

Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis

Sruti Shiva^{1,2}, Xunde Wang^{1,2}, Lorna A Ringwood^{1,2}, Xueying Xu³, Susan Yuditskaya^{1,2}, Vidhya Annavajhala¹, Hiroaki Miyajima⁴, Neil Hogg⁵, Zena Leah Harris³ & Mark T Gladwin^{1,2}

Nitrite represents a bioactive reservoir of nitric oxide (NO) that may modulate vasodilation, respiration and cytoprotection after ischemia-reperfusion injury. Although nitrite formation is thought to occur via reaction of NO with oxygen, this third-order reaction cannot compete kinetically with the reaction of NO with hemoglobin to form nitrate. Indeed, the formation of nitrite from NO in the blood is limited when plasma is substituted with physiological buffers, which suggests that plasma contains metal-based enzymatic pathways for nitrite synthesis. We therefore hypothesized that the multicopper oxidase, ceruloplasmin, could oxidize NO to NO⁺, with subsequent hydration to nitrite. Accordingly, plasma NO oxidase activity was decreased after ceruloplasmin immunodepletion, in ceruloplasmin knockout mice and in people with congenital aceruloplasminemia. Compared to controls, plasma nitrite concentrations were substantially reduced in ceruloplasmin knockout mice, which were more susceptible to liver infarction after ischemia and reperfusion. The extent of hepatocellular infarction normalized after nitrite repletion. These data suggest new functions for the multicopper oxidases in endocrine NO homeostasis and nitrite synthesis, and they support the hypothesis that physiological concentrations of nitrite contribute to hypoxic signaling and cytoprotection.

Recent data suggests that nitrite is a bioactive endocrine form of NO that regulates hypoxic physiological responses and has potential therapeutic applications^{1–5}. Nitrite is reduced to NO by several mechanisms *in vivo*, including acidic disproportionation and enzymatic reduction by xanthine oxidoreductase^{6–8}. Within the vasculature, nitrite is also converted to NO after reaction with deoxyhemoglobin along the physiological oxygen and pH gradient^{2,9}. These mechanisms have been suggested to contribute to hypoxic vasodilation, hypoxic NO-dependent signal transduction and modulation of mitochondrial respiration^{2–4}. In tissues, near-physiological concentrations of nitrite (<200 nM) protect against ischemia-reperfusion (I/R) injury in several organs and animal models^{10,11}. Though the precise mechanism of this cytoprotection is still being elucidated, nitrite can readily modify diverse biomolecules by protein and lipid nitration^{12,13}, N- and S-nitrosation¹⁴ and iron nitrosylation^{15,16}, and it regulates the expression of essential stress response genes^{1,14}. Given the role of nitrite as an important hypoxic signaling molecule, a better understanding of the mechanisms responsible for nitrite formation, transport and metabolism (that is, nitrite homeostasis) is essential. Furthermore, an understanding of the ways in which nitrite forms would allow for the design of experiments to test the hypothesis that basal physiological concentrations of nitrite contribute to hypoxic signaling and cytoprotection after I/R injury.

Although nitrite concentrations in human plasma and erythrocytes are known to be maintained within a narrow range (121 ± 9 nM and

288 ± 47 nM, respectively)¹⁷ and seem to be conserved across many mammalian species¹⁸, the mechanisms of nitrite formation in the vasculature are not clear. Traditionally, auto-oxidation of NO was thought to be the primary route of nitrite formation, based on the fact that this reaction occurs in oxygenated aqueous buffers. However, from a kinetic standpoint this is impossible in blood. The high concentrations (10 mM) of hemoglobin present in blood react with NO at nearly diffusion-limited rates ($k = 3.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and convert NO to metabolically inert nitrate¹⁹. Though the encapsulation of hemoglobin in red blood cells creates important diffusional barriers to NO that decrease the rate of reaction of hemoglobin with NO by approximately 1,000-fold compared with cell-free hemoglobin, the half-life of NO in whole blood is still calculated to be less than 2 ms. For comparison, the calculated half-time for the NO auto-oxidation reaction ($k = 2 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$)²⁰ at physiological NO and oxygen concentrations (400 nM and 150 μM, respectively) is greater than 30 min, which shows that NO auto-oxidation cannot occur before NO is scavenged by hemoglobin *in vivo*.

We therefore hypothesized that there must exist a mechanism for metal-based catalysis of nitrite synthesis and a specific NO oxidase activity in plasma that can effectively compete with the dioxygenase activity of erythrocytic oxyhemoglobin. In this study we investigate the NO oxidase activity of plasma and describe a previously unknown role for ceruloplasmin in nitrite homeostasis.

¹Vascular Medicine Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, USA. ²Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20892, USA. ³Department of Anesthesia and Critical Care Medicine, Johns Hopkins Hospital and School of Medicine, Baltimore, Maryland 21287, USA. ⁴First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan. ⁵Department of Biophysics and Free Radical Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, USA. Correspondence should be addressed to M.T.G. (mgladwin@nih.gov).

Received 14 April; accepted 14 July; published online 13 August 2006; doi:10.1038/nchembio813

RESULTS

Plasma has NO oxidase activity

Despite the severe kinetic constraints on NO reactivity in blood, nitrite somehow forms rapidly in blood *in vitro*²¹ and *in vivo*²², and acute increases in plasma nitrite concentrations are observed after pharmacological endothelial nitric oxide synthase activation^{17,22}. This suggests that mechanisms other than NO auto-oxidation exist in the vasculature to generate nitrite from NO at a kinetically favorable rate. Indeed, we previously found that even in the presence of cell-free hemoglobin (2 μM), a substantial concentration of nitrite is formed when NO is added to plasma²¹. To further explore the fate of NO and formation of nitrite in blood, we added 0–50 μM of the NO donor 2-(*N,N*-diethylamino)-diazeneolate-2-oxide nonate (DEANONOate, or DEANO; **1**) to plasma and phosphate-buffered saline (PBS) in the presence and absence of red blood cells (50% hematocrit). After 30 min incubation at room temperature (23–27 °C), we removed the erythrocytes by centrifugation and measured NO metabolites in the supernatant by reductive chemiluminescence in the presence and absence of acid sulfanilamide (Fig. 1a,b). As expected, 100% of the added NO was oxidized to nitrite in PBS in the absence of red blood cells (a result consistent with NO auto-oxidation), whereas approximately 90% was oxidized to nitrite in plasma in the absence of red blood cells, with the remaining 10% forming *S*-nitrosothiol. In the presence of erythrocytes, although nitrite formation was lower than in their absence, a substantial fraction of the added NO (up to 30% with 50 μM DEANO) was able to escape scavenging by hemoglobin and become oxidized to plasma nitrite, and a minor fraction (~4%) of the NO formed *S*-nitrosothiol (Fig. 1b,c), which is consistent with previous data²¹.

