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Elevated free tyrosine in rhinoceros erythrocytes

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Abstract

Red blood cells of African black rhinoceroses (*Diceros bicornis*) are highly sensitive to oxidant-induced hemolysis and they possess a number of enzymatic and biochemical features that differ radically from other mammals. Here we show concentrations of free tyrosine in rhinoceros red blood cells which can approach levels as high as 1 mM, 50-fold higher than in human red blood cells. Elevated levels of tyrosine are also observed in red blood cells of other members of the order Perissodactyla such as the horse and zebra. Captive black rhinoceroses have significantly lower levels of red blood cell tyrosine than black rhinoceroses in the wild. Tyrosine transport studies indicate that black rhinoceros red blood cells have lost the ability to transport tyrosine as efficiently as human red blood cells.

Keywords: Red blood cells; Rhinoceros; Tyrosine; Erythrocyte; Nucleotides; Taurine; Horse; Zebra

1. Introduction

Over the past three decades, habitat encroachment and poaching have progressively reduced the world-wide population of African black rhinoceroses (*Diceros bicornis*) from more than 60,000 to approximately 2500, less than 5% of which currently reside under captive conditions. This captive population is of sufficient size to have significant value to conservation of the species, but it has been threatened by several disease syndromes, including acute hemolytic anemia which is one of the most common causes of death in captivity. Despite extensive hematological investigation (Chaplin et al., 1986; Fairbanks and Miller, 1990; Miller and Boever, 1982; Paglia et al., 1986), the etiologies of this and most of the other frequently lethal disorders remain uncertain.

Metabolic studies on rhinoceros red cells have revealed a number of unique enzymatic and biochemical features that would be considered pathological deficiency states where they found in human or other mammalian erythrocytes (Paglia et al., 1986; Paglia, 1993). Among these, low levels of erythrocyte ATP and catalase activity (each ~2–5% relative to humans) have received considerable attention (Paglia et al., 1986; Paglia and Miller, 1992; Paglia, 1993), but both are characteristic of all members of the family *Rhinocerotidae* with no apparent differences between affected and unaffected animals, so neither has been unequivocally implicated in the etiology or pathogenesis of hemolytic anemia.

This report describes the new phenomenon of very high concentrations of free tyrosine in rhinoceros red blood cells and other members of the order Perissodactyla.

2. Materials and methods

2.1. Acquisition of blood samples

Blood samples were obtained opportunistically from apparently healthy wild black and white rhinoceroses in Kruger National Park, Hluhluwe-Umfolozi Park, and Addo Elephant National Park, South Africa (R.S.A.), when they were immobilised for translocation, radio-collaring, or other purposes, and from healthy human volunteers. Horse blood samples were obtained from Blue Cross Veterinary Hospital, Newlands, Cape Town and zebra blood samples from Mountain Zebra National Park (outside Cradock) and Kammanassie and Gamkaberg (Oudtshoorn) Nature Reserves, South Africa.

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2.2. Storage and extraction of red blood cells

Red blood cells were frozen as droplets into liquid nitrogen (Issitt, 1985). Rapidly thawed red cells, which undergo very little lysis under these freezing and storage conditions, were separated from other formed elements by centrifugation and successive washes with isotonic saline. They were then resuspended in Hanks balanced salt solution (HBSS, Sigma), and incubated for 1 h at 37 °C. Following incubation, the cells were rewashed and suspended in 1 vol. isotonic saline, then deproteinized by rapid addition of 2 vol. 1.2 M perchloric acid (PCA). After centrifugation, the supernatant was neutralised with 2.5 M K_2CO_3, and the $KClO_4$ precipitate removed by centrifugation.

