Endocrine and metabolic changes in Anguilla anguilla L. following exposure to h-naphthoflavone—a microsomal enzyme inducer

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Abstract

Anguilla anguilla L. were exposed during 24 and 48 h to 2.7 AM h-naphthoflavone (BNF), a known microsomal enzyme inducer. The BNF effects on thyroid-stimulating hormone (TSH), free triiodothyronine (T3), free thyroxine (T4) and cortisol plasma levels were investigated. Alterations on plasma glucose and lactate levels were also measured as an indication of energy-mobilizing hormones alterations. BNF showed to be able to decrease significantly A. anguilla plasma T4 levels, whereas TSH, T3 and cortisol plasma remained constant. However, plasma glucose levels were significantly increased, demonstrating that intermediary metabolism has been affected. These results demonstrate that BNF a PAH-like compound alters the normal functioning of the hypothalamo-pituitary-thyroid (HPT) axis in A. anguilla.

Keywords: h-Naphthoflavone; Plasma; Endocrine

1. Introduction

In the vast majority of living organisms, the induction of phases I and II biotransformation enzymes is a well-known response to organic xenobiotic exposure. However, the knowledge of the linkage between these activations and other biological functions, namely endocrine regulation, is still a challenge to environmental toxicology. The majority of studies carried out on this subject concern effects on sexual hormones. For instance, h-naphthoflavone (BNF), an aryl-hydrocarbon (AhR) prototype ligand and cytochrome P4501A (CYP1A) inducer, demonstrated to impair the systemic hormonal control of reproductive processes by acting both at the hepatic level (vitellogenin production) and at the pituitary-gonad axis (Navas et al., 2004). Nevertheless, the interference of this kind of xenobiotics with nonsexual endocrine responses in fish is still poorly understood.

Previous research work concerning mammals showed that typical microsomal enzyme inducers affected thyroid function. Thus, a decrease in plasma thyroxine (T4) and triiodothyronine (T3) concentrations, after exposure to phenobarbital, 3-methylcholanthrene, polychlorinated biphenyls (PCBs), and BNF was observed (Vansell and Klassen, 2002; Hood et al., 2003; Kato et al., 2003). Nevertheless, to our knowledge, there are no studies on fish concerning the effects of this type of chemicals over the hypothalamo-pituitary-thyroid (HPT) axis. The information on the effects of microsomal enzyme inducers on cortico-steroid hormones is also scarce. According to Wilson et al. (1998), BNF can affect fish pituitary-interrenal (HPI) axis since it was demonstrated that it abolishes interrenal sensitivity to adrenocorticotropic hormone (ACTH). Considering the previous statements, it seems relevant to investigate how chemicals that are not commonly regarded as endocrine disruptors can interfere at hormonal levels.

HPT and HPI axes play a central role on a wide range of important homeostatic mechanisms in fish. Thyroid hor-

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mones regulate growth, and hydromineral balance (Van Anholt et al., 2003), while cortisol is involved in the regulation of energy metabolism, anti-inflammatory response as well as immune competence (Hontela, 1997; Wenderlaar Bonga, 1997). Thyroid hormones and cortisol can both interact and influence carbohydrate metabolism (Hontela et al., 1995). Alterations in these hormone plasma concentrations, as well as on glucose and lactate levels can reflect endocrine alterations, reducing fish physiological competence and possibly survivorship. Thus, the previously mentioned parameters can also be useful tools on monitoring the impact of anthropogenic stressors in fish.

Previous studies have been performed with Anguilla anguilla concerning the effects of different xenobiotics on plasma cortisol, glucose and lactate levels (Santos et al., 1990, 1992, 1993, 1996; Pacheco and Santos, 2001; Teles et al., 2003a,b).

The choice of A. anguilla L. as test organism was based on its well-known resistance and sensitivity in the presence of adverse conditions making it a good option as an aquatic biological model for sublethal toxicological studies. A significant knowledge of eel's physiology was achieved by previous works namely those carried out in our laboratory. Moreover, the responses of A. anguilla to BNF in terms of biotransformation responses were extensively studied as an increase in microsomal EROD activity, despite the lack of knowledge concerning its endocrine and intermediary metabolic effects. Thus, the purpose of the present study was to investigate the effects of BNF on the A. anguilla L. plasma thyroid-stimulating hormone (TSH), free triiodothyronine (T3), free thyroxine (T4), cortisol, as well as glucose and lactate levels.

