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An investigation of activities and paraoxon sensitivities of hepatic aliesterases in β-naphthoflavone-treated rats

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Abstract

Aliesterases (carboxylesterases, EC 3.1.1.1) are serine esterases which may protect acetylcholinesterase during organophosphorus insecticide intoxication by providing alternative phosphorylation sites. Levels of hepatic aliesterase activity were investigated after the intraperitoneal administration of β-naphthoflavone (BNF) to female rats using nine 4-nitrophenyl esters as substrates (including straight and branched chain aliphatic and aromatic esters) and 1-naphthyl acetate. In addition, the in vitro sensitivities of aliesterases to inhibition by paraoxon, the active metabolite of the common insecticide parathion, were studied. Hepatic aliesterases from BNF-treated rats displayed lower activities than those from the controls with all substrates except 4-nitrophenyl phenylbutyrate and isovalerate. The aliesterases from BNF-treated rats were more sensitive to paraoxon inhibition with 4-nitrophenyl phenylbutyrate, valerate, and butyrate. Esterases hydrolyzing 4-nitrophenyl butyrate, valerate, and branched chain esters were most sensitive to paraoxon inhibition while those hydrolyzing 4-nitrophenyl hexanoate and aromatic esters were least sensitive. The results suggested that BNF-induced changes in hepatic aliesterases could alter responses to organophosphates.

Key words: Aliesterases; β-Naphthoflavone; Paraoxon; Organophosphate

1. Introduction

Organophosphates (OPs), such as paraoxon, the active metabolite of the insecticide parathion, cause toxicity by inhibiting acetylcholinesterase (AChE), which is a

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critical enzyme in peripheral and central nervous system function. Parathion causes little inhibition of acetylcholinesterase in vitro [1,2]. When parathion is administered in vivo, a substantial degree of AChE inhibition can occur as paraoxon is produced metabolically by cytochrome P450-mediated desulfuration [3,4]. Inhibition of aliesterases (carboxylesterases, EC 3.1.1.1) by OPs can inactivate some of the OP molecules and protect the target acetylcholinesterase [5,6]. The greater sensitivity of liver aliesterases than brain AChE to inhibition by a number of OPs suggests their importance in protection [7]. However, the physiological function, if any, of the aliesterases is not known, and prolonged inhibition of the aliesterases by OP poisoning had no apparent detrimental effect on the animal and was not involved in nervous system dysfunction [8].

β-Naphthoflavone (BNF) is a non-carcinogenic polycyclic aromatic hydrocarbon that is frequently used experimentally to induce P4501A enzymes. In previous studies in this laboratory, pretreatment of rats with BNF decreased hepatic aliesterase specific activity (as assayed using 4-nitrophenyl valerate as the substrate) by about 23% and reduced the level of inhibition of aliesterases after in vivo treatment of rats with parathion or paraoxon [9]. Reduction in aliesterase activity implies that fewer functioning protective esterases are present in BNF-treated rats. These previous results suggest that BNF exposure could alter the potential response of organisms to OP intoxication by altering levels and OP sensitivities of these protective aliesterases, as well as by potential effects on Phase I enzymes. Such an interaction could be of concern to those exposed to OP insecticides and who are also exposed to pollutants or other xenobiotics.

Studies done by Sterri et al. [10] indicated different aliesterases to be important for methyl butyrate and 4-nitrophenyl butyrate hydrolysis. Isoenzymes of aliesterases have been shown to have different specificity toward methyl butyrate and 4-nitrophenyl acetate [11]. Therefore, this study was designed to investigate the in vitro characteristics of hepatic aliesterases from rats treated with BNF assayed with several substrates to determine whether a variety of substrates would be useful in characterizing the BNF effect. Nine esters of 4-nitrophenol as well as 1-naphthyl acetate were used as substrates; the latter was used because of its historical significance in the assay of aliesterases and because of its utility in future electrophoretic studies. The sensitivity to inhibition by paraoxon was also assessed.

