CITY UNIVERSITY OF HONG KONG

香港城市大學

Production of Reactive Oxygen Species and Antioxidant Responses in Fish Exposed to Xenobiotics, *Chattonella marina* and Hypoxia

外源污染物、赤潮海洋褐胞藻及缺氧誘導魚產生之活性氧及抗氧化反應

Submitted to

Department of Biology and Chemistry

生物及化學系

In Partial Fulfillment of the requirements

For the degree of Doctor of Philosophy

哲學博士學位

by

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MARCH, 2005

二零零五年三月

Abstract

Numerous studies have been carried out to determine antioxidant responses and oxidative damages in aquatic organisms, and subsequently relating the observed changes to pollution. However, no direct scientific evidence has been provided thus far to show that the observed responses and damages are mediated through production of reactive oxygen species (ROS). Meanwhile, studies in mammalian models have provided substantial experimental evidence that ROS production is the major contributing factor for the toxicity of different stressors including xenobiotics, hypoxia and hydrogen peroxide. In this study, I hypothesize that xenobiotics (i.e. menadione, duroquinone and B[a]P), hypoxia and *Chattonella marina* (a red tide alga known to kill fish and capable of producing about 100 times higher concentration of hydrogen peroxide than most other algal species) share a common toxic mechanism by inducing the production of ROS, which will then cause antioxidant responses in fish. In case these antioxidant responses are not sufficient to combat the ROS produced, the excessive ROS will result in lipid peroxidation, protein oxidation and DNA damage. In order to test this hypothesis, the present thesis sets out to determine ROS production in fish exposed to these three different stressors, and to relate ROS levels to antioxidant responses and oxidative damages

A single *i.p*. injection of menadione, duroquinone and B[a]P (1.0 mg/kg body wt.) caused a significant increase of ROS level in both hepatic mitochondria and microsomes in the grouper (*Epinephelus tauvina*) (Tukey test, $P<0.05$). While significant decreases of hepatic total oxidant scavenging capability (TOSC) were observed at 24, 48 and 96 h upon menadione treatment, significant decrease of hepatic TOSC was only found at 96 h. upon B[a]P treatment (Tukey test, $P<0.05$). Protein carbonyl and lipid peroxide (LPO) in mitochondria and microsomes, as well as hepatic 8-OHdG level also markedly increased following enhanced ROS production. However, only B[a]P, but not menadione and duroquinone, could cause a significant induction of ethoxyresorufin-*O*-deethylase (EROD) activity, suggesting that the toxic mechanisms of menadione, duroquinone and B[a]P were different. For B[a]P, production of ROS (and hence toxicity) is mediated

through activation of CYP1A1, and dependent on binding with AhR. In contrast, toxic mechanisms of menadione and duroquinone are AhR independent. Menadione causes oxidative damages possibly through inducing ROS production during redox cycling, and affecting thiol levels via direct arylating to the thiol groups (protein and glutathione), while toxic action of duroquinone is mainly mediated through ROS production during redox cycling.

Experiments were further carried out to establish the dose response relationship between a variety of antioxidant responses and duroquinone. ROS, antioxidant capacity, oxidation of protein and lipid in liver, as well as DNA damage (measured by Comet assay) in red blood cells, were measured at 12, 24, 72 and 168 h. after a single ***i.p.*** injection of 0.1, 1.0 and 10 mg/kg body wt. duroquinone to the grouper (*Epinephelus tauvina*). A good dose-response was demonstrated for ROS production, protein carbonyl and LPO in both hepatic mitochondria and microsomes (Two-way ANOVA, $P<0.05$), and significant correlations were also found between levels of ROS and oxidative damages (***e.g.*** mitochondrial ROS and protein carbonyl) upon treatment of 10 mg/kg body wt. duroquinone ($r=0.979$ and 0.974 at 12 and 24 h respectively). Temporal changes in ROS production and oxidative damage were also evident, showing that oxidative damages were responsive to ROS production. The results therefore clearly demonstrated that oxidative damages induced by duroquinone are mainly mediated through ROS production, and levels of ROS can serve as a good predictor for subsequent oxidative damages.

Increase in TOSC was only found at the highest dose (i.e. 10 mg/kg body wt.), whereas mitochondrial and microsomal ROS, protein carbonyl and LPO were inducible at both 1.0 and 10 mg/kg body wt. of duroquinone (Tukey test, $P<0.05$). DNA damages in red blood cells appeared to be most sensitive, and readily observable even at the lowest dose administered (*viz*. 0.1 mg/kg body wt.) after 24 hour (Tukey test, $P<0.05$). Further studies however, are required to examine the tissue-specific response and the threshold value for induction of TOSC.

Grouper (*Epinephelus tauvina*) exposed to hypoxia (2.0 mg O_2/l) for 24, 72 and 120 h

showed no significant changes in their hepatic mitochondrial and microsomal ROS and LPO, as compared with the normoxic control (One-way ANOVA, $P<0.05$). However, microsomal ROS level in the hypoxic group was significantly lower than that of the normoxic control at 24 h (t test, $P<0.05$). The observed decrease may be due to the decrease in CYP1A1 enzyme induced by hypoxia. However, since antioxidant and oxidative responses to hypoxia may be tissue-specific, the absence of oxidative response in liver may not necessarily rule out the possibility of enhanced ROS production under hypoxic condition. It would be instructive to conduct further experiments to determine ROS levels, antioxidant responses and oxidative damages in other tissues.

