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Effects of iron on Vitamin C/copper-induced hydroxyl radical generation in bicarbonate-rich water

PATRIC J. JANSSON[1], URKO DEL CASTILLO[2], CHRISTER LINDQVIST[3], & TOMMY NORDSTRÖM[1]

[1]*Department of Biochemistry and Pharmacy, Åbo Akademi University, BioCity, P.O. Box 66, 20521 Turku, Finland,* [2]*University of Basque Country, Spain, and* [3]*Department of Biology, Åbo Akademi University, BioCity, P.O. Box 66, 20521 Turku, Finland*

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Abstract

The aim of this study was to evaluate whether iron, like copper, could support Vitamin C mediated hydroxyl radical formation in bicarbonate-rich water. By using the hydroxyl radical indicator coumarin-3-carboxylic acid, we found that iron, in contrast to copper, was not capable to support Vitamin C induced hydroxyl radical formation. However, when 0.2 mg/l iron and 0.1 mg/l copper were both added to bicarbonate supplemented Milli-Q water, the Vitamin C induced formation of 7-hydroxycoumarin, as measured by HPLC analysis, was inhibited by 47.5%. The inhibition of hydroxyl radical formation by iron was also evident in the experiments performed on copper contaminated bicarbonate-rich household drinking water samples. In the presence of 0.2 mg/l of ferric iron the ascorbic acid induced hydroxyl radical formation was inhibited by 36.0–44.6%. This inhibition was even more significant, 47.0–59.2%, when 0.8 mg/l of ferric iron was present. None of the other redox-active metals, e.g. manganese, nickel or cobalt, could support ascorbic acid induced hydroxyl radical formation and did not have any impact on the ascorbic acid/copper-induced hydroxyl radical generation. Our results show, that iron cannot by itself produce hydroxyl radicals in bicarbonate rich water but can significantly reduce Vitamin C/copper-induced hydroxyl radical formation. These findings might partly explain the mechanism for the iron-induced protective effect on various copper related degenerative disorders that earlier has been observed in animal model systems.

Keywords: *Vitamin C, water, iron, copper, bicarbonate*

Introduction

The structure of iron and its capacity to vary its oxidation state and bind to different ligands gives iron a unique biochemical role. Iron is a highly precious metal for the growth and viability of all cells and indispensable for human survival. It is primarily required for hemoglobin synthesis, but it has also a crucial role in e.g. DNA synthesis, electron transport and many enzymatical activities throughout the body. Low dietary intake of iron, results in iron deficiency and anemia [1–3].

Iron has also been implicated in the pathogenesis of a variety of neurodegenerative disorders e.g. Parkinson's disease, Alzheimer disease MS and EAE [4–6], liver and heart disease [7–9], cancer [10,11], diabetes [12–13] and immune abnormalities [14,15]. The toxicity of iron has generally been attributed to its ability to reduce molecular oxygen, thus forming reduced oxygen species. One of the most accepted mechanisms by which iron is involved in free radical production is the Fenton/Haber-Weiss reaction cycle. In this reaction, hydroxyl radicals can be easily

Correspondence: T. Nordström, Department of Biochemistry and Pharmacy, Åbo Akademi University, BioCity, P.O. Box 66, 20521 Turku, Finland. Tel: +358-2-2154004. Fax: +358-2-2154745. E-mail: tomnords@abo.fi

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generated by iron-catalyzed reduction of oxygen to superoxide that in turn can react with hydrogen peroxide [16]. Hydroxyl radicals can also directly be generated from hydrogen peroxide by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$ [17,18]. Various iron chelators, such as EDTA and NTA, have also been shown to promote hydroxyl radical generation very effectively via the Haber-Weiss cycle in an ascorbate-driven Fenton reaction [19–22].

