

Original Contribution

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

Niels B.J. Vollaard<sup>a,1</sup>, Brandon J. Reeder<sup>a</sup>, Jerry P. Shearman<sup>a</sup>, Patrick Menu<sup>b</sup>,  
Michael T. Wilson<sup>a</sup>, Chris E. Cooper<sup>a,\*</sup>

<sup>a</sup>*Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, CO4 3SQ, UK*

<sup>b</sup>*Laboratoire de Physiologie, Faculté de Pharmacie de Nancy, Université Henri Poincaré,  
5 rue Albert Lebrun, BP 403, 54001 Nancy Cedex, France*

Received 14 December 2004; revised 21 June 2005; accepted 21 June 2005

Available online 10 August 2005

## 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.

© 2005 Elsevier Inc. All rights reserved.

**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 electrons 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<sup>•</sup>). 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;  $\dot{V}O_{2\text{MAX}}$  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)-diazene-2-oxide; DETA/NO, (Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazene-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.

\* Corresponding author. Fax: +441206 872592.

E-mail address: [ccooper@essex.ac.uk](mailto:ccooper@essex.ac.uk) (C.E. Cooper).

<sup>1</sup> Present address: School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, Scotland, UK.

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:



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

Both the ferryl iron ( $\text{Fe}^{4+} = \text{O}^{2-}$ ) 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 pseudo-peroxidase activity [13,23–26]. A covalent heme protein bond only forms under extreme conditions in vivo (e.g.,  $\text{pH} < 6.5$ ). 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 methHb. 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  $\dot{V}\text{O}_{2\text{MAX}}$  was obtained using the Åstrand-Ryhming test [27].

### Exercise testing

$\dot{V}\text{O}_{2\text{MAX}}$  tests were performed on an electrically braked ergometer (Lode Excalibur Pro, Lode, Groningen, the Netherlands), and consisted of a  $30 \text{ W min}^{-1}$  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  $\pm$  SD)

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

## Reagents

Trifluoroacetic acid (TFA) was purchased from Fisher (Loughborough, Leicestershire, UK). HPLC-grade acetonitrile was from BDH (Poole, Dorset, UK). NADPH and glutathione reductase were from Merck Biosciences (Nottingham, UK). 2-(*N,N*-Dimethylamino)diazene-2-oxide (DEANO-NOate) and (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-aminoethyl)amino]diazene-1,2-diolate (DETA/NO) and Proli/NO were purchased from Alexis (Nottingham, UK). All other chemicals were acquired from Sigma-Aldrich (Poole, Dorset, UK). Hemoglobin was purified from human volunteer blood by the method of Antonini [28] with the addition of ion-exchange chromatography on DEAE Sephadex A50 [29] to remove contaminating catalase. Myoglobin and hemoglobin were converted to the ferric (met) states by the addition of excess ferricyanide, which was subsequently removed via a Sephadex G25 column.

## Blood sampling

Blood samples were drawn from an antecubital vein into 6-ml Vacutainer EDTA whole blood tubes (Becton-Dickinson, Oxford, UK). Aliquots for measurement of oxidatively modified heme (OxHm) and methHb were directly frozen in liquid nitrogen, whereas 0.50-ml aliquots of whole blood were incubated on ice for 10 min with 0.75 ml 6% TCA for analysis of total glutathione (TGSH), or with 0.75 ml 6% TCA/40 mM NEM for oxidized glutathione (GSSG). Samples were centrifuged at 13,500g for 6 min at 4°C, and clear supernatant was frozen in liquid nitrogen and stored at –80°C until analysis.

## OxHm

Whole blood was diluted 10 times with water and centrifuged at 9400g for 10 min, after which 0.50 ml of supernatant was transferred to a microcentrifuge tube with a 0.22-μm cellulose acetate membrane microcentrifuge filter (Sigma-Aldrich, Poole, Dorset, UK), and centrifuged at 850g for 10 min. For reverse-phase HPLC analysis (Agilent HP1100 HPLC fitted with a diode array spectrophotometer) 60 μl of sample was transferred to a vial. A Zorbax StableBond 300 C3 250 × 4.6-mm column fitted with a 12 × 4.6-mm guard column was used. Solvents consisted of 0.1% trifluoroacetic acid dissolved in water or acetonitrile. An initial 35% proportion of 0.1% TFA in acetonitrile increased to 37% after 10 min, to 40% after 15 min, and to 43% after 16 min. The remainder was 0.1% TFA in water. Flow rate was set at 1 ml min<sup>–1</sup>, temperature was 25°C, and injection volume was 12.5 μl. Heme B was determined from the 14.6 min eluant. Integrated area-under-the-curve of the chromatogram at 630 nm was converted to a concentration of heme B by comparing to a heme B standard of known concentration measured at 430 and 630 nm ( $\epsilon$  430 nm: 133,000 M<sup>–1</sup> cm<sup>–1</sup> [28]). OxHm was determined from the

8.3–8.4 min eluant by drawing a baseline from the troughs on either side of the cluster of three peaks with elution times between 8.0 and 8.7 min, after which the two peaks on the right were isolated with a drop line. Of these two peaks the integrated area-under-the-curve of the chromatogram at 400 nm was converted to a concentration of OxHm using an extinction coefficient of 76,000 M<sup>–1</sup> cm<sup>–1</sup> [22]. OxHm was expressed as a percentage of heme B.

## Mass spectrometry

Samples of oxidatively modified hemes were purified for mass spectrometry using an Agilent HP1100 HPLC fitted with an Agilent G1364 integrated fraction collector. The HPLC method was identical to that described for analysis and quantification of oxidatively modified hemes (above). Samples were collected between 7.9 and 8.8 min, and the eluate from several identical runs was pooled. Equal amounts of samples and matrix solution (20%  $\alpha$ -cyano-4-hydroxycinnamic acid (saturated in 90% acetone) in 0.1% TFA) were mixed and injected (2 μl) onto a plate and analysed by a Bruker Daltonics Reflex IV MALDI-TOF mass spectrometer.

## MetHb

Blood levels of methHb were measured as described previously [30], and expressed as a percentage of Hb (HemoCue B-Hemoglobin, Derbyshire, UK).

## TGSH

0.80 ml 200 mM sodium phosphate buffer (pH 7.6) was added to 0.20 ml sample and vortex-mixed. To this was added 0.10 ml 1 mM NADPH with 15 U ml<sup>–1</sup> glutathione reductase solution in 200 mM sodium phosphate buffer (pH 7.6). Samples were vortex-mixed, and incubated at room temperature for 15 min after which glutathione reductase activity was inhibited with 0.20 ml 1 M HCl. To 0.50 ml sample was added 0.50 ml 200 μM DTNB in 200 mM sodium phosphate buffer (pH 7.6). Absorbance (Cary 5E, Varian, Walton-on-Thames, UK) was read at 412 and 550 nm (to correct for baseline shifts). TGSH concentration was calculated using a standard curve created from GSH standards dissolved in phosphate-buffered saline (PBS).

## GSSG (adapted from [31])

0.80 ml 180 mM NaOH with 200 mM CAPS was added to 0.20 ml sample, vortex-mixed, and incubated at room temperature for 15 min. To a 0.50 ml sample was then added 0.35 ml 200 mM NaH<sub>2</sub>PO<sub>4</sub> in a 1.5-ml plastic microcuvette. Directly before measuring sample absorbance, 0.25 ml 1 mM NADPH in 200 mM sodium phosphate buffer (pH 7.6), and 0.25 ml 400 μM DTNB with 3 U ml<sup>–1</sup> glutathione reductase in 200 mM sodium phosphate buffer (pH 7.6) were added to the sample. The resulting increase in absorbance at 412 nm was measured every 30 s for 90 s

(Cary 5E, Varian, Walton-on-Thames, UK), and the slope of the regression equation of the increase in absorbance over time was used to determine GSSG concentration from a standard curve. Standard curves were created from GSSG standards dissolved in PBS, treated as described above.

#### *Glutathione redox status*

Postexercise TGSH and GSSG concentrations were corrected for changes in hematocrit. Concentration of GSH was calculated as the difference between TGSH and two times GSSG. Glutathione redox status was expressed as the ratio GSH/GSSG with GSSG in GSH equivalents.

