DNA adduct formation and induction of detoxification mechanisms in *Dreissena polymorpha* exposed to nitro-PAHs

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Received on January 28, 2014; revised on July 1, 2014; accepted on August 11, 2014

Derived polycyclic aromatic hydrocarbons (PAHs) such as nitro-PAHs are present in the environment and are known to be much more toxic than PAHs compounds. However, very few studies have analysed their effects on the aquatic environment and none have investigated the freshwater environment. In the present study, we determined whether 1-nitropyrene (1-NP), a model of nitro-PAHs, can induce DNA adducts in gills and digestive glands of the freshwater mussel *Dreissena polymorpha*. Two concentrations of 1-NP (50 and 500 μM) were tested. In addition, in order to understand the metabolic pathways involved in 1-NP genotoxicity, mRNA expression of genes implicated in biotransformation mechanisms was assessed by quantitative reverse transcription–PCR. Results showed the presence of DNA adducts in both gills and digestive glands, with highest levels obtained after 5 days of exposure to 500 μM. Metallothionein mRNA levels were enhanced in digestive glands exposed to 50 μM. Surprisingly, at the higher concentration (500 μM), aryl hydrocarbon receptor and HSP70 genes were only up-regulated in digestive glands while pgP mRNA levels were increased in both tissues. Results suggested a cytotoxic and genotoxic effect of 1-NP. Mussels seemed to be able to partially detoxify this compound, in view of the low amount of DNA adducts observed after 5 days exposure to 50 μM. For the first time, 1-NP biotransformation and detoxification systems have been characterised in *D. polymorpha*.

Introduction

Among contaminants found in urban environment, organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and their derivatives, including nitro-PAHs, are produced in high quantities by combustion of organic compounds (1). Nitro-PAHs such as nitropyrene (NP) and dinitropyrene are known to be carcinogens and mutagens (2,3). Due to their strong mutagenic activity at very low concentrations, their effects on humans have been investigated and DNA adducts have been controlled (4). The main source of nitro-PAHs results from incomplete combustion of organic compounds such as diesel and gasoline (5) but also from the reaction between PAHs and nitrogen oxides in ambient air (6). 1-1NP is the most abundant nitro-PAH in diesel exhaust particles and 30% of the direct mutagenicity of diesel is due to 1-NP (7). Nitro-PAHs have been reported in the atmosphere of Paris suburbs (8–13). Even though nitro-PAHs are mainly present in the atmosphere of urban areas, they have also been found in aquatic environments (14). However, very few studies have reported their effects in freshwater and seawater environments (14,15).

Genotoxicity end points have been demonstrated to be reliable markers of PAHs exposure in aquatic environment. Indeed, biomarkers of DNA alteration (DNA strand breaks, micronuclei or DNA adducts) have been reported to be trustworthy indicators of the genotoxic impact of pollutants in zebra mussels *Dreissena polymorpha*, a freshwater mussel largely used for biomonitoring in lakes and in rivers (16–19). DNA adducts are result from metabolically activated xenobiotics that covalently bind to DNA (20). Several studies have demonstrated interest in using DNA adducts (21–23) to evaluate biological effects of chemical contamination in water. DNA adduct formation was observed in both marine mussels (22,24,25) and freshwater mussels (21,22,26), exposed to model PAHs. However, nitro-PAH effects on aquatic organisms have not been studied so far even though they represent the main mutagenic contaminants from urban areas (4).

1-NP is known to be converted into reactive nitrenium ions in vertebrate and invertebrate cells. It has a strong affinity/reactivity with the C8 atom of guanine nucleotides of DNA and leads to bulky hydrophobic lesions in the genome (27,28) including N-(deoxyguanosine-8-yl)-1-aminopyrene (APG) (29). 1-NP is a direct mutagenic compound at low concentrations (pg/l). In brown trout and turbot, 1-NP causes DNA adducts in *in vitro* and *in vivo* as well as the activation of detoxification mechanisms (30). Further it was demonstrated that 1-NP caused mutations and apoptosis in liver cells through induction of Akt, ERK1/2, p38 and JNK phosphorylation (2). 1-NP has also been shown to increase intracellular levels of reactive oxygen species (ROS) in human cells (31).

In mussels, very few studies have shown the genotoxic effect of 1-NP (30). In two studies, invertebrates (marine mussels and oysters) were exposed to 1-NP. Of these, one was a laboratory study that analysed the toxicity of this compound (30), whereas the other was a field study where bioaccumulation of 1-NP in mussels and oysters was evaluated (32). In the marine mussel, it has been demonstrated that the cytochrome P450 inhibition prevented the formation of DNA strand breaks by 1-NP, indicating that 1-NP biotransformation via P450 led to DNA damage (30).

