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DIFFERENTIAL EFFECTS OF AGING ON HEPATIC MICROSOMAL MONOOXYGENASE INDUCTION BY PHENOBARBITAL AND β-NAPHTHOFLAVONE

LORA E. RIKANS* and BRUCE A. NOTLEY

Department of Pharmacology, University of Oklahoma, Health Sciences Center, Oklahoma City, OK 73190, U.S.A.

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Abstract—The influence of aging on hepatic microsomal monooxygenase induction by phenobarbital (PB) or β-naphthoflavone (BNF) was investigated in male Fischer 344 rats maintained in a constant environment. PB-induced increases in microsomal cytochrome P-450 content and NADPH-cytochrome *c* reductase activity were similar in rats aged 3–5 months (young-adult) and 24–25 months (old), but increases in benzephetamine *N*-demethylase activity were markedly diminished in the old rats. Separation of hepatic microsomal proteins by sodium dodecylsulfate gel electrophoresis demonstrated that aging decreased the induction by PB of a polypeptide with a molecular weight of 52,500. BNF-induced increases in microsomal cytochrome P-450 and nitroanisole *O*-demethylase activity were greater in old than in young-adult rats, and BNF induction of 55,000 and 57,000 molecular weight microsomal polypeptides was increased slightly in livers from old rats. The results indicate that age-related effects on monooxygenase induction vary with different inducers of the hepatic microsomal enzyme system.

Biological aging is often characterized by a diminished capacity for adaptation to environmental change. However, the consequence of aging on the induction response of the hepatic microsomal monooxygenase system has been a subject of controversy. An early study by Kato and Takanaka [1] demonstrated that phenobarbital treatment produced higher levels of hepatic microsomal cytochrome P-450, and of NADPH-cytochrome *c* reductase and drug metabolism activities in young rats than in old ones. Subsequently, Adelman [2] reported that the age-related impairment occurred only in the rate of induction and that age associated differences in induced cytochrome *c* reductase activity disappeared after 5 days of PB administration. On the other hand, the recent studies of Schmucker and Wang [3,4] demonstrated that both the rate and the degree of induction by PB were diminished in old rats. Age-dependent decreases in the inducibility of cytochrome P-450 content, cytochrome *c* reductase activity and ethylmorphine *N*-demethylation were evident after 2, 4, and 6 days of PB administration. Other investigators, however, have reported that livers of old and young rats respond in a similar manner to several exogenous drug metabolism inducers, including PB, 3-methylcholanthrene, β-naphthoflavone (BNF), pregnenolone-16 α-carbonitrile and polychlorinated biphenyls [5–9]. Mixed findings were reported by McMartin *et al.* [10] who showed that aging reduced the induction of cytochrome P-450 by phenobarbital but did not affect induction by BNF. Some of the variability in these results may be attributable to differences among rat strains and sexes or to differences in dosages and durations of drug treatment. However, some of the discrepancies cannot be reconciled on the basis of these differences, and it is clear that additional studies are necessary for understanding the effects of aging on microsomal monooxygenase inducibility.

We investigated hepatic microsomal monooxygenase induction as a function of age in male Fischer 344 rats maintained under strictly controlled environmental conditions. Monooxygenase components, drug metabolism activities and polypeptide profiles were analyzed in liver microsomes from control and from PB- and BNF-treated rats at 3–5, 14–15 and 24–25 months of age. Our results on the effects of aging from young adulthood to middle age have been reported [11].

EXPERIMENTAL PROCEDURES

Animals. Male Fischer 344 rats were purchased from the Charles River Breeding Laboratories, Wilmington, MA, at 7 weeks of age and maintained under constant environmental conditions as described previously [12]. They were fed a semipurified diet (AIN-76, ICN Nutritional Biochemicals, Cleveland, OH) in order to avoid seasonal variations in the composition of commercial laboratory rations. All rats used for these studies were clinically healthy. Liver sections were taken from each of the old rats and data are included only for those livers with no significant histologic lesions other than mild to moderate cytoplasmic vacuolation. There were no differences between control and treated groups in the incidence or extent of cytoplasmic vacuolation, and this finding was considered insignificant. Mild hepatic lesions (fatty change, hydropic degeneration, hepatocellular atrophy) in

* Address correspondence to: Dr. Lora E. Rikans, Department of Pharmacology, University of Oklahoma, Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190 U.S.A.

old male Fischer 344 rats have been observed previously [13].

