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Benzo[*a*]pyrene and *β*-Naphthoflavone Mutagenic Activation by European Eel (*Anguilla anguilla* L.) S9 Liver Fraction

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Is *Anguilla anguilla* L. (eel) liver ethoxyresorufin *O*-deethylase (EROD) induction absolutely necessary in order to convert promutagens as benzo[*a*]pyrene (BaP) into a mutagenic compound? Eels were exposed for 8 h to clean (control) and 0.3 μM *β*-naphthoflavone (BNF)-contaminated water. The 8-h exposure to 0.3 μM BNF brought about a very high EROD induction (10 pmol/min/mg protein) compared to control animals (1 pmol/min/mg protein). The Ames test (Maron and Ames, 1983) was carried out with *Salmonella typhimurium* TA 98 strain (TA98 His^-) and eel isolated S9 liver fraction was used as a metabolic BaP activator. The BaP and BNF dose range concentrations tested were 0 (blank), 0.015, 0.08, 0.15, 0.38, 0.75, 1.5, 3.8, and 7.5 μM/plate and 0 (blank), 0.412, 1.235, 3.704, 11.1, 33.0, and 100 nM BNF, respectively. A dose–response relationship between BaP concentration and mutagenic activity was observed in the presence of S9 fractions in control and 0.3 μM BNF-exposed eels. Significant positive results, as TA98 His^+ revertants, were observed at 0.38, 0.75, 1.5, 3.8, and 7.5 μM BaP/plate induced by BNF S9 fractions. Significant BaP mutagenic activation by liver control S9 was detected only at 1.5, 3.8, and 7.5 μM/plate. The BaP 1.5, 3.8, and 7.5 μM/plate mutagenic activation by BNF S9 and control S9 were not significantly different. Relative to BNF activation, it was only possible to detect His^+ reversion at 11.1 nM BNF concentration with 0.3 μM BNF-induced S9. The above results demonstrate that the eel S9 liver fraction has the capacity to biotransform high BaP concentrations and convert it into a mutagenic compound with or without previous liver BNF biotransformation induction. The same does not apply to low BaP concentrations, where liver S9 induction by BNF is necessary to promote mutagenesis. © 2002 Elsevier Science (USA)

Key Words: **mutagenesis; Ames test; Fish liver s9; BaP; BNF; EROD; *Anguilla anguilla* L.**

INTRODUCTION

A wide range of chemicals, such as polycyclic aromatic hydrocarbons (PAHs), require bioactivation by microsomal or cytosolic enzymes to produce mutagenic effects (Stegeman, 1981; Buhler and Williams, 1988,1989; López-Barea and Pueyo, 1998; Fourman, 1989). Since many of these catalysts are induced by environmental pollutants, an increased activation of promutagens is used to monitor pollution. The S9-activating capacity of several contaminants into mutagens which are detectable by bacterial genotoxicity assays has been validated in fish. Another detection method is the biotransforming activity of several different enzymes. These tests can be used as powerful biomarkers in field environment studies or in exposure studies with model compounds (Rodríguez-Ariza *et al*., 1994, 1995; Díaz-Méndez *et al*., 1998).

PAH compounds frequently found in the environment have been associated with areas that demonstrate high tumor prevalence in fish populations (Baumann, 1984; Black, 1983; Malins *et al*., 1987). The mutagenicity of these compounds is a well-known consequence of their metabolic activation (Thakker *et al*., 1985).

Benzo[*a*]pyrene (BaP), a model PAH compound, is classified as a promutagen, i.e., chemical substance that must be metabolically activated to become a DNA-damaging agent (Johnson, 1992). It is regarded as a clastogen and a potent animal carcinogen (Vainio *et al*., 1985; Varanasi, 1987; Hawkins *et al*., 1990). BaP must be activated and/or alternatively detoxified by metabolically favored pathways. The above findings suggest the need for a balance between the Phase I and Phase II detoxification process. Other compounds such as *β*-naphthoflavone (BNF) a (PAH-like compound) are strong ethoxyresorufin *O*-deethylase (EROD) inducers (Pacheco and Santos, 1998, Schlezinger and Stegeman, 2000). This synthetic flavonoid compound produces effects on the microsomal cytochrome *P*-450 (P450)-dependent monooxygenase system, which is involved in the metabolic activation and detoxification of a wide range of procarcinogenic contaminants (Wiebel, 1980).

