

Effects of Nitric Oxide on the Copper-Responsive Transcription Factor Ace1 in *Saccharomyces cerevisiae*: Cytotoxic and Cytoprotective Actions of Nitric Oxide

Kenneth T. Chiang,* Masaru Shinyashiki,* Christopher H. Switzer,* Joan S. Valentine,† Edith B. Gralla,† Dennis J. Thiele,‡ and Jon M. Fukuto*¹

*Department of Pharmacology, UCLA Medical School, Center for the Health Sciences, Los Angeles, California 90095;

†Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90095;

and ‡Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109

Received November 1, 1999, and in revised form February 18, 2000

Previous studies indicate that nitric oxide (NO) can serve as a regulator/disrupter of metal-metabolizing systems in cells and, indeed, this function may represent an important physiological and/or pathophysiological role for NO. In order to address possible mechanisms of this aspect of NO biology, the effect of NO on copper metabolism and toxicity in the yeast *Saccharomyces cerevisiae* was examined. Exposure of *S. cerevisiae* to NO resulted in an alteration of the activity of the copper-responsive transcription factor Ace1. Low concentrations of the NO donor DEA/NO were found to slightly enhance copper-mediated activation of Ace1. Since Ace1 regulates the expression of genes responsible for the protection of *S. cerevisiae* from metal toxicity, the effect of NO on the toxicity of copper toward *S. cerevisiae* was also examined. Interestingly, low concentrations of NO were also found to protect *S. cerevisiae* against the toxicity of copper. The effect of NO at high concentrations was, however, opposite. High concentrations of DEA/NO inhibited copper-mediated Ace1 activity. Correspondingly, high concentrations of DEA/NO (1 mM) dramatically enhanced copper toxicity. An intermediate concentration of DEA/NO (0.5 mM) exhibited a dual effect, enhancing toxicity at lower copper concentrations (<0.5 mM) and protecting at higher (≥0.5 mM) copper concentrations. Thus, it is proposed that the ability of NO to both protect against (at low concentrations) and enhance (at high concentration) copper toxicity in *S. cerevisiae* is, at least partially, a result of its effect on Ace1. The results of this study have implications for the role of

NO as a mediator of metal metabolism. © 2000 Academic Press

Key Words: nitric oxide; yeast; *Saccharomyces cerevisiae*; copper; transcription factor; Ace1; cytotoxicity.

Nitric oxide (NO)² is an endogenously generated species synthesized by a variety of cells. It has both physiological and pathophysiological roles (for a review, see 1). For example, NO generated at low levels by endothelial cells is an important regulator of vascular tone. Nitric oxide generated at high levels in an immune response is an important pathophysiological agent with cytotoxic/cytostatic actions against invading pathogens and tumor cells. Thus, there exists a spectrum of activity associated with NO which is a function of not only the site of its generation but also its levels. Some of the earliest studies regarding the biological effects of NO indicated that it is capable of altering metal homeostasis. High levels of NO were found to affect the iron status of cells (for example, 2), possibly by disruption of iron–sulfur (Fe–S) cluster proteins. Since then, others have described the ability of NO, and NO-derived species, to react with the Fe–S containing iron-response protein, altering its activity (for example, 3–6). Alteration of gene expression has also been proposed to occur via NO-mediated destruction of the zinc–thiol bonds in Zn-dependent DNA-binding

¹ To whom correspondence should be addressed. Fax: (310) 825-6267. E-mail: jfukuto@mednet.ucla.edu.

² Abbreviations used: NO, nitric oxide; Sod1, antioxidant enzyme copper–zinc superoxide dismutase; DEA/NO, diethylammonium 1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate; DDW, double-distilled water; ONPG, *o*-nitrophenyl β-D-galactopyranoside; SDC, synthetic dextrose medium.

proteins (7, 8), and the zinc-containing DNA repair enzyme Fpg is inhibited by NO both *in vitro* and *in vivo* (9). Finally, demetalation of the thiol-rich, metal-sequestering protein metallothionein by an NO-mediated process could lead to cellular toxicity associated with the released metal (8, 10). Based on these previous reports, it appears that proteins which ligate metals via thiol coordination may be particularly susceptible to reaction with NO and/or NO-derived species.