Injection of PB and BNF. PB in saline was administered intraperitoneally for 4 days at a daily dose of 40 mg/kg and rats were killed 24 hr after the last injection. This dose was used because an 80 mg/kg dose produced heavy sedation and some deaths in the young-adult rats, and old rats are thought to be more sensitive than young ones to the sedative-hypnotic effects of barbiturates. BNF in corn oil was administered intraperitoneally at a dose of 80 mg/kg 40 hr prior to killing. Control groups received corn oil, saline or no treatment. As there were no significant differences in hepatic microsomal monooxygenase components or enzyme activities between the control groups, the values were combined.

Preparation of microsomes. Rats were killed by stunning and exsanguination. Their livers were removed and microsomes were isolated by differential centrifugation as described previously [12]. The microsomal pellets were washed with a solution containing 1.15% KCl and 0.05 M Tris–HCl, pH 7.5.

Assay of microsomal proteins. Microsomal protein content was measured by a modification [14] of the Lowry method. Total microsomal cytochrome P-450 content was determined from the reduced CO difference spectrum according to Omura and Sato [15].

Enzymatic assays. Drug metabolism assays were performed using freshly prepared microsomes, and conditions were optimized for each reaction so that activity was linear with incubation time and amount of microsomal protein. The procedures for these assays have been described in detail [12]. Briefly, benzphetamine *N*-demethylase activity was determined by measuring the production of formaldehyde with Nash reagent [16]. One-fifth of the usual amount of microsomal protein was used for this assay when the microsomes were from PB-treated rats. Aniline hydroxylase activity was determined by measuring the production of *p*-aminophenol, essentially according to the procedure of Imai *et al.* [17]. A modification of the procedure of Netter and Seidel [18] was used to measure nitroanisole *O*-demethylation. Reaction mixtures were clarified with NaOH [19] prior to reading the absorbance of the product, *p*-nitrophenol, at 415 nm. The activity of NADPH-cytochrome *c* reductase was measured at 23° according to Vermilion and Coon [20], with an extinction coefficient for cytochrome *c* of 21 $mM^{-1} cm^{-1}$ at 550 nm.

SDS gel electrophoresis. Sodium dodecylsulfate (SDS) slab gel electrophoresis in 7.5% polyacrylamide gels was carried out according to Haugen and Coon [21] except that the samples contained 10 μg of microsomal protein. Reference proteins and their molecular weights were bovine serum albumin, 68,000; catalase, 58,000; glutamate dehydrogenase, 53,000; ovalbumin, 45,000; and carbonic anhydrase, 30,000. The gels were stained with Coomassie Blue and scanned at 540 nm on a Pye-Unicam SP8-100 spectrophotometer with the densitometer attachment. Staining for heme by tetramethylbenzidine-H_2O_2 [22] required some modifications in the electrophoresis procedure [23].

Statistics. Groups of data were compared by Student's *t*-test to determine whether a significant difference existed.

Chemicals. The following chemicals were used: acrylamide, J. T. Chemical Co., Phillipsburg, NJ; BNF, 3,3′,5,5′-tetramethylbenzidine, Aldrich Chemical Co., Milwaukee, WI; aniline hydrochloride, Coomassie brilliant blue R-250, *N,N*-methylenebisacrylamide, *p*-nitroanisole, *N,N,N′,N′*-tetramethylethylenediamine, Eastman Kodak Co., Rochester, NY; and phenobarbital sodium, American Pharmaceutical Co., New York, NY. All enzymes, cofactors and reference proteins were obtained from the Sigma Chemical Co., St. Louis, MO. Benzphetamine was donated by the Upjohn Co., Kalamazoo, MI.