A number of fish studies on the genotoxic effects of BaP and/or BNF have been performed using cytogenetic tests, such as the presence of micronuclei (Grinfeld *et al*., 1986),

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erythrocytic nuclear abnormalities (ENA) (Pacheco and Santos, 1997, 1998), chromosome aberrations (Hoofman, 1981), and tumor detection (Hendricks *et al*., 1985, Hawkins *et al*., 1988).

The capability of hepatic S9 fractions to activate known promutagens provides a better evaluation of xenobiotic metabolism than the determination of liver biotransformation enzymes. The Salmonella/microsome test has been used by Ames *et al*. (1975) and Paolini *et al*. (1991) to assess the potentially mutagenic and carcinogenic intermediate metabolites resulting from xenobiotic biotransformation. The adaptation of fish liver homogenates to the Salmonella/microsome (Kurelec *et al*., 1979; Rodríguez-Ariza *et al*., 1995) and SOS-umu tests (Bihari *et al*., 1990) has been reported, although not yet extensively characterized.

Previous research studies have reported metabolic activation of BaP by liver S9 fractions of *Mugil* sp. (grey mullet) from contaminated environments (Rodríguez-Ariza *et al*., 1994) and carp under laboratory experimental conditions (Britvic and Kurelec, 1986). The mutagenic potential of BaP and BNF on European eel (*Anguilla anguilla* L.) has not been sufficiently investigated, because it is a migrant fish which may have been exposed to a wide range of potentially genotoxic environmental contaminants. Pacheco and Santos (1997, 1998, 2002) have demonstrated that eel is a good model species for genotoxicity screening as well as for liver function response studies on PAHs and PAH-like compounds. In this perspective, the purpose of the current study was to determine whether pre-induction of EROD in eel liver is necessary for conversion of a promutagen into a mutagenic compound.

MATERIALS AND METHODS

Chemicals

BNF, BaP, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of analytical grade and were purchased from Sigma, Boehringer Mannheim GmbH (Germany), or E. Merck-Darmstadt (Germany).

Test Animals

The experiment was carried out on *A. anguilla* L. (eel), average weight 50 g, collected from the Aveiro lagoon. The eels were transported under anoxic conditions and acclimated to laboratory conditions in aerated, filtered, and dechlorinated tap water in 200-L aquaria for 1 week, at 20°C. Eels were exposed for 8 h to 0 (control) and 0.3 μM BNF. Control fish were exposed to DMSO. Two tanks containing 20 animals each were used for control and BNF exposure. Fish were killed by decapitation and livers were sampled, immediately frozen in liquid nitrogen, and stored at −20°C until homogenization.

Preparation of Liver Postmitochondrial Supernatant (S9) and S9 Mixture

The liver S9 fraction is based on the procedure of Maron and Ames (1983). All steps of the procedure are carried out at 0–4°C using cold, sterile solutions and glassware. Livers were homogenized with three volumes of 0.15 M KCl in a Potter–Elvehjem apparatus with a Teflon pestle and the homogenates centrifuged at 9000 *g* for 10 min. Supernatants (S9 fractions) were decanted, avoiding the superficial lipid layer. The freshly prepared S9 fractions were distributed (1 to 2-ml portions) in small sterile polystyrene tubes and immediately stored at −80°C.

The S9 mixture contained 5 mM glucose-6-phosphate, 4 mM NADP, 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer (pH 7.4), and 50 μl of the S9 fraction, in 0.5 ml total volume. This mixture was prepared immediately before each experiment and maintained at 4°C.

Bacterial Strain

Salmonella typhimurium TA98 strain was used as an indicator of genotoxic activity. The bacteria carrying the his auxothrophy (*his*D3052) required for the His assay contain the R-factor plasmid pKM101 and two mutations, *rfa* and *uvrb*. This strain was kindly provided by Dr. B. N. Ames, University of California, Berkley. The test strain genotypes were confirmed for each mutagenicity assay.