It is well established that much of the unique biology of NO is a result of its chemical interaction with thiol-containing peptides and proteins (for example, 11, 12). Considering the role of thiols in protein-metal binding, it is likely that NO disruption of the actions of metal-binding proteins via thiol modification may represent an important event in the pathophysiological actions (and possibly the normal physiological actions) of NO. An attractive way to address the idea that NO (and/or NO-derived species) can alter the actions of thiol-containing metal-binding proteins is to examine the effects of NO on the yeast transcription factor Ace1. Ace1 is a copper-responsive yeast transcription factor responsible for binding and transactivation of the *CUP1* gene promoter (for example, 13). The *CUP1* gene encodes for a protein (Cup1 or yeast metallothionein) which is functionally similar and bears some resemblance to the mammalian metallothioneins (14–16). It has also been found that the Ace1 controls the copper-mediated expression of the antioxidant enzyme copper-zinc superoxide dismutase (Sod1) (17) as well as another metallothionein-like protein, Crs5 (18).

Resistance to copper toxicity in yeast is mediated by the *CUP1* locus (19, 20), indicating that yeast metallothionein may play a major role in the detoxification of copper. Thus, copper-mediated activation of Ace1 leads to an increased expression of several major copper-binding proteins and to an increased level of protection against copper and reactive oxygen species. Yeast metallothionein binds copper in a trigonally coordinated geometry with cysteine thiolates similar to that seen in mammalian metallothioneins (21).

The copper-binding motif in Ace1 is similar to that found for the metallothioneins (22, 23). That is, copper binding in Ace1 occurs through ligation via protein thiols. Since it has been reported previously that NO can disrupt the metal-binding capability of metallothionein, it may be expected that NO may disrupt the actions of Ace1 in a similar manner. Furthermore, disruption of Ace1 metal binding by NO would be expected to dramatically alter the resistance of yeast toward copper toxicity. Therefore, studies are described herein which specifically examine the interaction of NO with Ace1 in *Saccharomyces cerevisiae*. The toxicological manifestation of the effects of NO on Ace1 is also examined by investigating the effects of NO on copper toxicity toward *S. cerevisiae*.

EXPERIMENTAL PROCEDURES

Chemicals and solutions. Diethylammonium 1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) was synthesized by the method of Drago and Paulik (24). Stock solutions of DEA/NO were prepared in double-distilled water (DDW) immediately prior to use. Diphenylthiocarbazone and *o*-nitrophenyl β -D-galactopyranoside (ONPG) were purchased from Sigma (St. Louis, MO) and bathocuproine disulfonic acid was purchased from Aldrich (Milwaukee, WI). All other chemicals and solutions were purchased from commercial suppliers and were of the highest purity available.

Yeast strains/mutants. The parental wild-type strain utilized was EG217 (W303A, *MATa leu2-3,112 trp1-1 his3-11,15 ura3-1 ade2-1*). A high copy number reporter plasmid construct harboring a *CUP1*- β -galactosidase fusion gene (YEPCUP-HSE-M-lacZ), an Ace1 overexpression plasmid (YEPCUP1), and an *ace1* Δ mutant (KKY7 (W303 1a containing the *ace1* Δ 255 complete deletion of the ACE1 open reading frame)) were constructed (25; D. Koch and D. J. Theile, unpublished).

Viability studies. Yeast cells from an overnight culture were inoculated at an OD₆₀₀ = 0.1 and grown to late-log phase (OD₆₀₀ = 3.0–3.5) at 30°C in synthetic dextrose medium (SDC) (26). Cell suspension was divided into 1-mL aliquots and exposed to CuSO₄ (0–1 mM) and/or DEA/NO (0–1 mM) for 4 h at 30°C in a shaking water bath. After the 4-h exposure, the cell suspension was diluted 1:1000 in water and 10 μ L was plated on nutrient-rich (YPD) plates (26). Viability was assessed by counting the colonies formed relative to control.

Yeast transformations. Yeast transformations were performed according to the general method of Elble (27). Transformation of EG217 with YEPCUP-HSE-M-LacZ and YEPCUP1 resulted in 217CUP and 217ACE, respectively.

Nitric oxide inhibition of Ace1 transcriptional activity. Following the method of Holmquist (28), SD medium with uracil omitted (SD-ura) was extracted with diphenylthiocarbazone, resulting in SD-ura-Cu. 217CUP yeast cells were inoculated in SD-ura-Cu containing 0.1 mM bathocuproine disulfonic acid (BCS) (Aldrich) at an OD₆₀₀ = 0.1 and grown to OD₆₀₀ = 3–4 at 30°C. To induce *CUP1* promoter-linked β -galactosidase activity, cells were washed twice with DDW and resuspended in 10 mL of SD-ura containing 0–250 μ M CuSO₄. After incubation at 30°C for 0–120 min in a shaking water bath, induction was stopped by placing on ice. Cells were then pelleted by centrifugation for 5 min at 850g. The pellet was washed once with DDW, followed by centrifugation and then frozen in liquid nitrogen and stored at –80°C until use. To test the effect of NO on the induction of β -galactosidase activity, the above-described procedure was carried out identically except in the presence of 0–1 mM of the NO donor DEA/NO and at a fixed copper sulfate concentration of 0.1 mM. DEA/NO was added to the samples at the same time as copper sulfate addition. Control experiments were run in parallel with distilled deionized water addition instead of CuSO₄ or DEANO.