Ascorbate (Vitamin C) has been reported, *in vitro*, to mediate hydroxyl radical formation in the presence of iron [23–25]. Based on this, and the known fact that ascorbic acid can redox-cycle with iron, we have here evaluated whether iron, like copper, could have hydroxyl radical formation properties in a drinking water environment. We demonstrate here, that iron cannot by itself produce hydroxyl radicals in such an environment but it has an inhibitory effect on Vitamin C induced hydroxyl radical formation in copper contaminated bicarbonate-rich household drinking water.

Materials and methods

Chemicals

Coumarin-3-carboxylic acid, 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA), coumarin and 7-hydroxycoumarin (umbelliferone) were from Fluka, Switzerland. Ascorbic acid, ferric chloride tetrahydrate, ferrous chloride hexahydrate, calcium chloride dihydrate and cupric chloride dihydrate were purchased from Fluka, Riedel-deHaen, Germany. Tris[hydroxymethyl]aminomethane (TRIS base), manganese chloride tetrahydrate, nickel chloride hexahydrate, cadmium chloride anhydrous, gallium nitrate hydrate, zinc chloride, aluminium chloride hexahydrate, cobalt chloride hexahydrate, diethyldithiocarbamic acid, ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine) and sodium bicarbonate were from Sigma, St. Louis, USA. Magnesium chloride hexahydrate were purchased from J.T. Baker, Denventer, Holland. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of vitamin C induced hydroxyl radical formation by using coumarin-3-carboxylic acid

To measure hydroxyl radical formation in household drinking water or bicarbonate supplemented Milli-Q water, 200 μl of the water samples were pipetted in triplicate onto a 96 well microtiter plate. After this, 200 μM coumarin-3-carboxylic acid was added to all wells by using a 8 channel multiwell pipett followed by 2 mM ascorbic acid that started the reaction. The microtiter plate was incubated at room temperature in dark for 3 h and the reaction was stopped by pipetting 10 mM TRIS base to all wells. Addition of TRIS buffer adjusted the pH in the samples to 9.0 that maximized the pH dependent fluorescence signal of 7-hydroxycoumarin-3-carboxylic acid. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. All measurements were done at room temperature. The fluorescence of the samples and standards were measured with a Victor plate reader, Wallac, Finland. The optical filter set used was excitation 380 nm and emission 460 nm.

Measurement of iron, copper and bicarbonate concentration in the water samples

The iron concentration in the drinking water samples was measured by using the iron specific reagent ferrozine [26]. For the assay, 200 μl aliquots in triplicate of the water samples were pipetted onto a 96 well microtiter plate followed by 400 μM of ferrozine and 100 μM ascorbic acid. Ascorbic acid was used to reduce the Fe(III) to the Fe(II) form. The colored Fe(II)-ferrozine complex formed was measured at 560 nm by using a Victor plate reader, Wallac, Finland. The absorbance values were converted to concentration by comparison with a standard curve. The standard curve was generated by adding known amounts of ferric chloride tetrahydrate, 100 μM ascorbic acid and 400 μM ferrozine to Milli-Q water buffered with 100 mg/l bicarbonate. The copper and bicarbonate concentration in the water samples were measured as previously described [27,28].

Measurement of hydroxyl radical formation by HPLC analysis

An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 μl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. The column used for the analysis was a Symmetry C18, 250 × 4.6 mm I.D, 10 μm particle size column (Waters). Chromatography was performed using isocratic elution using 150 mM phosphate buffer (KH_2PO_4) containing 30% methanol, pH 3.0 (H_3PO_4). The flow rate was 0.75 ml/min. The indicator molecule used in the assay was coumarin that readily forms 7-hydroxycoumarin (umbelliferone) when attacked by hydroxyl radicals. The peaks were detected at 200 nm and analyzed by using the Waters Breeze software. For peak identification and calibration we used standards of coumarin, 7-hydroxycoumarin (umbelliferone) in Milli-Q water. All separations were performed at room temperature.

