Reaction of vanadium(V) with thiols generates vanadium(IV) and thiyl radicals

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The in vivo toxicity of vanadium(V) has been found to correlate with the depletion of cellular glutathione and related non-protein thiols. With a view to understanding the mechanism for this observation, we have investigated the oxidation of glutathione, cysteine *N*-acetylcysteine and penicillamine by vanadium(V), using electron spin resonance (ESR) and ESR spin trapping methodology. The spin trap used was 5,5-dimethyl-1-pyrroline 1-oxide (DMPO). It is found that the oxidation of these thiols by vanadium(V) generates the corresponding thiyl radicals and vanadium- (IV) complexes. The results suggest that free radical reactions play a significant role in the depletion of cellular thiols by vanadium(V) and hence in vanadium(V) toxicity.

Vanadium(V); Non-protein thiol; ESR; Spin trapping; Thiyl radical; Vanadium toxicity

1. INTRODUCTION

This communication reports on the identification of thiyl radicals formed as intermediates in the reaction of vanadium(V) with glutathione (GSH) and related non-protein thiols. This study was undertaken because the biochemical mechanism of vanadium toxicity is still not fully understood, despite being a topic of current research activity [1-20]. As to possible clues, it is known that vanadium is most toxic when administered in its pentavalent state [21], and that vanadium(V) administration into laboratory mice reduces cellular levels of NAD(P)H and GSH [22]. While the oxidation of NAD(P)H by vanadium has been studied extensively [16-20], that of glutathione and related non-protein thiols has been paid less attention. On the other hand, GSH and related thiols are important cellular antioxidants [23]. Their functions include decomposing lipid peroxides and protecting cell membranes [23-25]. Thus any reduction in the cellular contents of such non-protein thiols after vanadium exposure may result in the accumulation of lipid peroxides which might ultimately lead to tissue injury. With a view of finding the mechanism for this thiol depletion process, we have investigated reactions of non-protein thiols with vanadium(V). Particular attention has been paid to the detection of thiyl radicals, since it has been postulated [1] (but not experimentally proven) that the glutathionyl radical ($GS^{\cdot}$) might be an intermediate in the reaction between vanadium(V), NADH and GSH. We have used electron spin resonance (ESR) and ESR spin trapping methodology for investigating the reactions of GSH, cysteine, *N*-acetylcysteine and penicillamine with vanadium(V). The results show that the corresponding thiyl radicals are indeed formed in the one-electron reduction of vanadium(V) by all of the above mentioned thiols.

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2. MATERIALS AND METHODS

Sodium metavanadate ($NaVO_3$), used as vanadium(V) source, was purchased from Aldrich and its solution was always freshly prepared. GSH, cysteine, *N*-acetylcysteine and penicillamine were purchased from Sigma. Spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich and was purified by charcoal decolorization. The phosphate buffer solutions of pH 7.2 were purchased from Fisher and those of pH = 3.0-6.0 were purchased from Aldrich.

ESR spectra were obtained at X-band (9.7 GHz) using a Bruker ER200 ESR spectrometer. The magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, Model ER035A) and the microwave frequency was measured with a digital frequency counter (Hewlett-Packard, Model 5340A). An ASPECT 2000 computer was used for data storage and analysis. The concentrations given in the figure legends are the final concentrations. All experiments were carried out in the buffer solution as indicated and at room temperature.

3. RESULTS

An aqueous solution of 0.1 M spin trap DMPO at pH 7.2, either with vanadium(V) alone or GSH alone, did not give a detectable ESR spectrum. When vanadium(V), GSH and DMPO were mixed at pH = 7.2, an ESR spectrum was observed which was a composite of a spin adduct signal, (the 1:2:2:1 quartet) and a broad (vanadium(IV)) peak as indicated by the arrow in Fig.1a). A computer simulation analysis of the quartet yielded the nitrogen hyperfine coupling $a_N = 15.4$ G and the proton hyperfine coupling $a_H = 16.2$ G, which com-

