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The roles of bone morphogenetic protein 2 in perfluorooctanoic acid induced developmental cardiotoxicity and L-carnitine mediated protection

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ABSTRACT

Perfluorooctanoic acid (PFOA), a wide spread environmental pollutant, was associated with developmental cardiotoxicity in chicken embryo, while the underlying molecular mechanism had not been fully elucidated. In the current study, 2 mg/kg (egg weight) PFOA and/or 100 mg/kg (egg weight) L-carnitine were exposed to embryonic day zero (ED0) chicken embryo via air cell injection, and then bone morphogenic protein 2 (BMP2) silencing lentivirus or BMP2 recombinant protein were introduced into ED2 embryo. Electrocardiography and histological methods were utilized to assess the cardiac function and morphology in hatchling chickens, respectively. Consistent with previous results, 2 mg/kg PFOA exposure at ED0 significantly elevated heart rate and thinned right ventricular wall in hatchling chickens, while L-carnitine co-treatment reverted such changes. BMP2 silencing induced very similar changes in hatchling chicken hearts as PFOA exposure, while co-exposure of recombinant BMP2 protein alleviated PFOA-induced changes. L-carnitine exposure alleviated the BMP2-silencing induced changes as well. Western blotting revealed that PFOA exposure enhanced BMP2 expression and suppressed pSMAD1 expression in ED15 chicken embryo hearts, while both changes were reverted by L-carnitine co-exposure. Furthermore, silencing of BMP2 significantly increased the expression level of PPAR alpha in ED15 chicken embryo hearts, while silencing of PPAR alpha did not have significant impact on BMP2 expression. In conclusion, BMP2/pSMAD1 signaling participates in the PFOA-induced developmental cardiotoxicity in chicken embryo, which is likely located upstream of PPAR alpha for this particular endpoint. Protection of BMP2 signaling might contribute to L-carnitine mediated protection against PFOA-induced developmental cardiotoxicity.

1. Introduction

Perfluorinated compounds (PFCs) are widely used in the production of fluoropolymers. One of the most prominent members of PFCs, perfluorooctanoic acid (PFOA), is ubiquitously used in the production of polymer products, including non-stick plastic coatings, water repellent coatings and flame retardants (Begley et al., 2005). PFOA used to be considered biologically inert because of its chemical inertness, however, starting from the late 1990s, it had been associated with various adverse health effects. Concerns were further raised because of its extreme stability in both environment and biota (Fromel and Knepper, 2010). The most well-known adverse health effects induced by PFOA exposure included carcinogenicity (Biegel et al., 2001), immunotoxicity (DeWitt et al., 2008), endocrine disruption (Vested et al., 2013) and developmental toxicity (Wolf et al., 2007).

The production of PFOA had been phased out in the United States in 2015 (Bjerregaard-Olesen et al., 2016), however, its production continues in other parts of the world, such as China (Liu et al., 2017). Thus, the investigation of PFOA-induced adverse health effects is still demanded and ongoing. Myriad efforts had been made to elucidate its mechanism of toxicity, but much still remain to be done. PFOA is generally considered as a proliferator-activated receptor alpha (PPAR alpha) agonist (Wolf et al., 2008), which is required for the PFOA-induced general developmental toxicity in mice (Abbott et al., 2007). However, as the PPAR alpha independent effects had been clearly demonstrated, many evidences also show that PFOA behaves more than PPAR alpha agonist (Buhrke et al., 2015; Filgo et al., 2015).

Among the developmental effects induced by PFOA exposure, our work focused on the developmental cardiotoxicity in chicken embryo. PFOA was found to be capable of inducing morphological and functional changes in developing chicken embryo hearts (Jiang et al., 2012), which was partially associated with PPAR alpha (Jiang et al., 2013).

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However, in that study, PPAR alpha agonist WY 14,643 did not induce identical response as PFOA, indicating that PPAR alpha independent effects are not ignorable in PFOA-induced developmental cardiotoxicity. In our further studies, both the PPAR alpha antagonist GW 6471 and PPAR alpha *in ovo* silencing partially blocked PFOA-induced effects, but not completely reverted the changes (Jiang et al., 2016; Zhao et al., 2017a), further confirming that PPAR alpha plays an important role in PFOA-induced developmental cardiotoxicity, but other signaling also have their roles.A potential target that had not been fully elucidated is the bone morphogenic protein 2 (BMP2), a critical regulator protein in development (Rogers et al., 2015). It was shown that PFOA developmental exposure interfered with BMP2 signaling (Jiang et al., 2013). However, this previous study only included descriptive data regarding to BMP2. In the current study, the lentivirus-based *in ovo* gene silencing technique developed in our lab (Zhao et al., 2017a) was utilized to silence BMP2 in developing chicken embryo heart. Additionally, recombinant active BMP2 protein was introduced into the developing embryo, so that both suppressed and enhanced expression of BMP2 were studied as potential molecular mechanisms of PFOA-induced developmental cardiotoxicity in chicken embryo. Furthermore, the PPAR alpha silenced samples were also utilized to demonstrate the potential interactions between PPAR alpha and BMP2 in developing chicken embryo hearts. Our data added to the knowledge base of PFOA induced developmental toxicity, and provided clues for potential protection against such effects.