RESULTS

Effect of age on the hepatic microsomal monooxygenase system. Microsomes isolated from livers of old rats (24 months of age) contained less cytochrome P-450 and NADPH-cytochrome *c* reductase activity than those from young-adult rats (3–5 months of age) (Table 1). However, drug metabolism activities were not uniformly decreased as a consequence of the age-related decline in the two major protein components of the microsomal monooxygenase system. Although aniline hydroxylase and benzphetamine *N*-demethylase activities

Table 1. Effects of age and treatment with inducers on hepatic microsomal cytochrome P-450 and NADPH–cytochrome *c* reductase activity*

Treatment	Age (months)	Cytochrome P-450 (nmoles/mg protein)	Cytochrome *c* reductase (μmoles/min/mg)
Control	3–5	0.79 ± 0.01	0.24 ± 0.01
	24–25	0.55 ± 0.04†	0.12 ± 0.01†
PB	3–5	1.33 ± 0.08†	0.29 ± 0.01‡
	24–25	1.07 ± 0.07‡§	0.17 ± 0.01†‡
BNF	3–5	1.28 ± 0.06‡	0.21 ± 0.01‡
	24–25	1.24 ± 0.04‡	0.12 ± 0.01†

* Treatments were phenobarbital (PB), 40 mg/kg, daily for 4 days and β-naphthoflavone (BNF), 80 mg/kg, 40 hr prior to killing the animals. Values are means ± S.E.; $N \geq 6$.

† Significantly different from 3–5 month values, $P < 0.01$.

‡ Significantly different from untreated rats of the same age, $P < 0.01$.

§ Significantly different from 3–5 month value, $P < 0.05$.

Table 2. Effects of age and treatment with inducers on hepatic microsomal monooxygenase activities*

Treatment	Age (months)	Activities (nmoles/min/mg protein) BND	NOD	AH
Control	3–5	4.89 ± 0.11	1.02 ± 0.04	0.72 ± 0.02
	24–25	1.22 ± 0.07†	1.78 ± 0.12†	0.36 ± 0.02†
PB	3–5	18.5 ± 0.6‡	2.90 ± 0.23‡	1.55 ± 0.08‡
	24–25	6.1 ± 0.9†‡	2.74 ± 0.16‡	0.94 ± 0.03†‡
BNF	3–5	1.59 ± 0.10‡	5.91 ± 0.76‡	0.71 ± 0.03
	24–25	0.76 ± 0.10†‡	8.95 ± 0.56†‡	0.72 ± 0.03‡

* Activities were benzphetamine *N*-demethylase (BND), nmoles formaldehyde formed per min per mg microsomal protein at 37°; nitroanisole *O*-demethylase (NOD), nmoles *p*-nitrophenol formed per min per mg protein at 37°; and aniline hydroxylase (AH), nmoles *p*-aminophenol formed per min per mg protein at 37°. Treatments were the same as in Table 1. Values are means ± S.E.; $N \geq 6$.

† Significantly different from 3–5 month values, $P < 0.01$.

‡ Significantly different from untreated rats of the same age, $P < 0.01$.

decreased 50 and 75%, respectively, nitroanisole *O*-demethylase activity increased 75% as a result of aging (Table 2).

Effect of PB and BNF treatment. The dose of phenobarbital used in these studies (40 mg/kg, daily for 4 days) produced a hepatomegalic response in the young-adult rats (35% increase in liver weight, 10% increase in microsomal protein content) but the response was less than that reported for higher doses of PB [3,8]. PB-treated old rats showed no increase in liver weight or microsomal protein content compared with untreated rats. PB-treatment increased cytochrome P-450 content, cytochrome *c* reductase activity and drug metabolism activities in both age groups (Tables 1 and 2). The largest induction effect was on benzphetamine *N*-demethylase activity, which increased about 4-fold in young-adults rats treated with PB. Treatment with BNF also resulted in increased concentrations of cytochrome P-450 in liver microsomes from young-adult and old rats. The activity that responded most to BNF treatment was nitroanisole *O*-demethylase, with about a 6-fold increase in the young-adult rats. The effects of PB and BNF treatment on monooxygenase components and activities were consistent with those reported by other investigators [6,8,10].

