SPECIES AND STRAIN DIFFERENCES IN THE BUTYLATED HYDROXYTOLUENE (BHT)-PRODUCING INDUCTION OF HEPATIC DRUG OXIDATION ENZYMES

Sumie KAWANO, Toshiko NAKAO and Kogo HIRAGA
Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo 160, Japan

Accepted June 23, 1980

Abstract—Five week-old, Wistar-JCL male and female rats and C57BL/6N male mice given a 0.5% butylated hydroxytoluene (BHT)-containing diet for 6 days produced a marked increase in hepatic weight and microsomal protein content. However, the augmentations of cytochrome P-450 content and drug oxidation activities were much more significant, i.e. 2.5-fold and more than three-fold increases were observed on a body weight basis, respectively. BHT-induced cytochrome P-450 cannot be distinguished from phenobarbital (PB)-induced cytochrome in many respects we have examined: i.e. 1) a broad substrate specificity; 2) absence of the blue shift in the CO-binding difference spectrum; 3) no rise in the peak height ratio of ethylisocyanide difference spectrum; 4) absence of α-naphthoflavone inhibition of p-nitroanisole demethylase activity; 5) marked increases of 50,000 and 54,000 molecular weight polypeptides in SDS-polyacrylamide gel electrophoresis. However, the induction of 46,000 molecular weight polypeptide by BHT in rats was more conspicuous than that by PB, and this induction was not observed in mice. In contrast to this marked induction, the administration of BHT to MC nonresponsive DBA/2N mice produced neither hepatic enlargement nor induction of cytochromes, but did produce an extremely high mortality.

Butylated hydroxytoluene (2,6-di-tert-butyl-4-methylphenol, BHT) has been a subject of an extensive toxicological investigation because of its abundant use in foods as an antioxidant. Recently, Takahashi and Hiraga (1, 2) in our laboratory reported a hemorrhagic phenomenon accompanied by hypoprothrombinemia in BHT-fed male rats. Gilbert et al. (3, 4) found that this compound, when given orally, produced hepatic enlargement and induction of drug-metabolizing enzymes in rodents.

In this decade, it has been clarified that multiple forms of cytochrome P-450 exist in liver microsomes, and that phenobarbital (PB) and 3-methylcholanthrene (MC), typical inducers of drug-metabolizing enzymes, increase the amount of different forms of cytochrome (5–7). The administration of polycyclic aromatic hydrocarbons such as MC and β-naphthoflavone to certain inbred strains of mice represented by C57BL/6N induces a spectrally distinct form of cytochrome, known as cytochrome P-448. However, in other strains, represented by DBA/2N, the administration of these drugs produces no induction. Thus, these strains are known as MC-responsive and -nonresponsive strains, respectively, and this responsiveness to MC administration is thought to be associated with specific regulatory genes, termed Ah loci (6).

The forms of cytochrome induced by BHT have not been reported. Recently, Suzuki
et al. (8) reported that liver DT-diaphorase activity was increased four to five times by the administration of BHT. We are most interested in this finding, because Lind and Ernster (9) and Kumaki et al. (10) previously suggested that the induction of this enzyme was associated with Ah loci. Thus, in the present report we investigated the induction of drug-metabolizing enzymes by BHT in rats and in two inbred strains of mice (C57BL/6N and DBA/2N), in an attempt to clarify the species of cytochrome induced and the relation of this induction to Ah loci.

**MATERIALS AND METHODS**

**Chemicals:** Materials and their sources are as follows: butylated hydroxytoluene (BHT) and α-naphthoflavone (7,8-benzoﬂavone) were obtained from Tokyo Kasei Kogyo Co.; disodium salt of glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from baker's yeast, Type V), bovine serum albumin, ovoalbumin, catalase and ethylisocyanide were purchased from Sigma Chemical Co.; glutamate dehydrogenase from Boehringer Mannheim; metyrapone (2-methyl-1,2-di-3-pyridyl-1-propane) from Aldrich Chemical Co.; NADP from Oriental Yeast Co.; acrylamide and N,N-methylene-bis-acrylamide from Seikagaku Kogyo Co.; sodium dodecyl sulfate (SDS), nicotinamide, p-nitroanisole and aniline from Wako Pure Chemical Industries; aminopyrine from Sanko Seikagaku Kogyo Co. p-Nitroanisole was recrystallized from ethanol and ethylisocyanide was distilled prior to use.

