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Generation of hydroxyl radicals from dissolved transition metals in surrogate lung fluid solutions

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Abstract

Epidemiological research has linked exposure to atmospheric particulate matter (PM) to several adverse health effects, including cardiovascular and pulmonary morbidity and mortality. Despite these links, the mechanisms by which PM causes adverse health effects are poorly understood. The generation of hydroxyl radical ($^{\bullet}OH$) and other reactive oxygen species (ROS) through transition-metal-mediated pathways is one of the main hypotheses for PM toxicity. In order to better understand the ability of particulate transition metals to produce ROS, we have quantified the amounts of $^{\bullet}OH$ produced from dissolved iron and copper in a cell-free, surrogate lung fluid (SLF). We also examined how two important biological molecules, citrate and ascorbate, affect the generation of $^{\bullet}OH$ by these metals. We have found that Fe(II) and Fe(III) produce little $^{\bullet}OH$ in the absence of ascorbate and citrate, but that they efficiently make $^{\bullet}OH$ in the presence of ascorbate and this is further enhanced when citrate is also added. In the presence of ascorbate, with or without citrate, the oxidation state of iron makes little difference on the amount of $^{\bullet}OH$ formed after 24 h. In the case of Cu(II), the production of $^{\bullet}OH$ is greatly enhanced in the presence of ascorbate, but is inhibited by the addition of citrate. The mechanism for this effect is unclear, but appears to involve formation of a citrate–copper complex that is apparently less reactive than free, aquated copper in either the generation of hydrogen peroxide (HOOH) or in the Fenton-like reaction of copper with HOOH to make $^{\bullet}OH$. By quantifying the amount of $^{\bullet}OH$ that Fe and Cu can produce in surrogate lung fluid, we have provided a first step into being able to predict the amounts of $^{\bullet}OH$ that can be produced in the human lung from exposure to PM containing known amounts of transition metals.

Keywords: Reactive oxygen species (ROS); Hydroxyl radical; Transition metals; Particulate matter; Fenton reaction

1. Introduction

Numerous epidemiological studies indicate that exposure to atmospheric particulate matter (PM) can cause cardiovascular and cardiopulmonary damage (Dockery et al., 1993; Pope et al., 2002, 1995). It has been estimated that in the United States between 20,000 and 50,000 people die each

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year from PM exposure (Mokdad et al., 2004). Researchers have also shown that PM exposure is linked to other human diseases such as asthma (Slaughter et al., 2005; Whittemore and Korn, 1980), lung cancer (Dellinger et al., 2001; Pope et al., 2002, 1995), and Alzheimer's (Calderon-Garciduenas et al., 2004). Although there are strong correlations between exposure to PM and adverse health effects, the actual mechanisms by which PM induces these effects are still poorly understood. One hypothesis is that the damage originates with the in vivo generation of reactive oxygen species (ROS) by the particles, followed by oxidative stress and cell damage (Dellinger et al., 2001; Imlay, 2003; Risom et al., 2005; Zhou et al., 2003).

Hydroxyl radical ($^{\bullet}OH$) is one of the most strongly oxidizing ROS species (Valavanidis et al., 2000). Past studies have shown that urban PM and particles from gasoline and diesel exhaust can generate $^{\bullet}OH$ in the presence of hydrogen peroxide (HOOH) and that the Cu-content of PM is correlated with $^{\bullet}OH$ generation (Valavanidis et al., 2005a, b). Smith and Aust (1997) found that Fe present in urban PM was responsible for most of the generation of ROS in their studies. Past studies have also found that exposure to diesel exhaust particles in mice produced $^{\bullet}OH$ in the lung through Fe-catalyzed reactions of superoxide and HOOH (Han et al., 2001). In our prior research we showed that $^{\bullet}OH$ is produced by ethylene flame soot in a cell-free surrogate lung fluid solution containing HOOH (Jung et al., 2006).

In addition to the work described above, a number of other past studies have also found that transition-metal-mediated pathways are most responsible for $^{\bullet}OH$ formation. PM contains trace amounts of metals, such as Fe, Cu, Ni, Co, V, and others (Deguillaume et al., 2005; Dreher et al., 1997). When PM is inhaled into the lung, the soluble transition metals can be mobilized into the lung lining fluid, where they can form ROS via Fenton or Fenton-like reactions (Deguillaume et al., 2005; Donaldson et al., 1997; Knaapen et al., 2002; Zepp et al., 1992; Zhou et al., 2003). In the Fenton reaction (R1), the reduced form of iron, Fe(II), reacts with HOOH to produce the oxidized form of iron and hydroxyl radical.

$$Fe(II) + HOOH \rightarrow Fe(III) + {}^{\bullet}OH + OH^{-}. \quad (R1)$$

Similar reactions can occur with Cu, Cr, and Ni. Furthermore, biological chelators and reductants can greatly enhance the production of ROS (Burkitt and Gilbert, 1991; Engelmann et al., 2003; Wenk et al., 2001). For example, in the presence of ascorbate (Asc), a biological reductant, the oxidized form of the transition metal produced by the Fenton reaction can be reactivated (R2 and R3), thus allowing additional ROS to be produced.

$$Fe(III) + Asc^{n} \rightarrow Fe(II) + Asc^{n+1}, \quad (R2)$$

$$Cu(II) + Asc^{n} \rightarrow Cu(I) + Asc^{n+1}. \quad (R3)$$

Given the amounts of Asc (160 μM) and citrate (330 μM) in human lung fluid (Greenwell et al., 2003; Smith and Aust, 1997) it is likely that these species play important roles in ROS generation from particulate transition metals.