Effect of age on monooxygenase induction. Although the qualitative aspects of PB and BNF induction were mostly similar in young-adult and old rats, there were quantitative differences in the induction response that occurred as a consequence of aging. PB-induced levels of hepatic microsomal cytochrome P-450 content and cytochrome *c* reductase activity were lower in old rats than in young-adult rats (Table 1). Nevertheless, the extent of induction produced by PB treatment was similar in the two age groups, because the non-induced concentrations of monooxygenase compounds were lower in old than in young control rats. On the other hand, BNF-induced concentrations of cytochrome P-450 were similar in liver microsomes from young-adult and old rats; thus, the increase produced by BNF was greater in old rats than in young-adult rats. Interestingly, the BNF-induced decrease in cytochrome *c* reductase activity observed in young-adult rats did not occur in old rats. Differences in the effect of aging on the ability of PB and BNF to induce microsomal drug metabolism activities are shown in Table 3. The results are given as absolute increases in activity (as opposed to percentage increases) because there were significant effects of aging on non-induced levels of activity. PB induction of monooxygenase activities was diminished in old rats, and benzphetamine *N*-demethylase was the activity that showed the greatest age-related loss in inducibility. In contrast to the results with PB,

Table 3. Effect of aging on elevation of microsomal cytochrome P-450-dependent activities*

Treatment	Age (months)	Increase in cytochrome P-450 (nmoles/mg)	Increase in Activity (nmoles/min/mg) BND	NOD	AH
PB	3–5	0.54	13.6	1.9	0.83
	24–25	0.52	4.9	1.0	0.58
BNF	3–5	0.49		4.9	0
	24–25	0.69		7.2	0.36

* Increases were calculated by subtracting mean values of control rats from mean values of induced rats. Treatments and activities are the same as in Tables 1 and 2.

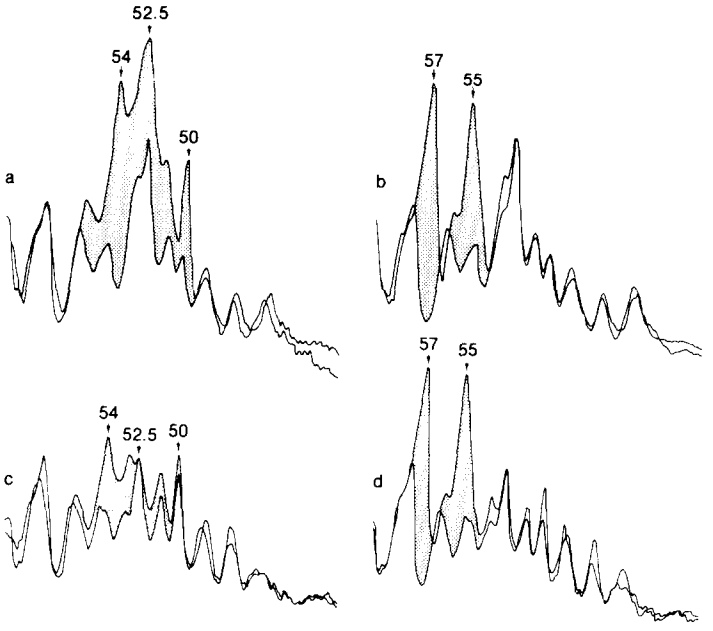

Fig. 1. Induction of hepatic microsomal polypeptides in young-adult and old rats. Densitometric scans are shown of a Coomassie blue-stained SDS polyacrylamide gel after electrophoresis of microsomes from (a) young-adult control and PB-induced, (b) young-adult control and BNF-induced, (c) old control and PB-induced and (d) old control and BNF-induced rats. The extent of induction is indicated by shading. Each sample contained 10 μg of protein, and migration was from left to right. The cytochrome P-450 contents, in nmoles/mg microsomal protein, were: young-adult control, 0.54; old control, 0.36; young-adult PB-induced, 1.24; old PB-induced, 0.93; young-adult BNF-induced 1.0; and old BNF-induced, 0.87. Numbers are molecular weights $\times 10^{-3}$, averaged from six or more gels; the reference proteins were bovine serum albumin, catalase, glutamate dehydrogenase, ovalbumin and carbonic anhydrase.

inducibility by BNF was greater in old than in young-adult rats. BNF produced a greater increase in nitroanisole *O*-demethylase activity in old than in young rats and, in addition, produced a 2-fold increase in aniline hydroxylase activity in old rats compared with no increase in young-adult rats (Table 3).

