Inhibition of the Catalytic Activity of Alcohol Dehydrogenase by Nitric Oxide Is Associated with S Nitrosylation and the Release of Zinc†

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ABSTRACT: Nitric oxide (NO) reacts with the sulfhydryl groups of proteins to form nitroso thiols. Alcohol dehydrogenase (ADH) plays an important role in the metabolism of ethanol. Chronic alcohol administration stimulates NO formation in the liver, and production of NO is increased in alcohol liver injury. The effect of exogenous and endogenous NO on rat or horse ADH activity was evaluated. Incubation of intact rat hepatocytes or cytosol isolated from hepatocytes with *S*-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide donor, resulted in a decrease in ADH activity. Endogenous NO synthesis was induced in rat hepatocytes by incubation with a mixture of cytokines and endotoxin in the presence of L-arginine. As NO production in hepatocytes increased over a 24 h time period, a significant decrease in ADH activity was observed. This effect was blocked by the competitive inhibitor of NO synthesis, N^{ω}-nitro-L-arginine methyl ester, indicating that ADH was also inactivated by endogenously generated NO. The decreased activity of ADH was not related to lowering of the ADH content as shown by Western blot analysis. To evaluate the mechanism of inhibition, purified ADH from equine liver was incubated with gaseous NO or NO released from NO donors such as the diethylamine/nitric oxide complex (DEA/NO) and SNAP. NO donors inactivated ADH in a dose- and time-dependent manner. Trapping of NO with hemoglobin resulted in protection of ADH against inactivation by NO. There was no effect by analogues of the NO donors which do not release NO. NAD afforded some protection against the NO inactivation of ADH. Measurements of thiol oxidation, S nitrosylation, and zinc release were used to assess the effect of NO on ADH activity. Thiol oxidation, *S*-nitroso thiol formation, and zinc release correlated with inactivation of ADH by NO, indicating that disruption of the zinc/thiolate active center due to S nitrosylation of ADH results in zinc release, followed by inactivation of the enzyme. Recovery experiments were performed by incubating the NO-treated enzyme with dithiothreitol (DTT) and/or Zn^{2+}. The inhibitory effect by NO was reversible since, after the nitrosylated enzyme was reduced with DTT followed by incubation with $ZnCl_2$ to allow reincorporation of Zn^{2+}, ADH activity was increased from 20% of control values to 70%. These results suggest that cysteine residues contained within the zinc/thiolate active center may be primary sites of NO interaction with ADH. NO may modulate the metabolism of ethanol and influence metabolic actions of ethanol via interaction with ADH.

Nitric oxide (NO),[1] an endogenously produced free radical, is synthesized from L-arginine by NO synthase by a variety of mammalian cells, including endothelium, smooth muscle cells, neuronal cells, macrophages, neutrophils, platelets, and hepatocytes (Moncada et al., 1991; Ignarro, 1990). NO plays important bioregulatory roles in a number of physiological processes such as vasodilation, neurotransmission, and platelet aggregation and in the cytostatic and cytotoxic action of macrophages and neutrophils (Moncada et al., 1991; Nathan, 1992; Stamler, 1994). Many of these effects are based on modulation of enzyme activity through the binding of NO to the heme or non-heme iron cofactor of proteins such as soluble guanylate cyclase (Ignarro, 1990), aconitase (Stadler et al., 1991), cytochrome *c* oxidase (Cleeter et al., 1994), cyclooxygenase (Kanner et al., 1992), and neuronal nitric oxide synthase (Abu-Saud et al., 1995).

NO or its derivatives also interact with the sulfhydryl groups of proteins and glutathione to form nitroso thiols (Stamler et al., 1992; Simon et al., 1996). Attachment of the NO group to sulfhydryl residues of active site thiols in enzymes (S nitrosylation) such as GAPDH (Molina y Vedia et al., 1992), PKC (Gopalakrishna et al., 1993), glutathione peroxidase (Asahi et al., 1995), or ecto-5-nucleotidase (Siegfried et al., 1996) is likely to be responsible for inhibition of their catalytic activity. NO was also proposed to play a role in endogenous ADP autoribosylation of GAPDH (Brune & Lapetina, 1989; Zhang & Snyder, 1992; Kots et al., 1992; Dimmeler et al., 1992) and in NAD attachment to this enzyme (McDonald & Moss, 1993a).

Chronic alcohol consumption stimulates production of LPS and several cytokines, which are all inducers of NO synthesis (Bode et al., 1987; Bigatello et al., 1987; Honchel et al., 1992). Circulating endotoxin and tumor necrosis factor are markedly raised in acute alcoholic hepatitis (Bird et al.,

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[1] Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; NAP, *N*-acetylpenicillamine; NO, nitric oxide; DEA/NO, diethylamine/nitric oxide complex; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; PAR, 4-(2-pyridylazo)-resorcinol; L-NAME, N^{ω}-nitro-L-arginine methyl ester; CM, cytokine mixture.

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1990). An antagonist of the actions of NO improved arterial pressure in severe, decompensated alcoholic liver disease, and it has been suggested that there is increased generation of NO in alcohol-induced liver failure (Midgely et al., 1991). Wang et al. (1995) have shown that chronic alcohol administration in rats stimulated NO formation in the liver and production of NO by Kupffer cells and hepatocytes was increased in patients with alcoholic hepatitis (Hunt & Goldin, 1992). These authors suggested that NO could play a role in the pathogenesis of alcoholic liver disease.

There have been few studies concerning the effects of NO on mammalian alcohol-metabolizing enzymes. The NO donor, sodium nitropruside, was shown to inhibit aldehyde dehydrogenase (McDonald & Moss, 1993b). Dimmeler and Brune (1993) reported an inhibitory effect of SIN-1 on the activity of yeast ADH, and recently, it has been found that peroxynitrite, a potent oxidant formed by reaction of superoxide with NO, inhibited yeast alcohol dehydrogenase (Crow et al., 1995). The inhibition of yeast ADH by peroxynitrite was associated with release of zinc and oxidation of thiol residues of the enzyme, and inhibition was also observed with other powerful oxidants such as hypochlorite and to a lesser extent H_2O_2 (Crow et al., 1995). The effect of NO on mammalian ADH has not been reported; peroxynitrite can inactivate certain enzymes, e.g. aconitase, in which NO has no effect (Hausladen & Fridovich, 1994; Castro et al., 1994). Therefore, in the current report, the effect of exogenous and endogenous NO on rat and equine ADH activity was determined. It was observed that exogenous and endogenous NO inhibited activity of ADH and that this inhibition could be related to the S nitrosylation of sulfhydryl residues followed by release of zinc from the catalytic zinc−thiolate active site of ADH. Inhibition by NO of ADH was reversed upon treatment with DTT and $ZnCl_2$.

MATERIALS AND METHODS

Materials. Alcohol dehydrogenase from equine liver, NADH, NAD^+, *N*-acetyl-DL-penicillamine (NAP), diethylamine (DEA), hemoglobin, dithiothreitol (DTT), sulfanilamide, ammonium sulfamate, *N*-(1-naphthyl) ethylenediamine, 5,5′-dithiobis(2-nitrobenzoic acid), 4-(2-pyridylazo)-resorcinol (PAR), zinc chloride, and N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO). SNAP was from Biomol (Plymouth Meeting, PA) and DEA/NO from Research Biochemical (Natic, MA). NO gas was from Aldrich (Milwaukee, WI). Mercuric chloride was from Fischer (Springfield, NJ).

