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HYDROXYL RADICAL GENERATION IN THE NADH/MICROSOMAL REDUCTION OF VANADATE†

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ESR spin trapping measurements demonstrate generation of hydroxyl (·OH) radical from reduction of vanadate by rat liver microsomes/NADH without exogenous H_2O_2. Catalase decreases the ·OH signal while increasing a vanadium(4+) signal. Addition of superoxide dismutase (SOD) or measurements under an argon atmosphere show decreased ·OH radical production. The results suggest that during the one-electron vanadate reduction process by microsomes/NADH, molecular oxygen is reduced to H_2O_2, which then reacts with vanadium(4+) to generate ·OH radical via a Fenton-like mechanism.

KEY WORDS: ȮH radical, vanadate reduction, O_2-reduction, Fenton-like mechanism.

INTRODUCTION

Vanadate (V(5+)) and related compounds are known to exert potent toxic effects on a wide variety of biological systems.[1–4] One of the pathways of V(5+) toxicity is thought to be mediated by oxygen-derived free radicals.[5–9] Keller and coworkers[5] studied V(4+)-induced lipid peroxidation and suggested that V(4+)-induced hydroxtl (·OH) radical formation may play an important role in V(5+) toxicity. Carmichael[6,7] investigated the reaction between a RNA/VO^{2+} complex and H_2O_2 with a view to probe the possible relationship between V(4+)-mediated ·OH radical generation and DNA damage. He suggested that in this reaction the ·OH radical adds to the C[5] carbon of uracil, causing nucleotide damage, similar to what occurs in the nucleotide/Fe^{2+}/H_2O_2 reaction and in γ-irradiated aqueous solutions of nucleic acids. Younes and coworkers[8] have shown that trolox C, a water soluble vitamin E analogue, inhibits vanadate-induced lipid peroxidation and protects against vanadate-induced mitochondrial damage via oxidative stress. Liochev and Fridovich[9] reported that, in the presence of H_2O_2, V(4+) causes NADH oxidation which was inhibitable by ethanol, suggesting the role of ·OH radical in the mechanism of enhanced NADH oxidation by V(4+) plus H_2O_2.

While it has been demonstrated that reaction of V(4+) with exogenous H_2O_2 generates ·OH radical,[6,7,9–11] the data concerning the generation of ·OH by V(5+)

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itself are scanty, especially in cellular systems without exogenous H_2O_2. A recent study[12] reported that reduction of Cr(6+) by microsomes in the presence of NADH yields Cr(5+), which in turn reacts with H_2O_2 to generate ·OH radical. It has also been reported that the NAD(P)H/microsomal system is endowed with vanadate reductase property and can cause one-electron reduction of vanadate to generate V(4+).[13] Since chromate is isoelectronic with vanadate, we investigated the possibility of ·OH generation in the course of NADH/microsomal reduction of V(5+).

MATERIALS AND METHODS

All chemicals were reagent grade as described elsewhere.[11–14] Bovine blood superoxide dismutase (SOD) was purchased from Sigma. Bovine liver catalase was purchased from Boehringer-Mannheim. The spin trapping ESR measurements were made using a Varian E-3 spectrometer and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap, essentially as reported earlier.[11–14]

Microsomes were prepared from livers of Sprague-Dawley rats. Microsome pellets were isolated from the livers by the differential centrifugation method.[15] Washed microsomes were suspended in a phosphate buffer (pH 7.2) solution, stored at 4°C, and used within 2 days. The microsomal protein concentration was measured colorimetrically by the method of Lowry *et al.*[16] Inactivated microsomes were obtained by keeping the microsome-containing glass tube in boiling water for about 5 minutes.

The concentrations given in the figure legends were the final concentrations.

RESULTS AND DISCUSSION

Figure 1(a) shows a typical spin adduct ESR spectrum obtained from a mixture of 4.8 mM $NaVO_3$, 4 mM NADH, 3.8 mg/ml microsomes, and 60 mM DMPO in a pH 7.2 phosphate buffer. The hyperfine splittings of this spectrum are $a_N = a_H = 14.9$ G, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogen and α-hydrogen, respectively. Based on these splitting constants,[17] this spectrum was assigned to a DMPO/OH adduct.

Spin trapping competition experiments were carried out to ascertain that the 1:2:2:1 quartet signal in Figure 1a was indeed due to the trapping of ·OH radicals. As shown in Figure 1b, addition of ethanol decreases the intensity of this signal and results in the appearance of a new spin adduct signal (as indicated by asterisks) with the splitting constants $a_N = 15.8$ and $a_H = 22.8$ G. These splittings are typical of those of the DMPO/·$CHOHCH_3$ adduct,[17] indicating the trapping (and, hence, generation) of ·OH radicals. Similar results were obtained using sodium formate in place of ethanol (Figure 1c). Together, these results demonstrate that the detection of DMPO/·OH spin adduct (Figure 1a) is the result of the trapping of ·OH radical generated in the reaction.