#### *Effects of $pO_2$ and NO levels on OxHm in vitro*

To examine the effects of changing oxygen tension ( $pO_2$ ) and hemoglobin saturation ( $SAO_2$ ) in vitro on levels of OxHm, a venous blood sample was split into three triplicates which were left untreated for 1 h ( $pO_2$ , 50 mm Hg;  $SAO_2$ , 55%); left for 1 h and then subsequently fully oxygenated by mixing in a syringe with 100%  $O_2$  ( $pO_2$ , 200 mm Hg;  $SAO_2$ , 100%); or fully oxygenated and left for 1 h ( $pO_2$ , 200 mm Hg;  $SAO_2$ , 100%).

The effect of NO on levels of OxHm was studied by adding a rapid releasing NO donor (17  $\mu$ l 14 mM proli/NO) to 1.5 ml venous blood, resulting in the rapid release of 320  $\mu$ M NO. Samples ( $n = 3$ ) were then incubated at 37°C for 1 h. Venous blood was also incubated for 1 h with different volumes (3, 15, and 30  $\mu$ l) of a slow-release NO donor (30.15 mM DETA/NO); the 3  $\mu$ l addition was observed to result in a steady-state concentration of 1  $\mu$ M NO when added to PBS (steady-state NO was undetectable in the presence of the NO scavenging of blood).

#### *Statistical analyses*

Multiple linear regression with stepwise selection was used to examine relationships between levels of OxHm (dependent variable) and age, gender, BMI,  $\dot{V}O_{2MAX}$ , cigarettes per day, antioxidant use, and servings of fruits and vegetables per day (independent variables). Data on gender and antioxidant use were entered as dummy values. Paired-samples  $t$  tests were used to examine the effects of exercise and exchange transfusion on levels of OxHm and the GSH/GSSG ratio. All analyses were performed using SPSS statistical software. Statistical significance was accepted at  $P < 0.05$ .

#### *Animal studies*

The animal protocol was approved by the French National Ethics Committee (licence No. 006101), and the experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, revised 1985) at the Université Henri Poincaré, Nancy. The animals were acclimatized for 1

week after arrival with free access to food and water. At the end of the experiments, the animals were sacrificed by an excess dose of pentobarbital.

Two hours before experimentation, male guinea pigs (Charles River laboratories, L'Arbelle, France) weighing  $400 \pm 40$  g, anesthetized under halothane (volatile anaesthetic, Blamont, Neuilly-sur-Seine, France), were chronically implanted with a venous and arterial heparin-filled polyethylene catheters. The venous catheter (internal diameter 0.3 mm, Biotrol Dianostic, Chennevieres les Louvres, France) was implanted inside the right jugular vein (for venous blood withdrawal). The arterial catheter (internal diameter 0.3 mm, Biotrol Dianostic) was implanted inside the right carotid artery (for arterial blood withdrawal). A heparin (Héparine Choay, Sanofi Winthrop, Gentilly, France) dose of 150 IU  $kg^{-1}$  bw was injected to prevent clotting inside the catheter. Throughout the study the animals were spontaneously breathing room air.

Conscious guinea pigs ( $n = 6$ ) were then subjected to acute partial exchange transfusion (50% of total blood volume) performed with 2-ml syringes. Acute normovolemic hemodilution (ANH) was performed gradually in six steps by exchanging 13 ml  $kg^{-1}$  bw of blood with an identical volume of the Oxyglobin [32] hemoglobin-based blood substitute (Biopure, Cambridge, MA). Blood was collected from the arterial catheter with a syringe and the hemoglobin solution was infused at the same rate. The total duration of blood withdrawal and volume expansion was 25 min. Blood samples were collected before ANH ( $t = 0$ ) and 5 and 120 min after the end of Oxyglobin perfusion. At the end of exchange, 2 ml of Oxyglobin was injected to compensate for the blood samples to be withdrawn. Of the 1.5 ml of blood withdrawn, 0.3 ml was taken for hematological assessment (not reported in this paper) and the remainder separated into a plasma and red cell fraction. Samples were then frozen in liquid nitrogen and transported from Nancy (France) to Colchester (UK) on dry ice for HPLC analysis of the OxHm content in the blood and plasma.

## **Results**

### *Peroxide induces covalent modifications in hemoglobin in vitro*

Reverse-phase HPLC (run at acid pH) separates the heme B in hemoglobin (5 min) from the polypeptide chains (14 min for free heme B,  $\approx 21$  min for the  $\beta$  chains and  $\approx 22$  min for the  $\alpha$  chains). Detection at 280 nm allows both heme and protein to be observed (Fig. 1A); detection at 400 nm predominantly measures the free heme (Fig. 1B). The addition of  $H_2O_2$  to purified methemoglobin at neutral pH has only a small effect on



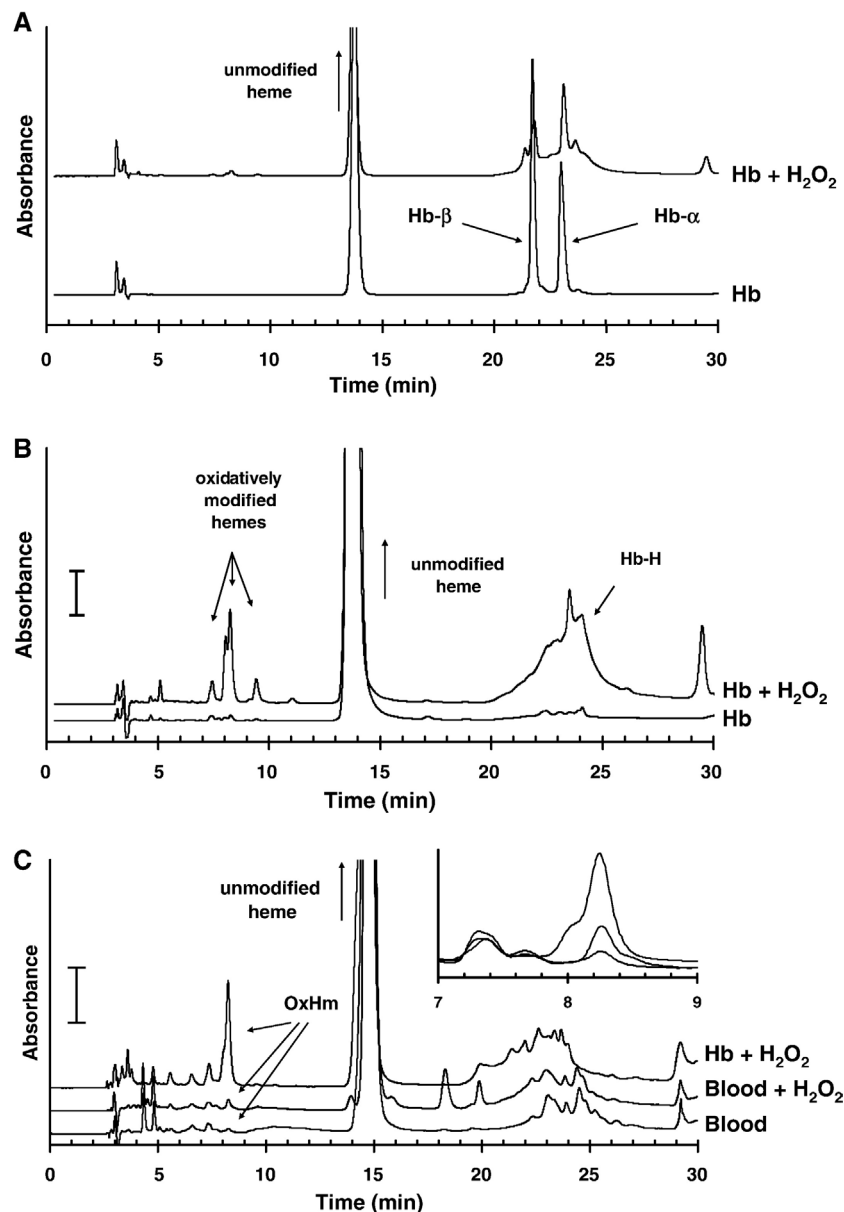


Fig. 1. Peroxide treatment of Hb and human blood modifies heme. HPLC chromatograms of 100  $\mu$ M metHb and 100  $\mu$ M metHb treated with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> detected at (A) 280 nm, and (B) at 400 nm; (C) HPLC chromatograms at 400 nm of normal blood; blood treated with 50 mM H<sub>2</sub>O<sub>2</sub> in 25 mM NaPi, pH 7.4, purified metHB (100  $\mu$ M) treated with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> in 25 mM NaPi, pH 7.4. Chromatograms off-set for clarity (+3 mAu). Peroxide treatment produced a modified heme species with an elution time of between 7 and 10 min. The inset in Fig. 1C shows the similarity of the elution time of OxHm under the different conditions (top line divided by a factor of 4 for clarity). The scale bars represent 10 mAu.

