

Heme Oxygenase Activity Correlates with Serum Indices of Iron Homeostasis in Healthy Nonsmokers



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ABSTRACT: Heme oxygenase (HO) catalyzes the breakdown of heme to carbon monoxide, iron, and biliverdin. While the use of genetically altered animal models in investigation has established distinct associations between HO activity and systemic iron availability, studies have not yet confirmed such participation of HO in iron homeostasis of humans. Carbon monoxide produced through HO activity will bind to hemoglobin in circulating erythrocytes, and therefore, blood carboxyhemoglobin (COHb) can be used as an index of HO activity. Using the second National Health and Nutrition Examination Survey, we tested the postulate that HO activity correlates with serum indices of iron homeostasis in healthy nonsmokers. The investigation included 844 lifetime nonsmokers (586 females) 18 years of age and older in the study population. Significant correlations were demonstrated between COHb and several indices of iron homeostasis including serum levels of both ferritin and iron and percentage iron saturation of transferrin. There was no significant association between COHb and hemoglobin, the largest repository of heme in the human body, which functions as the substrate for HO. We conclude that HO activity contributes to human iron homeostasis with significant correlations between COHb and serum ferritin and iron levels and percentage iron saturation of transferrin.

KEYWORDS: carbon monoxide, carboxyhemoglobin, ferritin, heme oxygenase, iron

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Introduction

Heme oxygenase (HO) is an essential, rate-limiting protein, which catalyzes the breakdown of heme to carbon monoxide (CO), iron, and biliverdin. The CO produced through HO activity diffuses out of the cell, binds to hemoglobin (with 200–250 times the affinity of oxygen) in circulating erythrocytes and can be measured as blood carboxyhemoglobin (COHb). Since endogenous CO results almost exclusively from the breakdown of heme by HO, COHb in circulating blood can be used as an index of its activity after controlling for variation in ambient CO concentrations.^{1,2} In the lungs, COHb dissociates and CO is excreted in the breath with measurable concentrations correlating with blood COHb.^{3,4} Compared to COHb, the measurement of breath CO can be used as an index of heme catabolism by HO provided that the effect of variability on the ambient CO exposure is considered.^{5,6}

Iron, also released through HO activity, can potentially impact metal homeostasis including the serum concentrations of ferritin and iron, total iron-binding capacity (TIBC), percentage iron saturation of transferrin, hematocrit, and hemoglobin. The daily requirement for iron in a healthy human exceeds the dietary intake (approximately 25 and 10 mg respectively in an adult).⁷ The majority of this metal is necessary for heme synthesis. Most of the iron required for this synthesis comes from

the recycling of heme from senescent erythrocytes catalyzed by HO.⁸ Accordingly, HO is regarded as the essential protein in the process of this reutilization of iron. HO can present in two forms, an inducible HO-1 and a constitutive HO-2. With the induction of HO-1 expression and activity in an animal model, serum iron approximately doubled supporting the role of this enzyme in mobilizing iron from heme.⁹ Mice deficient in HO-1 demonstrated a severe microcytic anemia with serum iron levels approximately half that of normal mice corroborating a role for this protein in the provision of this requisite metal.¹⁰

While the use of genetically altered animal models in investigation has established distinct associations between HO activity and systemic iron availability, studies have not yet confirmed such participation of HO in iron homeostasis of humans. Using COHb as a measure reflecting HO catalysis of heme, we tested the postulate that the activity of this enzyme correlates with serum indices of iron homeostasis in the healthy nonsmoking human.

Methods

The second National Health and Nutrition Examination Survey (NHANES II) is a cross-sectional survey to monitor the health and nutritional status of the civilian, noninstitutionalized population of USA.¹¹ The NHANES samples are



selected through a complex, multistage design in order to be nationally representative. NHANES II is a nationwide probability sample of 27,801 persons from six months to 74 years of age. From this sample, 25,286 people were interviewed, and 20,322 people were examined, resulting in an overall response rate of 73%. Interviews, health examinations, and laboratory tests are performed in mobile examination centers.