Although most of the prior research on $^{\bullet}OH$ production from PM has used extraction fluids that contain an initial amount of HOOH, HOOH does not have to be added in order for R1 to occur. This is because reduced forms of transition metals can react with dissolved oxygen to produce superoxide (R4), which can in turn react with another reduced metal atom to produce HOOH (R5). For example, in the case of Fe, these reactions are

$$Fe(II) + O_2 \rightarrow Fe(III) + {}^{\bullet}O_2^{-}, \quad (R4)$$

$$Fe(II) + {}^{\bullet}O_2^{-} + 2H^{+} \rightarrow Fe(III) + HOOH. \quad (R5)$$

While a number of studies have examined the ability of PM to form $^{\bullet}OH$ through Fenton or Fenton-like reactions (Lewis et al., 2003; Park et al., 2006; Shi et al., 2003; Winterbourn, 1981; Zepp et al., 1992), only a few have examined how biological reductants or chelators influence this chemistry. Furthermore, the concentrations of reactive components, such as ascorbate, in past surrogate lung fluids have typically been several times higher than levels found in the human body (Lewis et al., 2003; Smith et al., 2003; Valavanidis et al., 2005a, b). In addition, many past studies have not absolutely quantified the amounts of $^{\bullet}OH$ produced from these reactions, but rather have reported the relative amounts of $^{\bullet}OH$ generated from different metals or treatments.

We have addressed some of these issues in this current work by tackling four main objectives: (1) quantifying the amounts of $^{\bullet}OH$ produced in SLF solution from dissolved Fe(II), Fe(III), and Cu(II); (2) determining the influence of Asc and Cit on $^{\bullet}OH$ production; (3) examining how $^{\bullet}OH$ generation is affected when levels of Asc, Cit, and HOOH in the SLF are greater than physiologic concentrations;

and (4) determining the $^{\bullet}OH$ production reaction efficiency in the surrogate lung fluid solutions in order to assess the possible burden of particle-generated $^{\bullet}OH$ in human lungs.

2. Materials and methods

2.1. Chemicals

Sodium citrate (A.C.S. reagent grade) and sodium bisulfite (A.C.S.) were from GFS chemicals and *p*-hydroxybenzoic acid was from TCI America. Copper (II) chloride (99.999 + %), ferrous sulfate (99.9 + %), ferric chloride (99.9%), desferoxamine mesylate (95%), and ascorbic acid (Puriss p.a., 99.0%) were from Sigma. Chelex-100 molecular biology grade resin was from BioRad laboratories. Sodium benzoate (A.C.S.), potassium phosphate (HPLC grade), hydrogen peroxide (A.C.S.), sodium phosphate (A.C.S.), and sodium chloride (A.C.S.) were from Fisher Scientific. Purified water was obtained from a Milli-Q Plus system (Millipore; $\geqslant 18.2\,M\Omega\,cm$).

2.2. Surrogate lung fluid (SLF)

All of the experiments were performed in either one of two cell-free SLF solutions. SLF 1 consisted of 10 mM sodium benzoate (NaBA) as a $^{\bullet}OH$ scavenger, 114 mM sodium chloride (NaCl), 10 mM total phosphate (7.8 mM Na_2HPO_4 and 2.2 mM KH_2PO_4) to buffer the solution at pH 7.4, 1.0 mM HOOH and 1.0 mM of Asc and/or 1.0 mM of Cit. SLF 2 consisted of the same ingredients except it contained no HOOH and had 200 μM Asc and/or 300 μM Cit. After preparation, SLF solutions were refrigerated and generally used within a month. Stock solutions of Asc and Cit were prepared fresh on the day of each experiment. Metals were removed from all SLF solutions (prior to addition of Asc, Cit, or HOOH) using Chelex 100 resin.

2.3. Sample preparation

Twenty micromolar (200 nmol) of Fe(II), Fe(III), or Cu(II) were added to 10 ml of either SLF 1 or SLF 2 in a 125-ml acid-washed Teflon FEP bottle. The Teflon bottles were completely wrapped with Al foil and shaken for 24 h in a wrist-action shake table in the dark. Within 10 min after removing each solution from the wrist-action shaker we added 100 μM Desferoxamine (DSF) and 50 μM HSO_3^- to stop the generation of $^{\bullet}OH$. SLF samples were then acidified to pH 2 by adding 120 μl of 1.0 M H_2SO_4. The extract was then filtered using a syringe filter (0.22 μm pore Teflon filter; Pall Co.). Stock solutions of each metal solution were freshly prepared from the appropriate salt on the day of each experiment.

