 10[d]	1.92 ± 0.31
2	CDAA + PBN (low)	278 ± 18	4.65 ± 0.22	49 ± 10	63 ± 14	6.06 ± 1.07[e]	170 ± 28	0.33 ± 0.20[e]
3	CDAA + PBN (middle)	279 ± 14	4.57 ± 0.33	49 ± 10	65 ± 14	32.86 ± 4.81[e]	149 ± 15[e]	0.17 ± 0.06[e]
4	CDAA + PBN (high)	270 ± 9	4.70 ± 0.21	53 ± 9	62 ± 17	61.76 ± 8.90[e]	142 ± 10[e]	0.10 ± 0.05[e]
5	CSAA	356 ± 10	3.19 ± 0.20	49 ± 10	64 ± 16	0	0	
6	CSAA + PBN (high)	347 ± 7	3.44 ± 0.19	48 ± 11	64 ± 16	64.45 ± 9.53[d]	0	

[a] The effective number of rats in each group was five.
[b] Calculated from the weekly monitoring data.
[c] The values are means ± SD.
[d] Significantly different from the group 5 value ($P < 0.01$).
[e] Significantly different from the group 1 value ($P < 0.01$).

and fluorometry for peroxidase activity with hydrogen peroxide and dichlorofluorescein diacetate (both from Sigma) according to Brandt and Keston (14). The peroxidase activity assay was performed additionally on the cytosol-rich (supernatant) and membrane-rich (pellet) fractions after recentrifugation of the samples at 14,000 × *g* and 4°C for 10 min. The bands obtained in Western blotting were detected by an ECL system (Amersham International plc, Buckinghamshire, UK), recorded by an Eagle Sight II still video system (Stratagene, La Jolla, CA) and quantified using a Zero-Dscan software (Scanalysis, Palo Alto, CA). Protein values were determined using a Total Protein Assay kit (Sigma) when necessary. The remaining liver portions were immediately frozen under liquid nitrogen, stored at −80°C, and used for quantification of 8-OHG levels in liver DNA by electrochemical detection using high performance liquid chromatography as detailed elsewhere (15) and for Northern blotting for COX2 mRNA and rRNA of 28 s (for internal standard purpose) with ^{32}P-labeled oligonucleotide probes against rat sequences synthesized in our laboratories by a PCR technique. The Northern blots were autoradiographed at −80°C for 15 h and then quantified as for the Western blots. Also with portions of the frozen liver samples, PGE_2 was extracted according to the method of Powell (16), and its level was determined using an enzyme-immuno-assay kit for PGE_2 (Cayman Chemical, Ann Arbor, MI) and a Termo Max plate reader (Molecular Device, Sunnyvale, CA). Statistical analyses were carried out using an InStat software (GraphPad Software, Inc., San Diego, CA), in which Dunnett's multiple comparison test is used to assess statistical significance of intergroup differences of means for multiple groups after one-way ANOVA to determine variations among group means, followed by Bartlett's test to determine homogeneity of variance.

Results and Discussion

All rats survived until their scheduled deaths. Final body weights and relative liver weights of group 1 were significantly lighter and heavier, respectively, than those of group 5 in accordance with our earlier findings using male Fischer 344 rats (7–9). PBN did not affect these parameters in groups 2–4 and 6 (Table 1). There were no differences among groups 1–6 in terms of food or water intake when standardized by body weight throughout the experimental period (Table 1). Rats in groups 2–4 and 6 were thus exposed to PBN steadily during the study and proportionally to its administered concentrations. The calculated average exposure is presented in Table 1.

In the livers of group 1, intrahepatocellular fat accumulation, hepatocyte death, and connective tissue proliferation in line with borderline cirrhosis were observed (Fig. 1*A*). This result is in accordance with our earlier reports (7–9). Despite a lack of clear effects on fat accumulation and cell death, PBN drastically reduced the connective tissue proliferation in groups 2–4 (Fig. 1*B*). No particular histological changes were found in groups 5 or 6. GST-P-positive lesions were observed in the livers of all of the rats fed the CDAA diet either with or without PBN (groups 1–4), whereas no lesions were detected in groups 5 or 6. PBN at its middle and high doses (groups 3 and 4, respectively) significantly reduced the numbers of the lesions, whereas all of the three doses of PBN significantly and drastically decreased the lesion sizes (Table 1). These data thus indicate