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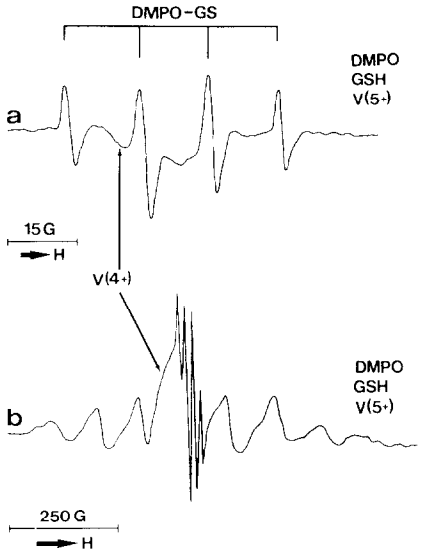

Fig. 1. (a) An ESR spectrum recorded 2 min after reaction initiation in a reaction mixture containing 12.5 mM GSH, 3.3 mM sodium metavanadate and 0.1 M DMPO in a pH = 7.2 phoshate buffer solution. The arrow indicates one of the 8 lines of vanadium(IV) ESR spectrum. Spectrometer settings were: receiver gain, 5.0×10^5; modulation amplitude, 1.25 G; scan time, 200 s; field, 3480 ± 75 G; time constant, 0.5 s. (b) Same as (a) but spectrum was recorded at modulation amplitude 6.3 G; field, 3480 ± 750 G.

pare very well with those of the DMPO-GS formed in other GSH-metal redox systems reported earlier [26–30]. The DMPO-GS spin adduct spectrum showed a rapid decrease with time (half-life ~ 10 min) essentially as described previously [26–30]. The possibility of the 1:2:2:1 quartet arising from the ·OH radical (rather than GS·) was ruled out by standard tests using radical scavengers. For example, reaction of ·OH with ethanol generates the ·$CHOHCH_3$ radical, which can be trapped by DMPO to give a well characterized DMPO-$CHOHCH_3$ spin adduct spectrum [31–34]. The addition of ethanol to the above mentioned system did not yield any detectable DMPO-$CHOHCH_3$ adduct signal (data not shown), indicating that ·OH radicals were not generated. When the reaction was carried out at pH = 3.0–6.0 the same DMPO-GS spin adduct spectrum with stronger intensity was obtained (data not shown) showing that acidity has a significant effect on the GS· radical formation. When the spectrum was recorded with a higher modulation amplitude and a wider scan width, an 8 line spectrum (Fig. 1b) was obtained, which is characteristic of vanadium(IV) (V^{51}, I = 7/2) [35]. The four sharp lines in the middle are due to DMPO-GS. Thus the broad peak indicated by the arrow in Fig. 1a is the fourth ($m_I = -1/2$) of the eight hyperfine lines of vanadium(IV) (I = 7/2) ESR spectrum. This result shows that a long-lived vanadium(IV) species is generated in the reduction of vanadium(V) by GSH.

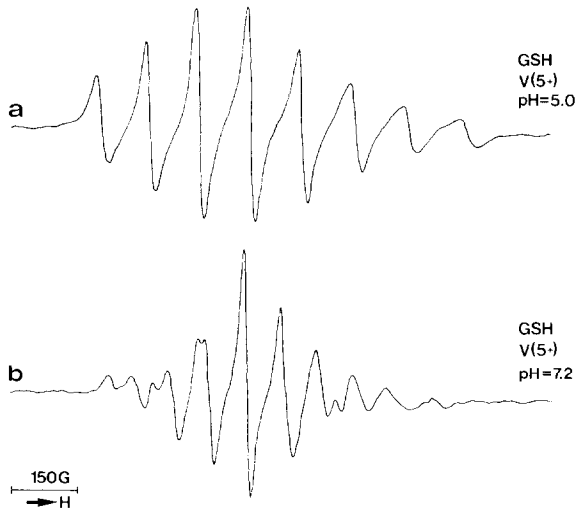

Fig. 2. (a) An ESR spectrum recorded in a reaction mixture containing 50 mM GSH and 10 mM sodium metavanadate at pH 5.0 buffer solution. The spectrometer settings were: receiver gain, 2.5×10^5; modulation amplitude, 1.6 G; scan time, 200 s; time constant, 0.5 s; field, 3480 ± 750 G. (b) Same as (a) but reaction was carried out at pH 7.2 in phosphate buffer solution.