When we replaced the plasma with PBS, the fraction of NO that was oxidized to nitrite (8–10%) was substantially lower than that in plasma (Fig. 1b). This suggests that plasma has NO oxidase activity (the ability to catalyze the oxidation of NO to nitrite), even in the presence of erythrocytic hemoglobin (50% hematocrit). Though increasing hematocrit decreased the absolute amount of nitrite formed, the ability of plasma to catalyze nitrite formation in comparison to that of PBS was independent of erythrocyte concentration and was observed at physiological hematocrits (Fig. 1d).

The bolus addition of NO to an aerated oxyhemoglobin or oxymyoglobin solution can yield artifactually high concentrations of nitrite, secondary to high regional NO concentrations during mixing²³. To make sure that the large proportion of nitrite formed in plasma was not an artifact of such bolus NO mixing, we incubated blood with the same concentration of several NO donors (100 μM NO release) that have varying half-lives and with authentic NO solution (100 μM). Although the absolute concentration of nitrite measured was slightly different in each NO donor owing to nitrite contamination, the amount of plasma nitrite formed in the presence of erythrocytes was approximately 30% of the total amount of nitrite formed in the absence of erythrocytes, regardless of the NO source (Fig. 1e).

NO oxidase is a high-molecular-weight redox-active protein

The formation of plasma nitrite in the presence of erythrocytes suggests that a rapid mechanism of NO oxidation, such as metal-based catalysis, must exist in plasma. To determine whether a metal or thiol is involved in plasma NO oxidase activity, we treated plasma containing erythrocytes (50% hematocrit) with oxidants, reductants and the thiol alkylating agent *N*-ethylmaleimide (NEM, **2**) and then tested NO oxidase activity. Whereas the NO oxidase activity of plasma was unchanged by the oxidants ferricyanide (1 mM) and cyanide (250 μM), or by NEM (500 μM), the reductants dithiothreitol (1 mM), glutathione (100 mM) and ascorbate (1 mM) decreased the yield of nitrite from $26 \pm 5 \mu\text{M}$ to less than 10 μM (Fig. 2a). Treatment of plasma with the metal chelators diethylenetriaminepentaacetate (DTPA; **3**; 100 μM), EDTA (250 μM), neocuproine (**4**; 200 μM) and bathocuproine (**5**; 200 μM) had no effect on NO oxidase activity (Table 1). Taken together, these data suggest that the protein of interest is a redox-active protein sensitive to reductants, but that thiols and free metals are not involved.

To determine the size of the plasma protein of interest, we then subjected plasma to fractionation by passage through a Sephadex G-25 column to remove low-molecular-weight proteins and ultrafiltration to separate the high-molecular-weight ($\geq 100 \text{ kDa}$) proteins. Whereas removal of the low-molecular-weight fraction had no effect on NO

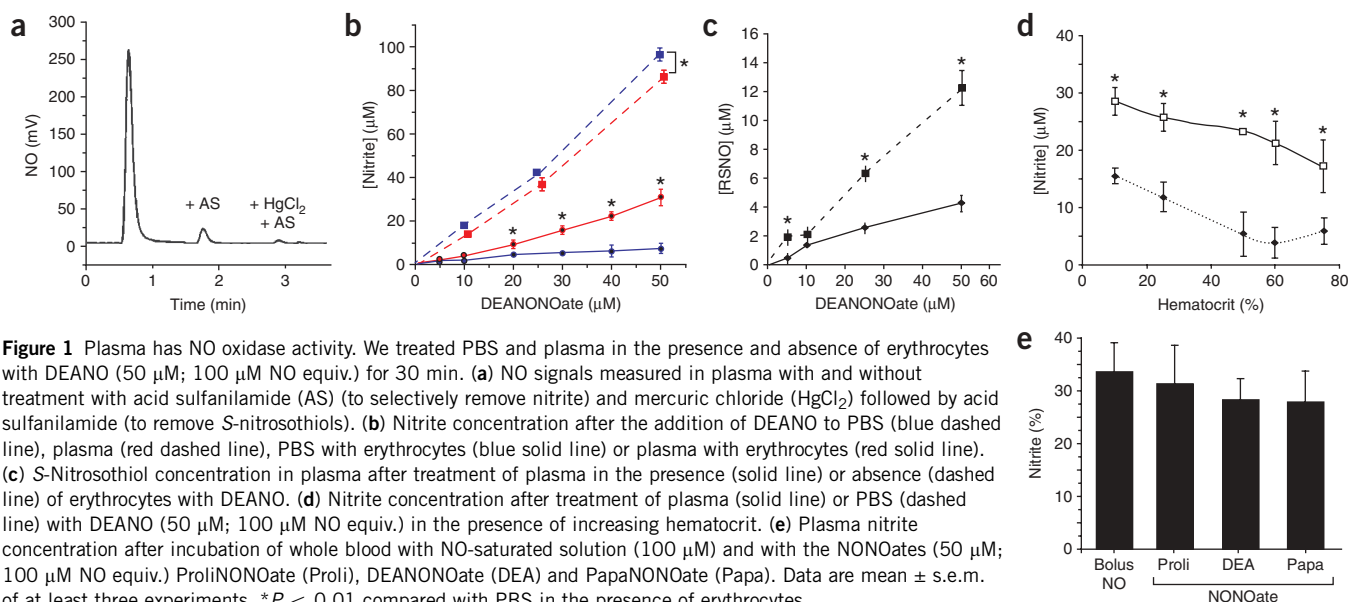


Figure 1 Plasma has NO oxidase activity. We treated PBS and plasma in the presence and absence of erythrocytes with DEANO (50 μM ; 100 μM NO equiv.) for 30 min. **(a)** NO signals measured in plasma with and without treatment with acid sulfanilamide (AS) (to selectively remove nitrite) and mercuric chloride (HgCl_2) followed by acid sulfanilamide (to remove *S*-nitrosothiols). **(b)** Nitrite concentration after the addition of DEANO to PBS (blue dashed line), plasma (red dashed line), PBS with erythrocytes (blue solid line) or plasma with erythrocytes (red solid line). **(c)** *S*-Nitrosothiol concentration in plasma after treatment of plasma in the presence (solid line) or absence (dashed line) of erythrocytes with DEANO. **(d)** Nitrite concentration after treatment of plasma (solid line) or PBS (dashed line) with DEANO (50 μM ; 100 μM NO equiv.) in the presence of increasing hematocrit. **(e)** Plasma nitrite concentration after incubation of whole blood with NO-saturated solution (100 μM) and with the NONOates (50 μM ; 100 μM NO equiv.) ProlinONOate (Prol), DEANONOate (DEA) and PapaNONOate (Papa). Data are mean \pm s.e.m. of at least three experiments. * $P < 0.01$ compared with PBS in the presence of erythrocytes.

