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Cobalt-mediated generation of reactive oxygen species and its possible mechanism

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Abstract

Electron spin resonance spin trapping was utilized to investigate free radical generation from cobalt (Co) mediated reactions using 5,5-dimethyl-l-pyrroline (DMPO) as a spin trap. A mixture of Co with water in the presence of DMPO generated 5,5-dimethylpyrroline-(2)-oxy(1) DMPOX, indicating the production of strong oxidants. Addition of superoxide dismutase (SOD) to the mixture produced hydroxyl radical (•OH). Catalase eliminated the generation of this radical and metal chelators, such as desferoxamine, diethylenetriaminepentaacetic acid or 1,10-phenanthroline, decreased it. Addition of Fe(II) resulted in a several fold increase in the •OH generation. UV and O_2 consumption measurements showed that the reaction of Co with water consumed molecular oxygen and generated Co(II). Since reaction of Co(II) with H_2O_2 did not generate any significant amount of •OH radicals, a Co(I) mediated Fenton-like reaction [Co(I) + H_2O_2 → Co(II) + •OH + OH^-] seems responsible for •OH generation. H_2O_2 is produced from $O_2^{\bullet-}$ via dismutation. $O_2^{\bullet-}$ is produced by one-electron reduction of molecular oxygen catalyzed by Co. Chelation of Co(II) by biological chelators, such as glutathione or β-ananyl-3-methyl-L-histidine alters, its oxidation–reduction potential and makes Co(II) capable of generating •OH via a Co(II)-mediated Fenton-like reaction [Co(II) + H_2O_2 → Co(III) + •OH + OH^-]. Thus, the reaction of Co with water, especially in the presence of biological chelators, glutathione, glycylglycylhistidine and β-ananyl-3-methyl-L-histidine, is capable of generating a whole spectrum of reactive oxygen species, which may be responsible for Co-induced cell injury. © 1998 Published by Elsevier Science Inc.

Keywords: Cobalt; Reactive oxygen species; Mechanism; Cell injury

1. Introduction

Cobalt (Co) is an essential trace element for mammalian nutrition. It is toxic and carcinogenic at higher concentrations [1–4]. The International Agency for Research on Cancer has classified this metal as possibly carcinogenic to humans [5]. Occupational exposure to hard metals, including Co, causes ''hard metal diseases'', such as cancer and asthma [6]. As stated in the National Occupational Research Agenda (NORA), investigation on occupational diseases caused by exposure to metals, such as cobalt, chromium and tungsten, is a NORA mission and appears on the National Toxicology Program priority list [6].

In epidemiological studies, workers exposed to cobalt in an electrochemical plant producing cobalt and workers exposed to cobalt-containing hard metal compounds exhibited significantly enhanced risk for lung cancer [7–9]. In laboratory studies Co(II) caused direct induction of DNA damage [10,11], DNA-protein crosslinking, and sister-chromatid exchange [1]. Besides the direct induction of DNA damage, Co(II) has been reported to interfere with DNA repair processes [1,11]. Co(II) enhances the frequency of UV induced mutations and sister-chromatid exchanges in V79 Chinese hamster cells [12]. Co(II) compounds have been shown to have a carcinogenic effects in animal studies [1,13]. Cobalt itself causes little or no DNA damage in vitro although Co(II) can bind to DNA [14]. While the mechanisms of cobalt-induced toxicity and carcinogenicity remains to be elucidated, cobalt-mediated free radical reactions have been

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suggested to be involved [10,15]. Co(II) alone does not efficiently generate hydroxyl radicals (•OH) from H_2O_2. However, in the presence of nitrilotriacetic acid as a chelating agent, Hanna et al. [16] have reported the formation of •OH radical from H_2O_2 by Co(II). Although nitrilotriacetic acid may not be a significant ligand for Co(II) in cells exposed to this cation, this study does suggest that the reactivity of Co(II) toward H_2O_2 could be enhanced by proper chelation. In a recent study [15], we have shown that Co(II) can generate •OH and lipid hydroperoxide-derived free radicals from H_2O_2 and model lipid hydroperoxides in the presence of biologically relevant ligands, such as glutathione and anserine. The cobalt-mediated radical generation from these reactions was suggested to be involved in the mechanism of Co(II)-related toxicity and carcinogenicity [11,15].

