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硕士学位论文

转录组学比较分析 17α-炔雌醇不同时间暴露的海水青鳉免疫毒性效应及其机制

Comparative transcriptome analysis on immunotoxic effect and mechanisms under different exposure time of EE2 on *Oryzias melastigma*

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目 录

Contents

摘 要

17α-炔雌醇（EE2）是一种广泛存在于海洋环境中的内分泌干扰物，具有多种毒性效应，影响鱼类的生殖生长和健康。EE2 对鱼类生殖毒性效应方面的研究已有较多报道，但关于 EE2 免疫毒性机制的研究相对较少。本研究选择典型的内分泌干扰物 EE2 为受试物，以海洋模式动物海水青鳉（*Oryzias melastigma*）为研究对象，采用 RNA-seq 测序技术构建了海水青鳉雄鱼肝脏转录组文库，以本实验室前期研究中克隆鉴定的抗菌肽（hepcidin）为目标免疫因子，选取表达变化最为显著的 12 h（*OM-hep2* 上调）和 48 h（*OM-hep1* 下调）两个时间点，以及有效的 EE2 暴露浓度（500 ng/L），通过对 EE2 暴露 12 h 和 48 h 后的海水青鳉雄鱼肝脏内分泌、应激反应以及免疫相关差异基因的表达情况筛选并分析，比较评价了 EE2 对于海水青鳉的免疫毒性效应。在此基础上，我们设置了先腹腔注射雌激素受体（ER）抑制剂 ICI 182780 再进行 EE2 暴露的实验组，比对并筛选出通过雌激素受体及其信号通路调控的靶基因，初步分析并推测其免疫致毒机制。本研究从转录组水平初步探讨了 EE2 对海水青鳉的免疫毒性效应及其通过 ER 介导的可能的致毒机制，该研究为深入研究雌激素受体如何在环境内分泌类污染物对海洋鱼类的免疫毒性效应过程中发挥潜在的调控作用提供重要的参考依据。研究结果如下：

（1）构建雄性海水青鳉肝脏的转录组文库 Medaka-lv，得到介于 201 bp-16,884 bp 之间，平均长度为 686 bp 的 122,987 条 unigenes，其中长度在 200 bp-500 bp 的序列 83,267 条，占 67.70%。38,233 条 unigenes（20.8%）注释到 NCBI 官方的蛋白序列数据库（NR）数据库，72,662 条 unigenes（4.5%）注释到 NCBI 官方的核酸序列数据库（NT）数据库。

（2）初步阐明了 EE2 暴露不同时间点（12 h 和 48 h）下海水青鳉雄性成鱼肝脏内分泌、内质网应激、免疫相关功能基因的变化情况：EE2 暴露 12 h 后，内分泌相关基因（如 *Esr1*、*Vtg*、*CHG* 等）以及雌激素信号通路相关基因（如 *Hsp90b1*、*Hspa4*、*Creb* 等）的诱导表达，表明 12 h 即可引起内分泌干扰效应；

内质网应激通路激活关键基因（例如 *Eif2ak3*、*Ire1*、*atf4*、*Xbp1* 等）表达量的显著增加表明 EE2 12 h 即可引起内质网应激反应；EE2 暴露激活抗菌肽基因 *OM-hep2* 表达，显著抑制先天性免疫相关信号通路（TNF、JAK-STAT、补体系统）和抗原呈递过程相关基因的表达，即 EE2 暴露 12 h 可对先天性免疫造成一定的毒性效应。EE2 暴露 48 h 后，内质网应激通路关键基因（如 *Hspa5*、*Eif2ak3*、*atf6*、*atf4* 等）及蛋白质加工运输功能相关的基因的表达量仍持续上调，即内质网应激反应持续激活。TNF、JAK-STAT、补体系统等先天性免疫相关信号通路仍被抑制，抗菌肽基因 *OM-hep1* 及 TLRs、细胞凋亡信号通路相关基因表达均下调，适应性免疫信号通路（如 T 细胞受体、B 细胞受体信号通路）相关基因表达也呈现下调，表明 EE2 暴露 48 h 可持续影响先天性免疫，并进一步对适应性免疫造成一定的毒性效应。综上所述，推测 EE2 暴露可能通过多种途径对鱼体产生免疫毒性效应。