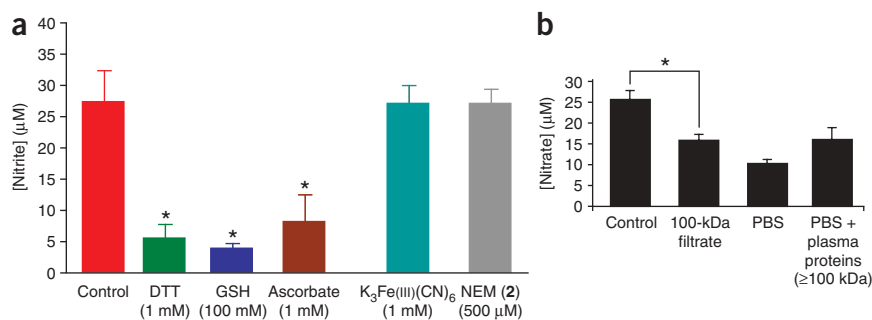


Figure 2 Redox-sensitive proteins of high molecular weight are responsible for plasma NO oxidase activity. **(a)** DEANOate (50 μ M; 100 μ M NO equiv.) added to plasma treated with DTT (1 mM), ascorbate (1 mM) or glutathione (GSH; 100 mM) in the presence of red blood cells (50% hematocrit) resulted in decreased nitrite concentration whereas treatment with ferricyanide (1 mM) or NEM (500 μ M) had no effect. **(b)** Nitrite concentration after DEANOate (50 μ M) exposure to native plasma, plasma after the removal of high-molecular-weight (> 100 kDa) proteins, PBS and PBS with high-molecular-weight plasma proteins added. Data are mean \pm s.e.m. of at least three experiments. * $P < 0.01$ compared to untreated plasma.

oxidase activity, removal of the high-molecular-weight fraction of the plasma caused nitrite yield to be significantly lower than that of unfractionated plasma ($16.8 \pm 2.4 \mu\text{M}$ versus $26.8 \pm 3.1 \mu\text{M}$, respectively; $P \leq 0.01$). When we added the high-molecular-weight plasma fraction to PBS in the presence of erythrocytes, DEANO addition resulted in a nitrite concentration that was higher than that in PBS without these proteins (16.3 ± 4.1 versus $10.2 \pm 2.4 \mu\text{M}$, respectively). The results of these experiments (Fig. 2b) suggest that nitrite formation in plasma is derived from a redox-active protein of high molecular weight that is sensitive to reductants. Because albumin has been reported to form nitrite via micellar catalysis²⁴, we also added 50 μM albumin to PBS; however, there was no significant increase in nitrite in the presence of albumin. Similarly, passing plasma through an Affy-blue (Bio-Rad) column to remove albumin did not decrease nitrite formation in albumin-depleted plasma with red cells (50% hematocrit) compared with that in normal plasma ($26.8 \pm 2.4 \mu\text{M}$ versus $24.8 \pm 3.1 \mu\text{M}$, respectively).

Ceruloplasmin catalyzes plasma nitrite formation

Taken together, these data suggest that the protein responsible for the NO oxidase activity must be a high-molecular-weight redox-active plasma protein that can function as an oxidase and generate nitrosative (NO^+) chemistry. This protein's unavoidable competition with the nearly diffusion-limited reaction of NO with hemoglobin requires a rapid reaction rate with NO, which suggests metal-based catalysis. These requirements led us to investigate the multicopper oxidase ceruloplasmin.

Ceruloplasmin, a 132 kDa plasma protein containing six copper centers, is expressed in plasma at concentrations of 1–5 μM and is known to oxidize amines in a process coupled to the reduction of molecular oxygen^{25,26}. Ceruloplasmin has ferroxidase activity that is responsible for the oxidation of ferrous iron to its ferric form, which is necessary for efficient iron efflux from the cell^{27,28}. The mechanism by which ceruloplasmin oxidizes its substrates is thought to involve a type 1 copper center that is closely associated with a tricluster of type 2 and type 3 copper ions. Substrates are oxidized at the first center, a reaction mediated by a one-electron reduction of Cu(II) , the cupric (2^+) ion, to Cu(I) , its cuprous (1^+) form. The electron is then transferred to the closely associated tricluster of type 2

and type 3 copper centers, which coordinate oxygen until electrons are present to reduce the oxygen to water^{26,29}.

The type 1 copper of ceruloplasmin has previously been considered a target for NO^30 and can catalyze *S*-nitrosothiol formation in cell culture media³¹. Furthermore, reduction of the copper sites would result in inhibition of the enzyme's oxidase activity, which would be consistent with our observations of lowered nitrite in reduced plasma samples. Hence, we hypothesized that ceruloplasmin could function as an NO oxidase in plasma, with its type 1 copper site oxidizing NO to NO^+ , which would be quickly hydrated to nitrous acid and then nitrite in aqueous solution (Fig. 3). To investigate this hypothesis, we removed ceruloplasmin from human plasma by immunoprecipitation and confirmed this depletion by western blot of the plasma (Fig. 4). We suspended red blood

cells in the immunodepleted plasma and incubated the depleted blood with DEANO (50 μM). Nitrite formation in the depleted plasma was decreased ($14.8 \pm 1.4 \mu\text{M}$) in comparison with normal plasma ($25.3 \pm 2.4 \mu\text{M}$) and comparable to the concentrations formed in PBS (with red cells at 50% hematocrit; $15.2 \pm 2.9 \mu\text{M}$) in this study. In these experiments, immunoprecipitation of plasma using the isotype-matched haptoglobin antibody had no effect on NO oxidase activity (Fig. 4a). We analyzed the immunoprecipitate by MALDI-TOF with subsequent LC-MS-MS and confirmed that the substance was indeed ceruloplasmin and addition of this immunoprecipitate (5 μM) or purified ceruloplasmin (5 μM) from another source (purchased from Vital Products Corp.) to the depleted plasma restored NO oxidase activity ($24.1 \pm 2.7 \mu\text{M}$). Consistent with these results, incubation of the NO donor with the immunoprecipitate resulted in copper reduction, which we monitored by electron paramagnetic resonance spectroscopy (Fig. 3b). Adding increasing concentrations of the immunoaffinity-purified ceruloplasmin to plasma (with red cells at 50% hematocrit) also increased nitrite formation from DEANO in a concentration-dependent manner (Fig. 4b). Notably, the concentration of plasma *S*-nitrosothiol ($5.3 \pm 0.4 \mu\text{M}$) was not significantly changed by the depletion ($5.2 \pm 0.7 \mu\text{M}$) or supplementation ($4.9 \pm 0.8 \mu\text{M}$) of ceruloplasmin, an observation consistent with the high instability of the nitrososonium cation in aqueous solutions (lifetime of 3×10^{-10} seconds)³²; NO^+ preferentially reacts with water (to form nitrite) over thiols (to form *S*-nitrosothiols).

