β-Naphthoflavone and benzo(a)pyrene treatment affect liver intermediary metabolism and plasma cortisol levels in rainbow trout

Onkorhynchus mykiss

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Abstract

To assess the effects of the polycyclic aromatic hydrocarbons (PAHs) β-naphthoflavone (β-NF) and benzo(a)pyrene (BaP) on liver intermediary metabolism and plasma steroid hormones, immature female rainbow trout (Onkorhynchus mykiss) were intraperitoneally injected (2 μg g⁻¹) with vegetable oil alone (control) or containing β-NF or BaP (10 mg kg⁻¹) and returned to their tanks; 3, 24, and 72 h after injection, 11 fish were sampled from each group. On each sampling time, plasma hormone levels (cortisol and 17β-estradiol) and metabolic parameters in plasma (glucose, lactate, and α-amino acid levels) and liver (glycogen, glucose, lactate, and α-amino acid levels, and HK, GK, PK, LDH, G6Pase, G6PDH, FBPase, GDH, Asp-AT, and HOAD activities) were assessed. Changes described for hormonal systems resulted in an increase in plasma levels of cortisol after 24 and 72 h of treatment with both PAHs whereas no changes were noticed for 17β-estradiol levels. Changes in intermediary metabolism described effects in several pathways due to treatment with both PAHs. These changes can be summarized as increased glucose and lactate levels in plasma, and increased glycogenolysis and gluconeogenesis in liver after 24 and 72 h of treatment with both PAHs. Furthermore, β-NF treatment stimulated amino acid catabolism in liver. These metabolic changes can be associated with increased levels of plasma cortisol, and suggest a different metabolic behavior depending on PAHs.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have been demonstrated to be mutagenic and carcinogenic precursors as well as to impair growth, reproduction, and osmor-
occur to overcome toxic stress (Frasco and Guilhermino, 2002). Thus, changes in plasma metabolites after PAHs exposure have been described in several studies such as for glucose and lactate (Teles et al., 2004, 2005), and lipids (Monteiro et al., 2000a).

The liver is the primary organ receiving PAHs (Deb et al., 2000). However, the studies carried out to assess the impact of PAHs exposure on liver energy metabolism are scarce (Vijayan et al., 1997; Roche et al., 2002; Stephensen et al., 2003). In a previous study, we have assessed the impact of naphthalene, the simplest PAHs, on intermediary metabolism in liver of rainbow trout (Tintos et al., 2006b, 2007). Since naphthalene is probably one of the less powerful inducers of detoxification processes, we aimed to assess the effects of more powerful PAHs in energy metabolism of liver in rainbow trout. Accordingly we have assessed in the present study the effects of \( \beta \)-naphthoflavone (\( \beta \)-NF) and benzo(a)pyrene (BaP). \( \beta \)-NF, a synthetic analog of a large series of naturally occurring flavonoid compounds, is an aromatic hydrocarbon (Ah) receptor agonist (Pacheco and Santos, 2002) that has been considered the most potent P450 mixed-function oxidase (MFO) inducer among a number of synthetic flavonoid compounds. BaP is a ubiquitous PAH, and has been also shown to be carcinogenic and MFO inducer in fish (Lemaire-Gony and Lemaire, 1992). The information regarding effects of both PAHs in liver energy metabolism is limited to several studies with \( \beta \)-NF (Vijayan et al., 1997; Morrow et al., 2004; Teles et al., 2005) and very few with BaP (Lemaire-Gony and Lemaire, 1992). Therefore, the specific objectives of the present study were to examine whether or not \( \beta \)-NF or BaP treatment induced in fish hepatic intermediary metabolism as well as levels of plasma steroid hormones more important changes than those observed after treatment with other PAHs like naphthalene.

2. Materials and methods

2.1. Fish

Sexually immature female rainbow trout (Oncorhynchus mykiss, 50 ± 6 g body weight) were provided by a hatchery in Soutorredondo (Noia, Spain) and transferred to the laboratory in the Faculty of Biology (Vigo, Spain). Fish were acclimated for 4 weeks in flow-through tanks providing a constant supply of freshwater before the experiments. Fish were kept under natural photoperiod (December 2005) and constant temperature (15 °C). Fish of three different groups (reared in separate tanks) were caught by netting, anaesthetized with MS-222 (50 mg l\(^{-1} \)) buffered with sodium bicarbonate, weighed, and intraperitoneally injected (2 \( \mu \)l g\(^{-1} \)) with vegetable (sunflower) oil alone (control) or containing \( \beta \)-NF or BaP (10 mg kg\(^{-1} \)) and returned to their tanks. Three, 24, and 72 h after injection, 11 fish were sampled from each group. Uninjected fish were also used to assess the vehicle effect.

