Jpn. J. Cancer Res. 81, 220–224, March 1990

Effect of Butylated Hydroxyanisole on the Level of DNA Adduction by Aristolochic Acid in the Rat Forestomach and Liver

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Administration of butylated hydroxyanisole (BHA) orally at either 0.5 g or 1 g/kg daily for 14 days to rats did not produce any DNA adducts in the forestomach as measured by the ^{32}P-postlabeling method using (1) limiting concentrations of ^{32}P-ATP; (2) nuclease P_1 enhancement; or (3) butanol extraction. Experiments were conducted to establish the effects of BHA administration on aristolochic acid (AA) DNA adduct formation in the forestomach and liver, when BHA was administered prior to, together with or after AA administration. Adduct levels per 10^9 nucleotides in the liver after oral dosing daily for 5 days with 1 mg/kg AA and BHA (1 g/kg) or corn oil (5 ml/kg) for 7 days were as follows: (a) BHA and AA given simultaneously; 235±71, (b) AA+corn oil; 63±39, (c) AA followed by BHA; 57±13, (d) AA followed by corn oil; 91±38, (e) BHA followed by AA; 90±12, (f) corn oil followed by AA; 83±24. For the forestomach the values were: (a) 236±86, (b) 77±25, (c) 367±97, (d) 296±47, (e) 217±81, (f) 70±64. These data suggest that BHA could have an enhancing effect on AA-induced lesions in the forestomach if dosed together with, or prior to, AA as adduct levels are significantly higher than in controls.

Key words: Forestomach — Carcinogenesis — Butylated hydroxyanisole — ^{32}P-postlabeling

Although most chemical carcinogens interact with DNA by the formation of electrophilic metabolites that react with DNA to form covalently-bound DNA adducts,[1] some important animals carcinogens, such as diethylhexylphthalate and nafenopin, are negative in short-term genotoxicity tests[2,3] and do not bind to DNA.[4] Another such non-genotoxic chemical, butylated hydroxyanisole (BHA), a synthetic antioxidant widely used in the food industry, has been shown to cause tumors in the forestomach of the rat[5] and hamster.[6,7] The mechanism of action of BHA in the forestomach is unclear but it has been demonstrated that BHA does not bind to DNA as determined using ^{14}C-labeled BHA *in vivo* or *in vitro*.[8] A much more sensitive technique for detecting DNA adducts is the ^{32}P-postlabeling test,[9] which has been used to study a wide range of carcinogen adducts *in vitro* or *in vivo*.[10,11] Subsequent enhancement modifications involving the use of the enzyme nuclease P1[12] or butanol extraction[13] to concentrate adducted nucleotides, increased the sensitivity to one adduct in 10^9–10^{10} nucleotides. These methods were used here to investigate possible formation of BHA adducts at low levels in forestomach and liver DNA of male Wistar rats dosed with 1 g/kg body weight of BHA by oral gavage, a dosage regime that has been shown to induce short-term proliferative changes in the forestomach of the rat, similar to those seen in the early stages of the carcinogenicity study.[14]

In addition, since BHA has been shown to exert a range of effects on the action of other carcinogens, depending on the target organ involved, and to enhance tumor formation initiated by known genotoxic forestomach carcinogens in the rat,[15,16] a study of the effects of BHA on aristolochic acid (AA) adduct formation using the nuclease P1 enhancement method was performed. AA is a potent genotoxic forestomach carcinogen in the rat[17] for which the ^{32}P-postlabeling profile has been reported.[18] This compound, which is a nitrophenanthrene derivative obtained from the plant *Aristolochia clematitis*, was used in a variety of pharmaceutical products in Germany until 1982.[19] As well as being carcinogenic in rats, it is also a direct mutagen in *Salmonella typhimurium*.[20] In this study, a series of experiments were performed to investigate comparative differences in AA adduct levels in the rat when BHA was dosed prior to, together with, or after, AA for 5–12 days, in both the forestomach (target organ) and liver (non-target organ).

MATERIALS AND METHODS

2,[3]-*tert*-Butyl-4-hydroxyanisole (mixed isomers) and aristolochic acid (95% crystalline, a mixture of AA1 and 2) were obtained from Sigma, Poole, Dorset, UK.