Although addition of the same concentration of the immunopurified ceruloplasmin substantially increased nitrite formation in PBS (from 15.2 ± 2.9 to $21.0 \pm 1.7 \mu\text{M}$), it did not increase it

Table 1 NO oxidase activity of plasma and PBS pretreated with various chelators

Chelator	Plasma	PBS
Control	19.2 ± 0.312	10.6 ± 0.112
DTPA (100 μM)	18.9 ± 0.101	9.11 ± 0.243
Neocuproine (200 μM)	17.6 ± 1.21	10.1 ± 0.732
Bathocuproine (200 μM)	18.1 ± 0.241	10.6 ± 0.414
EDTA (250 μM)	18.9 ± 0.344	10.1 ± 0.403

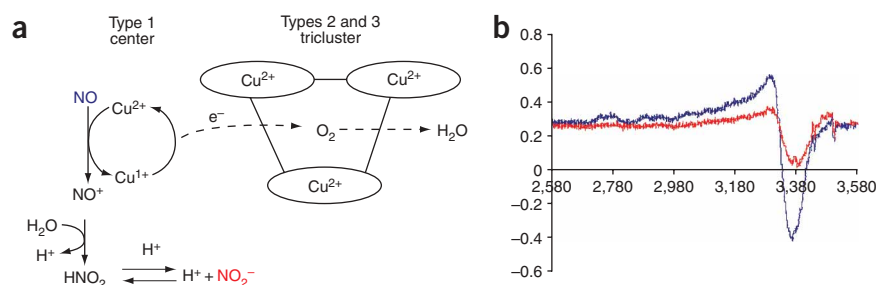


Figure 3 Ceruloplasmin as an NO oxidase. (a) NO is oxidized to NO⁺ at the type 1 copper site, which is reduced from Cu²⁺ to Cu¹⁺. NO⁺ is hydrated to nitrous acid, which is in equilibrium with nitrite. The electron gained by the copper center is transferred to the type 2 and 3 copper tricluster to return to the Cu¹⁺ state. Oxygen is coordinated by the copper tricluster and is ultimately reduced to water by four electrons. (b) EPR spectra of immunopurified human ceruloplasmin treated with (red) or without (blue) DEANONOate (25 μM).

to the level of formation in plasma, which suggests that other cofactors in plasma may be necessary for ceruloplasmin-dependent NO oxidase activity. To further investigate whether cofactors are needed for ceruloplasmin-dependent NO oxidase activity, we diluted ceruloplasmin-depleted plasma with PBS to different extents. We added immunopurified ceruloplasmin (3 μM) to each dilution and measured NO oxidase activity. Addition of increasing concentrations of ceruloplasmin-depleted plasma to PBS with 3 μM ceruloplasmin caused NO oxidase activity to increase in a dose-dependent manner (Fig. 4c).

NO oxidase activity is reduced in ceruloplasmin knockouts

Ceruloplasmin knockout mice serve as a model for aceruloplasminemia and develop many of the clinical abnormalities observed in the human disease, such as pathological iron accumulation in the liver, spleen and retina³³. Measurement of basal nitrite concentrations in the blood of these mice showed that plasma nitrite concentrations were significantly lower in both the heterozygous (0.53 ± 0.03 μM) and homozygous (0.51 ± 0.05 μM) animals compared with wild-type controls (0.79 ± 0.08 μM; *P* = 0.01 and *P* = 0.005, respectively; Fig. 5a). Though there was no significant difference in nitrite concentrations between the homozygous and heterozygous animals, this was not unexpected given that there was also no significant difference between the groups' amine oxidase activity, measured by the ability of plasma from these animals to oxidize *p*-phenylenediamine (heterozygotes, 0.022 ± 0.007 versus homozygotes, 0.015 ± 0.002 units ml⁻¹; *P* = 0.1; Fig. 5b). The levels of *p*-phenylenediamine oxidation reported here, particularly in the heterozygote mice, are lower

than those previously published (0.022 ± 0.007 versus 0.037 ± 0.010 units ml⁻¹, respectively)²⁷; this may be due to the relatively young age (3 months) of the mice used for the measurements in this study. These data suggest that in both heterozygotes and homozygotes the ceruloplasmin deficiency is sufficient to largely eliminate the NO oxidase activity in the presence of red blood cells. We then tested the NO oxidase activity of the mouse plasma *in vitro* by suspending freshly obtained human erythrocytes (50% hematocrit) in the plasma and incubating it with DEANO (100 μM NO) for 30 min. After incubation, the resulting plasma nitrite concentration of the ceruloplasmin knockout animals was significantly lower than that in the wild type (13 ± 5.13 versus 25.01 ± 4.85 μM; *P* = 0.05) and comparable to nitrite generated in PBS in the presence of erythrocytes (Fig. 5c). Notably, basal concentrations of plasma nitrite in each mouse significantly correlated with the measured NO oxidase activity from the same mouse, supporting the hypothesis that NO oxidase activity contributes to *in vivo* plasma nitrite formation (*R* = 0.62; *P* = 0.01; Fig. 5d). We performed these *in vitro* experiments in the presence of human erythrocytes in all groups, thereby confirming that the plasma compartment is indeed responsible for the NO oxidase activity and there is little contribution from erythrocytes.

Plasma NO oxidase activity is reduced in aceruloplasminemia

Aceruloplasminemia is a rare autosomal recessive disease in which a mutation in the ceruloplasmin gene results in the absence of circulating ceruloplasmin³⁴. Individuals with this condition develop iron accumulation in the liver, spleen, retina and central nervous system as a result of impaired iron efflux^{34,35}. Increased lipid peroxidation

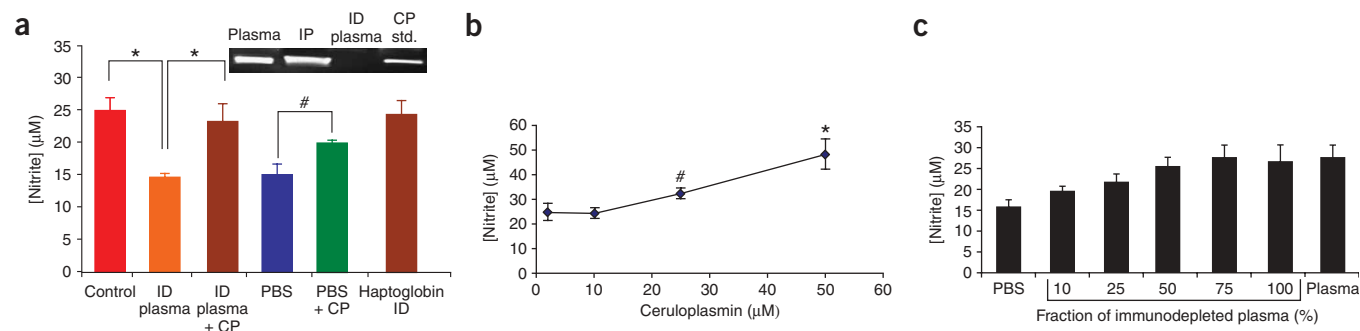


Figure 4 Ceruloplasmin is a plasma NO oxidase. (a) Nitrite concentrations in the supernatant after addition of DEANO (50 μM; 100 μM NO equiv.) to erythrocytes (50% hematocrit) suspended in plasma (control), plasma immunodepleted of ceruloplasmin (ID plasma), PBS, PBS with immunoprecipitated ceruloplasmin (PBS + CP; 5 μM) and immunodepleted plasma with immunopurified ceruloplasmin (ID plasma + CP; 5 μM). Haptoglobin ID is plasma immunodepleted of haptoglobin with same conditions. Inset shows a western blot of plasma before treatment, the immunoprecipitate, immunodepleted plasma and purified ceruloplasmin. (b) Plasma nitrite concentrations after the addition of increasing concentrations of ceruloplasmin to whole blood and subsequent incubation with DEANO. (c) NO oxidase activity of PBS alone, PBS with increasing percentage of ceruloplasmin depleted plasma added, and undepleted plasma. All conditions except for undepleted plasma have immunopurified ceruloplasmin (3 μM) added. Data represent means ± s.e.m. for at least three independent experiments. **P* < 0.01, #*P* < 0.05 (versus 0 μM in b).

