Aquatic Toxicology 94 (2009) 123–130

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Transcript profiling and DNA damage in the European eel (*Anguilla anguilla* L.) exposed to 7,12-dimethylbenz[a]anthracene

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ARTICLE INFO

Article history:
Received 3 April 2009
Received in revised form 8 June 2009
Accepted 9 June 2009

Keywords:
Anguilla anguilla
Biomarker
SSH
Genotoxicity
PAH

ABSTRACT

The molecular responses induced during and after an acute exposure to 7,12-dimethylbenz[a]anthracene (DMBA) were analysed in liver, gill and blood cells of juvenile *Anguilla anguilla* with the aim of developing molecular biomarkers of environmental PAH pollution. Changes in the mRNA expression levels of the cell cycle checkpoint-related *rad1* gene and the mRNAs of differentially expressed genes by suppression subtractive hybridization (SSH) were analysed in the liver, and related to well-established biomarkers: *cyp1A1* mRNA expression and assessment of the DNA integrity using the comet assay and flow cytometry. DMBA exposure resulted in increased *cyp1A1* mRNA levels, suggesting that cyp1A1 might be involved in the metabolism of DMBA. Global DNA damage, detected by the comet assay, was observed in the three tissues analysed but only blood cells showed chromosomal lesions as analysed by flow cytometry. Although DNA damage was found in the liver, no induction in *rad1* gene was observed in this organ. The global SSH approach revealed that mRNAs of genes related to xenobiotic metabolism, immune processes and cytoskeleton dynamics were differentially expressed in DMBA-exposed eel livers, highlighting the complexity in the response observed in fish exposed to a genotoxic agent and providing directions for new biomarker development.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are contaminants that may enter aquatic systems through spillage and seepage of fossil fuels, discharge of domestic and industrial wastes, atmospheric input and continental runoff (Hartl, 2002). Their presence in the environment is of concern since they induce acute toxicity in organisms and have been linked to liver neoplasms and other abnormalities in fish species (Jha, 2004). 7,12-Dimethylbenz[a]anthracene (DMBA) is a prototype synthetic PAH, cytotoxic, mutagenic and immunotoxic (Miyata et al., 2001). It is regarded as one of the most carcinogenic PAHs in mammalian and fish species (Miyata et al., 2001; Weimer et al., 2000). Like other PAHs, DMBA requires metabolic activation in order to produce potentially damaging effects. PAHs biotransformation is most effective in the liver of fish, where they are easily metabolized by the phase I enzymes of the cytochrome P450 monooxygenase system (e.g. CYP1A1 enzyme) to more hydrophilic products (van der Oost et al., 2003). Most of the resultant metabolites are excreted after conjugation by phase II enzymes. However, some of the intermediates produced during the biotransformation process, especially epoxides and dihydrodiols, are highly reactive and may bind to cellular macromolecules such as DNA, forming covalent bulky DNA adducts responsible for mutagenicity and carcinogenicity.

Cells have evolved a variety of systems to maintain the genomic stability by detecting and repairing damaged DNA. In response to genotoxic exposure, cells initiate cell cycle checkpoints that halt replication to allow time to repair damaged DNA. The mammalian Rad9-Rad1-Hus1 (9-1-1) checkpoint complex has been characterized as a sensor of DNA damage (Helt et al., 2005). Bulky adducts such as those induced by DMBA, elicit a checkpoint pathway involving the 9-1-1 complex (Bi et al., 2006), which is recruited to affected sites where it may attract specialized DNA polymerases and other DNA repair effectors (Helt et al., 2005). In fish, the role of rad1 and other mechanisms of detecting DNA damage are yet to be elucidated.

Exposure of organisms to chemicals may also result in complex changes in gene expression levels as a direct response or as a compensatory response to molecular damage or cellular dysfunction. Suppression Subtractive Hybridization (SSH) is a technique used to detect mRNAs of differentially expressed genes (Diatchenko

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; RT, reverse transcriptase; SSH, suppression subtractive hybridization; FCM, flow cytometry.

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doi:10.1016/j.aquatox.2009.06.007

et al., 1996). The method is particularly useful when the available sequence data of an organism is limited, which is most often the case for aquatic species. In the toxicology field, advances in transcript analysis have led to the recognition that altered gene transcription is potentially an early, rapid and sensitive means of stress response detection (Williams et al., 2003).

The aim of this study was to investigate global and specific molecular responses to DMBA exposure in juvenile *Anguilla anguilla* (European eel), with the broad aim of being able to develop these as molecular biomarkers of environmental PAH pollution. *A. anguilla* is widely distributed in estuaries and freshwater courses and, despite its description as a bottom dwelling fish, can move throughout the entire water column being thus a versatile indicator species with a high economic value. The animal is easy to capture and to maintain in a laboratory (Costa et al., 1992). However, since the 1980s, a decline in *A. anguilla* and *A. rostrata* populations have been recorded. Because of their unusual life cycle, they are considered particularly susceptible to pollutant exposure and this is postulated as one of the causes for their population decline (Robinet and Feunteun, 2002).

To develop such biomarkers, changes in the mRNA expression levels of the cell cycle checkpoint-related *rad1* gene and the detection of mRNAs of differentially expressed genes by SSH were analysed in the liver of DMBA-exposed eels. To anchor the novel molecular biomarkers described herein, well-established biomarkers were studied in parallel. These included hepatic *cyp1A1* gene transcription and assessment of DNA integrity by the comet assay and flow cytometry (FCM).

