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β-naphthoflavone induction of CYP1A in brain of juvenile lake trout (*Salvelinus namaycush* Walbaum)

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Summary

Many environmental pollutants induce expression of the cytochrome P450 (CYP) 1A subfamily of genes. We integrated cellular and molecular biological techniques to examine the effects of β-naphthoflavone (BNF) exposure in lake trout brain CYP1A distribution and dynamics. Over a 32-day time-course, real time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) results showed that CYP1A mRNA induction in response to BNF exposure occurred rapidly and continued to rise in the BNF-treated lake trout after 4 h, with a peak at or after 2 days. Messenger RNA levels fell after 4 days, and this trend continued after 16 days of exposure. *In situ* hybridization indicated that CYP1A mRNA was universally elevated in the brain of BNF-exposed fish and was mainly expressed in the endothelia and occasionally in the glial cells. CYP1A immunoreactivity was induced in the olfactory bulb and valvula cerebelli of BNF-treated fish. Other brain areas showed constitutive CYP1A immunoreactivity in both control and BNF-treated fish. Some BNF-treated fish contained multifocal hemorrhages in the brain tissue, and these fish had overall depressed CYP1A immunoreactivity in the brain. The relationship between transcriptional and translational effects of BNF exposure in the brain of juvenile lake trout is discussed.

Key words: lake trout, *Salvelinus namaycush*, CYP1A induction, brain, cytochrome P450, β-naphthoflavone.

Introduction

Cytochrome P450 (CYP) is a multi-gene family encoding constitutive and inducible heme-containing enzymes (Nelson et al., 1996). They function in the metabolism of a wide spectrum of xenobiotics and of numerous endogenous substances with physiological functions in inter- and intracellular signaling, such as steroid hormones, prostaglandins and fatty acids (Parkinson, 1995; Stegeman and Hahn, 1994). These enzymes are primarily found in the liver and adrenal glands; however, they are also distributed throughout the body in diverse areas such as the brain, heart, intestine, kidney, lung and skin (Norris et al., 1996; Sarasquete and Segner, 2000). CYP enzymes in the mammalian brain have been shown to be highly localized in discrete areas and may thus alter the local action or concentration of neuroactive drugs (Majewska et al., 1986). In addition, they may have homeostatic functions since CYP isoforms have been shown to participate in cerebral blood vessel tone and also in the synthesis of neuroactive steroids (Harder et al., 1997; Walther et al., 1987; Warner et al., 1994).

The most intensively studied P450 genes in fish are CYP1As (Nelson et al., 1996), which are inducible by a wide variety of persistent contaminants found in the Great Lakes, particularly polychlorinated biphenyls (PCBs) and dioxins. CYP1A nucleotide sequences have been determined in rainbow trout (Berndtson and Chen, 1994; Heilmann et al., 1988), plaice (Leaver et al., 1993), Atlantic tomcod (Roy et al., 1995), toadfish and scup (Morrison et al., 1995), killifish (Morrison et al., 1998), red sea bream (Mizukami et al., 1994), sea bass (Stien et al., 1998) and, more recently, Atlantic salmon (Rees et al., 2003). Current evidence shows that several CYP1A isoforms exist in fish (Teramitsu et al., 2000) and they are distributed in tissues such as the liver, gut, kidney, gill and heart (Goksøyr and Husoy, 1998; Sarasquete and Segner, 2000; Stegeman and Hahn, 1994). Few immunocytochemistry studies show that constitutive CYP1A proteins are present in the neurons and the endothelia of the fish brain (Sarasquete and Segner, 2000). Inducible CYP1A proteins are found mainly in the pituitary cells and brain endothelia in fish (Sarasquete and Segner, 2000). The CYP1A-mediated *in situ* metabolism and cellular toxicity of xenobiotics in the brain may have far-reaching consequences by causing disruption of neuronal and neuroendocrine function (Andersson et al., 1993; Huang et al., 2000; Morse et al., 1998).

Many chemically different compounds induce *de novo* synthesis of CYP1A protein (Nebert and Gonzalez, 1987; Nebert et al., 1989; Parkinson, 1995; Stegeman and Hahn, 1994). The inductive response in this subfamily is known to occur *via* the high-affinity binding of aromatic hydrocarbons

to an intracellular receptor complex (the *Ah* receptor), involving the 90-kDa heat shock protein (Hsp90) and a nuclear translocation factor. Translocation of the inducer–receptor complex to the nucleus results in the transcriptional activation of the genes in the *Ah* battery (Hoffman et al., 1991; Nebert and Jones, 1989; Nebert et al., 1989); therefore, levels of CYP1A mRNA and newly synthesized CYP1A proteins are increased and the CYP1A proteins subsequently undergo processing, heme insertion and folding to yield the catalytically active enzymes. Each of these steps, i.e. mRNA, protein and catalytic activity, can be analyzed with a suitable probe to detect induction (Goksøyr and Förlin, 1992). However, few studies have actually used all the above assays to examine the transcription and translation of CYP1A in fish simultaneously.

In the present study, we assessed the effects of sublethal β-naphthoflavone (BNF) exposure on both the distribution and dynamics of CYP1A mRNA and protein in lake trout brain, using quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), *in situ* hybridization and immunocytochemistry. BNF was chosen as the contaminant since it is a well-known *Ah* receptor agonist and CYP1A inducer (Smeets et al., 1999).

Materials and methods

Animals

Juvenile lake trout (*Salvelinus namaycush* Walbaum) (body length, 11.5±4 cm; body mass, 12.5±3 g) were acquired from Marquette Fish Hatchery (Marquette, MI, USA) and maintained at the Michigan State University Lower River Laboratory (East Lansing, MI, USA). Trout were acclimated for two weeks at 12°C in an 800-liter flow-through tank (well water; 600 l h^{-1}). During the acclimation period, lake trout were fed a diet of Purina AquaMax© Grower 400 (Purina Mills, Inc.; St Louis, MO, USA) twice daily to satiation. A photoperiod of 10 h light and 14 h dark was kept during the duration of the experiment.