2.4. $^{\bullet}OH$ measurements using high-performance liquid chromatography (HPLC)

Hydroxyl radicals were quantitatively trapped and measured using a benzoate chemical probe (Anastasio and McGregor, 2001; Jung et al., 2006) where $^{\bullet}OH$ reacts with benzoate to produce *p*-hydroxybenzoate (*p*-HBA), a stable product that is measured using HPLC:

$$\text{Benzoate} + {}^{\bullet}OH \rightarrow p\text{-HBA} + \text{other products.} \quad \text{(R6)}$$

The HPLC used in these experiments consisted of a Shimadzu LC10-AT pump, a Keystone Scientific C-18 Beta Basic reverse-phase column (250 × 3 mm, 5 μm beads) with an attached guard column, and a Shimadzu SPD-10AV UV–visible detector ($\lambda = 256$ nm). The eluent consisted of 70% H_2O and 30% CH_3CN adjusted to pH 2 with $HClO_4$ and was run at a flow rate of 0.60 ml min^{-1}. The amount of *p*-HBA produced in each sample solution was quantified using a calibration curve produced from *p*-HBA standards made in SLF and run on the same day of the experiments. The concentration of $^{\bullet}OH$ in each solution was determined using (Jung et al., 2006)

$$[{}^{\bullet}OH] = [p\text{-HBA}]/(Y_{p\text{-HBA}} \times f_{BA}), \quad \text{(R7)}$$

where [*p*-HBA] is the measured concentration of *p*-hydroxybenzoic acid, $Y_{p\text{-HBA}}$ is the molar yield of *p*-HBA produced from the reaction of $^{\bullet}OH$ with benzoate in SLF (0.215 ± 0.018) (Jung et al., 2006) and f_{BA} is the fraction of $^{\bullet}OH$ that reacts with benzoate in a given SLF. Based on published rate constants for $^{\bullet}OH$ (Bonifacic et al., 1994; Zepp et al., 1992), we calculate that values of f_{BA} in our SLF solutions are: 0.999 in the absence of Asc or Cit, 0.989 with Asc, 0.999 with Cit, and 0.988 with both Asc and Cit.

3. Results and discussion

3.1. Reaction time-course experiments

In order to determine the time dependence for $^{\bullet}OH$ formation from Fe(II), Fe(III), and Cu(II), we

performed several time-course experiments using SLF 2 with added Asc and/or Cit (Fig. 1). In the absence of Asc and Cit, only Fe(II) produces any noticeable amount of $^{\bullet}$OH, although the amount produced is rather small (Fig. 1a). Interestingly, $^{\bullet}$OH production in this SLF solution is essentially finished by the first time point (2 h). In the presence of just Cit, only Fe(II) produces noticeable amounts of $^{\bullet}$OH (Fig. 1b), but levels are only slightly higher than in the case without Cit (Fig. 1a). In the presence of only Asc, Fe(II) still shows an exponential rise (with most of the $^{\bullet}$OH being produced within the first 8 h) but now Fe(III) also produces $^{\bullet}$OH. After 24 h the amount of $^{\bullet}$OH from Fe(III) is only slightly lower than that of Fe(II) (Fig. 1c). It is interesting to note that while Asc can reduce Fe(III) and produce $^{\bullet}$OH (Fig. 1c), Cit does not appear to reduce dissolved Fe(III) over 24 h (Fig. 1b). The