β -Galactosidase activity was determined by the method of Thorvaldsen *et al.* (29). Briefly, the yeast pellet was defrosted on ice and 2–4 volumes of Z buffer (50 mM Na₂P₄, pH 7.0, containing 10 mM KCl, 1 mM MgSO₄, and 50 mM 2-mercaptoethanol). The cell suspension was vortexed for 30 s six times in 1 volume of glass beads (0.5-mm diameter) and then centrifuged for 5 min at 15,000g. The cell extract (5–10 μ g of protein) was incubated with 1 mL of Z buffer containing 0.63 mg/mL of ONPG at 30°C for 5 min. The reaction was stopped by adding 0.3 mL of 1 M Na₂CO₃ and absorbance was measured at 420 nm. Protein concentration was determined by the method of Bradford (30), using BSA as the standard. β -Galactosidase activity was calculated based on units defined as (Abs₄₂₀)/(1000)/minute/milligram of protein.

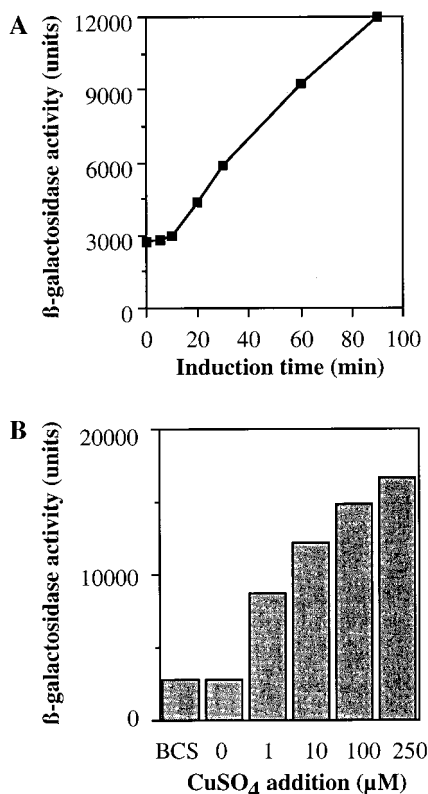


FIG. 1. Characterization of reporter plasmid system. 217CUP yeast transformed cells were exposed to 0.1 mM CuSO₄ for 0–90 min (A) or 0–0.25 mM CuSO₄ for 60 min (B) at 30°C. Ace1 transcriptional activity was determined as a function of β -galactosidase activity. Representative experiments are shown.

RESULTS

Effect of NO on Ace1 activity. Previous work by others indicates that metal-binding proteins, such as metallothionein, which utilize sulfur as a metal coordinating ligand may be susceptible to modification by NO (10). Since the metal-responsive yeast transcription factor Ace1 has homology to metallothionein, it is conceivable that Ace1 activity can be altered by exposure to NO.

The idea that NO can alter the copper-mediated activation of Ace1 was examined utilizing a *CUP1*- β -galactosidase (*lacZ*) gene fusion plasmid. Thus, this reporter gene plasmid was transformed into the EG217 yeast strain and its utility as a measure of copper-mediated Ace1 activation of β -galactosidase expression examined. β -Galactosidase activity was found to increase as the time of exposure to 0.1 mM copper sulfate increased (Fig. 1A). β -Galactosidase activity was also found to increase in the transformed yeast with increasing concentration of copper sulfate after a 60-min exposure, with maximum expression observed with 250 μ M copper sulfate (Fig. 1B). These results are consistent with the known ability of copper to bind/

activate Ace1, leading to transactivation of β -galactosidase expression in the reporter plasmid via *CUP1* promoter binding. The effect of NO on copper-mediated Ace1 activation was examined using the diazeniumdiolate NO donor diethylammonium 1-(*N,N*-diethylamino)-diazene-1-ium-1,2-diolate (DEA/NO). Thus, yeast cells (grown to late-log phase, OD₆₀₀ = 3–4) were exposed simultaneously to 0.1 mM copper sulfate and varying levels of DEA/NO for 1 h. Low concentrations of DEA/NO (0.1 mM) resulted in a small (8–10%), but reproducible, increase in copper-mediated Ace1 activity (Fig. 2A). However, increasing concentrations of DEA/NO (0.2–1 mM) resulted in a decrease in copper-mediated Ace1 activation. An approximate 75 and 90% decrease in copper-mediated Ace1 activation was observed at 0.5 and 1 mM DEA/NO exposure, respectively. Nearly identical results were also found with two other diazeniumdiolate NO donors, (*Z*)-1-[*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino]diazene-1-ium-1,2-diolate (SPER/NO) and (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazene-1-