**Animals and treatment:** Four week-old male and female Wistar-JCL rats (65-70 g) were purchased from CLEA Japan Co. The inbred strains of mice, C57BL/6N (13-15 g) and DBA/2N (14-18 g) were purchased from Charles River Japan Inc. The environment of the animal room was rigidly controlled, as previously described (11). The rats were housed in stainless steel wire cages, and the mice on cedar bedding in aluminium cages. The animals were housed in groups of five or six. After maintenance on a commercial stock diet (CLEA CE-2, CLEA Japan Co.) for one week, the animals were fed a test diet, *i.e.* powdered CLEA CE-2 containing 0.5% BHT, for 6 days, and thereafter the test diet was removed. PB-treated rats and mice were given the drug in drinking water (0.1%) for 6 days. All the animals were killed about 24 hr after the drugs were removed.

**Enzyme assays:** Microsomes were prepared as previously described (12) and stored at -80°C. Protein content was determined according to the method of Lowry et al. (13). The activities of p-nitroanisole demethylase, aminopyrine demethylase and aniline hydroxylase were determined, as previously described (11). The composition of assay mixture (1.25 ml) was as follows: glucose-6-phosphate 4 mM, NADP 0.8 mM, MgCl₂ 5 mM, glucose-6-phosphate dehydrogenase 0.2 U/ml, nicotinamide 5 mM, sodium phosphate buffer (pH 7.4) 56 mM, EDTA 0.1 mM, substrate (1.2 mM p-nitroanisole, 4 mM aminopyrine or 0.5 mM aniline) and the microsomal fraction derived from 62.5 mg rat liver or 25 mg mouse liver. The incubation was performed at 37°C for 10 min.

**Measurements of difference spectra:** The difference spectrum between dithionite-reduced and oxidized microsomes, CO-binding difference spectrum of dithionite-reduced
microsomes and ethylisocyanide difference spectrum of dithionite-reduced microsomes at pH 7.4 were determined, as previously described (12) using Cary Model 17 spectrophotometer. The calculations of cytochrome b₅ and P-450 contents and 455 nm/430 nm peak height ratio of ethylisocyanide difference spectrum were as previously described (12).

**SDS-polyacrylamide gel electrophoresis:** 0.1% SDS – 10% acrylamide slab gel electrophoresis was performed by the method of Laemmli (14). To gels (2.0 mm-thick) containing twelve 6.0 mm-wide tracks was applied 40 μg of liver microsomal protein per track. Electrophoresis was carried out at 30 mA/gel for 4 hr. Thereafter, the gels were stained with Coomassie brilliant blue R-250, according to the method of Weber and Osborn (15).

RESULTS

*Induction of drug-metabolizing enzymes by the administration of BHT in liver of male and female rats:* Five week-old male and female rats were given BHT in the diet for the first 6 days of the one week test period. During the last 24 hr the drug was removed from the diet to prevent the obstruction of enzyme assay by the drug remaining in microsomes.

**TABLE 1.** Hepatic enlargement and induction of drug-metabolizing enzymes in male and female rats following administration of butylated hydroxytoluene (BHT)

