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Effects of NADH and H_2O_2 on Chromate-Induced Human Erythrocytes Hemoglobin Oxidation and Peroxidation

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The effects of NADH and H_2O_2 on chromate-induced human erythrocyte hemoglobin oxidation and peroxidation were studied. It was observed that NADH decreases the levels of chromate-induced human erythrocyte hemoglobin oxidation and peroxidation. H_2O_2 decreases the levels of chromate-induced hemoglobin oxidation, but increases the levels of chromate-induced peroxidation. The ability of H_2O_2 to decrease the levels of chromate-induced hemoglobin oxidation is higher than that observed for NADH. Furthermore, H_2O_2 increases the inhibitory effect of NADH on chromate-induced hemoglobin oxidation, but decreases the NADH effect on chromate-induced peroxidation. The meaning of these results is discussed in terms of involvement of reactive chromium(V) species and reactive oxygen species in the mechanism by which chromate induces its effects in human erythrocytes. © 2000 Academic Press

Key Words: **chromate; human erythrocytes; hemoglobin oxidation; H_2O_2; NADH; peroxidation.**

INTRODUCTION

Chromium(VI) is a widespread toxic metal ion present in industrial effluents and wastes. The toxic and carcinogenic effects of chromate have been attributed to chromium(VI) compounds, which readily cross cell membranes and enter cells, being reduced through reactive intermediates such as chromium(V) and chromium(IV) to the more stable chromium(III) by intracellular reductants such as ascorbate, glutathione (GSH), and NADPH-dependent flavoenzymes (for review, see Shi *et al.*, 1999). It has been reported that the levels of chromium intermediates inside cells may be associated with the induction of chromium(VI)-induced damage. Thus, intensive studies have been carried out to examine the formation of reactive chromium intermediates during the reduction of chromium(VI) by cells (for review, see Shi *et al.*, 1999). Chromium(V) intermediates have been reported to induce DNA strand breaks *in vitro* and mutation in bacterial systems. Data indicate that these reactive chromium intermediates are capable of generating reactive oxygen species via Fenton or Haber–Weiss types of reactions (for review, see Shi *et al.*, 1999). Because reactive oxygen species can produce a number of toxic reactions including DNA damage and lipid peroxidation, the toxic and carcinogenic effects of chromium(VI) may be partially associated with the production of reactive oxygen species (for review, see Shi *et al.*, 1999).

In noncellular systems reactive chromium(V) species are easily chelated by NADH, giving rise to a relatively stable chromium(V)/NADH complex, which, in the presence of H_2O_2, is readily decomposed, leading to the formation of chromium(VI) and hydroxyl radicals (Shi and Dalal, 1990). Therefore, NADH and H_2O_2 can be used as tools to study the role of intracellular chromium(V) in the mechanism by which chromium(VI) induces toxicity and carcinogenesis.

Previous studies performed in this laboratory demonstrated that dichromate, in a broad concentration range (0.5–8 mM), induces high levels of human erythrocyte hemoglobin oxidation (Alpoim *et al.*, 1995; Fernandes *et al.*, 1999) and low levels of human erythrocyte peroxidation (Fernandes *et al.*, 1999); it was being hypothesized that chromium(V) species and reactive oxygen species may be involved in the effects induced by chromate on human erythrocytes (Fernandes *et al.*, 1999).

To gain more insight into the role of chromium(V) species and reactive oxygen species in the mechanism by which chromate induces its effects on human erythrocytes, the effects of NADH and H_2O_2 on chromate-induced human erythrocyte hemoglobin oxidation and peroxidation were studied.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri, except $Na_2Cr_2O_7$, which was purchased from Aldrich, Milwaukee, Wisconsin.

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Preparation of human erythrocyte suspensions. Venous blood (20 ml) was obtained from healthy volunteers into blood collection tubes containing acid citrate dextrose as anticoagulant. The blood was centrifuged at 600 g in a bench centrifuge (Sigma 302) for 5 min at 4°C. The plasma and buffy coat were removed and the erythrocytes were washed three times with 20 ml of 0.9% NaCl solution. The erythrocytes were subsequently diluted to 30 ml with Hepes buffer (10 mM Hepes, 10 mM glucose, 140 mM NaCl, pH 7.4), and enumerated with a Cell-Dyn 1600 supplied by Abbott. The number of erythrocytes in the suspension was adjusted to a concentration of approximately 3,000,000 erythrocytes per microliter with Hepes buffer, and hemoglobin content was measured using Drabkin's method (Drabkin and Austin, 1932).