2. Material and methods

2.1. Chemicals

h-Naphthoflavone (BNF), h-nicotinamide adenine dinucleotide (h-NAD), l-lactic dehydrogenase, and glutamic-pyruvic transaminase were purchased from Sigma (USA). All the other chemicals were of analytical grade.

2.2. Biochemical analysis

The determination of cortisol, TSH, T3 and T4 were performed in plasma, using diagnostic ELISA direct immunoenzymatic kits (Diametra, Italy). The absorbance in each well was measured at 450 nm in a microplate reader (ASYS Hitech).

The cortisol in the sample competes with horseradish peroxidase (HRP)-cortisol for binding onto the limited number of anti-cortisol sites in the microplate wells. The enzyme substrate (H_2O_2) and the TMB-substrate (TMB) are added, and after an appropriate time has elapsed for maximum color development, the enzyme reaction is stopped and the absorbances are determined. Cortisol concentration in the sample is calculated based on a series of standards and the color intensity is inversely proportional to the cortisol concentration in the sample.

The methods for free T3 and free T4 follow the same principles of the cortisol test, requiring immobilized T3 or T4 antibodies, as well as HRP-T3 or HRP-T4 conjugates.

Concerning TSH, an antibody specific to the h-chain of TSH molecule is immobilized on microwell plates and other antibodies to the TSH molecule are conjugated with HRP. TSH from the sample is bound to the plates. The enzymatic reaction is proportional to the amount of TSH in the sample.

Plasma glucose was measured according to the method modified from Banauch et al. (1975). Plasma lactate was determined according to the method modified from Noll (1974).

2.3. Test animals

The experiment was carried out using A. anguilla (European eel) collected from the Aveiro lagoon area—Murtosa, Portugal. The eels with a 25F3 cm (yellow eel) average length and weighing 30F5 g were acclimated to laboratory conditions in aerated (dissolved oxygen: 7.6F0.3 mg/l), filtered, dechlorinated tap water with pH 7.2F0.4, under a natural photoperiod at 20 8C, for 1 week prior to experimentation. Fish were neither fed under laboratory adaptation nor during the experimental procedure. The experiment was carried out in 20 l aquaria under the previous conditions.

2.4. Experimental design

The eels were exposed to BNF 2.7 AM during 24 and 48 h. The appropriate amount of BNF was previously dissolved in 1 ml of dimethyl sulfoxide (DMSO) and added to the experimental aquaria. The same volume of DMSO was added to the control aquaria. Fish blood was collected from the posterior cardinal vein using a heparinized Pasteur pipette and its plasma isolated using an Eppendorf centrifuge, 14,000 rpm during 5 min. Experiments were carried out using test groups of five eels (n=5). The BNF concentration and exposure time adopted were based on a previous study with the same species, where a significant increase in liver EROD activity was observed (Teles et al., 2003b).

2.5. Statistical analysis

MeanFstandard error (S.E.) was calculated for each experimental group, and data were analyzed for significance of differences between control and exposed groups according to the two-tailed Student's t-test (Bailey, 1959). Experiments were carried out using test groups of five eels (n=5). Differences between means were considered significant when Pb0.05.

3. Results

3.1. Hormonal responses

A. anguilla plasma T4 was significantly decreased after 24- and 48-h exposure to BNF 2.7 AM when compared to the control. The plasma T4 decrease was time-related since a 2.5- and 5.1-fold decrease was, respectively, detected after 24 and 48 h. However, BNF did not induce any significant alteration on plasma cortisol, TSH and T3 concentrations (Fig. 1).