Animal dosage

1) BHA adducts: Three groups of four male Alderley Park APfSD rats (150–250 g) were dosed by oral intubation daily for 14 days as follows: Group 1 received 5 ml/kg body weight arachis oil; group 2 received 0.5 g/kg body weight BHA in arachis oil (0.2 g/ml) and

group 3 received 1 g/kg body weight BHA in arachis oil (0.2 g/ml). Alderley Park APfSD rats are a Wistar-derived strain of rats bred in the ICI animal house. Rats were maintained on standard pelleted diet and water *ad libitum*. After 14 days rats were killed with CO_2. The liver and forestomach were removed and stored at −90°C.

2) Effect of BHA on AA adducts: Three experimental regimes were followed using groups of three male Wistar rats (160–200 g supplied by Charles River, UK), dosed by oral intubation with AA at 1 mg/kg body weight in 4 ml/kg water and/or BHA at 1 g/kg body weight in 5 ml/kg corn oil, depending on the dosage regime as follows. In experiment 1, group one received AA and BHA for 5 days, and group two received AA and corn oil for 5 days. In experiment 2, group one received BHA for 7 days followed by AA for 5 days and group two received corn oil for 7 days followed by AA for 5 days. In experiment 3, group one received AA for 5 days followed by BHA for 7 days and group two received AA for 5 days followed by corn oil for 7 days.

As negative controls, two rats received water and BHA, or water and corn oil for 7 days. At the end of each experiment animals were killed with CO_2. Forestomach and liver samples were removed and stored at −90°C.

DNA extraction DNA was extracted from liver and forestomach mucosal layer using an enzyme solvent extraction procedure[21] and concentration was estimated spectrophotometrically, assuming A_{260} of 1 mg/ml DNA=22.

^{32}P-postlabeling In the case of the BHA adduct study, DNA samples were labeled by three methods: (i) with limiting concentration of ATP,[11] ii) nuclease P1 enhancement[12] and iii) following butanol extraction.[13] In each case [γ-^{32}P]ATP was synthesized according to the method of Johnson and Walseth[22] to a specific activity of 37–54 TBq/mmol using ^{32}P-orthophosphate obtained from Amersham UK. For labeling experiments 3.7 MBq of ATP/sample was used. Excess ATP was hydrolyzed using 40 mU of potato apyrase and the whole reaction mixture (20 μl) was applied to the origin. In the AA/BHA experiments, all samples were labeled by the nuclease P1 method.

Adduct spots observed after autoradiography at −90°C, using Kodak X-OMAT AR film, for up to five days, were excised and counted by Cerenkov assay to determine the level of DNA adducts.

RESULTS

On postlabeling of DNA from the forestomach and liver of rats dosed with either 0.5 or 1 g BHA/kg body weight, no adduct spots were visible on autoradiography,

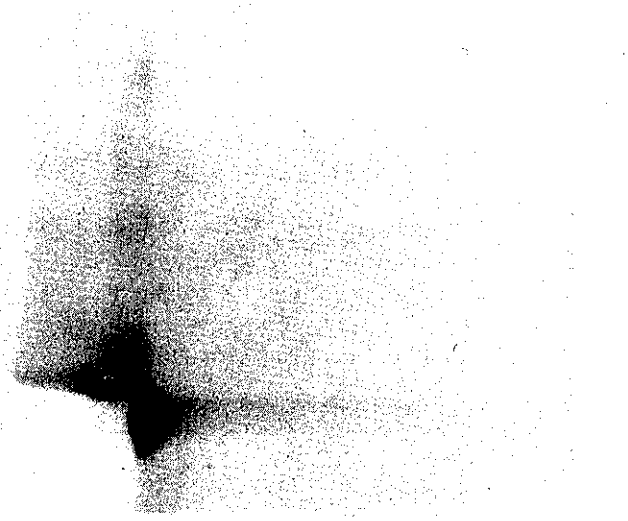

Fig. 1. Autoradiograph of postlabeled DNA from forestomach of a rat dosed with 1 g/kg body weight BHA for 14 days.