Mutagenicity and Metabolic Activation Assays

Mutagenicity and metabolic activation were performed using the Ames method (Maron and Ames, 1983). The assay was carried out in the absence and in the presence of the S9 mixtures from BNF-induced and uninduced eel liver. Three independent experiments were performed and duplicates were used for each tested dose. A bacterial suspension of 0.1 ml (14 h overnight culture in nutrient broth (NB) from Oxoid, approximate density $1\text{–}2 \times 10^8$ cells/ml) was pipetted into molten top agar. BaP doses and BNF concentrations used were 0 (blank), 0.015, 0.08, 0.15, 0.38, 0.75, 1.5, 3.8, and 7.5 μM/plate, and 0.412, 1.235, 3.704, 11.1, 33.0, and 100 nM, respectively. The BaP and BNF samples were dissolved in DMSO. BaP, BNF, biotin, L-histidine, NaCl, and 0.5 ml of S9 mixture were added to top agar, poured into plates containing minimal medium, and incubated 48 h at 37°C. After incubation, the number of histidine-independent revertants was counted. For a response to be considered positive the number of revertants has to double the number of spontaneous revertants (blank plates) and a clear relationship between mutagenic response and dose of mutagen or amount of S9 had to be observed.

Statistical Analysis

The results are expressed as means ± standard error (SE) and statistical analysis was performed using a two-tailed Student t test (Bailey, 1959).

RESULTS

Eels Exposure to BNF

The eel liver EROD response was significantly increased after 8 h exposure to 0.3 μM BNF ($P < 0.01$) compared to control level. Liver P450 was not significantly different from control.

Mutagenic Response of the His$^+$ Reverse Mutation Assay in Bacterial Strain TA98

The number of *S. typhimurium* TA98 strain His$^+$ revertants per selective plate, respectively, induced by increasing doses of BaP (μM/plate) 0, 0.015, 0.08, 0.15, 0.38, 0.75, 1.5, 3.8, and 7.5 (Fig. 1) and BNF (nM/plate) 0.412, 1.235, 3.704, 11.1, 33, and 100 nM (Fig. 2), was counted and expressed as means ± SE of two replicates in three independent experiments.

As seen in Fig. 1 and Table 1, there was a dose–response relationship between BaP dose and the number of His$^+$ revertants in eel BNF-induced and noninduced liver S9. BNF-induced S9 had an increased number of His$^+$ revertants in the 0.38, 0.75, 1.5, 3.8, and 7.5 μM BaP/plate doses, demonstrating its activation into a genotoxic compound. Induction potential, expressed as the ratio of BaP concentration to the number of blank revertants (without BaP, spontaneous revertants) was 2.2-, 2.8-, 3.2-, 3.6-, and 4.3-fold, respectively. In the control S9 fraction, the number of His$^+$ revertants was increased at 1.5, 3.8, and 7.5 μM/plate, demonstrating as 2.5-, 2.8-, and 3.6-fold BaP activation, respectively. When BaP was tested without the metabolic activator (S9), the number of His$^+$ revertants became very low, and were classified as spontaneous revertants.

Regarding BNF activation (Fig. 2 and Table 2) by BNF-induced liver S9, an increase (2.33-fold) in His$^+$ revertants at 11.1 nM BNF was observed. Control liver S9 did not demonstrate any BNF activation, for any BNF concentration tested.

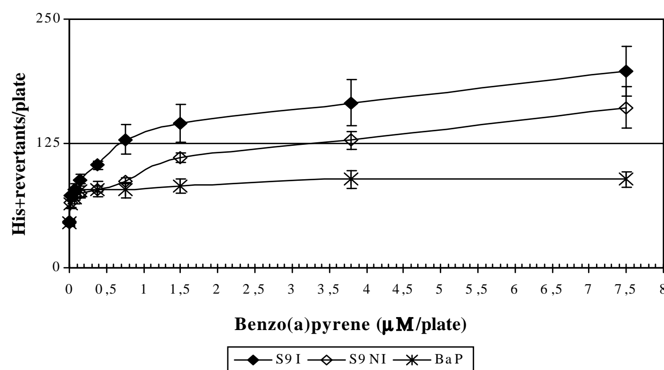

FIG. 1. Increasing doses of BaP (μM/plate) were assayed with 10^8 bacteria (TA 98) and 50 μL of liver S9. Assays without S9 (−S9) were included as controls. Data indicate the total number of His$^+$ revertants per plate, plotted as mean ± SE of two replicates of three independent experiments. **S9I**, S9 fraction BNF-induced; **S9NI**, S9 fraction noninduced.