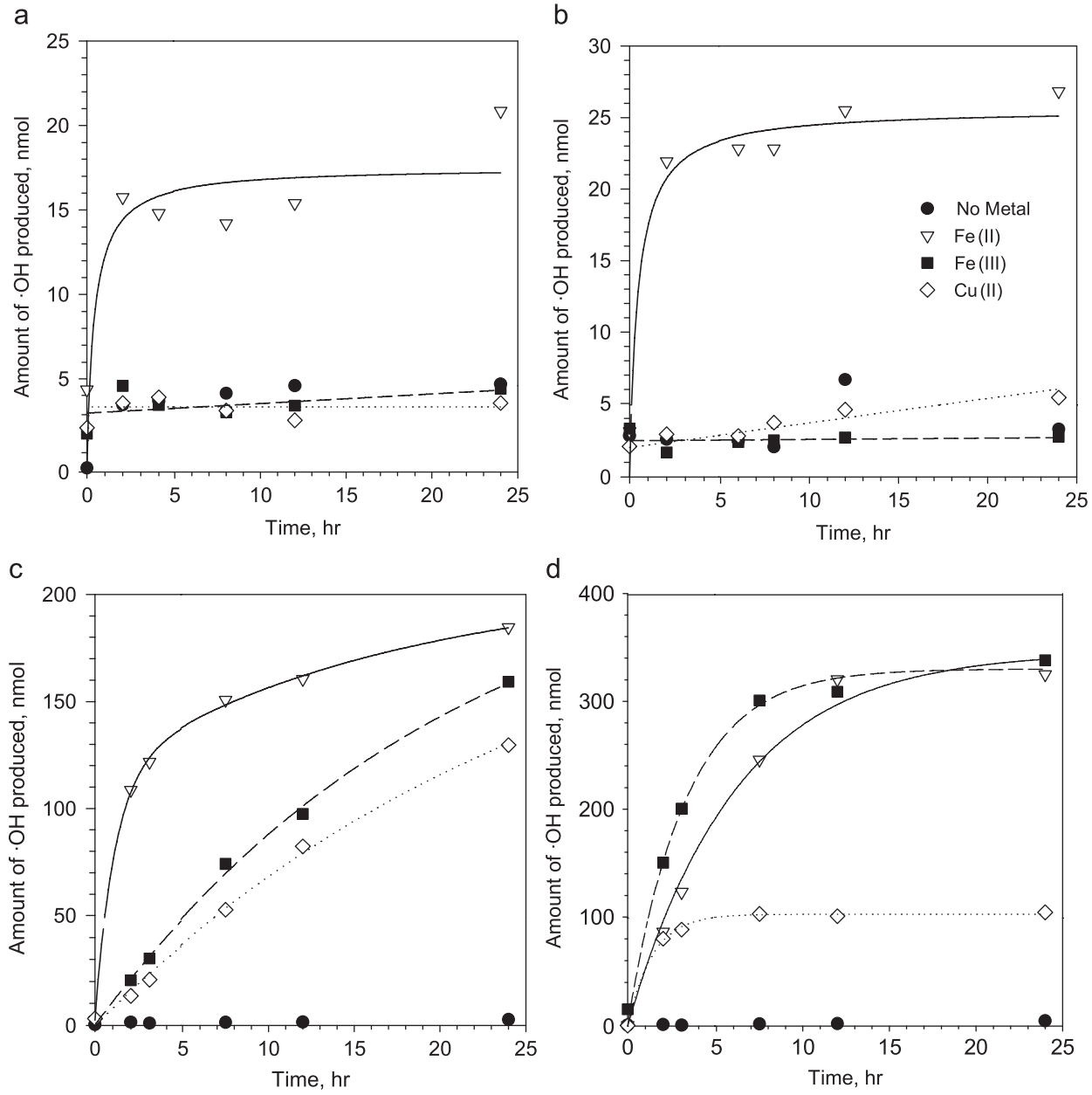

Fig. 1. Formation of $^{\bullet}$OH from a surrogate lung fluid containing 200 nmol ascorbate (Asc) and/or 300 nmol citrate (Cit) as a function of extraction time. (a) SLF2, (b) SLF + Cit, (c) SLF + Asc and (d) SLF + Asc + Cit.

production of •OH by Cu(II) in SLF 2 with only Asc exhibits an increase that is very similar in shape and magnitude to Fe(III) (Fig. 1c).

When both Cit and Asc are present in SLF 2, Fe(II) and Fe(III) have very similar behaviors, with almost identical amounts of •OH produced after 24 h (330 and 340 nmol, respectively) (Fig. 1d). The similarity of the rates of •OH production from both Fe(II) and Fe(III), and the fact that those rates were different in the presence of only Asc, indicates that Cit assists in the reduction and/or reactions of Fe(III) to make •OH. In the presence of Asc and Cit, the production of •OH by Cu(II) is rapid in the first 4 h but then stops.

3.2. •OH production from transition metals in SLF 1 (10 μmol HOOH with 10 μmol ascorbate and/or 10 μmol citrate)

In our initial studies we used SLF 1 (containing 10 μmol HOOH along with 10 μmol Asc and/or 10 μmol Cit), which is similar to the extraction fluid we used in our previous study (Jung et al., 2006). The amounts of •OH produced from Fe(II), Fe(III), and Cu(II) in SLF 1 are shown in Fig. 2. In the absence of both Asc and Cit, there is little •OH produced from either iron species (24 nmol from Fe(II) and 7 nmol from Fe(III)), but there is abundant •OH production from Cu(II) (290 nmol). When Cit is added to the SLF 1 solution, the amounts of •OH produced from both Fe(II) and Fe(III) increase by a factor of approximately 4 compared to the SLF without Cit, but the Cu(II) reactivity decreases by a factor of 14 (Fig. 2). Fig. 2 also shows that the addition of Asc to the SLF 1 solution dramatically increases the amount of •OH produced from each of three metals: nearly 700 nmol of •OH is produced from both Fe(II) and Fe(III), while Cu(II) increases to 2400 nmol of •OH, nearly a 10-fold increase compared to the case with no added Cit or Asc. Based on the fact that the Fe(II) and Fe(III) solutions both produced essentially the same amount of •OH in the presence of Asc, it is clear that the initial oxidation state of iron has little or no effect on •OH production under these conditions, suggesting that reduction of Fe(III) by Asc is quite efficient.

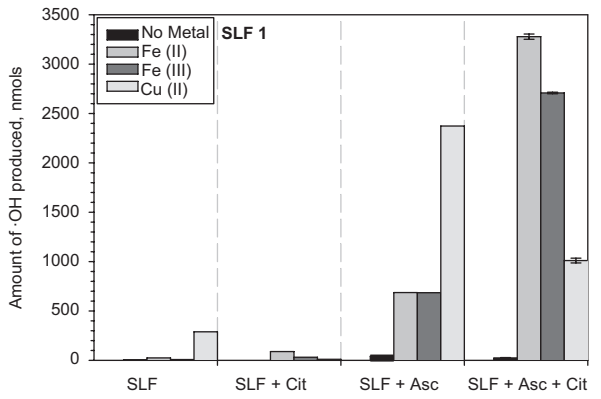

Fig. 2. Production of •OH from 200 nmol Fe (II), Fe (III), or Cu (II) in surrogate lung fluid solution containing 10,000 nmol HOOH along with 10,000 nmol ascorbate (Asc) and/or 10,000 nmol citrate (Cit). Bars represent the mean values ($n = 3$). Error bars are 1σ values calculated from replicate experiments.

In the presence of both Asc and Cit in the SLF 1 solution, the amount of •OH produced from Fe(II) and Fe(III) increased to 3300 and 2700 nmol, respectively. Under these conditions, the initial oxidation state of the iron does play a role in the production of •OH, but this effect is small as there is only an 18% difference between the two results. In the case of Cu(II), the addition of Cit decreased the amount of •OH produced from 2400 to 1100 nmol as compared to the case of SLF 1 with Asc only. This reduction further illustrates that Cit decreases the ability of copper to produce •OH under these conditions.

