Toxicology and Applied Pharmacology 309 (2016) 63–76

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

AHR2 morpholino knockdown reduces the toxicity of total particulate matter to zebrafish embryos

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ARTICLE INFO

Article history:
Received 31 May 2016
Revised 23 August 2016
Accepted 25 August 2016
Available online 27 August 2016

Keywords:
Cigarette smoke
Total particulate matter (TPM)
Zebrafish development
Oxidative stress
AHR pathway

ABSTRACT

The zebrafish embryo has been proposed as a 'bridge model' to study the effects of cigarette smoke on early development. Previous studies showed that exposure to total particulate matter (TPM) led to adverse effects in developing zebrafish, and suggested that the antioxidant and aryl hydrocarbon receptor (AHR) pathways play important roles. This study investigated the roles of these two pathways in mediating TPM toxicity. The study consisted of four experiments. In experiment I, zebrafish embryos were exposed from 6 h post fertilization (hpf) until 96 hpf to $TPM_{0.5}$ and $TPM_{1.0}$ (corresponding to 0.5 and 1.0 μg/mL equi-nicotine units) in the presence or absence of an antioxidant (*N*-acetyl cysteine/NAC) or a pro-oxidant (buthionine sulfoximine/BSO). In experiment II, TPM exposures were performed in embryos that were microinjected with nuclear factor erythroid 2-related factor 2 (Nrf2), AHR2, cytochrome P450 1A (CYP1A), or CYP1B1 morpholinos, and deformities were assessed. In experiment III, embryos were exposed to TPM, and embryos/larvae were collected at 24, 48, 72, and 96 hpf to assess several genes associated with the antioxidant and AHR pathways. Lastly, experiment IV assessed the activity and protein levels of CYP1A and CYP1B1 after exposure to TPM. We demonstrate that the incidence of TPM-induced deformities was generally not affected by NAC/BSO treatments or Nrf2 knockdown. In contrast, AHR2 knockdown reduced, while CYP1A or CYP1B1 knockdowns elevated the incidence of some deformities. Moreover, as shown by gene expression the AHR pathway, but not the antioxidant pathway, was induced in response to TPM exposure, providing further evidence for its importance in mediating TPM toxicity.

1. Introduction

Maternal smoking during pregnancy remains a serious problem for the developing fetus/newborn. In comparison to adults, the developing fetus has increased sensitivity to the chemicals present in tobacco smoke; this is likely due to intensive cell proliferation, lower immunocompetency, and a lower detoxification capacity (Votavova et al., 2011). Hence, prenatal tobacco exposure is associated with adverse effects during pre and postnatal development (Rogers, 2009). Specifically, maternal smoking is associated with low birthweight, increased risk of stillbirth, altered cardiovascular responses, increased asthma and wheezing (Knopik, 2009), as well as neonatal malformations (Hackshaw et al., 2011) and neurobehavioral effects (Rogers, 2009). Despite years of research on the toxicity of cigarette smoke, precise mechanisms that mediate early developmental toxicity are still not fully understood.

Recent studies argue that the zebrafish embryo model is a useful 'bridge model' between *in vitro* and *in vivo* mammalian models that enables the investigation of mechanistic aspects relating to cigarette smoke toxicity during early development (Ellis et al., 2014; Massarsky et al., 2015). These studies have demonstrated that total particulate matter (TPM) from cigarette smoke induces deformities and behavioral changes among other adverse effects. Oxidative stress, as evidenced by increased oxidized glutathione concentrations and glutathione reductase activity, could play a role in TPM-induced toxicity (Massarsky et al., 2015). The importance of the aryl hydrocarbon receptor (AHR) pathway, including phase I and phase II enzymes, in mediating TPM toxicity was also proposed (Ellis et al., 2014; Massarsky et al., 2015).

Consequently, the current study was designed to test the two hypotheses generated by these previous studies. The study consisted of four experiments. Experiment I aimed to elucidate the involvement of oxidative stress in TPM toxicity by performing co-treatments with the antioxidant *N*-acetyl cysteine (NAC) or pro-oxidant buthionine

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http://dx.doi.org/10.1016/j.taap.2016.08.024
0041-008X/

sulfoximine (BSO) (Fig. 1A). We predicted that BSO co-treatment would exacerbate, while NAC co-treatment would reduce TPM toxicity.

Experiment II utilized the morpholino knockdown approach to investigate the role of the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor that regulates several antioxidant genes (Jung and Kwak, 2010). Experiment II also included the knockdown of AHR2, cytochrome P450 1A (CYP1A), or CYP1B1 to elucidate the role of the AHR pathway (Fig. 1B). We predicted that Nrf2 knockdown would challenge the antioxidant system, exacerbating TPM toxicity, while AHR2 knockdown would suppress xenobiotic metabolism and the generation of harmful metabolites, thereby reducing TPM toxicity. As for CYP1A knockdown, it was predicted that TPM toxicity would be exacerbated, since CYP1A appears to have a protective role (see Discussion). Lastly, it was predicted that CYP1B1 knockdown would not affect TPM toxicity, since CYP1B1 has been shown not to alter the synergistic teratogenicity of polycyclic aromatic hydrocarbons (PAHs) (Timme-Laragy et al., 2008), which are abundant in TPM.