| Sex     | Male | BHT | Female | BHT |
|---------|------|-----|--------|-----|
| Treatment | None | BHT | None | BHT |
| Body weight (B.W., g) | | | | |
| (100) | 176 ± 13 | 166 ± 12 | (100) | 142 ± 7 | 137 ± 7 |
| Liver weight (g/100 g B.W.) | | | | |
| (100) | 4.95 ± 0.22 | 7.43 ± 0.45*** | (100) | 4.64 ± 0.29 | 6.39 ± 0.39*** |
| Liver protein content (mg/100 g B.W.) | | | | |
| (100) | 845 ± 39 | 1208 ± 50*** | (100) | 783 ± 50 | 1121 ± 81*** |
| Microsomal protein content (mg/100 g B.W.) | | | | |
| (100) | 94.3 ± 8.4 | 190 ± 6 + 17 6*** | (100) | 88 ± 9.2 | 155.1 ± 8.2*** |
| Hemoprotein content (nmole/100 g B.W.) | Cytochrome P-450 | | | |
| (100) | 86.8 ± 6.7 | 225 ± 41 5** | (100) | 64.5 ± 7.7 | 168.6 ± 17.4** |
| (259) | 301 | (100) | 5.17 ± 0.51*** |
| (392) | (100) | | | |
| Cytochrome b₅ | | | | |
| (100) | 37.8 ± 4.1 | 113.8 ± 9.6*** | (100) | 32.9 ± 5.1 | 85.4 ± 8.7*** |
| (301) | (100) | | | |
| Enzymatic activity (μmole of formed metabolite 10 min/100 g B.W.) | p-Nitroanisole demethylase | | | |
| (100) | 1.67 ± 0.15 | 6.51 ± 1.01*** | (100) | 1.32 ± 0.11 | 5.17 ± 0.51*** |
| (390) | (100) | | | |
| Aminopyrine demethylase | | | | |
| (100) | 16.2 ± 2.1 | 39.2 ± 5.0*** | (100) | 6.9 ± 0.3 | 25.1 ± 2.8*** |
| (242) | (100) | | | |
| Aniline hydroxylase | | | | |
| (100) | 0.82 ± 0.09 | 2.93 ± 0.60*** | (100) | 0.58 ± 0.05 | 2.44 ± 0.22*** |
| (357) | (100) | | | |

Five week-old, Wistar-JCL male and female rats were given a 0.5% BHT-containing test diet for 6 days, and after being maintained on a control diet for the following 24 hr, the animals were killed. Each value represents the mean ± SD for 5 or 6 animals. **Significantly different from nontreated control animals of both sexes, P < 0.001. Numbers in parentheses represent % of each control group.
The results were expressed on a body weight basis, which should be most appropriate to visualize the function of the whole animal. As shown in Table 1, orally administered BHT produced a remarkable enlargement in liver weight in both sexes. Total liver protein increased in content in parallel with the increase of liver weight by the administration of BHT, but the rate of increase in the hepatic microsomal protein content markedly exceeded the rate of hepatic enlargement. More than 150% increases were observed in cytochrome P-450 and b1 contents by the administration of BHT. In the activities of drug-metabolizing enzymes, more than three-fold increases were observed, except for aminopyrine demethylase activity in male rats.

The characteristics of cytochrome P-450 induced by BHT were similar to those of PB-type cytochrome, as shown in Table 2. This cytochrome seems to have a broad substrate specificity, because it metabolizes p-nitroanisole, aminopyrine and aniline equally well. Further, there was a trivial red shift rather than the blue shift in the CO-binding difference spectrum. A marked change was not observed in the peak height ratio of ethylisocyanide difference spectrum.

Table 3 shows the effects of α-naphthoflavone and metyrapone, which are specific inhibitors of MC-induced and PB-induced aryl hydrocarbon hydroxylases, respectively (6), on the p-nitroanisole demethylase activity in microsomes from nontreated and BHT-treated female rats. Both drugs inhibited only slightly at the lower two concentrations (10⁻⁶ and 10⁻⁵ M) and at the highest concentration of 10⁻⁴ M, only a moderate inhibition (smaller than 30%) was observed, irrespective of the sources of microsomes.

**Effect of dietary BHT on the induction of drug-metabolizing enzymes in liver of two different inbred strains of mice, C57BL/6N and DBA/2N:** As described above, BHT is a

| Table 2. Characteristics of rat hepatic drug-metabolizing enzymes induced by butylated hydroxytoluene (BHT) |
|---|---|---|---|
| Sex | Male | Female |
| Treatment | None | BHT | None | BHT |
| Drug-metabolizing activity of cytochromes | | | | |
| p-Nitroanisole demethylation | 19.2 ± 0.9 | 29.1 ± 2.2*** | 20.5 ± 1.3 | 30.7 ± 0.7*** |
| (100) | (152) | (100) | (150) |
| Aminopyrine demethylation | 181.7 ± 10.4 | 175.8 ± 17.5 | 107.3 ± 8.7 | 149.1 ± 4.6*** |
| (100) | (97) | (100) | (139) |
| Aniline hydroxylation | 9.4 ± 0.5 | 13.1 ± 1.9** | 9.0 ± 0.8 | 14.0 ± 0.5*** |
| (100) | (139) | (100) | (156) |
| CO-binding difference spectrum | | | | |
| Wavelength of Soret peak (nm) | 449.5 ± 0.2 | 449.6 ± 0.0 | 448.8 ± 0.1 | 449.4 ± 0.2* |
| Ethylisocyanide difference spectrum | | | | |
| 455 nm/430 nm peak height ratio | 0.26 ± 0.04 | 0.33 ± 0.03* | 0.65 ± 0.03 | 0.50 ± 0.05*** |