2. Materials and methods

2.1. Chemicals

All biochemicals were obtained from Sigma Chemical Company (St.Louis, MO). Paraoxon, with a purity of greater than 95%, was synthesized as described previously [9]. Intermediates for the synthetic reactions were obtained from Aldrich Chemical Co. (Milwaukee, WI).

All nitrophenyl esters were synthesized by similar procedures. One equivalent of 4-nitrophenol was dissolved in anhydrous benzene and 1.5 equivalents of the appropriate acyl chloride was added while mixing. Triethylamine (1.5 equivalents) was added slowly and the mixture was stirred for about 1 h. Progress of the reaction was monitored by thin layer chromatography (TLC) of samples of the reaction mixture.

For slower reactions the mixture was refluxed. When little or no nitrophenol was detectable by TLC, the reaction mixture was washed with distilled water, 0.1 N HCl, and finally with 2% $NaHCO_3$ until the aqueous phase was colorless. Benzene was removed under vacuum and the product was purified by recrystallization from *n*-hexane until a single spot was detectable by TLC. Detailed chemical characterization of products was considered unnecessary because: (1) the straightforward esterification process consistently yielded a single reaction product, (2) the R_f values of products relative to 4-nitrophenyl acetate and benzoate (Aldrich) were as expected, and (3) alkaline hydrolysis yielded the theoretical amount of 4-nitrophenol. The 4-nitrophenyl esters used contained a series of straight chain (acetate to octanoate) and branched chain (isobutyrate and isovalerate) alkyl and aryl (phenylacetate to phenylbutyrate) acidic moieties.

2.2. Animals and treatment

Adult female Sprague–Dawley (Crl:CD(SD)BR) rats (originally from Charles River) weighing between 320–370 g were used. The rats were housed in a controlled temperature (22°C) room with a 12:12 h light cycle and had free access to Purina laboratory rodent chow and tap water. All procedures were previously approved by the Institutional Animal Care and Use Committee.

2.3. β-Naphthoflavone treatment

The rats were injected intraperitoneally (i.p.) daily with BNF in corn oil (80 mg/kg/day; 1 ml/100 g body weight) for 3 days prior to sacrifice. Two control groups were used: untreated and vehicle controls injected with corn oil (1 ml/100 g body weight). Three rats were used for each treatment.

2.4. Tissue samples

On the fourth day, rats were sacrificed by decapitation. Samples of the right anterior lobe of the liver were rapidly removed and chilled on ice. The liver samples were homogenized in 0.05 M Tris–HCl buffer, pH 7.4, at 0.05 g/ml and stored frozen at −70°C prior to assays.

2.5. Aliesterase assays

The assay of the 4-nitrophenyl esters was similar to what has been described previously [9]. The homogenates were diluted to a 4 ml total volume with 0.05 M Tris–HCl buffer, pH 7.4, to an appropriate concentration to ensure linearity during the 15 min incubation time for each substrate. The 0.05 g/ml initial liver homogenate was diluted in Tris–HCl buffer to the following final concentration for the various 4-nitrophenyl ester substrates: 0.025 mg/ml, valerate; 0.05 mg/ml, butyrate and isobutyrate; 0.075 mg/ml, phenylpropionate, phenylacetate, and phenylbutyrate; 0.1 mg/ml, hexanoate and propionate; 0.35 mg/ml, isovalerate. After temperature equilibration, 4 μl of different concentrations of paraoxon in ethanol were added to the tissue suspensions at 15 s intervals to yield final concentrations of 1.0×10^{-10} M to 1.0×10^{-5} M. The control tube received 4 μl ethanol. The tubes were vortexed and were allowed to incubate for 15 min in a shaking water bath at 37°C. After 15 min, 40 μl of a 4-nitrophenyl ester in ethanol were added with an incubation time

of 15 min. The reaction was terminated with a 1 ml solution containing 2% Tris base (to alkalinize the solution for product visualization) and 2% sodium dodecyl sulfate. Absorbance of the product, 4-nitrophenol, was monitored at 400 nm in a Perkin Elmer Lambda 5 spectrophotometer. Samples were run in triplicate, and tubes were processed at 15 s intervals. The final concentrations of the substrates used were 0.25 mM for 4-nitrophenyl phenylacetate and 0.5 mM for all other substrates; these concentrations assured linearity. Correction for non-esterase hydrolysis was performed by subtracting the absorbance of the 10^{-5} M paraoxon assay from all others. 4-Nitrophenyl acetate was too unstable to assay with accuracy. The heptanoate, octanoate, and benzoate were insufficiently soluble to assay.

