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Fish Carboxyl Esterase Activity as a Possible Hypoxia Biomarker

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Research Article

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Abstract

Physiology and biochemistry adaptations studied in fish are important to comprehend how they endure the challenges of living in aquatic environments. Oxygen variations are a frequent natural and cyclic phenomenon that occurs in the Pantanal and Amazon watersheds and it is the limiting factor to survival of the aerobic species. The growing of pollution in these water basins threaten many species as anthropic hypoxia intensifies the production of reactive oxygen species (ROS) and lipid peroxidation inside the cells, leading organisms to a condition known as oxidative stress. Our research group, studying the effects of the oxygen variations on the carboxyl esterase (CarbE) enzyme of pacu, a typical fish from Pantanal, observed that CarbE activity diminished by half in pacus exposed to 42 hours of hypoxia. Based on this finding, we decided to investigate this phenomenon in other fish species, including hypoxia-tolerant fishes. CarbE activities were determined using p-nitrophenyl acetate (p-NPA) as a substrate. All five species of fish studied had both serum and liver CarbE activity significantly decreased. Pacus had its serum and liver activities decreased by approximately 50% under severe hypoxia.

Keywords: Oxidative Stress; Fish; Hypoxia; Biomarker; Enzyme; Carboxyl Esterase

Abbreviations: ROS: Reactive Oxygen Species; COBEA: Brazilian College For Animal Experimentation; OC: Oxygen Concentration.

Introduction

Fish and Oxidative Stress

Pacus (*Piaractus mesopotamicus*), Orcars (*Astronotus ocellatus*) and Piavussu, (*Leporinus macrocephalus*) are Neotropical fishes that inhabit Amazonia, Pantanal, Paraná and Paraguay wetland basins. Fish from these regions evolved to resist against hyperoxia and hypoxia conditions since these watersheds are characterized by flood pulses which causes seasonal and daily oxygen variations inside the water [1-3]. These variations happen in detriment of intense sunlight combined with rainfall, providing high photosynthesis rates during sunlight periods, in contrast to the high rates of oxygen consumption during the night. Oxygen variation can also be initiated by xenobiotic discharges into the aquatic environment which may lead to eutrophication followed by a trophic chain unbalance and depletion in oxygen supply in the water. The fact is that, abrupt changes in the amounts of oxygen inside the water can cause oxidative stress in aquatic organisms. Oxidative stress is characterized by an unbalance between oxidants and antioxidants molecules, which results in an increased production of reactive oxygen species (ROS) and/or reduction in the amounts of the antioxidants. Thus, ROS can be toxic to cells as higher intracellular levels favours alterations of proteins and nucleic acid, resulting in impairment of many cell functions. Biomembranes are also susceptible to ROS attack, resulting in lipid peroxidation followed by fragmentation of polyunsaturated fatty acids and aldehydes production [4,5]. Oxidative stress encompasses a wide variety of physiological and pathophysiological, endogenous and exogenous processes that directly or indirectly affects cellular homeostasis [6]. In order to survive, fishes developed some strategies such as swim up

to superficial waters to breathe [7], besides physiological responses also occurs, such as hyperventilation followed by the increase of the volume and the number of erythrocytes [8-10]. One of the most important biochemical mechanisms that make feasible the survival of certain species of fish during long periods under low oxygen availability is the depression of the metabolic rate [11-13]. Another biochemical response is extremely important: the enzyme defence system, an efficient strategy to prevent cell damage against the effects caused by xenobiotic and oxidative stress.

Enzymes

Carboxylesterases (CarbE) (EC 3.1.1.1) are enzymes classified as B-esterases that hydrolyse compounds containing ester, tioester and amide groups. Physiologically, CarbE isoenzymes exhibit lipase activities and are involved in lipid metabolism [14,15] as hydrolyses cholesterol esters and fatty acids [16-18]. It has an important role in the xenobiotic metabolism, pro-drugs activation, antitumoral drugs and narcotics, such as cocaine and heroin [19,20], including the control of the cholinergic impulse. It can also acts as a scavenger of organophosphates and carbamates agrochemicals, functioning as a barrier preventing cellular toxicity [18,19,21], and ROS production.