Experiment 1. Time-course Q-RT-PCR for BNF-induced CYP1A mRNA

Chemical exposure

Trout were not fed for 2 days prior to injections. Individuals were randomly sampled and anesthetized by immersion in buffered 100 ng l^{-1} tricaine methanesulfonate (MS-222; pH 7.0; Sigma Chemical Co., St Louis, MO, USA). Anesthetized fish were given an intraperitoneal injection of either β-naphthoflavone (BNF; Sigma; 50 mg kg^{-1} body mass) dissolved in corn oil (10 mg ml^{-1}) or corn oil alone (corn oil was autoclaved and sonicated prior to administration). Lake trout were divided into 40 liter experimental aquaria where flow rate (0.5 l min^{-1}) and temperature (12°C) were kept constant. Each aquarium received a group (N=8) of either BNF-induced individuals or control individuals. One control and one experimental group of lake trout were sampled prior to injection (time zero) and after each exposure period of 2 h, 4 h, 8 h, 24 h, 2 days, 4 days, 8 days, 16 days and 32 days.

Tissue collection and storage

After the appropriate exposure, lake trout were given an overdose of MS-222. Whole brain tissue was excised with a pair of small forceps, submerged in 0.5 ml RNALater© (Ambion, Austin, TX, USA) and immediately placed on ice. Samples were then frozen at –80°C for long-term storage.

RNA isolation, quantification and storage

For isolation of total RNA, brain tissue was removed from RNALater©, placed in 1 ml Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and homogenized. RNA pellets were reconstituted in varying amounts of diethylpyrocarbonate-treated water (DEPC-H_2O) dependent upon pellet size. Genomic DNA was digested by incubation at 37°C with 1 μl RNase-free DNase I (Roche Molecular Biochemicals, Mannheim, Germany) and 0.3 μl rRNasin (Promega Corp., Madison, WI, USA) per 100 μl total RNA. DNase was inactivated by heating samples to 70°C for 10 min. Total RNA was quantified (Sambrook et al., 1989) using a GeneQuant *pro* RNA/DNA calculator (Amersham Biosciences, Piscataway, NJ, USA). To verify that RNA concentrations and dilutions were accurate, A260/A280 ratios were produced in triplicate, and a quality assurance protocol was followed during spectrophotometer usage to reduce the chances of pipetting error. Following RNA quantification, 3 μl of each RNA sample was electrophoresed on a 1% ethidium bromide stained agarose gel to check the integrity and density of 18S and 28S ribosomal RNA bands (data not shown), increasing our confidence that each reverse transcription reaction would receive the same amount of total RNA. For long-term storage, RNA samples were supplemented with three volumes of 95% ethanol, a 10% volume of 3 mol l^{-1} sodium acetate and placed at –80°C (Sambrook et al., 1989).

RT-PCR

Reverse transcription (all reagents were from Invitrogen Life Technologies) was performed on all samples in a final volume of 20 μl containing a 1× concentration of First Strand Buffer, 0.01 mol l^{-1} dithiothreitol, 1 mmol l^{-1} of each deoxynucleotide triphosphate, 2.5 μmol l^{-1} oligo(dT), 5 units of MMLV reverse transcriptase, 1 unit of rRNasin (Promega Corp.) and 100 ng of total RNA. The reaction mixture was incubated at 37°C for 50 min and inactivated at 70°C for 15 min. Then, 1 μl of the cDNA sample was spiked into a PCR master mix. Each PCR reaction consisted of 12.5 μl of 2× TaqMan® Universal PCR master mix (Applied Biosystems, Branchburg, NJ, USA), 300 nmol l^{-1} of each primer (forward WML158 5′ CCA ACT TAC CTC TGC TGG AAG C 3′ and reverse WML159 5′ GGT GAA CGG CAG GAA GGA 3′), 100 nmol l^{-1} of the TaqMan® probe (WML160 5′ TTC ATC CTG GAG ATC TTC CGG CAC TC 3′) that contained a 3′ TAMRA quencher and a 6-FAM fluorescent label at the 5′ end, 1 μl of cDNA template, and deionized water to a final volume of 25 μl. Reactions were then analyzed on an ABI 7700 real-time PCR thermalcycler (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, and

40 cycles of 95°C for 15 s followed by 60°C for 1 min. Amplification plots were generated, and CYP1A mRNA levels were estimated against a standard curve.

Recombinant RNA standard and generation of standard curves

A CYP1A recombinant RNA standard was used to generate standard curves in each set of reactions. The entire 160 samples were analyzed in two plates. Both standard curves exhibited a correlation coefficient (r^2) of at least 0.995. Due to the high degree of sequence identity among salmonid CYP1A genes (>95% by comparing CYP1A GenBank sequences from brook trout, lake trout, Atlantic salmon and rainbow trout), the cRNA standard was synthesized as follows. A 491 bp conserved region of the CYP1A gene was amplified from an Atlantic salmon CYP1A clone (GenBank Accession Number AF361643) using the following primers and conditions: forward primer WML 169 5′ TAA TAC GAC TCA CTA TAG GCT GTC TTG GGC TGT TGT GTA CCT TGT G 3′, reverse primer WML 170 5′ TTT TTT TTT TTT TTT TTT GGA GCA GGA TGG CCA AGA AGA GGT AG 3′, conditions of 1 cycle at 94°C for 4 min, 40 cycles at 94°C for 5 s and 72°C for 2 min, and 1 cycle at 72°C for 5 min as added extension. The generated PCR product contained a 5′ T7 promoter, 454 bp of CYP1A sequence, including the region of the real-time amplicon, and a poly dT tail at the 3′ end. This product was then diluted 1/100 with deionized water, re-amplified and up-scaled with the same reaction conditions. The concentrated PCR product was cleaned using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA) and transcribed using the Riboprobe *In Vitro* Transcription System (Promega Corp.) according to standard protocol. The cRNA was then treated with RNase-free DNase to remove excess DNA template and was subsequently extracted with water-saturated (pH 4.9) phenol:chloroform (24:1). The aqueous phase was isolated and extracted with chloroform:isoamyl alcohol (24:1) followed by an overnight ethanol precipitation at –20°C. To remove free nucleotides, the precipitated sample was spun for 10 min at 12 000 ***g***, resuspended in 20 μl DEPC-H_2O and filtered through a NucAway™ Spin Column (Ambion). The size and quality of the cRNA standard was verified by analysis on an agarose gel and quantified at 260 nm using a spectrophotometer. This RNA standard was then used to generate standard curves useful for the real-time Q-RT-PCR analysis of CYP1A (C. B. Rees, J. E. Hinck, D. E. Tillitt and W. Li, manuscript submitted).