Liver samples were assayed for aliesterase activity with 1-naphthyl acetate by a modification of the method of Gomori [12]. The liver homogenates were diluted to 0.07 mg/ml in Tris–HCl buffer. Liver homogenate (3.9 ml) was added to each test tube. These tissue suspensions were temperature equilibrated to 37°C in a shaking water bath for 15 min. After temperature equilibration, 4 μl of different concentrations of paraoxon in ethanol were added at 15 s intervals to yield a final concentration of 1.0×10^{-10} M to 1.0×10^{-5} M. The tubes were vortexed and allowed to incubate at 37°C in a shaking water bath for 15 min. After 15 min, 30 μl of a 0.04 M solution of 1-naphthyl acetate in methoxyethanol were added to the tissue suspensions, vortexed and allowed to incubate for 15 min. One hundred μl 4% fresh Fast Blue RR salt in distilled water were added to each tube at 15 s intervals. The reaction was terminated with 1 ml of 2% sodium dodecyl sulfate solution. Absorbance of the product, 1-naphthol, complexed to the Fast Blue dye was immediately monitored at 500 nm. Samples were run in triplicate. Correction for non-esterase hydrolysis was performed by subtracting the absorbance of the 10^{-5} M paraoxon from all others.

2.6. Protein

Protein concentration was quantified by the method of Lowry et al. [13], using bovine serum albumin as the standard.

2.7. Statistical analysis

An analysis of variance using a completely randomized design was used to identify the differences in enzyme activity at the $P < 0.05$ level. I_{50} values were calculated by linear regression of the $\log_{10}$ of paraoxon concentration with the logit transformation of percentage inhibition data. Comparison of the log I_{50} values were by an analysis of variance followed by the Student-Newman-Keuls test at the $P < 0.05$ level.

3. Results

Limited studies with a representative straight chain aliphatic, branched chain aliphatic and aromatic ester (4-nitrophenyl valerate, isovalerate, and phenylbutyrate, respectively) indicated less than 15% of the total activity occurred within the cell membrane/debris and mitochondrial fractions of the cell, calculated on a wet weight basis. The majority of activity (about 70%) occurred within the microsomal fraction with 15–20% in the cytosolic fraction with the valerate and phenylbutyrate. Activity

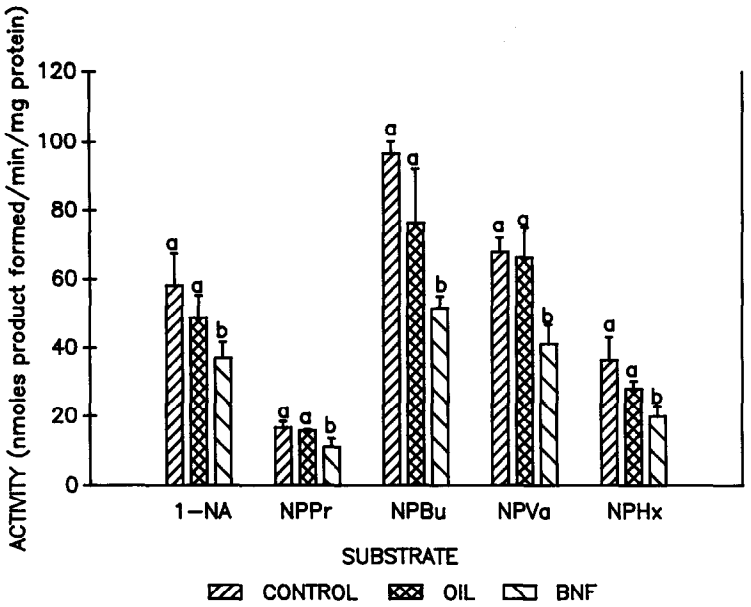

Fig. 1. Enzyme activities (mean and S.E.M.) of liver aliesterases with 1-naphthyl acetate and straight chain aliphatic esters of 4-nitrophenol after pretreatment of female rats with BNF. Means within the same substrate not followed by the same letter are significantly different at $P < 0.05$. 1-NA, 1-naphthyl acetate; NPPr, 4-nitrophenyl propionate; NPBu, 4-nitrophenyl butyrate; NPVa, 4-nitrophenyl valerate; NPHx, 4-nitrophenyl hexanoate.