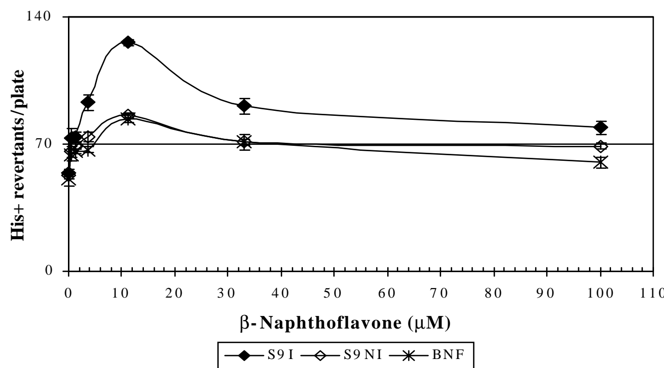

FIG.2. Increasing doses of BNF (nM/plate) were assayed with 10^8 bacteria (TA 98) and 50 μL of liver S9. Assays without S9 (−S9) were included as controls. Data indicate the total number of His$^+$ revertants per plate, plotted as mean ± SE of two replicates of three independent experiments. **S9I**, S9 fraction BNF-induced; **S9NI**, S9 fraction noninduced.

TABLE 1
Benzo[a]pyrene (BaP) Mutagenic Activation by *Anguilla anguilla* L. Liver S9 Fraction

BaP (μM/plate)	+S9		−S9
	S9I[a]	S9NI[b]	
0	46 ± 3 (1.0)[c]	45 ± 3 (1.0)	45 ± 3 (1.0)
0.015	73 ± 2 (1.6)	66 ± 5 (1.5)	64 ± 4 (1.4)
0.08	79 ± 5 (1.7)	73 ± 5 (1.6)	70 ± 6 (1.6)
0.15	88 ± 6 (1.9)	75 ± 3 (1.7)	77 ± 7 (1.8)
0.38	103 ± 4 (2.2)	79 ± 3 (1.8)	79 ± 8 (1.8)
0.75	129 ± 15 (2.8)	87 ± 3 (1.9)	78 ± 8 (1.7)
1.5	145 ± 19 (3.2)[d]	111 ± 5 (2.8)[d]	82 ± 7 (1.8)
3.8	166 ± 23 (3.6)[e]	128 ± 8 (2.8)[d]	89 ± 9 (1.9)
7.5	198 ± 25 (4.3)[d]	161 ± 21 (3.6)[d]	89 ± 8 (1.9)

Note. Assays without S9 (−S9) were included as controls. Data indicate the total number of His$^+$ revertants per plate, plotted as mean ± SE of two replicates of three independent experiments.

[a]Liver S9 isolated from *A. anguilla* L. at 8 h exposure to 0.3 μM BNF.

[b]Liver S9 isolated from *A. anguilla* L. at 8 h exposure to clean water (Control).

[c]The ratio BaP concentration *versus* blank revertants (without BaP, spontaneous revertants).

[d]$P < 0.01$ compared to control (−S9).

[e]$P < 0.02$.

TABLE 2
β-Naphthoflavone Mutagenic Activation by *Anguilla anguilla* L. Liver S9 Fraction

BNF (μM/plate)	+ S9		− S9
	S9I[a]	S9NI[b]	
0	54 ± 1 (1.0)[c]	53 ± 1 (1.0)	51 ± 2 (1.0)
0.412	73 ± 4 (1.4)	66 ± 2 (1.5)	64 ± 2 (1.3)
1.24	74 ± 2 (1.4)	69 ± 1 (1.3)	66 ± 1 (1.3)
3.7	93 ± 4 (1.7)	74 ± 2 (1.4)	67 ± 1 (1.3)
11.1	126 ± 2 (2.3)[d]	86 ± 1 (1.6)	84 ± 2 (1.7)
33	91 ± 4 (1.7)	71 ± 1 (1.4)	71 ± 3 (1.4)
100	79 ± 3 (1.5)	69 ± 1 (1.4)	60 ± 2 (1.2)

Note. Assays without S9 (− S9) were included as controls. Data indicate the total number of His$^+$ revertants per plate, plotted as mean ± SE of two replicates of three independent experiments.