the elution profile of the polypeptide chains, but produces a range of modifications in the heme group. In particular a band is now detected at  $\approx 24$  min that elutes close to the polypeptide chains. This band has a characteristic absorbance peak at 720 nm [13], indicative of the formation of an iron-chlorin product [33]. This species (which we have called HbH) is a covalently cross-linked heme-protein product; it is produced at much higher concentrations under acid conditions and has been detected *in vivo* under extreme pathological conditions

(e.g., in the CSF following subarachnoid hemorrhage [11]).

However, peroxide treatment also induces covalent modifications to the heme group that do not result in a heme:protein cross-link. These can be seen at 400 nm as free heme species eluting between 7 and 10 min (Fig. 1B). One of these species (eluting at 8 min) has the same characteristic 720-nm heme band indicative of an iron-chlorin species. We term this oxidative modification of the heme, OxHm.

*Hemoglobin in control blood contains oxidatively modified heme products (OxHm) identical to those formed by addition of peroxides to Hb in vitro*

To determine if OxHm can be detected *in vivo* we assayed control human blood from healthy volunteers (Fig. 1C). We then induced oxidative modifications to the heme *ex vivo* by treating blood with high (50 mM) concentrations of  $H_2O_2$  (necessary due to the presence of catalase in the red blood cell).

The 400 nm chromatograms in whole blood are obviously more complex. However, unlike following subarachnoid hemorrhage, we saw no peak corresponding to a heme:protein cross-link (HbH) eluting at  $\approx 24$  min; there is a complete absence of a 720-nm heme absorbance peak in this region, either pre or post *ex vivo* peroxide treatment. Peroxide does, however, induce an increase in a modified heme species with an elution time of 8.3 min, essentially identical to that seen when peroxide is added to purified hemoglobin. This species also has the same spectral feature at 720 nm as that seen following peroxide addition to purified methemoglobin (Fig. 2) confirming its identity as the iron chlorin, OxHm.

Closer inspection (Fig. 1C inset) of the control “non-peroxide-treated” blood indicated a compound also eluting at 8.3 min that had the same 720-nm band (Fig. 2); the presence of OxHm in control blood demonstrates that heme oxidative modification occurs in humans *in vivo* under normal conditions. We suggest that endogenous OxHm is produced by a mechanism essentially identical to that occurring following the *in vitro* addition of peroxides to hemoglobin.

*Reproducibility of the OxHm assay*

A simple quantitative assay for OxHm was developed (see Methods). Intraassay variability of 10 duplicated

samples was low (coefficient of variation: 6.0%). OxHm concentration determined in aliquots of a blood sample frozen in liquid nitrogen 1, 3, 6, or 15 min after taking the sample was within  $\pm 5\%$  of the concentration determined in the fresh blood sample without freezing/thawing, suggesting that OxHm is a stable species unaffected by sample preparation.

*OxHm normally occurs in human blood, but levels are not related to methHb, age, fitness, or selected behavioural health factors*

To determine the distribution of resting levels of OxHm in the blood of healthy human subjects we recruited 64 young adults (30 females, 34 males,  $23 \pm 3$  years old) and 26 older adults (12 females, 14 males,  $57 \pm 6$  years old). All subjects were apparently healthy, and refrained from drinking alcohol or performing vigorous exercise for a minimum of 24 h prior to collection of a venous blood sample for the analysis of OxHm. On average 1 in  $\sim 2000$  Hb molecules was covalently oxidatively modified (Fig. 3); this compares to an average of 1 in  $\sim 500$  Hb molecules oxidised to methemoglobin in the same population (results not shown). Distribution of OxHm levels was not significantly different from normal (Kolmogorov-Smirnov test:  $P = 0.68$ ).

For each individual subject we subsequently obtained an estimate of  $\dot{V}O_{2MAX}$  using the Åstrand-Rhyming test [27], the number of cigarettes smoked per day, antioxidant use, and servings of fruits and vegetables consumed per day, parameters which have all been previously linked to oxidative damage [34–36]. Multiple linear regression analysis revealed no significant correlations between levels of OxHm and any of these parameters. Gender or age also did not correlate with OxHm. Base levels of OxHm seem to be well controlled across this range of lifestyle factors. Although there is a correlation between absolute methHb

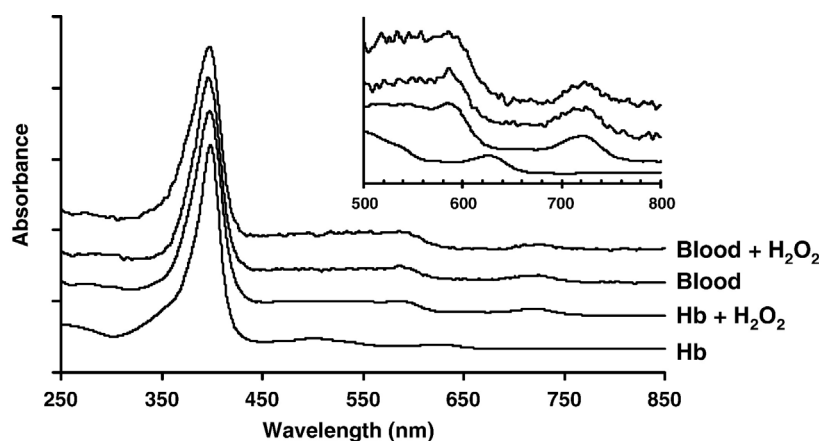


Fig. 2. Spectral properties of modified hemes. Spectral properties are shown for the species in Fig. 1 eluting at 8.3 min. Hemoglobin; hemoglobin treated with hydrogen peroxide; blood; blood + peroxide. Data indicate structural differences between heme B in control hemoglobin and in oxidatively modified hemoglobin (OxHm). The similarity of the characteristic peaks at 590–610 nm and 720–740 nm, clearly visible in the magnified inset, suggests the endogenous OxHm arises from peroxide modification of hemoglobin *in vivo*.

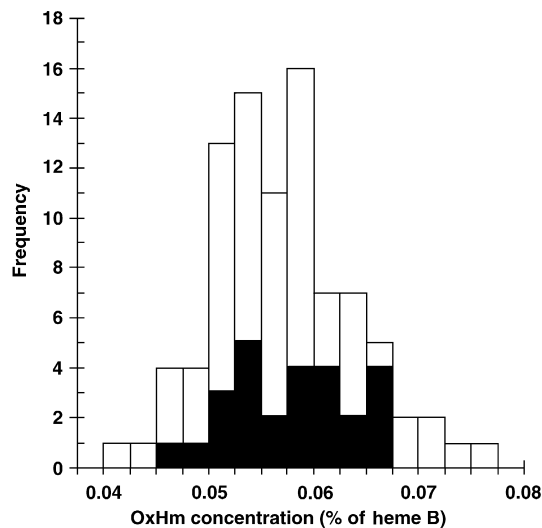


Fig. 3. Frequency distribution of OxHm levels. OxHm levels in a population of healthy young adults ( $n = 64$ ; white bars) and older subjects ( $n = 26$ ; black bars) appears to be normally distributed. See Table 1 for description of subjects.

levels and absolute OxHm levels ( $R^2 = 0.10$ ;  $P < 0.01$ ), this is solely due to the fact that both correlate with total hemoglobin. When both OxHm and metHb are expressed as percentage of total heme there is no correlation ( $R^2 = 0.03$ ,  $P = 0.11$ ); i.e., subjects with relatively high metHb levels do not have relatively high OxHm.