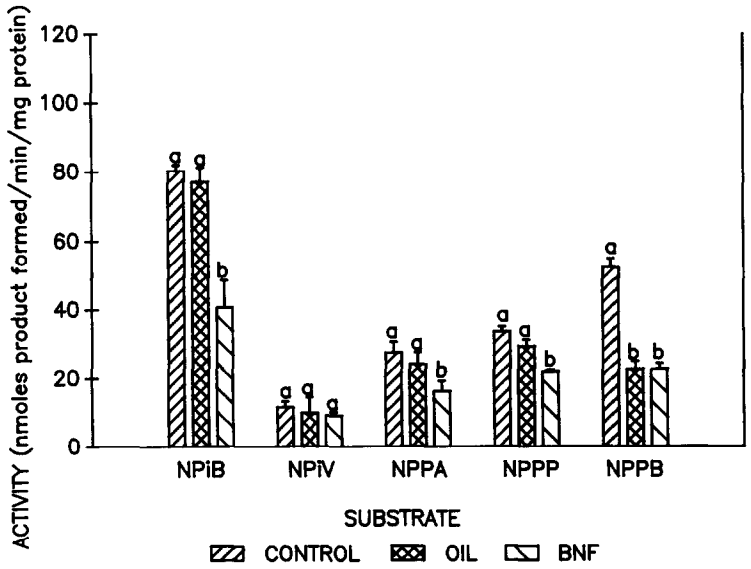

Fig. 2. Enzyme activities (mean and S.E.M.) of liver aliesterases with branched chain aliphatic and with aromatic esters of 4-nitrophenol after pretreatment of female rats with BNF. Means within the same substrate not followed by the same letter are significantly different at $P < 0.05$. NPiB, 4-nitrophenyl isobutyrate; NPiV, 4-nitrophenyl isovalerate; NPPA, 4-nitrophenyl phenylacetate; NPPP, 4-nitrophenyl phenylpropionate; NPPB, 4-nitrophenyl phenylbutyrate.

occurred equally (45% each) in the microsomal and cytosolic fractions with the isovalerate. Therefore, a whole homogenate was assayed to yield the most toxicologically relevant information.

Activity of hepatic aliesterases from control animals varied among substrates (Figs. 1 and 2). Although there was a trend toward decreasing activity with increasing size of the acidic moiety within the 4-nitrophenyl straight chain and branched chain aliphatic esters, the propionate did not support this trend. There was a trend toward increasing activity with increasing size of the 4-nitrophenyl aromatic esters.

Treatments caused no change in the protein concentration. Aliesterase activity of BNF-treated rats was significantly lower than controls (both naive and oil-treated) for all substrates except 4-nitrophenyl isovalerate and 4-nitrophenyl phenylbutyrate (Figs. 1 and 2). With the latter substrate, both BNF- and oil-treated animals had significantly lower activity than the naïve controls.

I_{50} values in control rats demonstrated a range of 136×10^{-9} to 2.95×10^{-9} M (Figs. 3 and 4). The esterases hydrolyzing 4-nitrophenyl butyrate, valerate, isobutyrate, and isovalerate were most sensitive to paraoxon inhibition, while the esterases hydrolyzing 4-nitrophenyl hexanoate and the aromatic esters were least sensitive. With 4 substrates (1-naphthyl acetate, 4-nitrophenyl propionate, and the 2 branched chain aliphatic esters) there was no difference in paraoxon sensitivity of esterases from BNF-treated animals and from either control. The esterases hydrolyzing 4-nitrophenyl butyrate, valerate, and phenylbutyrate were significantly more sensitive to paraoxon inhibition than those from either control. The esterases hydrolyzing 4-nitrophenyl hexanoate, phenylacetate, and phenylpropionate from