2. Materials and methods

2.1. Experimental exposure

Juvenile specimens of European eel (*A. anguilla*) with an average weight of 0.2 g (glass eels) were caught at the Mondego River mouth, Figueira da Foz (Portugal). Fish were acclimatised to laboratory conditions for a period of 2 weeks in recirculating, filtered, well aerated and dechlorinated tap water, at 20 °C, under a 12 h light/12 h darkness photoperiod. Animals were fed three times a week during the acclimation period. Eels were exposed in 40 l aquaria to DMBA (Sigma, USA) previously dissolved in dimethyl sulfoxide (DMSO, Sigma, USA). The DMSO concentration in water (0.0033%) has no detectable cytogenetic or toxic effect in juvenile eels (Pacheco and Santos, 2002). The same DMSO volume was added to the control aquarium. Groups of at least 150 animals were exposed to a nominal concentration of 0.39 and 3.9 μM (0.1 and 1 ppm, respectively) of DMBA for 24 h, and afterwards maintained in clean water and fed three times a week. Control fish were also transferred to clean water. DMBA concentrations were selected basing on previous studies which showed the ability of DMBA to bind to fish DNA and to induce neoplasic lesions (El-Zahr et al., 2002).

Eels were sampled before the exposure (T0), immediately after the exposure and at 1, 3, 7, 14, 28 and 90 days during the post-exposure period. At each sampling point, five fish were sacrificed by decapitation and the blood directly collected from the heart using heparinised capillary tubes, suspended in cold phosphate buffered saline (PBS) 1× and kept on ice until use. Liver and gills were also collected and kept in cold PBS 1×/EDTA 100 mM/DMSO 10% buffer until use in the comet assay. The liver from 15 other eels were collected, kept in RNAlater RNA Stabilization Reagent (Qiagen, UK) and frozen at −70 °C.

2.2. Traditional biomarkers

2.2.1. Evaluation of DNA integrity by comet assay

The comet assay, which detects DNA strand breaks and alkali-labile sites, was conducted with whole blood, liver and gill tissue, under yellow light to prevent UV induced DNA damage. Liver and gill cell suspensions were obtained by homogenizing the tissues in the presence of PBS 1×/EDTA 100 mM/DMSO 10% buffer, to reduce DNase activity and prevent oxidant-induced DNA damage (Hartmann et al., 2003). The comet assay protocol was performed as described in Nogueira et al. (2006). Slides were examined using a fluorescence microscope (Leica DMLS, Germany). Fifty randomly selected cells per slide (2 slides/animal) were analysed using the image analysis software Comet Assay IV (Perceptive Instruments, UK). DNA damage was expressed as percentage tail DNA (% tDNA) and presented as mean of medians ± standard error of the mean (SEM). The extent of DNA migration (% tDNA) is positively correlated with the amount of DNA damage in the cell (Hartmann et al., 2003).

2.2.2. Evaluation of DNA integrity by flow cytometry

One volume of citrate buffer (sucrose 250 mM, trisodium citrate 40 mM, pH = 7.6) with 10% DMSO was added to blood samples before frozen in liquid nitrogen and stored at −70 °C. Liver and gill cell suspensions (obtained as described in Section 2.2.1) were also frozen in liquid nitrogen and stored at −70 °C for flow cytometric analysis.

Frozen samples were thawed and suspended in LB01 buffer (Doležel et al., 1989), treated with 50 mg/ml RNase (Fluka, Buchs, Switzerland) and stained with 50 mg/ml propidium iodide (PI, Fluka). Samples were incubated for 10 min on ice before analysis. The fluorescence intensity of at least 5000 nuclei/sample was measured with a Coulter EPICS-XL flow cytometer (Beckman-Coulter®, Hialeah, FL, USA). The instrument was equipped with an air-cooled argon-ion laser (15 mW, operating at 488 nm) and PI fluorescence was collected through 645 nm dichroic long-pass and 620 nm band-pass filters. The results were acquired using the SYSTEM II software (v. 3.0, Beckman Coulter®) in the form of three graphs: fluorescence integral (FL) histogram (to analyse the FL intensity of nuclei); FL *vs*. time cytogram (to analyse the nuclei fluorescence stability during sample acquisition); and FL pulse integral *vs.* FL pulse height cytogram (to detect and eliminate, through region gating, doublets and other debris). Prior to data acquisition, the instrument was checked for linearity and resolution with fluorescent check beads (Beckman-Coulter®), and the amplification was adjusted so that the peak corresponding to sample's nuclei was positioned approximately at channel 200 of a 1024 scale. This setting was kept constant throughout the whole experiment. An increase in the amount of chromosomal damage is reflected by an increase in cell-to-cell variation in DNA content (Matson et al., 2004), so the half-peak coefficient of variation (HPCV) of the G_0/G_1 peak was calculated for each sample.

2.2.3. Determination of the mRNA expression of cyp1A1 gene

The mRNA expression of *cyp1A1* gene was determined by real-time quantitative PCR (qPCR) in the liver of control (non-exposed) and DMBA-exposed eels sampled during the post-exposure period at 3, 7 and 28 days, in order to have a temporal response overview. Total RNA was isolated from pools of two livers (5 pools/group) using RNeasy reagents (Qiagen, UK) and treated with RNA-free DNase I (Qiagen, UK) to remove genomic DNA. RNA concentrations were measured with the Quant-iT RNA assay kit (Invitrogen, UK) using a Qubit fluorometer (Invitrogen, UK). Reverse transcription of 1 μg of total RNA samples was carried out using SuperScript® Vilo™ cDNA synthesis kit (Invitrogen, UK). Real-time PCR reactions were performed in duplicate, in a final volume of 25 μl containing 12.5 μl of Express SYBR® GreenER™ qPCR SuperMix (Invitrogen, UK), 5 μl of diluted cDNA (1/100), 3.75 μM primers (Table 1) and 0.1 μl of ROX reference dye. A control lacking cDNA template was included in qPCR analysis to determine the specificity of target cDNA amplification. Amplification was detected with a Mx3005P real-time PCR

Table 1
Real-time PCR primer sequences.