Fig. 2a shows the spectrum recorded in the reaction of higher concentrations of GSH and vanadium(V) at pH 5.0 with smaller modulation. This is typical 8 line vanadium(IV) spectrum as mentioned earlier. When the same reaction was carried out at pH = 7.2, the spectrum (Fig. 2b) obtained was different in hyperfine splitting from that in Fig. 2a, indicating that the acidity of the reaction medium has significant effect on the structure of vanadium(IV) complexes generated, as shown earlier by Goda and coworkers [36].

To further examine the generality of the thiyl radical formation in the oxidation of biologically relevant thiols by vanadium(V), we have investigated the reac-

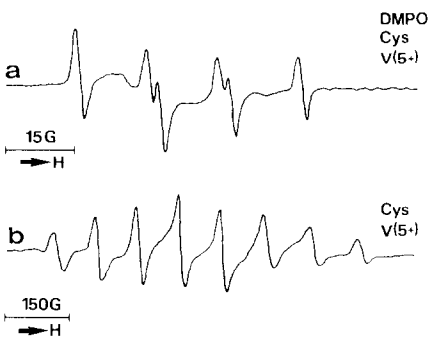

Fig. 3. (a) An ESR spectrum recorded 2 min after reaction initiation in a reaction mixture containing 25 mM cysteine, 2.5 mM sodium metavanadate and 0.1 M DMPO in a pH = 7.2 phosphate buffer solution. Spectrometer settings were: receiver gain, 5.0×10^5; modulation amplitude, 1.6 G; scan time, 200 s; field, 3480 ± 75 G; time constant, 0.5 s. (b) Same as (a) but no DMPO added and the spectrum was recorded at modulation amplitude, 5 G; field, 3480 ± 750 G.

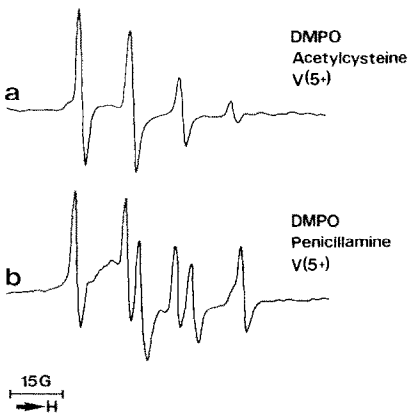

Fig. 4 ESR spectra recorded 2 min after reaction initiation in a reaction mixture of pH = 3.0 buffer solution containing 10 mM sodium metavanadate, 0.1 M DMPO and (a) 50 mM *N*-acetylcysteine; (b) 50 mM penicillamine. Spectrometer setting were the same as those in Fig. 1a.

tion of vanadium(V) with cysteine, *N*-acetylcysteine and penicillamine. Fig. 3a shows the ESR spectrum obtained in the reaction of cysteine with vanadium(V) containing DMPO at pH = 7.2. A computer simulation analysis of this spectrum yielded hyperfine splittings of $a_N = 15.1$ G and $a_H = 17.4$ G, which are essentially the same as those for the DMPO-cysteine adduct detected in the enzymatic oxidation of cysteine [37–41]. The intensity of DMPO-cysteine spin adduct signal increases with the decrease of pH in the reaction medium. The vanadium(IV) spectrum (Fig. 3b) was also obtained in the reaction of cysteine with vanadium(V) at pH 7.2. Unlike the above mentioned case for GSH, for cysteine the acidity (pH = 5.0–7.2) of the reaction medium does not have a significant effect on the hyperfine splitting of vanadium(IV) spectrum (data not shown). At pH = 7.2, we did not observe any spin adduct spectrum in the reaction of vanadium(IV) with *N*-acetylcysteine or penicillamine. However at lower pH we did observe ESR spectra of DMPO adducts in the reaction of vanadium(V) with *N*-acetylcysteine and penicillamine. The spectra obtained at pH 3.0 are shown in Fig. 4. The intensities of these spin adducts decrease with increase in pH. The hyperfine splittings are $a_N = 15.3$ G and $a_H = 16.8$ G for *N*-acetylcysteine (spectrum in Fig. 4a) and $a_N = 15.3$ G and $a_H = 20.0$ G for penicillamine (Fig. 4b). The spectra in Fig. 4a,b were assigned to the adducts of the *N*-acetylcysteine radical and penicillamine radical with DMPO because of their close similarity to the same adducts generated in the enzymatic oxidation of *N*-acetylcysteine [37–41] and penicillamine [37–41] respectively.