It is known that the DMPO/·OH spin adduct signal exhibits a 1:2:2:1 quartet.[17] However, as shown in Figure 1(a), the fourth (highest field) line of the DMPO-OH spectrum is substantially weaker than the first one. We conjectured that the decrease in spectral intensity during the measurement may be the result of the decomposition

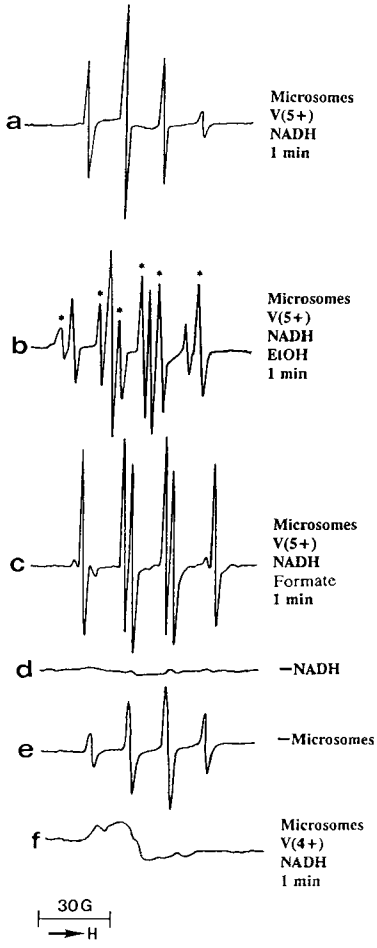

FIGURE 1 (a) ESR spectrum recorded at 1 minute after mixing, in 60 mM phosphate buffer (pH 7.2), 60 mM DMPO, 4.8 mM $NaVO_3$, 4 mM NADH, and 3.4 mg/ml microsomes; (b) same as (a) but with 0.3 M ethanol added; (c) same as (a) 0.8 M sodium formate; (d) same as (a) but without NADH; (e) same as (a) but without microsomes; (f) same as (a) but with $VOSO_4$ in place of $NaVO_3$. The spectrometer settings were: receiver gain, 1.25×10^5; modulation amplitude (100 kHz), 1.0 G; time constant, 0.5 second; scan time, 8 minutes; scan width, 3470 ± 100 G. The asterisks indicate the DMPO/·$CHOHCH_3$ spin adduct peaks.

of the DMPO/·OH adduct through its metabolism by the NADH/microsomal system, as is known to be the case for other cellular systems.[18,19] As shown in Figure 2a the intensity of the DMPO/·OH spin adduct signal indeed decreased as a function of time and became nondetectable within five minutes. Addition of $K_3Fe(CN)_6$, which impairs the microsomal electron transfer system,[20] enhanced the DMPO/·OH signal (Figure 2b). Addition of β-diethylaminoethyl diphenylpropyl acetate (SKF 525A), which is known to inhibit the mixed function oxidase of the microsomal system,[21] was more effective in enhancing the DMPO/·OH signal (Figure 2c). Additional support for such DMPO/·OH metabolism was obtained using the stable radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) as a model nitroxide

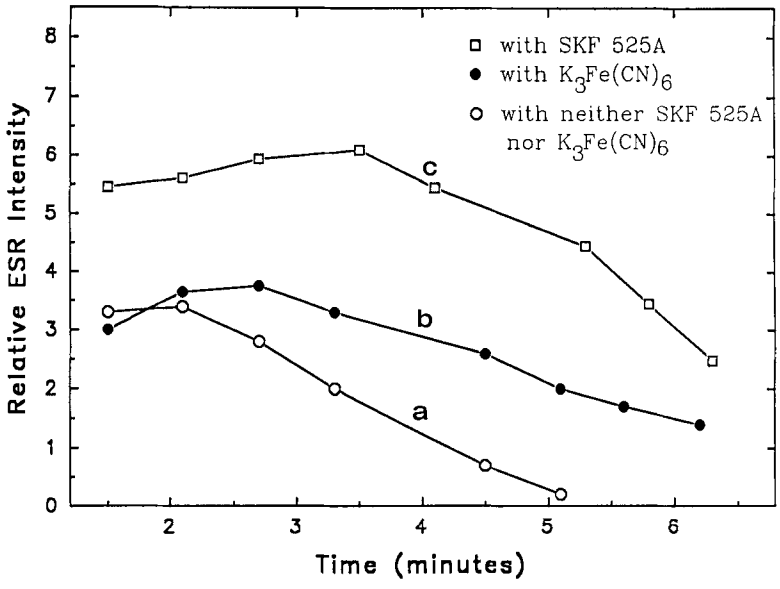

FIGURE 2 Time course of DMPO-OH generation: (a) reaction mixture containing 60 mM DMPO, 4.8 mM $NaVO_3$, 4 mM NADH and 3.48 mg/ml microsomes in 60 mM phosphate buffer (pH 7.2); (b) same as (a) but with 1 mM $K_3Fe(CN)_6$ added; (c) same as (a) but with 2 mM SKF 525A added.