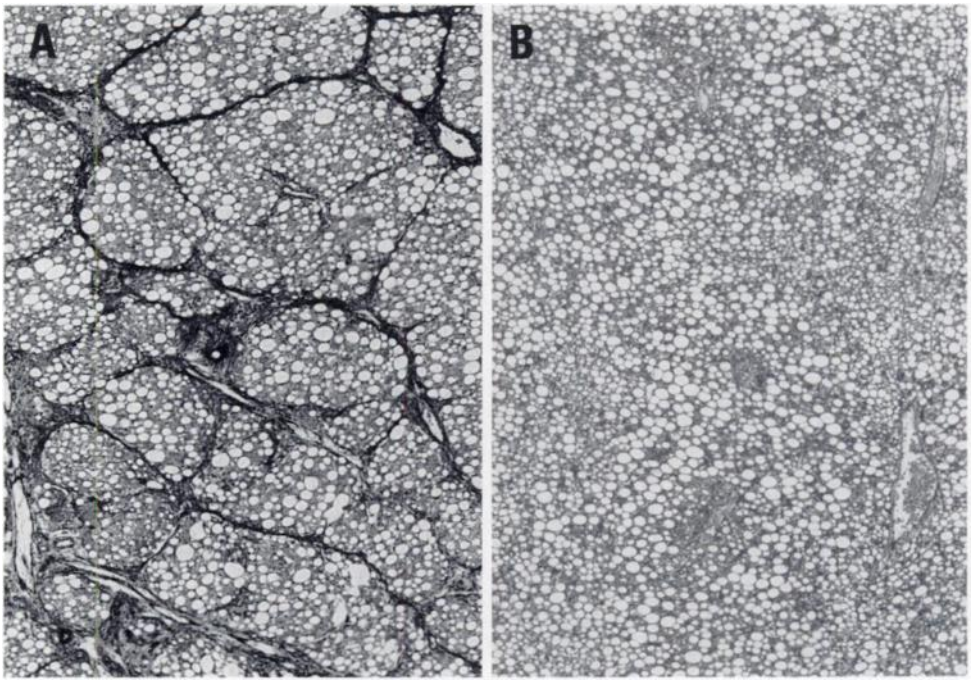

Fig. 1. Representative Azan-Mallory histology of the livers of rats fed the CDAA diet with the coadministration of: *A*, no PBN (group 1); and *B*, PBN at the high dose (group 4). ×10.

Table 2 *The data for GST activity, 8-OHG level, COX2 mRNA, and protein levels, PGE_2 level, and peroxidase activity*

Group[a]	Treatment(s)	GST activity (unit/mg protein)	8-OHG (8-OHdG/10^6 dG)[b]	COX2 (arbitrary unit)		PGE_2 (mg/g wet liver)	Peroxidase activity (pmole H_2O_2/mg protein/minute)		
				mRNA	Protein		Total	Pellet	Supernate
1	CDAA	4.2 ± 0.8[c,d]	18.92 ± 1.96[d]	880 ± 70[d]	890 ± 170[d]	16.25 ± 4.60[d]	43 ± 5[d]	5.3 ± 0.9[d]	2.9 ± 0.7
2	CDAA + PBN (low)	5.1 ± 0.6	13.10 ± 2.56[e]	NA	NA	NA	46 ± 8	7.2 ± 0.4	3.5 ± 0.4
3	CDAA + PBN (middle)	5.8 ± 0.5[e]	5.11 ± 1.24[e]	NA	NA	NA	37 ± 8	4.8 ± 0.9	3.9 ± 0.9
4	CDAA + PBN (high)	6.8 ± 0.4[e]	2.55 ± 0.83[e]	960 ± 80	810 ± 150	8.36 ± 1.59[e]	33 ± 4[e]	3.2 ± 0.5[e]	3.3 ± 0.2
5	CSAA	6.4 ± 0.6	1.00 ± 0.10	470 ± 100	240 ± 160	1.31 ± 0.35	27 ± 3	0.0 ± 0.1	3.4 ± 0.2
6	CSAA + PBN (high)	6.9 ± 0.4	1.00 ± 0.17	NA	NA	NA	24 ± 3	0.2 ± 0.2	2.9 ± 0.4

[a] The effective number of rats in each group was five.
[b] 8-OHdG, 8-hydroxydeoxyguanosine; dG, deoxyguanosine; NA, not assessed.
[c] The values are means ± SD.
[d] Significantly different from the group 5 value ($P < 0.01$).
[e] Significantly different from the group 1 value ($P < 0.01$).

that PBN can inhibit both the induction of putative preneoplastic lesions and, more prominently, their subsequent growth in the livers of rats fed the CDAA diet. This is the first report to demonstrate the inhibitory effects of PBN against carcinogenesis. Assuming its low toxicity (1), PBN may thus be a good candidate for cancer chemoprevention. Further evaluation on this compound is warranted.