CarbE are found in several tissues including liver, blood, kidney, lower intestine, lungs, adipose tissue and brain of many organisms, but not in human blood [22]. In mammals, the highest levels were found in the liver [19]. CarbE are anchored to the endoplasmic reticulum of hepatocytes trough a Lis-Asp-Glu-Leu (KDEL) amino acid sequence located at the C-terminal enzyme. This sequence can be cleaved to liberate the enzyme in the cytosol and then secreted to the blood [18,19]. Such importance prompted the esterases to be studied in several areas such as toxicology, biochemistry, pharmacology and medical clinics [19,23]. In this study we propose to investigate if CarbE enzyme activity from serum and liver of fishes exposed to hypoxia will diminish and suggest this enzyme activity as a possible hypoxia biomarker. We believe that these findings will be of great importance to aquaculture since suitable oxygen levels inside the tanks are essential to fish development, growth and reproduction. CarbE activity as hypoxia biomarker can still contribute to clinical medicine since many organism disturbances and diseases such as cancer, inflammation and embrionary development [24,25] are related with reduced oxygen levels in the cells.

Material and Methods

Animals and housing

Pacu specimens (*Piaractus mesopotamicus*) of both sexes, weighing 250 g were supplied by Morro Grande Fish Farm (Cachoeira de Macacu, Rio de Janeiro, Brazil). Carps (*Cyprinus carpio*) weighing 122 g, Oscars (*Astronotus ocellatus*) weighing 298 g and Goldfish (*Carassius auratus*) weighing 25 g were bought in a pet shop in the city of Nova Iguaçu (Rio de Janeiro, Brazil). Piavussu (*Leporinus macrocephalus*) weighing 888 g were supplied by São João Fish Farm located at Itaocara city, Rio de Janeiro, Brazil. Procedures of experiments with the fishes were carried out in conformity with the ethical principles in animal experimentation elaborated by the Brazilian College for Animal Experimentation (COBEA), which is in accordance with the uniform requirements for submission of manuscripts to biomedical journals. The fishes were acclimated in 1000 L water tanks and fed with standard fish food. Water temperature was kept at 23 ºC and dissolved oxygen concentration (OC) at 6.0 mg L-1. Water pH was maintained at 6.4 ± 0.2. Fishes were kept in these conditions for one week until submitted to hypoxia.

Hypoxia experiment

In order to investigate the effect of hypoxia on serum and liver CarbE activities, the fishes were distributed in two groups: normoxia and hypoxia. The oxygen concentration inside the normoxia tank was maintained at 6.0 mg L-1 along the whole experiment. Nitrogen gas was bubbled inside the hypoxia tank for five hours until the oxygen concentration reached 0.5 mg L^{-1} and the fishes were submitted to this oxygen concentration until the end of the experiment. Aerial surface respiration was avoided by covering all tanks flush at the water level with tight mesh nylon. The time of hypoxia exposure varied according to the species, as fish species do not exhibit the same resistance to hypoxia. All fish from the different groups were euthanatized by severing their spinal cords and had their liver excised.

Serum Preparation

Blood was collected from the caudal vein of the fishes in the end of each experiment. Afterwards, blood was kept inside the ice until coagulate. The coagulated blood was centrifuged at 3.500 rpm during 10 min and the supernatant (the serum) was used in CarbE assays.

Hepatic Preparation

Livers were weighted and homogenized using a Potter-Elvehjem type apparatus in four volumes of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.25 M of sucrose. The homogenate were stored at - 20 ºC until assays. Protein content was determined according to Peterson GLA, et al. [26] using bovine serum albumin as standard.