Primer name	GenBank accession no.	Primer forward (5′–3′)	Primer reverse (5′–3′)
ARP	AY63793	GTGCCAGCTCAGAACACTG	ACATCGCTCAAGACTTCAATGG
CYP	AF420257	GTGTGGGACTGGAGCGTACT	CAGTGCGGTGTCTTTAGTGG
RAD1	FJ438472	GGGAAGCACTACACCAGGAG	AATGGGATCCTCTGGCTCTT
ST	GO096062	ACCTGGAAGCAAGGGAAGAT	ACTCTATCAGCGGTGGCATC
VLIG	GH717880	CTGCTGTTTGGGTCATCTCA	AGTTGGGAGAAAACCCTGCT
COMT	GH717900	TGTGAGCAAAGGTGGCATTA	GCGAGTAAGCTCAGGTCGAT
ITIH3	GH717895	TTTCGCTTTGCCCTAACTGT	CCGTGCTGAAGTTTGTGATG

system (Stratagene, UK) and with the following cycling parameters: 50 °C for 2 min, 95 °C 10 min, 40 cycles of 30 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C. Melting curves were determined following the instrument instructions, to identify the presence of primer dimers and to analyse the specificity of the reaction. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA. Eel acidic ribosomal protein (ARP) gene was used as the internal reference (Weltzien et al., 2005). The relative mRNA expression levels of target genes were calculated according to the efficiency-corrected method described by Pfaffl (2001), using the non-treated group as calibrator.

2.3. Development of novel biomarkers

2.3.1. Determination of mRNA expression of rad1 gene

For the isolation of *rad1* gene cDNA, RNA was extracted from the pool of two livers of non-exposed eels using RNeasy reagents (Qiagen, UK) and treated with RNA-free DNase I (Qiagen, UK). First strand cDNA was synthesized from 1 μg of total RNA in the presence of random hexamer primers using Superscript First Strand cDNA Synthesis System for RT-PCR (Invitrogen, UK). This cDNA was the template for the amplification of a *rad1* gene fragment using the degenerated primers Rad1F (5′-GAAGAAGGWGGWGTGGTG-3′) and Rad1R (5′-TACTGTARDGAGAGGAAGCCTC-3′), designed based on published sequences of fish species. The PCR reaction was performed in reaction buffer with 400 μM each dNTP, 50 pmol each primer and 1 U platinum Taq DNA polymerase (Invitrogen, UK). The rad1 fragment was amplified in a Techne™ thermal cycler using 35 sequential cycles at 94 °C for 40 s, 50 °C for 40 s, 72 °C for 60 s, followed by a final 2 min extension at 72 °C. The sequence obtained from the cloned fragments subsequently served for specific primer design. The isolation of the complete 5′ and 3′ ends of *rad1* gene was performed with control *A. anguilla* liver total RNA (1 μg) using SMART™ RACE cDNA amplification reagents (Clontech, France) and the gene-specific primers RACE5rad1 (5′-CCTTGTTGGTGACATTGGTGCTGCAG-3′) and RACE3rad1 (5′-CTTTGGGGGAAGCACTACACCAGGA-3′). The RACE products obtained were analysed on agarose gel, excised and purified using a Qiaquick spin columns (Qiagen, UK). Purified DNA was ligated into a pGEM®-T Easy vector (Promega, UK). Recombinant plasmids were transformed and selected using ampicilin LB plates. Plasmid DNA was purified for DNA sequence analysis using commercial sequencing (Eurofins MWG Operon, Germany) to verify the identity of the products.

The mRNA expression of *rad1* gene was determined by real-time qPCR in control (non-exposed) and in DMBA-exposed eels sampled during the recovery period at 3, 7 and 28 days, as described in Section 2.2.3, and using the RAD1 primers (Table 1). Data were normalized to eel ARP gene and expressed as the fold difference from the control group.

2.3.2. Suppression subtractive hybridization (SSH)

The SSH procedure was used to isolate and enrich for gene sequences with mRNAs differentially expressed between the liver of control (non-exposed) and 3.9 μM DMBA-exposed eels sampled at day 7 of the post-treatment period, when the highest levels of DNA damage were observed in the liver, as measured by comet assay. For each group, equal amounts of the RNA extracted in Section 2.2.3 were pooled (five RNA samples in each group). The synthesis of cDNA was carried out with the SuperSMART PCR cDNA Synthesis kit (Clontech, France) and the generated cDNA was directly used for SSH. The forward- and reverse-subtracted libraries were produced using the PCR-Select cDNA Subtraction kit (Clontech, France) according to the manufacturer's protocol. The differential PCR products generated by SSH were inserted in a pGEM®-T Easy vector (Promega, USA) and cloned into JM109 bacterial cells. Sixty randomly selected colonies were inoculated in LB broth and screened for inserts by PCR using vector-based primers. A total of 20 clones per library were randomly selected for sequencing (Eurofins MWG Operon, Germany) directly from the PCR amplified product. Sequence identities were obtained by BLAST searches against the NCBI nucleic acid and protein databases. Sequence reads with *E*-value higher than 10^{-5} were filtered out. The differential screening result was confirmed by qPCR using four candidate genes: sulfotransferase (ST), similar to very large inducible GTPase1 (VLIG), inter-alpha globulin inhibitor H3 (ITIH3) and catechol-O-methyltransferase (COMT), as described in Section 2.2.3. Primer pairs used in qPCR are listed in Table 1.

2.4. Statistical analysis

For statistical analysis (SigmaStat 3.11 Software), all data were tested for normality and homogeneity of variances. Differences between control and exposed fish were determined using analysis of variance (ANOVA), followed by a Tukey test ($P < 0.05$). If an invalidation of normality and variances homogeneity was observed, data were analysed using a non-parametric Kruskal–Wallis ANOVA on ranks, followed by a Mann–Whitney *U* pairwise comparison test (SigmaStat 3.11 Software). A $P < 0.05$ was considered significant.

