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Purine metabolism in response to hypoxic conditions associated with breath-hold diving and exercise in erythrocytes and plasma from bottlenose dolphins (*Tursiops truncatus*)

Iris del Castillo Velasco-Martínez [a], Claudia J. Hernández-Camacho [b], Lía C. Méndez-Rodríguez [a], Tania Zenteno-Savín [a,*]

[a] Centro de Investigaciones Biológicas del Noroeste, S.C., Programa de Planeación Ambiental y Conservación, Instituto Politécnico Nacional 195, Playa Palo Santa Rita Sur, La Paz, Baja California Sur C.P. 23096, México

[b] Centro Interdisciplinario de Ciencias Marinas—Instituto Politécnico Nacional, Laboratorio de Ecología de Pinnípedos, Av. Instituto Politécnico Nacional s/n, Playa Palo Santa Rita, La Paz, Baja California Sur C.P. 23096, México

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ABSTRACT

In mammalian tissues under hypoxic conditions, ATP degradation results in accumulation of purine metabolites. During exercise, muscle energetic demand increases and oxygen consumption can exceed its supply. During breath-hold diving, oxygen supply is reduced and, although oxygen utilization is regulated by bradycardia (low heart rate) and peripheral vasoconstriction, tissues with low blood flow (ischemia) may become hypoxic. The goal of this study was to evaluate potential differences in the circulating levels of purine metabolism components between diving and exercise in bottlenose dolphins (*Tursiops truncatus*). Blood samples were taken from captive dolphins following a swimming routine ($n = 8$) and after a 2 min dive ($n = 8$). Activity of enzymes involved in purine metabolism (hypoxanthine guanine phosphoribosyl transferase (HGPRT), inosine monophosphate deshydrogenase (IMPDH), xanthine oxidase (XO), purine nucleoside phosphorylase (PNP)), and purine metabolite (hypoxanthine (HX), xanthine (X), uric acid (UA), inosine monophosphate (IMP), inosine, nicotinamide adenine dinucleotide (NAD^+), adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), ATP, guanosine diphosphate (GDP), guanosine triphosphate (GTP)) concentrations were quantified in erythrocyte and plasma samples. Enzymatic activity and purine metabolite concentrations involved in purine synthesis and degradation, were not significantly different between diving and exercise. Plasma adenosine concentration was higher after diving than exercise ($p = 0.03$); this may be related to dive-induced ischemia. In erythrocytes, HGPRT activity was higher after diving than exercise ($p = 0.007$), suggesting an increased capacity for purine recycling and ATP synthesis from IMP in ischemic tissues of bottlenose dolphins during diving. Purine recycling and physiological adaptations may maintain the ATP concentrations in bottlenose dolphins after diving and exercise.

1. Introduction

Bottlenose dolphins (*Tursiops truncatus*) perform activities during diving and at the surface (Perrin et al., 2002; Lusseau, 2006). This species usually dives from 2 to 10 min and to depths between 4 and 500 m (Stewart, 2002). During breath-hold diving (apnea) in bottlenose dolphins the use of oxygen stores is maximized by a decrease in heart rate (bradycardia) and peripheral vasoconstriction, which reduces blood flow (ischemia) to visceral organs, skin and muscles in favor of the most oxygen-dependent tissues, such as the central nervous system (Kooyman et al., 1980; Panneton, 2013). In consequence, muscles become hypoxic, oxygen partial pressure decreases, and the oxygen bound to myoglobin (Mb) dissociates. If apnea persists, oxygen concentration in the muscles can eventually be depleted (Davis, 2014). With surface activities, including jumps and tail slappings which are related with prey capture as well as intra- and interspecific communication, swimming speeds can be up to 8.3 m s^{-1} (Fish and Hui, 1991; Lusseau, 2006). These activities carry a higher energy cost and can be compared with exercise in land mammals (Williams et al., 1993). During exercise, the increasing energy demand resulting from muscular contraction generates an increase in ventilation and a higher oxygen consumption (Nadel, 1977). In addition, there is an increase in heart rate (tachycardia) and peripheral vasodilation, which rise blood flow and oxygen

* Corresponding author. Tel.: +52 612 123 8502; fax: +52 612 125 3625.
E-mail addresses: idelcastillo@pg.cibnor.mx (I. del Castillo Velasco-Martínez), cjhernandez78@gmail.com (C.J. Hernández-Camacho), lmendez04@cibnor.mx (L.C. Méndez-Rodríguez), tzenteno04@cibnor.mx (T. Zenteno-Savín).