In order to quantify CYP1A levels, each plate of samples was normalized against a set of standard curve reactions. To generate standard curves, RT-PCR was carried out on a dilution series (10^{10}–10^3 molecules) of the CYP1A cRNA molecule. Amplification plots were analyzed on the ABI 7700, and Ct values for each of the reactions in the dilution series were calculated. Ct values were plotted against starting quantity of RNA template to generate the standard curve. Additional control reactions were also run on each plate including a no template (water) negative control, a no amplification (RNA) control, a negative reverse-transcription (water added) control and a CYP1A positive control by adding 1 μl of a plasmid containing a full-length CYP1A cDNA sequenced from lake trout (GenBank Accession Number AF539415) to the PCR reaction mixture.

Statistical analysis

All data were log-transformed to fulfill normality requirements and analyzed using a two-way analysis of variance (Statistical Analysis Systems, Cary, NC, USA). Simple effects were determined for each factor using the SLICE procedure (Statistical Analysis Systems v. 8). All pairwise comparisons were tested for significance using a Tukey–Kramer adjustment (Statistical Analysis Systems v. 8).

Experiment 2. In situ hybridization for BNF-induced CYP1A mRNA in the brain

After a 4-day BNF induction (as described in experiment 1), 12 (6 control and 6 induced) juvenile lake trout were anesthetized with 0.05% MS-222 and perfused with 20 ml saline and decapitated to excise their brains. The tissues were fixed in 4% paraformaldehyde [in 0.1 mol l^{-1} phosphate buffer saline (PBS)] for 3 h. Following cryoprotection in 0.1 mol l^{-1} PBS (with 25% sucrose and 4% paraformaldehyde) overnight at room temperature, the tissues were embedded in Tissue Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) and stored in a –80°C freezer. The brain was sectioned into 20 μm slices using a Leica CM1850 cryostat, adhered to Superfrost Plus microslides (Fisher, Orangeburg, NY, USA) and stored at –80°C.

Synthesis of digoxingenin-labeled cRNA probes

The digoxingenin-labeled antisense RNA probe (500 bp) was generated from lake trout CYP1A full-length cDNA clone using the Riboprobe *In vitro* Transcription Systems (Promega). In brief, 2 μg of linearized vectors were transcribed in the presence of 700 nmol digoxigenin-11-UTP. The cRNA was collected by ethanol precipitation and resuspended in DEPC-H_2O. The sense RNA was prepared with a similar procedure and used as the negative control.

Hybridization

Tissue sections were brought to room temperature, treated with proteinase K (20 μg ml^{-1} in PBS) for 5 min and post fixed for 15 min in 4% paraformaldehyde (in 0.1 mol l^{-1} PBS). Sections were rinsed three times for 10 min each in PBS before a 2 h incubation in prehybridization solution, containing 50% deionized formamide, 1× Denhart's solution, 750 mmol l^{-1} sodium chloride, 25 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 25 mmol l^{-1} piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (PIPES), 0.25 mg ml^{-1} calf thymus DNA, 0.25 mg ml^{-1} poly A acid and 0.2% sodium dodecyl sulfate (SDS). Sections were then hybridized with antisense or sense RNA probes in hybridization solution (prehybridization solution with 5% dextran sulfate) at 60°C for 16–20 h. After hybridization, sections were washed three times for 10 min each in 2× SSC

[containing 0.3% polyoxyethylenesorbitan monolaurate (Tween-20)] followed by three washes in 0.2× SSC (containing 0.3% Tween-20) at 65°C.

Immunovisualisation of digoxigenin

For detection of digoxigenin-labeled probes, the sections were blocked for 1 h in 4% dry milk, 2% bovine albumin and 0.3% triton. The sections were incubated for 3 h with alkaline phosphatase-conjugated sheep-anti-digoxigenin Fab fragments (1:1000 in blocking solution; Boehringer Mannheim, Indianapolis, IN, USA) followed by nitroblue tetrazolium chloride and 5-bromo-4-chloro-3 indolyl phosphate substrate (NBT/BCIP; Boehringer Mannheim) for 20–30 min, and counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA, USA).

Experiment 3. Immunocytochemistry for BNF-induced CYP1A protein in the brain

Some sections from experiment 2 were selected for immunocytochemistry study. Sections were washed in Tris buffer saline (TBS: 50 mmol l^{-1} Tris buffer, 150 mmol l^{-1} NaCl, pH 7.2) three times (5 min each) in between each step. All the procedures followed the manufacturer's instruction. Unless otherwise specified, all the reactions were performed at room temperature. Sections were reacted with 0.01% H_2O_2 (DAB substrate kit; Vector) for 10 min to eliminate the endogenous peroxidase activity, followed by avidin- and then biotin-blocking solutions for 10 min each (Avidin–Biotin Blocking Kit; Vector) to eliminate endogenous biotin. Sections were incubated at 4°C overnight in the primary antibody solution (1:200 rabbit-anti-rainbow trout CYP1A, CP-226; Cayman Chemical, Ann Arbor, MI, USA) in TBS with 0.05% Triton X-100 and normal goat serum (Vectastain ABC kit for rabbit IgG; Vector). Sections were reacted with the biotinylated secondary antibody (goat-anti-rabbit, Vectastain ABC kit; Vector) for 2 h, incubated in ABC solution (Vectastain ABC kit; Vector) for 2 h, reacted with 3,3′-diaminobenzidine and $NiCl_2$ (DAB substrate kit; Vector) for 15 min, counterstained with hematoxylin (Sigma) for 5 min, dehydrated through an ethanol series (70%, 95%, 100%; 2 min each), clarified twice by xylene (5 min and 10 min) and covered with glass using DPX mounting media (Sigma).