Results

Copper, but not iron, can support ascorbic acid induced hydroxyl radical formation in bicarbonate-rich water

We have earlier shown that addition of ascorbic acid to tap water samples contaminated with copper ions can trigger an ongoing production of hydroxyl radicals that can be detected by using coumarin-3-carboxylic acid [27,28]. In Figure 1, ascorbic acid (2 mM) was added to bicarbonate buffered Milli-Q water supplemented with different concentrations of either copper or iron. Even very low concentrations of copper (0.01–0.05 mg/l) were sufficient to give a detectable hydroxyl radical signal. On the contrary, when iron was used in the assay, no hydroxyl radical formation could be detected. Neither ferrous nor ferric iron could support any hydroxyl radical formation. Manganese, cadmium, nickel, cobalt, aluminum, magnesium, calcium and zinc (as chloride salts) or gallium (as nitrate salt) did not result in any detectable hydroxyl radical formation (tested by using the highest amount of the contaminants that is allowed in drinking water, Maximum Contaminant Level, MCL, data not shown).

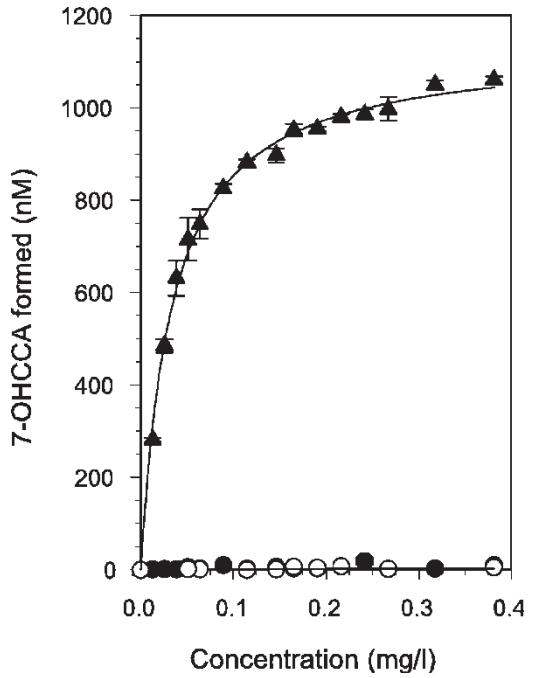

Figure 1. Ascorbic acid induced hydroxyl radical formation in bicarbonate buffered Milli-Q water supplemented with copper or iron. 200 μM coumarin-3-carboxylic acid followed by 2 mM ascorbic acid were added to Milli-Q water samples buffered with 100 mg/l bicarbonate and various concentrations of copper (▲), ferrous iron (•) or ferric iron (○). After 3 h incubation in dark at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. Data points are mean ± SD of triplicates from one representative experiment out of three conducted. Where absent, error bars were smaller than the symbol.

Inhibition of ascorbic acid/copper-catalyzed hydroxyl radical formation by iron

To further elucidate the effects of iron on Vitamin C induced hydroxyl radical formation in the presence of copper and bicarbonate, we used HPLC analysis. For the assay, coumarin was chosen as the target molecule. As shown in Figure 2A, 2 mM ascorbic acid, in the presence of 0.1 mg/l copper and 100 mg/l bicarbonate, promoted the formation of a family of hydroxylated coumarin compounds. We focused our analysis on one of these hydroxylated compounds, namely 7-hydroxycoumarin. Within 3 h, 5.5 μM of 7-hydroxycoumarin was formed. When the copper ion was substituted with 0.2 mg/l ferric iron, no hydroxylated coumarin compounds appeared in the chromatogram (Figure 2B). In our experiments, 0.2 mg/l iron was used since this is the MCL for iron in drinking water in Finland. When 2 mM ascorbic acid was added to Milli-Q water that has been supplemented with 0.2 mg/l iron, 100 mg/l bicarbonate and 0.1 mg/l copper, a 47.5% reduction in the 7-hydroxycoumarin formation was observed (Figure 2C). In these chromatograms, based on the retention time for the standard, the peak that appeared at 22.5 min was identified as 7-hydroxycoumarin (Figure 2D). When manganese, cadmium, nickel, cobalt, gallium, aluminum, magnesium, calcium and zinc salts (chloride salt) were tested at their MCLs no inhibitory effect of the ascorbic acid/copper-mediated hydroxyl radical formation could be seen (data not shown).