3. Results

3.1. Traditional biomarkers

3.1.1. Evaluation of DNA integrity by comet assay

DNA damage was analysed in liver, gill and blood cells of *A. anguilla* exposed for 24 h to two different concentrations of DMBA and sampled at different times post-exposure. Levels of damage were compared with the respective control group (Fig. 1). The highest level of DNA damage in liver cells was observed in eels exposed to 3.9 μM DMBA at 7-day post-exposure. A significant increase ($P < 0.05$) was also observed in liver cells at 28-day post-exposure. The gill cells were affected by both concentrations of DMBA in eels sampled at different days post-exposure. The group exposed to the higher concentration showed significant increases in DNA damage levels at 7 (near fivefold) and 28-day post-exposure. The DNA damage detected in the gill cells of eels exposed to 0.39 μM was observed at 1- and 28-day post-exposure. Interestingly, the values observed in this exposed group following 1 day in clean water

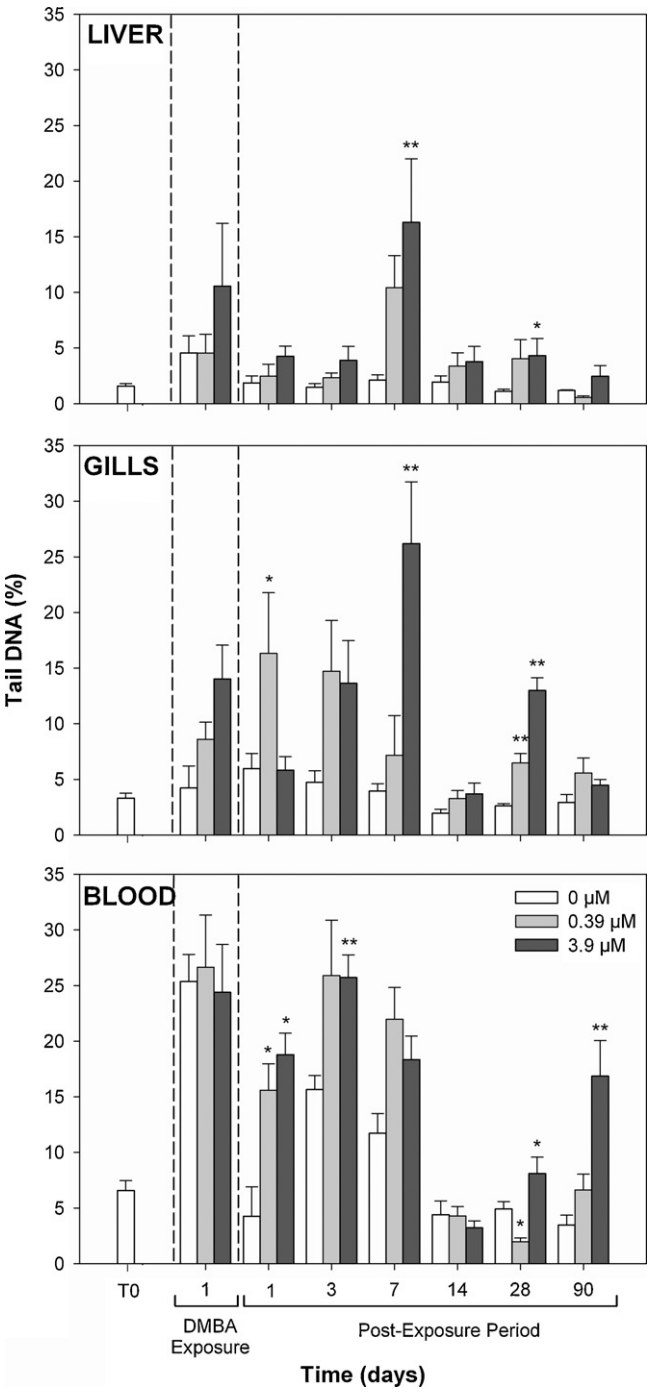

Fig. 1. DNA damage detected by the comet assay and expressed as tail length (%) in liver, gill and blood cells isolated from *A. anguilla* exposed for 1 day to two different concentrations of DMBA (0.39 and 3.9 μM) and sampled before the exposure (T0), after 1 day of exposure (sampling time = 1) and at 1-, 3-, 7-, 14-, 28- and 90-day post-exposure. Data are expressed as mean of median ± SEM ($n = 5$ animals). Asterisks denote statistically significant differences from the control group: $^{*}P < 0.05$; $^{**}P < 0.01$.