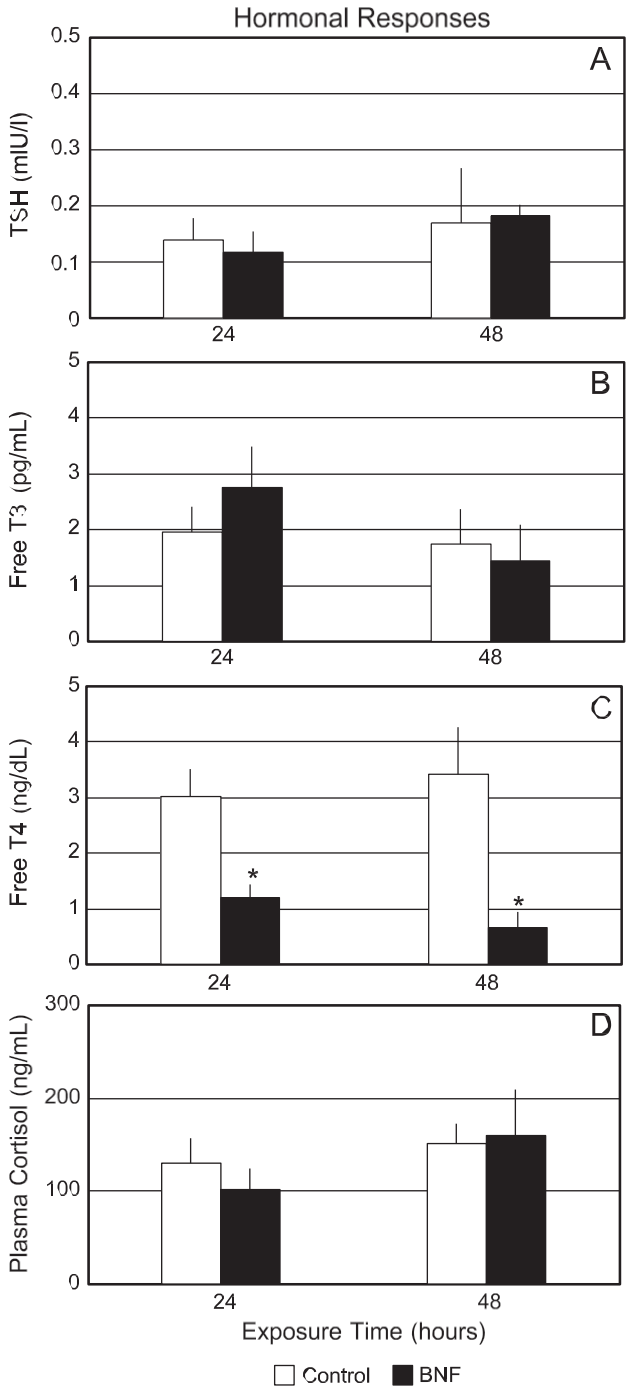

Fig. 1. Plasmatic concentrations of TSH (A), free T3 (B), T4 (C) and cortisol (D) after 24 and 48 h of exposure to BNF 2.7 AM. Values represent the means and S.E. (n=5/treatment). Differences from control: *Pb0.05.

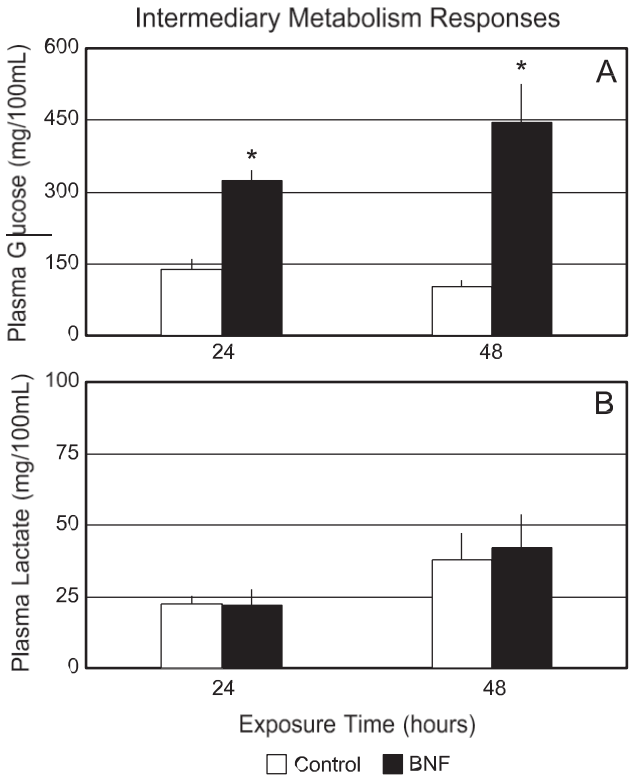

Fig. 2. Plasmatic concentrations of glucose (A) and lactate (B) after 24 and 48 h of exposure to BNF 2.7 AM. Values represent the means and S.E. (n=5/treatment). Differences from control: *Pb0.05.