Experiment III used non-NAC/BSO/morpholino-treated embryos to test the hypothesis that TPM constituents and/or their metabolites lead to oxidative stress, thus upregulating the antioxidant system, in order to enhance the antioxidant capacity. Therefore, mRNA transcript abundance of several genes associated with the antioxidant system [Nrf2, glutathione peroxidase (GPx1), superoxide dismutase (CuZnSOD, MnSOD), and glutamate-cysteine ligase (GCLc)] was assessed. It was also hypothesized that TPM exposure would upregulate the AHR pathway, in order to increase the metabolism and elimination of chemicals present in TPM as well as their metabolites. Thus, the transcript abundance of several genes associated with the AHR pathway [AHR2, AHR repressors (AHRR1, AHRR2), CYP1A, CYP1B1, CYP1C1, and glutathione-*S*-transferases (GSTp1, GSTp2)] was assessed.

Lastly, experiment IV assessed the activity of CYP1A. Our previous study reported that TPM reduced CYP1A activity in 72 hpf larvae. Since the AHR pathway is the focus of this study, we thought it would be essential to verify CYP1A activity with alternative methods. The protein levels of CYP1A and CYP1B1 were also assessed.

2. Materials and methods

2.1. Chemicals

TPM was prepared at Labstat International Inc. (Kitchener, ON, Canada) by mechanical smoking of 3R4F reference cigarettes (University of Kentucky) following the ISO standard 3308:2012, which defines a puff volume of 35 mL, an interval of 60 s between puffs, and a puff duration of 60 s (Johnson et al., 2009). TPM was collected onto Cambridge glass fiber filter pads. These filter pads typically retain at least 99.9% of TPM, and the particle size of TPM is reported to be ≥0.3 μm in diameter (Johnson et al., 2009). The collected TPM was then dissolved in DMSO at a concentration of 20 mg TPM/mL to generate the stock solution and stored at −80 °C until used. The partial analysis of the TPM stock solution was performed at Labstat International as reported previously (Massarsky et al., 2015). All other reagents were purchased from Sigma Aldrich unless otherwise specified.

2.2. Fish husbandry and zebrafish embryo collection

Adult EkkWill zebrafish (EkkWill Waterlife Resources, Ruskin, FL, USA) were maintained in holding tanks on a 14:10 h light-dark cycle at 28 °C in circulating AHAB system (Aquatic Habitats, Apopka, FL, USA) in 60 mg/L salt water (Instant Ocean, Foster & Smith, Rhinelander, WI, USA). Fish were fed brine shrimp in the morning and a 1:1 mixture of Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats) and Cyclop-eeze (Argent Chemical Laboratories, Redmond, WA, USA) in the afternoon. Breeding tanks were set at 1700, and embryos were collected the following morning within half an hour of spawning. All procedures were approved by the Duke University Institutional Animal Care & Use Committee (A279-08-10).

2.3. Experimental set-up

The study was composed of four experiments. For experiments I and II, 15 embryos were randomly assigned into 6 cm diameter glass Petri dishes, containing a total volume of 15 mL 30% Danieau (in mM: 58

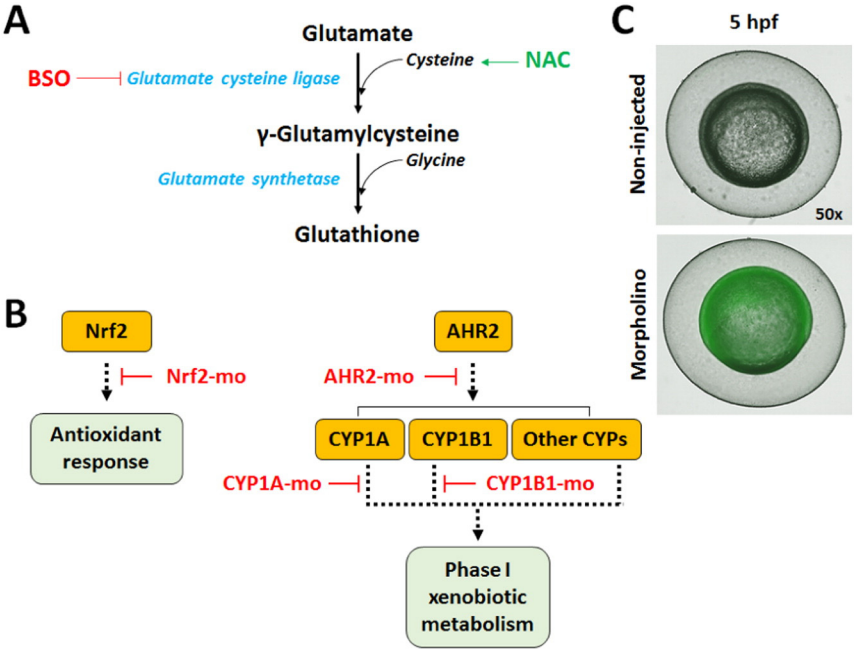

Fig. 1. A. The antioxidant function of NAC involves its conversion into cysteine, thereby increasing the generation of glutathione. BSO inhibits the action of glutamate cysteine ligase, thereby decreasing the generation of glutathione. B. Morpholinos that were used in this study: Nrf2 is a transcription factor that binds to the antioxidant response element to increase the antioxidant capacity; AHR2 is a receptor that upon ligand binding translocates into the nucleus, where it binds to the xenobiotic response element to upregulate xenobiotic metabolism, including CYP enzymes. C. Morpholino-injected embryos were screened for fluorescence to confirm incorporation. Merged bright-field and fluorescent images are presented.