Enzymatic Assays

The carboxylesterase (CarbE) activities were determined in liver homogenate and serum from each fish using a Shimadzu UV-160 A spectrophotometer according to a kinetic reaction [27]. The p-NPA hydrolyses was measured at 400 nm by absorbance increase of the product reaction (p-nitrophenol) along 1 min. CarbE activities were calculated using the extinction coefficient of 13,000 $M^{-1}.cm^{-1}$.

Serum CarbE Assays

The reaction was started by adding the substrate p-nitrophenyl acetate (p- NPA) in a final concentration of 3 mM in a cuvette containing 1µL of serum and a volume of a potassium phosphate buffer 0.1 M, pH 7.7 at a 25 ºC to reach 0.5 mL. Serum CarbE activities were expressed in $U.mg^{-1}$ protein and $U.mL^{-1}$.

Liver CarbE Assays

The reaction was started by adding p-NPA in a final concentration of 3 mM in a cuvette that contained 5 µL of liver homogenate and a volume of a potassium phosphate buffer 0.1 M, pH 7.7 at a 25 ºC to reach 0.5 mL. The CarbE liver activities were expressed in U mg-1 protein or U g-1 wet tissue.

Statistical Analysis

Data are presented as mean ± SD of CarbE assays carried out using one sample from one serum or homogenate prepared from each tissue. Comparisons between data were made by nonparametric T-test analysis ($p < 0.05$) using the software GraphPad Prism, San Diego, California, USA.

Results

Blood partial oxygen pressure analysis

The oxygen levels were determined in the blood of two fish species (*Astronotus ocellatus* and *Piaractus mesopotamicus*) using AVL / Roche OMNI 7 instruction. These analyses were made to assure that fishes were indeed submitted to hypoxia during the experiments (Tables 1 & 2).

Group	Blood partial oxygen pressure
Normoxia	65.0 ± 9.0 mm Hg
Hypoxia	18.0* ± 2.0 mm Hg

Table 1: Blood partial oxygen pressure in oscar, *Astronotus ocellatus*. *Denotes statistical significance ($p < 0.05$) from control after Student's non-parametric "t" test.

Group	Blood partial oxygen pressure
Normoxia	30.25 ± 4.25 mm Hg
Hypoxia	1.16* ± 0.6 mm Hg

Table 2: Blood partial oxygen pressure in pacu, *Piaractus mesopotamicus*.*Denotes statistical Significance ($p < 0.05$) from control after Student's non-parametric "t" test.

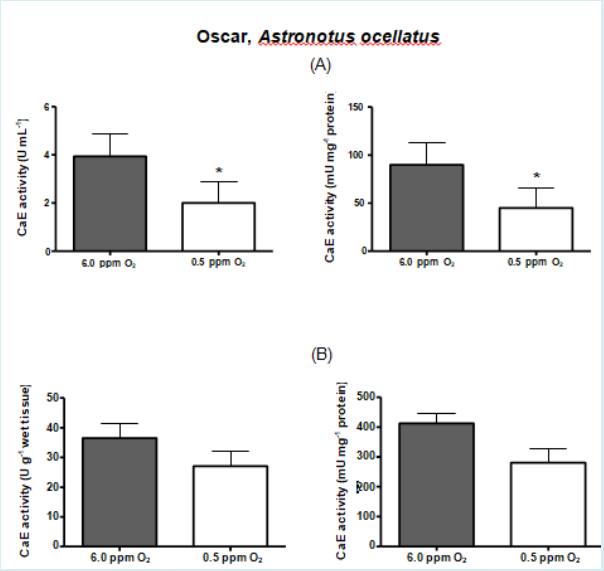

Figure 1: CarbE activity in serum (A) and liver (B) of oscar, *Astronotus ocellatus*, submitted to 24 h of hypoxia. Bars represent the mean ± S.D. * Denotes statistical significance ($p < 0.05$) from control after Student's non-parametric "t" test.