Hepatocyte Isolation and Cell Culture. Hepatocytes were isolated from male Sprague−Dawley rats (175−225 g) by collagenase perfusion (Wu et al., 1990) and purified by low-speed differential centrifugation to give cells of at least 95% viability and 95% purity. Hepatocytes (4×10^6) were plated on 100 mm collagen-coated culture plates (Corning, Palo Alto, CA) in 10 mL of Williams medium E (Gibco, Grand Island, NY), supplemented with insulin (1 μM), Hepes (15 mM), L-glutamine, penicillin, streptomycin, and 10% fetal calf serum (Sigma). Cells were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37 °C for 3 h to allow adherence. Nonadherent cells were removed by washing the plates with PBS, and fresh medium was added to the cultures.

To study the effect of endogenous NO on ADH activity in hepatocytes, the medium was changed after a 24 h incubation to include a cytokine mixture (CM) containing 10 μg/mL LPS (*Escherichia coli* 0111:B4; Sigma, Purchase, NY), 500 u/mL rhTNF α, (Intergen, Purchase, NY), and 100 u/mL rhIFNγ (Genzyme, Boston, MA) as described by Geller et al. (1993). Where indicated, 1 mM L-arginine (Gibco) with or without 1 mM N^{ω}-nitro-L-arginine methyl ester (L-NAME) was added to the cultures with the CM. At the indicated time points, cell free supernatants were collected for nitrite determination and hepatocytes were harvested, and the cytosol was isolated and analyzed for ADH activity.

Preparation of Cytosolic Fractions. Cells with stimulated and nonstimulated iNOS or after incubation with or without SNAP were pelleted at 250*g* for 5 min and lysed for 20 s with sonication on ice using the microtip probe of a W-375 sonicator (Heat Systems-Ultrasonics, Inc.). Lysates were centrifuged at 4 °C at 100000*g* for 30 min, and the supernatant was used for ADH activity measurement after evaluating the concentration of protein. Equal amounts of protein from all samples were incubated in a reaction system containing 1.2 mg of cytosolic protein, 8 mM NAD, and 30 mM ethanol in 1 mL of PBS. Formation of NADH was determined at 340 nm over a 200−300 s time interval at 37 °C. Absorbance was linear with time over this period, and an extinction coefficient ϵ_{340} of 6220 M^{-1} cm^{-1} was used to calculate enzyme activity, which was expressed as micromolar per minute since an equivalent amount of protein was used in the same series of experiments.

Determination of Nitrite. To determine the amount of NO produced by the hepatocytes, the culture supernatant was assayed for the stable oxidative metabolite of NO, nitrite, as described by Green et al. (1982). At the end of the incubation periods, 150 μL of the culture medium was mixed with an equal volume of Griess reagent (one part 0.1% naphthylethylenediamine dihydrochloride and one part 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 15 min. The absorbance at 550 nm was measured, and the amount of nitrite was determined using a curve calibrated with sodium nitrite as the standard.

Inactivation of ADH by NO and ADH Activity Assay. ADH from equine liver (typically 1.66 mg/mL) was incubated with or without aliquots of gaseous NO or the NO-generating compounds SNAP or DEA/NO, or their parent molecules (which do not produce NO) NAP or DEA, in 1 mL of 100 mM PBS at pH 7.4 and room temperature for 20 min. Measurement of the activity of ADH was carried out in a reaction system containing ADH preincubated as described above (typically 0.048 mg or 0.069 u/mL), 60 mM ethanol, and 1.6 mM NAD in 1 mL of 100 mM PBS at pH 7.4. NADH formation was monitored at 340 nm (ϵ_{340} = 6220 M^{-1} cm^{-1}) for approximately 60−120 s, at either 25 or 37 °C. The NO gas stock solution was prepared by bubbling of NO gas (Aldrich, Milwaukee, WI) for 20 min into argon-saturated 0.01 M PBS at pH 7.4. Any NO_2 was eliminated by first bubbling the NO through 5 M KOH. The concentration of NO was determined by measuring the oxidation of oxyhemoglobin to methemoglobin according to Murphy and Noack (1994).

Determination of S Nitrosylation of ADH. Equine ADH (1.5 mg per 0.4 mL) in 100 mM PBS and in the absence or presence of 0.5 mM EDTA at pH 7.4 was incubated with buffer or with either 5 mM DEA/NO, SNAP, DEA, or NAP. At the indicated times, samples were desalted over Sephadex G-25 columns to remove the NO donor and analyzed for

free thiol and *S*-nitroso thiol content. The formation of *S*-nitroso thiol in ADH was assessed by diazotization with sulfanilamide and azo coupling with *N*,*N*-ethylenediamine in the presence and absence of Hg^{2+} ions according to the procedure of Saville (1958). Briefly, the sample containing *S*-nitroso-ADH (0.2 mL) was first mixed with 40 μL of 0.5% ammonium sulfamate in H_2O for 1 min. A solution (40 μL) containing 2.7% sulfanilamide and 0.25% $HgCl_2$ in 4% H_3PO_4 was than added, followed by 320 μL of 1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in H_2O. The Hg^{2+} releases NO from *S*-nitroso thiols, which under acidic conditions gives a positive Griess reaction. The concentration of azo compound was determined after 15 min by measuring the absorption at 570 nm. The content of *S*-nitroso thiol was calculated as being equivalent to the difference of absorption readings of Hg^{2+}-containing versus Hg^{2+} free samples. The nitroso content was quantified according to a standard curve constructed with $NaNO_2$. Rates of S nitrosylation were the same whether reactions were carried out in the absence or presence of EDTA.

Determination of the Thiol Content of ADH. The thiol content of ADH was determined by a modified Ellman's assay (Ellman, 1959). Briefly, ADH (0.1 mg) in 0.1 M PBS and 2% SDS was incubated with an excess of 5,5′-dithiobis-(2-nitrobenzoate) (prepared in ethanol before use) for 15 min at room temperature. The reaction was followed by measuring the increase in absorbance at 412 nm. The concentration of thiols was calculated from the molar extinction coefficient of the thio nitrobenzoate anion ($\epsilon_{412} = 13\,600\ M^{-1}\ cm^{-1}$) (Riddles et al., 1983)

Protein was determined by a modified Lowry procedure using the Bio-Rad DC protein assay kit with BSA as the standard.

Determination of Zinc Release from ADH. Release of zinc was determined as described by Crow et al. (1995). ADH (0.8 mg or 1 unit) was dissolved in 1 mL of PBS buffer at pH 7.4 and incubated with or without DEA/NO, SNAP, DEA, or NAP for 20 min at room temperature. PAR (200 μM) was added to the solutions, and after 15 min of incubation, formation of the zinc/PAR complex was measured at 500 nm (Hunt et al., 1985). Addition of 1 mM EDTA, which chelates zinc more tightly than PAR, resulted in a rapid loss of absorbance, and the difference in absorbance at 500 nm between samples treated with or without 1 mM EDTA was taken to represent the zinc/PAR complex. The amount of zinc release was calculated according to a standard curve using $ZnCl_2$.