At present, most of the studies concerning the cobalt-mediated free radical generation have focused on Co(II). There is only limited study of free radical generation mediated by cobalt metal [17]. The goal of the present study is to investigate the possible free radical generation by cobalt metal and elucidate possible mechanisms of generation.

2. Materials and methods

2.1. Materials

Cobalt metal powder (trade name, Xtra Fine Cobalt) was obtained from Newcomer Products (Latrobe, PA). The material is used as the metal binder in the industrial production of WC–Co hard metals. Xtra Fine Cobalt was analyzed at 99.3 purity of Co (O_2, 0.6%; Ni, 0.1%; C, 0.3%; Na, 0.2%; Si, 0.01%; Ca, 0.01%; Mg, 0.01%; Mn, 0.01%; Cu, 0.01%; Fe, 0.01%; Mg, 0.01%). The range of particle size is 0.1–1.5 μm. Cobalt suspension, instead of solution, is used.

Diethylenetriaminepentaacetic acid (DTPA), desferoxamine, 1,10-phenanthroline, 5,5-dimethyl-l-pyrroline *N*-oxide (DMPO), glutathione (GSH), glycylglycylhistidine (Gly–Gly–His) and *β*-ananyl-3-methyl-L-histidine (anserine) sodium formate, H_2O_2, and $FeCl_2$ were purchased from Sigma (St. Louis, MO). Superoxide dismutase (SOD) was purchased from Boehringer Mannheim (Indianapolis, IN). The spin trap, DMPO, was purified by charcoal decolorization and vacuum distillation. DMPO solution thus purified did not contain any ESR detectable impurities. Chelex 100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA). The phosphate buffer (pH 7.4) was treated with chelex 100 to remove transition metal ion contaminants.

All ESR measurements were conducted using a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{pp})^2$, where ΔH_{pp} represents peak-to-peak width. An EPRDAP 2.0 program was used for data acquisitions and analyses.

Reactants were mixed in test tubes in a final volume of 450 μl. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. All experiments were performed at room temperature and under ambient air.

2.2. Oxygen consumption measurements

Oxygen consumption measurements were carried out using Gilson oxygraph. Measurements were made on a system containing 10 mg/ml cobalt particles and 5 μg/ml SOD in pH 7.4 phosphate buffer.

2.3. Scanning electron microscope measurements

For scanning electron microscopy measurements the cobalt particles were scattered on double-side carbon tape, then sputter coated with gold/palladium and imaged on a JEOL 6400 scanning electron microscope.

3. Results

Fig. 1 contains the scanning electron microscope image of cobalt particles. As shown by this figure, the par-

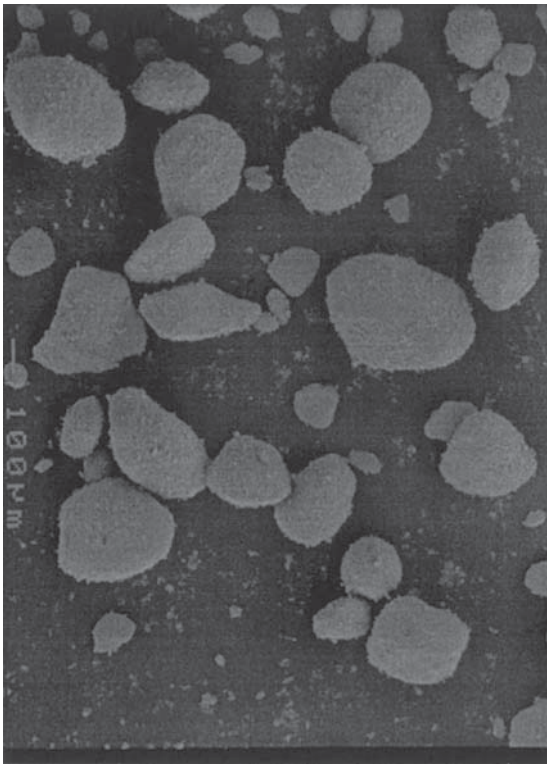
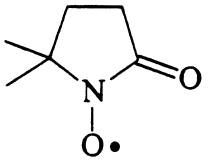


