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Red blood cell metabolism shows major anomalies in Rhinocerotidae and Equidae, suggesting a novel role in general antioxidant metabolism

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Abstract. The black rhinoceros, *Diceros bicornis*, shows some striking anomalies in red cell biochemistry compared with humans: many enzyme levels are grossly different, ATP levels are very low, and the red cells contain very high levels of free tyrosine. On exposure to oxidative stress dityrosine, a substance never previously described in free form in cells can be detected. Uric acid, another soluble free radical scavenger, can also on occasions be readily demonstrable, often at very high concentrations, in rhinoceros and equine red cells. Assays for oxygen radical absorptive capacity (ORAC) in intact red cells, and in cell free preparations containing tyrosine or urate, give results consistent with a role for tyrosine and purine derivatives as additional defence mechanisms against reactive oxygen intermediates. The integration of these in vitro and in vivo analyses reveals insights and mechanisms which should be exploitable for the development of preventative or therapeutic measures against hemolytic and other free radical-induced disorders in both rhinoceros and other mammals, including man.

Keywords: Rhinoceros; Horse; Tyrosine; Uric acid; Erythrocyte; Antioxidant; Free radical

1. Introduction

Over the past three decades, habitat encroachment and poaching have progressively reduced the worldwide 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 has been threatened by several disease

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syndromes, including acute hemolytic anaemia which is one of the most common causes of death in captivity. Despite extensive haematological investigation [1], the causes of this remained uncertain. However, the black rhinoceros does show some striking peculiarities in respect to its normal red cell biochemistry: many enzyme activities are very different, catalase especially being only about 2% of levels in human red cells, and ATP levels are remarkably low relative to other mammals [2,3].

We have also demonstrated diminished erythrocyte glycolysis through the hexose monophosphate shunt [4], and this, together with the presence of Heinz bodies in erythrocytes of normal individual rhinoceroses [2], suggests that impairments in antioxidant capacity may contribute to the hemolytic tendency.

2. Materials and methods

Blood samples were obtained opportunistically from apparently healthy wild black and white rhinoceroses immobilised for translocation, radio-collaring, or other purposes, in the Kruger National Park, Hluhluwe-Umfolozi Park, and Addo Elephant National Park, South Africa. Red blood cells were either used fresh or were frozen as droplets into liquid nitrogen [5] for later analysis or experimentation.

Analysis of acid extracts of red cells by HPLC and identification of metabolites are as described previously [6].

Dityrosine was synthesised in vitro from unlabelled or ^{14}C-labelled tyrosine using horseradish peroxidase [7].

Oxygen radical absorptive capacity (ORAC) was assayed according to Cao et al. [8], where the prevention of fluorescent decay of 0.04 μM oxidant-sensitive β-phycoerythrin in the presence of 40 mM of the free-radical generator 2,2′ -Azobis(2-amidinopropane) dihydrochloride (AAPH) (Sigma-Aldrich) by either a standard scavenger (Trolox) or the test solution of tyrosine, urate, plasma, or cell extract was followed at 5-min intervals in an Aminco SPF 500 fluorimeter at an emission of 565 nm and an excitation of 540 nm.

3. Results

Whilst investigating nucleotide profiles of acid-extracted rhinoceros red blood cells using anion-exchange and reversed-phase HPLC, and confirming that all rhinoceros species show very low ATP levels, we observed a major U/V absorbing peak with a distinctive 260/280 ratio. Comparisons with other U/V absorbing metabolites showed the elution positions, and 260/280 ratio of this substance to correspond to the amino-acid tyrosine. Confirmation of this identity was provided by the characteristic absorption profile given by diode array scan, the retention time and ninhydrin positivity on cation-exchange amino acid analysis, the excitation and emission properties on fluorescence spectrometry and mass number identity with tyrosine on mass spectrometry.