2.2. Experimental design

Fish of three different groups (reared in separate tanks) were caught by netting, anaesthetized with MS-222 (50 mg l\(^{-1} \)) buffered with sodium bicarbonate, weighed, and intraperitoneally injected (2 \( \mu \)l g\(^{-1} \)) with

2.3. Sampling

At each sampling time, fish were quickly caught by netting, and anaesthetized with MS-222 (50 mg l\(^{-1} \)) buffered with sodium bicarbonate. Blood was obtained in ammonium-heparin treated syringes by puncture of the caudal veins. Plasma samples were obtained by centrifugation of blood, and divided into two aliquots. One aliquot was immediately frozen on liquid nitrogen for the assessment of plasma hormone levels, whereas the other aliquot, for the assessment of plasma metabolites, was deproteinized immediately with 0.6 N perchloric acid and neutralized with 1 mol l\(^{-1} \) potassium bicarbonate, frozen in liquid nitrogen and stored at −80 °C until further assay. Liver was removed quickly from each fish, freeze-clamped in liquid nitrogen, and stored at −80 °C until assay.

2.4. Analytical techniques

Plasma cortisol and E2 levels were measured in plasma by ELISA (Tintos et al., 2006a, 2007). Plasma glucose and lactate levels were measured using commercial kits from Spinreact (Spain). Plasma total z-amino acid levels were assessed colorimetrically using the nynhidrin method of Moore (1968) with modifications to adapt the assay to a microplate format.

The frozen liver was finely minced on a chilled Petri dish, vigorously mixed and divided into two portions to assess enzyme activities and metabolite levels. The frozen tissue used for the assessment of metabolite levels was homogenized by ultrasonic disruption with 7.5 vols of iced-cooled 0.6 N perchloric acid, neutralized (using 7.5 vols of 1 mol l\(^{-1} \) potassium bicarbonate), centrifuged (2 min at 13,000 g), and the supernatant used to assay tissue metabolites. Glycogen levels were assessed using the method of Kepler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Spinreact, Spain). Lactate, and total z-amino acid levels were assessed as described above for plasma samples.

The portions of liver used for the assessment of enzyme activities were homogenized by ultrasonic disruption with 10 vols of ice-cold stopping-buffer containing (in mmol l\(^{-1} \)): 50 imidazole-HCl (pH 7.5), 1 mercaptoethanol, 50 NaF, 4 EDTA, 120 sucrose, and a protease inhibitor cocktail (Sigma, P-2714). The homogenate was centrifuged and the supernatant used in enzyme assays.

The activities of several enzymes representative of major pathways of energy metabolism (HK, GK, PK, G6PDH, G6Pase, FBPase, HOAD, GDH, Asp-AT, and LDH) were assessed. Reaction rates of enzymes were determined using a microplate reader SPECTRAFluor (Tecan, Grödig, Austria). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (15 μl), at a pre-established protein concentration, omitting the substrate in control wells (final volume 265–295 μl), and allowing the reactions to proceed at 20 °C for pre-established times (3–10 min). Enzyme activities are expressed per mg protein. Protein was assayed in homogenates using the bicinechonic acid method with bovine serum albumin (Sigma, USA) as standard. Enzymatic analyses were all carried out at maximum rates, with the reaction mixtures set up in preliminary tests to render optimal activities (Sangiao-Alvarellos et al., 2003, 2005a,b).

2.5. Statistic

The effect of treatment and time of exposure, as well as their possible interaction in parameters assessed was analyzed using a two-way ANOVA with treatment (control, \( \beta \)-NF and BaP) and time (3, 24, and 72 h) as main factors.
factors. Multiple comparisons were carried out using the Student–Newman–Keuls test. Significance level was set at $P < 0.05$.

3. Results

No mortality, health disturbances or any alterations in behavior were observed in any group of fish throughout the experiments. No differences were observed between un.injected fish (data not shown) and those fish implanted with vehicle alone (control) for any parameter assessed.

Plasma cortisol levels increased after 24 or 72 h in fish treated with $\beta$-NF or BaP with the increase being higher in fish treated with $\beta$-NF (Fig. 1A) while no changes were noticed in plasma 17$\beta$-estradiol levels (Fig. 1B). Plasma glucose levels increased after 24 or 72 h in fish treated with $\beta$-NF or BaP (Fig. 2A) whereas plasma lactate levels increased after treatment with both PAHs at all times assessed (Fig. 2B). Plasma $\alpha$-amino acid levels increased after 3 and 24 h of treatment with $\beta$-NF whereas BaP produced an increase only after 3 h (Fig. 2C).