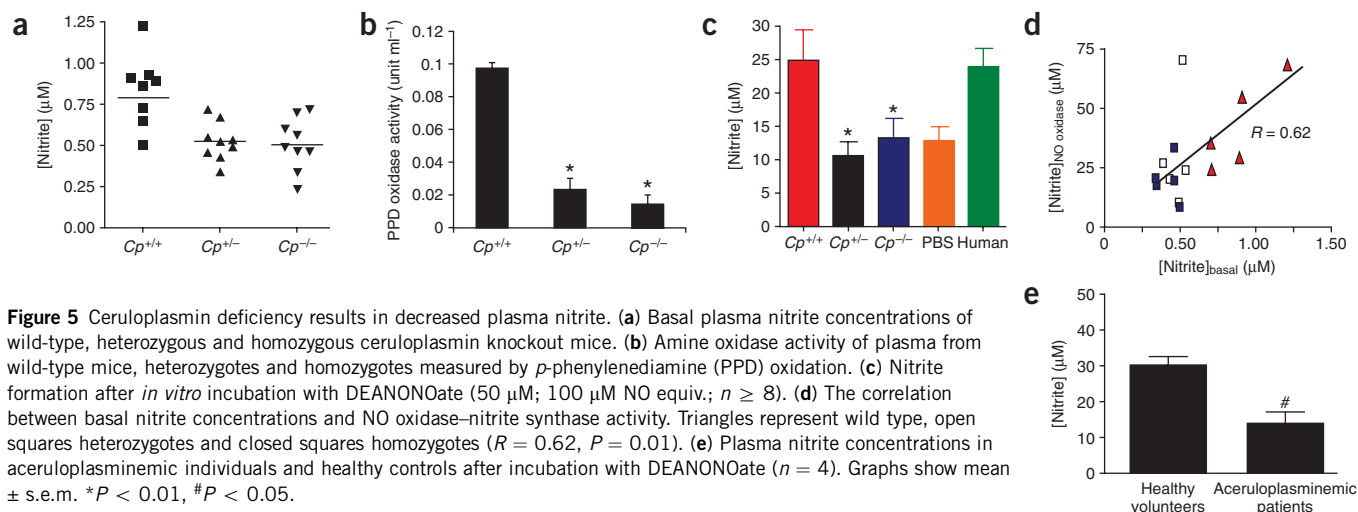


Figure 5 Ceruloplasmin deficiency results in decreased plasma nitrite. **(a)** Basal plasma nitrite concentrations of wild-type, heterozygous and homozygous ceruloplasmin knockout mice. **(b)** Amine oxidase activity of plasma from wild-type mice, heterozygotes and homozygotes measured by *p*-phenylenediamine (PPD) oxidation. **(c)** Nitrite formation after *in vitro* incubation with DEANONOate (50 μ M; 100 μ M NO equiv.; $n \geq 8$). **(d)** The correlation between basal nitrite concentrations and NO oxidase–nitrite synthase activity. Triangles represent wild type, open squares heterozygotes and closed squares homozygotes ($R = 0.62$, $P = 0.01$). **(e)** Plasma nitrite concentrations in aceruloplasminemic individuals and healthy controls after incubation with DEANONOate ($n = 4$). Graphs show mean \pm s.e.m. * $P < 0.01$, # $P < 0.05$.

and mitochondrial abnormalities occur secondary to iron deposition and are thought to contribute to the observed profound neurodegeneration^{36,37}. We hypothesized that if ceruloplasmin is responsible for plasma nitrite generation, individuals with aceruloplasminemia should have lower NO oxidase activity of plasma than healthy controls. To test this hypothesis, we collected blood samples from four aceruloplasminemic individuals in heparinized syringes and centrifuged the samples to separate the plasma. We then suspended normal erythrocytes in this plasma and added DEANO (50 μ M). After 30 min of incubation, plasma nitrite concentrations were significantly lower in plasma from aceruloplasminemic individuals than in plasma from healthy controls (12.2 \pm 6 μ M versus 30.1 \pm 2.4 μ M; $P = 0.01$; **Fig. 5e**).

Ceruloplasmin and nitrite concentrations modulate I/R injury

We have previously shown that near-physiological concentrations of nitrite are cytoprotective in a mouse model of hepatic I/R¹⁰. Indeed, as little as 2.4 nmol of nitrite, which only increases plasma nitrite concentrations from 700 to 900 nM, reduces liver and heart infarction by 50%¹⁰. These findings suggest that endogenous nitrite concentrations may modulate the cellular response to ischemic stress. Because ceruloplasmin knockout mice have a 300 nM reduction in basal plasma nitrite compared with the wild type, we hypothesized that they would sustain more injury after I/R than wild-type mice and that

nitrite repletion would normalize this response. To test this hypothesis, we subjected wild-type and homozygous knockout mice to 45 min of hepatic ischemia during which we gave half of the animals one intraperitoneal dose of nitrite (48 nmol). After 5 h of reperfusion, we measured the circulating concentrations of the liver transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to assess the extent of hepatocellular apoptosis and necrosis. We observed a gender divergence in injury similar to that reported previously³⁸ in which females in all groups had milder injuries than males. Indeed, whereas no female mice and no control mice died after reperfusion, two untreated male knockout mice died 45 min after the initiation of reperfusion, and one male nitrite-treated knockout mouse died 4 h into reperfusion. Of the surviving mice, ceruloplasmin knockout mice sustained significantly greater injuries than wild-type animals regardless of gender (1,100 \pm 51 versus 817 \pm 45 international units (IU) I⁻¹ ALT in males; 737 \pm 88 versus 535 \pm 23 IU I⁻¹ AST in females). Consistent with this being a nitrite-dependent effect, exogenous nitrite administration during ischemia reduced the severity of liver cytotoxicity in the knockout mice to that of the wild-type controls (**Fig. 6**).

DISCUSSION

In summary, we found that in the presence of a physiological hematocrit, up to 30% of NO added to blood is oxidized to plasma nitrite in a process catalyzed by the multicopper oxidase ceruloplasmin. Ceruloplasmin is a 132-kDa protein present in micromolar concentrations in plasma and upregulated as an acute-phase protein in inflammatory conditions, as well as in pregnancy and diabetes and after myocardial infarction^{26,35,39}. This complex protein contains six copper centers with ferroxidase activity that oxidizes iron and facilitates iron efflux from cells²⁸. However, many questions remain concerning the biological role of ceruloplasmin *in vivo*; the possibility that ceruloplasmin may use and oxidize alternative substrates has received little attention.