Effects of iron on Vitamin C/copper-induced hydroxyl radical formation in household drinking water samples

Next we evaluated whether iron has any impact on ascorbic acid induced hydroxyl radical formation in copper contaminated bicarbonate-rich drinking water samples. The copper concentration in the different water samples varied from 0.13 to 0.02 mg/l. The bicarbonate concentration varied between 77.6 and 130.1 mg/l. The drinking water samples had been sampled in the same way, directly drawn from the tap, but they originated from four different municipal water suppliers. The water samples used in the assay did not contain any detectable iron. When Vitamin C was added to these samples more than 900 nM of 7-hydroxycoumarin-3-carboxylic acid was formed (Table I). When 0.2 mg/l ferric iron was added to the tap water samples the ascorbic acid induced hydroxyl radical formation was inhibited by 36.0–44.6%. When the water samples were supplemented with 0.8 mg/l ferric iron the inhibition was significantly higher, 47.0–59.2%.

Discussion

We have previously shown that ascorbic acid can drive a hydroxyl radical generating process in copper and bicarbonate containing household drinking water

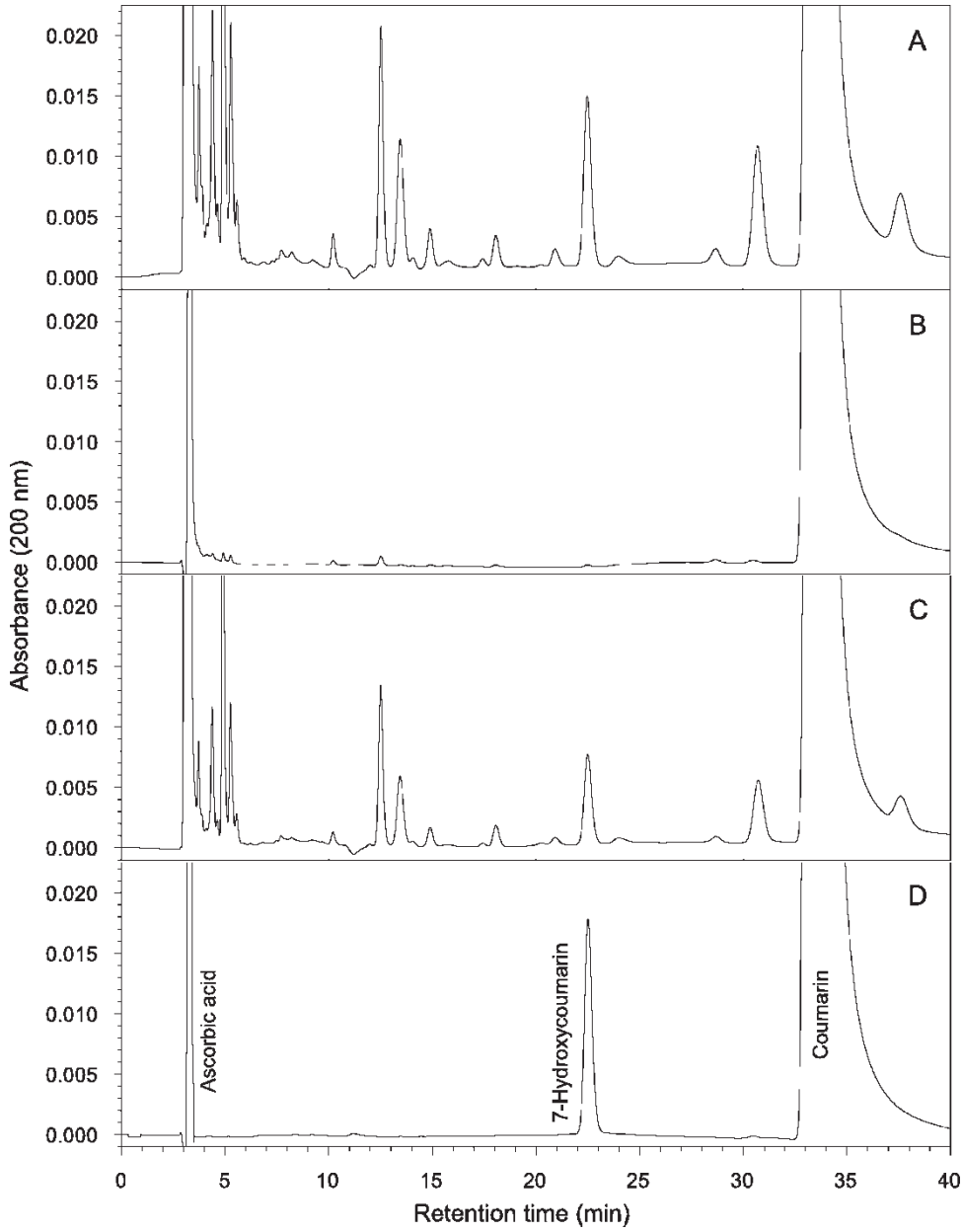

Figure 2. HPLC analysis of ascorbic acid induced hydroxylation of coumarin (100 μM) in the presence of copper or iron. 2 mM ascorbic acid was added to Milli-Q water buffered with 100 mg/l bicarbonate containing (A) 0.1 mg/l copper (B) 0.2 mg/l ferric iron. (C) Both 0.1 mg/l copper and 0.2 mg/l ferric iron present. (D) Ascorbic acid standard (2 mM), 7-hydroxycoumarin standard (7 μM) and coumarin standard (100 μM) in Milli-Q water. Reaction time was 3 h.