We have also found that GST activity was significantly decreased, whereas 8-OHG levels were significantly increased by the CDAA diet (group 1) and that such changes were significantly inhibited by PBN (Table 2). The presently used assay for GST activity covers A, B, and C isozymes of GSTs (13). Therefore, inasmuch as these phase II detoxifying activities against toxic substances including reactive oxygen species are suppressed by chronic feeding of the CDAA diet, this again is supportive for oxidative mechanisms underlying this diet-associated carcinogenesis. This then suggests that the preventive effect of PBN not only is due to its direct scavenging of reactive oxygen species but may also be due to the maintenance of its nonspecific decomposition of such species by phase II detoxifying enzymes. 8-OHG is an established promutagenic oxidative DNA lesion involved in carcinogenesis (17), and its formation is essential for the induction of GST-P-positive lesions in the livers of rats fed the CDAA diet (7, 9). The reduction of the 8-OHG levels by PBN is considered, therefore, to be a direct cause of the decrease of the lesion numbers by virtue of the antioxidative effects of this free radical trapping compound.

The above profile of the preventive activities of PBN on the CDAA diet-induced rat liver carcinogenesis closely resembles that obtained in the case of the administration of nonsteroidal, anti-inflammatory drugs such as acetylsalicylic acid and piroxicam (8), both of which are COX2 inhibitors (18). A 12-week feeding of the CDAA diet indeed elevated COX2 mRNA as well as protein levels in the livers of group 1 rats, but the high dose of PBN did not affect these levels in group 4 (Fig. 2; Table 2). Nevertheless, the liver PGE_2 level, a parameter of COX2 activity, which was significantly increased in rats fed the CDAA diet (group 1; shown previously in the livers of rats fed the semipurified choline-deficient diet; Ref. 19), was significantly reduced by the high dose of PBN (see group 4, Table 2). Moreover, similar results were obtained for the peroxidase activity detected both in the total liver homogenate and in

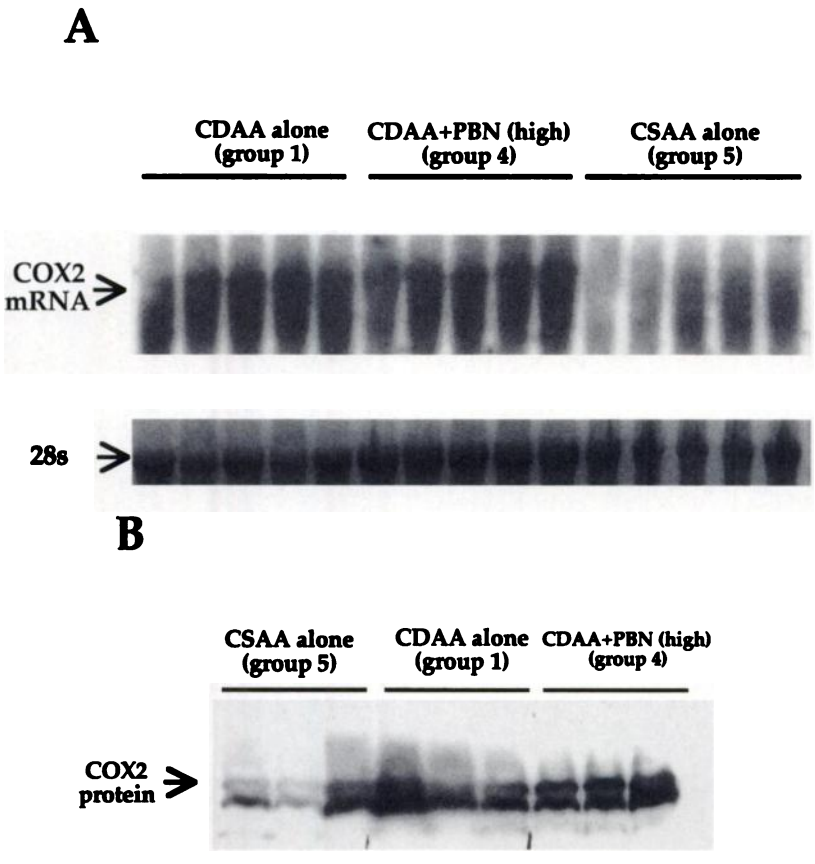

Fig. 2. Electrophoresis of: *A*, Northern blotting for COX2 mRNA (*upper panel*) and ribosomal RNA of 28s (internal standard; *lower panel*) using liver samples obtained from rats fed the CDAA diet alone (group 1, *left five lanes*) and with coadministration of PBN at the high dose (group 4; *middle five lanes*) and those from rats fed the CSAA diet alone (group 5; *right five lanes*; presenting all of the 5 samples for each group); and *B*, Western blotting for COX2 protein using liver samples from groups 1 (*middle three lanes*), 4 (*right three lanes*), and 5 (*left three lanes*; presenting representative 3 samples for each group).