compound stimulating DMPO/·OH. In a phosphate buffer solution (pH 7.2), TEMPO gave a stable 1:1:1 triplet signal (Figure 3a). Addition of NADH/microsomes decreased its intensity to approximately 20% within 3 minutes (Figure 3b), indicating that the NADH/microsomal system does indeed metabolize the nitroxyl radical.

As controls, it can be noted from Figure 1d that omission of NADH suppressed the spin adduct signal. Additionally, omission of microsomes reduced the intensity of the spin adduct signal (Figure 1e), showing that reaction of NADH and V(5+) alone does generate a minor amount of ·OH radicals. This observation corroborates an earlier indirect evidence leading to the same conclusion.[22] Note, however, that the amount of ·OH radicals generated in the NADH/microsomal reduction of V(5+) (Figure 1a) is much higher than from reaction of NADH with V(5+) (Figure 1e). The amount of the ·OH radicals generated by NADH and vanadate alone (Figure 1e) was nearly the same as that obtained using microsomes inactivated by keeping the microsomes-containing glass tube in boiling water for about 5 minutes (data not shown). An important result was that replacement of V(5+) by V(4+) did not generate any ESR detectable amount of ·OH radicals (Figure 1f), indicating that the one-electron reduction of V(5+) is essential in the mechanism of V(5+) mediated ·OH generation. The broad peak in Figure 1e is the fourth ($m_I = -1/2$) component of the eight V(4+) hyperfine lines.[23]

As shown in Figure 4a–c, the DMPO/·OH spin adduct signal disappeared in approximately 5 minutes after reaction initiation. As may also be noted from Figure 4a–c, the disappearance of the DMPO/·OH signal takes place simultaneously with the appearance of the broad V(4+) peak. The full V(4+) spectrum is shown in

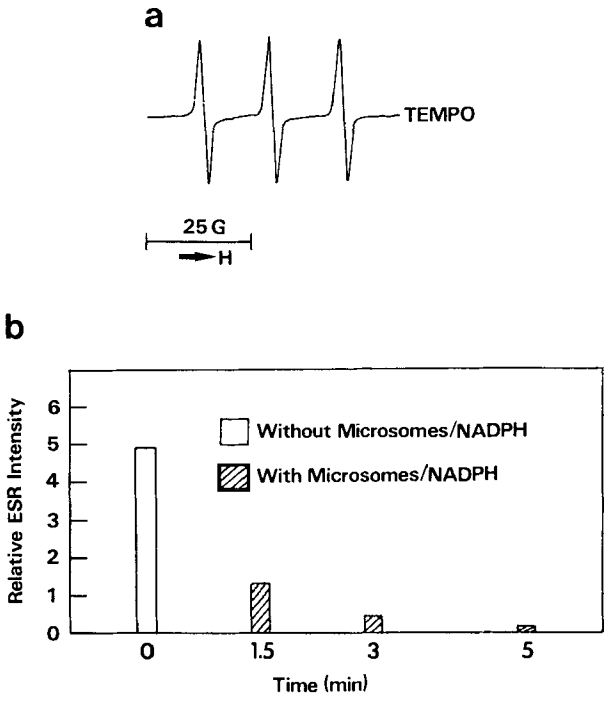

FIGURE 3 (a) ESR spectrum recorded from phosphate buffer (pH 7.2, 60 mM) containing 2 μM TEMPO; (b) time course of relative ESR intensity measured from phosphate buffer (pH 7.2, 60 mM) containing 2 μM TEMPO, 3.4 mg/ml microsomes and 4 mM NADPH. The spectrometer settings were the same as those in Figure 1. No significant time dependence was detected in the absence of microsomes/NADH.

Figure 5a. Its similarity to that obtained using $VOSO_4$ (V(4+)) (Figure 5b) supported the assignment of the spectrum in Figure 5a to a VO^{2+}-type of species.

The following measurements yield clues to the mechanism of ·OH generation. Addition of catalase to a reaction mixture containing NADH, microsomes, V(5+), and DMPO diminished ·OH generation, but increased V(4+) formation (Figure 4d–f), indicating that the reaction of V(4+) with H_2O_2 is involved in the mechanism of ·OH radical generation. SOD also inhibited ·OH formation (Figure 4g–i), indicating that O_2^- is an important intermediate in the mechanism of ·OH generation. Measurements under an argon atmosphere yielded much weaker DMPO/·OH signals (Figure 4j–l), showing that molecular oxygen (O_2) is involved in the ·OH generation mechanism. Together, these data indicate that during the microsomal/NADH reduction of V(5+), O_2 is reduced to generate H_2O_2, probably via O_2^- as an intermediate. This H_2O_2 reacts with V(4+) to generate ·OH radical via a Fenton-type mechanism.