To describe the ability of a given combination of metal and SLF to make •OH over our 24-h reaction time, we define the •OH reaction efficiency as

$$\text{Reaction efficiency} = \frac{\text{Moles of } {}^{\bullet}\text{OH produced}}{\text{Moles of transition metal in SLF}}. \quad \text{(R8)}$$

While this definition has •OH as its endpoint, it should be kept in mind that the reduction of dissolved O_2 to make •OH requires 3 electrons, i.e., the overall reaction is

$$O_2 + 2H^+ + 3e^- \rightarrow {}^{\bullet}OH + OH^-. \quad \text{(R9)}$$

Thus in the absence of initial HOOH (i.e., in SLF 2), a reaction efficiency of 1 for Fe(III) or Cu(II) corresponds to 3 electron transfers (e.g., the sequence (R2), (R4), (R5), (R1)), while greater values correspond to multiple passes through this cycle or related cycles. In SLF 1, where HOOH is present initially, production of •OH from the oxidized metal requires 2 electrons.

As shown in Table 1, •OH generation from Cu(II) is most efficient in the SLF 1 solution with

Table 1
Reaction efficiencies for •OH formation from metal solutions in two different surrogate lung fluids (SLF)[a]

	SLF 1			SLF 2		
	Fe(II)	Fe(III)	Cu(II)	Fe(II)	Fe(III)	Cu(II)
SLF	0.12	0.04	1.4	0.05	0.00	0.00
SLF + Cit	0.44	0.16	0.05	0.10	0.01	0.01
SLF + Asc	3.4	3.4	12	0.83	0.70	0.90
SLF + Cit + Asc	16	14	5.0	1.8	1.7	0.48

[a]•OH reaction efficiencies represent the moles of •OH produced per mole of metal (see (R8)). Solutions contained 200 nmol Fe(II), Fe(III), or Cu(II) in one of two surrogate lung fluid (SLF) solutions. SLF 1 contained 10,000 nmol HOOH and 10,000 nmol of Asc and/or Cit. SLF 2 contained no HOOH and 200 nmol of Asc and/or Cit.

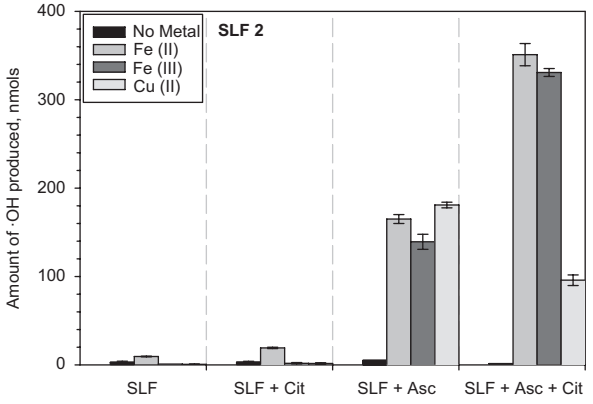

Fig. 3. Production of •OH from 200 nmol Fe (II), Fe (III), or Cu (II) in a surrogate lung fluid solution containing 200 nmol ascorbate (Asc) and/or 300 nmol citrate (Cit) (and no initial HOOH). Bars represent the mean values ($n = 3$) with error bars of 1σ.

the addition of Asc only, which increases the reactivity by a factor of 9 compared to SLF 1 with no Asc or Cit. Furthermore, reaction efficiencies in SLF 1 are large in the presence of both Asc and Cit, with values of 16, 14, and 5 for Fe(II), Fe(III), and Cu(II), respectively. Compared to values in SLF 1 without Asc or Cit, adding Asc and Cit increases the •OH reaction efficiencies by factors of 160, 140, and 3.6 for Fe(II), Fe(III), and Cu(II), respectively. These results indicate that Asc greatly enhances •OH production, likely by reducing the oxidized transition metal formed in the Fenton reaction (see (R1)–(R3)). Furthermore, the impact of Asc under the SLF 1 conditions is significantly greater for copper compared to iron. While these results are interesting and provide some insights into the impacts of Asc and Cit on •OH generation, the levels of these species (and of the initial HOOH) are much greater than biological amounts. Thus, we carried out the rest of our experiments in this study using SLF 2, which contains lower, and more biologically relevant, concentrations of Asc and Cit and no initial HOOH.

3.3. *•OH production from transition metals in SLF 2 (no initial HOOH, 2.0 μmol Asc and/or 3.0 μmol Cit)*

Fig. 3 illustrates the amounts of •OH produced from the three dissolved metals in SLF 2 solutions. In the absence of both Asc and Cit, there is little or no •OH production from any of the metal solutions. The addition of Cit to SLF 2 leads to an approximately 2-fold increase in •OH production from Fe(II), but has no significant effect on •OH production from Fe(III) or Cu(II). The presence of Asc greatly enhances •OH production from iron: both Fe(II) and Fe(III) produce approximately 150 nmol of •OH under these conditions, an increase of a factor of 15 and 30, respectively, compared to results from the SLF 2 solution in the absence of Asc and Cit (Fig. 3). With addition of both Asc and Cit to the SLF 2, •OH production from both Fe(II) and Fe(III) increases, from approximately 150 nmol in the SLF 2 with Asc only to approximately 340 nmol with both Cit and Asc. The similarities of the •OH values from both Fe(II) and Fe(III) in this latter solution indicates that the initial iron oxidation state has only a small effect on the total amount of •OH produced under these conditions, which is similar to the case in the SLF 1 experiments.

