Effect of Butylated Hydroxytoluene on Cell Population in Rat Hepatocytes

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Abstract: In order to examine the effect of 3,5-di-tert-butyl-4-hydroxytoluene (BHT) on hepatocellular population, BHT was given orally to male rats for 7 consecutive days at dose levels of 75 or 450 mg/kg/day. BHT induced hepatocellular proliferation, increase in apoptosis, and elevated immunoreactivity for transforming growth factor (TGF) -β during the treatment, and hepatocellular mitoinhibition following the withdrawal. The induction of mitoinhibition, apoptosis, and TGF-β might be adaptive changes in response to cell proliferation; thus the homeostasis of hepatocellular population was preserved. Although both the mechanism and the biological significance of mitoinhibition observed following the withdrawal of BHT, after a 7-day treatment period, were not elucidated, its mechanism seemed to be related to the elevation of TGF-β immunoreactivity and its biological significance might be similar to those with non-genotoxic hepatocarcinogens in chronic treatments. (J Toxicol Pathol 2001; 14: 145–150)

Key words: BHT, liver, mitoinhibition, rats, TGF-β1

Introduction

The synthetic phenolic antioxidant 3,5-di-tert-butyl-4-hydroxytoluene (BHT) is widely used as an additive to protect food products against free radical-induced damage. Numerous genotoxicity studies of BHT have been uniformly negative. BHT induces hepatocellular hypertrophy, which is attributed to increase in microsomal enzyme activity and proliferation of smooth endoplasmic reticulum. Large doses of BHT produce centrilobular necrosis, increased serum transaminase activities, and hemorrhage in the liver. BHT has also been found to increase in the mitotic activity of hepatocytes in rats and act as a promoter for hepatocarcinogenesis. Olsen et al., following a two-generation study in rat, described BHT as a putative rat liver carcinogen, yet there was no significant evidence to indicate a hepatocarcinogenic potential for BHT when it was originally tested in either rats or mice.

In addition, BHT also inhibits chemical carcinogenesis in various organs when fed before or concurrently with a carcinogen. Thus, the effects of BHT on experimental animals are still controversial. Induction of cell proliferation and suppression of apoptosis would contribute to the process of hepatocarcinogenesis in non-genotoxic hepatocarcinogens, such as cyproterone acetate, nafenopin, phenobarbital (PB), α-hexachlorocyclohexane, etc. Therefore, it is important to assess the abilities of chemicals to disrupt cell population, in the investigation of their hepatocarcinogenic potential. On the other hand, it is known that transforming growth factor (TGF) -β is a potent inhibitor of hepatocyte proliferation and induces apoptosis in rat liver. A loss of sensitivity to TGF-β in tumorigenic cells is a factor in carcinogenesis. Thus, TGF-β is a useful marker for the regulation of hepatocellular population. In order to investigate the effect on the regulation of hepatocellular population by BHT, the levels of hepatocyte proliferation, TGF-β, and apoptosis in the liver were histopathologically examined during and after consecutive treatment of BHT.

Materials and Methods

Seventy-two male specific pathogen-free Fischer 344 rats, approximately 8.5 weeks old, were purchased from Japan Charles River Laboratories (Tsukuba, Japan).
Animals of this age were selected because of the stability of their hepatocyte proliferative activities. The animals were single-housed in wire-mesh cages in an air-conditioned room (temperature, 22 ± 2°C; humidity, 55 ± 10%; light cycle, 12 hr/day). Food (Oriental Yeast Co., LTD., Japan) and water were available ad libitum.

All animals were acclimatized for 5 days prior to the first treatment. BHT was suspended in corn oil and administered orally (1 ml/100 g body weight of rats). Animals were treated daily at dose levels of 0 (corn oil only), 75 or 450 mg/kg for 7 days. In previously reported studies, hepatocellular necrosis was seen in male rats given BHT at 500 mg/kg/day for 7 days. Four rats from each group were sampled after 1, 3, 7, 9, 11, and 14 days of treatment. The experimental design is shown in Fig. 1.