An important observation was that SOD did not significantly affect V(4+) generation. This observation indicates that O_2 is not directly involved in the mechanism of vanadate reduction.

As mentioned in the Introduction, the ·OH radical has been suggested to be involved in the mechanism of V(5+) related cellular damage.[5–9] In this context, we

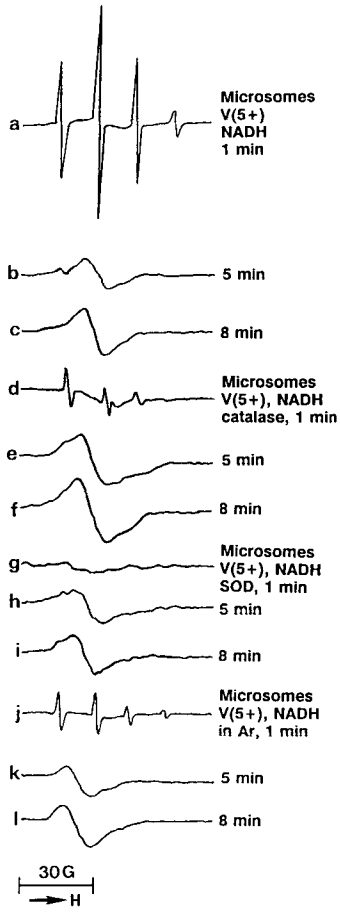

FIGURE 4 (a) ESR spectrum recorded at 1 minute after mixing, in 60 mM phosphate buffer (pH 7.2), 60 mM DMPO, 4.8 mM $NaVO_3$, 4 mM NADH, and 3.4 mg/ml microsomes; (b) and (c) same as (a) but the spectra were recorded 5 and 8 minutes after reaction initiation; (d) same as (a) but with 2000 units/ml catalase added; (e) and (f) same as (d) but the spectra were recorded 5 and 8 minutes after reaction initiation; (g) same as (a) but with 100 units/ml SOD added; (h) and (i) same as (g) but the spectra were recorded 5 and 8 minutes after reaction initiation; (j) same as (a) but the reaction was carried out under an argon atmosphere; (k) and (l) same as (j) but the spectra were recorded 5 and 8 minutes after reaction initiation. The experimental parameters are the same as those in the legend to Figure 1.

note that many transition metal ions are strongly implicated in the pathobiology of oxygen free radicals.[24–28] It has been suggested that much of the ·OH radical generation *in vivo* originates from the Fe(2+)-dependent breakdown of cellular H_2O_2 according to the following general equations:[24–28]

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \cdot OH + OH^- \quad [a]$$

$$M^{(n+1)+} + \text{reductant} \rightarrow M^{n+} + \text{oxidized product} \quad [b]$$

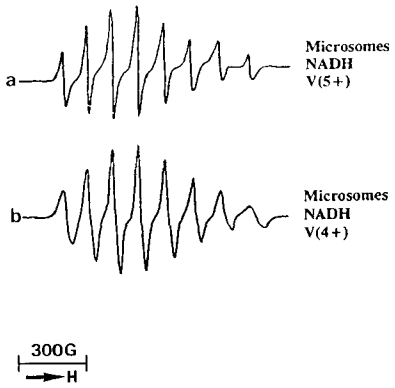

FIGURE 5 (a) ESR spectrum recorded at 1 minute after mixing, in 60 mM phosphate buffer (pH 7.2), 4.8 mM $NaVO_3$, 4 mM NADH, and 3.4 mg/ml microsomes; (b) same as (a) but using $VOSO_4$ (2 mM) instead of $NaVO_3$. The spectrometer settings were: receiver gain, 1.25×10^5; modulation amplitude (100 kHz), 4.0 G; time constant, 1 second; scan time, 8 minutes; scan width, 3470 ± 1000 G.

The present investigation indicates that V(5+) follows the above equations and that such ·OH radical generation may be involved in the mechanism of V(5+) induced cellular injury.

In conclusion, this work demonstrates that one-electron reduction of V(5+) by NADH/microsomes generates ·OH radical without any exogenous H_2O_2. The proposed mechanism involves the reaction of V(4+) with H_2O_2, the latter being generated in the microsomal reduction of molecular oxygen. The microsomal electron transfer system appears to play a key role in the mechanism of vanadate reduction and, hence, ·OH radical generation.

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