In the SLF 2 solution containing only Asc, Cu(II) produces 180 nmol of •OH. However, when both Cit and Asc are present in SLF 2, Cu(II) only produces 95 nmol of •OH. This reduction in •OH production due to Cit is similar to that seen for Cu(II) in SLF 1 (Fig. 2). Based on chemical speciation modeling in our solutions (MINEQL+ (Schecher and Drew, 1992)) the addition of Cit dramatically shifts the copper speciation, from $Cu(H_2O)_6$ in SLF 2 without Cit, to a copper–citrate complex ($Cu_2C_6H_4O_7$) in the presence of Cit. In combination with our •OH results, this suggests that the copper–citrate complex is less reactive than the free, aquated Cu, either in the generation of

HOOH from dissolved O_2, or in the generation of $^{\bullet}OH$.

The reaction efficiencies for Fe(II) and Fe(III) in SLF 2 are greatest in the presence of both Asc and Cit, with efficiency values of 1.8 and 1.7, respectively (Table 1). These efficiency values are 35 and 165 times higher, respectively, than the Fe(II) and Fe(III) values for SLF 2 solution without Asc or Cit. These efficiencies indicate that nearly 2 moles of $^{\bullet}OH$ are produced per mole of dissolved Fe; the fact that the Fe(II) and Fe(III) efficiencies are essentially the same indicates that reduction by Asc is more important than the initial oxidation state of the dissolved iron. Asc plays a similarly important role in the generation of $^{\bullet}OH$ by Cu(II), where the reaction efficiency increased by a factor of ~90 with Asc compared to when Asc is not present.

3.4. Effect of HOOH on $^{\bullet}OH$ production from Cu and Fe

Based on results from the SLF 1 and SLF 2 solutions, the production of $^{\bullet}OH$ from Cu(II) is greatly dependent on the initial presence of HOOH. In the absence of Cit and Asc, Cu(II) produced 290 nmol $^{\bullet}OH$ in SLF 1 (containing 10 μmol of initial HOOH) but less than 1 nmol of $^{\bullet}OH$ in SLF 2 (with no initial HOOH). The large difference in the amounts of $^{\bullet}OH$ produced from these two SLF solutions, in which only the HOOH concentration was different, suggest that hydroxyl production from Cu(II) is very sensitive to the initial presence of HOOH. To examine this issue further, we measured the production of $^{\bullet}OH$ from Cu(II) over a range of initial HOOH concentrations in SLF 2 (with 200 nmol Asc, 300 nmol Cit, and 200 nmol Cu(II)). As shown in Fig. 4, in the absence of HOOH, Cu(II) produced 96 nmol of $^{\bullet}OH$. In the presence of 100 nmol of HOOH the amount of $^{\bullet}OH$ produced increased only slightly (to 110 nmol), while 1000 nmol of added HOOH with Cu(II) produced 150 nmol of $^{\bullet}OH$. At the highest concentration of 10,000 nmol of HOOH, Cu(II) produced 380 nmol of $^{\bullet}OH$, nearly 4 times as much as in the absence of HOOH. Based on these results it is clear that production of $^{\bullet}OH$ by Cu(II) is enhanced by the presence of large initial levels of HOOH, but is not affected much by lower levels. The difference between the amount of $^{\bullet}OH$ produced in Fig. 4 with 10 μmol HOOH (380 nmol $^{\bullet}OH$) and in SLF 1 with Asc and Cit (1010 nmol $^{\bullet}OH$; Fig. 2) indicates that the higher amounts of Asc and Cit

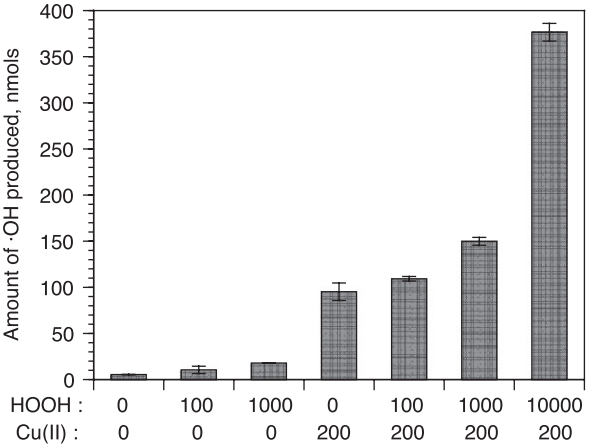

Fig. 4. Production of $^{\bullet}OH$ from 200 nmol Cu(II) with various initial amounts of HOOH (in units of nmol) in a surrogate lung fluid solution containing 200 nmol ascorbate (Asc) and 300 nmol citrate (Cit). Bars represent the mean value ($n = 3$) with error bars of 1σ.

(10,000 nmol each) in SLF 1 enhance $^{\bullet}OH$ formation by approximately a factor of 2.6 compared to SLF 2 with the same amount of HOOH.

Comparing the results from SLF 1 (Fig. 2) and SLF 2 (Fig. 3) in the absence of both Asc and Cit, i.e., where the presence of 10 μmol of HOOH in SLF 1 is the only difference between these solutions, shows that initial HOOH also enhances $^{\bullet}OH$ production from both Fe(II) and Fe(III) (by factors of 3 and 9, respectively). However, under both of these conditions Fe produces very little $^{\bullet}OH$ compared to solutions with added Asc. Based on our results, it appears that Fe(II) is less dependent on the initial presence of HOOH than is Cu(II), which much more efficiently makes $^{\bullet}OH$ in the presence of large initial amounts of HOOH.