At sampling, all animals were given a single intraperitoneal injection of 5-bromo-2’-deoxyuridine (BrdU: Tokyo Kasei Kogyo Co., Japan) at 100 mg/kg body weight, two hours prior to sacrifice. Animals were weighed, sacrificed under diethyl ether anesthesia, and necropsied. The livers were removed, weighed, and examined for the presence of grossly visible lesions. A portion of the central median lobe of the liver was immediately excised, fixed in 10% neutral buffered formalin for 24 hr, embedded in paraffin, sectioned at 4-µm thickness, and routinely stained with hematoxylin and eosin (HE) for histopathological evaluation. Immunohistochemical staining was performed according to the avidin-biotin complex (ABC) method (VECTSTAIN ABC Kit: Vector Laboratories Inc., Canada). To demonstrate BrdU labeling index, liver sections were deparaffinized and treated with 0.3% H2O2 (22°C, 30 min.), 2 N HCl (37°C, 30 min.), trypsin (0.05% in phosphate-buffered saline, 37°C, 3 min.), antibody to BrdU (DAKO, Japan) (dilution 1:800, 4°C, overnight), ABC and hematoxylin. To demonstrate TGF-β positive area, liver sections were deparaffinized and treated with 0.3% H2O2 (22°C, 30 min.), antibody to TGF-β1, -β2 or -β3 (Santa Cruz Biotechnology, Inc., USA) (dilution 1:50, 4°C, overnight), ABC and hematoxylin. As negative control, normal rabbit or mouse serum was used instead of the first antibody. A standard in situ TUNEL (Apoptosis in situ Detection Kit Wako: Wako Pure Chemical Industries, Japan) method was used for detection of DNA fragmentation in apoptosis. After treatment with proteinase K (37°C, 5 min.), deparaffinized sections were treated with TdT and digoxigenin DNA labeling mixture (37°C, 10 min.), 0.3% H2O2 (22°C, 30 min.), peroxidase-conjugated anti-digoxigenin (37°C, 10 min.), DAB, and hematoxylin. For BrdU labeling index (LI%), labeled nuclei and total nuclei were scored in approximately 2,000–2,300 hepatocytes per liver in a TUNEL reaction, by light microscopy with a ×40 objective and an image analyzer. The number of ACs found was expressed as a percentage of intact cells. For TGF-β1, -β2, and -β3 positive area ratio, four randomly selected microscopic fields from each liver were analyzed with a ×10 objective. The positive and the total area were measured with an image analyzer. The positive area ratio, expressed as a percentage of labeled area versus total area, was calculated for each animal. Means and standard error (SE) were calculated.

Statistical differences from control values for the data were determined using Dunnett’s multiple comparison test.

**Results**

Between Day 1 and Day 7, hypoactivity, soiled fur, and loose stool were observed in rats at 450 mg/kg. Statistically significant decreases in body weights were seen in rats at 450 mg/kg throughout the study (data not shown). These clinical signs and the decline in body weights were the most evident on Day 3. There was no apparent change in clinical sign and body weights at 75 mg/kg. Liver to body weight ratios (LBR) are shown in Fig. 1. In treated animals, dosage-related increases in LBRs were seen and peaked on Day 7. Although LBRs of both dose levels gradually returned to those of controls following the withdrawal of BHT, they were both higher than those of control values on Day 14. Histopathologically, centrilobular and mid-zonal hypertrophy of hepatocytes was seen in treated animals between Day 3 and Day 14. The degree of hypertrophy was...
greater at 450 mg/kg than that at 75 mg/kg. Nuclear enlargement of hepatocytes was also observed at 450 mg/kg on Day 3. Neither necrosis nor degeneration of hepatocytes was observed in any rats. Statistically or biologically significant increases in LI% during the treatment, being peaked on Day 7 (3.8-fold and 10.5-fold), were seen at 75 mg/kg and 450 mg/kg, respectively (Fig. 2a). However, on Day 3 transient decrease of LI% was seen at 450 mg/kg. The toxicological effects of BHT might probably cause this, as the worst physical condition was recorded on Day 3. The BrdU-labeled hepatocytes were mainly observed at the periportal region (Fig. 3). Following the withdrawal of BHT, significant decrease in LI% was seen in all treated animals on Day 9 and Day 11. The LI% of both dose levels returned to that of the control values by Day 14. As shown in Fig. 2b, increase in the number of ACs during the treatment, being peaked on Day 3 (2.6-fold), were seen at 450 mg/kg. Subsequently, the number of ACs decreased and returned to the control values on Day 7. There was no change of ACs at 75 mg/kg. Centrilobular positive staining for TGF-β1 was slightly seen in control livers (Fig. 4). In the treated liver, statistically or biologically significant increases in positive area ratios at the centrilobular region were dose-dependently seen above that of the control value, on Day 1 at 75 mg/kg, and from Day 1 to Day 9 at 450 mg/kg, being peaked on Day 1 (Fig. 5). The positive area ratios decreased thereafter and returned to control values by Day 3 and Day 11 at 75 mg/kg and 450 mg/kg, respectively. Moderate staining for TGF-β2 and -β3 was seen in all rats and there was no apparent change (data not shown).