Western Blot Analysis. Western blot analysis of ADH was carried out using 25 μg of cytosol protein. The immunoblots were developed using a rabbit-derived anti-mouse ADH polyclonal antibody provided by Dr. M. Felder (Department of Biology, University of South Carolina) and horseradish peroxidase-conjugated goat anti-rabbit IgG as the second antibody.

Reversibility of ADH Activity after NO Treatment. ADH from equine liver (3.5 mg) was incubated in the presence or absence of 3 mM SNAP or NAP in 400 μL of 0.1 M PBS at pH 7.4 for 20 min at room temperature. After being desalted over a Sephadex G-25 column to remove the NO donor, samples were incubated for 30 min with or without 5 mM DTT and again gel filtered on a Sephadex G-25 column to remove excess DTT. The gel-filtered samples (typically 0.7 mg/mL) were incubated in 100 mM PBS at pH 7.4 for 15 min at room temperature in the presence or absence of 0.5 mM $ZnCl_2$ in order to reinsert zinc back into ADH in attempts to restore ADH activity. Measurement of the activity of ADH was carried out as described above with identical concentrations of ADH protein in all samples (0.021 mg/mL).

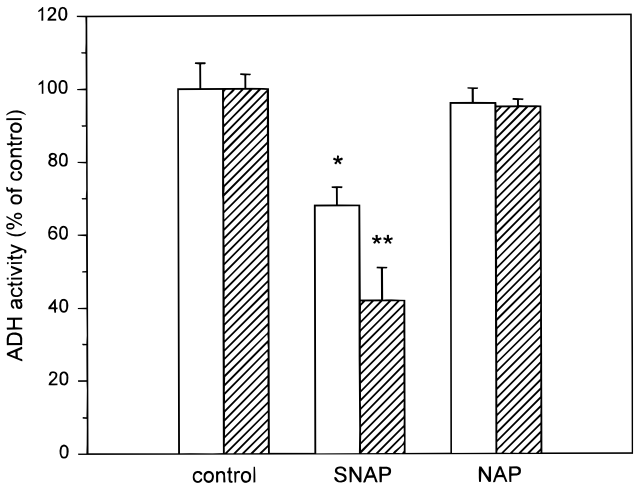

FIGURE 1: Effect of SNAP on ADH activity. The ADH activity was determined after incubation of cytosol fractions isolated from rat hepatocytes (hatched bars) or intact hepatocytes (open bars) for 30 min at 22 °C in the presence of the indicated additions: 1 mM SNAP, 1 mM NAP, or control (absence of SNAP or NAP) as described in Materials and Methods. The amount of liver cytosolic protein used to assay ADH activity for all samples was 1.2 mg in a 1 mL final volume. The activity was expressed as the percent of the control, which was 4.6 ± 0.17 and 4.3 ± 0.3 μM/min in experiments with cytosolic fractions and hepatocytes, respectively. Values represent mean ± SE from three experiments. *$p < 0.05$ versus control. **$p < 0.01$ versus control.

RESULTS

Effect of Exogenous NO on ADH Activity in Rat Hepatocytes. To study the effect of NO on activity of rat liver ADH, the NO-generating compound SNAP, as well as the parent molecule NAP, was added to the cytosolic fraction isolated from rat hepatocytes. Cytosolic fractions were incubated with 1 mM SNAP or 1 mM NAP for 30 min, and activity of ADH was evaluated. The incubation with 1 mM SNAP resulted in a 60% inhibition of ADH activity, whereas only minor inhibition (about 5%) was detected in cytosolic fractions incubated with NAP (Figure 1, hatched bars).

In the experiments with intact cells, hepatocytes were directly incubated in the presence of 1 mM SNAP or NAP for 30 min and the cytosolic fraction was isolated and ADH activity determined in the absence of any further additions. As shown in Figure 1 (open bars), incubation of hepatocytes with 1 mM SNAP resulted in 32% inhibition of ADH activity whereas, no inhibition was detected in the cytosolic fraction isolated from hepatocytes incubated with NAP. The greater extent of inhibition of ADH activity with cytosolic fractions than with intact hepatocytes by SNAP could reflect the presence of a higher concentration of NO-trapping agents, e.g. cytochromes, in the intact cells than in the isolated cytosolic fractions.

Effect of Endogenous NO on ADH Activity in Rat Hepatocytes. To study the effect of endogenous NO on ADH activity, rat hepatocytes were incubated with a combination of LPS and cytokines in the presence of L-arginine; this

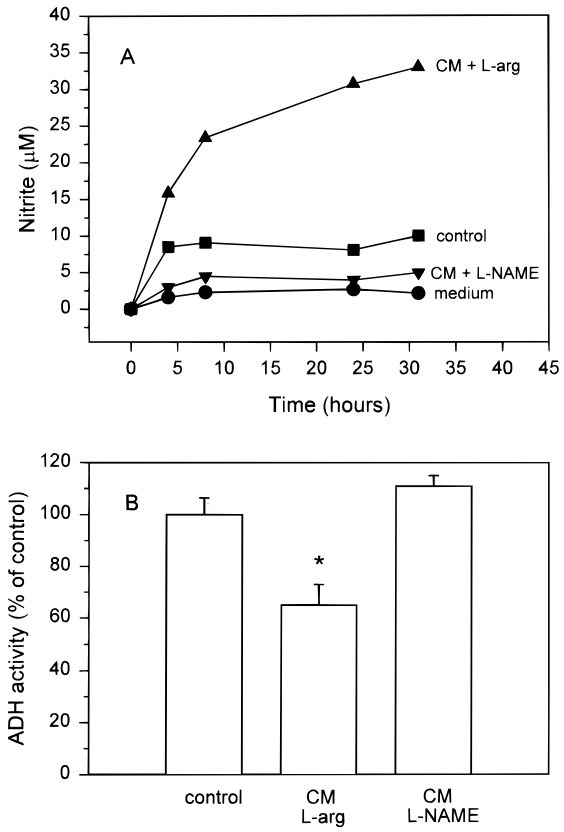

FIGURE 2: Effect of endogenous NO on ADH activity. (A) Rat hepatocytes were incubated in the absence (control, ■) or presence of a cytokine mixture (CM) consisting of 10 μg/mL LPS, 500 u/mL rhTNFα, 100 u/mL rhIFNγ, plus either 1 mM L-arginine (▲) or 1 mM L-NAME (▼) for the indicated time periods, and nitrite levels in the medium were measured as described in Materials and Methods. Medium alone, in the absence of the hepatocytes, was also analyzed (●). (B) Effect of endogenous NO production on ADH activity. Rat hepatocytes were incubated without (control) or with cytokines plus LPS (CM) in the presence of either 1 mM L-arginine (L-arg) or 1 mM L-NAME (L-NAME) for 28 h as described in Materials and Methods. After isolation of cytosol, ADH activity was determined using 1.2 mg of cytosolic protein for all samples. The activity of the control was 3.3 ± 0.19 μM/min. Values represent mean $\pm$SE from three experiments. *$p < 0.05$ versus control.

mixture is known to stimulate iNOS in hepatocytes (Geller et al., 1993). This treatment within a few hours increased the production of NO, measured as nitrite accumulation (Figure 2A). ADH activity measured in cytosolic fractions isolated from these hepatocytes was significantly lower than activity of control cells (Figure 2B). In the presence of 1 mM L-NAME, an NO synthase inhibitor, nitrite accumulation and ADH activity remained at control levels, suggesting the participation of NO produced by iNOS in the inhibition of ADH activity in the rat hepatocytes.