Liver glycogen levels decreased (Fig. 3A), and glucose levels increased (Fig. 3B) in fish treated with $\beta$-NF or BaP after 24 and 72 h of treatment. Liver lactate levels increased in fish treated with $\beta$-NF after 3 and 24 h of treatment, whereas in fish treated with BaP, levels increased only after 3 h (Fig. 3C). Liver $\alpha$-amino acid levels increased after 72 h of treatment with $\beta$-NF (Fig. 3D).

Fig. 1. Changes in the levels of cortisol (A) and 17$\beta$-estradiol (B) in plasma of rainbow trout after treatment with $2 \mu$g$^{-1}$ of vegetable oil alone (control) or containing $\beta$-naphthoflavone or benzo(a)pyrene (10 mg$^{-1}$ body weight) that were sampled 3, 24, and 72 h after injection. Each value is the mean ± SEM of 11 fish. *, Significantly different ($P < 0.05$) from control fish. #, significantly different ($P < 0.05$) from fish treated with $10$ mg $\beta$-naphthoflavone$^{-1}$ body weight. Different letters indicate significant differences among times within each treatment.

Fig. 2. Changes in the levels of glucose (A), lactate (B), and $\alpha$-amino acids (C) in plasma of rainbow trout after treatment with $2 \mu$g$^{-1}$ of vegetable oil alone (control) or containing $\beta$-naphthoflavone or benzo(a)pyrene (10 mg$^{-1}$ body weight) that were sampled 3, 24, and 72 h after injection. Each value is the mean ± SEM of 11 fish. *, Significantly different ($P < 0.05$) from control fish. #, significantly different ($P < 0.05$) from fish treated with $10$ mg $\beta$-naphthoflavone$^{-1}$ body weight. Different letters indicate significant differences among times within each treatment.

Liver glycogen levels decreased (Fig. 3A), and glucose levels increased (Fig. 3B) in fish treated with $\beta$-NF or BaP after 24 and 72 h of treatment. Liver lactate levels increased in fish treated with $\beta$-NF after 3 and 24 h of treatment, whereas in fish treated with BaP, levels increased only after 3 h (Fig. 3C). Liver $\alpha$-amino acid levels increased after 72 h of treatment with $\beta$-NF (Fig. 3D).
Liver enzyme activities are displayed in Table 1. FBPase and G6Pase activities increased in fish treated with β-NF or BaP after 24 and 72 h. In the case of G6Pase the increase observed after 72 h was higher in fish treated with BaP than in fish treated with β-NF. G6PDH activity decreased in fish treated with β-NF or BaP at all times assessed. GDH and Asp-AT activities increased in fish treated with β-NF after 24 and 72 h with activities being higher than those observed in BaP-treated fish. No significant effects of treatment were observed for HK, GK, PK, LDH, and HOAD activities (data not shown).

4. Discussion

Since PAHs were administered intraperitoneally at a single dose instead of dietary or water exposure routes, the present study is limited relative to environmental extrapolations. To better understand the effects of the PAHs assessed, more studies are needed including dietary or water exposure, dose–responses, sampling additional time points, and assessing different life stages or body sizes.

The administration procedure used in the present study was similar to others described in literature for β-NF or BaP delivery (Hutz et al., 1999; Morrow et al., 2004; Kennedy et al., 2004; Aluru and Vijayan, 2004). Furthermore, the doses used were also similar to those used in previous studies with i.p. injections of β-NF (Vijayan et al., 1997; Hutz et al., 1999; Aluru and Vijayan, 2006) or BaP (Lemaire-Gony and Lemaire, 1992; Au et al., 1999; Kennedy et al., 2004).

Neither β-NF nor BaP induced any significant changes in plasma levels of E2. In other studies either decreases (Afonso et al., 1997; Hutz et al., 1999) or increases (Navas et al., 2004) have been observed after β-NF treatment. Moreover, the absence of changes in fish treated with BaP also disagrees with the decrease observed in flounder treated with BaP (Monteiro et al., 2000b). The difference could be attributed to the different time of exposition used in those studies suggesting that β-NF or BaP need longer periods of exposition to alter levels of E2 compared with other PAHs like naphthalene (Tintos et al., 2007).