#### *Both exhaustive and submaximal exercise enhance formation of OxHm*

Unlike the more gradual, long-term effects on oxidative damage of factors such as age, antioxidant intake, and smoking, exercise poses an acute oxidative stress, generally believed to be caused by the increase in oxygen consumption, and/or possible hypoxia/reperfusion mechanisms. We have recently reviewed this field [37]. Exercise therefore provides an ideal system to test whether heme protein-mediated oxidative stress is increased by a known acute oxidative stress inducer in humans *in vivo*.

To investigate the effects of exercise on levels of OxHm, we studied nine well-trained triathletes, who performed two 1-h exhaustive cycling tests [38] spaced 1 week apart. The effectiveness of the protocol was indicated by the use of an independent marker of general oxidative stress (the GSH/GSSG ratio). Exercise significantly increased OxHm levels in postexercise venous blood samples from  $0.0575 \pm 0.0071$  to  $0.0596 \pm 0.0070\%$  ( $P < 0.05$ ) and from  $0.0565 \pm 0.0067$  to  $0.0596 \pm 0.0063\%$  ( $P < 0.05$ ) in the first and second trials, respectively (Fig. 4A); the GSH/GSSG ratio decreased significantly following both trials (Fig. 4B).

We observed exercise-induced increases in OxHm levels of the same magnitude (Fig. 4C) in untrained, physically active subjects cycling for 30 min at 65% of  $\dot{V}O_{2\text{MAX}}$ , performing an exhaustive, incremental treadmill test (10 kph

for 5 min increased by 1 kph every 2.5 min), or performing an exhaustive incremental cycling test (30 W  $\text{min}^{-1}$  ramp protocol, starting at 30 W). The fact that the exercise-induced increase in OxHm was similar in both trained and untrained subjects, and in both submaximal and maximal exercise, suggests the existence of an active defence mechanism that prevents increases above a threshold level.

#### *OxHm appears to be actively removed from the circulation*

To test the hypothesis that levels of oxidatively modified heme were actively controlled, we performed an additional experiment to determine whether a consistent exercise-induced increase in OxHm levels was still present 1 h after cessation of exercise. Eight untrained, physically active subjects cycled for 30 min at either a set intensity of 65% of  $\dot{V}O_{2\text{MAX}}$ , or alternating 1 min at 80% of  $\dot{V}O_{2\text{MAX}}$  and 1 min at 40% of  $\dot{V}O_{2\text{MAX}}$ . The significant increase observed directly after exercise was followed by a decrease to baseline levels within 1 h under both conditions (Fig. 4D). These data seem to imply that a repair or removal mechanism is in place in the body to limit the levels of OxHm.

#### *In vivo removal of OxHm in an animal model of exchange transfusion*

To explore the importance of the erythrocyte to the “repair” of OxHm we measured the levels of OxHm inside the erythrocyte and in the plasma following a 50% exchange transfusion with the artificial hemoglobin based oxygen carrier, Oxyglobin, in a guinea pig model. In this case the erythrocyte OxHm reports on the animal’s own hemoglobin and OxHm in the plasma reports on damage to the Oxyglobin. Fig. 5 shows that there is an increase in OxHm inside the erythrocyte following the transfusion. However, as in the exercise model this returns to control levels after 2 h. Perhaps surprisingly, however, a similar decrease was seen in the plasma; Oxyglobin OxHm decreases relative to unmodified Oxyglobin-derived heme B, implying a mechanism independent of red cell enzymes.

#### *Effects of $pO_2$ and NO levels on OxHm in red cells *in vitro**

During exercise and recovery there are large changes in  $pO_2$  and  $pNO$  that might be responsible for the changes in OxHm levels. We therefore determined the effect of varying  $pO_2$  (and consequently the hemoglobin autooxidation rate) on OxHm *ex vivo*. A sample was divided into three. One was kept at venous  $pO_2$ ; one was oxygenated to 100% saturation for 1 h; one was kept at venous  $pO_2$  for 1 h and then oxygenated to 100%. No effect was seen on the levels of OxHm (OxHm levels of  $0.0705 \pm 0.0007$ ,  $0.0701 \pm 0.0003$ , and  $0.0708 \pm 0.0008\%$ , respectively). The effect of varying NO was studied by adding a bolus of a fast release NO donor to venous blood *ex vivo* (Proli NO, releasing 320  $\mu\text{M}$  NO).

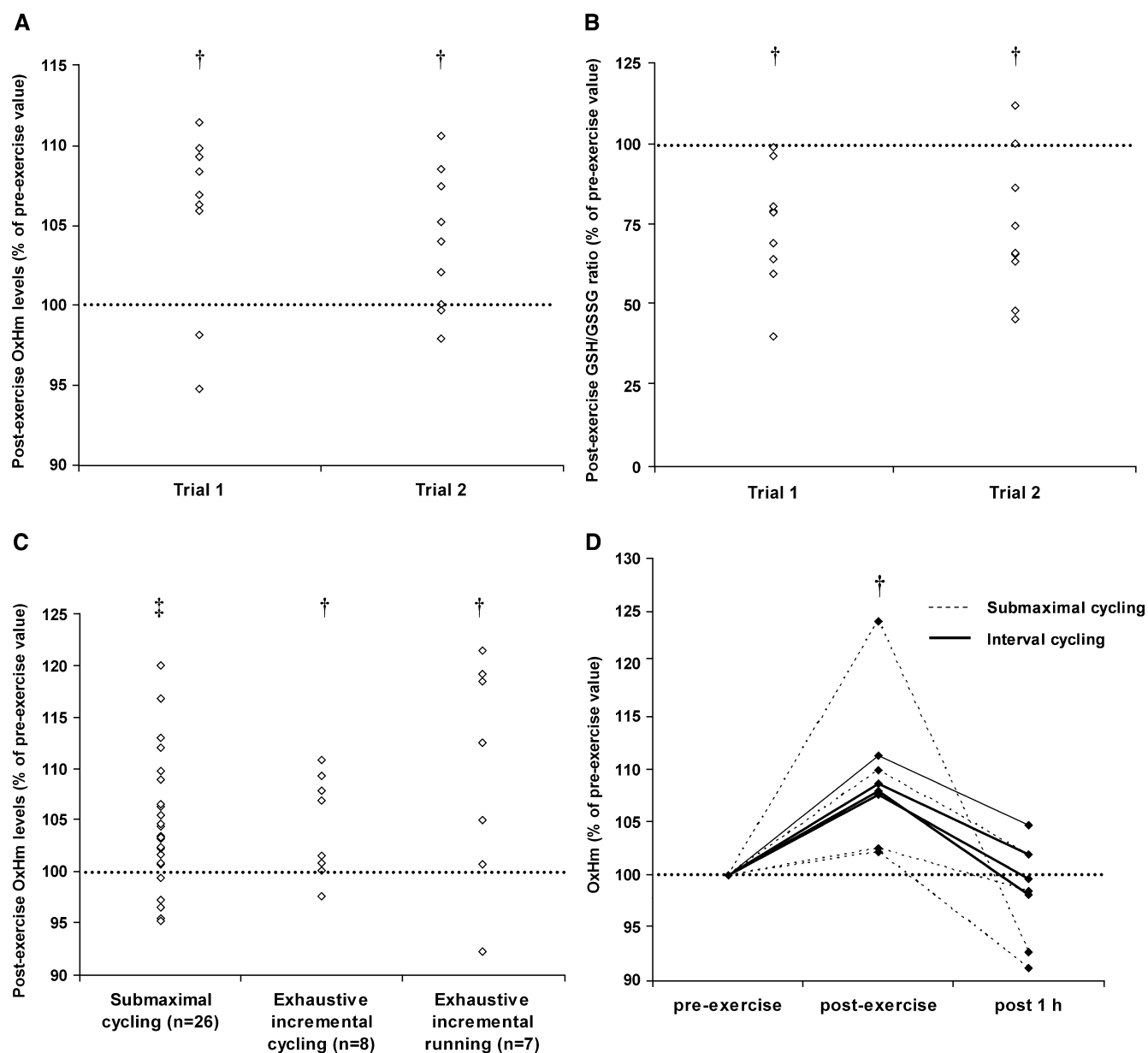


Fig. 4. Exercise-induced changes in levels of OxHm. In two independent trials endurance athletes show a postexercise increase in OxHm (A) accompanied by a decrease in the ratio GSH/GSSG (B). In untrained subjects there is also an increase in OxHm levels following exercise (C) which was reversible (D). † $P < 0.05$ ; ‡ $P < 0.001$ .