To examine the specificity of the antibody used for immunocytochemistry, western blot was performed. The brain and liver tissues of non-treated and BNF-treated lake trout were homogenized separately in 200 µl ice-cold 10 mmol l^{-1} Tris buffer (pH 7.4 containing 25 µg ml^{-1} leupeptin, 5 µg ml^{-1} aprotonin, 40 µg ml^{-1} phenylmethylsulfonyl fluoride, 50 µg ml^{-1} benzamidine and 0.5 µg ml^{-1} pepstatin) at 0°C. Protein concentration was determined using a DCA protein analysis kit (Pierce, Rockford, IL, USA). 25 µg of protein were then applied to 10% acrylamide/*N*,*N*′-methylene-bis-acrylamide (29:1 mix; Bio-Rad Laboratories, Hercules, CA, USA) SDS-PAGE at 150 V for 45 min (Laemmli, 1970). Gels were transferred to polyvinylidene difluoride (PVDF) membranes (Immobion-P; Millipore, Billerica, MA, USA) by electroblotting. The PVDF membranes were then blocked with 5% (w/v) nonfat dry milk in TBST (20 mmol l^{-1} Tris-HCl, 150 mmol l^{-1} NaCl, 0.04% Tween 20) overnight. The PVDF membranes were incubated in the primary antibodies (1:200, Cayman) in 5% nonfat dry milk for 1 h. After washing three times with TBST, the PVDF membranes were then reacted with goat-anti-rabbit antibody conjugated with horseradish peroxidase (Pierce) at a 1:10 000 dilution for 1 h. Protein signal was detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent kit (Pierce).

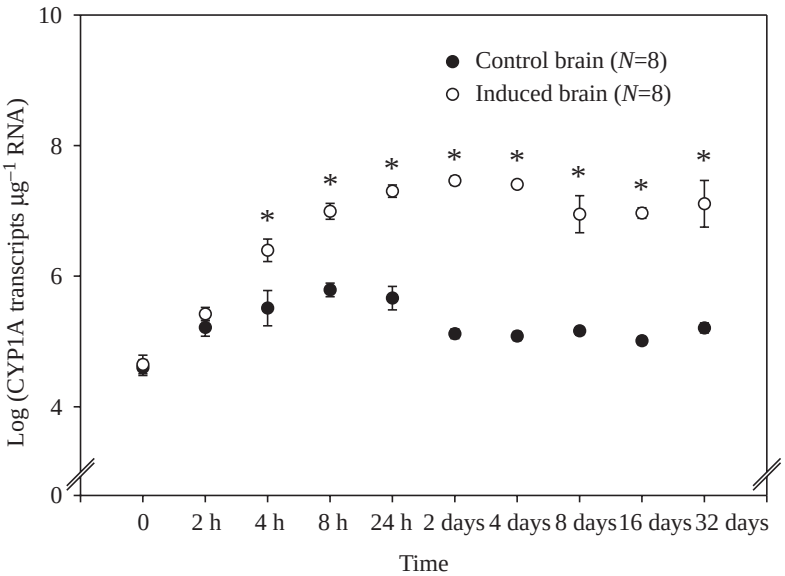

Fig. 1. Time course for β-naphthoflavone (BNF)-induced CYP1A mRNA measured by Q-RT-PCR. Juvenile lake trout were randomly assigned to treatment groups (*N*=8 for each treatment group) and given an intraperitoneal injection of BNF in corn oil (50 mg kg^{-1} body mass) or corn oil alone. Whole brain tissue was taken at 10 different time periods between time zero and 32 days. Total RNA was extracted and analyzed for CYP1A mRNA levels using Q-RT-PCR. Data were analyzed using a two-way analysis of variance followed by a Tukey–Kramer adjustment for all pairwise comparisons. At most time periods, significantly higher levels of CYP1A expression were found in each induced group over its respective control group (designated by *; Tukey–Kramer, $P<0.0001$).

Results

Experiment 1. Time course of CYP1A mRNA induction in the brain

Fig. 1 shows the induction time course of brain CYP1A mRNA in the control and BNF-treated lake trout. Over a 32-day time-course, data revealed that CYP1A mRNA induction in brain by BNF exposure occurred rapidly. Both control and induced lake trout groups at time zero showed nearly identical mean CYP1A levels at 3.98×10^4