Only those individuals with ages of ≥ 18 years and with known values of the variables analyzed were included in this investigation ($n = 3134$). If the subject responded *yes* to having either *ever smoked at least 100 cigarettes or currently smoking cigars or a pipe*, they were excluded from the study population. Variables used in the analysis included COHb, ferritin, iron, TIBC, percentage iron saturation of transferrin, hematocrit, hemoglobin, and white blood cell (WBC) count. Specific analyses for COHb and all indices of iron homeostasis have been detailed.¹² As provided by NHANES II, two of these endpoints are calculated: TIBC and percentage iron saturation of transferrin. TIBC is the additive product of unsaturated iron-binding capacity and iron concentration and measures the blood's capacity to react iron with transferrin, the major iron-binding protein in the blood. Percentage iron saturation of transferrin is calculated as follows: (serum [iron]/TIBC) $\times 100$; it reflects how much of the iron-binding sites presented by transferrin are occupied by serum iron.

To assess the relationships between COHb, serum indices of iron homeostasis, and WBC counts, Spearman correlation coefficients were calculated (SAS Institute). Males and females were separately analyzed for relationships between COHb and indices of iron homeostasis since gender affects numerous endpoints of iron homeostasis.¹³ Significance was assumed at $P < 0.05$.

Results

The total study population included 844 lifetime nonsmokers (586 females). The mean (standard deviation) age was 42 (19) and 46 (19) years for males and females, respectively. Subjects were predominantly Caucasian (84% in the total study population and 83% and 84% in males and females, respectively). Measured endpoints are provided (Table 1). Mean values of the measured endpoints for the study population are all included within those values recognized as normal. However, the range of each endpoint included values residing outside of accepted normal values. Similarly, indices of iron homeostasis and WBC counts could reside outside the normal range with both low and high values.

In the total study population, initial multivariate regression analysis showed that COHb was significantly associated with gender and race but not with age. Subsequently, age was excluded from the analyses. For ease of interpretation, correlations are reported with adjustment for race. Among males, the concentrations of COHb did not correlate with any endpoint of iron homeostasis (Table 2A). However, among females, significant associations of COHb with (1) serum iron

Table 1. Mean, standard deviation, and range for endpoints measured in the total study population ($n = 844$).

	TOTAL ($n = 844$)	MALE ($n = 258$)	FEMALE ($n = 586$)
COHb (%)	0.91 (0.79) [0.00–11.53]	0.93 (0.70) [0.00–8.02]	0.90 (0.83) [0.00–11.53]
Ferritin (ng/mL)	87 (100) [1–823]	122 (112) [6–766]	71 (90) [1–823]
Iron ($\mu\text{g/dL}$)	97 (35) [16–259]	102 (34) [36–217]	94 (36) [16–259]
TIBC ($\mu\text{g/dL}$)	368 (60) [204–670]	352 (49) [204–508]	375 (63) [235–670]
Percentage saturation (%)	27 (10) [3–86]	30 (10) [10–70]	26 (10) [3–86]
Hematocrit (%)	40 (4) [20–52]	43 (4) [20–52]	39 (3) [25–51]
Hemoglobin (g/dL)	13.6 (1.5) [6.9–18.1]	14.7 (1.5) [6.9–18.1]	13.1 (1.2) [8.8–16.9]
WBC count ($\times 10^3$)/mm ³	6.7 (2.0) [2.6–24.4]	6.7 (1.8) [3.0–14.9]	6.7 (2.1) [2.6–24.4]

and (2) percentage iron saturation of transferrin were observed (Table 2B). In addition, the correlation between COHb and serum ferritin approximated significance. These were positive associations so that as COHb increased, serum ferritin and iron concentrations and percentage iron saturation of transferrin increased.