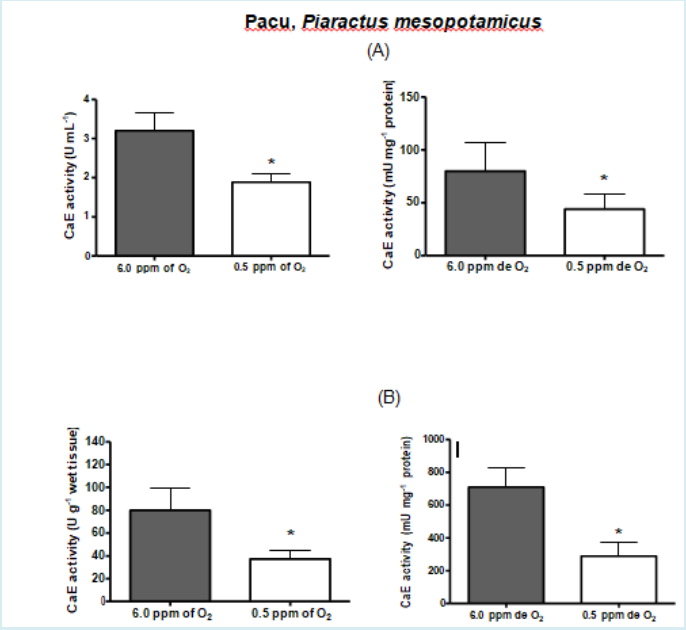

Figure 2: CarbE activity in serum (A) and liver (B) of pacu, *Piaractus mesopotamicus*, submitted to 42h of hypoxia. Bars represent the mean ± S.D. * Denotes statistical significance ($p < 0.05$) from control after Student's non-parametric "t" test.

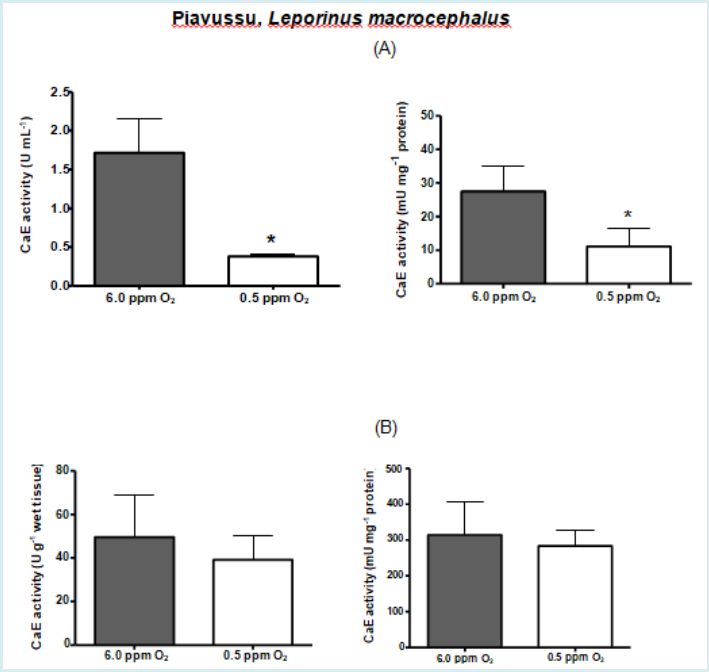

Figure 3: CarbE activity in serum (A) and liver (B) of piavussu, *Leporinus macrocephalus*, submitted to 24 h of hypoxia. Bars represent the mean ± S.D. * Denotes statistical significance ($p < 0.05$) from control after Student's non-parametric "t" test.