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supply to skeletal muscle (McArdle et al., 2006; Davis and Williams, 2012). However, during vigorous exercise, energy demand exceeds oxygen supply to tissues through respiratory and circulatory system, which can generate hypoxia in muscular tissue (Hill et al., 2004; Muller et al., 2012). Therefore, breath-hold diving and surface exercise can cause hypoxic conditions in bottlenose dolphin tissues (Davis et al., 2004; Davis and Williams, 2012). Hypoxia reduces adenosine triphosphate (ATP) synthesis and the consequent decrease in intracellular ATP concentration promotes degradation of purine nucleotides (Fox, 1981; Moriwaki et al., 1999). Purine nucleotide phosphorylase (PNP) and xanthine oxidase (XO) are involved in purine degradation (Moriwaki et al., 1999). PNP catalyzes the reversible conversion of nucleosides (guanosine, xantosine, inosine) to purine bases (guanine, xanthine (X), hypoxanthine (HX)) (Dudzinska et al., 2006). XO oxidizes HX to X, and the latter to uric acid (UA) (Moriwaki et al., 1999). In non-primate mammals, including dolphins, uricase catalyzes the conversion of UA to allantoin (Balinsky, 1972). Degradation of X to UA, as well as the oxidation of the later to allantoin, results in reactive oxygen species (ROS) formation and the potential for oxidative stress (Glantzounis et al., 2005; Chawla, 2014). It has been suggested that the ischemia–reperfusion associated to breath-hold diving in marine mammals increases ROS generation (Vázquez-Medina et al., 2011). However, the enhanced antioxidant capacity in dolphins, and marine mammals in general, appears to contribute to avoidance of oxidative damage (Wilhelm-Filho et al., 2002; Zenteno-Savín et al., 2002; Vázquez-Medina et al., 2006; Cantú-Medellín et al., 2011). Increased HX concentration was reported in heart and kidney of two seal species (*Phoca hispida*, *Pagophilus groenlandicus*) following a period of *in vitro* ischemia (Elsner et al., 1995). In northern elephant seal (*Mirounga angustirostris*), apnea induces an increase in XO activity and X concentration (Vázquez-Medina et al., 2011). In humans, the exercise response is characterized by a decrease in plasma ATP concentration, higher levels of IMP in erythrocytes, and increased HX and UA concentration in plasma (Sutton et al., 1980; Dudzinska et al., 2010). HX accumulation is associated to tissue injury due to increased ROS production (Rao et al., 1990; Collard and Gelman, 2001; López-Cruz et al., 2014). An enhanced purine salvage pathway has been suggested for marine mammals, catalyzed by hypoxanthine guanine phosphoribosyl transferase (HGPRT), which is capable of recycling HX and direct it towards ATP synthesis (Elsner et al., 1998; Soñanez-Organis et al., 2012). Purine metabolism and associated diseases caused by enzymatic deficiencies have been studied in humans (Dudzinska et al., 2006). However, studies on the synthesis and degradation of purines, as well as the efficiency of purine salvage pathway under hypoxic conditions associated to diving and exercise in marine mammals, are still scarce. The goal of this study was to analyze and compare the activity of enzymes (PNP, XO, HGPRT, inosine monophosphate dehydrogenase (IMPDH)) and the concentration of metabolites (HX, X, UA, inosine monophosphate (IMP), inosine, nicotinamide adenine dinucleotide (NAD^+), adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), ATP, guanosine diphosphate (GDP), guanosine triphosphate (GTP)), involved in purine metabolism after diving and exercise in erythrocytes and plasma from bottlenose dolphin (*T. truncatus*). Blood samples are a relatively non-invasive method to study wildlife and captive organisms. Several metabolites and enzymes from different tissues may be released to the plasma and, thus, may be quantified as a proxy for conditions in muscle or other organs (Skotnicka et al., 2008). Erythrocytes cannot produce ATP either by oxidative phosphorylation or by the *de novo* pathway, but are permeable to several purine metabolites, such as purine bases and nucleosides, which could constitute substrates for salvage reactions (Craik et al., 1997). Therefore, the salvage pathway may represent a crucial role for energy source in erythrocytes (Dudzinska et al., 2006). Further, erythrocytes have been suggested to provide ATP to muscle under hypoxic conditions (Ellsworth et al., 2015).