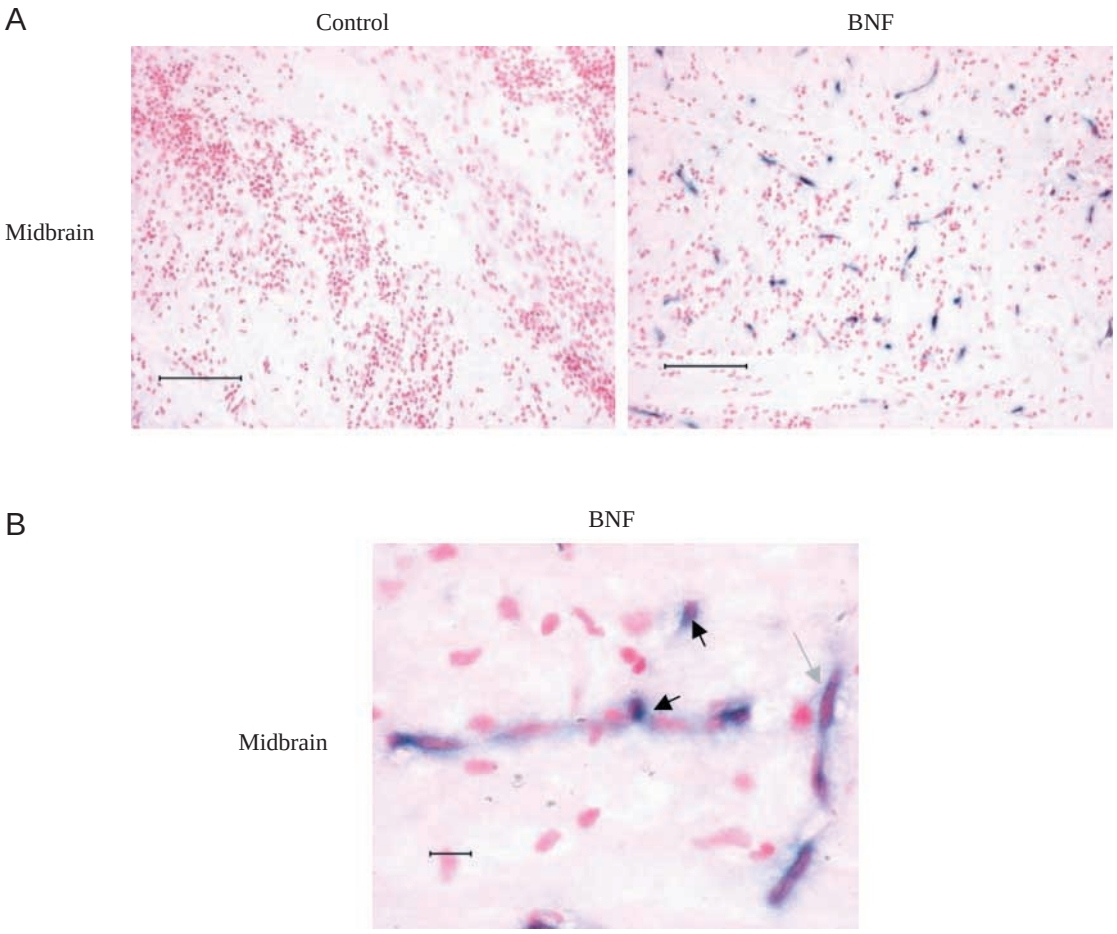

Fig. 2. (A) *In situ* hybridization expression patterns of CYP1A mRNA in juvenile lake trout midbrain of control fish and fish exposed to β-naphthoflavone (BNF) for 4 days. CYP1A mRNA-positive cells are denoted by blue stain. Control fish were injected with corn oil vehicle daily for 4 days before sacrifice. Scale bars, 100 μm. (B) Higher magnification of BNF-exposed fish midbrain showing blue-stained glial cells (black arrows) and endothelia (gray arrow). Scale bar, 10 μm. Horizontal sections were counterstained with nuclear Fast Red.

and 4.44×10^4 CYP1A transcripts μg^{-1} total RNA, respectively. After two hours of exposure, both control and BNF-induced groups showed significantly higher CYP1A levels than those at time zero (Tukey–Kramer, $P<0.05$). However, no difference was found between the control and BNF-induced group at 2 h. CYP1A levels continued to rise in the BNF-treated lake trout after 4 h, 8 h and 24 h, with a peak in CYP1A mRNA expression after 2 days at 2.90×10^7 transcripts CYP1A μg^{-1} total RNA. At all time points after 4 h, significantly higher levels of CYP1A expression were found in each induced group over their respective control groups (Tukey–Kramer, $P<0.0001$). CYP1A mRNA expression began a trend of reduction after 4 days in BNF-induced individuals. This trend continued after 16 days of exposure, where five times fewer CYP1A transcripts were found compared with maximal levels. Control levels during this time were statistically the same in all time groups. After 32 days, a large degree of individual variation in CYP1A expression was found. Messenger RNA levels in some trout returned to basal levels at that time period while others remained elevated. To make sure this was not due to errors in RNA measurement or RNA degradation, total RNA samples were separated on agarose gels and analyzed by densitometry comparisons and ribosomal band integrity. All RNA samples were found to be similar in RNA integrity and band density (data not shown). The standard curves exhibited a correlation coefficient (r^2) of at least 0.995.

Experiment 2. Distribution of BNF induced CYP1A mRNA in brain

Fig. 2 shows histological sections of lake trout midbrain in control fish and in fish subjected to a 4-day BNF exposure. As shown in Fig. 2A, no CYP1A mRNA was detectable in control lake trout midbrain. After a 4-day exposure to BNF, CYP1A mRNA was highly induced and universally expressed in the midbrain, mainly in the endothelia and rarely in the glia. Fig. 2B illustrates a higher magnification of mRNA-positive glia that were in direct contact or close vicinity to the blood vessels. No CYP1A mRNA-positive cells showed neuronal morphology in the samples examined. In other brain regions, no detectable CYP1A mRNA was found in the control fish whereas BNF-induced CYP1A

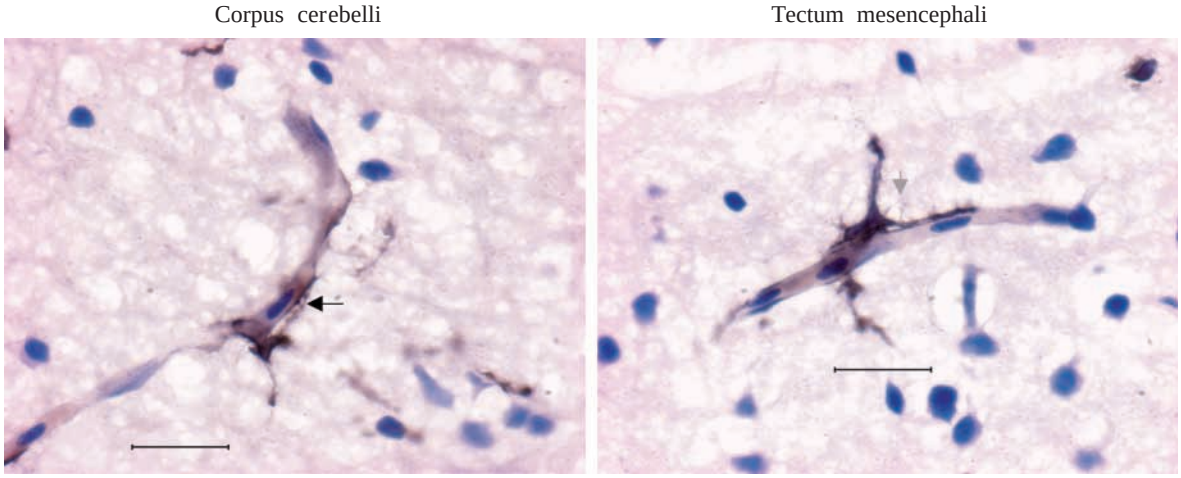

Fig. 3. CYP1A-immunoreactive (black) glial cells in the corpus cerebelli and the tectum mesencephali of the control juvenile lake trout. Note that the glial cell courses along the blood vessels, and the end feet (black arrow) and dendritic spines (grey arrow) are visible. Horizontal section (20 μm thick) is counterstained with hematoxylin nuclear stain (purple). Scale bars, 20 μm.

mRNA expression was evenly distributed throughout the whole brain (data not shown).