No effect was seen on OxHm levels (treated,  $0.0695 \pm 0.0013\%$ ; control,  $0.0687 \pm 0.0013\%$ ); the incubation of venous blood samples for 1 h with different concentrations of the slow NO donor DETA/NO also had no effect (control,  $0.0675 \pm 0.0020\%$ ; 60  $\mu\text{M}$  DETA/NO,  $0.0671 \pm 0.0025\%$ ; 300  $\mu\text{M}$  DETA/NO,  $0.0675 \pm 0.0018\%$ ; 600  $\mu\text{M}$  DETA/NO,  $0.0664 \pm 0.0022\%$ ).

#### Effects of reductants on OxHm production from Hb in vitro

To confirm that the ferryl iron and protein radicals were required for OxHm formation we added  $\text{H}_2\text{O}_2$  to methemoglobin in the presence of compounds known to reduce these

haemoglobin-oxidising equivalents (Fig. 6). Ascorbate prevented OxHm formation as long as it was higher than the peroxide concentration (Fig. 6A). As both ascorbate and peroxide are two electron oxidants this suggests that ascorbate's ability to reduce both protein radicals and ferryl iron is the mechanism by which it prevents OxHm formation. NO can also reduce ferryl hemoglobin [39]. Consistent with this, the NO donor, DEANO-NOate, also decreased OxHm formation (Fig. 6B). Given the difficulties of maintaining an aerobic steady-state NO concentration the exact stoichiometry is harder to determine, although the data suggest that superstoichiometric concentrations are necessary for preventing OxHm formation. At very high DEANO levels



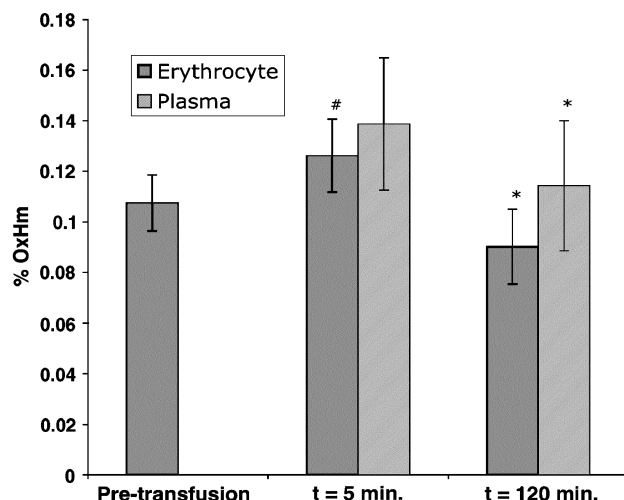


Fig. 5. Reversal of OxHm formation following exchange transfusion in vivo. Levels of OxHm formation following a 50% exchange transfusion with Oxyglobin in vivo. Erythrocyte (HbA<sub>0</sub>) OxHm levels were measured pretransfusion and 5 and 120 min posttransfusion. Oxyglobin OxHm levels were measured in plasma samples taken 5 and 120 min postexchange transfusion. \**P* < 0.05 versus *t* = 5 min; #*P* < 0.05 versus pretransfusion.

OxHm levels actually began to increase again, suggesting a role for NO-derived oxidants (e.g., N<sub>2</sub>O<sub>3</sub>) produced under these conditions. The antioxidant iron-chelator, desferrioxamine, is known to reduce ferryl heme and protein radicals [16,40]. It was also able to prevent OxHm formation (Fig. 6C). In this case a 2:1 desferrioxamine:H<sub>2</sub>O<sub>2</sub> ratio was required, consistent with desferrioxamine being a single electron donor. Its iron chelation properties were irrelevant to these effects; the redox inactive iron chelators, transferrin, had no effect on OxHm formation (Fig. 6D).

#### Structure of OxHm prepared from myoglobin and hemoglobin

We compared the OxHm produced from H<sub>2</sub>O<sub>2</sub> treatment of myoglobin and hemoglobin (Fig. 7). Identical mass spectroscopy and optical data between the two proteins confirm that OxHm produced from hemoglobin and myoglobin are identical species. The structure of the oxidized heme derived from peroxide treatment of myoglobin was previously determined by Sugiyama et al. [33] to be an iron chlorin. The mass of our hemoglobin-driven product (632.2) corresponds to Sugiyama's product II (632.5) and this structure is illustrated in Fig. 7.

#### Discussion

We have developed an assay that allows for a simple and reproducible measure of the extent of hemoglobin that has been specifically oxidatively modified in vivo. Using it we have determined that hemoglobin is oxidatively modified in humans in vivo, that this modification increases following a

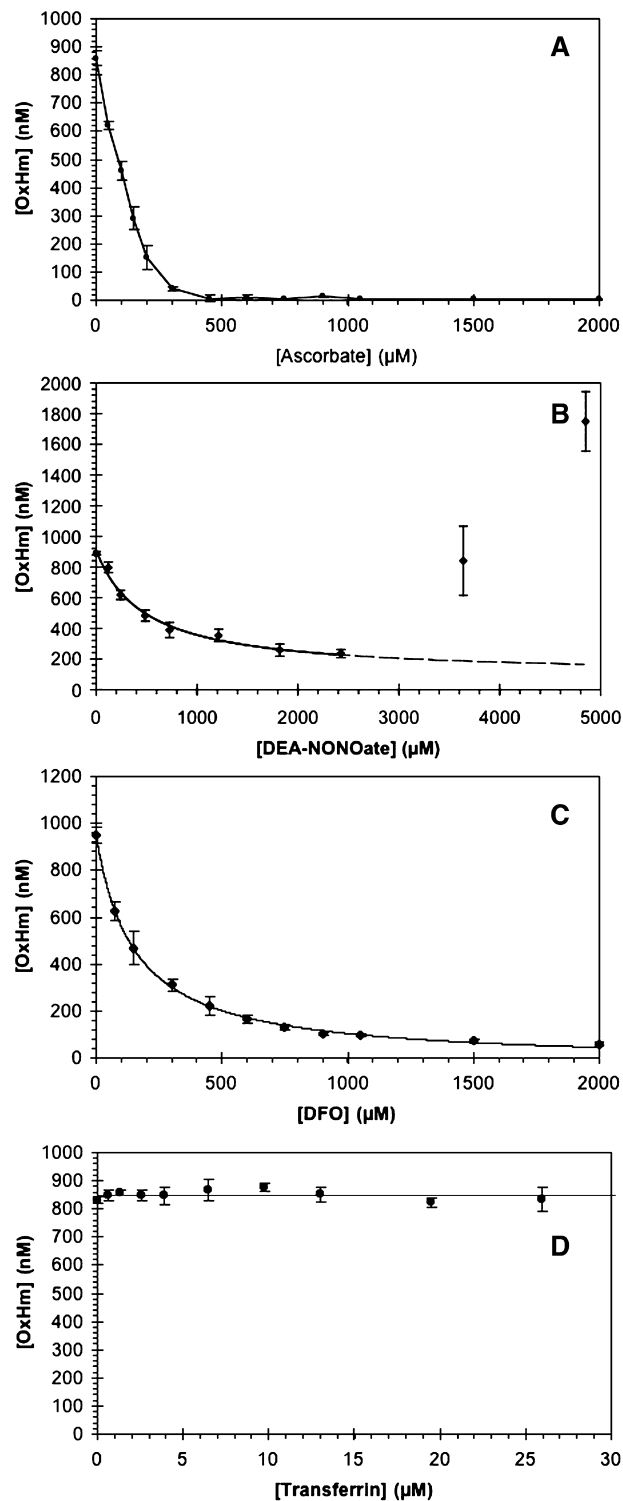


Fig. 6. Effect of antioxidants and iron chelators on the formation of OxHm in vitro. Methemoglobin (100 μM) was reacted with hydrogen peroxide (300 μM) in PBS buffer, pH 7.4, for 2 h at 37°C in the presence and absence of solutions indicated. Oxidatively modified heme (OxHm) was quantified by HPLC as described under Experimental Procedures (*n* = 3). (A) Ascorbate; (B) 2-(*N,N*-dimethylamino)-diazene-2-oxide (DEA/NO-NOate), under these conditions 1.5 NO molecules are released for every DEANO-NOate molecule; (C) desferrioxamine mesylate (DFO); (D) apotransferrin. In b the addition of peroxide was subsequent to DEANO-NOate addition by 0, 2, and 5 min No significant differences were observed between the three data sets.