[a]Liver S9 isolated from *A. anguilla* L. at 8 h exposure to 0.3 μM BNF.

[b]Liver S9 isolated from *A. anguilla* L. at 8 h exposure to clean water (Control).

[c]The ratio BaP concentration Versus blank revertants (without BNF, spontaneous revertants).

[d]$P < 0.01$ compared to control.

DISCUSSION

Metabolic activation of known promutagens can be easily carried out with bacterial genotoxicity assays. Promutagen *in vitro* activation by the mammalian S9 seems to be closely related to the *in vivo* Phase I biotransformation pathways without a convenient and effective Phase II. However, promutagen metabolic activation by fish S9 fractions has not been extensively studied (Rodríguez-Ariza *et al*., 1994).

According to the current results (Fig. 1 and Table 1), eel S9 liver fractions have the ability to promote biotransformation of the highest BaP doses into reactive metabolites which induce a His$^+$ reversion in *Salmonella* TA98 strain. However, the presence of the S9 fraction induced by BNF seems to increase the mutagenic effects of BaP doses ranging from 0.38 up to 7.5 μM/plate, since the number of revertants was higher than those found with control liver S9. A dose-related mutagenic response was also found as a His$^+$ reversion in *Salmonella* TA98 strain at a BaP concentration range between 1.5 and 7.5 μM/plate for control liver S9. In the absence *A. anguilla* L. S9, the TA 98 strain did not revert. This is due to the fact that BaP requires metabolic activation for its biotransformation and cofactors in the medium, such as glucose-6-phosphate and NADP, which promote NADPH generation. The results suggest that an increase of BaP mutagenic activity in eel BNF-induced liver S9 fraction, at 0.38, 0.75, 1.5, 3.8, and 7.5 μM/plate BaP dose levels, is due to the excessive production of BaP reactive metabolites as well as to its lack of conjugation. One possible explanation may be the suppression of a Phase II reaction *in vitro* when there is a high concentration of substrate interacting with the monooxygenase-dependent P450 system. However, at the lowest BaP concentration range, 0.015–0.08 μM/plate, no mutagenic activity was observed for BNF-induced S9 or for noninduced S9.

Previous research has reported that the use of hepatic microsomes from BNF-treated mice as an enzyme source in mutagenicity tests produced marked increases in the activation of BaP to mutagenic products (Raina *et al.,* 1985).

The assessment of the BNF mutagenic potential (Fig. 2 and Table 2) with BNF-induced S9 fraction revealed that the 11.1 nM BNF concentration promoted the appearance of His$^+$ *S. typhimurium* TA98 (2.33-fold), whereas concentrations lower and higher than 11.1 nM did not induce mutagenic activity. Furthermore, it was also observed that for the two highest concentrations, 33 and 100 nM BNF, there was a decrease in the number of the revertants, 1.7- and 1.5-fold, respectively. In the S9 control fraction, the mutagenic effect of BNF was not observed.

Using cytochrome *P*-4501A as an indicator (Goksoyr and Forlin, 1992), the effects of BNF biotransformation induction on *Sparus aurata* and *Liza aurata* were analyzed by Cousinou *et al.* (2000). They observed an increase in EROD activity (9.1- and 3.7-fold higher than controls, respectively). These authors also observed BNF biotransformation induction in *S. aurata* and *L. aurata* resulting from the ability of the S9 liver fraction to convert 2-aminoanthracene (2-AA) into mutagens, which were detectable by the *S. typhimurium* strain BA149. In the BNF-treated fish the activation of 2-AA into genotoxins was dramatically increased compared to controls.

CONCLUSIONS

From the current study, the following conclusions are made: (1) *A. anguilla* L. liver S9 has the capacity to convert a promutagen such as BaP into a mutagenic compound. (2). *A. anguilla* L. liver S9 induced by BNF is required to convert very low concentrations of a promutagen such as BaP into a mutagenic compound. The results suggest that previous liver EROD induction is required for low concentrations of promutagens to be converted into mutagenic compounds. The results concerning BNF seem inconclusive, since low concentrations have no mutagenic effect, high concentrations are inhibitory, and an intermediate concentration is mutagenic.

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