There were no significant associations between (1) COHb and hemoglobin, the substrate for HO, and (2) COHb and hematocrit, the volume percentage of red blood cells in blood, which correlates closely with hemoglobin (Table 2A). Serum iron concentrations and percentage iron saturation of transferrin correlated with both hemoglobin and hematocrit among males (Table 2A), while serum ferritin and iron concentrations and percentage iron saturation of transferrin correlated with both hemoglobin and hematocrit among females (Table 2B).

Inflammation frequently impacts both HO activity and indices of iron homeostasis.¹⁴ Accordingly, confounding by inflammation was considered. The WBC count was used as an index of inflammation. There was a correlation between COHb and the WBC count, which approximated significance in females, but the value was negative (Table 2B); that is, COHb decreased as WBC count increased. The WBC count similarly demonstrated negative correlations with serum iron and percentage iron saturation of transferrin among males and percentage iron saturation of transferrin among females (Tables 2A and 2B). Among females, there were positive correlations of the WBC count with TIBC, hematocrit, and hemoglobin in the total study population (Table 2B).

To further delineate the association between HO activity and indices of iron homeostasis, the study population was modified to include only those with a COHb of < 0.79 (the median value of COHb among males). This was predicted to diminish the impact of environmental sources of CO on COHb values

**Table 2A.** Correlations between COHb, indices of iron homeostasis, and WBC count among males in the total study population ($n = 258$).

	FERRITIN	IRON	TIBC	% SATURATION	HEMATOCRIT	HEMOGLOBIN	WBC
COHb	-0.043 $P = 0.49$	0.098 $P = 0.12$	-0.036 $P = 0.68$	0.110 $P = 0.08$	-0.028 $P = 0.65$	-0.003 $P = 0.96$	-0.091 $P = 0.15$
Ferritin		-0.009 $P = 0.88$	-0.194 $P < 0.01$	0.063 $P = 0.31$	0.052 $P = 0.41$	0.043 $P = 0.49$	0.092 $P = 0.14$
Iron			0.042 $P = 0.50$	0.912 $P < 0.01$	0.289 $P < 0.01$	0.285 $P < 0.01$	-0.204 $P < 0.01$
TIBC				-0.329 $P < 0.01$	0.235 $P < 0.01$	0.214 $P < 0.01$	0.079 $P = 0.21$
% Saturation					0.166 $P < 0.01$	0.173 $P < 0.01$	-0.214 $P < 0.01$
Hematocrit						0.915 $P < 0.01$	0.075 $P = 0.23$
Hemoglobin							0.064 $P = 0.30$

Table 2B. Correlations between COHb, indices of iron homeostasis, and WBC count among females in the total study population ($n = 856$).

	FERRITIN	IRON	TIBC	% SATURATION	HEMATOCRIT	HEMOGLOBIN	WBC
COHb	0.082 $P = 0.05$	0.196 $P < 0.01$	-0.136 $P = 0.74$	0.190 $P < 0.01$	0.017 $P = 0.68$	-0.006 $P = 0.89$	-0.081 $P = 0.05$
Ferritin		0.080 $P = 0.05$	-0.342 $P < 0.01$	0.194 $P < 0.01$	0.208 $P < 0.01$	0.207 $P < 0.01$	0.005 $P = 0.91$
Iron			0.055 $P = 0.19$	0.904 $P < 0.01$	0.225 $P < 0.01$	0.225 $P < 0.01$	-0.045 $P = 0.28$
TIBC				-0.327 $P < 0.01$	-0.022 $P = 0.59$	-0.044 $P = 0.29$	0.149 $P < 0.01$
% Saturation					0.214 $P < 0.01$	0.219 $P < 0.01$	-0.106 $P = 0.01$
Hematocrit						0.866 $P < 0.01$	0.085 $P = 0.04$
Hemoglobin							0.107 $P < 0.01$

