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Expression analysis of the molluscan p53 protein family mRNA in mussels (*Mytilus* spp.) exposed to organic contaminants

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ABSTRACT

In this study, we report the tissue expression analysis of the p53 protein family mRNA in mussels (*Mytilus* spp.) by means of quantitative RT-PCR. The tissue specific response was evaluated after 24 h exposure to a sublethal benzo[a]pyrene (B[a]P) concentration (75 nM), showing a 2.6 fold induction in digestive gland cells and a dramatic gene down regulation in circulating hemocytes. The comet assay and DNA gel diffusion tests showed significant effects in hemocytes and negligible differences in the digestive gland nuclei, implicating p53 in DNA damage of molluscan hemocytes. Finally, the kinetics of p53 protein family mRNA expression in the digestive gland of animals exposed to B[a]P and crude oil (0.5 ppm) showed partially overlapping trends, characterised by a common down regulation after 1 week exposure. These data should be carefully considered in view of the biological effects of organic pollutants and particularly following spills.

1. Introduction

Human P53 and its two homologues P63 and P73 form a protein family involved in the development, differentiation, cell transformation and response to stress (Bourdon, 2007). Human P63 and P73 proteins display a high structural homology to P53. The most prominent degree of homology with P53 is found in the TAD and DNA binding domains. Furthermore, the critical residues for the proper folding of the entire domain, as well as for the binding to the target DNA sequence, are strictly conserved (Strano et al., 2001). The P53 protein family displays a high degree of complexity, encompassing multiple alternatively spliced C-terminal and N-terminal truncated isoforms (Bourdon et al., 2005).

Polycyclic aromatic hydrocarbons, such as B[a]P, are products of incomplete combustion of organic matter and are widespread in the environment (Phillips, 1981). Carcinogenic effects of B[a]P have been well documented in mammalian models, but also in aquatic organisms (Hylland, 2006). B[a]P may induce apoptosis through P53 activation (Kim et al., 2005).

Homologues of the P53 family have been described also in invertebrates with roles in apoptosis and cell death as in the mammalian counterparts, i.e. the case of the symbiont-induced morphogenesis of the *Euprymna scolopes* light organ (Goodson et al., 2006). Instead, in several bivalve mollusks these proteins seem to be involved in the occurrence of hemocytic neoplasia. Barker et al. (1997) have demonstrated that leukemia in clam (*Mya arenaria*) hemocytes is associated with mutant P53 expression. Kelley et al. (2001) reported that P53 and P73 are absent in the nuclei of cancerous clam hemocytes. Increased mRNA levels of the p53 and ΔNp63/p73-like that lacks the full TA domain (Muttray et al., 2008), as well a raised cytosolic expression of the P73 protein (St-Jean et al., 2005) were observed in leukemic hemocytes of mussels. A common origin of the molluscan P53 family isoforms from a unique gene by means of alternative transcription start sites and splice variants has been proposed (Van Beneden et al., 1997; Kelley et al., 2001; Muttray et al., 2007).

The worldwide distribution of *Mytilus* spp. together with their ecological conditions (sessile, filter-feeding behaviour and ability to accumulate pollutants) makes them ideal species for: (i) investigation of the effect of toxic chemicals (Viarengo et al., 1990), (ii) environmental monitoring (Viarengo et al., 2007) and (iii) alternative model to study relationships between the environment and disease (Kelley et al., 2001; Auffret et al., 2006).

The present work aims for the first time to study the effects of organic compounds on the expression of the p53 protein family mRNA in mussel tissues and its possible implication in DNA damage.

2. Material and methods

2.1. Animals and treatments

Specimens of *Mytilus galloprovincialis* (Lam.) 4–5 cm shell length were purchased from an aquaculture farm in La Spezia (Italy)

Abbreviations: B[a]P, Benzo[a]pyrene; DMSO, dimethylsulfoxide; PCB, polychlorobiphenyls; PCR, polymerase chain reaction; RT, Reverse Transcription; TAD, Trans-Activation domain.

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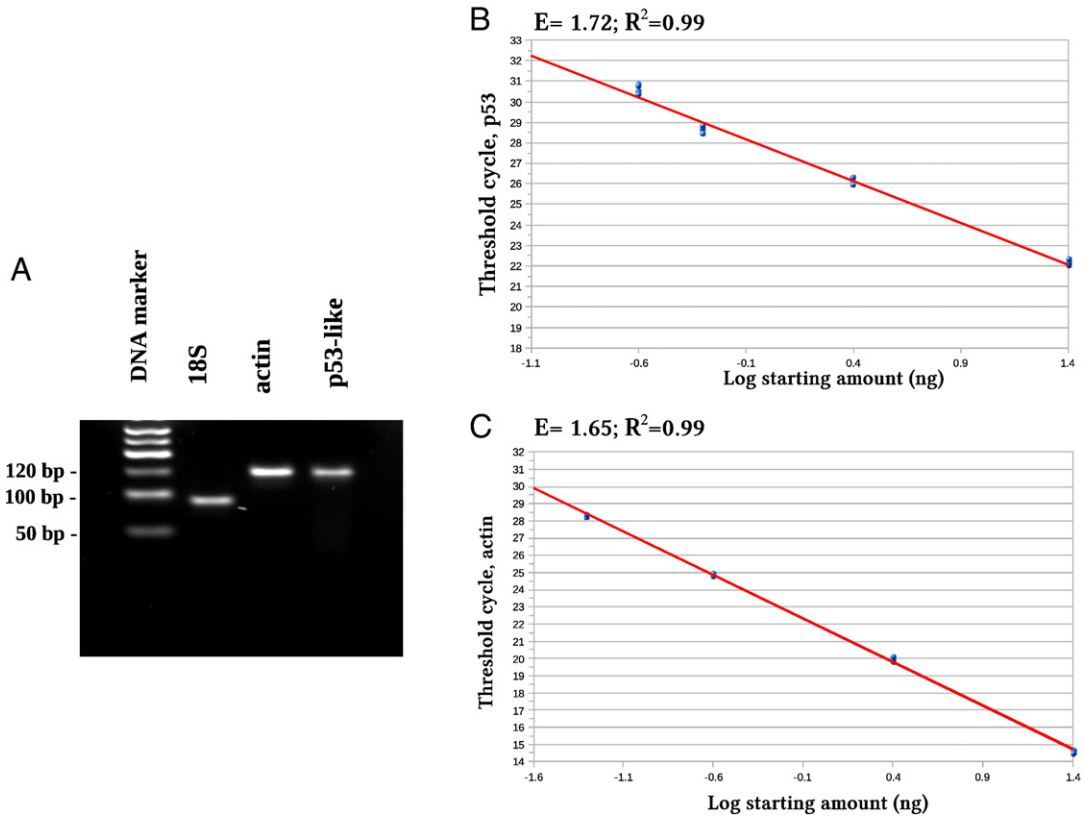