its membrane-rich fraction (pellet), whereas no differences among groups 1–6 were observed in the cytosol-rich fraction (supernatant; Table 2). Because the peroxidase activity considered is inducible and exists in the membrane-rich fraction, it is suggested that it is due mainly to COX2. These data then indicate that *COX2* gene expression is up-regulated by chronic feeding of the CDAA diet and that PBN disturbs the enhanced COX2 activity at the catalytic level. In fact, we have preliminarily found that PBN reduced PGE_2 production in primarily isolated peritoneal macrophages from male BALB/c mice and treated with lipopolysaccharide and IFN-γ.[4] The present results, therefore, confirm our earlier suggestion (8) that COX2-mediated reactions may participate in the mechanisms underlying rat liver carcinogenesis associated with chronic feeding of the CDAA diet. The results also indicate that the disturbance of such reactions by PBN may be another preventive mechanism of this free radical trapping compound against carcinogenesis.

As described earlier, COX2-related reactions have become recognized as parts of the comprehensive signal transduction system in which reactive oxygen and nitrogen species participate. It is, therefore, necessary to investigate whether it is this system that is involved in the mechanisms underlying the CDAA diet-associated carcinogenesis and in the cancer chemopreventive activity of PBN. The involvement of NF-κB should be considered a critical factor to explore with regard to the major role it may play in the transcription of both *iNOS* (3) and *COX2* (5) genes, and because acetylsalicylic acid, a COX2 inhibitor (18) preventing the CDAA diet-associated carcinogenesis (8), inhibits the activation of NF-κB (20). Indeed, pertinent to this point, we have shown that 1′-acetoxychavicol acetate inhibits rat liver carcinogenesis by the CDAA diet *in vivo* (9), whereas it inhibits NF-κB activation and iNOS induction *in vitro* (10). Furthermore, we have also preliminarily demonstrated that PBN inhibited NF-κB activation *in vitro*[4]. Investigations to assess the status and changes in iNOS and NF-κB as well as various cytokine levels using the present experimental protocols are in progress in our laboratories.

Dietary choline (or methyl donors) deficiency is known to cause specific loss of methylation at CpG islands in rat liver DNA, which is also considered to play a significant role in the hepatocarcinogenic mechanisms (21). For instance, a number of growth-related genes such as *c-myc*, *c-Ha-ras* and *c-fos* are indeed specifically hypomethylated, which may causally relate to their mRNA overexpression during dietary choline deficiency (21). Because it has been suggested that certain transcription factor(s) may be required for these changes (21), the perturbation of methylation pattern of CpG islands of DNA could also participate in the above mentioned signal transduction system. In fact, we have shown that *c-myc* and *c-Ha-ras* mRNAs are overexpressed in rat liver by the short-term feeding of the CDAA diet (22). Assuming the importance of the continuous hepatocyte proliferation in rat liver carcinogenesis by dietary choline deficiency (23), it is necessary to assess the status and changes in the methylation pattern and the relationship of these changes with the other factors that are described above in our future investigations about the mechanisms underlying rat hepatocarcinogenesis caused by chronic feeding of the CDAA diet and the cancer chemopreventive activity of PBN.

In conclusion, decrease in GST activity coupled with COX2 induction along with 8-OHG formation are involved in the development of putative preneoplastic lesions in the livers of rats fed the CDAA diet. A nitrone-based free radical trapping agent, PBN, effectively inhibits rat liver carcinogenesis associated with the CDAA diet in its early phase. The disturbance of COX2 activity at the catalytic level may be as important as the known radical scavenging activity for the presently demonstrated cancer chemopreventive effects of PBN.

[4] Y. Kotake, H. Sang, T. Miyajima, and G. L. Wallis. Inhibition of NF-κB, *iNOS* gene induction, *COX2* gene induction, and COX2 catalytic activity by the spin trapping agent, phenyl *N-tert*-butyl nitrone, submitted for publication.

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