3.2. Intermediary metabolism responses

A. anguilla plasma glucose concentration significantly increased after 24- and 48-h BNF exposure. A 2.4-fold increase was observed after 24 h, while after 48-h exposure the increase was 4.3-fold. On the other hand, plasma lactate was not significantly altered (Fig. 2).

4. Discussion

The effects of different microsomal enzyme inducers (Liu et al., 1995; Hood and Klaassen, 2000), including BNF (Johnson et al., 1993), on the thyroid hormone dynamics have been extensively studied in rats, unanimously revealing plasma T4 decrease. To our knowledge, no studies were performed on this issue concerning fish. Current results revealed decreased A. anguilla plasma T4 levels after exposure to BNF, confirming the data previously obtained with mammals. This similarity is not surprising considering that the morphofunctional organization of the thyroid gland is similar in fishes and in rats (Kornienko and Kozhin, 1997).

A wide range of mechanisms can be implicated in the xenobiotic-induced alterations of plasma thyroid hormones, corresponding to changes on thyroid status and/or alterations upstream or downstream the hormone production. These mechanisms include alterations on the: (i) hypothalamus and/or pituitary status (Alkindi et al., 1996), (ii) biosynthesis and secretion steps of T3 and T4 (Capen, 1997), (iii) uptake by peripheral tissues, (iv) hepatic 5V-monodeiodinase activity (Waring et al., 1996), or (v) hormone catabolism and clearance rates (Saito et al., 1991; Hontela et al., 1995). Among all the possible mechanisms involved in plasma T4 decrease following liver microsomal enzyme inducers exposure, it has been frequently proposed that UDP-glucuronyl-transferase (UDP-GT) inducers can increase T4 glucuronidation and biliary excretion (mechanism v), reducing plasma T4 concentration (McClain, 1992; Johnson et al., 1993; Liu et al., 1995). In fact, it was demonstrated that BNF induces liver UDP-GT activity in Dicentrarchus labrax (Novi et al., 1998; Gravato and Santos, 2002). Thus, besides the similarity between mammals and fish responses, a mechanism resemblance can also be proposed, applying the previous explanation to the current responses observed in A. anguilla. Plasma T3 concentrations were unaffected by the current BNF fish treatment, which is consistent with the effects of other hepatic microsomal enzyme inducers on plasma T3 levels in rats (Hood et al., 1999, 2003; Liu et al., 1995). The ability to maintain plasma T3 concentrations is stronger than the ability to maintain T4 levels (Hood et al., 2003), probably due to an activation of homeostatic mechanisms, such as increased synthesis of T3 by the thyroid or by extra-thyroidal tissues (5V monodeiodinase activity increase), recovery of T3 from T3-SO_4 by sulfatases, and increased enterohepatic circulation (Alkindi et al., 1996; Hood et al., 2003). According to Sapin and Schlienger (2003), plasma T3 is a less reliable reflection of thyroid hormone production than T4 since most of circulating T3 (around 80%) is produced extra-thyroidally from T4 deiodination.

In response to reduced plasma T4, an increase in plasma TSH would be expected; however, in the present study plasma TSH levels remained unaltered. Previous studies concerning mammals exposed to microsomal enzyme inducers revealed divergent responses, i.e., increased (Hood et al., 2003) or unaltered (Liu et al., 1995; Hood and Klaassen, 2000) plasma TSH concomitantly with decreased plasma T4. According to Hood and Klaassen (2000), these mechanisms are still poorly understood.

Previous fish studies (Anderson et al., 1996; Navas et al., 2004) suggested a disruptive action of BNF upon the hypothalamus-pituitary-gonad axis through the disappearance of the negative feedback control on pituitary luteinizing hormone (LH) release, explained through its absence of effect on estrogen receptors (ER) at the pituitary and hypothalamus level. Considering the current data, the occurrence of alterations on thyroid hormone receptors (TR) at the hypothalamus/pituitary level is not excluded; though it cannot be adopted as an explanation for the absence of any TSH response to plasma T4 depression. Additionally, an alteration on thyrotrophin releasing hormone (TRH) receptors at pituitary cells could also be suggested.