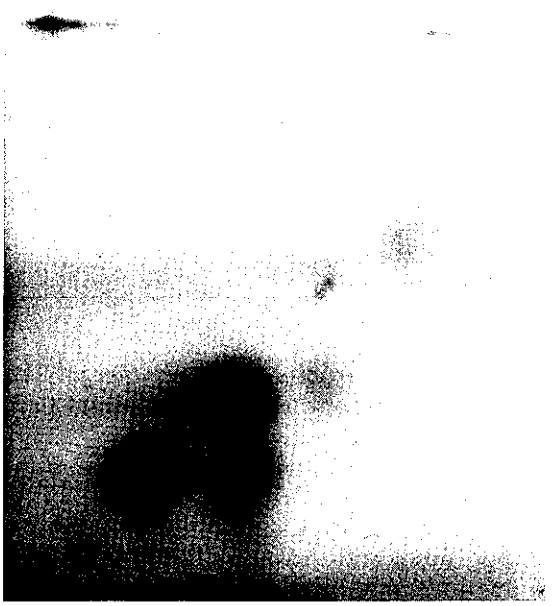

Fig. 2. Autoradiograph of postlabeled DNA from forestomach of a rat dosed with 1 mg/kg body weight AA for 5 days. Chromatography was performed in 3.5 *M* lithium formate, 8.5 *M* urea, pH 3.5 (bottom to top) and 8.5 *M* urea, 0.8 *M* HCl, 0.5 *M* Tris-HCl, pH 8 (left to right).

Table I. Levels of Aristolochic Acid Adducts in Rat Forestomach and Liver DNA

Dosage	Adducts/10^9 nucleotides[a)]	
	Forestomach	Liver
AA+BHA (5 days)	236±86	235±71
AA+corn oil (5 days)	77±25	63±39
BHA (7 days) followed by AA (5 days)	217±81	90±12
Corn oil (7 days) followed by AA (5 days)	70±64	83±24
AA (5 days) followed by BHA (7 days)	367±97	57±13
AA (5 days) followed by corn oil (7 days)	296±47	91±38

a) Values are means of results from three rats; each sample was labeled in triplicate.

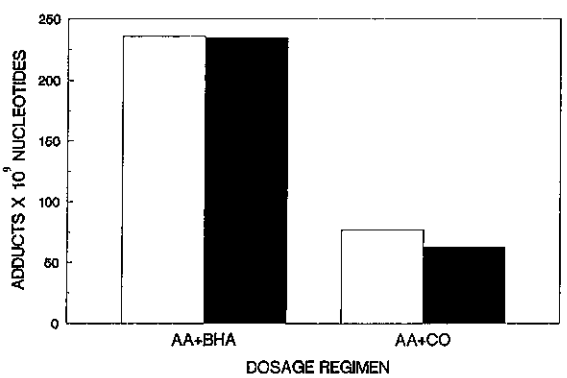

Fig. 3. Levels of AA-DNA adducts in rat forestomach and liver when dosed with BHA+AA, as compared to AA+corn oil. Each column represents the averaged DNA adduct levels from three rats; each sample was labeled in triplicate. □, Forestomach; ■, liver.

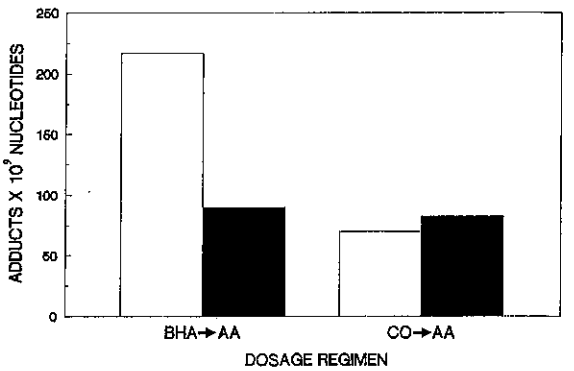

Fig. 4. Levels of AA-DNA adducts in rat forestomach and liver when dosed with BHA prior to AA, as compared to corn oil prior to AA. Format as in Fig 3. □, Forestomach; ■, liver.

