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Effects of Anthropogenic Pollution on the Oxidative Phosphorylation Pathway of Hepatocytes from Natural Populations of *Fundulus heteroclitus*

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ABSTRACT

Persistent organic pollutants (POPs), including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), potentially target mitochondria and cause toxicity. We compared the effects of POPs on mitochondrial respiration by measuring oxidative phosphorylation (OxPhos) metabolism in hepatocytes isolated from lab-depurated *Fundulus heteroclitus* from a Superfund site contaminated with PAHs (Elizabeth River VA, USA) relative to OxPhos metabolism in individuals from a relatively clean, reference population (King's Creek VA, USA). In individuals from the polluted Elizabeth River population, OxPhos metabolism displayed lower LEAK and lower activities in complex III, complex IV, and E State, but higher activity in complex I compared to individuals from the reference King's Creek population. To test the supposition that these differences were due to or related to the chronic PAH contamination history of the Elizabeth River population, we compared the OxPhos functions of undosed individuals from the polluted and reference populations to individuals from these populations dosed with a PAH {benzo [α] pyrene (BaP)} or a PCB {PCB126 (3,3′,4,4′,5-pentachlorobiphenyl)}, respectively. Exposure to PAH or PCB affected OxPhos in the reference King's Creek population but had no detectable effects on the polluted Elizabeth River population. Thus, PAH exposure significantly increased LEAK, and exposure to PCB126 significantly decreased State 3, E state and complex I activity in the reference King's Creek population. These data strongly implicate an evolved tolerance in the Elizabeth River fish where dosed fish are not affected by PAH exposure and undosed fish show decreased LEAK and increased State 3 and E state.

1. Introduction[1]

Persistent organic pollutants (POPs) are some of the most prevalent pollutants because of their resistance to environment degradation and propensity to bioaccumulate (Fisher, 1999; Arnot et al., 2011; Ruzzin 2012). Both polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are POPs of major concern. PAHs, which are released into the environment primarily through incomplete organic matter combustion (Walker et al., 2005), are organic carbon compounds composed of fused aromatic ring structures (Jung et al., 2011). They are a toxicologically important class of pollutants because some compounds have been identified as carcinogenic or mutagenic (Srogi, 2007), and their environmental significance has been increasing partially due to the elevated rate of fossil fuel consumption (Van Metre et al., 2000). PCBs, which were commercially manufactured and marketed in the U.S. from 1929 to 1977, are chemically stable, have high boiling points, low solubility, and their nonconductive nature cause them to persist in the environment and bioconcentrate. These traits create potential hazards that affect natural biota and human populations (Weaver, 1984). Understanding the biochemical impact of PAHs and PCBs on natural populations provides insight into their toxicology and a greater understanding of their involvement in health and disease (Whitehead et al., 2011).

POPs contribute significantly to human diseases: they are associated with cancers and mutagenesis and affect arteriosclerosis, intrauterine growth retardation and neurological development (Fisher, 1999; Jones and de Voogt 1999; Li et al., 2006; Porta et al., 2008; Lim et al., 2010; Arnot et al., 2011; Ruzzin 2012). Initially, the primary health concerns about POP exposure focused on carcinogenicity and mutagenicity (Fisher, 1999; Li et al., 2006; Yu et al., 2010; Arnot et al., 2011; Ruzzin 2012; Lee et al.,

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[1] POP, persistent organic pollutant; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; OxPhos, oxidative phosphorylation; BaP, benzo [α] pyrene; ANT, adenine nucleotide translocase; UCP, uncoupling protein; HRR, high-resolution respirometry; ER, Elizabeth River; KC, King's Creek; RCR, respiratory control ratio; UCR, uncoupling control ratio; QC, quality control.

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2014). More recently, POP exposure has been associated with metabolic diseases, including type 2 diabetes, obesity, and energy metabolism (Lee et al., 2006; Lim et al., 2010; Airaksinen et al., 2011; Karami-Mohajeri and Abdollahi 2011; Lee et al., 2011; Ruzzin, 2012). These associations link increased POP body burdens with increased metabolic disorder incidences. However, there is little experimental evidence demonstrating that POPs directly affect the biochemical pathway responsible for most cellular energy production – the oxidative phosphorylation (OxPhos) pathway.

OxPhos is the metabolic pathway that produces most of the ATP in aerobic animals. This mitochondrial respiratory chain pathway consists of 89 proteins that form five multisubunit OxPhos enzyme complexes imbedded in the inner mitochondrial membrane (Hatefi 1985; Smeitink et al., 2001; Pagliarini and Rutter 2013). During OxPhos, complexes I and II accept reduction equivalents from NADH and $FADH_2$ respectively, and energy harvested from electron transfer drives respiratory complexes I, III, and IV to pump protons into the mitochondrial intermembrane space. This pumping activity creates a proton gradient across the inner membrane that powers complex V to generate ATP (Hatefi, 1985; Boyer, 1997; Schultz and Chan, 2001). Since protons can leak across the inner membrane thus relieving the proton gradient, OxPhos is considered incompletely coupled (Divakaruni and Brand 2011). Two protein families that influence proton leak are adenine nucleotide translocase (ANT) and uncoupling proteins (UPC) (Divakaruni and Brand 2011). Genetic variation in these proteins or their expression levels could alter proton leak and affect mitochondrial membrane potential.