Western Blot Analysis of the Content of ADH. To evaluate the possibility that NO decreased the activity of ADH by lowering the protein level or content of ADH or to determine whether the NO-inactivated ADH was rapidly degraded, Western blot analysis was carried out. The content of ADH was not decreased in the hepatocytes with an elevated production of endogenous NO (Figure 3, lane 3) and was similar to the content of ADH in hepatocytes incubated in the presence of L-NAME (lane 4) as well as that of control samples (lane 2).

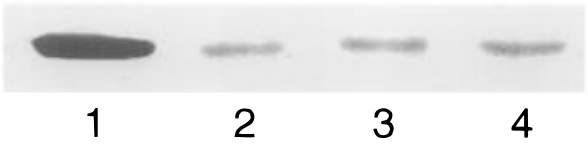

FIGURE 3: Western blot analysis of the content of ADH in cytosol isolated from rat hepatocytes. Experiments were carried out as described in Materials and Methods. Cytosolic fractions were from hepatocytes incubated with CM in the presence of 1 mM L-arginine (lane 3), CM in the presence of 1 mM L-NAME (lane 4), and control cytosolic fractions (lane 2). Lane 1 contained ADH from equine liver as a standard.

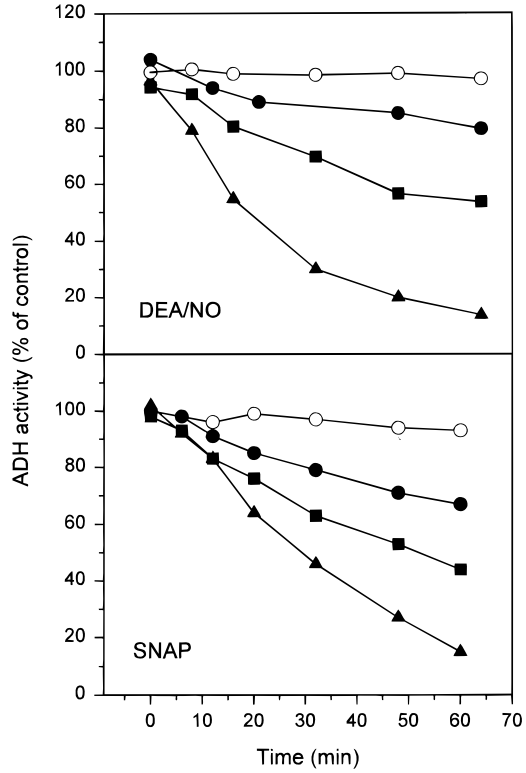

FIGURE 4: Effect of NO donors on ADH activity. The ADH activity was determined after incubation of the equine liver enzyme (1.66 mg/mL) with 1 mM (▲), 0.3 mM (■), or 0.1 mM (●) DEA/NO (upper panel) or SNAP (lower panel) or in the presence of the parent molecule, 3 mM NAP, or 3 mM DEA (○) for the indicated time periods. The amount of ADH protein used to assay ADH activity was about 0.05 mg/mL. The activity of control samples was 33.7 μM/min.

Inactivation of ADH from Equine Liver by SNAP, DEA/NO, and NO Gas. To study the mechanism of inactivation of ADH and whether NO can directly affect ADH, ADH from equine liver was incubated with different NO-generating compounds or with gaseous NO, followed by assay of ADH activity. The NO-generating compounds SNAP and DEA/NO inhibited ADH activity in a dose- and time-dependent manner (Figure 4). Similar to the situation with NO donors, preincubation of ADH for 20 min in the presence of gaseous NO also inhibited the activity of ADH in a concentration-dependent manner (Figure 5). Bubbling the stock solutions of NO gas with N_2 or O_2 for 5 min before addition to the ADH preincubation mixture abolished the inhibitory effect of NO, indicating that inhibition of ADH was mediated by the volatile NO gas (Figure 5). The parent molecules of the NO donors, NAP and DEA (3 mM), as well as nitrite did not inhibit ADH activity, indicating that it was the released NO and not the donor molecule itself or a major

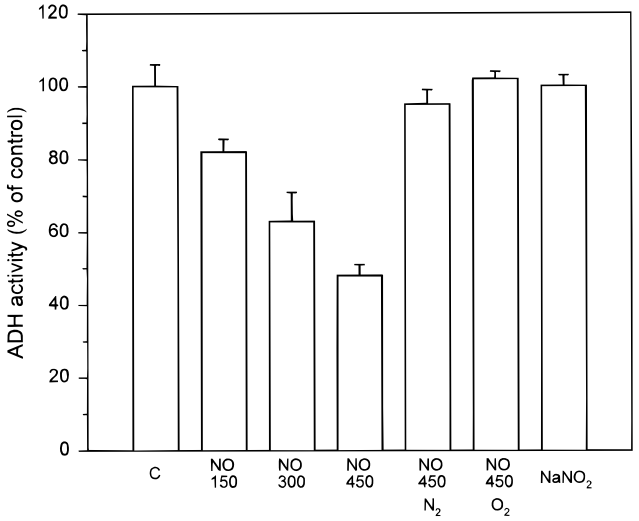

FIGURE 5: Effect of NO gas on ADH activity. The ADH activity was determined after 20 min of incubation of the equine liver enzyme (1.66 mg/mL) in the absence (C) or presence of the indicated concentrations (μM) of NO gas solution (NO). Some samples of NO were bubbled with oxygen (O_2) or nitrogen (N_2) for 5 min at 22 °C. The activity of control samples was 36 ± 2.28 μM/min. Values represent mean ± SE from four experiments.

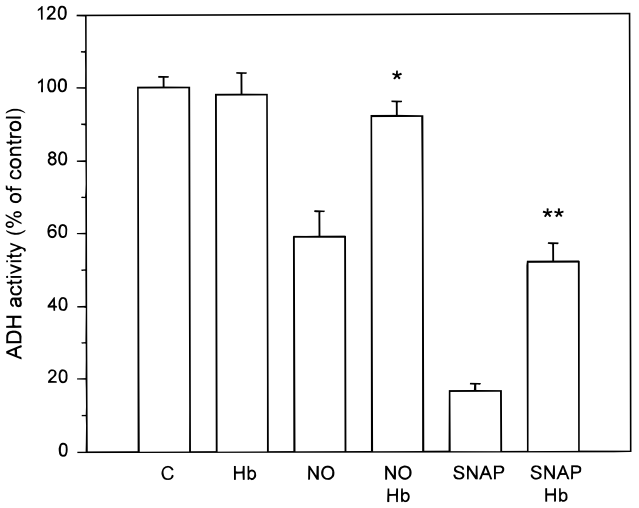

FIGURE 6: Effect of hemoglobin on the inhibition of equine liver ADH activity by NO. The protective effect of 30 μM hemoglobin (Hb) was determined after 20 min of incubation of ADH (1.66 mg/mL) with 300 μM NO gas (NO) or after 60 min of incubation with 1 mM SNAP (SNAP) in the absence or presence of Hb. The activity of control samples (C) was 37.3 ± 1.1 μM/min. Values represent mean ± SE from three experiments. *$p < 0.05$ versus NO. **$p < 0.05$ versus SNAP.

oxidative metabolite of NO which blocked the activity of ADH (Figures 4 and 5).