[27,28]. Here we show, by using coumarin-3-carboxylic acid as a fluorescent probe for detection of hydroxyl radical formation, that even very low concentrations of copper (≤0.1 mg/l) are sufficient to give a significant hydroxyl radical signal. However, when copper was substituted by iron, ascorbic acid was not capable to stimulate hydroxyl radical formation (Figure 1). This was also demonstrated by using HPLC analysis. The HPLC data clearly showed that coumarin, in Milli-Q water supplemented with 100 mg/l bicarbonate, was strongly hydroxylated by ascorbic acid in the presence of copper ions but not in the presence of 0.2 mg/l iron alone (Figure 2A and B).

Our results demonstrate that iron partly can inhibit the ascorbic acid/copper driven hydroxyl radical formation in a drinking water environment. When 0.2 mg/l iron was added to the Milli-Q water that had been supplemented with 100 mg/l bicarbonate and 0.1 mg/l copper, the ascorbic acid induced formation of 7-hydroxycoumarin was inhibited by 47.5% (Figure 2C). Our results are in agreement with the recent report by White *et al.*, demonstrating that iron can impair reductant-mediated copper and H_2O_2 generation and neurotoxicity [29]. Moreover, our results are in line with the recent findings by Munday *et al.* showing that copper-catalyzed cysteine oxidation

Table I. Effects of iron on Vitamin C/copper induced hydroxyl radical formation in drinking water.

			Iron supplementation				
			7-OHCCA formed (nM)			Percentage inhibition (%)	
Sample No.	Copper (mg/l)	Bicarbonate (mg/l)	0.0 (mg/l)	0.2 (mg/l)	0.8 (mg/l)	0.2 (mg/l)	0.8 (mg/l)
1	0.15 ± 0.00	92.2 ± 2.6	965.8 ± 7.2	593.4 ± 3.6	454.3 ± 9.8	38.6 ± 0.4	52.9 ± 1.0
2	0.20 ± 0.02	130.1 ± 5.7	990.2 ± 58.9	575.5 ± 53.9	428.1 ± 16.4	41.9 ± 5.6	56.8 ± 1.7
3	0.15 ± 0.01	77.6 ± 4.3	1093.8 ± 94.7	699.6 ± 19.6	579.8 ± 4.7	36.0 ± 1.8	47.0 ± 0.4
4	0.13 ± 0.01	101.4 ± 2.1	904.6 ± 20.9	501.2 ± 51.6	369.2 ± 6.8	44.6 ± 5.7	59.2 ± 0.8