Each value represents the mean ± SD for 5 or 6 animals except for wavelength measurements of the CO-binding difference spectrum (2 or 3 animals). Significantly different from nontreated control animals of both sexes, *P < 0.05; **P < 0.01; ***P < 0.001. Numbers in parentheses are % of nontreated control groups.
TABLE 3. Inhibition of p-nitroanisole demethylase activity by α-naphthoflavone (NF) and metyrapone (MP) in microsomes obtained from control and BHT-treated female rats

| Inhibitor | Concentration (M) |          |          |          |          |          |          |
|-----------|-------------------|----------|----------|----------|----------|----------|----------|
|           |                   | Control rat | BHT-treated rat |          |          |          |          |
|           |                   | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 |          |          |
| NF        | 0                 | 100 (13.2) | 100 (13.0) | 100 (32.8) | 100 (41.8) |          |          |
|           | 10⁻⁶              | 95.1      | 98.8     | 98.9      | 100.4     |          |          |
|           | 10⁻⁵              | 86.3      | 90.0     | 93.0      | 98.8      |          |          |
|           | 10⁻⁴              | 89.0      | 70.8     | 73.3      | 82.3      |          |          |
| MP        | 0                 | 100 (13.2) | 100 (13.0) | 100 (32.8) | 100 (41.8) |          |          |
|           | 10⁻⁶              | 90.1      | 100.0    | 96.0      | 100.0     |          |          |
|           | 10⁻⁵              | 87.5      | 95.0     | 91.0      | 93.7      |          |          |
|           | 10⁻⁴              | 80.0      | 87.3     | 72.4      | 71.3      |          |          |

Each value represents mean of 3 determinations. Numbers in parentheses are nmole of formed metabolite/ml of incubation mixture/10 min.

TABLE 4. Effect of butylated hydroxytoluene (BHT) on the drug-metabolizing enzyme system in liver of male C57BL/6N and DBA/2N mice

| Strain       | Treatment | C57BL/6N | DBA/2N |
|--------------|-----------|----------|--------|
|              |           | None     | BHT    |
|              |           | None     | BHT    |
| Body weight  |           | 18.6±0.8 | 19.1±1.1 |          | 21.4±1.6 | 16.3±2.8*** |
| (B.W., g)    |           | (100)    | (103)  |          | (100)    | (76)     |
| Liver weight |           | 5.84±0.18| 7.42±0.30*** | 5.44±0.20 | 5.52±0.97 |
| (g/100 g B.W.)|           | (100)    | (127)  |          | (100)    | (101)    |
| Liver protein content | | 1051±65 | 1333±44*** | 1140±43 | 1117±125 |
| (mg/100 g B.W.)| | (100) | (127) |          | (100) | (98) |
| Microsomal protein content | | 96.8±12.1 | 149.1±12.3*** | 105.6±3.0 | 103.0±25.2 |
| (mg/100 g B.W.)| | (100) | (154) |          | (100) | (98) |
| Hemoprotein content | | 67.8±12.1 | 170.4±16.5*** | 85.5±7.0 | 91.6±36.3 |
| (nmole/100 g B.W.)| | (100) | (251) |          | (100) | (107) |
| Cytochrome P-450 | | 21.9±3.8 | 59.1±6.2*** | 18.7±4.7 | 33.9±10.3*** |
| (nmole/100 g B.W.)| | (100) | (270) |          | (100) | (181) |
| Enzymatic activity (nmole of formed metabolite/10 min/100 g B.W.) | | 3.24±0.51 | 10.22±1.17*** | 4.03±0.61 | 6.75±2.00* |
| p-Nitroanisole demethylase | | (100) | (315) |          | (100) | (167) |
| Aminopyrine demethylase | | 15.3±3.2 | 53.2±7.9*** | 19.3±3.1 | 35.2±12.4* |
| (nmol/100 g B.W.)| | (100) | (348) |          | (100) | (182) |
| Aniline hydroxylase | | 0.96±0.17 | 2.43±0.28*** | 1.30±0.23 | 2.31±0.79* |
| CO-binding difference spectrum | Wave length of peak (nm) | 449.6±0.15 | 450.0±0.00*** | 449.4±0.18 | 449.9±0.18*** |
|               | Ethylisocyanide difference spectrum | 455 nm/430 nm peak ratio | 0.39±0.05 | 0.31±0.05* | 0.51±0.04 | 0.34±0.06** |