Experiment 3. BNF induced CYP1A immunoreactivity in specific brain regions

Fig. 3 illustrates constitutive expression of CYP1A proteins in glial cells. CYP1A immunoreactive glial cells course along and directly contact the blood vessels in the control juvenile lake trout.

Figs 4, 5 demonstrate that BNF induces CYP1A immunoreactivity in selected regions of lake trout brain. As shown in Fig. 4, CYP1A immunoreactivity was clearly induced in the olfactory bulb and the valvula of the cerebellum, a folded structure tucked in the ventricle within the optic tectum. BNF appeared to increase CYP1A immunoreactivity in endothelia, glia, neurons and the nerve fibers in the olfactory bulb (Fig. 4). In some fish, BNF also increased CYP1A immunoreactivity in endothelia, glia and neurons in the valvula of the cerebellum (Fig. 4). However, increased CYP1A immunoreactivity in this brain region showed individual variation. On the contrary, control and BNF-treated juvenile lake trout showed similar constitutive CYP1A immunoreactivity in endothelia, glia and neurons in the tectum mesencephali (part of the midbrain), corpus cerebelli and torus longitudinalis, an accessory cerebelloid structure that lies at the medial edge of the optic tectum in the midbrain (Fig. 5).

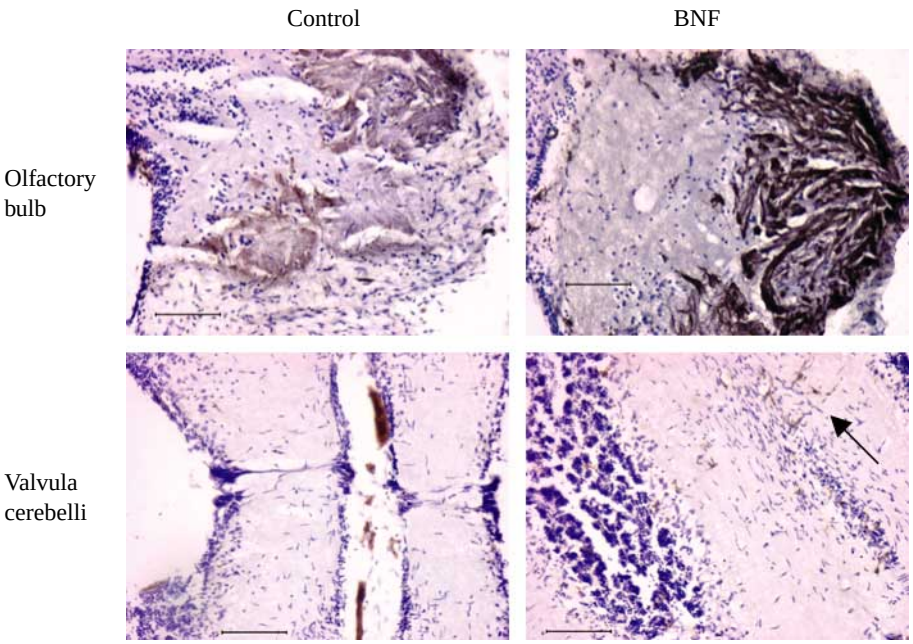

Fig. 4. CYP1A immunoreactivity (brown/black) in the olfactory bulbs and valvula cerebelli of the control and β-naphthoflavone (BNF)-treated juvenile lake trout. The black arrow points to an area with CYP1A-immunoreactive cells in the valvula cerebelli of BNF-treated fish. Horizontal sections (20 μm thick) are counterstained with hematoxylin nuclear stain (purple). Scale bars, 100 μm.

Fig. 6 demonstrates that hemorrhage depresses CYP1A immunoreactivity in the brain. As shown in Fig. 6A, BNF-treated fish with hemorrhages contained depressed CYP1A immunoreactivity in the brain compared with the control and BNF-treated fish without hemorrhages. Fig. 6B indicates that CYP1A immunoreactivity-depressed brain regions contained sporadic hemorrhage sites.

Western blot analysis showed that

CYP1A antisera specifically identified a protein at 65 kDa and revealed a robust increase of this protein in the liver tissues of BNF-exposed fish (data not shown).