（3）初步揭示了 EE2 暴露通过 ER 介导的免疫毒性效应及作用机制。抑制剂 ICI 处理组中内质网应激关键基因（如 *Hspa5*、*Dnajc3*、*Hsp90b1* 等）及内质网中与蛋白质加工与运输相关基因（如 *Ergic53*、*Sec61*、*Osts* 等）的表达量在 EE2 暴露 48 h 后显著下降，暗示 EE2 引起的内质网应激反应是由 ER 介导的；Ti 组中抗菌肽基因 *OM-hep1* 表达量显著回升，部分先天性免疫信号通路相关基因的表达抑制在 ICI 作用下消除：如 TLRs 信号通路相关的 *Tlr5*、*Map3k8*、*Junb*，JAK-STAT 信号通路相关的 *Socs3*、*Pik3r1*、*Spry4*，补体系统的 *C4*、*Cfh*、*Masp3b* 等。上述结果表明，EE2 可能通过 ER 及其信号通路调控部分免疫相关基因的转录表达，进而对鱼体产生免疫毒性效应。

综上所述，本研究利用 RNA-seq 技术，结合腹腔注射雌激素受体抑制剂，从转录水平比较评价了 EE2 对海水青鳉的免疫毒性效应，并初步揭示了 EE2 暴露可能通过雌激素受体及其信号通路调控的相关机制。本研究为深入探讨海洋环境中内分泌干扰物的免疫毒性效应，及海洋鱼类内分泌系统和免疫系统相互作用的机制研究奠定了基础。

关键词： 17α-炔雌醇；海水青鳉；免疫毒性效应；雌激素受体；

Abstract

17α-ethinylestradiol (EE2) is an endocrine disrupting chemical (EDC) that exists extensively in marine environment. It possesses various toxic effects which can impact on the reproduction and immunity health of fish. There are some studies mainly focus on the reproductive and immune toxicity effects of EE2 on fish, however, few researches on the immunotoxicity mechanism of EE2 are reported at present. In our study, a typical endocrine disrupting chemical EE2 was chosen as tested substance, and marine model animal *Oryzias melastigma* was chosen as study objects. The liver transcriptome library of male *O.melastigma* was constructed using RNA-seq. The exposure time (12 h and 48 h) and concentration of EE2 (500 ng/L) were set referring to the preliminary experiment result of which the gene expression of hepcidin, an important immune factor, were significantly effected. We compared and analyzed genes expression patterns related to endocrine, stress response and immune in liver of male *O.melastigma* at time point 12 h and 48 h post exposure. The result offers overall evaluation of toxic effect of EE2 and the time sequence of the process. Then we set a experimental group of fish exposed to EE2 after injection of ICI 182780, an inhibitor of estrogen receptor (ER), screened out target genes regulated by ER and its signal pathway, then further analyzed and surmised its potential ER-mediated immunotoxication mechanism on transcriptome level. This study contributes to intensive understanding on the potential regulatory role of ER in the process of EDCs' immunotoxic effect on marine fish. The main results for this study are as follows:

1. The liver transcriptome library of male *O.melastigma* was constructed using RNA-seq. High-throughput sequencing resulted in 122,987 unigenes, with a mean length of 686 bp, distribute in the range of 201 bp to 16884 bp. In total, 67.70% (83267) of these unigenes have the length between 200 bp to 500 bp, and 38,233 (20.8%) unigenes were annotated from NR database and 72,662 unigenes (4.5%) were annotated from NT database.

2. Illustrated the gene expression patterns related to endocrine, endoplasmic reticulum stress and immunity in liver of male *O.melastigma* at different time point post EE2 exposure. We screened 839 (at 12 h) and 1361 (at 48 h) differential expression genes after EE2 exposure. The expression of endocrine related genes (such as *Esr1*, *Vtg1*, *Vtg2*, *CHG-H* and *CHG-L*) and genes participate in estrogen receptor signaling pathway (such as *Hsp90b1*, *Hspa4*, *Creb*, *Adcy6 and Shc1/2*) was induced significantly by EE2 exposure, indicating that 12 h-exposure could induce endocrine disrupting activity. The expression of genes related to endoplasmic reticulum stress (such as *Eif2ak3*, *Ire1*, *atf4*, *Xbp1* and so on) was significantly up-regulated after 12 h, suggesting 12h-exposure could cause ER response. Antimicrobial peptide gene OM-hep2 was activated by EE2 exposure, while signaling pathways related to innate immunity (TNF, JAK-STAT, complement system) and genes involved in antigen-presenting process were significantly surppressed, indicating that 12h-exposure of EE2 had a certain toxic effect on innate immunity. After 48 h exposure of EE2, some of genes related to endoplasmic reticulum stress (*Hspa5*, *Eif2ak3*, *atf6*, *atf4* and so on) and genes related to protein processing and trafficking were still induced, indicating the continuously activation of ER response. Innate immunity signaling pathways (TNF, JAK-STAT, complement system, etc.), antimicrobial peptide gene OM-hep1 and genes invloved in TLRs and apoptosis were down-regulated, suggesting EE2 exposure exerted persistently impact on innate immunity which could further cause certain toxic effect on adaptive immunity. Above all, EE2 exposure might pose its immunotoxic effect on fish through multiple ways.