Thyroid hormones deiodination, conjugation as well as transport and their receptors interaction, besides measuring its plasma levels, should also be evaluated in order for a better understanding of the HPT axis dynamic, following xenobiotic exposure.

Fish respond to stress with characteristic acute increases in plasmatic levels of catecholamines, and slower but more sustained increases in plasmatic levels of the corticosteroid cortisol (Grutter and Pankhurst, 2000). Thus, alterations on the levels of plasma cortisol could provide valuable information on the fish stress condition. BNF is a potent cytochrome P450 1A inducer in fish tissues, including interrenal (Husøy et al., 1994). CYP1A1 isozyme of fish does not catalyze oxidative metabolism of cortisol; however, CYP1A1 inducing compounds may interfere with steroid plasma levels through different mechanisms namely the increase of phase II conjugation of steroids, as suggested by Forlin and Haux (1985) for the effects of BNF on h-estradiol levels.

Furthermore, according to Wilson et al. (1998) a BNF interference on steroid biosynthetic pathways through a substrate competition or alterations on key mitochondrial enzymes involved in cortisol synthesis would be expectable, resulting in lower cortisol production. However, the present results did not confirm this hypothesis since A. anguilla plasma cortisol levels were not altered by the BNF treatment. It is well known that the stress of capture and handling induces an acute rise in plasma cortisol levels (Hontela, 1997); therefore, when control and treated fish present similar plasmatic levels of cortisol it means that both groups were equally able to elevate cortisol under stress.

A previous study concerning D. labrax exposure to BNF 0.9 AM during 24 h revealed no plasma cortisol alteration (Teles et al., 2004). In addition, no changes were found, after BNF injection, in Oncorhynchus mykiss plasma cortisol concentration, despite the ACTH interrenal sensitivity abolishment demonstration (Wilson et al., 1998). The authors suggested that the ability of BNF-treated fish to normally increase plasma cortisol concentration could be due to other hormonal pathways, in addition to ACTH sensitivity abolishment, as well as alterations in the plasma cortisol clearance rate. Therefore, the absence of significant plasma cortisol alterations cannot ensure that the cortisol dynamics was unaffected, remaining the possibility alterations of HPI axis as well as plasma cortisol uptake by the target cells.

In the present study, plasma glucose increased after BNF exposure, despite the unaltered plasma cortisol and lactate levels. Vijayan et al. (1997) stated that besides interrenal cortisol release, other mechanisms might be controlling glucose availability in fish. Moreover, under

acute stress catecholamines could be rapidly released resulting in increased glycogenolysis. However, this explanation cannot be applied to the current glucose response, since the persistence of catecholamine effects up to 48 h is not likely. Van der Boon et al. (1991) stated that the influence of plasma cortisol on fish carbohydrate metabolism is not very comprehensive and thus the establishment of a consistent relation between plasma cortisol, glucose and lactate seems difficult. However, the authors suggest that the current plasma glucose increase observed following BNF exposure may be related to an increased liver gluconeogenesis induced by the previously uptaken plasma cortisol.

Plasma T4 frequently follows a response pattern similar to that one of plasma cortisol, and T4 may also activate the interrenal function (Hontela et al., 1995). On the other hand, cortisol can induce T4 deiodination originating T3, as well as increase the clearance rate of T3 (Redding et al., 1991) and T4 (Leatherland, 1987).

Finally, the present results confirm the studied parameters as important biomonitoring tools to assess the presence of stressors in aquatic environment contributing to a broader knowledge of fish responses as integration at different physiological levels. Moreover, these findings highlight the need of a careful interpretation of field data, since the occurrence of sequential or simultaneous exposures to different classes of chemicals is likely.

5. Conclusions

- A. anguilla displayed a significant decrease in plasma T4 levels after BNF exposure, revealing a similarity with the known mammal responses. These results demonstrate that typical microsomal enzyme inducers, namely BNF, can also interfere with neuroendocrine processes.
- BNF affected the intermediary metabolism since plasma glucose levels were significantly increased; however, it seems difficult to establish a correlation between cortisol, thyroid hormones and carbohydrate metabolism.
- In the future, it would be of interest to assess thyroid hormones conjugation and deconjugation processes.
- The above results confirm the studied parameters as important biomonitoring tools to assess the presence of stressors in aquatic environment, providing significant information related to fish intermediary metabolism and endocrine responses to complex environmental mixtures.

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