Fig. 1. Validation of Q-PCR probes. (A): High resolving agarose gel electrophoresis of the three investigated amplified cDNA targets. The 84 bp 18S, 100 bp actin, and 120 bp p53 protein family mRNA Q-PCR products, amplified from the digestive gland cell cDNA of B[a]P exposed mussels (lanes 2, 3, 4). (B): Standard curve for the p53 protein family mRNA target amplification obtained on mussel digestive gland tissue. (C): Standard curve for the actin target amplification obtained from the muscle tissue. Actin was included to get clues on house-keeping gene expression. The max Ct variation observed, expressed as coefficient of variation (CV), was 5%. Data represent the means of four independent experiments.

and further acclimatised to aerated clean seawater in an aquarium for 15 days at 16 °C (35‰ salinity, 1 L/animal). Mussels were then exposed in semi-static conditions to a sublethal concentration of B[a]P (75 nM, delivered in dimethylsulfoxide (DMSO) at the final concentration of 0.001%) for 6 days (water and pollutant renewed every day). In other experiments, the mussels (*Mytilus edulis*) were exposed in same semi-static conditions to 0.5 ppm North Sea crude Oil (NSO) dispersed in seawater for 8 days. A set of control animals for each treatment, was kept in same condition. Female individuals (scored by microscopic inspection of gonad biopsies) were selected for analysis. After treatments, circulating hemocytes were collected with a 5 mL syringe and a 17 gauge needle from the adductor muscle and washed with Ca^{2+}–Mg^{2+} free physiological solution (CMFS: 20 mM HEPES; 500 mM NaCl; 12.5 mM KCl; EDTA 0.005 M; pH 7.4) and pelleted by centrifugation at 500 ×*g* for 5 min at 4 °C. The mussel tissues (gills, mantle, abductor muscle and digestive gland) were rapidly removed out, washed into artificial seawater buffered with 20 mM Hepes, pH 7.4, flash frozen into liquid nitrogen, and stored at −80 °C until further analysis.

Digestive gland cells were prepared by mechanical dissociation of the tissue as previously described (Dondero et al., 2005). Cell preparations were scored for viability as described in Dondero et al. (2006), with the addition of the cell membrane impermeable DNA binding dye Fluoplus (IKZUS, Italy), instead of Sybr Green I.

2.2. Q-PCR analysis

The different mussel tissues and hemocytes were collected from 10 individuals for each treatment to be analyzed. About 10 mg of tissue and the entire hemolymph pellet were homogenized with 1 mL of the Trizol reagent (Sigma-Aldrich—St. Louis, USA) and total RNA extraction was performed according to the manufacture's instructions. A total amount of 0.5 to 1 µg of total RNA was reverse transcribed in a 20 µl reaction mixture using random hexamer primers (Roche) and [200 U] of M-MuLV H⁻ RT (Fermentas, Vilnius, LI), 0.5 mM dNTPs

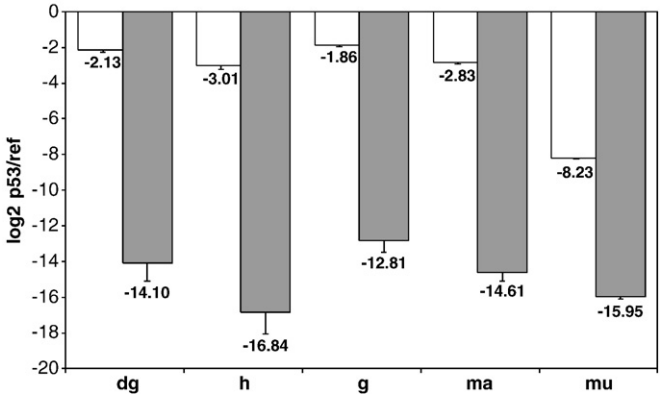

Fig. 2. Tissue expression analysis of the p53 protein family mRNA. Data are expressed in relative expression mode as the log2 ratio of the p53 RNA level with either actin (open bars) or 18S rRNA (solid bars). In the abductor muscle actin presented a clear bias due to its physiological role. Legend: dg, digestive gland; h, hemocytes; g, gills; ma, mantle; mu, muscle. Ratio have been calculated essentially according to Pfaffl (2001) as $^{p53}E^{-Ct}/{}^{ref}E^{-Ct}$, where ^{p53}E and ^{ref}E represent respectively PCR efficiencies for reference targets (18S or actin) and p53 protein family mRNA; Ct represents the threshold cycle of each corresponding target. Data represent the mean ± SEM (n = 10).