3.5. $^{\bullet}OH$ production from mixtures of Fe and Cu

PM contains trace amounts of a wide variety of transition metals (Dreher et al., 1997; Song et al., 2003; Valavanidis et al., 2005a, b, 2006). As a result, the transition-metal-mediated production of $^{\bullet}OH$ will depend on the amounts of each metal (especially iron) as well as their interactions. In ambient PM, Fe is typically the most abundant metal while the amount of Cu is typically 4–15% that of Fe (Tolocka et al., 2001). In order to understand how mixing Fe and Cu affects $^{\bullet}OH$ production, we performed experiments using 200 nmol Fe(II) and various amounts of Cu(II). Fig. 5 illustrates the results from these experiments in three variations of

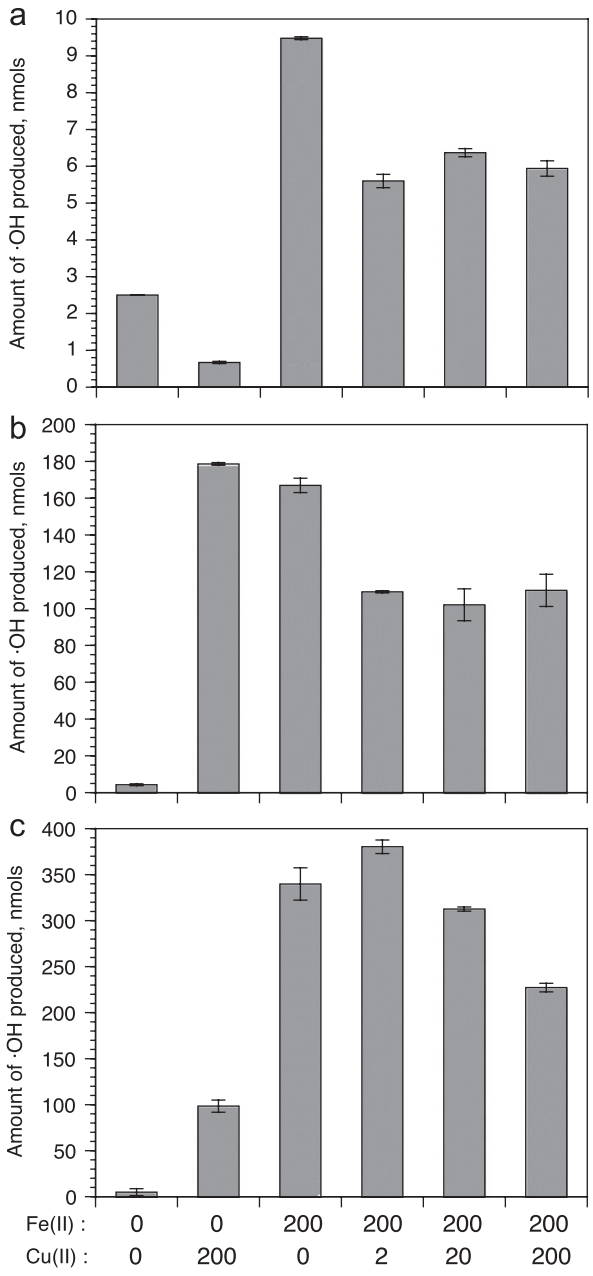

Fig. 5. Production of $^{\bullet}$OH from mixtures of Fe(II) and Cu(II) (in units of nmol) with and without 200 nmol ascorbate (Asc) and 300 nmol citrate (Cit). Bars represent the mean value ($n = 3$) with error bars of 1σ. (a) SLF, (b) SLF + Asc and (c) SLF + Asc + Cit.

SLF 2: (1) without Asc or Cit, (2) with 200 nmol Asc, and (3) with 200 nmol Asc and 300 nmol Cit. In the SLF 2 solution in the absence of Asc and Cit, addition of 2.0 nmol Cu(II) to a solution containing 200 nmol Fe(II) decreases $^{\bullet}$OH production from 9.4 nmol (in the solution containing only Fe(II)) to 5.2 nmol. In similar solutions containing higher levels of Cu(II) the amount of $^{\bullet}$OH produced remains essentially unchanged at this lower level (Fig. 5a). In the SLF 2 solution with Asc the amounts of $^{\bullet}$OH produced are much greater, but the same general behavior is observed. Whereas separate solutions containing 200 nmol Cu(II) and 200 nmol Fe(II) each produce approximately 170 nmol of $^{\bullet}$OH, mixtures of 2–200 nmol Cu(II) with 200 nmol Fe(II) produce only approximately 110 nmol of $^{\bullet}$OH, which is just 30% of the amount expected from the summation of the individual 200 nmol Fe(II) and Cu(II) solutions (Fig. 5b). However, in the SLF 2 solution in the presence of Asc and Cit, the behavior is somewhat different. Because of the impact of Cit on copper chemistry, Fe(II) here produces approximately 3.4 times more $^{\bullet}$OH than does Cu(II) in solutions containing only one of the metals (Fig. 5c). And adding 2 nmol of Cu(II) to the Fe(II) solution increases the amount of $^{\bullet}$OH produced (by approximately 9%) instead of reducing $^{\bullet}$OH production as seen in the other solutions. However, adding higher amounts of Cu(II) decreases $^{\bullet}$OH production by Fe(II): 34% less $^{\bullet}$OH is produced in the solution with 200 nmol Cu(II) (and 200 nmol Fe(II)) compared to the solution with only Fe(II).