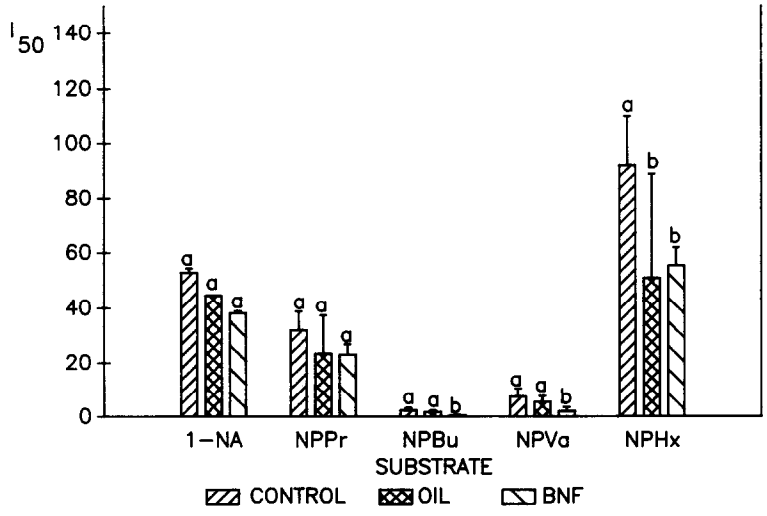

Fig. 3. Enzyme inhibition of liver aliesterases (mean and S.E.M.) with 1-naphthyl acetate and straight chain aliphatic esters of 4-nitrophenol after pretreatment of female rats with BNF. I_{50} values are expressed as nM, mean and S.E.M. Means within the same substrate not followed by the same letter are significantly different at $P < 0.05$. 1-NA, 1-naphthyl acetate; NPPr, 4-nitrophenyl propionate; NPBu, 4-nitrophenyl butyrate; NPVa, 4-nitrophenyl valerate; NPHx, 4-nitrophenyl hexanoate.

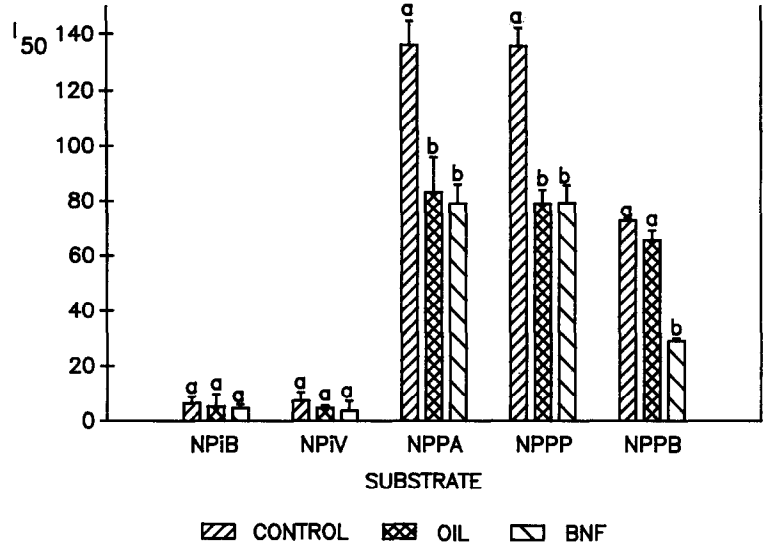

Fig. 4. Enzyme inhibition of liver aliesterases (mean and S.E.M.) with branched chain aliphatic and with aromatic esters of 4-nitrophenol after pretreatment of female rats with BNF. I_{50} values are expressed as nM, mean and S.E.M. Means within the same substrate not followed by the same letter are significantly different at $P < 0.05$. NPiB, 4-nitrophenyl isobutyrate; NPiV, 4-nitrophenyl isovalerate; NPPA, 4-nitrophenyl phenylacetate; NPPP, 4-nitrophenyl phenylpropionate; NPPB, 4-nitrophenyl phenylbutyrate.

oil- and BNF-treated rats were more sensitive to paraoxon inhibition than those from the naïve controls.