The specific role of NO in the inhibition of ADH was examined with the use of the NO scavenger, Fe^{2+} hemoglobin. ADH was incubated for 60 min with SNAP in the absence or presence of Hb or for 20 min with gaseous NO in the absence or presence of Hb. In the absence of NO gas or NO donor, Fe^{2+}/hemoglobin itself had no effect on ADH activity (Figure 6). However, in the presence of 30 μM Fe^{2+}/hemoglobin, the inhibitory effect of SNAP (3 mM) was reduced from a value of 20% of control to approximately 60% of control activity, and the inhibitory effect of NO gas was almost completely prevented (Figure 6). The protection by Fe/hemoglobin is consistent with NO being responsible for the blocking of ADH activity.

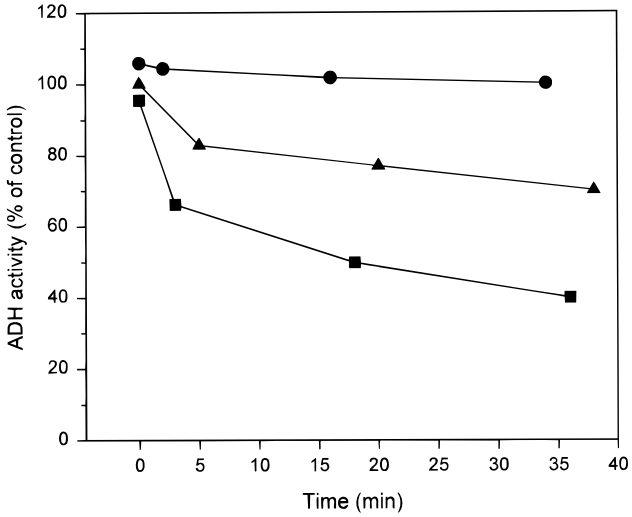

FIGURE 7: Effect of NAD on the inhibition of ADH activity by SNAP. The ADH activity was determined after incubation of the equine liver enzyme (1.66 mg/mL) in the absence (●) or the presence of 1.3 mM SNAP (■) or with SNAP plus 2 mM NAD (▲). Reactions were initiated by the addition of ethanol. The activity of the control was 31.3 μM/min. NAD had no effect on control ADH activity.

Effect of Substrates on the Inhibitory Effect of NO. To determine whether the inhibitory effect of NO can be affected by substrates of ADH, samples were preincubated in the presence of SNAP alone or SNAP plus either 2 mM NAD or 300 mM ethanol, prior to addition of the other substrate to initiate the reaction. NAD decreased but did not completely prevent the inhibitory effect of SNAP (1.3 mM) on ADH (Figure 7). Preincubation of SNAP and ADH in the presence of ethanol had no effect on the inhibition of ADH (data not shown).

Thiol Oxidation and S Nitrosylation. To evaluate the mechanism of inactivation of ADH by NO, the effect of NO on the content of free SH groups as well as the S nitrosylation of ADH by NO were evaluated. Incubation of ADH in the presence of DEA/NO decreased the SH content in a time-dependent manner (Figure 8). The decrease in SH content paralleled the S nitrosylation of ADH as the content of SNO increased over the time course (Figure 8). The stoichiometry between these two reactions was approximately two SH groups lost per nitroso group formed. Crow et al. (1995) reported that peroxynitrite-induced inactivation of yeast ADH was associated with a loss of six thiols per mole of enzyme. Inactivation of mammalian ADH by NO was associated with a loss of about eight thiols per mole of enzyme (Figure 8).

Zinc Release. Liver ADH is a metalloenzyme, with four zinc molecules per active ADH dimer. The zinc atoms are chelated to SH groups of cysteine residues, and this helps provide the active center of ADH. Interaction of NO with ADH thiols could disrupt the zinc/thiol complex and thus inactivate ADH. Therefore, the release of zinc after preincubation of ADH with SNAP and DEA/NO was determined. Zinc release from ADH was induced by DEA/NO in a concentration-dependent manner and was paralleled by a decrease in ADH activity (Figure 9A). Similar results were observed with SNAP (Figure 9B). The parent molecules of the NO donors, NAP and DEA, had no effect on zinc release (data not shown). Under these reaction conditions, a 40−50% loss of ADH activity was associated with release of

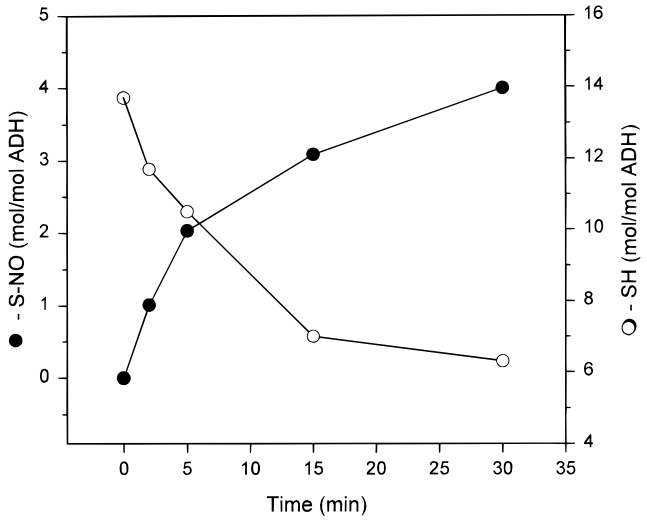

FIGURE 8: Effect of DEA/NO on *S*-nitroso thiol formation and on the content of SH groups in ADH. S nitrosylation (●) and SH group content (○) were determined after incubation of the equine liver enzyme (1.5 mg per 0.4 mL) with 5 mM DEA/NO for the indicated time periods at 22 °C as described in Materials and Methods. Values are expressed as the molar ratio of SNO or SH/moles of ADH protein.