In addition to circulating in plasma, ceruloplasmin is synthesized in the liver and in macrophages, in which concentrations of nitrite exist in the micromolar range^{27–29,40}. Although approximately 95% of the copper found in the human body is contained in ceruloplasmin, several other multicopper oxidase enzymes are distributed throughout the body. These enzymes may also participate in NO metabolism and nitrite synthesis. Indeed, a role for mitochondrial cytochrome *c* oxidase in the oxidation of NO to nitrite has previously been

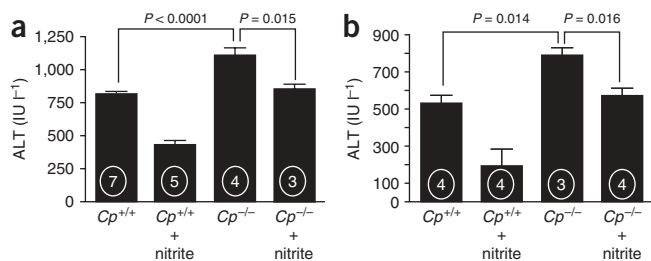


Figure 6 Ceruloplasmin knockout mice sustain more I/R-induced injury than wild-type mice, and nitrite is cytoprotective. **(a,b)** Circulating concentrations of ALT in male **(a)** and female **(b)** wild-type and ceruloplasmin knockout mice with and without nitrite treatment after 45 min of hepatic ischemia and 5 h of reperfusion. Graphs show mean \pm s.e.m. Circled numbers in each bar represent the number of animals in the corresponding group.

proposed⁴¹. Further studies are needed to investigate whether the ferroxidase hephaestin or the yeast homolog *fet3* has such a role.

Consistent with previous studies from our laboratory, we found that 10% of added NO formed plasma *S*-nitrosothiol in the absence of erythrocytes, whereas that fraction decreased to 4% when we added red blood cells (50% hematocrit). Formation of *S*-nitrosoglutathione in the media of HepG2 hepatic carcinoma cells is dependent on ceruloplasmin released by these cells³¹. However, in our experiments in whole blood, the concentration of plasma *S*-nitrosothiol remained unchanged when we added or removed ceruloplasmin from plasma. The nitrosonium cation (NO⁺) that is produced after NO oxidation by ceruloplasmin can nitrosate thiols or react with water to generate nitrite. Although plasma contains high concentrations of thiol targets such as albumin and glutathione, our data suggest that NO⁺ hydration and nitrite formation is the favored reaction in human plasma. This metal-catalyzed nitrosonium chemistry suggests that protein thiol concentration and nucleophilicity, together with microenvironment hydrophobicity and proximity to the NO oxidase, modulates nitrite and *S*-nitrosothiol yields in biological compartments.

In these experiments we found that the addition of purified ceruloplasmin to plasma increased the NO oxidase activity and amount of nitrite formed from NO. *In vivo*, ceruloplasmin is an acute-phase reactant that is transcriptionally upregulated during inflammation to concentrations as high as 15 μM⁴⁰. Furthermore, the ceruloplasmin gene contains hypoxia-responsive elements to which hypoxia-inducible factor 1α (HIF-1α) binds, increasing transcription during hypoxia. This suggests that ceruloplasmin is a member of a large group of proteins that are upregulated as an adaptation to hypoxia^{42,43}. With increasing data showing that nitrite may have a tissue-protective effect during hypoxia and a potential role in hypoxic vasodilation and signaling, this increase in ceruloplasmin may represent an adaptive mechanism to increase nitrite concentrations. Additionally, induction of ceruloplasmin has been observed after acute myocardial infarction⁴⁴, and changes in plasma nitrite as small as 200 nM protect tissue after experimental I/R injury¹⁰. Indeed, consistent with these results, we have shown that ceruloplasmin knockout animals sustain more I/R injury than wild-type animals. Additionally, supplementation of nitrite during I/R reduces the injury level in ceruloplasmin knockout mice to that of wild-type controls. These studies suggest that increases in blood and tissue nitrite after ischemic or hypoxic preconditioning that are associated with diets rich in leafy green vegetables may mechanistically account for the associated cardioprotective effects⁵.

The role of ceruloplasmin in the progression of cardiovascular disease is unknown. Conflicting evidence has been presented: some studies have shown ceruloplasmin to act as an oxidant, enhancing oxidation of low-density lipoprotein (LDL) in the vasculature^{45,46}, whereas other data suggests that ceruloplasmin is an antioxidant, decreasing iron concentrations (which drive Fenton chemistry) in cells. In a study comparing thiobarbituric acid-reactive products (TBARS) in plasma, plasma from aceruloplasminemic individuals was more susceptible to copper-mediated lipid peroxidation *in vitro* than plasma from healthy family members; the addition of ceruloplasmin to the plasma inhibited the formation of TBARS⁴⁷. People deficient in ceruloplasmin reportedly also develop increased plasma lipid peroxidation *in vivo* as well as accumulation of long-chain fatty acids in erythrocytic membranes^{47,48}. In the heart, aceruloplasminemic individuals have higher concentrations of the cholesterol peroxidation products 7-hydrocholesterol and 7-ketocholesterol than healthy subjects⁴⁹. Clearly, further investigation is warranted to

determine whether nitrite formed from ceruloplasmin modulates oxidant stress or cardiovascular disease.

In conclusion, we have shown that ceruloplasmin is an NO oxidase that generates nitrite in plasma and that physiological concentrations of ceruloplasmin and nitrite modulate tissue response to ischemia. We suggest a new paradigm for intravascular NO signaling in which NO can function either as a paracrine signaling molecule, with endothelial NO synthase-derived NO activating smooth muscle guanylate cyclase, or as an endocrine signaling molecule, with plasma (or tissue) ceruloplasmin-mediated NO oxidation generating nitrite, which can modulate hypoxic stress responses. According to this paradigm, the NO oxidase plays a critical role in the bioactivation of intravascular and tissue NO by limiting NO dioxygenation reactions with hemoglobin, myoglobin and other globin proteins. Thus the concentration and subcellular localization of the NO oxidase determines NO disposition. We speculate that coupled NO synthase-NO oxidase enzyme activities in plasma, macrophages, erythrocytes and other tissue compartments may redirect NO to nitrosative and endocrine nitrite-signaling pathways.

METHODS

Materials. We purchased all chemicals from Sigma-Aldrich unless otherwise stated. We obtained human purified ceruloplasmin from Vital Products and G-25 columns from Amersham Bioscience. We purchased NONOates from Sigma Aldrich or Cayman Chemicals.

Blood collection and processing. We collected blood from healthy volunteers in accordance with a protocol that was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and all volunteers gave written informed consent. We collected blood by venipuncture using methods to limit *ex vivo* hemolysis and immediately treated it with heparin. We obtained plasma from aceruloplasminemic patients from Hamamatsu University College of Medicine, and it was shipped to the National Institutes of Health on dry ice in tubes without patient identifiers.