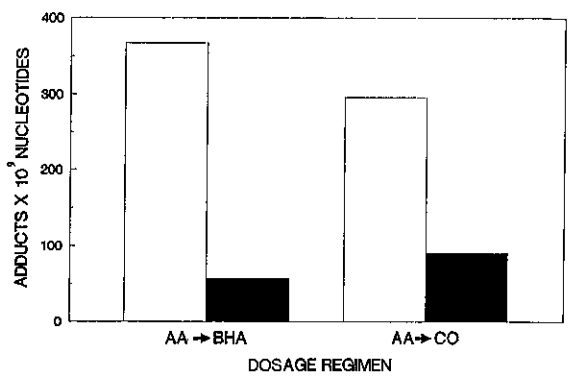

Fig. 5. Levels of AA-DNA adducts in rat forestomach and liver when dosed with AA prior to BHA, as compared to AA prior to corn oil. Format as in Fig 3. □, Forestomach; ■, liver.

even after five days' exposure time (see Fig. 1). This was the case for all three enhancement procedures that were used.

In those experiments combining AA dosage with BHA dosage, the adduct pattern seen in each case was due to AA, as seen by comparing animals dosed with AA+corn oil to those dosed with AA and BHA. Up to five adduct spots due to AA were visible on autoradiographs (see Fig. 2). Although the adduct pattern was basically the same for all three experimental protocols, in the case of AA pre-dosage the minor adduct spots were very faint and not always quantifiable. In order to maintain comparable results between the three experiments, therefore, only the two major adducts were quantified in each case (see Table I).

Experiment 1: AA+BHA In the forestomach the mean adduct levels from the rats dosed with BHA and AA were about three-fold higher than in the rats dosed with AA+corn oil (see Fig. 3). Statistical analysis using a two-sample *t*-test showed this increase to be significant ($P<0.05$). In the liver mean adduct levels were 3.7 times higher in rats dosed with BHA+AA, as compared to those dosed with AA+corn oil ($P<0.01$).

Experiment 2: BHA followed by AA In the forestomach, mean AA adduct levels were about three-fold higher in rats dosed with BHA prior to AA as compared to those dosed with corn oil prior to AA ($P<0.05$) (see Fig. 4). In the liver there was no significant difference between adduct levels in the two groups.

Experiment 3: AA followed by BHA In the forestomach there was no significant difference between the AA adduct levels in the two groups. In the liver the adduct levels were 1.6 times higher, on average, in the rats dosed with AA followed by corn oil, as compared to those with BHA after AA dosage ($P<0.05$). (see Fig. 5)

DISCUSSION

The enhancement techniques of the ^{32}P-postlabeling assay used in the present study to investigate possible adduct formation by BHA are sensitive enough to detect one adduct in 10^9 nucleotides. The finding that no adduct spots were visible on autoradiographs of labeled DNA from the liver (non-target organ) or forestomach (target organ) exposed to BHA *in vivo* confirms previous findings, using less sensitive methods, that BHA does not bind to DNA *in vivo*.[8] As BHA is a non-genotoxic compound, this finding is in line with results from ^{32}P-postlabeling studies on other non-genotoxic carcinogens which did not form DNA adducts, such as diethylhexylphthalate[23] and methapyrilene.[24] However, it must be noted that BHA administration in this study was only for a two-week time period. Recent work with the peroxisome proliferator ciprofibrate has shown that DNA adducts were detectable only after periods of 2–8 months' dosing.[25]

Further work presented here has been carried out to gain information on the manner in which BHA affects the adduct formation of a known genotoxic forestomach carcinogen. Aristolochic acid was chosen as a model compound for which the postlabeling profile was already known. Published work on aristolochic acid has shown that administration of AA, following the protocol used here, leads to the formation of tumors in the forestomach, kidney and bladder after 3 months.[17] Although the liver is a non-target organ for AA tumor formation, it has previously been demonstrated that DNA adducts due to AA can be detected using ^{32}P-postlabeling in both target organs and in non-target organs, including the liver.[18] The postlabeling profiles for AA adducts in forestomach and liver DNA presented here are in agreement with those previously published.