2.3. HPLC analysis

2.3.1. Anion-exchange chromatography

Erythrocyte extracts were fractionated on a Phenomenex Hypersil 5-μm NH2-2 anion-exchange column (250 × 3.2 mm, 5 μm diameter particles). Mobile phase buffers consisted of buffer A, 5 mM KH_2PO_4, pH 2.8, and buffer B, 0.5 M KH_2PO_4 + 1.0 M KCl, pH 3.5. Metabolites were eluted by a gradient consisting of 99% buffer A and 1% buffer B for 1 min, followed by 99% buffer B and 1% buffer A over 15 min, maintained for 1 min before returning to initial conditions over a period of 10 min. Flow rate was 0.5 ml/min, and eluting metabolites were monitored by absorbance at 260 and 280 nm using a Beckman System Gold Model-168 detector.

2.3.2. Reverse phase chromatography

This was performed on a Phenomenex Luna C18 column (250 × 4.6 mm, 5 μm diameter particles). Mobile phase buffers consisted of 0.01 M KH_2PO_4, pH 5.6 (buffer A) and 50% methanol (buffer B). Elution used a flow rate of 1 ml/min using a gradient with an initial composition of 99% buffer A and 1% buffer B for 5 min, followed by a change to 30% buffer B and 70% buffer A over 3 min. This state was maintained for 10 min before returning to initial conditions over 3 min.

2.3.3. Identification of HPLC species

Diode array scans using a Beckman Model-168 detector were used to compare the spectrum of the unknown with that of candidate species, including an authentic tyrosine standard showing a λ_{max} at 274 nm.

Amino acid analysis was performed on a Beckman System 6300 High Performance Analyser using a 10 cm cation-exchange column and lithium based buffers according to a standard method developed by the manufacturers.

For fluoroscopic analyses using a SPEX FluoroMax spectrofluorometer, red cell extracts were diluted 1:4 with distilled water. Tyrosine has a λ_{ex} and a λ_{em} maximum of 280 and 300–305 nm, respectively.

For mass spectrometry, fractions containing the unknown compound were isolated using reversed-phase HPLC and freeze-dried. Analysis used matrix-assisted laser desorption mass spectrometry with time-of-flight detection (Maldi-Tof) on a Perseptive Biosystems DE-PRO MALDI mass spectrometer.

2.3.4. Tyrosine uptake

Human blood samples were centrifuged at 1500 rpm for 10 min to pellet the red blood cells. The plasma was aspirated and the red cell pellet washed three times with isotonic saline. Human and black rhinoceros red blood cells, at a haematocrit of 50%, were incubated in the presence of 1 mM tyrosine (Sigma) for 0, 15, 30 and 60 min at 37 °C. The incubation medium used was the tyrode solution described by Widmer et al. (1990). These samples were acid extracted and analysed by reverse phase HPLC to determine the concentration of tyrosine in the red blood cells.

3. Results

3.1. Identification and quantification of intracellular tyrosine

Although it is known that ATP concentrations in rhinoceros red cells are very low, little is known about levels of other red cell nucleotides. Since such information has diagnostic value in a number of diseases (Simmonds et al., 1988), neutralised PCA extracts of human and black rhinoceros erythrocytes were analysed by anion-exchange HPLC to provide a comparison of ultraviolet (UV) absorbing metabolites. Fig. 1 compares typical profiles obtained from a human and from 1 of over 50 wild rhinoceroses studied. Taking the different ordinate scales into account, the marked relative reduction in ATP concentration (to between 20 and 50 μM) in the rhinoceros preparation is an obvious feature. Other peaks in the rhinoceros profile represent nucleotides, such as the indicated UDP-glucose and NADPH, which are found in most mammalian species, including humans (Simmonds et al., 1988).

An early eluting peak of UV absorbing material (labelled " × " in Fig. 1) provided another consistent feature of HPLC profiles and absorption spectra of rhinoceros erythrocyte extracts. A search of candidate UV absorbing compounds showed that on anion-exchange and reversed-phase HPLC it coincided with the elution position of the amino acid tyrosine. Confirmation of the identity of this species was provided by the following: the characteristic absorption profile given by diode array scan, retention time and ninhydrin positivity on cation-exchange amino acid analysis, excitation and emission properties on fluorescence spectrometry (Lehrer and Fasman, 1967; Malencik et al., 1996), and mass number identity with tyrosine on mass spectrometry.