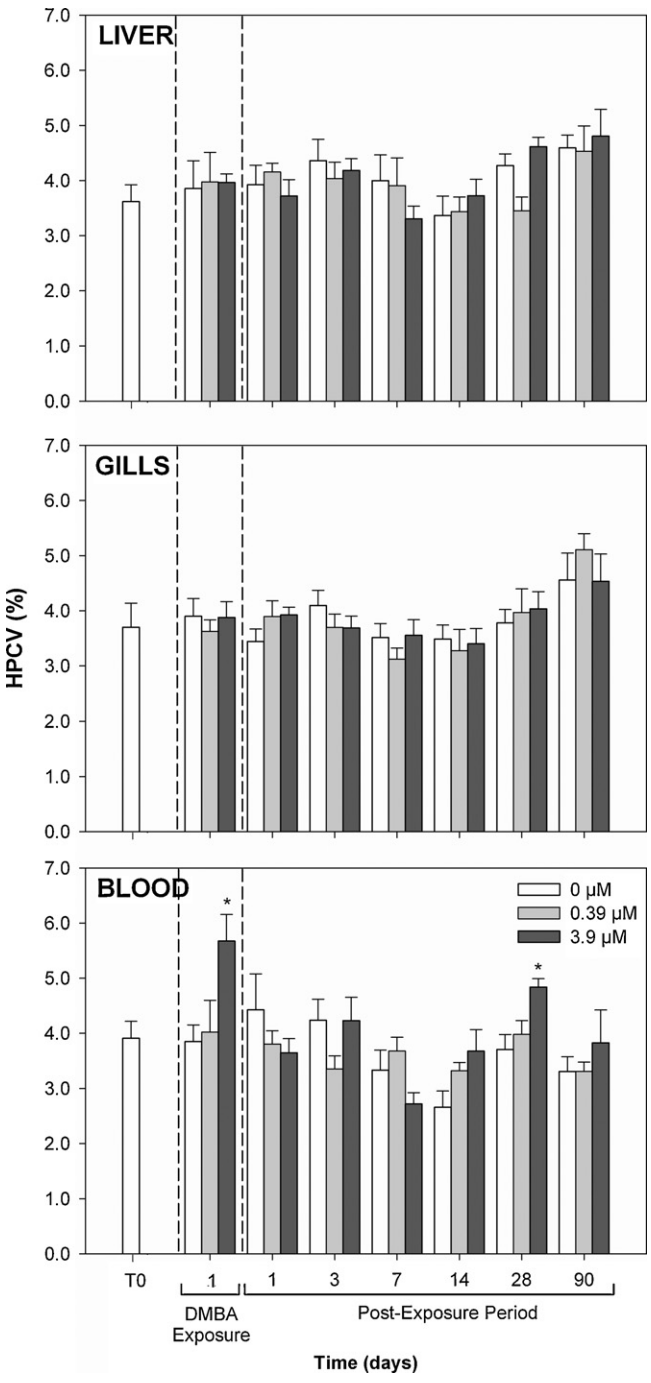

Fig. 2. DNA damage detected by flow cytometry and expressed as HPCV (%) in liver, gill and blood cells isolated from *A. anguilla* exposed for 1 day to two different concentrations of DMBA (0.39 and 3.9 μM) and sampled before the exposure (T0), after 1 day of exposure (sampling time = 1) and at 1-, 3-, 7-, 14-, 28- and 90-day post-exposure. Data are expressed as mean ± SEM ($n = 5$ animals). Asterisks denote statistically significant differences from the control group: $^{*}P < 0.05$.

were significantly higher than those observed in the group subjected to the higher concentration. Blood cells showed increased levels of DNA damage during the first week post-exposure, in both DMBA-treated groups. DNA damage in these cells was also observed 28- and 90-day post-exposure for the highest DMBA concentration. A significant ($P < 0.05$) decrease in tDNA values of blood cells was observed at 28-day post-exposure in eels exposed to 0.39 μM DMBA.

3.1.2. Evaluation of DNA integrity by flow cytometry

The DMBA exposure did not induce significant changes in the cell cycle or any major ploidy changes (e.g. aneuploidy) in any of the tested tissues. For putative chromosomal damage evaluation, mean HPCV values were calculated for liver, gill and blood cells and varied between 2.7 and 5.7% (Fig. 2). The variations in the mean HPCV of liver and gill cells were not statistically significant ($P > 0.05$) between the eels exposed and non-exposed to DMBA. Blood cells showed higher variation during the experiment than liver and gill

Table 2
Relative mRNA expression of *cyp1A1* and *rad1* genes in the liver of *A. anguilla* exposed to 3.9 μM DMBA and sampled 3-, 7- and 28-day post-exposure. Data were normalized to eel ARP gene and expressed as the fold difference from the control group. Asterisk (*) denotes a statistically significant difference from the control group ($P < 0.05$).

Gene	Condition	Relative expression
cyp1A1	3 days	0.90
	7 days	6.90*
	28 days	0.92
rad1	3 days	1.17
	7 days	1.06
	28 days	1.19

cells. Significant ($P < 0.05$) increases in the mean HPCV value of blood cells were found in eels exposed to the highest concentration and analysed after 1 day of exposure and at 28-day post-exposure.

3.1.3. Determination of mRNA expression of cyp1A1 gene

The relative mRNA expression of *cyp1A1* gene was determined in liver cells exposed to 3.9 μM DMBA and sampled at 3, 7 and 28 days during the post-exposure period. The hepatic *cyp1A1* transcript levels increased significantly ($P < 0.05$) nearly sevenfold at 7 days, and remained close to the control values at days 3 and 28 (Table 2).

3.2. Development of novel biomarkers of exposure

3.2.1. Determination of mRNA expression levels of rad1 gene

The isolated *A. anguilla rad1* cDNA with a nucleotide sequence of 1281 bp (excluding the polyA tail) included an ORF of 840 nucleotides, encoding 279 amino-acids (GenBank Accession No. FJ438472). The predicted amino-acid sequence showed conserved exonuclease domains and leucine zippers found in *rad1* genes (Fig. 3). The alignment of the *A. anguilla* deduced amino-acid sequence with other fish species and human rad1 sequences is shown in Fig. 3, revealing a high identity between eel and other fish species (from 86% to 91%) and with the human *rad1* sequence (75%). The relative mRNA expression of *rad1* gene was studied in the same samples used for mRNA expression levels of *cyp1A1* but no alterations were observed in the relative *rad1* transcript levels (Table 2).