In the three experimental protocols described, AA was dosed in the presence of corn oil as this was the vehicle used for BHA dosage. This ensured that the corn oil was not responsible for any of the differences seen when BHA was given as compared to when it was not.

BHA is a stimulator of enzyme activity in the liver[26] and a similar effect in the forestomach could be responsible for the observed three-fold average increase in the level of AA adducts when BHA was given together with, or prior to, AA. An increase in enzyme activity, resulting in an increase in the metabolism of AA to its reactive intermediates, could explain the increased adduct levels. In the case of BHA dosage, after AA dosage, there was no significant change in adduct levels. However, it must be noted that the levels of AA adducts in the control group were unusually high, compared with the levels of AA adducts in the other control groups. The reason for this is unclear, although there is a difference between this control group and the others, in that the rats were killed seven days after the final dose of AA. As AA is a lipophilic compound it is possible that a build-up of AA occurred in fat tissue, leading to a gradual release over the following seven days. However, this possibility has not been investigated further.

The observed situation in the liver appears to be more complicated as, although an increase in adduct level similar to that in the forestomach was found when AA was dosed together with BHA, the effects seen in the two other dosage regimes were different to those seen in the forestomach. This finding, that AA levels are influenced by BHA in various ways depending on the organ involved and the dosage regime followed, reflects differences seen in experiments investigating the effect of BHA on tumorigenesis by other genotoxic carcinogens.[27] These differences probably relate to different enzyme systems involved in metabolism, detoxification and DNA repair in different tissue types. In order to establish whether the changes in adduct levels in the forestomach could affect tumorigenesis, further work is in progress involving longer-term dosage with BHA following acute AA dosage.

ACKNOWLEDGMENTS

We would like to acknowledge the generous assistance of David H. Phillips and Alan Hewer of the Chester Beatty Institute, London, in the initial setting up of the postlabeling assay. MR is an ICI SERC/CASE student.

(Received September 22, 1989/Accepted January 11, 1990)

REFERENCES

1) Miller, E. C. and Miller, J. A. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. *Cancer*, **47**, 2327–2345 (1981).
2) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 24. "Some Pharmaceutical Drugs," pp. 125–133 (1980). IARC, Lyon.
3) Warren, J. R., Simmon, V. F. and Reddy, J. K. Properties of hypolipidemic peroxisome proliferators in the lympho-