2. Material and methods

2.1. Sample collection

Blood samples were taken from eight bottlenose dolphins (5 females, 3 males; 9.46 ± 3.11 years old) housed at Cabo Dolphins, Cabo San Lucas, Baja California Sur, México. The mean estimated weight was 218.31 ± 12.35 kg. Distress effect during sample collection was minimized due to routine training and veterinary examination. None of the sampled individuals were pregnant or lactating. Daily diet consisted in fish, and dolphins were continuously fed during training routines. Each dolphin was sampled after two experimental conditions, (1) diving (sedentary breath-holding), and (2) exercise (swimming and jumping at the surface). For the first condition, the dolphin was requested to perform a 2 min dive to a depth of 1.5 m. At the surface, the trainer indicated the dolphin to begin diving by a hand signal and a whistle sound; the dolphin was signaled to stay still during diving. Another whistle sound indicated the dolphin to return to the surface. To analyze the exercise response, the dolphin was cued to perform a swimming and jumping routine at the water surface for 15 min. The duration of diving and exercise periods was based on the protocol reported by Houser et al. (2010) and Williams et al. (1993) for sample collection. Blood samples were drawn following each routine (diving (n = 8) or exercise (n = 8)) from the fluke vein using a butterfly needle BD™ (21 gauge). The samples were collected in Vacutainer® tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. To separate plasma from erythrocytes, samples were centrifuged in a field centrifuge (Mobilespin, Grandview, MO, USA) at 1500 × *g* for 10 min. The buffy coat was discarded and erythrocytes were washed using cold saline solution (0.9%). Plasma and erythrocyte samples were kept on ice during transportation to the laboratory, where they were stored at −80 °C until analyzed. Prior to the analyses, erythrocytes were lyzed by osmotic and mechanic shock with cold distilled water, followed by two cycles of freezing/thawing to obtain intraerythrocyte content.

2.2. Enzyme activity

2.2.1. Inosine monophosphate dehydrogenase (IMPDH)

IMPDH activity was measured in erythrocytes and plasma by quantifying xanthine monophosphate (XMP) concentration using high performance liquid chromatography (HPLC), according to the protocols reported by Glander et al. (2001) and Montero et al. (1995). Erythrocytes were lysed by diluting the sample in a solution of cold distilled water and dithiothreitol (DTT, 4 mM). Samples were incubated in a solution of IMP (0.5 mM), NAD^+ (0.25 mM), sodium phosphate buffer (40 mM) and potassium chloride (50 mM) at pH 7.4 for three hours at 37 °C. Reaction was stopped with cold perchloric acid ($HClO_4$, 4 M), neutralized with potassium carbonate (KCO_3, 5 M) and incubated at −80 °C for 30 min. To obtain the extract of interest the neutralized reaction mixture was filtered through a Millex GV membrane of 0.22 mm (Merck Millipore, Bedford, MA, USA), and was placed in a HPLC vial and analyzed in duplicate. Stationary phase consisted of a Hypersil 125 × 4.6 mm, 3 μm particle size column (Thermo Scientific, USA), while mobile phase consisted of a binary gradient from 100% buffer A (KH_2PO_4 0.1 M, and tetrabutylammonium (TBA) 8 mM, pH 6.0) to 100% buffer B (KH_2PO_4 0.1 M, TBA 8 mM and methanol (30%), pH 6.0) in a total run time of 26 min. XMP detection was based on retention time and absorption spectrum of the peak. XMP concentration (μM mL^{-1}) was calculated using a standard curve (1.56 μM to 50 μM) and the area of the peak in the chromatogram. One unit of IMPDH activity is defined as the amount of enzyme needed to produce 1 mM of XMP per min at 37 °C. IMPDH activity detection limit was 4.28 U mg^{-1} protein h^{-1}. IMPDH activity is expressed as μM mg^{-1} protein h^{-1}.