Despite ROS levels measured in *Chattonella marina* culture (20 μM H_2O_2) being some 20 times higher than those in the seawater and the algal (*Dunaliella tertiolecta*) controls, ROS in mitochondria, as well as TOSC and LPO in the gill of gold-lined sea bream (*Rhabdosargus sarba*) did not change upon exposure to both low and high (blooming) cell density of *C. marina*. The results therefore showed that fish exposed to high ROS level in the external medium (at least at the level used in the present experiment) is not able to induce ROS formation, antioxidant responses and oxidative damages in fish gill. Results of this experiment therefore refute the current postulation that ROS is the culprit of fish kills in *C. marina* blooms.

This study presents, for the first time, direct evidence to demonstrate *in vivo* ROS production and their correlation with oxidative responses in fishes upon treatment of xenobiotics (i.e. menadione, duroquinone and B[a]P). Hypoxia can neither induce ROS production nor cause oxidative damages in fish liver. Enhanced ROS production is also unlikely to be the main cause of oxidative damage and mortality when fish are exposed to *C. marina*. Overall, this thesis refutes the hypothesis that xenobiotics, hypoxia and *Chattonella marina* share a common mechanism mediated through ROS production.

DOCTOR OF PHILOSOPHY

THESIS ACCEPTANCE

NAME OF STUDENT		:	Liu Wenhua
THESIS TITLE	(English)	:	Production of Reactive Oxygen Species and Antioxidant Responses in Fish Exposed to Xenobiotics, *Chattonella marina* and Hypoxia
	(Chinese)	:	外源污染物、赤潮海洋褐胞藻及缺氧誘導魚產生之活性氧及抗氧化反應

This is to certify that the student's thesis (as presented above) has been examined by the following examiners and has received full approval for acceptance in partial fulfillment of the requirements for the Degree of Doctor of Philosophy:

Prof. Rudolf S S Wu — Department of Biology and Chemistry, City University of Hong Kong

Dated this day 23 of March, 2005

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Dr Peter S W Leung
Chair
Panel of Examiners
City University of Hong Kong

DECLARATION

I here declare that apart from the sources cited, this thesis represents my own work under the supervision of my supervisor, Prof. Rudolf Wu Shiu Sun. The materials presented in this thesis have not been submitted to City University of Hong Kong or any other institution in application for admission to a degree or other qualifications.

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Liu Wenhua

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS AND ABBREVIATIONS

ABAP	2-2'-Azo-bis-(2 methylpropion- amidine)-dihydrochloride
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
B[a]P	Benzo[a]pyrene
BCF	Bioconcentration factor
Body wt.	Body weight
BPDE	B[a]P-7,8-Dihydro-diol-9,10-Epoxide
BSA	Bovine serum albumin
$\int$ CA	The integrated areas from the curve defining the control reactions
CAT	Catalase
CG_{Head}	The centre of gravity of the head
CG_{Tail}	The centre of gravity of the tail
CM	*Chattonella marina*
CoQ	Coenzyme Q
CYP	The cytochrome P450
DCF	dihydrofluorescein
DCF-DA	dihydrofluorescein diacetate
dG	Deoxyguanosine
$\%DNA_{Tail}$	The percent of migrated DNA in the tail compared to the head
DNP	Dinitrophenyl
DNPH	2,4-Dinitrophenylhydrazine
DQ	Duroquinone
DT	*Dunaliella tertiolecta*
Duroquinone	2,3,5,6-tetramethyl-1,4-benzoquinone
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
EROD	Ethoxyresorufin-*O*-deethylase
ETC	Electron transfer chain

FFA	Free fatty acid
FMOs	Flavin monooxygenases
GC/FID	Gas chromatography coupled with flame ionization detector
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
H	Hour
HAHs	halogenated aromatic hydrocarbons
HIF	Hypoxia inducing factor
HIF-1α	Hypoxia inducing factor 1α
HIF-1β	Hypoxia inducing factor 1β
H_2O_2	Hydrogen peroxide
$HO^{\cdot}$	Hydroxyl radicals
HPLC-EC	High-performance liquid chromatography equipped with electrochemical detection
Hsp70	Heat shock protein 70
i.p.	*Intra-peritoneal*
KMBA	2-Keto-4-thiomethylbutyric acid
LMAgarose	Low melting point agarose
I/R	Ischemia and reperfusion
LDL	Low density lipoprotein
LPO	Lipid peroxide
MD	Menadione
MDA	Malondialdehyde
Menadione	2-methyl-1,4-naphthaquinone
MS-222	Tricaine Methanesulfonate
M_{Tail}	The Oliver tail moment
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NADH	Nicotinamide adenine dinucleotide hydrogen

NEM	N-ethylmaleimide in redox quenching buffer
NS	No significant change
$O2^{-\cdot}$	Superoxide anion
8-OHdG	8-dihydroxy-2'-deoxyguanosine
OPA	o-Phthalaldehyde
8-oxoG	8-oxoguanine
PAHs	Polycyclic aromatic hydrocarbons
PC	Protein carbonyl
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dioxins
PCDFs	Polychlorinated dibenzofurans
PeCB	3,3′,4,4′,5-pentachlorobiphenyl
PPP	Pentose phosphate pathway
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RQB	Redox quenching buffer
∫ SA	The integrated areas from the curve defining the sample reactions
SCGE	Single cell gel electrophoresis
SE	Standard error
SOD	Superoxide dismutase
SW	Seawater control
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TCA-RQB	Trichloroacetic acid in redox quenching buffer
TOSC	Total oxidant scavenging capability
UV	Ultraviolet
UVA	Ultraviolet A radiation
UVB	Ultraviolet B radiation
UVC	Ultraviolet C radiation