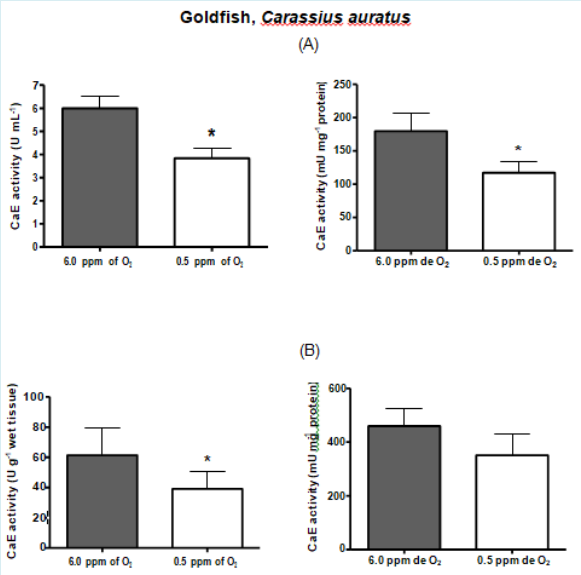

Figure 4: CarbE activity in serum (A) and liver (B) of goldfish, *Carassius auratus*, submitted to 8 h of hypoxia. Bars represent the mean ± S.D. * Denotes statistical significance ($p < 0.05$) from control after Student's non-parametric "t" test.

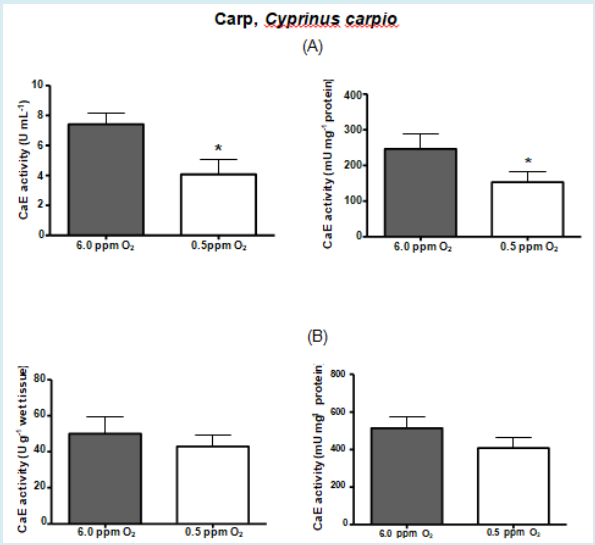

Figure 5: CarbE activity in serum and liver of carp, *Cyprinus carpio*, submitted to 24 h of hypoxia. Bars represent the mean ± S.D. * Denotes statistical significance ($p < 0.05$) from control after Student'snon-parametric "t" test.

	Serum				Liver			
FISH	**NORMOXIA U.mL^{-1}**	**HIPOXIA U.mL^{-1}**	**NORMOXIA mU.mg-1 ptn**	**HIPOXIA mU.mg-1 ptn**	**NORMOXIA U.g-1 w t**	**HIPOXIA U.g-1 w t**	**NORMOXIA mU.mg-1 ptn**	**HIPOXIA mU.mg-1 ptn**
OSCAR	3.93	2.0*	89.68	45.03*	36.53	27.02	412.25	280.93
PACU	3.2	1.88*	79.9	43.94*	79.84	37.27*	709.58	288.80*
PIAVUSSÚ	1.71	0.38*	27.48	11.11*	49.63	39.22	314.36	283.6
GOLDFISH	6	3.84*	179.73	117.3*	61.46	39.1*	459.63	351.1
CARP	7.41	4.06*	246.58	153.1*	50.1	43	513.58	407.1

Table 3: Comparison between serum and liver carboxylesterase activities among the fish species.* Denotes statistical significance ($p < 0.05$) from control after Student's non-parametric "t" test.