5 week-old male C57BL/6N and DBA/2N mice were given a 0.5% BHT-containing test diet for 6 days. Each value represents the mean±SD for 5 or 6 animals. Significantly different from nontreated control groups, *P<0.05; **P<0.01; ***P<0.001. Numbers in parentheses mean % of each control group.
potent inducer of drug-metabolizing enzymes in rats and all the characteristics examined fit well PB-type induction. Since Suzuki et al. (8) showed a marked induction of DT-diaphorase by BHT, we first assumed that BHT was a MC-type inducer. Thus, we examined the inducibility of BHT in two inbred strains of mice, i.e. C57BL/6N and DBA/2N, which were MC-responsible and non-responsible strains, respectively. The results are shown in Table 4.

In C57BL/6N mice, a marked induction of drug-metabolizing enzymes was observed. The substrate specificity and the spectral properties, i.e. peak wavelength of CO-binding difference spectrum and peak height ratio of ethylisocyanide difference spectrum of the induced cytochromes did not differ from those of PB-type cytochrome. However, in the DBA/2N mice, neither hepatic enlargement nor increase in cytochrome P-450 content was observed with the administration of BHT, nevertheless a moderate increase was brought about in the enzymatic activities and cytochrome b₅ content.

**SDS-polyacrylamide gel electrophoresis of microsomes obtained from BHT-treated animals (Fig. 1):** Control and BHT-induced microsomes showed five distinct polypeptide bands in the range of molecular weight between 45,000 and 55,000, in the electrophorogram. In microsomes from BHT-treated male and female rats, three polypeptide bands, the
molecular weights of which were tentatively calculated to be 54,000, 50,000 and 46,000, according to the method of Shapiro et al. (16), were markedly increased. These electrophoretic patterns were similar to those of PB-induced microsomes. Nevertheless, in the BHT-induced microsomes, increase in the 46,000 molecular weight band was more marked than that seen in the PB-induced microsomes. In C57BL/6N mice, only the former two polypeptide bands were markedly increased without any significant enlargement in the 46,000 molecular weight band. In DBA/2N mice, none of the bands increased remarkably, as anticipated from the results of Table 4, i.e. the absence of a significant induction of cytochrome P-450.

DISCUSSION

It is well-known that several different forms of cytochrome P-450 exist in liver microsomes, and that they have specific inducers such as PB, MC and pregnenolone-16α-carbonitrile (7, 17). We performed this experiment with the purpose of clarifying which forms of cytochrome are induced by a different inducer, BHT. Much of the evidence we found lends support to the fit for PB-type induction: 1) the cytochromes seem to have a broad substrate specificity; 2) the blue shift of the Soret peak in the CO-binding difference spectrum was not observed; 3) no rise was observed in the peak height ratio of ethylisocyanide-binding difference spectrum; 4) α-naphthoflavone, which was a specific inhibitor of MC-type cytochrome, did not show a marked inhibition of BHT-induced p-nitroanisole demethylase; 5) SDS-polyacrylamide gel electrophoretic patterns of BHT-induced microsomes showed a marked induction of 50,000 and 54,000 molecular weight polypeptides in rats and C57BL/6N mice. However, SDS-polyacrylamide gel electrophoretic patterns of rat microsomes induced by BHT showed only a slight difference from the PB-induced ones: BHT-induced microsomes showed a prominent increase in the 46,000 molecular weight polypeptide.