Cortisol, an energy mobilizing catabolic hormone is part of a generalized stress response system in fish after xenobiotics (including PAHs) exposure (Hontela, 2005). In the present study, both β-NF and BaP induce an increase in plasma cortisol levels. In literature, β-NF treatment on rainbow trout is reported to produce in plasma cortisol levels either increases (Vijayan et al., 1997; Hutz et al., 1999; Aluru and Vijayan, 2006) or BaP (Lemaire-Gony and Lemaire, 1992; Au et al., 1999; Kennedy et al., 2004).

Fig. 3. Changes in the levels of glycogen (A), glucose (B), lactate (C), and α-amino acids (D) in liver of rainbow trout after treatment with 2 μg/g of vegetable oil alone (control) or containing β-naphthoflavone or benzo(a)pyrene (10 mg kg⁻¹ body weight) that were sampled 3, 24, and 72 h after injection. Each value is the mean ± SEM of 11 fish. *, Significantly different (P < 0.05) from control fish. #, significantly different (P < 0.05) from fish treated with 10 mg β-naphthoflavone. kg⁻¹ body weight. Different letters indicate significant differences among times within each treatment.
Table 1
Changes in enzyme activities in liver of rainbow trout after treatment with 2 μg e⁻¹ of vegetable oil alone (control) or containing β-naphthoflavone or benzo(a)pyrene (10 mg kg⁻¹ body weight) that were sampled 3, 24, and 72 h after injection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Time after treatment (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>FBPase activity (U mg⁻¹ protein)</td>
<td>Control</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>β-Naphthoflavone</td>
<td>0.21 ± 0.03a</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>G6Pase activity (U mg⁻¹ protein)</td>
<td>Control</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>β-Naphthoflavone</td>
<td>0.38 ± 0.03a</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>0.35 ± 0.03a</td>
</tr>
<tr>
<td>G6PDH activity (U mg⁻¹ protein)</td>
<td>Control</td>
<td>0.94 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>β-Naphthoflavone</td>
<td>0.68 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>0.53 ± 0.04*</td>
</tr>
<tr>
<td>GDH activity (U mg⁻¹ protein)</td>
<td>Control</td>
<td>2.93 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>β-Naphthoflavone</td>
<td>3.44 ± 0.14a</td>
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<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>3.27 ± 0.14</td>
</tr>
<tr>
<td>Asp-AT activity (U mg⁻¹ protein)</td>
<td>Control</td>
<td>1.92 ± 0.14</td>
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<tr>
<td></td>
<td>β-Naphthoflavone</td>
<td>2.16 ± 0.09a</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>2.22 ± 0.10</td>
</tr>
</tbody>
</table>

Each value is the mean ± SEM of 11 fish. *, Significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from fish treated with 10 mg β-naphthoflavone kg⁻¹ body weight. Different letters indicate significant differences (P<0.05) among times within each treatment.

Aluru and Vijayan, 2004) or no changes (Wilson et al., 1998; Morrow et al., 2004). In contrast, there are no available studies in literature reporting any effect of BaP on plasma cortisol levels in fish. Changes in the levels of cortisol could potentially lead to a disturbance of cortisolegulated cellular and physiological processes in fish. Considering that cortisol is known to produce changes in fish intermediary metabolism (Mommsen et al., 1999), variations in energy metabolism could therefore be expected in β-NF and BaP-exposed fish.

Changes observed in glucose and lactate levels in plasma support an enhanced availability of those metabolites in fish exposed to β-NF or BaP. The increase in plasma glucose levels was higher in fish treated with β-NF than in those treated with BaP. The increase in plasma glucose levels was similar to that addressed after β-NF treatment in eel (Teles et al., 2005), though in other cases no changes were noticed (Vijayan et al., 1997; Teles et al., 2004; Aluru and Vijayan, 2004; Morrow et al., 2004). As for BaP treatment, there are no comparable studies in literature. The increase observed in plasma lactate is in contrast with the absence of changes observed after β-NF treatment in rainbow trout (Morrow et al., 2004) and eel (Teles et al., 2005), though increases in this parameter had been observed in rainbow trout after treatment with naphthalene (Tintos et al., 2007).

In fish liver, PAHs are known to induce a general metabolic increase as a stress response as has been observed in several species by an enhancement of the transamination capacity (Pacheco and Santos, 2001b; Gravato and Santos, 2002) as well as by an enhancement of the antioxidant and detoxification systems (Anderson et al., 1996; Pacheco and Santos, 2001a, b, 2002; Teles et al., 2003). These toxic effects, together with changes observed in hormone levels could produce changes in energy metabolism in fish liver after PAHs exposure.