Measurement of human erythrocyte hemoglobin oxidation. Human erythrocyte hemoglobin oxidation was measured, as described previously by Martinek (1965), in cells treated with dichromate, NADH, and H_2O_2 as follows: Aliquots of human erythrocyte suspensions containing 2.5 mg dm^{-3} hemoglobin were transferred to spectrophotometric cuvettes and resuspended in Hepes buffer, of appropriate osmolality, at 30°C, in a final volume of 2 ml. The reactions were initiated by addition of 4 mM dichromate and the levels of hemoglobin oxidation measured after 35 min of incubation. When the reactions were carried out in the presence of NADH, the human erythrocyte suspensions were preincubated for 5 min with 2 mM NADH prior to the addition of 4 mM dichromate. When the reactions were carried out in the presence of H_2O_2, 4 mM dichromate was added to the human erythrocyte suspensions followed by the addition of 3 mM H_2O_2 2 min later.When the reactions were carried out in the presence of both NADH and H_2O_2, 4 mM dichromate was added to the human erythrocyte suspensions previously treated with 2 mM NADH, followed by the addition of 3 mM H_2O_2 2 min later. Controls with and without dichromate, NADH, and H_2O_2 were carried out simultaneously, and the values obtained were subtracted from the values of the overall reactions. The results are expressed as percentages of methemoglobin (% Methemoglobin).

Measurement of human erythrocyte peroxidation. The measurement of human erythrocyte peroxidation was performed using 10 ml of human erythrocytes suspended in Hepes buffer (3×10^6 cells/μl) incubated at 30°C and treated with dichromate, NADH, and H_2O_2. The reactions were initiated by the addition of 8 mM dichromate, and peroxidation levels were measured after 4 h of incubation. Human erythrocyte peroxidation was measured using 0.5-ml aliquots of human erythrocyte suspensions treated as previously described. Briefly, 0.5-ml aliquots of human erythrocyte suspensions were cooled to 0°C and 0.5 ml of 20% trichloroacetic acid was added. The samples were vortexed and centrifuged at 600g in a bench centrifuge (Sigma-302) for 3 min at 4°C, and the supernatant was carefully removed. Then, 2 ml of 0.75% thiobarbituric acid in 0.1 M HCl was added to the supernatant; the mixture was heated at 90°C for 15 min, recooled in ice for 10 min, and centrifuged again for 15 min at 850g; and the supernatant was carefully removed. Peroxidation levels were evaluated by measuring thiobarbituric acid-reactive substances, as described elsewhere (Ernster and Nordenbrand, 1967). The results are expressed as nanomoles of malonaldehyde per million of cells (nmol MDA/10^6 cells).

Statistical analysis. All experiments were performed using at least three different blood samples and conducted in duplicate. The values are expressed as means ± SD. Means of the results were compared using ANOVA. Multiple comparisons with the Fisher PLSD and Scheffe F tests were performed when significant results were observed during ANOVA. Statistical significance was set at $P < 0.05$ for all tests.

RESULTS

The effects of NADH and H_2O_2 on dichromate-induced human erythrocyte hemoglobin oxidation are presented in Fig. 1. It is observed that NADH (2 mM) and H_2O_2 (3 mM) reduce significantly the levels of hemoglobin oxidation induced by 4 mM dichromate ($P = 0.0001$, Fisher PLSD and Scheffe F tests). The effect of H_2O_2 on dichromate-induced hemoglobin oxidation is significantly higher than the effect of NADH ($P = 0.0001$, Fisher PLSD and Scheffe F tests).

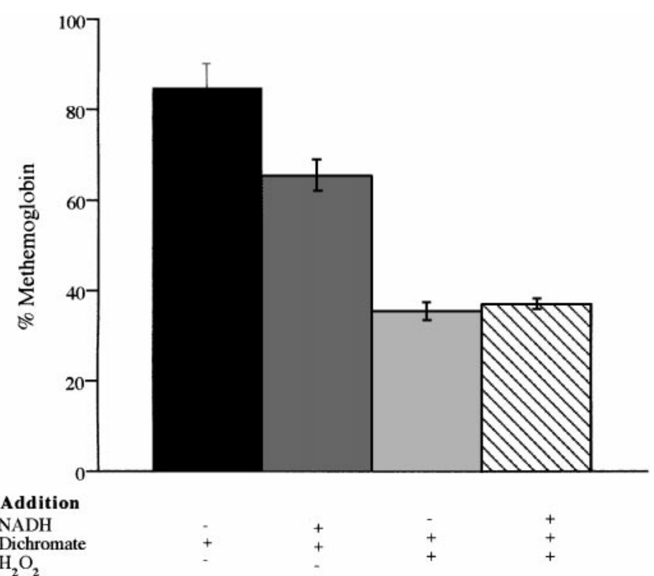


FIG. 1. Effects of NADH and of H_2O_2 on 4 mM dichromate-induced human erythrocyte hemoglobin oxidation. The results are means ± SD of at least three samples carried out in duplicate.

