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Original Contribution

A new sensitive assay reveals that hemoglobin is oxidatively modified in vivo

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Abstract

Free radical formation in heme proteins is recognised as a factor in mediating the toxicity of peroxides in oxidative stress. As well as initiating free radical damage, heme proteins damage themselves. Under extreme conditions, where oxidative stress and low pH coincide (e.g., myoglobin in the kidney following rhabdomyolysis and hemoglobin in the CSF subsequent to subarachnoid hemorrhage), peroxide can induce covalent heme to protein cross-linking. In this paper we show that, even at neutral pH, the heme in hemoglobin is covalently modified by oxidation. The product, which we term OxHm, is a "green heme" iron chlorin with a distinct optical spectrum. OxHm formation can be quantitatively prevented by reductants of ferryl iron, e.g., ascorbate. We have developed a simple, robust, and reproducible HPLC assay to study the extent of OxHm formation in the red cell in vivo. We show that hemoglobin is oxidatively damaged even in normal blood; approximately 1 in 2000 heme groups exist as OxHm in the steady state. We used a simple model (physical exercise) to demonstrate that OxHm increases significantly during acute oxidative stress. The exercise-induced increase is short-lived, suggesting the existence of an active mechanism for repairing or removing the damaged heme proteins.

Keyword: Free radical; Hemoglobin; Myoglobin; Peroxide; Exercise; Oxidative stress; Blood substitute; Oxidative modification; Assay; Chlorin

Introduction

Oxidative stress has been implicated in a wide range of diseases including atherosclerosis, hypertension, diabetes, ischemia–reperfusion injury, and a range of neurodegenerative disorders [1]. The fact that superoxide dismutase and catalase mimetics increase lifespan strongly suggests that oxidative stress is also a major contributor to ageing in general [2], as well as the more specific pathologies that accompany the ageing process [3,4]. Metal interactions with hydrogen peroxide have been frequently implicated in catalysing free radical-mediated damage. Redox-active transition metals, containing unpaired elections in their *d*-orbitals, are able to generate, or remove, reactive species with unpaired electrons. In particular free ferrous iron [5] has been suggested to catalyse tissue damage by reacting with hydrogen peroxide to generate the highly reactive hydroxyl radical ($OH^{\bullet}$). However, the relevance of this "Fenton" chemistry to systems where strong redox-inactive chelators (e.g., transferrin) exist to bind iron is not clear.

Abbreviations: Hb, hemoglobin; Mb, myoglobin; OxHm, oxidatively modified hemoglobin; metHb, methemoglobin; $\dot{V}O_{2MAX}$ tests, test of maximal whole body oxygen consumption; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; NEM, *N*-ethylmaleimide; TGSH, total glutathione; GSSG, oxidised glutathione; GSH, reduced glutathione; DEANO-NOate, 2-(*N*,*N*-dimethylamino)-diazenolate-2-oxide; DETA/NO, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PBS, phosphate-buffered saline; ANH, acute normovolemic hemodilution; MALDI-TOF, matrix-assisted laser desorption ionisation-time of flight.

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Recently there has been accumulating evidence that an additional mechanism to generate reactive oxygen species occurs in vivo [6–12]. This involves the interaction of the ferric state of heme proteins with hydrogen peroxide:

$$R + Fe^{3+} + H_2O_2 \leftrightarrow R^{\bullet+} + Fe^{4+} = O^{2-} + H_2O \quad (1)$$

(where R represents the polypeptide chain and Fe^{3+} the iron bound to the heme group).

Both the ferryl iron ($Fe^{4+} = O^{2-}$) and the protein-bound free radical ($R^{\bullet+}$) are able to initiate a range of oxidative chemistry with similar reactivity to the hydroxyl radical [13,14]. Heme proteins like hemoglobin (Hb) and myoglobin (Mb) contain redox-active transition metal iron that makes them susceptible to causing oxidative damage. Although the structure of the globin chain allows heme to bind oxygen with minimal oxidation of ferrous to ferric iron, autoxidation is not entirely prevented; low concentrations of ferric heme are normally present in vivo (methemoglobin, metmyoglobin). These can then react with the peroxides formed during the autoxidation process itself or elsewhere in the protein's vicinity (Eq. (1)).