Eight fresh red blood cell specimens taken from free-ranging black rhinoceroses gave a mean intracellular concentration of tyrosine of 0.78 ± 0.11 mM, levels which are at least 50-fold greater than those found in normal human red cells. Despite this, plasma levels of tyrosine were far lower, and similar to those found in human plasma. Examination of human, canine, feline, lepine, and bovine erythrocytes showed no detectable tyrosine on

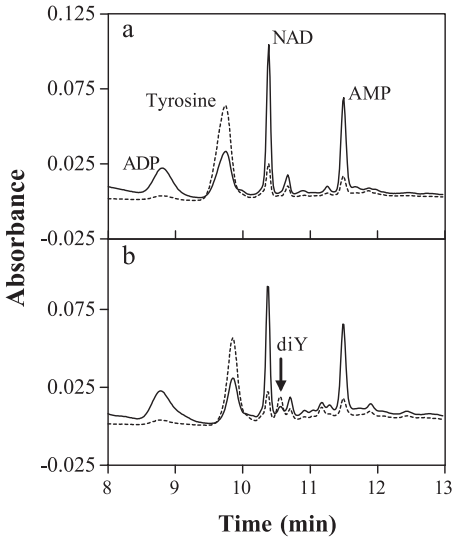

Fig. 1. Reversed-phase chromatography of neutralised acid-soluble extracts of rhinoceros red cells. Red cells were incubated for 30 min at 37 °C with either (a) Hank's balanced salt solution with no additions, or (b) with the addition of 2 mM H_2O_2; diY, dityrosine.

HPLC analysis, but equine erythrocytes from both horse and Plains zebra showed tyrosine at about one tenth of the levels found in rhinoceros.

Protein-bound dityrosine is known to form nonenzymatically from interactions between H_2O_2 and hemoglobin [9], but free dityrosine production has not been documented as a biological process. We therefore undertook to see whether we could identify free dityrosine after exposure of rhinoceros red cells to H_2O_2, and detected a novel UV absorbing species (Fig. 1) on reversed-phase HPLC with the known fluorescent properties of dityrosine [7]. Although this was readily detected in both black and white rhinoceros

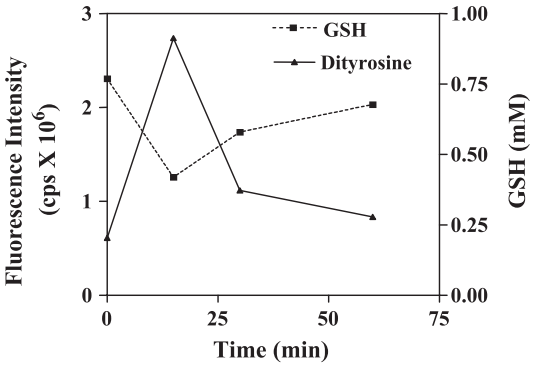

Fig. 2. Variation in levels of dityrosine and GSH over time after treatment of rhinoceros red cells incubated at 37 °C in Hank's balanced salt solution after addition of H_2O_2 to a final concentration of 2 mM at time 0. Each point shows the mean of duplicate measurements.

Table 1
In vitro studies using dialysed cell-free preparations (DCFP)

Additions	Dityrosine detected (μM)
Tyrosine+H_2O_2	no (<0.5)
Tyrosine+H_2O_2+DCFP	yes (4.1)
Tyrosine+H_2O_2+DCFP+GSH	no (<0.5)
Tyrosine+H_2O_2+heated DCFP	yes (149.3)
Tyrosine+H_2O_2+Fe^{++}	no (<0.5)
Tyrosine+H_2O_2+hemoglobin	yes (20.5)
Tyrosine+H_2O_2+hemin	yes (49.5)

Tyrosine (0.5 mM) and H_2O_2 (2 mM) were incubated with heat-inactivated or non-heat-inactivated dialysed cell-free preparations (DCFP) of rhinoceros red cell lysates at 37 °C for 0, 15, 30, 60, and 90 min before quantitation of dityrosine production by fluorescence spectroscopy. Results are the mean of duplicate assays at the time of maximum dityrosine accumulation. Additions were 0.5 mM GSH, 0.1 mM ferrous sulfate (BDH), or 10 μM bovine hemin.

red cells, it was not detected in human erythrocytes identically treated. Confirmation of the identity of this species as o,o'dityrosine was provided by fluorescence properties, U/V absorbance properties, co-elution with synthetic dityrosine, and mass spectrometry. This increase in H_2O_2-induced dityrosine production coincided with a decrease in reduced glutathione (GSH) concentrations (Fig. 2), with GSH concentrations rising again as dityrosine levels fell during the recovery phase.