Effect of age on induction of microsomal polypeptides. SDS gel electrophoresis indicated that the monooxygenase inducers increased the quantities of certain polypeptides migrating in the molecular weight region of the cytochromes P-450. In young-adult rats PB treatment increased polypeptide bands with molecular weights of 50,000, 51,000, 52,500, 53,000 and 54,000. The increases were most predominant for the 50,000, 52,500 and 54,000 molecular weight bands. BNF induced microsomal polypeptides with molecular weights of 55,000 and 57,000. Both PB and BNF treatment increased the intensity of heme staining in the cytochrome P-450 region of the gels. However, under the conditions required for heme staining, polypeptides in the 50,000–60,000 molecular weight region were poorly resolved, and it was not possible to identify all of the induced bands as cytochromes P-450. The effects of aging on induction by PB were variable except for the effect on the 52,500 molecular weight polypeptide band. Induction of this band was consistently decreased to a marked extent in old rats; in fact, no detectable increase was observed in most cases (Fig. 1). On the other hand, BNF induction of the 55,000 and 57,000 molecular weight bands in microsomes from old rats was equal to or greater than the induction in young-adult rats.

DISCUSSION

Age-related changes in the induction response of the hepatic microsomal monooxygenase system were different when the inducer was PB than when it was BNF. PB-induced increases in monooxygenase activities were less extensive whereas BNF-induced increases were more extensive in livers from old compared with young-adult rats. The results confirm and extend our findings with middle-aged rats [11]. Thus, it appears that changes in responsiveness to specific monooxygenase inducers occur in middle and old age which are analogous to the maturational changes in inducibility that occur early in life [24,25].

The specificity of the age-related effects on microsomal monooxygenase induction suggests that aging selectively affects the inducibility of certain forms of cytochrome P-450. It is well documented that PB and BNF induce distinct forms of rat liver microsomal cytochrome P-450 which differ in a number of properties, including substrate specificity. The major form induced by PB has a subunit molecular weight of about 53,000 and preferentially catalyzes the metabolism of benzphetamine, while the major form induced by BNF has a molecular weight of about 56,000 and preferentially metabolizes

benzo(*a*)pyrene, along with several other substrates [26–30]. Our results demonstrating a marked decrease in benzphetamine *N*-demethylase and 52,500 molecular weight polypeptide induction in PB-treated old rats probably indicate that aging diminishes the inducibility of the major PB-inducible form of liver microsomal cytochrome P-450. In contrast, induction by BNF of monooxygenase activity, total cytochrome P-450 and microsomal polypeptides was somewhat increased with age. The molecular weight of the polypeptides induced by BNF (55,000 and 57,000) were similar to reported values for cytochromes P-450 induced by other agents belonging to the same class of inducers [31,32]. However, because of the poor resolution obtained with the electrophoretic procedure used for heme-stained gels and because polypeptide bands in the 50,000–60,000 molecular weight region of SDS gels undoubtedly contain other proteins, we cannot conclude unequivocally that the age-related changes observed involved differences in cytochromes P-450. Our results on the effects of aging on microsomal monooxygenase inducibility are consistent with those obtained by McMartin *et al.* [10]. Using regioselective metabolism of R-warfarin as a probe of the forms of cytochrome P-450 present in liver microsomes from variously aged rats, they showed that the inducibility of two PB-inducible forms was diminished in old rats but inducibility of the major BNF-inducible form was unattenuated by aging.

We have also considered the possibility that age-related changes in the fatty acid composition of microsomal phospholipids may have contributed to the changes in the inducibility of microsomal monooxygenases. Our studies show that aging is associated with alterations in the relative percentages of microsomal fatty acids, with liver microsomes from old rats having a greater percentage of longer fatty acids (18 carbons or more) and a higher ratio of saturated to unsaturated fatty acids than microsomes from young-adult rats [33]. These changes could have significant effects on monooxygenase inducibility, especially in view of the studies of Becker *et al.* [19] which suggest that a particular fatty acid composition is necessary for the induction of maximal activity of certain enzymes in the microsomal monooxygenase system.

We conclude that the ability of the hepatic microsomal enzyme system to respond to phenobarbital is diminished with aging while the ability to respond to BNF is enhanced. Age-dependent factors may control the induction of different forms of cytochrome P-450. In addition, there may be changes in the structure and composition of the microsomal membrane which contribute to the specific effects of aging on monooxygenase inducibility.

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