In Table 3, we examined the effects of α-naphthoflavone and metyrapone added to the in vitro assay system on p-nitroanisole demethylase activity in the non-induced and BHT-induced microsomes. These drugs are well-known inhibitors of MC-induced and PB-induced aryl hydrocarbon hydroxylases, respectively (6). A recent study of Thorgeirsson et al. (18) showed a specific inhibition of cytochrome P-448-associated other hydroxylases in MC-induced animals by α-naphthoflavone, and we also have shown the inhibition of p-nitroanisole demethylase activity (12). Thus, the lack of inhibition by α-naphthoflavone in BHT-induced p-nitroanisole demethylase activity indicates that BHT-induced cytochromes are different from the MC-induced ones. In the present experiment, metyrapone showed merely a slight inhibition of p-nitroanisole demethylase activity not only in BHT-induced microsomes but also in the non-induced ones. In previous work (12) we found no remarkable inhibition by this drug in the PB-induced p-nitroanisole demethylase. Thus, in the case of p-nitroanisole demethylase, metyrapone might not be a specific inhibitor of PB-induced cytochromes.

In rats, BHT administration brought about a marked increase of 54,000, 50,000 and
46,000 molecular weight polypeptides in SDS-polyacrylamide gel electrophorogram, while in C57BL/6N mice a remarkable increase was not observed in the last mentioned polypeptide. At present, it is not clear whether all of these polypeptides are actually cytochrome P-450. Judging from the position on the electrophorogram, 54,000 and 46,000 molecular weight polypeptides seem to correspond to cytochrome P-450, and P-450, respectively, reported by Ryan et al. (19). A prominent increase of the 46,000 molecular weight polypeptide in rats seems to be a characteristic of BHT-producing induction. Using two-dimensional electrophoresis and 3,3',5,5'-tetramethylbenzidine-H2O2 staining method we confirmed that this polypeptide possesses the nature of a hemoprotein (unpublished data).

Atlas et al. examined the ontogenesis of the induction of aryl hydrocarbon hydroxylase in MC-administered rabbits, and found that 57,000 molecular weight protein was always associated with the induced aryl hydrocarbon hydroxylase activity without any shift of Soret peak wavelength in the CO-binding difference spectrum (20). Guenther and Nebert also confirmed the presence of a similar protein having the molecular weight of 56,000 in 2,3,7,8-tetrachlorodibenzo-p-dioxin-administered rats (21). Although the main cytochromes induced by BHT are PB-type polypeptides, it is unclear whether or not MC-inducible minor components are induced by BHT.

In the present experiment, two strains of mice, i.e. MC-responsible C57BL/6N and MC-nonresponsible DBA/2N mice, responded differently to the administration of BHT: in the former strain a marked induction was brought about, while in the latter strain no increase was observed in the cytochrome P-450 content and only a moderate increase in the enzymatic activities. In this respect, BHT markedly contrasts with PB which shows a marked induction in both strains of mice. It is considered that aryl hydrocarbon 'responsiveness' in mice is associated with specific genes termed Ah loci, and that one of the gene products is a receptor protein which can bind specific aryl hydrocarbon inducers (6, 22). Lind and Ernster (9) and Kumaki et al. (10) suggested that the induction of a cytosol enzyme, i.e. DT-diaphorase was associated with Ah loci, and further, Suzuki et al. reported that this enzyme was also induced by BHT (8). Thus, it may be inferred that BHT-producing induction of cytochrome P-450 is also mediated by Ah loci. However, considering that BHT brought about a marked growth repression in DBA/2N mice, there may be another explanation, e.g. in such growth-repressed animals, the ability of the liver to synthesize new enzymes in response to ingested BHT may be lacking.

Although the data were not shown, another remarkable phenomenon caused by BHT administration is the high mortality peculiar to DBA/2N mice. In this strain, 0.5% BHT-containing diet brought about death in five of ten mice examined during the one week test period, while in the other strain, i.e. C57BL/6N and in Wistar-JCL male and female rats, none of the animals died during this period. Such a high mortality in the DBA/2N mice should be given serious attention, toxicologically, as this compound is widely used in foods for both humans and livestock, and relationships may exist between this high mortality and the above-mentioned lack of inducibility of cytochrome P-450.
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