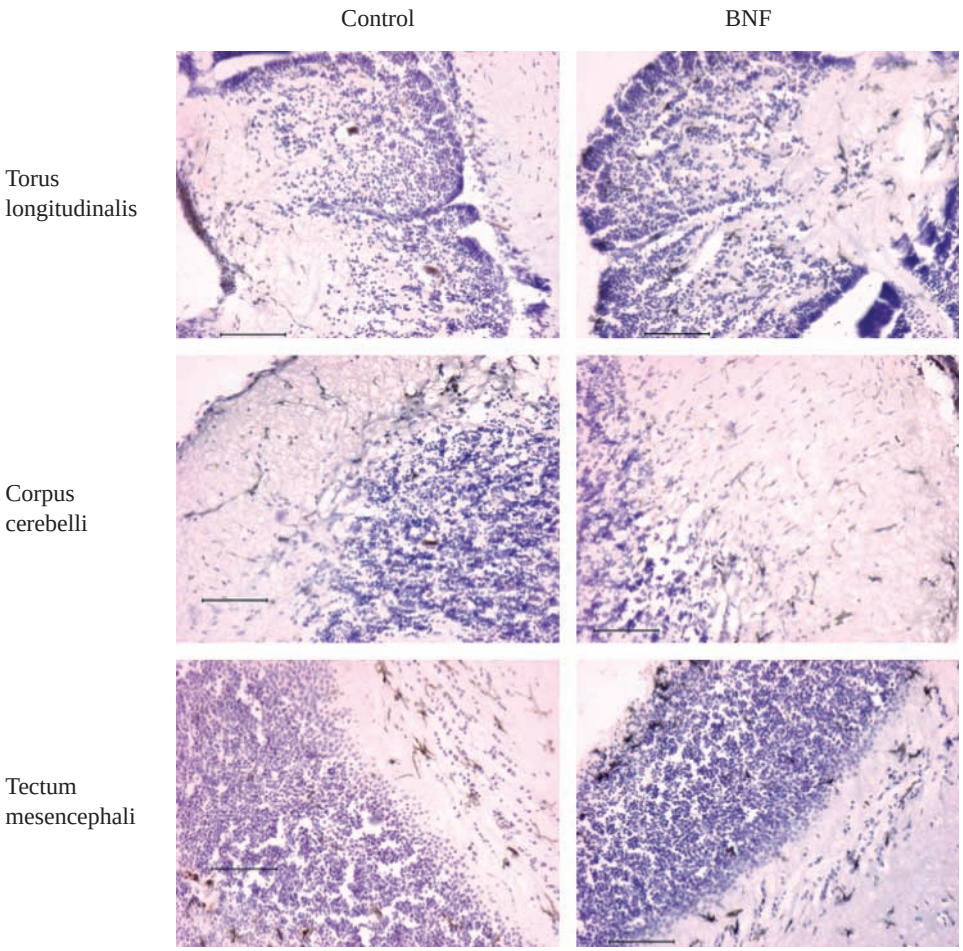

Fig. 5. CYP1A immunoreactivity (brown/black) in the torus longitudinalis, corpus cerebelli and tectum mesencephali in the control and β-naphthoflavone (BNF)-treated juvenile lake trout. Horizontal sections (20 μm thick) are counterstained with hematoxylin nuclear stain (purple). Scale bar, 100 μm.

Discussion

The results of Q-RT-PCR showed that the induction of CYP1A mRNA in lake trout brain was substantial (more than 100-fold), rapid (increased within 4 h) and sustained for days. BNF induction of CYP1A expression lasted for an extended period of time (32 days) in lake trout brain. The cause of this prolonged induction is likely to be due to a higher concentration of BNF accumulated in the blood during the later exposure time periods. The prolonged induction of CYP1A expression has also been shown in lake trout gill and liver and is discussed in detail by Rees et al. (submitted). Apparent individual difference in returning to steady-state CYP1A mRNA levels was observed among fish after prolonged BNF treatment. The difference might be explained by individual differences in their hormonal status (i.e. sex of the fish) and their genetic makeup. There was a significant initial rise in brain CYP1A mRNA levels of the control group after 2 h, 4 h and 8 h of the start of the experiment. CYP1A expression in the control group returned to basal levels after 2 days of exposure. This small induction may be due to the effects of handling stress. Stress-induced increases of CYP1A-mediated activities (i.e. EROD activity) when coupled with tetrachlorobiphenyl (TCB) injection have been reported previously (Blom and Förlin, 1997). Likewise, in rainbow trout, BNF exposure when coupled with cortisol treatment resulted in synergistic CYP induction (Devaux et al., 1992).

CYP1A protein was constantly produced at a low level in the brain and was only induced in specific brain regions such as the olfactory bulb and the valvula of the cerebellum. It is advantageous to have highly inducible CYP1A proteins in the olfactory bulb and the valvula of the cerebellum since both regions are readily accessed by xenobiotic compounds, as described below.

The olfactory bulb serves the first protection line in the brain for xenobiotics. The peripheral olfactory system is readily exposed to a wide variety of xenobiotic compounds since the olfactory receptor neurons (*via* their apical dendrites) are in direct and continuous contact with the external environment. In addition, olfactory receptor neurons innervating the olfactory bulbs provide direct access to the central nervous system for certain toxicants (Gottofrey and Tjalve, 1991; Hastings and Evans, 1991). Therefore, CYP protein in the olfactory bulbs should be constitutively expressed and also highly inducible in response to any acute xenobiotic exposure. Indeed, the presence of constitutive CYP1A1 in fish olfactory systems has been confirmed (Andersson and Goksøyr, 1994; Monod et al., 1994, 1995; Ortiz-Delgado et al., 2002), and CYP1A1 immunoreactivities and enzyme activities are induced in the olfactory organs in fish exposed to xenobiotics (Goksøyr and Förlin, 1992; Smolowitz et al., 1992).

The valvula (small folding doors) of the cerebellum in the lake trout is a folded structure tucked in the ventricle within the optic tectum (Butler and Hodos, 1996). The function of the valvula in the lake trout is not known, but in weak electric fish such as mormyrids it plays a major role in electroreception (Butler and Hodos, 1996). Since the whole valvula is immersed