Fig. 1. Scanning electron microscope image showing Xtra Fine Cobalt particles.

ticle sizes are distributed in the range of 0.1–1.5 μm. Fig. 2 shows a typical ESR spectrum obtained from an aqueous solution of 10 mg/ml cobalt particles at pH 7.4. The spectrum is centered at $g = 2.0065$, which indicates oxygen involvement. Computer stimulation shows that the hyperfine splittings are $a_N = 7.1$ G and $a_H = 4.2$, where a_N and a_H denote hyperfine splitting constants of nitroxyl nitrogen and the two α-hydrogens, respectively. According to the lineshape and the hyperfine splittings, the spectrum in Fig. 2 was assigned to 5,5-dimethyl-pyrrolidone-(2)-oxy-(1) (DMPOX), whose structure is shown below [18,19]:



DMPOX

The DMPOX formation reached its saturation level in about 20 min, showing a relative fast reaction.

The formation of DMPOX indicates that Co(I)-OO• is likely to be generated as a radical intermediate. The spectrum in Fig. 2 shows that only DMPOX is detected. It is possible that Co(I)-OO• itself is not converted to H_2O_2, a precursor of •OH radical. SOD was added to a reaction mixture containing Co and DMPO and this enzyme catalyzed the decomposition of Co(I)-OO• to H_2O_2. As shown in Fig. 3(a), a reaction mixture containing Co, DMPO and SOD generated a 1:2:2:1 quartet with splittings of $a_N = a_H = 14.9$ G, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogen and α-hydrogen. Based on these splittings and 1:2:2:1 lineshape [20,22], this spectrum was assigned to DMPO/•OH, thus providing evidence of •OH generation.

The DMPO/•OH signal reached its saturation level in about 10 min. Addition of catalase eliminated the ESR signal (Fig. 3(b)), showing an important role of H_2O_2 in •OH generation. Metal chelators, desferoxamine, DTPA or 1,10-phenanthroline also eliminated the signal (Fig. 3(c)–(d)).

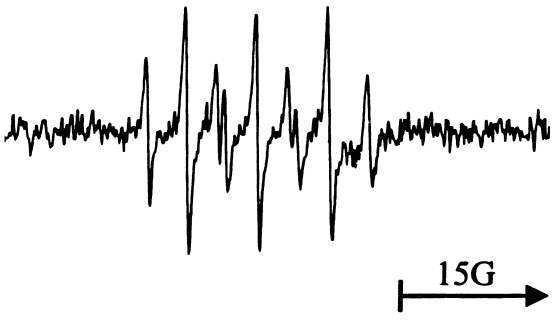


Fig. 2. ESR spectrum, recorded 5 min after reaction initiation, from a pH 7.4 phosphate buffer solution containing 10 mg/ml cobalt particles and 50 mM DMPO. The ESR spectrometer settings were: receiver gain, 2.5×10^5; time constant, 0.3 s; modulation amplitude, 0.25 G; scan time, 8 min; magnetic field, 3470 ± 100 G.