Proteins known to be implicated in PAHs biotransformation in mammals have also been characterized in mussels, including aryl hydrocarbon receptor (AHR), cytochrome P450 (CYP1a) (phase I), glutathione S-transferase (GST) (phase II), superoxide dismutase (SOD) and catalase (CAT) (33,34). It has been
accepted in mammals that PAHs enter cells through AHR owing to the lipophilic properties of this nuclear receptor. Once in cells, the AHR receptor/PAH complex acts as a transcription factor for many genes. Treatment of a hepatocyte human cell line with 1-NP resulted in an increase of both CYPIA gene expression and CYPIA protein activation via the Akt pathway but induced AHR activity to a lesser extent than benzo[a]pyrene (B[a]P) did (35), indicating that the 1-NP signalling pathway did not implicate AHR recruitment.

Genes encoding phase I and II proteins were recently sequenced in *D. polymorpha* (34). SOD represents the first defensive system against ROS production since it catalyses the dismutation of O$_2^-$ to H$_2$O$_2$. CAT catalyses the reduction of H$_2$O$_2$ into H$_2$O, whereas GST represents among others, one of the most important detoxification phase II enzymes (33), in terms of quantity. It catalyses conjugation reaction of glutathione with xenobiotics but also plays a role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (36). The HSP70 and the transmembrane protein transporter P-gp1 act by effluxing xenobiotics out of cells (37–39).

In invertebrates, metallothionein (MT) is also widely thought to play an important role in metal detoxification and in protecting cells against oxidative stress, notably nitrosation (40–44).

The objective of this study was to evaluate the relevance of DNA adducts as a biomarker of urban pollution in freshwater mussels.

For the first time in the present study, the effects of the major nitro-PAH, 1-NP, on *D. polymorpha* were investigated. Mussels were exposed in the laboratory to 1-NP and detection of DNA adducts was assessed using the $^3$H-postlabelling assay. Moreover, in order to characterise which parts of the metabolic pathways were involved in 1-NP-mediated genotoxicity, expression of genes implicated in phase I and II detoxification mechanisms were analysed by quantitative reverse transcription–PCR (qRT–PCR).

Materials and methods

Mussel sampling and maintenance conditions

Adult *D. polymorpha*, 18-22 mm long, were collected in March 2011 at a reference site Vertuzey (France) (48°55′33″N, 5°36′03″W). Animals were transferred to the laboratory, cleaned of all fouling organisms and kept in water (16°C) for 10 days acclimation before the experiments.

In vivo exposure of *D. polymorpha* to 1-NP

Mussels were exposed to 1-NP (N22959; Sigma) (50 and 500 μM) diluted in 0.001% dimethyl sulphoxide (DMSO) for 48 h or 5 days in glass tanks containing 12 l of Aquarel water (Nestlè), under artificial light and without feeding. Control mussels were incubated in water containing 0.01% DMSO as a solvent carrier. Media were completely renewed every 2 days. Digestive glands and gills (20 mussels per condition) were dissected at 48 h and 5 days after the beginning of exposure. The animals that had not attached to the tank were removed regularly. Tissues were then stored at −80°C for further analysis.

$^3$H-postlabelling analysis of DNA adducts

DNA isolation. DNA isolation was done as described previously (45). In brief, digestive glands or gills were homogenised in a solution containing NaCl (0.1 M), EDTA (20 mM) and Tris–HCl, pH 8 (50 mM) (SET). Proteins were precipitated by addition of sodium dodecyl sulphate and potassium acetate (6 M, pH 5). The supernatant, which contained nucleic acids, was collected and nucleic acids were precipitated overnight at −20°C. DNA purity was checked by recording ultraviolet spectra between 220 and 320 nm.

Radioactive spots were detected by autoradiography on Kodak super X-ray film. Autoradiography was carried out in the presence of an intensifying screen at −80°C for 48 h (47). The radioactivity was measured by a phosphor imager as described below.

Quantitation of total DNA adducts. For the quantification of total DNA adducts, the TLC plates were then placed in a cassette containing a storage phosphor screen (Amersham) overnight. Results were digitised using a storage phosphor imaging system (Typhoon™ 9210; Amersham) and quantified using ImageQuant™ 5.0 software. After background subtraction, the levels of DNA adducts were expressed as relative labelling adduct in total nucleotides. To calculate the levels of screen response (screen pixel) in dpm (disintegration per minute), samples of $^3$H-ATP at different concentrations from 10 to 500 dpm were appropriately diluted and spotted on a TLC plate. This TLC plate was then analysed on the Typhoon with the samples to obtain a radioactivity scale. The sensitivity allows detection of nitro-PAH adduct as low as 1 adduct/10$^6$ nucleotides.