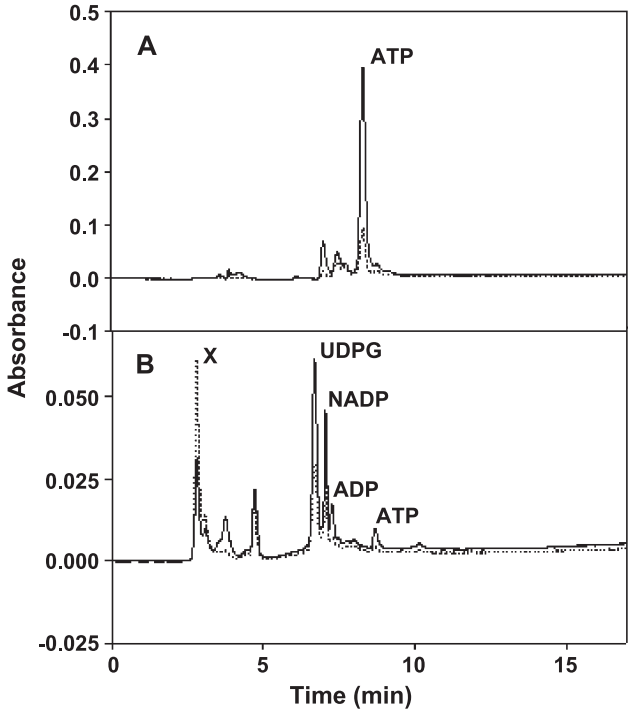

Fig. 1. Anion-exchange HPLC of neutralised acid-soluble extracts. Extracts are from (A) human and (B) black rhinoceros (*D. bicornis*) red blood cells. X marks the unknown species in rhinoceros extract; UDPG, UDP-glucose.

The mean intracellular concentration of tyrosine in eight fresh red blood cell specimens taken from wild free-ranging black rhinoceroses was 0.78 ± 0.11 mM. These levels are up to 50-fold greater than those found in normal human red cells. These high red blood cell tyrosine levels were not reflected in plasma levels of tyrosine. Dierenfeld (1995) reported plasma tyrosine concentrations in captive rhinoceroses that were indistinguishable from human values, and this was confirmed by our own analyses on plasma specimens from free-ranging black rhinoceroses.

Red blood cell tyrosine levels in 30 specimens taken in similar fashion from captive rhinoceroses in the USA averaged 0.37 ± 0.14 mM, a value significantly lower ($p < 0.0001$) than that in the wild animals. In other respects, the anion-exchange HPLC nucleotide profiles in the captive animals did not differ significantly from wild individuals.

Samples from three southern white rhinoceroses (*Ceratotherium simum simum*) gave a mean red cell tyrosine content of 0.75 ± 0.07 mM, one Asian greater one-horned rhinoceros (*Rhinoceros unicornis*) a value of 0.17 mM, and three Sumatran Rhinoceros (*Dicerorhinus sumatrensis*), a mean value of 0.21 ± 0.04 mM. All these species of rhinoceros therefore demonstrate varying degrees of elevated red cell tyrosine.

Free tyrosine was also observed in red blood cell extracts from members of the family Equidae (order Perissodactyla), namely the horse (*Equus cabalus*) and zebra (*Equus quagga*) (Fig. 2).

The mean tyrosine concentration in 6 horse red blood cell samples was 68.18 ± 4.10 μM and in four zebra red blood cell samples, 54.57 ± 2.77 μM. Although this was considerably lower than the levels observed in rhinoceros red blood cells it was still above levels found in normal human red blood cells (~ 20 μM; Proenza et al., 1994). A feature observed in some equid samples (as shown here) was a major peak of uric acid. This was a highly variable feature and is the subject of an investigation, the details of which will be described elsewhere.