Vitamin C (2 mM) induced hydroxyl radical formation was measured in tap water samples (numbered 1–4) supplemented with either 0.2 or 0.8 mg/l of ferric iron by using the coumarin-3-carboxylic acid assay. The values shown are the concentration of 7-hydroxycoumarin-3-carboxylic acid formed after 3 h incubation in dark at room temperature. Data are expressed as means ± SD of triplicates of one representative experiment out of three conducted.

can be partly inhibited by low concentrations of iron salts [30]. In this context, it can also be mentioned that Menditto *et al.* showed that loading of seminal plasma with either ferrous or ferric iron up to a concentration of 50 μM only modestly affected the rate of ascorbic acid oxidation [31]. The low oxidation rate of ascorbic acid by iron was also seen in our *in vitro* experiments. Low concentrations of copper, however, as shown here, induces rapid oxidation of ascorbic acid [31,32]. Interestingly, it was recently reported that, feeding trace amounts of copper (0.12 mg/l) in drinking water to cholesterol-fed rabbits could induce signs of Alzheimer's disease [33]. Moreover, injection of iron into cholesterol-fed rabbits has recently been reported to cause iron accumulation in the cerebral cortex [34]. One question to be addressed is then whether simultaneous administration of iron could slow down the copper mediated degenerative process.

The data shown in Table I, clearly demonstrate how iron can affect hydroxyl radical formation in copper contaminated, bicarbonate rich household drinking water samples. The formation of hydroxyl radicals in the drinking water samples, in the presence of ascorbic acid, was inhibited by 36.0–44.6% when 0.2 mg/l of ferric iron was present. This inhibition was even more significant, 47.0–59.2%, when 0.8 mg/l of ferric iron was present during the 3 h incubation period with ascorbic acid. Thus, as shown here, iron can to some extent prevent copper/reductant-induced formation of harmful hydroxyl radicals. The exact mechanism by which iron inhibits ascorbic acid/copper-induced hydroxyl radical formation in our water samples is not clear. The inhibition is unlikely to result from an experimental artifact since it is well known that coumarin-3-carboxylic acid can be used to detect iron driven hydroxyl radical reactions. [22,35,36] Moreover, our HPLC experiments using coumarin as the target molecule gave similar results. A plausible explanation for the iron-induced inhibition could be that ferric iron reacts with the superoxide generated from the copper/ascorbate redox reaction. Ferrous iron might also react with hydrogen peroxide and generate water and ferryl ions according to the Bray-Gorin reaction [37].

Iron is an essential micronutrient and the presence of iron in household drinking water is therefore not considered to be harmful. In fact, the intake of iron from drinking water, partly contributes to our daily iron intake. However, due to its offensive taste, color, foaming, odor, corrosion and staining of the drinking water, iron is considered by the water plants as a secondary contaminant. These characteristics are also the reason why excess iron in the drinking water is normally removed or adjusted to very low levels. Our results, however, indicate that complete removal of iron from the raw water in the water plants can to some extent increase the redox activity of copper, and the formation of reactive oxygen species in the drinking water. Moreover, iron deficiency can also increase the intestinal absorption of more harmful metals such as cadmium, lead, and aluminum [38].

In conclusion, our results demonstrate that iron cannot support ascorbic acid induced hydroxyl radical formation in a simple bicarbonate environment but unexpectedly displayed an inhibitory effect on the ascorbic acid induced hydroxyl radical formation process when copper was present. This phenomenon was also evident in our experiments performed in household drinking water samples. Thus, in the presence of bicarbonate, iron might function as an important regulator of copper/reductant-induced hydroxyl radical formation and copper mediated tissue damage. Our results might, to some extent, explain the mechanism for the iron induced protective effect that earlier has been seen in animal model systems.

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