Discussion and Conclusion

There are some organisms that are able to withstand changes in the amount of dissolved oxygen in their blood for months. This is possible thanks to physiological and biochemical adaptations that occurs in their organs to overcome hypoxia [28-31]. Hypoxia induces a family of transcriptional factors, called hypoxia induced factors (HIF) that is linked to the hypoxia response elements (HRE) to do proteins transcription and genes regulation. These genes are associated to erythropoietin, endothelial vascular growth factor, glucose transport, glycolytic enzymes and also enzymes related to iron metabolism and cell's survival [32,33]. In Goldfish, for example, its gills excrete ethanol produced in its muscles from lactate originated from glucose, which avoids the disadvantageous acidosis that would result if lactate were accumulated in hypoxia [34]. Severe hypoxia is known to produce the release of catecholamines and other stress hormones that are responsible for the hyperglycemic response through stimulation of glucogenolysis and/or gluconeogenesis [35-37]. Indeed, some fishes survive by mobilizing carbohydrate reserves to generate ATP molecules by anaerobic glycolysis. Carps tissues contain large glycogen stores and also show some important additional metabolic adaptations in their use [13]. A depression in the levels of free fatty acids of carps and rainbow trout plasma were also observed during hypoxia, and the mechanism for that is most probably a reduction of free fatty acids oxidation and mobilization, a consequence of the high plasma glucose concentration along hypoxia [37]. More recently, hypoxic inhibition of lipolysis has been demonstrated in humans [38] and results suggest increased concentration of glucose and fatty acid mobilization is also present in fish [39].

Hypoxia is thought to produce a general shift in oxidative fuel preference, from predominantly lipids and proteins to carbohydrates. Whereas carboxylesterase (CarbE) is the main nervous tissue lipase [16], a diminished flux of fatty acids and its reduced levels, could be linked to a decrease in the CarbE enzymatic activity during hypoxia. Indeed, human hepatocytes (HEPG2) maintained in hypoxia for 24 hours had several proteins with their gene expression depleted, including the carboxyl esterase [40]. Another experiment with human tumor cells, and tumor cells suffer from hypoxia, shows a reduction in the CarbE expression [41]. Moreover, studies realized by Suto D, et al. [42] demonstrated that singlet oxygen, generated during hypoxia, is a strong inhibitor of cysteine and serine proteases and can possibly inhibit CarbE activities as catalytic CarbE sites exhibits serine and histidine residues. In this present study, we attempted to submit the fish species to equal time of hypoxia, however, this was not possible. Each fish species exhibit different tolerance to the low concentrations of oxygen in the water and some species eventually died along the experiment. Therefore, the time of hypoxia exposure for each fish species was settled particularly and was the limit time between the fish death and the experiment accomplish. Pacu was the most resistant fish species, surviving 42 hours of hypoxia and had their both serum and liver CarbE activities diminished almost by half. Oscars also had their activities diminished by half but only endured 24 h of hypoxia. Piavussu and carps also endured 24 h of hypoxia. Piavussu exhibited the lowest CarbE activities among all fish species while carps exhibited the highest activities. Goldfish also exhibit high CarbE activities, however they only stud up for 8 hours of hypoxia. The relevance of this study is that all fish species submitted to hypoxia in this present study had their both serum and liver CarbE activities diminished. We believe that serum CarbE activities decreased significantly because fewer liver CarbE enzymes were secreted to the blood. Perhaps, the CarbE are more useful in the liver rather than in the blood, since lipid metabolism could be modified in response to hypoxia.

The decreases in oxidation and mobilization of fatty acids have been previously related to the high glycemic levels during hypoxia [37]. Since CarbE activities are essential for mobilize and oxidizelipids, it is understandable that these activities are also diminished during hypoxia. In addition, such depletion is a convenient adaption to the lipid metabolism because fishes submitted to hypoxia produce ethanol. Ethanol is a substrate of CarbE to form ethyl esters of fatty acids, a chaotropic product of mitochondrial membranes, resulting in the formation of reactive oxygen species. Thus, as CarbE activity decreases, the formation of the ethyl fatty acid esters also decreases and the integrity of membranes are preserved during low oxygen periods. Our goal in this study was achieved. All species had both liver and serum carboxyl esterase (CarbE) activity diminished, but serum activities were mightily decreased. These findings suggest that CarbE activities, especially in serum, can be used as a hypoxia biomarker in fish. What´s more, to draw the blood, it will not be necessary to kill the fish for enzyme analyses, an advantage for ecotoxicology studies and environmental preservation [43]. Another important conclusion is that fish may be more susceptible to xenobiotics when abiding in waters with low oxygen content, since their serum and hepatic CarbE, an important drug metabolism enzyme, decreases during hypoxia.

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