3.2.2. Suppression subtractive hybridization (SSH)

SSH methodology was employed in liver cells to screen differential mRNA expression of genes between non-exposed group and the group exposed to 3.9 μM DMBA and sampled 7-day post-exposure, where the DNA damage in the liver was highest as revealed by the comet assay. Twenty randomly selected clones were sequenced for each subtracted library and compared to publicly available sequences. Ten sequences from the forward library (genes with upregulated mRNAs, Table 3) and nine from the reverse (genes with downregulated mRNAs, Table 3) could be matched to genes from different organisms, mainly from other fish species. The remaining sequenced clones showed homology to genes encoding unidentified hypothetical or novel proteins or showed no homology with other sequences deposited in the database.

The SSH results were validated by real-time PCR, normalized to *ARP* gene, using two of the genes spotted for each library (Table 3). mRNA upregulation of *sulfotransferase* and *very large inducible GTPase1* genes, and down regulation of *inter-alpha globulin inhibitor H3* and *catechol-O-methyltransferase* genes were confirmed by qPCR (Table 3).

```
A.anguilla      MPLSTQSEAG-DERYVLVASLDNVRNLSNILKAITFKDHALFNATPNGLKVTVEDSKCLQ 59
D.rerio         MPLSTQSQSD-VDQYILIASLDNARNLSNILKAISFKDHAIFNATQNGLKVTVEDSKCLQ 59
G.aculeatus     MPLSTQSQAD-DEQYVLVASLDNARNLSNLLKAITFKDHAIFSATPNGLKVTVEESKCLQ 59
H.sapiens       MPLLTQQIQDEDDQYSLVASLDNVRNLSTILKAIHFREHATCFATKNGIKVTVENAKCVQ 60
                **: **   .  ::* * .****.****.:**** *::**   ** **:*****::**:*

                                        I                          II
A.anguilla      ANAFIQAEIFQEFTIQEDSVSFQVNLTVLLDCLTIFGGSTTPGVTTALRMCYNGYGYPLT 119
D.rerio         ANAFIQADIFQEYIIKEDAVGFQVNLTVLLDCLTIFGGSTVPGVCTALRMCYNGYGYPLT 119
G.aculeatus     ANAFIQAEIFQEFTIREDSVGFQVNLTVLLDCLSIFGGSTVPGVSTALRMCYRGYGYPLT 119
H.sapiens       ANAFIQAGIFQEFKVQEESVTFRINLTVLLDCLSIFGSSPMPGTLTALRMCYQGYGYPLM 120
                ******* ****: ::*::* *::*********:***.*. **. ***:***.***:**

                                                    III
A.anguilla      LFLEEGGVVTVCKINTEEPEDPIDFEFCSTNVTNKVILQSDSLREAFSELDMTSEILQIT 179
D.rerio         LFLEEGGVVTVCKINTQEPEEPIDFDFCSTNVTNKVILQSDSLKEAFSELDMTSEVLQIT 179
G.aculeatus     LFLEEGGVVTVCKINTQEPEEPVDFEFCSTNVTNKVILQSESLKEAFSELDMTSEVLQIT 179
H.sapiens       LFLEEGGVVTVCKINTQEPEETLDFDFCSTNVINKIILQSEGLREAFSELDMTSEVLQIT 180
                ***************:*:***:.:**:****** **:****:.*:***:******:.***

                                                                IV
A.anguilla      MSPSQPYFRLSTFGNSGNAHYDYPKDSDMMELFQCTKTQTNRYKMSLLKPSTKALALSCK 239
D.rerio         MSPSHPYFRLSTFGNSGNAHYDYPKDSDMMELFQCTKIQTNRYKMSLLKPSTKALALSCK 239
G.aculeatus     MSPSQPYFRLSTFGNSGNAHYDYPKDSDMMELFRCDKTQTNRYKMSLLKPSTKALALSCK 239
H.sapiens       MSPDKPYFRLSTFGNAGSSHLDYPKDSDLMEAFHCNQTQVNRYKISLLKPSTKALVLSCK 240
                ***.:**********:*.:* *******:* *:* : *.****:**********.****

                VSVRTDTRGFLSLQYLVRNDDGQICFVEYYCCPDEEVEEE-- 279
D.rerio         VSVRTDSRGFLSLQYLVRNDDGQICFVEYYCCPDEEVEEE-- 279
G.aculeatus     VSVRTDSRGFLSLQYLVRNDDGQICFVEYFCCPDEEVEEE-- 279
H.sapiens       VSIRTDNRGFLSLQYMIRNEDGQICFVEYYCCPDEEVPESES 282
                **:***.********::**:*********:******* *
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Fig. 3. Comparison of the deduced *A. anguilla* rad1 sequence (GenBank accession no. FJ438472) with selected vertebrate rad1 sequences: *Danio rerio* (GenBank accession NO. AAH49464); *Gasterosteus aculeatus* (Ensembl Gene ENSGACP00000021769) and *Homo sapiens* (GenBank accession no. AAC95466). Areas showing homology are marked with an asterisk. The conserved exonuclease domains (I, II and III) and leucine zippers (III and IV) are overlined.

Table 3
Genes with differentially expressed mRNAs in liver of *A. anguilla* exposed to 3.9 μM DMBA detected by SSH analysis (sequences with no similarity to genes with known functions are not included). Asterisk (*) denotes a statistically significant difference from the control group ($P < 0.05$).

Clone accession. no.	Species	Accession no.	Putative identity	*E*-value	Relative expression
Upregulated genes					
GO096062	*Danio rerio*	AAO49010	Sulfotransferase	5E−93	4.61*
GH717880	*Danio rerio*	XP_684086	Similar to very large inducible GTPase1	1E−79	2.52*
GH717881	*Ctenopharyngodon idella*	M25013	Beta-actin gene	4E−45	
GH717890	*Danio rerio*	XP_001919426	Similar to cysteine/glutamate transporter	7E−60	
GH717889	*Danio rerio*	BC164301	Similar to ribosomal protein S8	7E−19	
GH717888	*Paralichthys olivaceus*	BAE92861	Tributyltin binding protein type 1	3E−12	
GH717887	*Oncorhynchus mykiss*	ABD84407	CCAAT/enhancer-binding protein beta2	1E−34	
GH717886	*Crassostrea gigas*	ABQ18234	Bindin precursor 5 repeat variant	9E−15	
GH717885	*Salmo salar*	BT043871	Ribosomal protein L37a-1 mRNA	8E−14	
GH717883	*Danio rerio*	XP_001919639	Similar to enteropeptidase-2	2E−27	
GH717884	*Salmo salar*	ACI69743	Membrane-spanning 4-domains subfamily A member 12	6E−53	
Downregulated genes					
GH717891	*Danio rerio*	CAQ14913	Similar to vertebrate NCK-associated protein 1	7E−104	
GH717892	*Oncorhyncus mykiss*	CAA67764	Pentraxin	3E−36	
GH717894	*Tamia sibiricus*	BAD06478	Alpha 1-antitrypsin-like protein	5E−41	
GH717895	*Danio rerio*	XP_693183	Similar to inter-alpha-trypsin inhibitor heavy chain3	1E−59	0.35*
GH717897	*Danio rerio*	AAI53542	Serpin peptidase inhibitor, clade A, member 7	5E−49	
GH717898	*Platichthys flesus*	CAE53392	Serine proteinase inhibitor	1E−33	
GH717900	*Danio rerio*	XP_696495	Catechol-O-methyltransferase domain containing 1	2E−59	0.46*
GH717901	*Anguilla anguilla*	ABW96975	Cytochrome *b*	7E−131	