by decreasing in the study population the number of those (1) actively smoking (cigarettes, cigars, or pipes), (2) exposed to environmental tobacco smoke, and (3) occupationally exposed to CO (eg, truck drivers and police officers). Subsequently, with a decreased effect of environmental sources on COHb, a more valid relationship of HO activity with indices of iron homeostasis would result. Values of ferritin, iron, TIBC, percentage iron saturation of transferrin, hematocrit, hemoglobin, and WBC count did not appear to be influenced by the inclusion of only those with COHb $<0.79\%$ (Table 3). In this smaller population, the correlation coefficients between COHb and indices of iron homeostasis for the group with COHb $<0.79\%$ were higher than those for the total study population. Among males ($n = 128$), concentrations of COHb correlated with the percentage iron saturation of transferrin and the association of COHb with serum iron approached significance (Table 4A). Limiting the analysis to those with COHb $<0.79\%$ also provided increased correlation coefficients between COHb and

serum ferritin and iron and percentage iron saturation of transferrin among females (Table 4B). There was a correlation between COHb and the WBC count, which approximated significance in males, and the value was negative (Table 4A).

Discussion

Increased HO activity with hemoglobin breakdown can result in the elevation of serum ferritin concentrations supporting a role for this storage protein in the iron transport and/or storage following the release of metal from the heme.¹⁵ The results of this study support significant associations between HO activity, reflected by COHb, and indices of iron homeostasis (serum iron, serum ferritin, and percentage iron saturation of transferrin) in human. The correlations were positive with an elevation of available iron from HO activity contributing to serum concentrations of the metal and its storage protein in humans. Such associations define HO as a pivotal protein in systemic iron homeostasis.



Table 3. Mean, standard deviation, and range for endpoints measured in the study population with COHb less than 0.79% ($n = 444$).

	TOTAL ($n = 444$)	MALE ($n = 128$)	FEMALE ($n = 316$)
COHb (%)	0.54 (0.18) [0.00–0.78]	0.54 (0.18) [0.00–0.78]	0.53 (0.17) [0.00–0.78]
Ferritin (ng/mL)	81 (91) [1–656]	130 (123) [6–656]	61 (64) [1–390]
Iron ($\mu\text{g/dL}$)	93 (33) [16–220]	101 (33) [36–189]	90 (33) [16–220]
TIBC ($\mu\text{g/dL}$)	372 (64) [204–670]	355 (51) [204–508]	378 (67) [235–670]
Percentage saturation (%)	26 (10) [3–71]	29 (10) [10–70]	25 (9) [3–71]
Hematocrit (%)	40 (4) [20–52]	43 (4) [20–52]	39 (3) [25–47]
Hemoglobin (g/dL)	13.6 (1.5) [6.9–19.9]	14.8 (1.5) [6.9–17.9]	13.2 (1.2) [8.8–15.9]
WBC count ($\times 10^3/\text{mm}^3$)	6.9 (2.0) [2.9–24.4]	6.8 (1.9) [3.0–14.9]	6.9 (1.2) [2.9–24.4]

Confounding of the results was demonstrated. While COHb was used as an index of HO activity, other endogenous and exogenous sources of CO react with hemoglobin to also produce COHb. Despite endogenous CO resulting almost exclusively from the breakdown of hemoglobin by HO,^{2,16} enzymatic and nonenzymatic forms of autooxidation, photooxidation, and lipid peroxidation and *in vivo* oxidation of halomethanes can infrequently account for substantial CO generation.^{2,16–18} In healthy subjects, this contribution should be negligible. Regarding exogenous sources of CO, there are many. The most important exogenous source of CO is tobacco smoking, which can elevate COHb to values above 5% and even 10%.^{19,20} For nonsmokers, CO exposure can result from environmental tobacco smoke, which can increase COHb levels to values nearly 2.5 times higher than that of the control group.^{1,21} Further sources contributing to environmental CO include automobile exhaust, industrial combustion, solid waste, gas heaters and stoves, other home appliances, and ambient air pollution.^{21–23} Umbilical cord blood COHb in newborns supports a relationship between COHb and ambient air concentrations of CO.^{24,25} Accordingly, the utilization of COHb as an index of HO activity requires control for exogenous sources of CO.^{1,2} In this investigation, such control was attempted through the inclusion of only self-reported nonsmokers. Despite this effort, it is evident that the study population included active cigarette smokers with the values of COHb exceeding 10% in some self-reported nonsmokers among NHANES II participants. It is not uncommon that self-reported nonsmokers provide the values of COHb exposing active smoking.²⁶ Other sources of COHb were also not controlled for using data available in NHANES II. Such sources (eg, environmental tobacco smoke, occupational