4. DISCUSSION

The above results show that the oxidation of several biologically related thiols by vanadium(V) generates the corresponding thiyl radicals. This observation points to a pathway for the depletion of the cellular thiol pool after vanadium exposure [22]. The GSH-related thiols are known to function directly or indirectly in many important biological reactions, including the protection of cell membranes against oxidative damage [23]. The latter involves the reduction of cellular H_2O_2 level via GSH peroxidase [23]. This is an important detoxification pathway, and is of relevance here because of current reports [1,6,42] that in the reaction of vanadium(IV) with H_2O_2 the ·OH radical is generated (through a Fenton-like mechanism). The ·OH radical generation might be playing an important role in the vanadium toxicity [1,6,42]. Thus the depletion of cellular thiol pool by vanadium(V) might lead to reduced protection against H_2O_2, lipid peroxides and other toxic species. As a consequence, these toxic species themselves or the additional species generated by their subsequent reactions, such as the ·OH radical generated by vanadium(IV)-H_2O_2 reaction, could accumulate within the cellular system, rendering it vulnerable to oxidative damage. Moreover, it has been reported very recently that thiyl radicals have the ability to directly peroxididize lipids [43]. Thus the detection of the thiyl radicals here helps explain the recent experimental observation of vanadium(V) induced lipid peroxidation in human erythrocytes [44] and in animals treated acutely or chronically with vanadium(V) [45].

Recently, it has been reported that thiols increase the vanadium(V) induced oxidation of NADH in vitro [1]. In this reaction the formation of thiyl radicals was postulated but not proven experimentally [1]. Our successful detection here of the thiyl radicals helps understand the mechanism of vanadium(V) induced NADH oxidation in the presence of thiols. The thiyl radicals were suggested to oxidize NADH to generate NAD· radicals [1,38,46]. The NAD· radicals could participate in the chain mechanism of vanadium(V) induced oxidation. We have tested the effect of NADH on the formation of GS· in the oxidation of GSH by vanadium(V). It was found (unpublished results) that, upon addition of NADH (0.1 M) in the reaction of vanadium(V) with GSH, the intensity of the DMPO-GS spin adduct signal decreases by 40%. This result implies that NADH does have an ability to react with GS· radical confirming earlier hypotheses [1,38,46]. In addition thiyl radicals might react with another thiol molecule to generate the superoxide ($O_2^{\cdot-}$) radical as follows [1,46]:

$$RS^{\cdot} + RSH \rightarrow RSSR^{\cdot-} + H^{+} \quad (a)$$
$$RSSR^{\cdot-} + O_2 \rightarrow RSSR + O_2^{\cdot-} \quad (b)$$

The generation of the $O_2^{\cdot-}$ radical might lead to the formation of H_2O_2 and other reactive oxygenated species [1,47], which could eventually react with NADH and other biological species to interrupt normal cellular functions.

In conclusion this work demonstrates for the first time, the formation of thiyl radicals in the oxidation of biologically important thiols by vanadium(V). The detection of the thiyl radical formation provides an explanation for the earlier report of the depletion of cellular thiol pool due to vanadium(V) exposure [22]. In addition, thiyl radicals themselves might directly or indirectly participate in the mechanism of vanadium toxicity as outlined in equations (a) and (b). Thus we believe that the present study suggests a new mechanism for the toxic reactions of vanadium(V) compounds, and hence clues to strategies for the biochemical control of toxicity due to vanadium exposure.

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