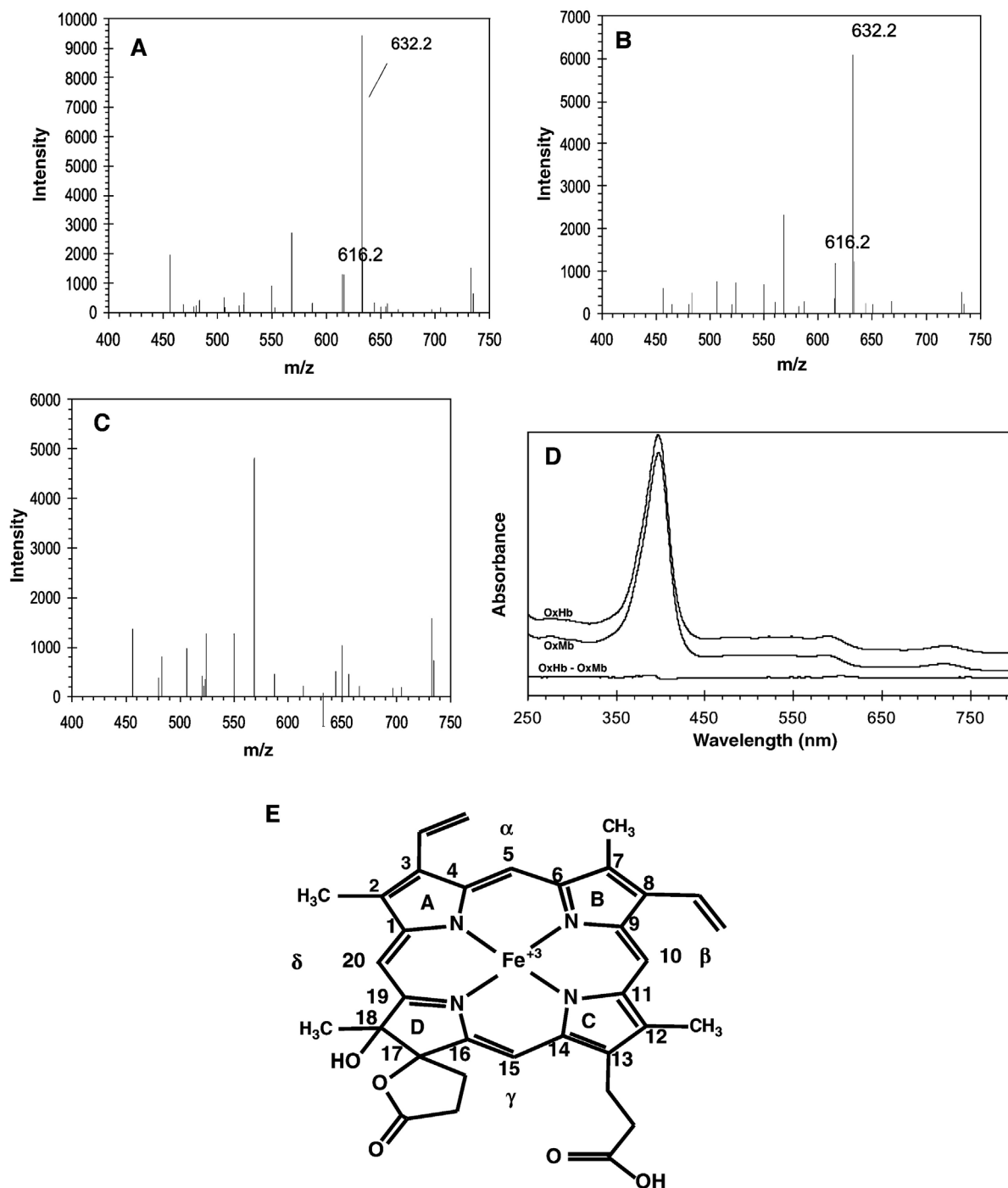


Fig. 7. Structure of OxHm. Optical and MALDI-TOF mass spectra of oxidatively modified heme from myoglobin and hemoglobin following peroxide damage. Metmyoglobin or methemoglobin (100  $\mu$ M) was reacted with hydrogen peroxide (1 mM) in PBS buffer, pH 7.4, at 37°C for 12 h. The oxidatively modified hemes were purified by HPLC and analysed by MALDI-TOF MS. The heme generated by peroxide damage to myoglobin (A) is identical to that from peroxide-induced hemoglobin damage (B) with a  $[M+H]^+$  of 632.2. Another heme is also present with  $m/z$  616.2; this could be a dehydration product of the damaged heme ( $[M+H]^+-H_2O$ ) but also coincides with the parent molecular mass of unmodified heme. A control sample of myoglobin without peroxide damage shows no appreciable amounts of the 632.2 or 616.2 heme species eluting at this time (C). UV/Vis spectra of OxHm derived from hemoglobin and myoglobin are identical (D). The predicted structure of OxHm consistent with this data (E)—figure adapted from Sugiyama et al. [33].

known inducer of oxidative stress (exercise), and that the body has a system for removing/repairing the damage.

Other methods have been used in vivo to detect the peroxidative chemistry of hemoglobin and myoglobin.

Equation (1) results in the production of both ferryl heme and a free radical. We have previously determined that the tyrosyl free radical produced by the interaction of peroxides with globin is readily detectable in human blood using low-

temperature electron paramagnetic resonance spectroscopy [41]. However, this technology is expensive and not always readily available. Ferryl iron can be detected optically in vivo, e.g., as ferryl myoglobin following ischemia reperfusion in the heart [42] or as ferryl hemoglobin following peroxide addition to blood [43]. However, its low concentration means that addition of sulfide (to generate the sulf species) is required for its detection [44]. The toxicity of sulfide limits the measurement in humans to ex vivo conditions.

Detection of ferryl iron in normal human blood (in the absence of peroxide addition) has not been observed. More problematic than simple detection issues, however, is the fact that the measured concentrations of ferryl hemoglobin and the globin radical are in a dynamic steady state. An increase in ferryl hemoglobin could mean that there is an increase in the potential for oxidative damage, but could just as readily mean that conditions are *less* conducive for ferryl-induced damage to occur. The most obvious situation where this occurs is the effect of pH. Lowering pH results in a highly reactive (more oxidatively damaging) protonated ferryl species, with a consequently much shorter half-life [23]. The ferryl concentration would therefore decrease, but as a result of *increased* oxidative stress to the body. Similar problems occur in interpreting changes in the concentration of the (much more readily detectable) methemoglobin species.

We have therefore chosen to look for a relatively stable species that will indicate whether hemoglobin is undergoing (and initiating) oxidative stress in vivo. In healthy volunteers we could not detect the heme-covalent cross-linked product (HbH) that we see in disease states. However, we could detect a range of additional modifications to the heme. We chose to target a species with a peak at 720 nm (which we termed OxHm). In myoglobin this species has been well characterised as due to a heme chlorin [13,33]. Analysis of the mass spectroscopic data confirms that the same species is formed in hemoglobin. Clearly there are a range of modified hemes in vivo, eluting at different times. However, using the 720-nm band as the key feature of a covalent heme modification enables us to say confidently that peroxide has interacted with hemoglobin, with resulting ferryl/free radical chemistry. This can modify the hemoglobin itself (as we show here), but also has the ability to oxidise other key biological molecules; lipid peroxidation is likely to be the most damaging in the vasculature [8,16]. Indeed heme-mediated peroxidation of arachidonic acid may be responsible for the cyclooxygenase-independent production of prostaglandin F<sub>2</sub>-like compounds in vivo in humans [45].