NO oxidase activity measurements. We centrifuged heparinized blood at 750g for 5 min and separated plasma from erythrocytes. We used healthy human erythrocytes in all experiments. We washed erythrocytes twice with PBS to remove any residual plasma and then resuspended them in PBS or plasma at 50% hematocrit. We added NO donors to the suspension and incubated the sample at room temperature for 7 half-lives of the donor with intermittent mixing. We then centrifuged samples again at 750g for 5 min to separate erythrocytes, and we measured nitrite concentration in the supernatant by triiodide-based reductive chemiluminescence as previously described⁵⁰. We treated samples with and without acidic sulfanilamide (0.5% in 0.1 M HCl final concentration) to determine *S*-nitrosothiol concentration as previously described⁵⁰. We initially observed that the absolute concentrations of nitrite formed with different donors were variable, likely owing to differing amounts of contaminating nitrite in various NO donors⁵¹. However, when we added these donors to PBS in the absence of erythrocytes, we found that the resulting nitrite concentration was also variable.

Though we report that significant concentrations of plasma nitrite are formed from added NO in this study, the absolute concentration of nitrite may have been underestimated owing to experimental design. The addition of bolus NO to experimental systems has been reported to form artifactual NO oxides²³. To avoid these artifacts, we used an NO donor (DEANO) with a half-life of 4 min. To ensure complete decay of the donor, we allowed the reaction mixture to incubate for up to 30 min. However, we have previously shown that the half-life of nitrite in blood is 11 min¹⁷, which suggests that within our experimental reaction time, a substantial decay of nitrite may have occurred.

Measurement of amine oxidase activity. We measured ceruloplasmin activity spectrophotometrically by monitoring the oxidation of *p*-phenylenediamine at 530 nm in the presence of plasma from knockout and wild-type mice (3 months of age) as described previously⁵².

Immunoprecipitation of ceruloplasmin. We immunoprecipitated human ceruloplasmin by passing plasma over gel beads (Pierce Biotechnology) conjugated to antibodies raised against ceruloplasmin (Sigma) or antibodies raised against haptoglobin (Sigma) as a control. We performed western blot analysis of depleted plasma by electrophoresis in 12% Bis-Tris precast gels (Invitrogen) and probed with antibodies raised against ceruloplasmin (Sigma). We eluted immunoprecipitated ceruloplasmin from the gel beads, quantified it and added it back to depleted plasma or PBS as specified in the Results. We also added human purified ceruloplasmin to depleted plasma.

Ceruloplasmin knockout mice. We obtained wild-type ($Cp^{+/+}$), heterozygous ($Cp^{+/-}$) and homozygous ceruloplasmin knockout ($Cp^{-/-}$) mice (males and females) from Z.L. Harris at Johns Hopkins Hospital and School of Medicine. The mice are on a pure *C57/BL6* background and backcrossed more than 12 generations. We allowed them food and water *ad libitum* and kept them on a 12 h–12 h light-dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Johns Hopkins College of Medicine. We obtained blood by puncture of the retro-orbital plexus under anesthesia with ketamine and xylazine. We measured NO oxidase activity in knockout mice in the presence of healthy human red blood cells in all groups. We measured oxidation of *p*-phenylenediamine in mice 3 months of age, whereas mice in which we measured I/R injury ranged from 3 to 8 months in age.

Treatment of plasma with oxidants and reductants. In experiments involving the pretreatment of plasma with cyanide, ferricyanide, dithiothreitol, glutathione, ascorbate and NEM, we incubated plasma with the agent for 10 min at room temperature and then passed it through a Sephadex G-25 column to remove excess reagent; we then incubated the plasma with red cells at 50% hematocrit for NO oxidase activity experiments. We removed high-molecular-weight proteins from plasma using ultraspin columns (Millipore) with a molecular weight cutoff of 100 kDa.

EPR spectroscopy. We took spectra at 20 K using an X-band Bruker Elexsys EPR spectrometer. Collection parameters were as follows: microwave power, 2 mW; modulation amplitude, 5 G, scan width 1,000 G; sweep time 42 s; time constant, 20 ms; number of averaged spectra, 8.

Hepatic I/R. We carried out hepatic I/R as previously described¹⁰. Briefly, we anesthetized mice by intraperitoneal administration of ketamine (100 mg kg⁻¹) and xylazine (8 mg kg⁻¹) before surgery. We made a midline incision to expose the liver, and we heparinized (100 µg kg⁻¹) the mice to prevent blood clotting. We clamped the hepatic artery and portal vein with microaneurysm clamps to cause ischemia in the left lateral and median lobes of the liver, and we left the clamp in place for 45 min, during which we kept the liver moist using gauze soaked in 0.9% saline. We removed the clamp after 45 min and sutured the midline to allow recovery. Five hours later, we drew blood from the inferior vena cava and measured liver transaminase concentrations in the plasma.

ACKNOWLEDGMENTS

We would like to thank D. Lefer and M. Duranski for generous instruction in the performance of the hepatic I/R protocol. We thank P. Fox for helpful discussions about ceruloplasmin.

AUTHOR CONTRIBUTIONS

S.S., acquisition, analysis and interpretation of data, and drafting and revision of the manuscript; X.W., L.A.R., X.X., S.Y. and V.A., acquisition of data; H.M., supplying of critical samples; N.H., acquisition, analysis and interpretation of data; Z.L.H. and M.T.G., analysis and interpretation of data, and drafting and critical review of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Chemical Biology* website for details).

Published online at <http://www.nature.com/naturechemicalbiology/>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