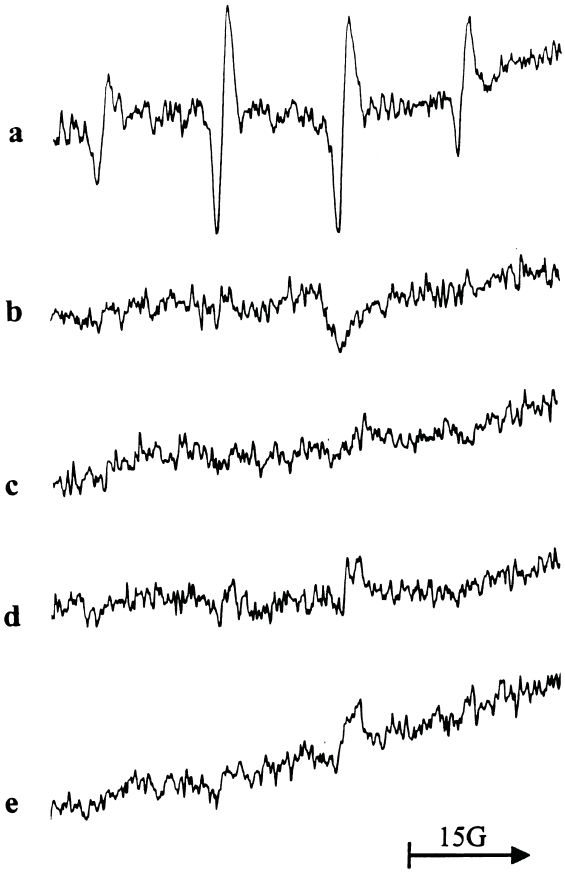


Fig. 3. ESR spectra, recorded 3 min after reaction initiation, from a pH 7.4 phosphate buffer solution of 50 mM DMPO and the following reactants: (a) 10 mg/ml cobalt particles and 5 μg/ml SOD; (b) 10 mg/ml cobalt particles, 5 μg/ml SOD and 2000 U/ml catalase; (c) 10 mg/ml cobalt particles, 5 μg/ml SOD and 0.5 mM desferoxamine; (d) 10 mg/ml cobalt particles, 5 μg/ml SOD and 0.5 mM DTPA; (e) 10 mg/ml cobalt particles, 5 μg/ml SOD and 5 mM 1,10-phenanthroline. The ESR spectrometer settings were: receiver gain, 2.5×10^5; time constant, 0.3 s; modulation amplitude, 0.25 G; scan time, 8 min; magnetic field, 3470 ± 100 G.

The inhibitory effect of catalase on Co-mediated •OH radical generation indicates that H_2O_2 is generated in the mixture containing Co and SOD and plays a key role in •OH generation. If this is the case, addition of Fe(II) to the Co/SOD mixture would result in enhanced generation of •OH radical via the Fenton reaction ($Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^-$). As shown in Fig. 4(a), a mixture of Co, Fe(II) and SOD generated a strong DMPO/•OH signal. Since DMPO/•OH adduct could, in principle, be produced from sources other than •OH trapping, we performed spin trapping competition experiments in which the •OH radical abstracts a hydrogen atom from formate, with the trapping of a new radical. As expected, addition of sodium formate decreased the intensity of the DMPO/•OH signal and resulted in the appearance of a new spin adduct signal with the splitting constants $a_H = 15.7$, 18.7 G (Fig. 4(b)). These splitting constants are typical of those of the DMPO/