Total RNA from control and exposed mussels was extracted using TRIzol Reagent as described (48). RNA concentration and purity were measured by spectrophotometric absorption at 260 and 280 nm. First strand complementary DNA (cDNA) synthesis was carried out on 1 μg of total RNA extract with oligo-dT primers according to Invitrogen (Promega). Preparations of digestive glands and gills cDNA were used to quantify specific transcripts in LightCycler 480 Real Time PCR System (Biorad) using SYBR Green Power Master Mix (Invitrogen) with the primers pairs listed in Table I. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp). Cp values for target genes (TG) were compared with the corresponding values for a reference gene (ribosomal S3 gene) to obtain ΔCp values ($\Delta C_p = C_{pTG} - C_{pRef}$) (49). PCR efficiency values for reference and tested genes were calculated as described (49).

Statistical analysis

Adduct and RT–qPCR results are given as mean values ± SD of three values (three mussels per condition pooled and three repetitions for each test). The measured values were compared among different groups using the non-parametric test Mann–Whitney. Statistical significance was accepted at a *P* < 0.05 (*), *P* < 0.01 (**) and *P* < 0.001 (***)

Results

DNA adduct formation

An example of DNA adduct patterns is presented in Figure 1. In gills of mussels treated with 1-NP, five different adducts...
Expression of genes implicated in detoxification mechanisms was investigated using quantitative RT–PCR. S3 ribosomal gene was chosen as the reference gene for gene expression normalization as demonstrated previously (26,50).

Generally, exposure of mussels to the lower concentration of 1-NP only caused an increase of PgP mRNA levels in gills and of MT mRNA levels in digestive glands. Following exposure to the higher dose resulted in only an increase of AHR, HSP70 and PgP gene expression in digestive glands, whereas in gills all of the genes studied were enhanced.

In gills exposed to 50 and 500 μM (Figure 3A) 1-NP, AHR mRNA levels were significantly decreased compared to control. The same result was also observed in digestive glands exposed to 50 μM. In contrast, a 500 μM 1-NP concentration up-regulated AHR mRNA as compared to control mussels (Figure 4A).

Regarding CAT gene expression, the difference from the control levels was not significant in gills in contrast to digestive glands where its expression was significantly down-regulated after exposure to both 50 and 500 μM 1-NP, with lowest levels registered after 5 days of treatment (Figures 3B and 4B).

Whatever the concentration and the duration of exposure to 1-NP, SOD mRNA expression was also significantly decreased in gills and digestive glands (Figures 3C and 4C).

GST mRNA level was significantly diminished in gills exposed to 1-NP for 48 h while in digestive glands, the decrease was significant for both concentrations at 48 h and 5 days (Figures 3D and 4D).

MT mRNA expression was significantly reduced in gills exposed to 50 and 500 μM 1-NP for 48 h and 5 days as well as in digestive glands after exposure to 500 μM. However, mRNA expression was slightly increased in mussels exposed to 50 μM for 48 h (Figures 3E and 4E).

In gills, HSP70 mRNA levels were significantly lower after 5 days of exposure to 50 μM 1-NP. In digestive glands, HSP70 mRNA levels were significantly reduced after 5 days of exposure to 50 and 500 μM 1-NP, but an increase was observed after 48 h exposure to 500 μM 1-NP (Figures 3F and 4F).

Finally, in the digestive glands, PgP mRNA levels were greatly increased after 48 h and 5 days exposure to 500 μM 1-NP while in gills, 5 days was necessary to see that change (Figures 3G and 4G).
Discussion

The present study highlights for the first time the effects of 1-NP on the formation of bulky DNA adducts in a freshwater organism, the zebra mussel *D. polymorpha*. In addition, in order to get a better understanding of the signalling pathways leading to this genotoxicity, expression of genes implicated in detoxification mechanisms was assessed by quantitative RT–PCR. All biomarker responses are summarised in Table II.

DNA adduct formation

In the present study, we demonstrated that 1-NP induces DNA adducts in zebra mussels. The amount of total DNA adducts detected in gills and digestive glands, ranging from below the limit of quantification to $7.18 \times 10^9$ nucleotides, are comparable to those obtained with BaP for zebra mussels (21,26) and for marine mussels (24,51,52).

These results demonstrate that freshwater mussels are able to rapidly biotransform 1-NP into compounds that have a strong affinity to DNA, hence forming DNA adducts. Biotransformation can lead to major adduct, APG adduct (29), and to other minor adducts (53); however, the DNA adducts spots reported here cannot be identified due to lack of adduct standards.

This study is the first that analyse the impact of 1-NP on DNA adduct formation in invertebrates. In fishes, such as brown trout and turbot, 1-NP has been demonstrated to induce DNA adducts both in vitro and in vivo as well as activation of detoxification mechanisms (30).