Addition of H_2O_2 to NADH-treated cells decreases significantly the levels of dichromate-induced hemoglobin oxidation as compared with the levels observed in erythrocytes treated with NADH alone ($P = 0.0001$, Fisher PLSD and Scheffe F tests). No significant difference is observed in the levels of dichromate-induced hemoglobin oxidation between erythrocytes treated with NADH to which H_2O_2 was added and erythrocytes treated with H_2O_2.

The effects of NADH and H_2O_2 on dichromate-induced human erythrocyte peroxidation are illustrated in Fig. 2. It is observed that NADH (2 mM) reduces significantly ($P = 0.0001$, Fisher PLSD and Scheffe F tests), while H_2O_2 (3 mM) increases significantly ($P = 0.0001$, Fisher PLSD and Scheffe F tests), the levels of 8 mM dichromate-induced peroxidation. Addition of H_2O_2 to NADH-treated erythrocytes increases significantly ($P = 0.0001$, Fisher PLSD and Scheffe F tests) the levels of dichromate-induced peroxidation as compared with the levels observed for erythrocytes treated with NADH alone. A significant difference is observed in the levels of dichromate-induced peroxidation between erythrocytes treated with NADH to which H_2O_2 was added and erythrocytes treated with H_2O_2 alone. However, no significant difference is observed in the levels of dichromate-induced peroxidation between erythrocytes treated with NADH to which H_2O_2 was added and erythrocytes treated with dichromate alone.

It is important to note that, first, under the conditions used in this study (pH > 7, dichromate concentration $< 10^{-1}$ M), dichromate gives rise to chromate (Mahan and Myers, 1987), which readily crosses erythrocyte membranes and enters cells (Connett and Wetterhahn, 1983); and second, human erythrocyte hemoglobin oxidation was studied using 4 mM dichromate. To study human erythrocyte peroxidation, 8 mM dichromate was used, instead of 4 mM dichromate, because the levels of peroxidation induced by 4 mM dichromate were very low and were difficult to measure accurately (results not provided).

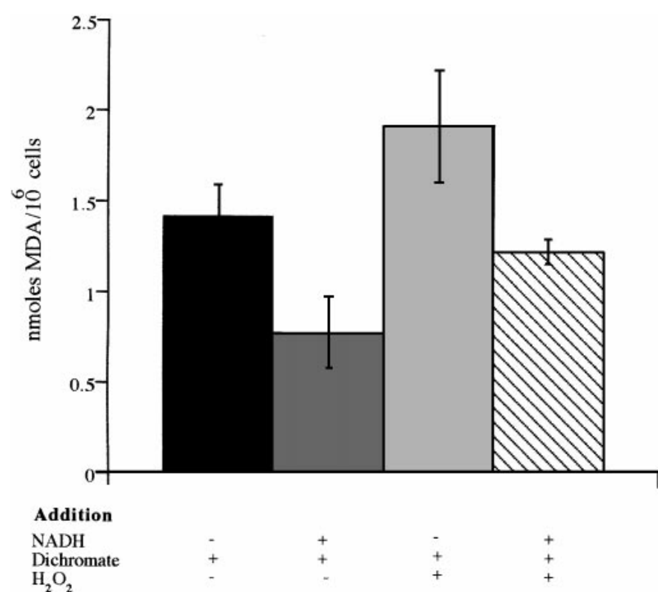


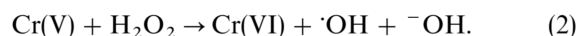
FIG. 2. Effects of NADH and of H_2O_2 on 8 mM dichromate-induced peroxidation in human erythrocytes. The results are means ± SD of at least three samples carried out in duplicate.

DISCUSSION

The results of this study demonstrate the follwing: first, NADH and H_2O_2 protect human erythrocytes against chromate-induced hemoglobin oxidation (Fig. 1), but the effect of H_2O_2 is more pronounced than that of NADH. H_2O_2 also potentiates the protective effect of NADH against chromate-induced hemoglobin oxidation (Fig. 1), suggesting that NADH and H_2O_2 protect human erythrocytes against chromate-induced hemoglobin oxidation through a common mechanism. Second, NADH protects human erythrocytes against chromate-induced peroxidation. In contrast, H_2O_2 potentiates chromate-induced peroxidation in human erythrocytes, in either the presence or the absence of NADH (Fig. 2). These results suggest that NADH inhibits, while H_2O_2 stimulates, chromate-induced reactive oxygen species generation. In fact, it has been reported that NADH reacts with chromium(VI), forming a relatively stable chromium(V)/NADH complex (Shi and Dalal, 1990), inhibiting generation of reactive oxygen species,

$$\mathrm{Cr(VI)} + 2\mathrm{NADH} \rightarrow [\mathrm{Cr(V)(NADH)_2}]^-, \quad (1)$$

whereas H_2O_2 reacts with chromium(V) via a Fenton-like reaction (for review, see Shi *et al.*, 1999) giving rise to chromium(VI) and hydroxyl radicals,