In order to determine whether the dityrosine production was an enzyme-catalysed process, dialysed cell free lysates were incubated with tyrosine, H_2O_2, and other additions (Table 1). Dityrosine could be easily detected with unheated and, more surprisingly, also with heated cell extracts. Dissecting the components of the cell extract showed then that the presence of either hemoglobin or heme was sufficient for dityrosine production, indicating that the heme ring alone could provide catalysis.

During the course of these experiments, an additional U/V absorbing species was intermittently encountered in some rhinoceros and some equine red cell extracts and sometimes it was the most prominent of the U/V absorbing species. Its absorption characteristics, elution positions on anion-exchange and reversed-phase HPLC, and disappearance from HPLC analyses after uricase treatment identified the species as uric acid. The puzzling quantitative variability compromised adequate investigation until a series of eight horses undertaking an endurance race were studied (Table 2), when the results suggested that red cell urate levels were strongly influenced by intense muscular activity. Complementary in vitro studies indicated that although red cell urate levels were not stable over periods in excess of a few hours at body or room temperature, transport in or

Table 2
Levels of intracellular red cell urate (μM)

	A	B	C	D	F	G	H	J
Twenty-four hours before exercise	56.4	0.0	0.0	0.0	67.6	0.0	0.0	1.7
Immediately after exercise	118.4	0.0	422.0	7.6	0.0	0.0	0.0	2.0
One hour after exercise	108.8	0.0	824.0	35.2	157.6	0.0	32.4	35.5

Urate was measured in red cell extracts from eight horses (A–J). Blood was taken before and after undergoing an endurance exercise of 80 km, with a mean duration for the exercise of 5.1 h.

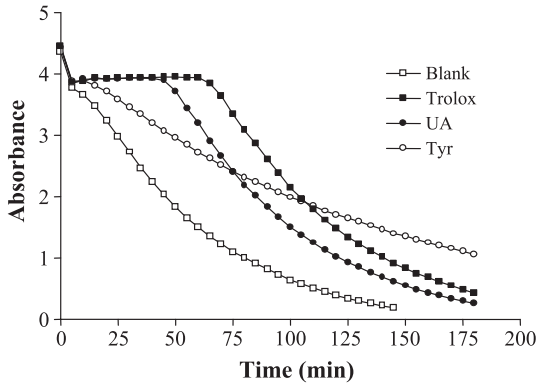

Fig. 3. ORAC of urate and tyrosine: time-dependent fluorescence decay of β-phycoerythrin after addition of AAPH in the presence of: Blank, no additions; Trolox, 5 μM Trolox; UA, 12.5 μM urate; Tyr, 12.5 μM tyrosine.

out of the red cell was slow and inadequate to explain the rapid increase or decrease in levels over the short-term in vivo, suggesting that the urate was being synthesised and destroyed within the red cell, perhaps by oxidative processes.

Since we had shown heme to catalyse oxidation of tyrosine in vitro, a similar test was tried with urate. Urate incubated with either heme or H_2O_2 alone gave no change, but all three together resulted in rapid urate degradation. Since urate is well-known as an effective scavenger of oxygen free radicals, and since tyrosine has also been proposed to serve as an antioxidant in seminal fluid [10] we compared the oxygen radical absorptive capacities (ORAC) of urate and tyrosine, and found that both exhibited similar antioxidant capacities, with ORAC values of 14550 and 12680 mmol/l for 5 mmol/l solutions of tyrosine and uric acid, respectively, although kinetics differed, with urate showing higher affinity for the oxidant (Fig. 3). Plasma extracts of both rhino and human samples showed ORAC kinetics similar to that of free tyrosine, probably due to the tyrosine in serum proteins. We then