**Discussion**

The present study showed that BHT induced hepatocellular proliferation, increase in apoptosis, and elevated immunoreactivity for TGF-β1 during the treatment and hepatocellular mitoinhibition following the withdrawal in a dose-dependent manner. It is known that TGF-β is an active polypeptide, which has a bifunctional role in modifying cell proliferation. It stimulates fibroblast growth but inhibits epithelial cells growth, including hepatocytes. TGF-β leads epithelial cells to lengthen or arrest the G1 phase of cell cycle and exists as three distinct homologous forms, TGF-β1, -β2, and -β3 in mammals. TGF-β1 and -β2 inhibit cell proliferation to approximately the same degree and may be more potent than TGF-β3. TGF-β1 is the most abundant isoform and might represent a general response to acute and chronic liver injury. The induction of TGF-β1 is observed in the short-term treatments of some peroxisome proliferators, and might represent an adaptive response to limit the initial hyperplastic effects of these compounds. In this study, the degree of the induction of TGF-β1 during the treatment appeared to correlate to their degree of cell proliferation during the treatment. Thus, the induction of TGF-β1 might be an adaptive response to hepatocyte proliferation of BHT. However, it was not clear whether mitoinhibition following the withdrawal was associated with the induction of TGF-β1, since the high level of TGF-β1 was seen during the treatment. One possible explanation is that the mitoinhibitory stimulating effect of TGF-β1 was induced by the proliferative stimulus of BHT, thus keeping the balance of hepatocellular population during the treatment. In the absence of the proliferative stimulus following the withdrawal, the residual stimulus of TGF-β1 might have induced hepatocellular mitoinhibition. In addition, since it is known that TGF-β induces apoptosis in rat liver, these data suggested that increase in apoptosis during the treatment at 450 mg/kg might be also associated with the induction of TGF-β1. Therefore, we consider that these were adaptive responses towards cell proliferation and BHT dosages used in the present study did not disturb the homeostasis of hepatocellular population.

**Fig. 2.** Time courses of BrdU-labeling index (a) and apoptotic cells (b) in livers with BHT. The treatment schedule is indicated by the arrows. The sampling schedule is indicated by the arrowheads. *, **, *** Significantly different from control at the p<0.05, p<0.01, p<0.001, respectively (Dunnett’s test).
immunoreactivity was seen in hepatocytes of the centrilobular region, in contrast to cell proliferation, which was observed in hepatocytes of the periportal region. It is known that the pattern of hepatocyte proliferation is in the periportal region with Wy-14,643 and in the centrilobular region with PB26. The region of hepatocyte proliferation varies in accordance with the potential of compounds27. However, the mechanistic significance of the patterns is unknown26. In this study, since the regions of BrdU-labeled hepatocytes differed from those of elevated immunoreactivity for TGF-β1, it seemed that the pattern of hepatocyte proliferation was related to the induction of TGF-β1.
On the other hand, it was not clear that elevations of TGF-β2 and -β3 were not induced in this study. It is known that the signals for TGF-β2 mRNA and TGF-β3 mRNA are much weaker when compared to TGF-β1 mRNA in normal liver. However, the level of TGF-β2 mRNA and TGF-β3 mRNA expression increases in fibrotic liver or after partial hepatectomy. Thus, the mRNA assay for TGF-β would be required to ascertain the biological significance of each TGF isoform.

In chronic exposure of PB to rats, the elevation in hepatic concentration of TGF-β1 reduces the proliferation of normal hepatocytes, but not of initiated hepatocytes within the preneoplastic foci to respond to mitogenic stimuli. Mutations in the TGF-β receptor II gene are reported to occur in rat colon cancers. Thus, the selective mitoinhibitory effect of TGF-β1 on normal hepatocytes, and the mutations in the TGF-β receptor II gene could be playing important roles in promotion of hepatocarcinogenesis. In our study, we have not investigated the link between hepatocellular mitoinhibition following the withdrawal of BHT after the short treatment period and that with chronic exposure of PB. We previously reported that hepatocellular mitoinhibition following the withdrawal of stimulus with non-genotoxic hepatocarcinogens, PB and clofibrate (CF) after a 7-day treatment period, was observed as a reaction to excess of hepatocytes in rat liver. Compared to BHT, PB and CF are showing much stronger effects in mitoinhibition, and less potent effects on hepatocellular proliferation. On the other hand, these compounds are more potent than BHT in their abilities to promote or induce liver carcinogenesis. Consequently, the biological significance of mitoinhibition following the withdrawal after the short treatment period might be similar to that of mitoinhibition seen in chronic treatment with these non-genotoxic hepatocarcinogens, and associated with tumor promotion in rat hepatocarcinogenesis. However, further studies on some growth factors and TGF-β receptors are necessary to disclose the mechanism of mitoinhibition following the withdrawal of BHT.

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References