3. Preliminarily revealed immunotoxic effect and mechanism of EE2 mediated by ERα. Compared with group T, the genes related to endoplasmic reticulum stress (such as *Hspa5*, *Dnajc3*, *Hsp90b1* and so on) and genes function for processing and transportation of protein (such as *Ergic53*, *Sec61, Osts* and so on) were significantly down-regulated by EE2 exposure in group Ti after 48 h. The expression of *OM-hep1* increased in group Ti. Meanwhile, EE2 exposure effected on the expressions of the

genes involved in TLRs-signaling pathway (*Tlr5, Map3k8, Junb*), JAK-STAT-signaling pathway (*Socs3, Pik3r1, Spry4*) and complement system (*C4, Cfh, Masp3b*) in group T were eliminated by ICI in group Ti. These results indicated that EE2 mediated the expressions of immune related genes through ERα and its signaling pathways and caused immunotoxic effect on fish.

In summary, RNA-seq accompanied with intraperitoneal injection technique were applied to evaluate the toxic effect of EE2 on *O.melastigma*in this study. Moreover, we illustrated the function mechanism of the effect on the target genes from EE2 mediated by ER and its signaling pathway using estrogen inhibitor. It will contribute to the further research on the relationship between immunotoxicity of marine environmental endocrine disruptors. In addition , it will lay foundation for the mechanism study of the relationship between endocrine system and immune system of marine fish.

Key Words: 17α-ethinyloestradiol (EE2); *Oryzias melastigma*; immunotoxicity; estrogen receptor (ER);

缩略词中英文对照表

英文缩写	英文全称	中文全称
APC	Antigen-presenting cell	抗原呈递细胞
AP-1	Actiator protein-1	激活蛋白-1
BCR	B-cell receptor	B 细胞抗原受体
BMP	Bone morphogenetic protein	骨形态发生蛋白
caspase	cysteinyl aspartate specific proteinase	含半胱氨酸的天冬氨酸蛋白水解酶
CHG	Choriogenin	卵壳前体蛋白
DMSO	Dimethyl sulfoxide	二甲基亚砜
EDCs	Endocrine Disrupting Compounds	环境内分泌干扰物
ERE	Estrogen response elements	雌激素受体响应区域
ER	Estrogen receptor	雌激素受体
EGF	Epidermal growth factor	表皮生长因子
EE2	17α-ethinyloestradiol	17α-炔雌醇
FADD	Fas-associated protein with death domain	Fas 相关死亡域蛋白
GH	Growth hormone	生长激素
GHR	Growth hormone receptor	生长激素受体
GPER	G protein-coupled estrogen receptor	G 蛋白耦联雌激素受体
HSP	Heat shock protein	热休克蛋白
HGP	Human Genome Project	人类基因组计划
IP3	Inositol trisphosphate	三磷酸肌醇
Ig	Immunoglobulin	免疫球蛋白
ICI 182780	Faslodex	氟维斯群
JAK	Janus kinase	Janus 激酶
MyD88	Myeloid differentiation factor 88	髓样分化因子
MHC	Major histocompatibility complex	主要组织相容性复合体

MAP3K8	Mitogen-activated protein kinase kinase kinase 8	激活蛋白激酶激酶激酶 8
PTP	Protein tyrosine phosphatases	蛋白质酪氨酸磷酸酶
PLC-γ1	Phospholipase C gamma 1	磷酸脂酶 C-γ1
RNA-seq	RNA Sequencing	转录组测序技术
SOCS	Suppressors of cytokine signaling	
STAT	Signal transducer and activator of transcription	信号传导及转录激活因子
TIR	Toll-IL-1 receptor domain	TIR 结构域
TNF	Tumor necrosis factor	肿瘤坏死因子
TNFR	TNF receptor	肿瘤坏死因子受体
TLRs	Toll-like receptors	Toll 样受体
TRAF6	TNF receptor associated factor 6	肿瘤坏死因子受体相关因子 6
TRADD	Tumor necrosis factor receptor type I-associated Deach domain protein	肿瘤坏死因子受体型相关死亡结构域蛋白
TCR	T cell receptor	T 细胞受体
Vtg	Vitellogenin	卵黄蛋白原