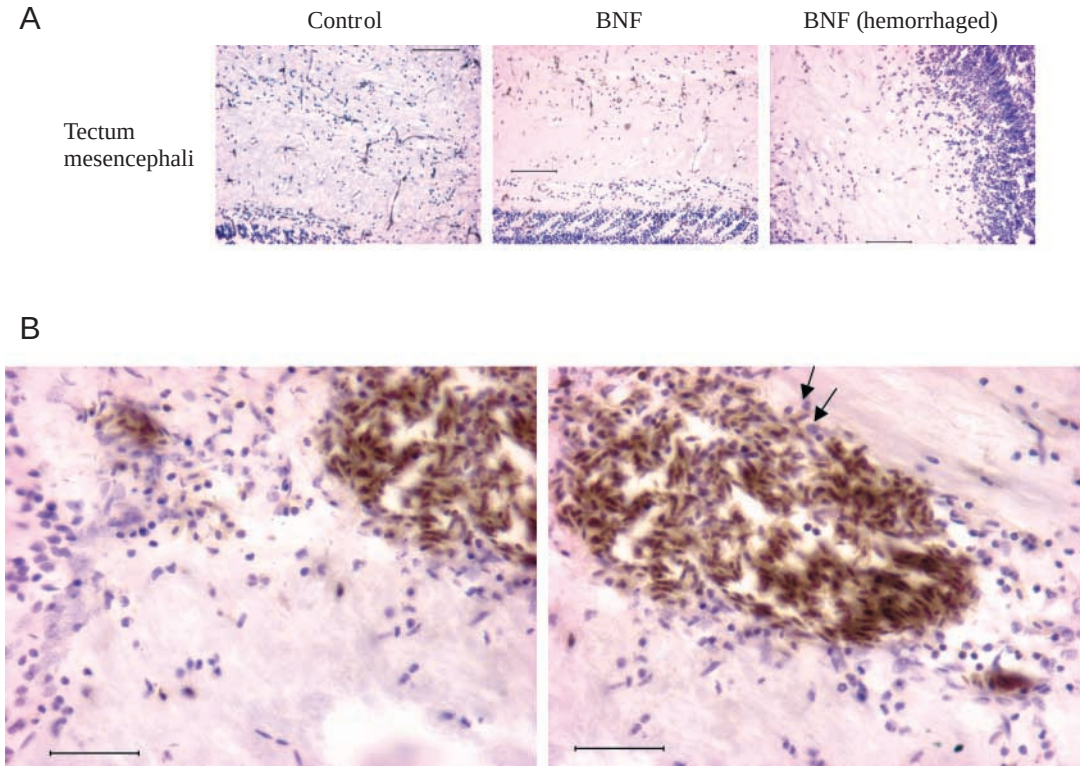

Fig. 6. (A) CYP1A immunoreactivity (brown/black) in the tectum mesencephali of the control and β-naphthoflavone (BNF)-treated lake trout. Hemorrhage depressed the immunoreactivity of CYP1A in BNF-treated juvenile lake trout. (B) Multifocal hemorrhage (brown) in the midbrain of the BNF-treated juvenile lake trout. Note that some blood cells have infiltrated into tissues. Black arrows indicate multinucleated white blood cells. Horizontal sections (20 μm thick) are counterstained with hematoxylin nuclear stain (purple). Scale bars, 100 μm.

in the ventricle, it is more readily exposed to xenobiotics that can penetrate the blood–brain barrier and enter the cerebrospinal fluid. Clearly, it is advantageous to have inducible CYP1A protein in this brain area to detoxify xenobiotics.

Constitutive CYP1A immunoreactivity in endothelia, glia and neurons was observed in juvenile lake trout brain, which was consistent with the immunohistochemical studies by Smolowitz et al. (1991) and Stegeman et al. (1991) in that CYP1A proteins were localized at vascular endothelia of the fish brain. Other studies in fish also provided the evidence that cerebral CYP1A immunoreactivity was not restricted to the endothelia but was also localized in neuronal tissue (Reinecke and Segner, 1998; Sarasquete et al., 1999). Since our results indicate that most of the glial cells that showed CYP1A immunoreactivity were attached to the blood vessels, it is likely that these glial cells can absorb xenobiotic chemicals from the blood vessels and they constitutively produce CYP1A proteins to serve a protective function by eliminating xenobiotics from the central nervous system.

It is surprising that some BNF-treated juvenile lake trout showed depressed CYP1A immunoreactivity in the brain compared with the control fish. In these fish, we found sporadic hemorrhage sites. It is known that BNF mimics the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in altering local brain circulation (Dong et al., 2002). TCDD has been shown to cause multifocal hemorrhages in zebrafish and lake trout embryos (Andreasen et al., 2002; Spitsbergen et al., 1991; Toomey et al., 2001). It is evident that 4-day BNF treatment caused multifocal hemorrhages in some juvenile lake trout; however, these pathological effects varied among individuals.

Studies in other species also indicated that hemorrhage may depress BNF-induced CYP1A immunoreactivity. Local hemorrhages in the brain trigger the inflammatory response (Perry et al., 1993; Rothwell et al., 1996) in which glial cells, specifically astrocytes and microglia, become activated and stimulate the acute phase immuno-response, which downregulates microsomal CYP protein level in cultured cells, humans and other animals (Nicholson and Renton, 2001; Paton and Renton, 1998; Renton et al., 1999; Renton and Nicholson, 2000; Shimamoto et al., 1998; Stanley et al., 1991). Most cytokines that are found to decrease basal CYP production can counteract *Ah* receptor-mediated increase of CYP1A protein and its associated EROD activity. Our discovery of depressed CYP1A immunoreactivity in the BNF-treated fish with hemorrhages was consistent with the CYP1A immunodepression in the inflammatory response.

BNF differentially induced CYP1A mRNA and protein in juvenile lake trout brain. CYP1A mRNA was not constantly produced in the brain. Once CYP1A mRNAs were induced, they distributed universally throughout the endothelia of the whole brain. Occasionally, induced CYP1A mRNAs were

found in the glial cells. On the contrary, CYP1A protein was constantly produced at a very low level in endothelia, glia and neurons in lake trout brain. Only specific brain regions showed increased CYP1A immunoreactivity, and the increase was not as robust as that of the CYP1A mRNA. This may be due to the immunological privilege of the brain (Perry et al., 1993; Rothwell et al., 1996). The mechanism to depress CYP1A protein apparently requires protein synthesis since treatment with cycloheximide in combination with *Ah*-receptor agonist led to superinduction of CYP1A mRNA (Abdel-Razzak et al., 1994). It is likely that CYP1A protein level is more tightly regulated in the brain than is CYP1A mRNA. Therefore, BNF can induce CYP1A mRNA universally in the brain endothelia whereas CYP1A protein only increased in specific cells and in specific brain regions. On the contrary, in other tissues such as livers, a transient CYP1A mRNA induction could be followed by a prolonged induction of CYP1A protein level (Kloepper and Stegeman, 1992, 1994).

To summarize, BNF differentially induces CYP1A mRNA and protein expression in juvenile lake trout brain. The induction of CYP1A mRNA is universally distributed throughout the endothelia of the whole brain while the increase of CYP1A protein is less robust and area specific. BNF may induce hemorrhage in some individuals and may cause the immunodepression of CYP1A protein in the brain.

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