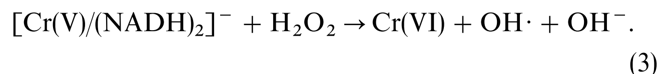
$$\mathrm{Cr(V)} + \mathrm{H_2O_2} \rightarrow \mathrm{Cr(VI)} + {}^{\cdot}\mathrm{OH} + {}^{-}\mathrm{OH}. \quad (2)$$

It is interesting to note that, although different, both mechanisms involve chromium(V) species [Eqs. (1) and (2)].

Therefore, the mechanism by which NADH and H_2O_2 protect human erythrocytes against chromate-induced hemoglobin oxidation may involve chromium(V) species. Since chromate is reduced to chromium(V) by intracellular reductants (for review, see Shi *et al.*, 1999), it is possible to hypothesize that NADH and H_2O_2 protect human erythrocytes against chromate-induced hemoglobin oxidation by decreasing reactive chromium(V) species inside the cells, rendering less probable the electron transfer reactions between chromium(V) and hemoglobin Fe^{2+}. According to this hypothesis the great ability of H_2O_2 to protect human erythrocytes against chromate-induced hemoglobin oxidation as compared with NADH (Fig. 1) may be related to a more accentuated decrease in chromium(V) species inside

human erythrocytes treated with H_2O_2 than in those treated with NADH [Eqs. (1) and (2)].

It has also been reported that H_2O_2 readily decomposes the relatively stable chromium(V)/NADH complex, leading to the formation of chromium(VI) and hydroxyl radicals (Shi and Dalal, 1990):



$$[Cr(V)/(NADH)_2]^- + H_2O_2 \rightarrow Cr(VI) + OH\cdot + OH^-. \quad (3)$$

If, as discussed above, H_2O_2 leads to a more accentuated decrease in reactive chromium(V) species than NADH, then the levels of chromate-induced hemoglobin oxidation in erythrocytes treated with NADH to which H_2O_2 was added and in erythrocytes treated with H_2O_2 alone may be identical, and very low. In fact, no significant difference was observed between the levels of chromate-induced hemoglobin oxidation in cells treated under these two conditions (Fig. 1), and the hemoglobin oxidation levels were very low (Fig. 1). According to Eqs. (2) and (3), the reduction in reactive chromium(V) species may be associated with reactive oxygen species generation; i.e., the levels of chromate-induced erythrocyte hemoglobin oxidation and peroxidation may be correlated. In fact, in erythrocytes treated with H_2O_2 the lowest levels of chromate-induced hemoglobin oxidation correspond to the highest levels of chromate-induced peroxidation (Figs. 1 and 2). Similarly, in erythrocytes treated with NADH the high levels of chromate-induced hemoglobin oxidation correspond to low levels of chromate-induced peroxidation (Figs. 1 and 2).

The observation that the levels of chromate-induced peroxidation in erythrocytes treated with NADH to which H_2O_2 was added are not significantly different from the levels of those treated with dichromate alone (Fig. 2) indicates that H_2O_2 decomposes the relatively stable chromium(V)/NADH complex *ex vivo*, like *in vitro* (Shi and Dalal, 1990). These results further suggest that reactive chromium(V) and reactive oxygen species are involved in the mechanism by which chromate induces its effects in human erythrocytes; i.e., in human erythrocytes treated with NADH to which H_2O_2 was added, chromium(V)/NADH complex and reactive chromium(V) species will be oxidized to chromium(VI) with the simultaneous generation of hydroxyl radicals [Eqs. (2) and (3)]. Chromium(VI) species will be reduced again to reactive chromium (V) species and its levels lowered by reaction with both NADH and H_2O_2. Concomitantly, reactive oxygen species will be generated [Eqs. (2) and (3)]. This cycle will be completed when the cells become deplected of their intracellular reductants. In fact, levels of GSH, glutathione reductase, and methemoglobin reductase are decreased in human erythrocytes treated with 8 mM dichromate (Fernandes *et al.*, 1999).

CONCLUSION

NADH protects human erythrocytes against chromate-induced hemoglobin oxidation and peroxidation; H_2O_2 protects human erythrocytes against chromate-induced hemoglobin oxidation, potentiating chromate-induced peroxidation. These observations reenforce the idea that chromate induces human erythrocyte hemoglobin oxidation and peroxidation through a mechanism that involves chromium(V) species and reactive oxygen species.

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