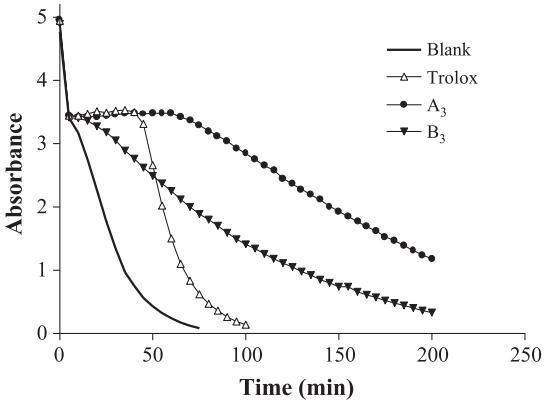

Fig. 4. ORAC of horse red cell extracts either lacking or containing urate, together with a blank and a Trolox control. A_3 is a high urate containing sample from horse A (see Table 2) and B_3 is a sample lacking detectable urate from horse B, both at 1 h after exercise.

compared the ORAC of two horse red cell extracts (Fig. 4), one with no detectable urate (horse B one h after exercise) and the other with high urate levels (horse A, one h after exercise, with 108 μM urate) demonstrating the markedly increased ORAC of the urate containing sample.

4. Discussion

The finding of high levels of tyrosine in mammalian red cells is unprecedented and its presence in both rhinocerotidae and equidae implies a long evolutionary history and presumably a useful physiological role. During an inflammatory response, activated macrophages produce myeloperoxidase and H_2O_2. The latter can then form the antimicrobial agents hypochlorous acid (HOCl) and peroxynitrite, as a consequence of which tyrosine in the vicinity can be converted to tyrosine chloramine, and nitrotyrosine, in reactions in which the tyrosyl radical is proposed to play a central role.

The amino-acid taurine is found in human neutrophils and epithelial cells and is assumed to have a role in protecting cellular components from strong oxidants such as the HOCl produced during phagocytic killing of microorganisms by the myeloperoxidase system [11]. It is possible that the physiological elevation of tyrosine in red blood cells in rhinoceroses might have an analogous role, and complements the similar protective role of urate. The generation of urate in red cells, as well as its removal, have at present no straightforward explanation, but it is possible that free-radical processes are involved in both formation of urate from, for example, hypoxanthine, and in the degradation to substances such as allantoin; both these pathways occur normally in some mammalian tissues, and both can be oxidative processes, the former catalysed by xanthine oxidase, and the latter by uricase. The (presumed) absence of both these enzymatic processes in red cells may be offset by the results of the in vitro experiments reported here which imply that heme may catalyse similar reactions in the presence of strong oxidants, analogous to the way heme can catalyse the oxidation of tyrosine.

It has recently been shown [12] that black rhinoceroses in the USA become progressively iron overloaded with time in captivity. The iron overload is likely to arise from the change from a natural diet (browse) rich in powerful iron-binding substances, for which the black rhinoceros would have had to have evolved equally powerful iron-absorptive mechanisms, to a captive diet of available fodder items unlikely to restrict iron availability to the same extent. Iron catalyses free radical production by the Fenton and Haber–Weiss reactions, so the cause of the hemolytic anaemia in these individuals may well be a decompensation of red cell antioxidant mechanisms in red cells with unusual antioxidant defences in the face of excess iron-induced free radical production. A feature of this idea is that it suggests novel therapeutic or preventative approaches such as reintroduction of more natural browse or addition of iron chelators to the diet.

These results leave a number of unresolved problems, including (1) how does tyrosine accumulate in rhinoceros red cells?, (2) what is the physiological role of heme with respect to oxidant production or removal?, (3) what is the explanation for the unusual dynamics of red cell urate production and destruction, and (4) do levels of red cell tyrosine, urate (and ORAC) vary under different dietary or exercise regimes, or in response to antioxidant treatments?

These results also, however, provide some intriguing new insights and suggestions for new physiological processes and mechanisms. These are: (1) Comparative studies on mammalian red blood cells can demonstrate novel, and generally unpredictable, physiological phenomena. (2) Tyrosine and uric acid in red blood cells both have the capability of scavenging oxidants and/or oxygen free radicals. (3) Given the magnitude and variety of antioxidant mechanisms in red blood cells, the question arises as to whether these mechanisms are primarily there to protect the red blood cell itself, or is their main purpose the provision of a convenient "package" of scavengers and enzymes for delivery to other tissues where free radicals need to be removed? If the latter were correct, then it would define a new pathophysiological role for the red blood cell of general relevance to veterinary and perhaps human medicine.

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