cyte [^{3}H]thymidine and *Salmonella* mutagenesis assays. *Cancer Res.*, **40**, 36–41 (1980).
4) Daniel, J. W. and Bratt, H. The absorption, metabolism and tissue distribution of di(2)-ethylhexylphthalate in rats. *Toxicology*, **2**, 51–65 (1974).
5) Ito, N., Fukushima, S., Hagiwara, A., Shibata, M. and Ogiso, T. Carcinogenicity of butylated hydroxyanisole in F344 rats. *J. Natl. Cancer Inst.*, **70**, 343–352 (1983).
6) Ito, N., Fukushima, S., Imaida, K., Sakata, T. and Masui, T. Induction of papilloma in the forestomach of hamsters by butylated hydroxyanisole. *Gann*, **74**, 459–461 (1983).
7) Ito, N., Hirose, M., Kurata, Y., Ikawa, E., Mera, Y. and Fukushima, S. Induction of forestomach hyperplasia by crude butylated hydroxyanisole, a mixture of 3-*tert* and 2-*tert* isomers, in Syrian golden hamsters is due to 3-*tert*-butylated hydroxyanisole. *Gann*, **75**, 471–474 (1984).
8) Hirose, M., Asamoto, M., Hagiwara, A., Ito, N., Kaneko, H., Saito, K., Takamatsu, Y., Yoshitake, A. and Miyamoto, J. Metabolism of 2- and 3-*tert*-butyl-4-hydroxyanisole(2- & 3-BHA) in the rat (ii): metabolism in forestomach and covalent binding in tissue macromolecules. *Toxicology*, **45**, 13–24 (1987).
9) Randerath, K., Reddy, M. V. and Gupta, R. C. ^{32}P-postlabelling test for DNA damage. *Proc. Natl. Acad. Sci. USA*, **78**, 6126–6129 (1981).
10) Reddy, M. V., Gupta, R. C., Randerath, E. and Randerath, K. ^{32}P-postlabelling test for covalent DNA binding of chemicals *in vivo*; application to a variety of aromatic carcinogens and methylating agents. *Carcinogenesis*, **5**, 231–243 (1984).
11) Watson, W. B. Post-radiolabelling for detecting DNA damage. *Mutagenesis*, **2**, 319–331 (1987).
12) Reddy, M. V. and Randerath, K. Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543–1551 (1986).
13) Gupta, R. C. Enhanced sensitivity of ^{32}P-postlabelling analysis of aromatic carcinogen DNA adducts. *Cancer Res.*, **45**, 5656–5662 (1985).
14) Altmann, H-J., Western, P. W., Mattiaschk, G., Grunow, W. and van der Heiyden, C. A. Induction of early lesions in the forestomach of rats by 3-*tert*-butyl-4-hydroxyanisole (BHA). *Food Chem. Toxicol.*, **23**, 723–731 (1985).
15) Ito, N., Fukushima, S. and Tsuda, H. Carcinogenicity modification of the carcinogenic response by BHA, BHT and other antioxidants. *CRC Crit. Rev. Toxicol.*, **15**, 109–150 (1985).
16) Fukushima, S., Sakata, T., Tagawa, Y., Shibata, M., Hirose, M. and Ito, N. Different modifying response to butylated hydroxyanisole butylated hydroxytoluene and other antioxidants for oesophagus and forestomach carcinogenesis. *Cancer Res.*, **47**, 2113–2118 (1987).
17) Mengs, U., Lang, W. and Pock, J. A. The carcinogenic action of aristolochic acid in rats. *Arch. Toxicol.*, **51**, 107–119 (1982).
18) Schmeiser, H. H., Schoepe, K-B. and Weissler, M. DNA adduct formation of aristolochic acid i and ii *in vitro* and *in vivo*. *Carcinogenesis*, **9**, 297–363 (1988).
19) Schmeiser, H. H., Pool, B. L. and Wiessler, M. Identification of metabolites of aristolochic acid formed by rat liver. *Carcinogenesis*, **7**, 59–63 (1986).
20) Goggelmann, W., Robisch, G. and Schimmer, O. Aristolochic acid is a direct mutagen in *S. typhimurium*. *Mutat. Res.*, **105**, 201–204 (1982).
21) Marten, C. and Garner, R. C. The identification and assessment of covalent binding *in vitro* and *in vivo*. *In* "Biochemical Toxicology: A Practical Approach," ed. K. Snell and B. Mullock, pp. 109–126 (1967). IRL Press, Oxford.
22) Johnson, R. A. and Walseth, T. F. The enzymatic preparation of [α-^{32}P]ATP, [α-^{32}P]GTP, [^{32}P]cAMP, [^{32}P]-cGMP and their use in assay of adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterases. *Adv. Cyclic Nucleotide Res.*, **10**, 135–167 (1979).
23) Gupta, R. C., Goel, S. K., Earley, K., Singh, B. and Reddy, J. K. ^{32}P-postlabelling analysis of peroxisome proliferator-DNA adduct formation in rat liver *in vivo* and hepatocytes *in vitro*. *Carcinogenesis*, **6**, 933–936 (1985).
24) Lijinsky, W. and Yamashita, K. Lack of binding of methapyrilene and similar antihistamines to rat liver DNA examined by ^{32}P-postlabelling. *Cancer Res.*, **48**, 6475–6477 (1988).
25) Randerath, E., Randerath, K., Reddy, R., Rao, M. S. and Reddy, J. K. Rat liver DNA alterations induced by the peroxisome proliferator ciprofibrate. *Proc. Am. Assoc. Cancer Res.*, **30**, 146 (1989).
26) Cummings, S. W. and Prough, R. A. Butylated hydroxyanisole-stimulated NADPH oxidase activity in rat liver microsomal fractions. *J. Biol. Chem.*, **258**, 12315–12319 (1983).
27) Ito, N. and Hirose, M. The role of antioxidants in chemical carcinogenesis. *Jpn. J. Cancer Res.*, **78**, 1011–1026 (1987).