3.2. Tyrosine transport

In order to address the question as to how tyrosine achieves such high levels in the rhinoceros red blood cell

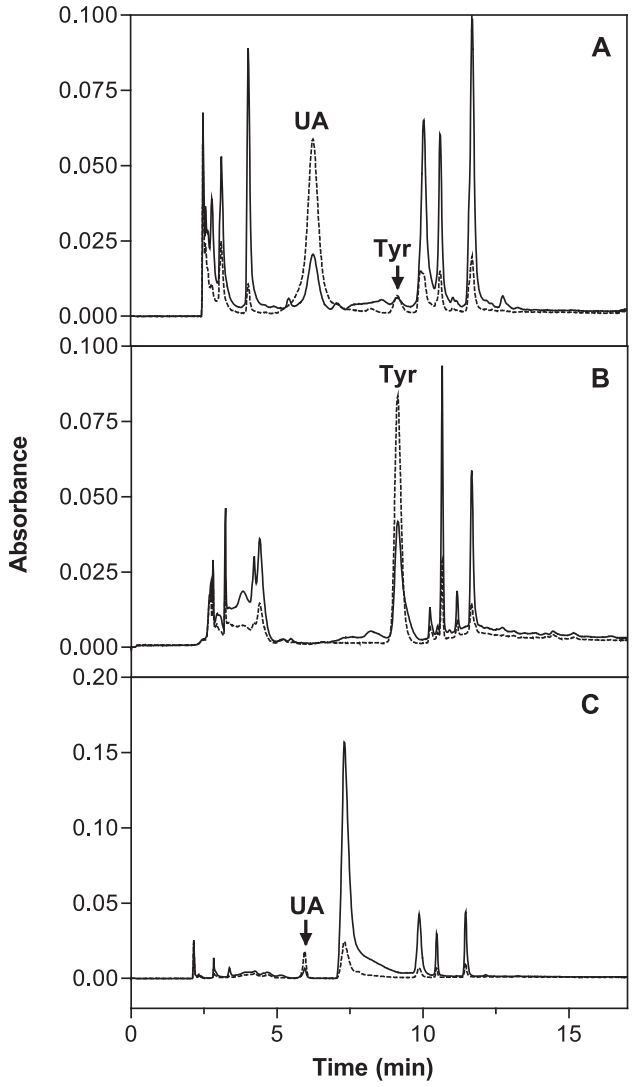

Fig. 2. Reverse phase HPLC of neutralised acid-soluble extracts of (A) horse, (B) black rhinoceros (*D. bicornis*) and (C) human red cells, demonstrating the elevated levels of free red blood cell tyrosine and uric acid at 9.1 and 6.2 min, respectively. The volume of sample analysed was fivefold less for the human sample as for horse and rhinoceros.

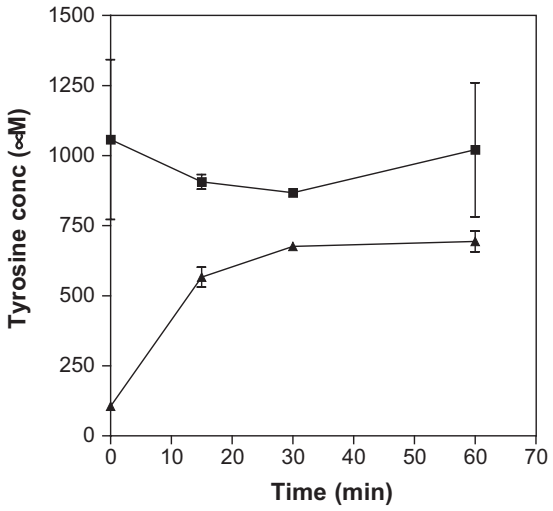

Fig. 3. Tyrosine transport in human and black rhinoceros (*D. bicornis*) red blood cells. Human (▲) and black rhinoceros (■) red blood cells at 50% haematocrit were incubated at 37 °C in the presence of 1 mM tyrosine. Transport of tyrosine across the red blood cell membrane was monitored by reverse phase HPLC.

we compared the ability of rhinoceros and human cells to accumulate tyrosine from the medium. There is a known transport system for tyrosine uptake in human red cells (Rosenberg et al., 1980) and use of this is illustrated in Fig. 3 where in the presence of 1 mM external tyrosine, human red cells accumulated tyrosine to a concentration in red cell water of 693.9 ± 36.88 μM. Under similar conditions rhinoceros red cells showed no significant uptake and accumulation of tyrosine.