Gills, as the first tissue in contact with water, are constantly exposed to dissolved contaminants and are capable of metabolizing mutagens and carcinogens such as PAHs into reactive products in the digestive glands (30,54). Globally more 1-NP-DNA adducts are formed in digestive glands especially after 5 days of exposure. Moreover the pattern is different. This indicates that the detoxifying pathway is less effective in digestive glands than in gills, and that the biotransformation pathways are different. This could be due also to a bioaccumulation of the pollutant in digestive glands. Similar results were also observed in the marine mussel *Mytilus galloprovincialis* exposed to BaP (55). It has been suggested that tissue-specific enzymes such as cytochrome P450s could induce the formation of different metabolites during pollutant biotransformation processes and hence contribute to forming different DNA adducts in gills and in digestive glands (24), explaining the differences observed between these two tissues in terms of total quantity and adduct profiles.

As a whole, in both tissues, it appears that DNA repair mechanisms were not sufficient enough to remove DNA adducts. Among the DNA repair pathways existing in mammals, nucleotide excision repair is mainly involved in removing bulky DNA adducts (56), and especially proliferating cell nuclear antigen, activated in mussels exposed to PAHs, is known to bind to DNA for DNA repair (56–58).

Expression of metabolic activation and detoxification genes

Since studies concerning mechanisms implicated in 1-NP detoxification in invertebrates have not been reported, gene expression analysis in the freshwater mussel exposed to 1-NP was been performed using quantitative RT–PCR, using published primer sequences (34,50). As referred to in the latter work, S3 ribosomal gene was chosen as the reference gene for gene expression normalisation and t0 was used as control of the experiment.
Results showed that in gills AHR, CAT, SOD, GST, HSP70 and MT mRNA were down-regulated after exposure of the mussels to 1-NP. In contrast, in digestive glands, at the higher concentration, AHR and HSP70 genes were up-regulated whereas MT expression was enhanced at both concentrations. Only PgP mRNA expression was increased at the higher concentration of 1-NP in both organs.

1-NP is reported to increase the intracellular level of ROS and the expression of pro-inflammatory cytokines in human lung epithelial cells (31,59,60). It appears in our conditions...
that at low concentration, proteins implicated in detoxification mechanisms already present in cells (e.g. CAT, SOD, GST) might not be sufficient to detoxify 1-NP, assessed by the presence of DNA adducts and by a down-regulation of all gene expressions except MT in digestive glands. Even though MT is known to play an important role in detoxification mechanisms and particularly through oxygen free radical scavenging actions (41), its expression is not correlated with a decrease in DNA adducts nor with an induction of the other genes implicated in oxidative stress.

On the contrary, highest doses of 1-NP increase the metabolic activation/detoxification gene expression, which does not allow...
DNA adduct removal. Those high levels of DNA adducts in both organs could be due to mutations of DNA repair enzymes as demonstrated in *Escherichia coli* strains (61).

It has been observed that there is an induction of apoptosis in an hepatocyte cell line exposed in *vitro* to 10 and 30 μM 1-NP, through activation of AMP-dependent protein kinase and caspase 3 (62). Other authors demonstrated that 1-NP induced p53 expression, apoptosis and S-phase arrest in the same cell line (63).

Nevertheless, in this study, it seems that 1-NP effects on digestive gland cells occurred through the recruitment of AHR, as previously demonstrated for BaP in the same species (26). The inactive AHR, present in the cytoplasm, is bound to two molecules of HSP90 stress protein. Ligand binding to AHR results in the dissociation of HSP90 and the association with ARNT (AH receptor nuclear translocator) and the complex hence formed acts as a transcription factor for many genes such as CYP450 family and other enzymes (64,65). In mammalian systems, the major pathway of 1-NP has been suggested to occur through the recruitment of the cytochrome P450 (31,66).

HSP70 gene expression was also induced with high concentration of 1-NP in digestive glands. Its expression was enhanced in marine and freshwater mussels exposed to PAHs such as BaP (26,58), with a correlation to the formation of DNA adducts and oxidative damage (26,67).

PgP is a part of the multixenobiotic resistance mechanism, an established marker of xenobiotic exposure and especially PAH exposure (68). In this study, PgP gene expression was increased in both gills and digestive glands exposed to the higher concentration of 1-NP. PgP is involved in the excretion of xenobiotics and operates non-specifically, exporting non-metabolised parent xenobiotics, but also their metabolites and waste products derived from potential cell damage (69). In general, PAHs have been shown to induce PgP expression (70).

**Conclusion—perspectives**

For the first time, the genotoxic impact of 1-NP on the freshwater mussel *D. polymorpha* was analysed. We have shown (i) that this species is able to metabolise this compound, assessed by the formation of DNA adducts and (ii) that there are tissue-specific differences in biotransformation mechanisms. Henceforth, future investigations are needed to identify in particular apoptosis or repair mechanism induction under the same conditions so as to clearly understand detoxification mechanisms activated in response to 1-NP exposure in *D. polymorpha*.

Conflict of interest statement: None declared.

### References