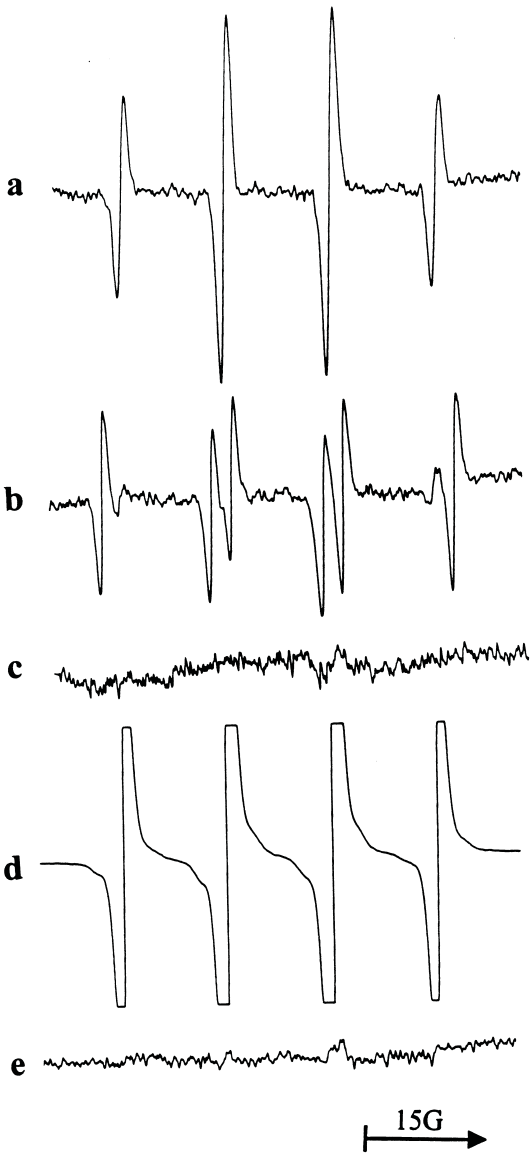


Fig. 4. ESR spectra, recorded 3 min after reaction initiation, from a pH 7.4 phosphate buffer solution of 50 mM DMPO and the following reactants: (a) 10 mg/ml cobalt particles, 5 μg/ml SOD and 0.2 mM Fe(II); (b) 10 mg/ml cobalt particles, 5 μg/ml SOD, 0.2 mM Fe(II), and 25 mM sodium formate; (c) 10 mg/ml cobalt particles, 5 μg/ml SOD, 0.2 mM Fe(II), and 2000 U/ml catalase; (d) 10 mg/ml cobalt particles, 5 μg/ml SOD, 0.2 mM Fe(II), and 2 mM H_2O_2; (e) 10 mg/ml cobalt particles and 0.2 mM Fe(II). The ESR spectrometer settings were the same as those described in the legend to Fig. 3.

$^{\bullet}COO^{-}$ [20–22], providing evidence for the $^{\bullet}OH$ radical generation in the original reaction. Addition of catalase suppressed the radical generation (Fig. 4(c)) and H_2O_2 enhanced it (Fig. 4(d)). The formation of $^{\bullet}OH$ radical generation depended on the presence of SOD. In the absence of SOD, a mixture of Co and Fe(II) did not generate any observable DMPO/$^{\bullet}OH$ signal (Fig. 4(e)).

Oxygen consumption measurement shows that a cobalt suspension rapidly consumed molecular oxygen

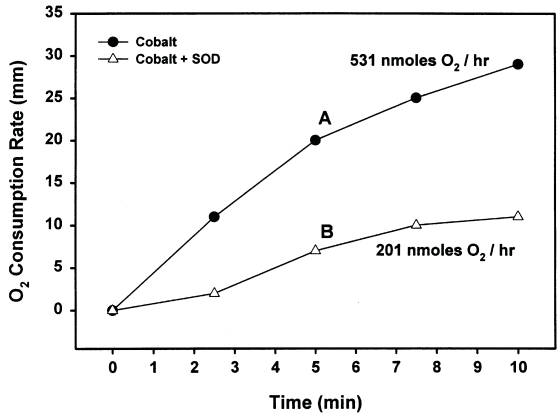


Fig. 5. (a) Oxygen consumption of a pH 7.4 phosphate buffer solution containing 10 mg/ml cobalt particles; (b) same as (a) but with 5 μg/ml SOD added.

(Fig. 5(a)). Addition of SOD decreased the oxygen consumption due to SOD-catalyzed dismutation of $O_2^{\bullet-}$ to generate O_2 ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) (Fig. 5(b)).

Fig. 6 shows the $^{\bullet}OH$ generation from a mixture of Co(II) and H_2O_2 and the effect of several biologically available chelators. As shown in this figure, GSH, Gly–Gly–His or anserine significantly enhanced the $^{\bullet}OH$ radical generation.

A UV absorption spectrum of Co(II) was obtained (data not shown) from a pH 7.4 phosphate buffer solution containing 10 mg/ml cobalt particles and 0.5 mM desferoxamine. The function of desferoxamine is to stabilize the Co(II) generated from the oxidation of Co by molecular oxygen.