The level of oxidatively modified heme appears well controlled in vivo. Although we showed that an increase in oxidative stress increased the levels detected, these returned to baseline within an hour. Our assay measures the level of modified heme relative to normal heme and therefore cannot distinguish between “repair” or “removal” of the altered heme product. We suggest that

the most likely mechanism is to remove the damaged heme, rather than have an enzymatic mechanism for regenerating heme B; removal might occur spontaneously if OxHm had a decreased affinity for the globin moiety. Normal scavenging systems could then remove the toxic heme [46]. In support of this “passive” mechanism, the OxHm formed in the plasma during transfusion of a haemoglobin-based oxygen carrier decreased to the same extent as OxHm formed within the erythrocyte.

The detailed mechanism of formation and removal of OxHm formation in vivo in the particular models we have chosen to illustrate these phenomena is beyond the scope of this paper. Nevertheless the in vitro studies on pure hemoglobin confirm that it is the oxidants formed in the protein that are responsible for the heme damage. Reductants known to reduce ferryl iron and protein radicals prevent OxHm formation stoichiometrically. The relative effects of ascorbate and nitric oxide suggest that the former may play a more important role in modifying the oxidative reactions of hemoglobin, especially given its much greater concentration in both plasma and erythrocytes in vivo. In the presence of iron chelators with no redox activity, OxHm formation is unchanged. Therefore the effect of desferrioxamine in preventing heme damage can be entirely explained by its direct antioxidant radical and ferryl-scavenging properties, rather than by iron chelation.

How does OxHm form during exercise? The chemistry suggests peroxide reactivity as the primary event. Although we also have doubts as to whether in vitro studies on erythrocytes can realistically mimic the effects of exhaustive exercise, we did modify the *pO*<sub>2</sub> and *pNO* in an attempt to mimic at least some of the in vivo changes expected during exercise. Raising the hemoglobin autooxidation rate by raising *pO*<sub>2</sub> and hemoglobin saturation did not affect OxHm levels ex vivo. Nor was there any effect following the addition of an NO donor. The level of OxHm formed in vivo appears resistant to change ex vivo. Although this might suggest that there are in vivo processes occurring that we cannot readily mimic in vitro (e.g., lipid peroxidation, shear stress), we would not want to overemphasise these preliminary studies (although we note it does improve the resistance of the assay to differences in sample handling ex vivo).

If peroxide is the cause of OxHm formation in vivo it might appear surprising that to mimic the effect ex vivo we have to add high (mM) levels of H<sub>2</sub>O<sub>2</sub> to overcome the effects of catalase and glutathione peroxidase in the erythrocyte. However, it is not unusual for “external” peroxide to be less effective than “internal” peroxide in initiating radical reactions. In particular the erythrocyte is a rather unique environment (being almost crystalline hemoglobin) and it is not clear that peroxidases and catalases can prevent all intracellularly generated peroxides from interacting with at least a fraction of hemoglobin given the very high concentration of the latter. Microheterogeneity may also play a part; if catalase cannot access the peroxide before the hemoglobin then there is always a chance of ferryl

formation. Our in vivo assay is over a longer time scale than that in vitro and these small effects could accumulate to generate the detectable low concentrations of OxHm we observe. Alternatively catalase could be bypassed if lipid peroxides were responsible for OxHm formation as they can initiate ferryl chemistry, but are poor substrates for catalases. In any event OxHm formation in vivo is consistent with our previous detection of the peroxide-derived hemoglobin tyrosyl radical in normal human blood [41].

In conclusion we have shown here that oxidative modification to hemoglobin is a normal occurrence in vivo in human blood, and is enhanced by exercise. As OxHm is exclusively produced in erythrocytes by peroxidation of hemoglobin, changes in its concentration may provide direct mechanistic and diagnostic information. We therefore propose that, more than general indicators such as glutathione redox status and TBARS, OxHm may be useful as an indicator of a specific form of oxidative stress. Although an enhanced prooxidant activity of heme to protein cross-linked species has previously been demonstrated [8,16], it is unknown whether noncovalently bound oxidatively modified hemes like OxHm are similarly prooxidant. The detailed relevance of OxHm to human physiology or pathophysiology remains to be elucidated; however, the fact that a mechanism exists for its removal/ degradation/repair in vivo suggests there is a desire by the body to keep its levels low.

## Acknowledgments

We thank the University of Essex Research Promotion Fund, the Wellcome Trust (Biomedical Research Collaboration Fund), and BBSRC for financial assistance. We thank Peter Nicholls (University of Essex, UK) and Dr. Abdu Alayash (FDA, Bethesda, MD) for helpful comments.