Mitochondrial membrane potential loss, ATP production decreases, and mitochondrial morphology changes (Zhu et al., 1995; Li et al., 2002; Ko et al., 2004; Xia et al., 2004) have been linked to one POP class, polycyclic aromatic hydrcarbons (PAHs). Exposure to another POP class, polychlorinated biphenyls (PCBs), has been reported to inhibit electron transfer, respiratory enzymes and mitochondrial respiration and increase OxPhos uncoupling (Pardini, 1971; Sivalingan et al., 1973; Chesney and Allen, 1974). Identifying the steps in the OxPhos pathway targeted by pollutants is essential to understanding the molecular basis of chronic pollutant toxicity, especially its involvement in metabolic health and disease.

We examined POP effects on OxPhos enzyme function in a population that has adapted to high POP concentrations compared to a reference "clean" population. A population of the salt marsh minnow, *Fundulus heteroclitus*, from the Elizabeth River, VA inhabits a Superfund site (an uncontrolled or abandoned site in the United States where hazardous waste is located) highly contaminated with PAHs (average ~200–400 μg/g sediments) and is resistant to the developmental toxicity of the sediments (Meyer and Di Giulio, 2002; Ownby et al., 2002; Meyer and Di Giulio, 2003; Wassenberg and Di Giulio, 2004; Burnett et al., 2007). In contrast, a nearby *F. heteroclitus* population from King's Creek, VA has been used as a genetically similar reference population. This reference population has much lower sediment PAH levels (<0.4% of Elizabeth River PAH concentrations) (Jung et al., 2011; Clark et al., 2013) and is sensitive to the toxicity of the polluted sediments from the Elizabeth River Superfund site (Meyer and Di Giulio 2002; Meyer and Di Giulio, 2003; Burnett et al., 2007; Whitehead et al., 2011). We tested the hypothesis that *F. heteroclitus* from a site chronically polluted with PAHs (Elizabeth River, VA) would have altered OxPhos metabolism as compared to *F. heteroclitus* from a nearby reference site (King's Creek, VA). We speculated that the polluted Elizabeth River population would compensate for chronic exposure to PAHs with altered OxPhos metabolism to cope with any disrupted membrane lipid bilayer dependent functions affecting ATP production.

To better understand potentially genetic effects as opposed to physiologically induced effects, we compared OxPhos enzyme function in fish that had been depurated in the laboratory for six months. To better understand physiologically induced POP effects on OxPhos functions, we dosed fish with two different POPs, benzo[α]pyrene (BaP) and polychlorinated biphenyl-126 (PCB-126). We dosed with two different POP classes because *F. heteroclitus*' adapted populations are resistant to a broad range of pollutants not found in their native habitats (Elskus et al., 1999; Nacci et al., 1999a,b; Bello et al., 2001; Meyer and Di Giulio, 2002; Clark and Di Giulio 2012). This dosing experiment allowed us to address two important questions: (i) Could PAH or PCB dosing induce acute, direct effects on the OxPhos functions of natural *F. heteroclitus* populations? (ii) Did the polluted Elizabeth River population and reference King's Creek population respond similarly to PAH and PCB dosing?

2. Materials and methods

2.1. Fish husbandry and treatments

F. heteroclitus were collected from Elizabeth River, VA (36°48'26.20"N, 76°17'9.83"W, [EPA ID VAD990710410]) and a nearby reference site, King's Creek, VA (37°15'43.38"N, 76°29'4.57"W), by minnow traps in June 2012. Fish were depurated in re-circulating aquatic system tanks for 6 months with controlled temperature (20° C) and salinity (15 ppt). Fish were checked for health and fed daily (brine shrimp flake, blood meal flake, and Spirulina flake– FOD, Aquatic Biosystems).

Fish for the dosing experiment were similarly collected from Elizabeth River, VA and King's Creek, VA in May 2013. Fish were depurated in re-circulating aquatic system tanks for 4 weeks with controlled temperature (20° C) and salinity (15 ppt) and then dosed by intraperitoneal injection (IP injection) with either 50 mg/kg body weight (198.2 moles/kg) PAH (BaP) or 10 mg/kg body weight (30.6 moles/kg) PCB126 dissolved in corn oil with an injection volume of 5 μL/g body weight (Fig. 1). Twenty-four hours later, fish hepatocytes were harvested and OxPhos functions were quantified *via* high resolution respirometry. Control or undosed groups for each pollutant were injected with corn oil only for 24 h. Doses were based on previous studies (Willett et al., 1995; Karami et al., 2011) and showed an effect on OxPhos functions in a preliminary time course experiment (data not shown). Experimental procedures were carried out following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami.

We used BaP as a representative PAH in the dosing because BaP is one of the most intensively studied PAHs. Importantly, it is of high concentration in sediments and water from the Elizabeth River Superfund site (Vogelbein et al., 1990; Bozinovic and Oleksiak 2010) and thus relevant for investigating the Elizabeth River population. We also used the PCB congener, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) as a representative PCB. This congener represents contaminants that are mediated through the aryl hydrocarbon receptor (AHR) pathway and such contaminants encompass major categories of toxic, organic anthropogenic pollutants (Nacci et al., 2010).

2.2. Hepatocyte isolation and permeabilization

We used isolated liver hepatocytes because of the liver's importance in regulating many metabolic and physiological processes, particularly xenobiotic metabolism (Segner 1998). To measure OxPhos functions requires either (1) isolated mitochondria or (2) permeabilized cells so that substrate and inhibitor can be introduced to mitochondria. We used permeabilized hepatocytes