exposures, and air pollution) are likely to account for observed higher values of COHb. When the study population included only those individuals with COHb values $<0.79\%$, the correlation coefficient with percentage iron saturation of transferrin in males became significant supporting a dependence of the results on environmental sources of CO. When limited to those individuals with COHb values $<0.79\%$, correlation coefficients among females also increased indicating some effect of exogenous sources of CO. Accordingly, the measures of the strength of association between HO activity (ie, COHb) and indices of iron homeostasis should be viewed as minimal estimates only.

Correlations between COHb and indices of iron homeostasis used in this investigation were dependent on gender. When results in the total study population were evaluated by gender, males did not demonstrate significant relationships between COHb and indices of iron homeostasis. However, with the evaluation of those with COHb <0.79 , the association with percentage iron saturation of transferrin among males was significant. This suggests that the environmental sources of CO confounded the evaluation by influencing COHb disproportionately in males. Misclassification of smoking can be impacted by gender.²⁷ Similarly, occupations that can predict exposure to higher concentrations of environmental CO can be male dominated (eg, truck driver, welder, industrial laborer, police officer, and firefighter).^{22,28} Subsequently, confounding by gender may reflect an increased environmental exposure of males to CO.

Inflammation can also function as a confounder in this study by impacting both COHb and indices of iron homeostasis measured. Expression and activity of HO can be affected (positively) by the diagnoses of inflammatory injury, including chronic obstructive pulmonary disease, trauma, sepsis, and shock.^{29–31} Similarly, serum iron, percentage iron saturation of transferrin will be decreased with inflammatory disease, while serum ferritin will be increased.^{32,33} Confounding of the results by inflammation was evaluated using the WBC count as an index of inflammation. Unexpectedly, the relationship between COHb and the WBC count supported decrements in HO activity with inflammation. Accordingly, it was concluded that inflammation did not account for the relationship between COHb and iron indices observed in this study.

Prior investigation attempted to define HO as prooxidative and to support inflammation through a liberation of catalytically active iron,^{34–36} but elevation of cell and tissue levels of this protein is now considered to be associated with an antioxidative response and cytoprotection.^{37–40} The results of our investigation support that, rather than viewing HO as either an antioxidant or a prooxidant, it is most specific to define the protein by its participation in iron homeostasis. HO is foremost a protein with an essential involvement in iron homeostasis. Altered expression and/or activity of the protein will reflect changes in iron homeostasis following exposure,

**Table 4A.** Correlations between COHb, indices of iron homeostasis, and WBC count among males in the study population with COHb less than 0.79% (*n* = 128).

	FERRITIN	IRON	TIBC	% SATURATION	HEMATOCRIT	HEMOGLOBIN	WBC
COHb	0.027 <i>P</i> = 0.77	0.176 <i>P</i> = 0.05	-0.107 <i>P</i> = 0.23	0.217 <i>P</i> = 0.01	0.078 <i>P</i> = 0.39	0.089 <i>P</i> = 0.32	-0.178 <i>P</i> = 0.05
Ferritin		-0.053 <i>P</i> = 0.56	-0.109 <i>P</i> = 0.22	-0.008 <i>P</i> = 0.93	0.016 <i>P</i> = 0.86	0.007 <i>P</i> = 0.93	0.044 <i>P</i> = 0.62
Iron			0.002 <i>P</i> = 0.98	0.919 <i>P</i> < 0.01	0.179 <i>P</i> = 0.04	0.208 <i>P</i> = 0.02	-0.180 <i>P</i> = 0.04
TIBC				-0.357 <i>P</i> < 0.01	0.201 <i>P</i> = 0.02	0.217 <i>P</i> = 0.01	0.120 <i>P</i> = 0.18
% Saturation					0.083 <i>P</i> = 0.35	0.103 <i>P</i> = 0.24	-0.214 <i>P</i> = 0.02
Hematocrit						0.936 <i>P</i> < 0.01	0.259 <i>P</i> < 0.01
Hemoglobin							0.233 <i>P</i> < 0.01

Table 4B. Correlations between COHb, indices of iron homeostasis, and WBC count among females in the study population with COHb less than 0.79% (*n* = 316).