GH717903	*Anguilla anguilla*	AJ244826	Mitochondrial 16S rRNA gene	0.0	

4. Discussion

Here, we have observed that DMBA exposure significantly increases DNA damage (as measured by comet assay) in the gills, blood and, to a lesser extent, liver of juvenile eel, in a one to 7-day post-exposure time frame. We also observe that mRNA expression of *cyp1A1* gene is similarly increased at 7-day post-exposure in the liver of exposed eels. In contrast, however, the flow cytometry and mRNA expression of *rad1* gene, the product of which is a component of the DNA repair mechanism in mammals, display no such significant increases in the liver following DMBA exposure. These targeted specific biomarkers of DMBA exposure thus provide contrasting results. On adopting an alternate global approach in parallel with the same samples, we observed that numerous mRNA expression changes of genes occur on exposure to DMBA, including a number of novel genes that may in future serve as possible biomarkers of hydrocarbon exposure.

The interaction of DMBA metabolites with DNA leads to the formation of adducts, contributing to the genotoxicity and carcinogenicity of this compound in fish (Weimer et al., 2000). Here we observe that DMBA exposure in *A. anguilla* resulted in an increase in DNA damage, and thus has similar genotoxic potential. CYP1A enzymes catalyse the first step of the DMBA detoxification process (van der Oost et al., 2003), and, again, the increase in mRNA expression of *cyp1A1* gene observed is consistent with that observed in the liver of different fish species after PAH exposure (Levine and Oris, 1999). Elevated *cyp1A1* and DNA damage both peak in the liver at day 7 suggesting that reactive metabolites take a few days to accumulate and result in actual DNA damage.

Little is known about the DNA repair efficiency of hepatic cells in fish. The possibility that the DNA repair *rad1* gene is inducible was investigated yet no alterations in its mRNA expression levels were found in hepatic cells of eels exposed to the highest concentration of DMBA at any sampling time point, including the day 7 post-exposure where a significant increase in the DNA damage level was observed. Similarly, Weimer et al. (2000) also reported a lack repair of DMBA-DNA adducts in trout liver cells, 48 h after DMBA exposure. Both findings seem to be in agreement that teleosts have a low DNA damage repair rate (Wirgin and Waldman, 1998).

In terms of tissue-specific effects, gill tissue showed high levels of DNA damage compared to the other tissues, suggesting that either unmetabolized DMBA or its' metabolites are affecting gill DNA. Pacheco and Santos (2001) observed no significant increases in the gill EROD activity levels in eels exposed to β-naphthoflavone (a PAH-type chemical), suggesting that the metabolism capacity of eel gill cells is reduced. Subsequently, the production of reactive oxygen species due to DMBA exposure and a low antioxidant defence capacity of gill cells (Levine and Oris, 1999) are possibly the main contributors for the DNA damage observed in these cells during the first 7-day post-exposure. This hypothesis, however, does not explain the DNA damage levels detected at 28-day post-exposure since the elimination of PAHs is generally very efficient in fish and no bioaccumulation of these compounds has generally been demonstrated (van der Oost et al., 2003). Genome instability and vulnerabilities in the DNA detection/repair systems may have possibly occurred in gill cells due to DMBA exposure and could explain the delayed damage observed in these cells. The replacement of damaged cells by new ones could have occurred after that period, as a consequence of the high mitotic rate of gill cells (Al-Sabti and Metcalfe, 1995), explaining the absence of DNA damage at 90-day post-exposure. The apparent higher genotoxic effect at the lowest DMBA concentration tested (0.39 μM) observed in gill cells after 1-day post-exposure, is possibly explained by a decrease in the gill metabolism of the parental DMBA to reactive intermediates in the high concentration-exposed eels, due to the saturation of metabolic enzymes or inhibition/inactivation of CYP1A.

Blood cells showed increased levels of DNA damage throughout the experiment even in control eels. The temporal variation of the damage baseline is dependent on non-contaminant related factors. Blood is a "buffered" tissue which receives and transports many substances, including contaminants and metabolites of exogenous and endogenous compounds produced in different tissues. The analysed response may thus be a result and reflection of the global organism condition rather than of the blood cells isolated. The blood DNA damage observed at 28 and 90 days may constitute the late effects of DMBA exposure resulting from the conjugation of different factors in blood and other tissues, in particular hematopoietic tissues (kidney and spleen). These factors include the toxicant accumulation/depuration ratio, DNA repair rate, antioxidant levels and cell turnover rate.

The consequence of structural perturbations on the DNA molecule, such as adducts and DNA strand breaks detected by comet

assay, may result in genotoxic lesions that become permanent, including chromosomal aberrations or variations of nuclear DNA content. FCM is a useful method to analyse changes in nuclear DNA content induced by genotoxic agents and has already been used in fish to evaluate the effects of environmental pollutants (Goanvec et al., 2008). Here, the chromosomal damage induced by DMBA exposure was evaluated using FCM in liver, gill and blood cell nuclei. Liver and gill cells showed no statistically significant differences between exposed and non-exposed eels. Gill cells have a high mitotic rate (Al-Sabti and Metcalfe, 1995) and may have eliminated and replaced the cells with high levels of damage. Liver cells, in contrast, have a lower mitotic rate and, therefore, the DNA repair capacity of cells may have a more determinant role in the removal of lesions that may have led to irreversible chromosomal damage.