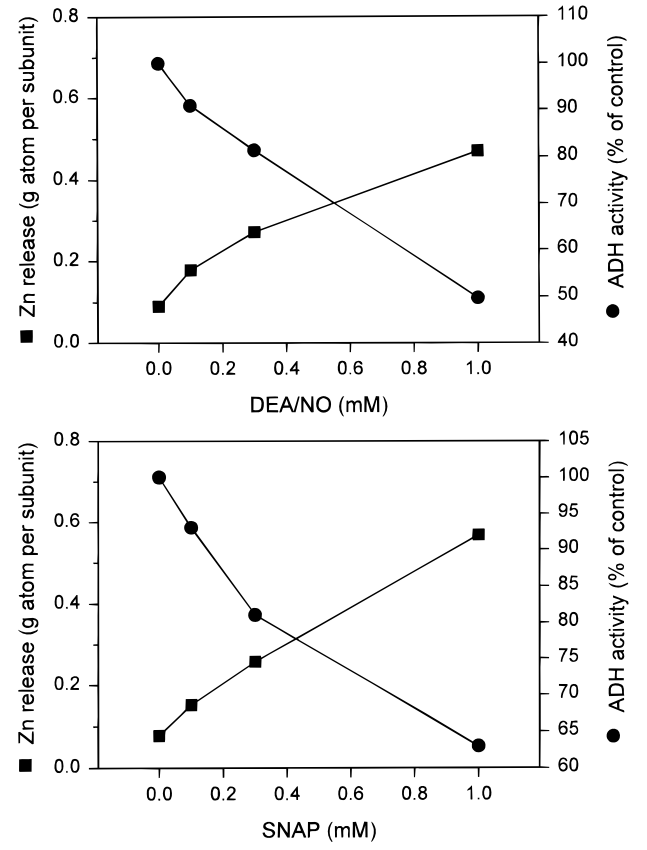

FIGURE 9: Effect of SNAP or DEA/NO on zinc release and loss of ADH activity. The zinc release (■) and ADH activity (●) were determined after incubation of the equine liver enzyme (0.8 mg/ml) in the presence of the indicated concentrations of DEA/NO (upper panel) or SNAP (lower panel) for 20 min at 22 °C. The amount of ADH protein used to assay ADH activity was 0.023 mg/mL. The activity of the control was 18 μM/min.

about 0.5−0.6 mol of zinc per ADH subunit (25−30% loss of total zinc).

Reversibility of the NO Inhibition of ADH Activity. Since the inactivation of ADH was accompaned by SH group oxidation and release of zinc, it was of interest to evaluate the possible reversibility of the inhibition of ADH activity by using thiol-reducing agents and incubation with $ZnCl_2$ to allow reincorporation of zinc into ADH. The enzyme was first incubated for 20 min in the presence or absence of 3 mM SNAP and then desalted on a Sephadex G-25 column. As shown in Figure 10, incubation of the enzyme with 3 mM SNAP resulted in an 80% inhibition of ADH activity. The addition of 5 mM DTT to the SNAP-inactivated enzyme and incubation of this mixture for 30 min followed by gel filtration (to remove DTT) did not restore the ADH activity (Figure 10, open bar). The addition of $ZnCl_2$ (0.5 mM) had no effect on control ADH activity, nor did it reverse the inhibition of ADH by SNAP in samples not previously treated with DTT (Figure 10, hatched bars). However, if $ZnCl_2$ was added to the SNAP-inactivated ADH which was first treated with DTT, followed by gel filtration and incubation for 15 min at room temperature, ADH activity increased from about 20% of control activity in the absence of $ZnCl_2$ to about 68% of activity after $ZnCl_2$ addition (Figure 10, hatched bars). Thus, a thiol reductant such as DTT and incubation with zinc helped to reverse the inhibition of ADH by NO.

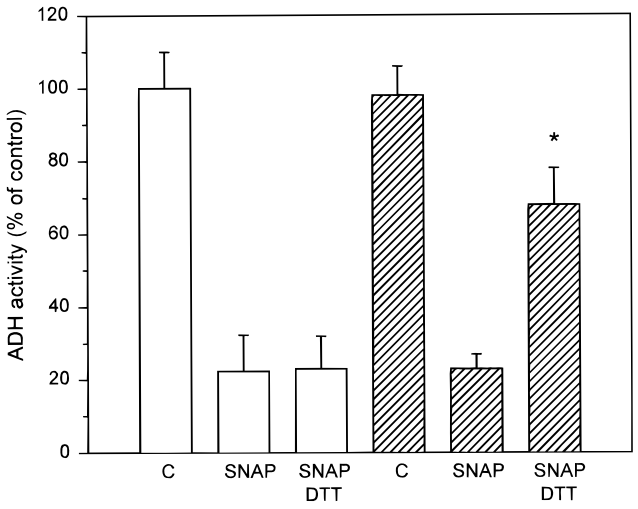

FIGURE 10: Effect of DTT and $ZnCl_2$ on the reversibility of the inhibition of ADH activity after NO treatment. Equine liver ADH was preincubated without (C) or with 3 mM SNAP (SNAP) for 20 min at 22 °C. The reversibility of the inhibition of ADH activity was determined after gel filtration to remove the SNAP, reduction with 5 mM DTT (DTT) to restore thiols, gel filtration to remove DTT, and incubation without (open bars) or with 0.5 mM $ZnCl_2$ (hatched bars) as described in Materials and Methods. The amount of ADH protein used to assay ADH activity was 0.021 mg/mL. The activity of the control was 15.9 ± 1.7 μM/min. Values represent mean ± SE from three experiments. *p < 0.05 versus SNAP (hatched bars).

DISCUSSION

Chronic alcohol administration in rats stimulates NO formation in the liver (Wang et al., 1995), and production of NO by Kupffer cells and hepatocytes is increased in patients with alcoholic hepatitis. (Hunt & Goldin, 1992). Moreover, circulating endotoxin and TNFα which activate iNOS in Kupffer cells and hepatocytes are markedly elevated in acute alcoholic hepatitis (Bird et al., 1990) and after chronic alcohol consumption (Bode et al., 1987; Bigatello et al., 1987; Honchel et al., 1992). How increased production of NO in the liver after alcohol intake influences the activity

of ethanol-metabolizing enzymes such as ADH, ALDH, or CYP2E1 is not known. Dimmeler and Brune (1993) have shown that NO spontaneously released from SIN-1 inhibited enzymatic activity of yeast alcohol dehydrogenase.

The effect of exogenous and endogenous NO on activity of mammalian liver ADH, the association of loss of thiol groups with S nitrosylation and zinc release, possible protection by substrates, and reversibility of the inhibition by NO were among the goals of the current study. Initial studies investigated the effect of NO on the activity of rat liver ADH. Cytosolic fractions isolated from rat hepatocytes as well as intact hepatocytes were incubated with a chemical NO donor, SNAP. It was observed that exogenous NO inhibited the activity of ADH. To study the effect of endogenously produced NO on intracellular activity of ADH, a cytokine mixture was used to induce iNOS activity in the hepatocytes. A significant decrease in intracellular ADH activity was observed in CM-treated cells in the presence of L-arginine. This inhibition appears to be due to induction of NO synthase and formation of NO because ADH activity was protected in the presence of L-NAME which blocked NO production (Figure 2). Western blot analysis showed that the content of ADH was not affected by endogenous NO, suggesting that the NO-mediated decrease of ADH activity is not due to inhibition of synthesis of ADH or to increased degradation of ADH by NO. ADH from equine liver was also found to be sensitive to inhibition by NO donors and NO gas. That NO was the agent responsible for blocking ADH activity was validated by the protective effect of hemoglobin, a scavenger of NO, as well as by the inability of non-NO analogues of NO donors and nitrite, an oxidized metabolite of NO, to inhibit the activity of ADH.