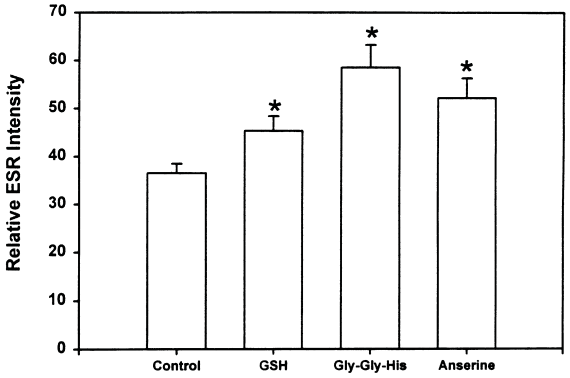


Fig. 6. Effect of biologically available peptides on relative radical concentration generated from a pH 7.4 phosphate buffer solution containing 10 mg/ml cobalt particles, 5 μg/ml SOD and 1.0 mM of GSH, Gly–Gly–His or anserine as indicated. Values are means of ± of three experiments. Asterisks indicate a significant elevation of $^{\bullet}OH$ generation. Tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{pp})^2$, where ΔH_{pp} represents peak-to-peak width.

4. Discussion

The results obtained in the present study show that reaction of metallic Co in aqueous suspension with dissolved oxygen is able to generate a strong oxidant as shown by the formation of DMPOX. According to Rosen and Rauckman [19], the DMPOX signal is indirect evidence for peroxy radical (ROO•) generation and its trapping by DMPO. While further studies are required to elucidate the detailed mechanism, the steps outlined in Fig. 7 may best explain the pathways of DMPOX formation.

When SOD was present, Co in suspension is able to react with dissolved oxygen to generate •OH radical as measured by ESR spin trapping. The generation of •OH radical is likely to involve the reaction of Co(I) with H_2O_2 [$Co(I) + H_2O_2 \rightarrow Co(II) + {}^{\bullet}OH + OH^-$] as shown by the following. (i) Reaction of Co(0) with water did not generate •OH radical, showing that the reaction, $Co(0) + H_2O_2 \rightarrow Co(I) + {}^{\bullet}OH + OH^-$, did not occur at a significant rate. (ii) In the presence of SOD, Co(I)-OO• decomposed to generate Co(I) and H_2O_2, leading to the generation of •OH radical. (iii) Catalase, which scavenges H_2O_2 inhibited the •OH generation.

The results obtained in the present study also show that in the presence of SOD, Co is able to generate H_2O_2. This is important because H_2O_2 can react with various metals to generate •OH radicals via the Fenton or Fenton-like reaction. For example, as shown in the present study, reaction of Fe(II) with Co-generated H_2O_2 produced •OH radicals. In cellular systems, H_2O_2 may also react with Cu(I)/Cu(II) to generate •OH radical.

H_2O_2 is also involved in a variety of pathological processes. It can induce activation of activator protein-1 (AP-1) and nuclear transcription factor κB (NF-κB) [23,24]. Ap-1 and NF-κB regulate various cytokines and oncogenes [24–26]. It is possible that Co-mediated H_2O_2 generation may significantly be involved in the mechanism of Co-induced toxicity and carcinogenicity.

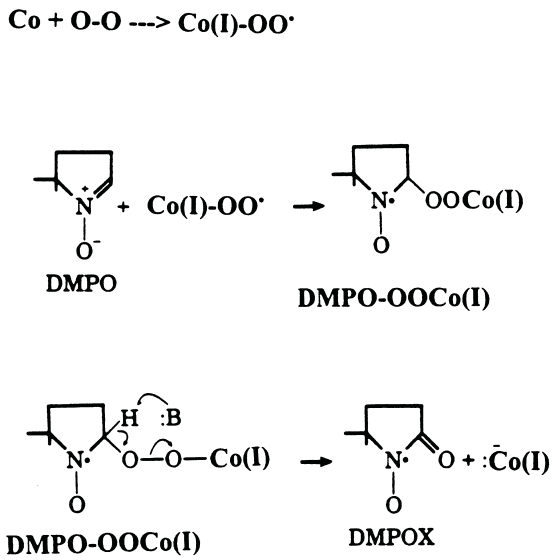


Fig. 7. Schematic representation of possible mechanism of DMPOX generation from cobalt-mediated reactions.