4. Discussion

Such high levels of a free aromatic amino acid in mammalian red cells are, as far as we know, unprecedented. Identical analyses of human, canine, feline, lepine and bovine erythrocytes failed to reveal similar elevations, although in the latter a prominent early eluting compound with similar UV absorptive properties displayed elution characteristics consistent with previously described urate riboside (Bartlett, 1969).

Tyrosine uptake studies have shown that human red blood cells readily take up tyrosine from the medium (Fig. 3), whereas rhinoceros red cells show poor uptake of tyrosine under identical conditions. An interesting possibility is that the physiological elevation of tyrosine in red blood cells in rhinoceroses might have an analogous role to the high concentrations of taurine found in human neutrophils and epithelial cells (Raschke et al., 1995). Taurine protects cellular components from highly potent oxidants, such as HOCl, produced during phagocytic killing of microorganisms by the myeloperoxidase–H_2O_2–halide system (Grisham et al., 1984; Cantin, 1994).

Taurine has no transport system in human red cells but reacts with HOCl to form taurine chloramine and taurine dichloramine, both of which are more readily reduced by GSH than is HOCl (Thomas et al., 1985). Taurine enters the (human) erythrocyte in the oxidised form, and is retained therein after GSH dependant reduction. Since the potent oxidants HOCl and $ONOO^-$ can react with tyrosyl residues to form chloro-tyrosine and nitro-tyrosine, respectively (Domigan et al., 1995; Van der Vliet et al., 1995), it is possible that tyrosine enters the rhinoceros erythrocyte in a similar oxidised form. Alternatively, oxidation of tyrosine by stimulated human neutrophils has been shown to generate peroxide adducts (Winterbourn et al., 1997) in significant amounts. One or other of such mechanisms could provide a link with established processes of free radical metabolism.

Examples of a role for tyrosine in physiological protection against oxidants are (a) the observation by Lupo et al. (1997) that a strain of *Saccharomyces* deficient in tyrosine synthesis was more susceptible to H_2O_2 than the wild type, and (b) the presence of high concentrations of tyrosine in seminal fluid, which has been proposed to serve as an antioxidant (van Overveld et al., 2000).

This description of such high levels of an amino acid in the rhinoceros red cell, apparently family or order specific (Figs. 2 and 4), together with previously described low levels of catalase activity, imply novel physiological processes in this order of mammals and may provide important clues to the hemolytic problem of these animals in captivity. The finding that captive rhinos have lower mean levels of red cell tyrosine than in those still free-ranging in natural African habitats indicates at the least an association of tyrosine with the mechanism of the hemolytic process.

At a more fundamental level, the elucidation of an unexpected new metabolic pathway of free tyrosine metabolism would open a door for investigation of novel processes of free radical and antioxidant metabolism in general, and selective evolutionary metabolic processes in red cells, in particular.

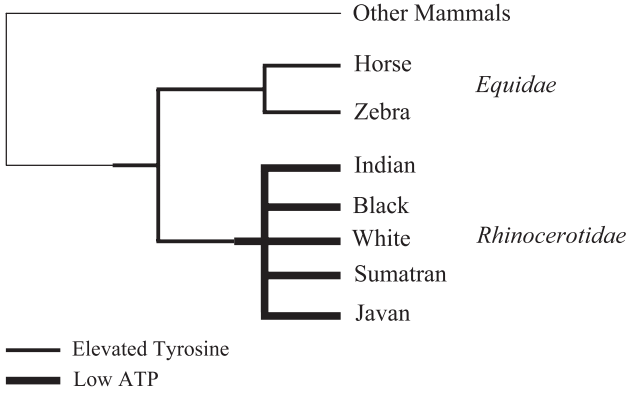

Fig. 4. Dendrogram of two metabolic "characters", ATP and tyrosine in two families of the order Perissodactylae vs. other mammals.

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