It was previously suggested that inactivation by NO of enzymes such as PKC (Gopalakrishna et al., 1993), glutathione peroxidase (Asahi et al., 1995), and ecto-5-nucleotidase (Siegfried et al., 1996) is probably due to nitroso thiol formation since enzyme inactivation was reversed by addition of DTT. Indeed, coincubation of ADH with DTT in the presence of NO did protect against enzyme inactivation; however, this is probably due to competition of sensitive thiols of ADH with DTT for NO as DTT reacts readily with NO (data not shown). Addition of DTT after incubation of ADH with NO (i.e. after forming inactive ADH) to attempt to restore activity was ineffective, although DTT did decrease the nitroso thiol content and increased the thiol content. This is probably due to release of zinc from the catalytic site of ADH once zinc/thiolate ligands have been disrupted by NO (see the discussion below).

NO may play a role in the endogenous ADP autoribosylation of GAPDH which is associated with a loss of enzymatic activity (Brune & Lapetina, 1989; Zhang & Snyder, 1992; Kots et al., 1992; Dimmeler et al., 1992). Dimmeler and Brune (1992) suggested that nitric oxide modification of SH groups (S nitrosylation) is a prerequisite for this reaction. The ADP autoribosylation of GAPDH appears in fact to be a covalent binding of the entire NAD molecule to GAPDH in the presence of NO (McDonald & Moss, 1993a,c). Inhibition of GAPDH cannot be explained solely by the extent of modification of the enzyme with NAD (McDonald & Moss, 1993) and appears to be correlated with the known S nitrosylation reaction of the active site cysteine residues with NO (Molina y Vedia et al., 1992). Mohr et al. (1996) have recently shown that S nitrosylation of GAPDH is responsible for reversible enzyme inhibition, whereas attachment of NAD accounts for irreversible enzyme inactivation. Dimmeler and Brune (1993) reported that, although SIN-1 inhibited activity of yeast ADH, ADP autoribosylation of ADH in comparison with that of GAPDH was stimulated to only a minor extent and lactate dehydrogenase was unaffected.

In our study, NAD was not present during the pretreatment of ADH with NO. Moreover, the combination of NAD plus NO did not result in potentiation of the effect of NO but rather produced partial protection against the inhibitory effect. This result suggests that there is no correlation between inhibition of ADH with NAD attachment under our reaction conditions. The partial protection by NAD (but not ethanol) against the inhibition by NO may reflect the protection of NO sensitive thiols from interaction with NO upon binding of NAD at the active site.

While it is likely that enzymes with active thiol groups will become S-nitrosylated upon interaction with NO, different enzymes may display variable sensitivities. For instance, the amount of *N*-ethylmaleimide necessary to inhibit GAPDH is about 100 times lower than the amount necessary to inhibit lactate dehydrogenase or ADH (Molina y Vedia et. al., 1992). Arenaemycin specifically reacts with thiol groups present in GAPDH; however, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, malate dehydrogenase, and glutamate dehydrogenase remained completely unaffected by the antibiotic (Hartmann et al., 1978). Although GAPDH is nitrosylated by NO and ADH has active thiol groups similar to those of GAPDH, the nitrosylation of liver ADH has not been reported. Treatment of equine liver ADH with NO donors resulted in the nitrosylation of ADH (Figure 8). The nitrosylation was accompanied by a loss in free thiol groups and inhibition of ADH activity mediated by NO correlated with *S*-nitroso thiol formation. This suggests that the modified thiol groups are probably related to cysteine residues at the active center of ADH.

Nitric oxide in an aerobic environment generates an intermediate NO_x (presumably N_2O_3 or N_2O_4) which is capable of oxidizing metal complexes or nitrosating amines and thiol complexes (Morley et al., 1993). It has been shown that NO in an aerobic environment readily forms thionitrosyl complexes (Stamler et al., 1992; Gaston et al., 1993; Jia et al., 1996). S nitrosylation of liver ADH may have occurred due to the formation of nitrogen oxide and higher oxides of NO_x or, in the case when SNAP was used, transnitrosylation (Arnelle & Stamler, 1995) since the direct reaction of NO with thiol is very slow (Pryor et al., 1982).

Many enzymes utilize the thiolate functional groups of cysteine residues to coordinate the zinc atom (Vallee & Falchuk, 1993) in formation of "zinc fingers" ($Zn_1Cys_2His_2$) or "zinc clusters" (Zn_1Cys_4). ADH is an enzyme having the zinc/thiolate ($Zn_1Cys_2His_1H_2O$) moiety which is structurally similar to the zinc finger ($Zn_1Cys_2His_2$). ADH is a dimer containing two zinc atoms per subunit; one zinc atom is found in the catalytic site of the enzyme bound to three ligands (Cys 46, His 67, and Cys 174), and the second zinc, which plays a structural role, is bound to four cysteine residues (97, 100, 103, and 111) (Vallee & Auld, 1990). Enzymatic activity of ADH is dependent on maintaining the integrity of zinc coordination at the catalytic site and perhaps at the noncatalytic zinc site. Removal of the active site zinc

ions by chelating agents (Maret, 1979) leads to inactivation of liver ADH (Reynolds & McKinley-McKee, 1970; Li & Vallee, 1964; Dahl & McKinley-McKee, 1981). Recently, Crow et al. (1995) found that the powerful oxidant peroxynitrite inactivated yeast ADH in association with zinc release and thiol/thiolate oxidation. Wink and Laval (1994) have shown that NO generated by DEA/NO irreversibly inhibited the activity of the zinc finger-containing DNA repair enzyme, formamido pyrimidine-DNA glycolyse. Kroncke et al. (1994) reported that NO destroys zinc/sulfur clusters inducing zinc release from metallothionein via oxidation of SH groups and formation of *S*-nitroso thiols. They also reported that NO inhibits DNA binding activity of the yeast transcription factor LAC9, which contains a zinc finger-like DNA binding domain.

Our results suggest that the inhibitory effect of NO on liver ADH activity can probably be explained by the ability of NO to disrupt zinc/thiolate centers upon attachment of NO to critical thiols. Nitrosylation of these thiols subsequently causes the release of zinc. This suggestion is supported by the observed nitrosylation, loss of thiols, and loss of zinc and the observation that this inhibition after NO treatment is reversible upon restoring free thiols with DTT followed by incubation with $ZnCl_2$ to allow reincorporation of the metal into the enzyme. We observed that a 25−30% loss of zinc (0.5−0.6 mol of zinc released per subunit of ADH) was associated with a 40−50% loss of catalytic activity. It is not known whether the zinc is released from the catalytic or the structural site. If all or most of the zinc was released from the catalytic site, then release of 50−60% of the catalytic site zinc was associated with a 40−50% loss of catalytic activity. However, the structural site zinc may also be critical for maintaining ADH activity.

It is interesting to speculate as to whether NO may influence the metabolism of ethanol and modify the metabolic effects produced as a consequence of the metabolism of ethanol by ADH, e.g. lowering of the hepatic NAD^+/NADH redox state or formation of acetaldehyde. These effects would be dependent upon the concentration of NO generated in vivo and the total exposure period to these levels of NO. Further studies are in progress to evaluate these possibilities, as well as to determine the effects of NO on other alcohol-metabolizing enzymes such as CYP2E1.

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REFERENCES

Abu-Soud, H. M., Wang, J., Rousseau, D. L., Fukuto, J. M., Ignarro, L. J., & Stuehr, D. J. (1995) *J. Biol. Chem. 270*, 22997−23006.