In exposed organisms, genes that are differentially transcribed may be useful as early warning molecular biomarkers to indicate exposure to toxicants. Here, DMBA-responsive gene transcripts were isolated from eel liver cells by SSH. The results represent a preliminary approach to the analysis of gene expression change in DMBA-exposed eels. Of particular note, and their relevance discussed in turn, are genes encoding a sulfotransferase, a number of protein translation machinery proteins, and immune response proteins.

mRNA expression of sulfotransferase gene was upregulated in the liver of DMBA-exposed eels. The reactions of sulfonation catalysed by this enzyme play a key role by facilitating the inactivation and elimination of potent endogenous chemicals and xenobiotics (Martin-Skilton et al., 2006). In mammalian liver, it is involved in metabolic activation of DMBA producing reactive metabolites that may react with DNA bases (Watabe, 1983). This result suggests that the sulfotransferase is also involved in the metabolism of DMBA in fish, possibly contributing to the DNA damage observed.

mRNAs of genes involved in protein translation machinery (ribosomal protein S8 and L37a-1) were upregulated in the liver of exposed eels. This suggests that new proteins are required to cope with the stress response. Specifically, mRNA of cysteine/glutamate transporter gene was upregulated in the exposed eels. This plasma membrane transporter is important for the uptake of cysteine used for the synthesis of glutathione which is essential for cellular protection from oxidative stress (Lo et al., 2008) and an important endogenous polar ligand for the biotransformation phase II reactions which contribute for the elimination of xenobiotics (van der Oost et al., 2003). Moreover, there is evidence that this transporter plays an important role in the growth and progression of cancers (Lo et al., 2008). As no tumours were observed (data not shown), the role of cysteine/glutamate transporter in DMBA-exposed eels is likely to be related to the DMBA phase II detoxification and/or the antioxidant defence, and consequently, protection of liver cells against oxidative damage. This result is in accordance to the low levels of DNA damage observed in liver cells when compared to those detected in gill and blood cells.

mRNAs of genes involved in the inflammatory and innate immune response were also upregulated in exposed eels. The presence of DNA strand breaks in mammalian cells has been related to immune suppression (O'Connor et al., 1996). In fish, although the PAH-induced immunotoxicity has been widely described (reviewed in Reynaud and Deschaux, 2006), little is known about the mechanism of induction. Here, mRNAs of the immune-related pentraxin, antitrypsin and serine protease genes were found downregulated in DMBA-exposed eels supporting the PAHs-mediated immunosuppression. Other upregulated mRNAs included the very large inducible GTPase1 (VLIG), tributyltin (TBT) binding protein and CCAAT/enhancer-binding protein beta2 (C/EBP).

The expression of NCK-associated protein 1 (Nap1) transcript was lower in DMBA-exposed eels than in controls. In humans, Nap1 protein plays an essential role in the regulation of actin cytoskeleton dynamics and its reduced expression has been linked to the induction of apoptosis of neuronal cells (Suzuki et al., 2000). Nap1 expression has also been found reduced in the liver and brain of benzo[a]pyrene-exposed fish (Brzuzan et al., 2007) suggesting that PAHs may affect the cytoskeleton dynamics of the cell or induce apoptosis in the affected fish.

In conclusion, DMBA exposure induced a toxic response in eels, as indicated by the study of traditional biomarker responses alongside with novel early-warning molecular biomarkers. The tissue-specific genotoxic responses obtained in the present work are related to organ location and function, tissue regeneration rate, capacity of protection against DNA damaging agents and DNA repair ability. The additional analysis performed with liver, revealed that the transcription of the checkpoint *rad1* gene, involved in the detection and repair of DNA lesions, is not induced by the levels of DNA damage observed in the hepatic cells. Additionally, no irreversible damage was detected by FCM in liver throughout the 90-day post-exposure, although important cell activities seemed to respond to DMBA exposure, including immune processes, protein translation and cytoskeleton dynamics. Alterations in these important cell processes may lead to future repercussions at organism-level of biological organization. The SSH approach also provided directions for new molecular biomarkers development. Finally, the application of novel molecular biomarkers allowed for a better understanding of the genotoxic implications of DMBA exposure in fish.

Acknowledgements

This work was supported by *Fundação para a Ciência e Tecnologia* through grants to Patrícia Nogueira (SFRH/BD/16763/2004) and Eleazar Rodriguez (SFRH/BD/27467/2006). The authors would like to express gratitude to Joana Torres and Eng[a] Olga Mayan from the *Centro de Saúde Ambiental e Ocupacional* (Porto, Portugal) for the assistance with comet assay slide analysis.

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