Previous studies have shown that Co(II) itself is unable to generate •OH radical [15,16]. In the presence of proper chelators, the reactivity of Co(II) toward H_2O_2 can be modulated [15,16]. Co(II) is capable of generating •OH radicals in the presence of such chelators. The results obtained in the present study show that GSH, Gly–Gly–His or anserine enhance the Co-mediated •OH generation. It may be noted that GSH and anserine have been classified as endogenous antioxidants [27]. The present study shows that these peptides are able to enhance the generation of •OH radical. In this situation, these peptides may function as pro-oxidants. GSH is the major non-protein thiol in cellular systems, where it may reach concentrations as high as 10 mM [28]. Likewise, anserine has been reported to be present in the range of 1–20 mM in the skeletal muscles of many vertebrates [29]. Thus GSH and anserine as well as other biologically available peptides may be the likely cellular chelators for Co(II) in its reaction toward H_2O_2 to generate •OH radicals. Hence, in the presence of Co(II), the function of cellular antioxidants, such as GSH and anserine, against reactive oxygen species may be significantly attenuated. They may, in fact, be diverted or converted into agents that actually accelerate oxidant formation. This change from antioxidants to pro-oxidants may which result in cellular damage mediated by Co and other transition metals such as nickel.

In contrast to GSH, Gly–Gly–His and anserine, several typical metal chelators, such as desferoxamine, DTPA or 1,10-phenanthroline inhibited Co-mediated •OH generation from H_2O_2. It may be noted that chelation has been used to prevent cellular damage induced by various metals. For example, 1,10-phenanthroline is a membrane-permeable chelating agent, which has been reported to inhibit Cr(V)- and Cr(IV)-mediated free radical generation and H_2O_2-induced DNA damage [30,31], mutation and transformation. Desferoxamine is widely used for the prevention and treatment of iron overload [32,33] as well as for combating the toxic effect of vanadium [32,34]. It appears that proper chelation may prevent or attenuate Co-mediated cellular damage.

In conclusion, in aqueous suspension, Co is able to reduce molecular oxygen to generate Co(I)-bound O_2^- radical adduct, which exhibits a strong oxidizing property. In the presence of SOD, this adduct generates H_2O_2 which reacts with Co(I) to produce •OH radical via Co(I)-mediated Fenton-like reaction [$Co(I) + H_2O_2 \rightarrow Co(II) + {}^{\bullet}OH + OH^-$]. Proper chelation of Co(II) generated by molecular oxygen oxidation of Co metal using biological chelators, such as GSH, Gly–Gly–His and anserine, alters the oxidation–reduction potential of this metal and makes it capable of generating •OH via a Co(II)-mediated Fenton-like reaction [$Co(II) + H_2O_2 \rightarrow Co(III) + {}^{\bullet}OH + OH^-$]. Thus, the reaction of Co with water, especially in the presence of biological chelators, such as GSH, Gly–Gly–His and anserine, is capa-

ble of generating a whole spectrum of reactive oxygen species. These species can cause cellular injury through various mechanisms, such as DNA damage, protein modification, induction of oncogene expression, and nuclear transcription factor activation. Thus, Co-mediated free radical reactions may play an important role in the mechanisms of the occupational diseases caused by these metals.