Arnelle, D. R., & Stamler, J. S. (1995) *Arch. Biochem. Biophys. 318*, 279−285.

Asahi, M., Fujii, J., Suzuki, K., Seo, H. G., Kuzuya, T., Hori, M., Tada, M., Fujii, S., & Taniguchi, N. (1995) *J. Biol. Chem. 270*, 21035−21039.

Bigatello, L. M., Broitman, S. A., Fattori, L., Di-Paoli, M., Pontello, M., Bevilacqua, G., & Nespoli, A. (1987) *Am. J. Gastroenterol. 82*, 11−15.

Bird, G., Sheron, N., Goka, J., Alexander, G. J. M., & Williams, R. (1990) *Ann. Intern. Med. 112*, 917−920.

Bode, C., Kugler, V., & Bode, J. C. (1987) *J. Hepatol. 4*, 8−14.

Brune, B., & Lapetina, E. G. (1989) *J. Biol. Chem. 264*, 8455−8458.

Castro, L., Rodriguez, M., & Radi, R. (1994) *J. Biol. Chem. 269*, 29409−29415.

Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., & Shapira, A. H. V. (1994) *FEBS Lett. 345*, 50−54.

Crow, J. P., Beckman, J. S., & McCord, J. M. (1995) *Biochemistry 34*, 3544−3552.

Dahl, K. H., & McKinley-McKee, J. S. (1981) *Eur. J. Biochem. 118*, 507−513.

Dimmeler, S., & Brune, B. (1992) *Eur. J. Biochem. 210*, 305−310.

Dimmeler, S., & Brune, B. (1993) *FEBS Lett. 315*, 21−24.

Dimmeler, S., Lottspeich, F., & Brune, B. (1992) *J. Biol. Chem. 267*, 16771−16774.

Ellman, G. (1959) *Arch. Biochem. Biophys. 82*, 70−77.

Gaston, B., Reilly, J., Drazen, J. M., Fackler, J., Ramdev, P., Arnelle, D., Mullins, M. E., Sugarbaker, D. J., Chee, C., Singel, D. J., Loscalzo, J., & Stamler, J. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10957−10961.

Geller, D., Nussler, A. K., DiSilvio, M., Lowenstein, Ch. J., Shapiro, R. A., Wang, S. C., Simmons, R. L., & Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. U.S.A., 90*, 522−526.

Gopalakrishna, R., Chen, Z. H., & Gundimeda, U. (1993) *J. Biol. Chem. 268*, 27180−27185.

Green, L. C., Wagner, D. A., Glogovski, J., Skipper, P. L., Withnok, J. S., & Tannenbaum, S. R. (1982) *Anal. Biochem. 126*, 131−138.

Hartmann, S., Neeff, J., Heer, U., & Mecke, D. (1978) *FEBS Lett. 93*, 339−342.

Hausladen, A., & Fridovich, I. (1994) *J. Biol. Chem. 269*, 29405−29408.

Honchel, R., Ray, M., Marsano, L., Cohen, D., Lee, E., Shedlovsky, S., & McClain, C. J. (1992) *Alcohol.: Clin. Exp. Res. 16*, 665−669.

Hunt, J. B., Neece, S. H., & Ginsburg, A. (1985) *Anal. Biochem. 146*, 150−157.

Hunt, N. C. A., & Goldin, R. D. (1992) *J. Hepatol. 14*, 146−150.

Ignarro, L. J. (1990) *Pharmacol. Toxicol. 67*, 1−7.

Jia, L., Bonaventura, C., Bonaventura, J., & Stamler, J. S. (1996) *Nature 380*, 221−226.

Kanner, J., Harel, S., & Granit, R. (1992) *Lipids 27*, 46−49.

Kots, A. Ya., Skurat, A. V., Sergirnko, E. A., Bulgarina, T. V., & Severin, E. S. (1992) *FEBS Lett. 300*, 9−13.

Kroncke, K. D., Fehsel, K., Schmidt, T., Zenke, F. T., Dasting, I., Wesener, J. R., Bettermann, H., Breunig, K. D., & Bachofen, V. K. (1994) *Biochem. Biophys. Res. Commun. 200*, 1105−1110.

Li, T.-K., & Valle, B. L. (1964) *Biochemistry 3*, 869−873.

Maret, W. (1979) *Eur. J. Biochem. 98*, 501−502.

McDonald, L. J., & Moss, J. (1993a) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6238−6241.

McDonald, L. J., & Moss, J. (1993b) *J. Biol. Chem. 268*, 17878−17882.

McDonald, L. J., & Moss, J. (1993c) *Trans. Assoc. Am. Physicians 106*, 155−161.

Midgely, S., Grant, I. S., Haynes, W. G., & Webt, D. J. (1991) *Lancet 338*, 1590−1593.

Mohr, S., Stamler, J. S., & Brune, B. (1996) *J. Biol. Chem. 271*, 4209−4214.

Molina y Vedia, l., McDonald, B., Reep, B., Brune, B., Di Silvio, M., Billiar, T. R., & Lapetina, E. G. (1992) *J. Biol. Chem. 276*, 24929−24932.

Moncada, S., Palmer, R. M., & Higgs, E. A. (1991) *Pharmacol. Rev. 43*, 109−142.

Morely, D., Maragos, C. M., Zhang, X.-Y., Biognon, M., Wink, D. A., & Keefer, L. K. (1993) *J. Cardiovasc. Pharamacol. 21*, 670−676.

Murphy, M. E., & Noack, E. (1994) *Methods Enzymol. 233*, 240−250.

Nathan, C. (1992) *FASEB J. 6*, 3051−3064.

Pryor, W. A., Church, D. F., Govindan, C. K., & Crank, G. (1982) *J. Org. Chem. 47*, 156−159.

Reynolds, C. H., & McKinley-McKee, J. S. (1970) *Biochem. J. 119*, 801−802.

Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 81*, 49−60.
Saville, B. (1958) *Analyst 83*, 670−672.
Siegfried, G., Amiel, C., & Friedlander, G. (1996) *J. Biol. Chem. 271*, 4659−4664.
Simon, D. I., Mullins, M. E., Lia, L., Gaston, B., Singel, D. J., & Stamler, J. S. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4736−4741.
Stadler, J., Billiar, T., Curran, R. D., Stuehr, D. J., Ochoa, J. B., & Simmons, R. L. (1991) *Am. J. Physiol. 260*, C910−916.
Stamler, J. S. (1994) *Cell 78*, 931−936.
Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., & Localzo, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 444−448.
Vallee, B. L., & Auld, D. S. (1990) *Biochemistry 29*, 5647−5659.
Vallee, B. L., & Falchuk, K. H. (1993) *Physiol. Rev. 73*, 79−118.
Wang, J. F., Greenberg, S. S., & Spitzer, J. J. (1995) *Alcohol.: Clin. Exp. Res. 19*, 387−392.
Wink, D. A., & Laval, J. (1994) *Carcinogenesis 15*, 2125−2129.
Wu, D., Clejan, L., Potter, B., & Cederbaum, A. I. (1990) *Hepatology 12*, 1379−1389.
Zhang, J., & Snyder, S. H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9382−9385.

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