## References

- [1] Halliwell, B.; Gutteridge, J. M. C. Oxygen free-radicals and iron in relation to biology and medicine—Some problems and concepts. *Arch. Biochem. Biophys.* **246**:501–514; 1986.
- [2] Melov, S.; Ravenscroft, J.; Malik, S.; Gill, M. S.; Walker, D. W.; Clayton, P. E.; Wallace, D. C.; Malfroy, B.; Doctrow, S. R.; Lithgow, G. J. Extension of life-span with superoxide dismutase/catalase mimetics. *Science* **289**:1567–1569; 2000.
- [3] Raha, S.; Robinson, B. H. Mitochondria, oxygen free radicals, disease and ageing. *Trends Biochem. Sci.* **25**:502–508; 2000.
- [4] Sohal, R. S.; Toy, P. L.; Allen, R. G. Relationship between life expectancy, endogenous antioxidants and products of oxygen free radical reactions in the housefly, *Musca domestica*. *Mech. Ageing Dev.* **36**:71–77; 1986.
- [5] Gutteridge, J. M. C. Iron promoters of the Fenton reaction and lipid preoxidation are released from haemoglobin by peroxides. *FEBS Lett.* **201**:291–295; 1986.
- [6] Turner, J. J.; Rice-Evans, C. A.; Davies, M. J.; Newman, E. S. The formation of free radicals by cardiac myocytes under oxidative stress and the effects of electron-donating drugs. *Biochem. J.* **277**:833–837; 1991.
- [7] Moore, K. P.; Holt, S.; Patel, R. P.; Svistunenko, D. A.; Zackert, W.; Goodier, D.; Reeder, B. J.; Clozel, M.; Anand, R.; Cooper, C. E.; Harvey, S.; Morrow, J. D.; Wilson, M. T.; Darley-Usmar, V.; Roberts, L. J. I. A causative role for redox cycling of myoglobin and its inhibition by alkalization in the pathogenesis and treatment of rhabdomyolysis-induced renal failure. *J. Biol. Chem.* **273**:31731–31737; 1998.
- [8] Holt, S.; Reeder, B.; Wilson, M.; Harvey, S.; Morrow, J. D.; Roberts, L. J., II; Moore, K. Increased lipid peroxidation in patients with rhabdomyolysis. *Lancet* **353**:1241; 1999.
- [9] Holt, S.; Moore, K. Pathogenesis of renal failure in rhabdomyolysis: the role of myoglobin. *Exp. Nephrol.* **8**:72–76; 2000.
- [10] Alayash, A. I.; Patel, R. P.; Cashion, R. E. Redox reactions of hemoglobin and myoglobin: biological and toxicological implications. *Antioxid. Redox Signal.* **3**:313–327; 2001.
- [11] Reeder, B. J.; Sharpe, M. A.; Kay, A. D.; Kerr, M.; Moore, K.; Wilson, M. T. Toxicity of myoglobin and haemoglobin: oxidative stress in patients with rhabdomyolysis and subarachnoid haemorrhage. *Biochem. Soc. Trans.* **30**:745–748; 2002.
- [12] Arduini, A.; Eddy, L.; Hochstein, P. Detection of ferryl myoglobin in the isolated ischemic rat heart. *Free Radic. Biol. Med.* **9**:511–513; 1990.
- [13] Reeder, B. J.; Svistunenko, D. A.; Sharpe, M. A.; Wilson, M. T. Characteristics and mechanism of formation of peroxide-induced heme to protein cross-linking in myoglobin. *Biochemistry* **41**:367–375; 2002.
- [14] Sutton, H. C.; Winterbourn, C. C. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radic. Biol. Med.* **6**:53–60; 1989.
- [15] Newman, E. S. R.; Rice-Evans, C. A.; Davies, M. J. Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochem. Biophys. Res. Commun.* **179**:1414–1419; 1991.
- [16] Kanner, J.; Harel, S. Desferrioxamine as an electron donor. Inhibition of membranous lipid peroxidation initiated by H<sub>2</sub>O<sub>2</sub>-activated met-myoglobin and other peroxidizing systems. *Free Radic. Res. Commun.* **3**:309–317; 1987.
- [17] Rogers, M. S.; Patel, R. P.; Reeder, B. J.; Sarti, P.; Wilson, M. T.; Alayash, A. I. Pro-oxidant effects of cross-linked haemoglobins explored using liposomes and cytochrome c oxidase vesicle model membranes. *Biochem. J.* **310**:827–833; 1995.
- [18] Nath, K. A.; Haggard, J. J.; Croatt, A. J.; Grande, J. P.; Poss, K. D.; Alam, J. The indispensability of heme oxygenase-1 in protecting against acute heme protein-induced toxicity in vivo. *Am. J. Pathol.* **156**:1527–1535; 2000.
- [19] Alayash, A. I. Oxygen therapeutics: can we tame haemoglobin? *Nature Rev. Drug. Disc.* **3**:152–159; 2004.
- [20] George, P.; Irvine, D. H. The reaction between metmyoglobin and hydrogen peroxide. *Biochem. J.* **52**:511–517; 1952.
- [21] Fox, J. B., Jr.; Nicholas, R. A.; Ackerman, S. A.; Swift, C. E. A multiple wavelength analysis of the reaction between hydrogen peroxide and metmyoglobin. *Biochemistry* **13**:5178–5186; 1974.
- [22] Catalano, C. E.; Choe, Y. S.; Ortiz de Montellano, P. R. Reactions of the protein radical in peroxide-treated myoglobin. Formation of a heme-protein cross-link. *J. Biol. Chem.* **264**:10534–10541; 1989.
- [23] Reeder, B. J.; Wilson, M. T. The effects of pH on the mechanism of hydrogen peroxide and lipid hydroperoxide consumption by myoglobin: a role for the protonated ferryl species. *Free Radic. Biol. Med.* **30**:1311–1318; 2001.
- [24] Vuletic, J. L.; Osawa, Y.; Aviram, M. Enhanced lipid oxidation by oxidatively modified myoglobin: role of protein-bound heme. *Biochem. Biophys. Res. Commun.* **269**:647–651; 2000.
- [25] Osawa, Y.; Korzekwa, K. Oxidative modification by low levels of HOOH can transform myoglobin to an oxidase. *Proc. Natl. Acad. Sci. USA* **88**:7081–7085; 1991.
- [26] Osawa, Y.; Williams, M. S. Covalent crosslinking of the heme prosthetic group to myoglobin by H<sub>2</sub>O<sub>2</sub>: toxicological implications. *Free Radic. Biol. Med.* **21**:35–41; 1996.

- [27] Astrand, P. O.; Ryhming, I. A nomogram for calculation of aerobic capacity (physical fitness) from pulse rate during sub-maximal work. *J. Appl. Physiol.* **7**:218–221; 1954.
- [28] Antonini, E.; Brunori, M. Hemoglobin and myoglobin and their reactions with ligands. *Hemoglobin and myoglobin and their reactions with ligands*. North Holland Publishing Company, Amsterdam, p. 436; 1971.
- [29] Bonaventura, J.; Schroeder, W. A.; Fang, S. Human erythrocyte catalase: an improved method of isolation and a reevaluation of reported properties. *Arch. Biochem. Biophys.* **150**:606–617; 1972.
- [30] Svistunenko, D. A.; Sharpe, M. A.; Nicholls, P.; Wilson, M. T.; Cooper, C. E. A New Method for Quantitation of Spin Concentration by EPR Spectroscopy: Application to Methemoglobin and Metmyoglobin. *J. Magn. Reson.* **142**:266–275; 2000.
- [31] Sacchetta, P.; Di Cola, D.; Federici, G. Alkaline hydrolysis of N-ethylmaleimide allows a rapid assay of glutathione disulfide in biological samples. *Anal. Biochem.* **154**:205–208; 1986.
- [32] Driessen, B.; Jahr, J. S.; Lurie, F.; Griffey, S. M.; Gunther, R. A. Effects of haemoglobin-based oxygen carrier hemoglobin glutamer-200 (bovine) on intestinal perfusion and oxygenation in a canine hypovolaemia model. *Br. J. Anaesth.* **86**:683–692; 2001.
- [33] Sugiyama, K.; Highet, R. J.; Woods, A.; Cotter, R. J.; Osawa, Y. Hydrogen peroxide-mediated alteration of the heme prosthetic group of metmyoglobin to an iron chlorin product: evidence for a novel oxidative pathway. *Proc. Natl. Acad. Sci. USA* **94**:796–801; 1997.
- [34] Church, D. F.; Pryor, W. A. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health. Perspect.* **64**:111–126; 1985.
- [35] McCall, M. R.; Frei, B. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic. Biol. Med.* **26**:1034–1053; 1999.
- [36] Eastwood, M. A. Interaction of dietary antioxidants in vivo: how fruit and vegetables prevent disease? *QJM* **92**:527–530; 1999.
- [37] Cooper, C. E.; Vollaard, N. B.; Choueiri, T.; Wilson, M. T. Exercise, free radicals and oxidative stress. *Biochem. Soc. Trans.* **30**:280–285; 2002.
- [38] Jeukendrup, A.; Saris, W. H.; Brouns, F.; Kester, A. D. A new validated endurance performance test. *Med. Sci. Sports Exerc.* **28**:266–270; 1996.
- [39] Herold, S.; Rehmann, F. J. Kinetics of the reactions of nitrogen monoxide and nitrite with ferryl hemoglobin. *Free Radic. Biol. Med.* **34**:531–545; 2003.
- [40] Cooper, C. E.; Green, E. S. R.; Rice-Evans, C. A.; Davies, M. J.; Wrigglesworth, J. M. A hydrogen-donating monohydroxamate scavenges ferryl myoglobin radicals. *Free Radic. Res.* **20**:219–227; 1994.
- [41] Svistunenko, D. A.; Patel, R. P.; Voloshchenko, S. V.; Wilson, M. T. The globin-based free radical of ferryl hemoglobin is detected in normal human blood. *J. Biol. Chem.* **272**:7114–7121; 1997.
- [42] Eddy, L.; Arduini, A.; Hochstein, P. Reduction of ferrylmyoglobin in rat diaphragm. *Am. J. Physiol.* **259**:C994–C997; 1990.
- [43] Giulivi, C.; Davies, K. J. A novel antioxidant role for hemoglobin. The comproportionation of ferrylhemoglobin with oxyhemoglobin. *J. Biol. Chem.* **265**:19453–19460; 1990.
- [44] Berzofsky, J. A.; Peisach, J.; Blumberg, W. E. Sulfheme proteins: I. Optical and magnetic properties of sulfmyoglobin and its derivatives. *J. Biol. Chem.* **246**:3367–3377; 1971.
- [45] Morrow, J. D.; Hill, K. E.; Burk, R. F.; Nammour, T. M.; Badr, K. F.; Roberts, L. J. d. A series of prostaglandin F<sub>2</sub>-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA* **87**:9383–9387; 1990.
- [46] Bui, L.; Rish, K.; Jaronecnyk, K.; Bourque, S.; McLaughlin, B. E.; Brien, J. F.; Marks, G. S.; Smith, A.; Nakatsu, K. The source of heme for vascular heme oxygenase I: heme uptake in rat aorta. *Can. J. Physiol. Pharmacol.* **82**:209–217; 2004.