4. Discussion

Aliesterases are a family of enzymes with several isozymes having different affinities toward substrates with varying inhibitor specificities [11,14–16]. A reason for this could be the result of marked differences in specificity or different quantities of a specific isozyme [11]. Aromatic and aliphatic esters reflect different activities with 2 different groups of carboxylesterases within the same tissue. Enzyme activity was lower with the substrate 4-nitrophenyl butyrate than methyl butyrate, in the presence of the inhibitor bis-*p*-nitrophenyl phosphate [10]. The differences in patterns of activity among the treatment groups and the differences in paraoxon sensitivity observed here suggest the participation of different mixtures of isozymes with the various substrates as well as a different isozyme composition in each of the 3 experimental groups. The substrate 1-naphthyl acetate was used to determine if a different pattern was observed with a structurally unrelated substrate; however, its profile of activity was similar to that of the majority of the 4-nitrophenyl esters. The differences in patterns of the aliesterase activity with the several substrates suggest the possible utility of using a battery of substrates to help characterize the isozymes of aliesterases and consequently the response of aliesterases to BNF exposure and possibly exposure to other xenobiotics.

In several instances, the corn oil vehicle, and not BNF, seemed to be responsible

for the changes in aliesterases. The reason for this is not apparent. Although some vegetable oils do possess estrogenic properties, it seems unlikely that this short-term treatment would have allowed a mild hormone-like effect to be manifest. Perhaps more likely is the possibility that the presence of the lipid altered membrane properties sufficiently to affect the configuration of the aliesterase molecules.

BNF is a polycyclic aromatic hydrocarbon that causes induction of some of the cytochrome P450 activities. Pretreatment of rats with BNF had been previously shown to decrease the hepatic aliesterase enzyme activity as well as reduce the level of inhibition after in vivo treatment of rats with parathion or paraoxon [9]. In vivo BNF treatment lowered the in vitro enzymatic activity of the hepatic aliesterases with most substrates. The reduction of aliesterase activity following BNF treatment was surprising because BNF is usually regarded as an inducer. BNF at concentrations up to its solubility limit does not inhibit aliesterase activity as assayed in vitro with 4-nitrophenyl valerate [9]; therefore, results observed reflect an in vivo response and not a direct inhibition.

The results in Fig. 1 show the substrate 4-nitrophenyl propionate, the shortest 4-nitrophenyl ester tested, yielding the lowest activity of the straight chain esters. Such a small substrate may not bind well to the active site of the aliesterases. The highest specific activity was observed with 4-nitrophenyl butyrate with decreasing activity as the carbon chain length increased. Similar trends were reported by Sabouri and Newcombe [17]; they observed that only short chain carbon esters were hydrolyzed by human monocyte aliesterases with the 4 carbon esters yielding the highest activity. These results suggest that 4-nitrophenyl butyrate might possess the closest configuration to the physiological substrate for the enzymes. The branched chain 4-nitrophenyl esters showed a decrease in activity with an increase in chain length. The aromatic esters also yielded relatively low activity. These results indicate that the active site cannot accommodate an extremely large substrate; thus, lipases are not significant participants in the hydrolysis of these substrates.

BNF exposure seems to have shifted the hepatic aliesterase population to isozymes with somewhat different substrate-specificities. With most substrates, the BNF treatment decreased activity; if this reduced activity reflects fewer enzyme molecules, a BNF-treated rat could possess lesser protection during organophosphate poisoning because of availability of fewer phosphorylation sites. However, with those substrates with altered paraoxon sensitivity, the I_{50}s of aliesterases from BNF-treated rats were lower than those of controls, indicating a greater sensitivity to phosphorylation. This greater sensitivity could counterbalance any reduction in phosphorylation sites. However, since BNF can have other in vivo effects, such as induction of P450 which is involved in important biotransformations for some organophosphorus compounds, it is impossible to predict how much the BNF-induced changes in aliesterases would alter in vivo toxicity. This phenomenon is currently under further investigation.

5. Acknowledgments

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