Both the globin-bound radical and ferryl heme iron can cause tissue damage, for example, by initiating lipid peroxidation reactions [15–17]. Recent evidence supports the view that kidney damage following crush injury (rhabdomyolysis) is caused by a heme (myoglobin) peroxidative mechanism (Eq. (1)), rather than free iron-catalysed Fenton chemistry as was previously thought [7,9]. There is evidence of oxidative damage specifically induced by myoglobin [7] and the enzyme that breaks down heme (heme oxygenase) is indispensable in protecting against this damage [18]. Hemoglobin can cause similar damage when it is released from the red blood cell in, for example, subarachnoid hemorrhage [11]. Heme protein-mediated oxidative stress may be implicated in a range of acute clinical scenarios, including toxic effects of blood substitutes [10,19], and therefore an understanding of the physiological processes involved in the prooxidant activities of the heme species is essential.

Reactions of peroxides with heme proteins in vitro have been well-characterized. One unique "green heme" product was observed as early as the 1950s [20]. This was later characterised [21,22] as incorporating a covalent heme-to-protein cross-link. This cross-linked species has been demonstrated to exhibit high prooxidant and pseudoperoxidase activity [13,23–26]. A covalent heme protein bond only forms under extreme conditions in vivo (e.g., pH < 6.5). Although other oxidatively modified heme species can be produced under less extreme conditions, these have until now received less attention. We have developed a novel HPLC assay that can measure a unique form of oxidatively modified hemoglobin (OxHm) that is produced solely through interaction of Hb with peroxides. This species is formed in vivo and its concentration increases under conditions of oxidative stress. We propose that OxHm can be used to address the role of heme protein-mediated oxidative stress in vivo and the normal physiological defence mechanisms that exist to prevent heme toxicity.

Experimental procedures

Subjects

Volunteers recruited for the various parts of this study were apparently healthy, and signed an informed consent form prior to participation. Subject characteristics for the participating volunteers are shown in Table 1. All procedures were approved by the Ethical Committee of the University of Essex.

Factors affecting resting levels of OxHm

Blood samples were obtained from 90 healthy subjects (42 females, 48 males; Table 1), and analysed for levels of OxHm and metHb. Smoking habits (cigarettes per day) and antioxidant supplement use over the past 3 months were determined through interviews. Subjects were (arbitrarily) classified as antioxidant users if taking any antioxidant supplements at least once a week. Average intake of 61 items of fruits and vegetables over the past 3 months was determined using a food-frequency questionnaire. Standard serving sizes were used, and intake was expressed as servings per day. An estimate of $\dot{V}O_{2MAX}$ was obtained using the Åstrand-Ryhming test [27].

Exercise testing

$\dot{V}O_{2MAX}$ tests were performed on an electrically braked ergometer (Lode Excalibur Pro, Lode, Groningen, the Netherlands), and consisted of a 30 W min^{-1} ramp to volitional exhaustion, starting at 30 W (untrained subjects), or 120 W (triathletes). Expired air samples were analysed using an online breath-by-breath gas analysis system (Oxycon Pro, Jaeger, Hoechberg, Germany). Further exercise tests were performed on the same cycling ergometer or on an HP Cosmos Quasar treadmill (Nussdorf, Germany).

Table 1
Subject characteristics (mean ± SD)

	Age (years)	Mass (kg)	Stature (m)	$\dot{V}O_{2MAX}$ (ml kg^{-1} min^{-1})
Distribution of resting levels				
Younger adults (n = 64)	23 ± 3	68.7 ± 11.9	1.72 ± 0.10	47.3 ± 16.8
Older adults (n = 26)	57 ± 6	73.0 ± 12.0	1.69 ± 0.10	34.6 ± 18.1
Exercise studies				
Triathletes (n = 9)	31 ± 7	70.7 ± 13.2	1.75 ± 0.09	63.8 ± 5.8
Untrained subjects (n = 31)	21 ± 3	76.6 ± 16.8	1.76 ± 0.10	48.6 ± 8.6