2. Materials and methods

2.1. Materials

PFOA (77,262, CAS 3825-26-1), and L-carnitine (C0158, CAS 541-15-1) were purchased from Sigma Aldrich (St. Louis, MO, US). Fertile chicken (*Gallus gallus*) eggs were purchased from Linwen Trading Co. Ltd. (Jining, Shandong, China). Lentivirus (Control lentivirus, BMP2 silencing lentivirus or PPAR alpha silencing lentivirus) were designed and synthesized by Genechem (Shanghai, China). The detailed information about the BMP2 silencing lentivirus is reported in Table 1. Information about the PPAR alpha silencing lentivirus can be found in Zhao et al. (2017a). Recombinant human BMP2 protein was purchased from Cusabio (College Park, MD, US). According to Haugen and Johnson (2010), human BMP2 shares 96% identity with chicken and is effective in chicken cells/tissues. Primers for BMP2 and PPAR alpha were designed and synthesized by Sangon Biotech (Shanghai, China), the sequences were reported in Table 2. The primer for GAPDH was purchased from BBI Life Sciences (Shanghai, China). Antibodies against PPAR alpha and BMP2 were purchased from Bioss (Beijing, China). Antibody against GAPDH was purchased from ZSbio (Beijing, China). Other chemicals and consumables were of the highest grade obtainable.

Table 2
Primer sequences used in qRT-PCR for BMP2 and PPAR alpha[a].

NO.	Sequence	Product size
BMP2-Forward	TGGATTGCACACGGACAACCTAAC[a]	148
BMP2-Reverse	CTAACAACGGCCTGAGCTGAGAC	148
PPAR alpha-Forward	CATCCACATCCAGGCTTCGCTAC	91
PPAR alpha-Reverse	GCGTCTCCGTGAAGGCAGTTC	91

[a] Primers were designed and synthesized by Sangon Biotech (Shanghai, China).

2.2. Methods

2.2.1. Chicken embryo incubation

Fertile chicken eggs (embryonic day zero, ED0) were ordered and shipped to the laboratory overnight in protective foam boxes. Upon arrival, surface of the eggs was cleaned with povidone iodide solution and carefully dipped dry with tissue papers. Eggs were then candled in dark room, the air cell area was marked with pencil on the shell. Broken eggs were detected and removed at this stage. Cleaned and candled eggs were weighed and assigned to different treatment groups evenly based on egg weight. A Keyu incubator (Dezhou, Shandong, China) was used to incubate the eggs. The incubation conditions were controlled by the incubator automatically. Briefly, the temperature was 37.9 degree Celsius at the beginning of incubation, which gradually decreased as incubation progressed, and was 37.1 degree prior to hatch. Humidity started at 50% and gradually increased along the incubation, and was 70% prior to hatch. The incubator automatically turned the eggs every 3 h until ED19. Between ED19 and ED20, eggs were moved to individual hatch boxes to hatch, allowing the correct identification of individual animals. Once hatched, chickens were kept in a warm box and supplied with clean water until use. All the procedures used in this study followed national institutes of health guide for the care and all animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao University.

2.2.2. Air cell injection

Air cell injection was performed as described in Jiang et al. (2012). According to the groups they were assigned to, fertile chicken eggs (ED0) were injected respectively with vehicle (sunflower oil), 2 mg/kg (egg weight) PFOA, 100 mg/kg (egg weight) L-carnitine or 2 mg/kg (egg weight) PFOA + 100 mg/kg (egg weight) L-carnitine. The dosing suspensions contained PFOA 20 mg/ml and/or L-carnitine 1000 mg/ml in sunflower oil, so that the 0.1 ul/g (egg weight) injection achieved the desired final doses. Note that 20% distilled water and 10% ethanol were added to the L-carnitine containing suspensions to improve the stability of L-carnitine. Corresponding volume of oil was subtracted from the L-carnitine containing suspensions so that the total volume remains identical.

The air cell area of fertile chicken eggs was sterilized with 70% ethanol, and then a metal probe was used to drill small holes (approximately 1 mm in diameter) in the center. The dosing suspensions

Table 1
siRNA sequences of the BMP2 silencing le ntivirus used in transfection.

NO.	5′	STEM	Loop	STEM	3′
BMP2-RNAi(59847-1)-a	Ccgg	CTGCCAGAAACAAGTGGGAAA	CTCGAG	TTTCCCACTTGTTTCTGGCAG	TTTTTg
BMP2-RNAi(59847-1)-b	aattcaaaaa	CTGCCAGAAACAAGTGGGAAA	CTCGAG	TTTCCCACTTGTTTCTGGCAG	
BMP2-RNAi(59848-1)-a	Ccgg	CGCCACAAATACAGTTGCAAA	CTCGAG	TTTGCAACTGTATTTGTGGCG	TTTTTg
BMP2-RNAi(59848-1)-b	aattcaaaaa	CGCCACAAATACAGTTGCAAA	CTCGAG	TTTGCAACTGTATTTGTGGCG	
BMP2-RNAi(59849-1)-a	Ccgg	ATAATGCAAGTAAATGGGAAA	CTCGAG	TTTCCCATTTACTTGCATTAT	TTTTTg
BMP2-RNAi(59849-1)-b	aattcaaaaa	ATAATGCAAGTAAATGGGAAA	CTCGAG	TTTCCCATTTACTTGCATTAT	

BMP2 silencing lentivirus were designed and synthesized by Genechem (Shanghai, China).
The lentivirus vector used was GV248, with the components: hU6-MCS-Ubiquitin-EGFP-IRES-puromycin. For details, please refer to http://www.genechem.com.cn:8080/Zaiti.aspx?zt=GV248