	FERRITIN	IRON	TIBC	% SATURATION	HEMATOCRIT	HEMOGLOBIN	WBC
COHb	0.121 <i>P</i> = 0.03	0.239 <i>P</i> < 0.01	-0.002 <i>P</i> = 0.97	0.225 <i>P</i> < 0.01	0.071 <i>P</i> = 0.21	0.052 <i>P</i> = 0.35	-0.018 <i>P</i> = 0.76
Ferritin		0.073 <i>P</i> = 0.20	0.339 <i>P</i> < 0.01	0.173 <i>P</i> < 0.01	0.192 <i>P</i> < 0.01	0.232 <i>P</i> < 0.01	-0.061 <i>P</i> = 0.28
Iron			0.021 <i>P</i> = 0.70	0.894 <i>P</i> < 0.01	0.284 <i>P</i> < 0.01	0.272 <i>P</i> < 0.01	-0.127 <i>P</i> = 0.02
TIBC				-0.378 <i>P</i> < 0.01	-0.034 <i>P</i> = 0.55	-0.060 <i>P</i> = 0.29	0.153 <i>P</i> < 0.01
% Saturation					0.259 <i>P</i> < 0.01	0.0259 <i>P</i> < 0.01	-0.186 <i>P</i> < 0.01
Hematocrit						0.877 <i>P</i> < 0.01	0.054 <i>P</i> = 0.34
Hemoglobin							0.073 <i>P</i> = 0.20

injury, and disease. This is analogous to bacteria and plants, which use HO in the acquisition and utilization of iron.⁴¹⁻⁴³ When grown in iron-replete conditions, a microbial demonstrates a diminished expression of HO, but expression of this protein is increased after iron depletion⁴⁴; the elevation in the catalysis of heme by higher concentrations of HO allows survival of the microbe after it makes available more requisite metal. Exposures, injuries, and disease can alter iron homeostasis effectively reducing metal concentrations available to the cell.⁴⁵ Any protective role for HO that might reveal under these conditions can be attributed to its impact on iron homeostasis. Iron is absolutely required for life, and loss of this metal would challenge cell survival.⁴⁶ By catalyzing the deconstruction of heme to iron, HO functions to increase metal concentrations absolutely pivotal to the continued survival of the cell, tissue, and living system.

There are limitations to this investigation. Confounding of the COHb by environmental sources is an important limitation delineated by the results. In addition, inadequate numbers of non-Caucasians precluded the evaluation of race as a determinant of COHb. Furthermore, the use of WBC as the only available index of inflammation restricts the interpretation of its association with COHb. Finally, the absolute value of some of the correlations between COHb and indices of iron homeostasis was small.

Conclusion

We conclude that HO activity contributes to systemic iron homeostasis in humans with significant correlations between COHb and serum ferritin and iron levels and percentage of iron saturation of transferrin. As a result of confounding in the measurement of COHb by environmental sources of CO,



the quantification of its relationship with the indices of iron homeostasis cannot be accomplished with absolute accuracy. Therefore, the measures of strength of the association provided should be viewed as minimal with increases expected following the control of environmental CO.

Author Contributions

Conceived and designed the experiments: AJG, DMS. Analyzed the data: DMS. Wrote the first draft of the article: AJG. Contributed to the writing of the article: AJG, DMS. Agreed the article results and conclusions: AJG, DMS. Jointly developed the structure and arguments for the article: AJG, DMS. Made the critical revisions and approved the final version: AJG, DMS. Both authors reviewed and approved the final article.

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