An enhanced glycogenolytic potential was observed in livers of fish exposed to β-NF or BaP based on decreased glycogen levels. The mobilization of liver glycogen resulted in glycosyl units ready to be used within liver as demonstrated the simultaneous rise of liver glucose levels. There are few studies in literature to compare with these data. Thus, no changes in liver glycogen and glucose levels were observed in rainbow trout treated with β-NF (Vijayan et al., 1997a) whereas BaP treatment is known to produce decreased liver glycogen levels in eel (Lemaire-Gony and Lemaire, 1992) and sole (Au et al., 1999). Moreover, treatment with other PAHs including naphthalene is able to enhance liver glycogenolysis (Dange, 1986; Tintos et al., 2007). The capacity of liver for use of exogenous glucose was unchanged due to treatment based on the absence of changes in HK and GK activities in contrast with the increase in the activity of both enzymes occurring in rainbow trout treated with naphthalene (Tintos et al., 2007). This is suggesting that in BaP- and β-NF-treated fish, liver is more involved in a general metabolic stress response rather than in an increased use of carbohydrates as fuel to cope with detoxification processes.

Gluconeogenesis, another pathway involved in glucose production in liver, appears to be activated in fish treated with β-NF or BaP based on the increase observed in FBPase activity. Since cortisol levels are known to stimulate gluconeogenesis in fish liver (Mommsen et al., 1999), the increased gluconeogenic capacity of rainbow trout liver
exposed to β-NF or BaP could be partially attributed to the increase observed in cortisol levels in plasma of exposed fish. Furthermore, since gluconeogenic enzymes are important in the metabolic adjustments associated with stress, the increase in this pathway may favor the animal’s ability to elicit a metabolic response to stress. Accordingly, enhanced liver gluconeogenesis had been also suggested by Vijayan et al. (1997) in rainbow trout treated with β-NF. We have also observed an increased capacity for oxidation of amino acids in liver of β-NF-exposed fish based on increased GDH and Asp-AT activities. Since ketoacids obtained from oxidation of amino acids can be used through gluconeogenesis this is also supporting the increased gluconeogenic capacity reported above. A similar increase after β-NF treatment in the activity of another transaminase (Ala-AT) was observed in eel (Pacheco and Santos, 2002) and rockfish (Jee et al., 2006). Since no changes were noticed in amino acid oxidation capacity of BaP-treated fish this may suggest a lower necessity of synthesizing glucose in BaP- compared with β-NF-treated fish.

Therefore, a higher glucose production occurs in liver of β-NF- or BaP-treated fish via enhanced hepatic glycogenolytic and gluconeogenic capacities, pathways that have been demonstrated to be activated by increased levels of plasma cortisol (Vijayan et al., 2003). The enhanced availability of glucose in livers of β-NF or BaP-exposed fish appears not to be increasingly used in situ through glycolysis due to the absence of changes noticed in PK and LDH activities in exposed fish, which in the case of β-NF is in agreement with similar lack of changes in LDH activity in the same (Vijayan et al., 1997) and other fish species (Frasco and Guilhermino, 2002) after β-NF treatment. The increased availability of glycolyl units in liver appears to be used to export into plasma based on the concomitant increase in plasma glucose levels in fish treated with β-NF or BaP, and with the increase noticed in the activity of G6Pase, which is involved in the capacity for exporting glycolyl units into plasma.

The pentose phosphate shunt appears to be depressed by β-NF or BaP treatment based on the decrease observed in liver G6PDH activity. This is in agreement with the decreased G6PDH activity already observed in flounder hepatocytes exposed to β-NF (Winzer et al., 2002). Since G6PDH is a regulatory enzyme in NADPH-dependent xenobiotic biotransformation and defences against oxidative stress, reduction of G6PDH activity may result in decreased NADPH production, which is a limiting factor for biotransformation (Winzer et al., 2002).

5. Conclusions

In summary, the results obtained in the present study support an increase in plasma levels of cortisol after β-NF or BaP exposure. Moreover, several changes were observed in several pathways of liver energy metabolism including increased mobilization of glycogen stores as well as in increased glucose production through gluconeogenesis whereas β-NF also stimulated amino acid catabolism. The activation of those pathways can be attributed to cortisol action and result in increased glucose export capacity into plasma. The effects of β-NF and BaP are different in some cases (lack of changes in the use of exogenous glucose and glycolysis, and increased catabolism of amino acids) to those previously described for naphthalene demonstrating that the action of PAHs on energy metabolism is specific of the PAH assessed.

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The experiments described comply with the guidelines of the European Union Council (86/609/EU) and the Spanish Government (RD 1201/2005) for the use of animals in research.

References