These measurements suggest that low levels of dissolved copper have little effect on $^{\bullet}$OH formation from iron in lungs, but that higher amounts of copper have an antagonistic effect on $^{\bullet}$OH production from Fe(II). This Cu(II) inhibition of $^{\bullet}$OH production by iron agrees with past results (Maestre et al., 1992), in which it was observed that at low concentrations, Cu(II) ions inhibited $^{\bullet}$OH radical generation from the iron-driven Fenton reaction. We do not know the mechanism for this antagonistic effect of copper, but it is interesting that it does not require the formation of a copper–citrate complex.

4. Implications

Although past researchers have examined the generation of $^{\bullet}$OH from particulate transition metals, there have been few quantitative estimates of the possible burden of particle-generated $^{\bullet}$OH in human lungs. Here we estimate this $^{\bullet}$OH burden from inhalation of airborne particulate Fe based on the $^{\bullet}$OH production reaction efficiencies (RE) from Table 1. To a first approximation, the amount of $^{\bullet}$OH generated in human lungs from particulate

iron can be estimated by

$$\text{Vol. of air inhaled } (\text{m}^3\,\text{day}^{-1}) \times \text{PM Fe conc. } (\text{ng}\,\text{m}^{-3}) \times (1/\text{MW}_{\text{Fe}}) \times \text{Fraction of PM Fe that is water soluble} \times {}^{\bullet}\text{OH RE } (\text{nmol } {}^{\bullet}\text{OH}\,\text{nmol}^{-1}\text{ Fe}) = {}^{\bullet}\text{OH load } (\text{nmol } {}^{\bullet}\text{OH}\,\text{day}^{-1}). \quad \text{(R10)}$$

Using an RE of 1.7 (Table 1), an average volume of air inhaled by an adult of $20\,\text{m}^3\,\text{day}^{-1}$, typical Fe levels in PM of $250\,\text{ng}\,\text{m}^{-3}$ (Majestic, et al., 2007), and estimating that 5% of the particulate Fe is water soluble (See et al., 2007), we calculate a lung burden of approximately 8 nmol $^{\bullet}$OH per day. It is important to note that to form this amount of $^{\bullet}$OH requires 2–3 times as much oxidation of biological molecules (e.g., Asc) in order to reduce dissolved O_2 to $^{\bullet}$OH (see (R9)). Thus, the formation of $^{\bullet}$OH in our estimate corresponds to 16–24 nmol of oxidation during the formation of $^{\bullet}$OH, followed by 8 nmol of oxidation by $^{\bullet}$OH, to yield a total amount of Fe-mediated oxidation of approximately 24–32 nmol day^{-1}.

As described previously (Jung et al., 2006), the generated $^{\bullet}$OH will react with antioxidants (primarily Asc, urate, and reduced glutathione) as well as other biological molecules. Given that each of these three antioxidants is present in the lung lining fluid at a concentration of approximately 200 μM (Greenwell et al., 2003), and based on an estimated adult lung lining fluid volume of 25 ml (Walters, 2002), this corresponds to approximately 15 μmol of total antioxidant. This antioxidant loading is much greater than the daily 20–30 nmol of oxidant burden due to inhalation of soluble, particulate Fe that we calculate above. However, this oxidant burden is likely an underestimate since $^{\bullet}$OH will be produced from other metals and there are other factors (described in Jung et al., 2006) that can also enhance ROS formation in the lungs.

5. Conclusions

We have quantified the amounts of $^{\bullet}$OH formed from aqueous metals in two types of SLF solutions with and without Asc and Cit. In both SLF solutions Asc greatly enhances $^{\bullet}$OH production from Fe(II) and Fe(III) and the addition of Cit further enhances the generation of $^{\bullet}$OH by Fe. Under these conditions the oxidation state of Fe makes little or no difference on the amount of $^{\bullet}$OH formed after 24 h. The $^{\bullet}$OH generating reaction efficiencies for Fe (i.e., moles $^{\bullet}$OH/moles Fe) ranged from <0.01 (with no Asc or Cit) to 1.8 (with both Asc and Cit) in the SLF with no initial HOOH. Because the formation of each mole of $^{\bullet}$OH in these solutions requires 2 to 3 electron transfers (e.g., the sequence (R2), (R4), (R5), (R1)), these reaction efficiencies indicate that Fe efficiently redox cycles in the presence of Asc and Cit.

In the case of dissolved Cu, $^{\bullet}$OH production is enhanced by Asc but inhibited by Cit, apparently because the citrate–copper complex is less reactive than free, aquated Cu. In addition, Cu(II) production of $^{\bullet}$OH is relatively insensitive to low levels of initial HOOH but at high levels $^{\bullet}$OH production is increased by nearly 4-fold. Furthermore, it was found that the addition of Cu to Fe solutions causes an antagonistic effect: solutions with Cu/Fe ratios of 1/100 and greater produced less $^{\bullet}$OH than the sum of the corresponding individual copper and iron solutions.

To our knowledge this is the first research that has quantified the production of $^{\bullet}$OH from dissolved transition metals in surrogate lung fluid solutions. These results are a first step towards being able to predict the amounts of $^{\bullet}$OH that can be produced in human lungs from exposure to PM containing transition metals. In order to better understand this process, future work should examine the solubility of transition metals, and the generation of $^{\bullet}$OH and other ROS, in more complicated surrogates for lung lining fluid that contain surfactants, proteins, and antioxidants in addition to Asc.

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