References

[1] D. Beyersmann, A. Hartwig, Toxicol. Appl. Pharmacol. 115 (1992) 137.
[2] J.C. Health, Nature 173 (1954) 822.
[3] A.A. Shabaan, V. Marks, M.C. Lancaster, G.N. Dufeu, Lab. Anim. 11 (1977) 43.
[4] G. Kazantzis, Environ. Health Perspect. 40 (1981) 143.
[5] International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 52, International Agency for Research on Cancer, Lyon, 1991, p. 363.
[6] National Occupational Research agenda, US Department of Health and Human Services, DHHS (NIOSH) Publication No. 96-115, 1996.
[7] G. Lasfargues, P. Wild, J.J. Moulin, B. Hammon, B. Rosmorduc, B. Rondeau du Noyer, M. Lavandier, J. Moline, Am. J. Ind. Med. 26 (1994) 585.
[8] C. Hogstedt, R. Alexanderson, Scand. J. Work Environ. Health 13 (1987) 177.
[9] J.M. Mur, J.J. Moulin, M.P. Charruyer-Seinarra, J. Lafitte, Am. J. Ind. Med. 11 (1987) 75.
[10] K.S. Yamamoto, S. Inoue, A. Yamazaki, T. Yoshinaga, S. Kawanishi, Chem. Res. Toxicol. 2 (1989) 234.
[11] Y. Mao, K.J. Liu, X.J. Shi, Toxicol. Environ. Health 47 (1996) 233.
[12] A. Hartwig, R.S. Snyder, R. Schlepegrell, D. Beyersmann, Mutation Res. 248 (1991) 177.
[13] D. Lison, Critical Rev. Toxicol. 26 (1986) 585.
[14] G.L. Eichhorn, Y.A. Shin, J. Am. Chem. Soc. 90 (1968) 7323.
[15] X. Shi, N.S. Dalal, K.S. Kasprzak, Chem. Res. Toxicol. 6 (1993) 277.
[16] P.M. Hanna, M.B. Kadiiska, R.P. Mason, Chem. Res. Toxicol. 5 (1992) 109.
[17] D. Lison, P. Carbonnelle, L. Mollo, R. Lauwerys, B. Fubini, Chem. Res. Toxicol. 8 (1995) 600.
[18] R.A. Floyd, L.M. Soong, Biochem. Biophys. Res. Commun. 74 (1977) 79.
[19] G.M. Rosen, E.J. Rauckman, Mol. Pharmacol. 17 (1980) 233.
[20] G.R. Buettner, Free Radical Biol. Med. 3 (1987) 259.
[21] X. Shi, N.S. Dalal, Arch. Biochem. Biophys. 277 (1990) 342.
[22] X. Shi, N.S. Dalal, Biochem. Biophys. Res. Commun. 163 (1990) 627.
[23] M. Meyer, H.L. Pahl, P.A. Baeuerle, Chem. Biol. Interactions 91 (1994) 91.
[24] Y. Sun, L.W. Oberley, Free Radical Biol. Med. 21 (1996) 335.
[25] P.A. Baeuerle, T. Henkel, Annu. Rev. Immunol. 12 (1994) 141.
[26] D. Bohmann, T.J. Bos, A. Admon, T. Nishimura, P.K. Vogt, R. Tjian, Science 238 (1987) 1386.
[27] R. Kohen, Y. Yamamoto, K.C. Cundy, B.N. Ames, Proc. Natl. Acad. Sci. USA 85 (1988) 3175.
[28] D.J. Reed, in: M.W. Anders (Ed.), Bioactivation of Foreign Compounds, Academic Press, Orlando, FL, 1985, p. 71.
[29] C.R. Scriver, T.L. Perry, W. Nutzenadel, in: J.B. Stanbury, J.B. Wyngaarden, J.L. Goldstein, M.S. Brown (Eds.), The Metabolic Basis of Inherited Disease, 5th ed., McGraw-Hill, New York, 1983, p. 570.
[30] Y. Mao, L. Zang, X. Shi, Biochem. Mol. Biol. Intl. 36 (1995) 327.
[31] M. Sugiyama, K. Tsuzuki, N. Haramaki, Arch. Biochem. Biophys. 305 (1993) 261.
[32] B. Halliwell, Free Radical Biol. Med. 7 (1989) 645.
[33] G.D. McLaren, W.A. Muri, R.W. Kellermeyer, CRC Crit. Rev. Clin. Lab. Sci. 19 (1983) 205.
[34] R.J. Keller, J.D. Rush, T.A. Grover, J. Inorg. Biochem. 41 (1991) 269.