1. Bryan, N.S. *et al.* Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat. Chem. Biol.* **1**, 290–297 (2005).

2. Cosby, K. *et al.* Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat. Med.* **9**, 1498–1505 (2003).
3. Crawford, J.H. *et al.* Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. *Blood* **107**, 566–574 (2006).
4. Gladwin, M.T. *et al.* The emerging biology of the nitrite anion. *Nat. Chem. Biol.* **1**, 308–314 (2005).
5. Lundberg, J.O. & Weitzberg, E. NO generation from nitrite and its role in vascular control. *Arterioscler. Thromb. Vasc. Biol.* **25**, 915–922 (2005).
6. Li, H., Samouilov, A., Liu, X. & Zweier, J.L. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *J. Biol. Chem.* **276**, 24482–24489 (2001).
7. Modin, A. *et al.* Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol. Scand.* **171**, 9–16 (2001).
8. Zweier, J.L., Wang, P., Samouilov, A. & Kuppusamy, P. Enzyme-independent formation of nitric oxide in biological tissues. *Nat. Med.* **1**, 804–809 (1995).
9. Nagababu, E., Ramasamy, S., Abernethy, D.R. & Rifkin, J.M. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J. Biol. Chem.* **278**, 46349–46356 (2003).
10. Duranski, M.R. *et al.* Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J. Clin. Invest.* **115**, 1232–1240 (2005).
11. Webb, A. *et al.* Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc. Natl. Acad. Sci. USA* **101**, 13683–13688 (2004).
12. O'Donnell, V.B. *et al.* Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12**, 83–92 (1999).
13. Hazen, S.L. *et al.* Formation of nitric oxide-derived oxidants by myeloperoxidase in monocytes: pathways for monocyte-mediated protein nitration and lipid peroxidation in vivo. *Circ. Res.* **85**, 950–958 (1999).
14. Bryan, N.S. *et al.* Cellular targets and mechanisms of nitrosylation: an insight into their nature and kinetics in vivo. *Proc. Natl. Acad. Sci. USA* **101**, 4308–4313 (2004).
15. Huang, K.T. *et al.* The reaction between nitrite and deoxyhemoglobin. Reassessment of reaction kinetics and stoichiometry. *J. Biol. Chem.* **280**, 31126–31131 (2005).
16. Huang, Z. *et al.* Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *J. Clin. Invest.* **115**, 2099–2107 (2005).
17. Dejam, A. *et al.* Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood* **106**, 734–739 (2005).
18. Kleinbongard, P. *et al.* Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic. Biol. Med.* **35**, 790–796 (2003).
19. Kim-Shapiro, D.B., Schechter, A.N. & Gladwin, M.T. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler. Thromb. Vasc. Biol.* **26**, 697–705 (2006).
20. Ford, P.C., Wink, D.A. & Stanbury, D.M. Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* **326**, 1–3 (1993).
21. Wang, X. *et al.* Biological activity of nitric oxide in the plasmatic compartment. *Proc. Natl. Acad. Sci. USA* **101**, 11477–11482 (2004).
22. Lauer, T. *et al.* Direct biochemical evidence for eNOS stimulation by bradykinin in the human forearm vasculature. *Basic Res. Cardiol.* **98**, 84–89 (2003).
23. Zhang, Y. & Hogg, N. Mixing artifacts from the bolus addition of nitric oxide to oxymyoglobin: implications for S-nitrosothiol formation. *Free Radic. Biol. Med.* **32**, 1212–1219 (2002).
24. Rafikova, O., Rafikov, R. & Nudler, E. Catalysis of S-nitrosothiols formation by serum albumin: the mechanism and implication in vascular control. *Proc. Natl. Acad. Sci. USA* **99**, 5913–5918 (2002).
25. Hellman, N.E. & Gitlin, J.D. Ceruloplasmin metabolism and function. *Annu. Rev. Nutr.* **22**, 439–458 (2002).
26. Musci, G., Polticelli, F. & Calabrese, L. Structure/function relationships in ceruloplasmin. *Adv. Exp. Med. Biol.* **448**, 175–182 (1999).
27. Harris, Z.L. *et al.* Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. *Proc. Natl. Acad. Sci. USA* **92**, 2539–2543 (1995).
28. Sarkar, J., Seshadri, V., Tripoulas, N.A., Ketterer, M.E. & Fox, P.L. Role of ceruloplasmin in macrophage iron efflux during hypoxia. *J. Biol. Chem.* **278**, 44018–44024 (2003).
29. Osaki, S., Johnson, D.A. & Frieden, I. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. *J. Biol. Chem.* **241**, 2746–2751 (1966).
30. Torres, J. & Wilson, M.T. The reactions of copper proteins with nitric oxide. *Biochim. Biophys. Acta* **1411**, 310–322 (1999).
31. Inoue, K. *et al.* Nitrosothiol formation catalyzed by ceruloplasmin. Implication for cytoprotective mechanism in vivo. *J. Biol. Chem.* **274**, 27069–27075 (1999).
32. Hughes, M.N. Relationships between nitric oxide, nitroxyl ion, nitronium cation and peroxynitrite. *Biochim. Biophys. Acta* **1411**, 263–272 (1999).
33. Meyer, L.A., Durley, A.P., Prohaska, J.R. & Harris, Z.L. Copper transport and metabolism are normal in aceruloplasminemic mice. *J. Biol. Chem.* **276**, 36857–36861 (2001).
34. Miyajima, H. Aceruloplasminemia, an iron metabolic disorder. *Neuropathology* **23**, 345–350 (2003).
35. Harris, Z.L. Aceruloplasminemia. *J. Neurol. Sci.* **207**, 108–109 (2003).
36. Xu, X., Pin, S., Gathinji, M., Fuchs, R. & Harris, Z.L. Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. *Ann. NY Acad. Sci.* **1012**, 299–305 (2004).

37. Yoshida, K. *et al.* Increased lipid peroxidation in the brains of aceruloplasminemia patients. *J. Neurol. Sci.* **175**, 91–95 (2000).
38. Harada, H. *et al.* Selected contribution: effects of gender on reduced-size liver ischemia and reperfusion injury. *J. Appl. Physiol.* **91**, 2816–2822 (2001).
39. Fox, P.L., Mukhopadhyay, C. & Ehrenwald, E. Structure, oxidant activity, and cardiovascular mechanisms of human ceruloplasmin. *Life Sci.* **56**, 1749–1758 (1995).
40. Gitlin, J.D. Transcriptional regulation of ceruloplasmin gene expression during inflammation. *J. Biol. Chem.* **263**, 6281–6287 (1988).
41. Torres, J., Sharpe, M.A., Rosquist, A., Cooper, C.E. & Wilson, M.T. Cytochrome *c* oxidase rapidly metabolises nitric oxide to nitrite. *FEBS Lett.* **475**, 263–266 (2000).
42. Martin, F. *et al.* Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. *Blood* **105**, 4613–4619 (2005).
43. Mukhopadhyay, C.K., Mazumder, B. & Fox, P.L. Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. *J. Biol. Chem.* **275**, 21048–21054 (2000).
44. Singh, T.K. Serum ceruloplasmin in acute myocardial infarction. *Acta Cardiol.* **47**, 321–329 (1992).
45. Ehrenwald, E., Chisolm, G.M. & Fox, P.L. Intact human ceruloplasmin oxidatively modifies low density lipoprotein. *J. Clin. Invest.* **93**, 1493–1501 (1994).
46. Fox, P.L., Mazumder, B., Ehrenwald, E. & Mukhopadhyay, C.K. Ceruloplasmin and cardiovascular disease. *Free Radic. Biol. Med.* **28**, 1735–1744 (2000).
47. Miyajima, H., Takahashi, Y., Serizawa, M., Kaneko, E. & Gitlin, J.D. Increased plasma lipid peroxidation in patients with aceruloplasminemia. *Free Radic. Biol. Med.* **20**, 757–760 (1996).
48. Miyajima, H. *et al.* Increased very long-chain fatty acids in erythrocyte membranes of patients with aceruloplasminemia. *Neurology* **50**, 130–136 (1998).
49. Miyajima, H. *et al.* Increased oxysterols associated with iron accumulation in the brains and visceral organs of aceruloplasminemia patients. *QJM* **94**, 417–422 (2001).
50. Yang, B.K., Vivas, E.X., Reiter, C.D. & Gladwin, M.T. Methodologies for the sensitive and specific measurement of S-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. *Free Radic. Res.* **37**, 1–10 (2003).
51. Lim, M.D., Lorkovic, I.M. & Ford, P.C. The preparation of anaerobic nitric oxide solutions for the study of heme model systems in aqueous and nonaqueous media: some consequences of NO x impurities. *Methods Enzymol.* **396**, 3–17 (2005).
52. Sunderman, F.W., Jr & Nomoto, S. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. *Clin. Chem.* **16**, 903–910 (1970).