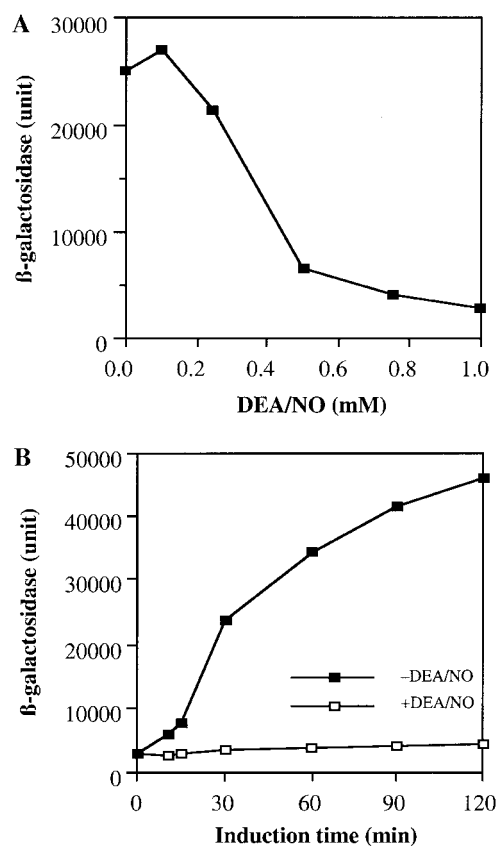


FIG. 2. Effect of DEA/NO on *CUP1-lacZ* activity. (A) Concentration-dependent inhibition of Ace1 transcriptional activity by 0–1 mM DEA/NO in medium containing 0.1 mM CuSO₄ and incubated for 60 min. (B) Time course of inhibition of 1 mM DEA/NO on Ace1 transcriptional activity in medium containing 0.1 mM CuSO₄ and incubated for 0–120 min. Representative experiments are shown.

ium-1,2-diolate] (PAPA/NO). That is, low (0.1 mM) concentrations of these donors resulted in a small, but significant, increase in copper-mediated Ace1 activation while increased concentrations resulted in a decrease in copper-mediated Ace1 activation (data not shown). Simultaneous addition of 1 mM DEA/NO and 0.1 mM copper sulfate followed by a 10- to 120-min incubation showed that the inhibition of copper-mediated Ace1 activation was essentially irreversible for the 2-h exposure (Fig. 2B). The half-life of DEA/NO under the conditions of these experiments was approximately 4–8 min, indicating that the NO generated from DEA/NO was likely to be completely dissipated after 120 min. Analysis of experimental solutions of 1 mM DEA/NO after 1 h showed no detectable ($<0.1 \mu\text{M}$) NO (data not shown). Thus, the inhibition of copper-mediated Ace1 activation by NO appears to last far beyond the solution lifetime of NO. Interestingly, previous work indicates that the ability of NO to inhibit copper-dependent Ace1 activation does not require the simultaneous presence of NO and copper since pre-treatment of yeast with DEA/NO prior to treatment with copper gives the same effect (31).

Effect of NO on yeast susceptibility to copper toxicity. The above results indicate that NO is able to quickly and irreversibly inhibit the activation of Ace1 by copper. Moreover, this inhibition appears to be independent of the presence of NO after the initial exposure. Of significance is the small, but reproducible, ability of low levels of NO to increase copper-mediated activation of Ace1. Since Ace1 activity is vital to yeast resistance against the toxicity of copper, the above results would predict that NO exposure may modify yeast susceptibility to copper toxicity. Therefore, the effect of NO on yeast copper toxicity was examined.

In order to investigate whether the effect of NO on Ace1 activation is manifested in a change in yeast susceptibility toward copper toxicity, yeast cells were exposed simultaneously to 0, 0.1, 0.5, and 1 mM DEA/NO and 0, 0.01, 0.05, 0.1, 0.5, and 1 mM copper sulfate for 4 h. Following the 4-h exposure period, cells were replated on YPD plates and colony formation used as a measure of cell viability. As shown in Fig. 3A, a low concentration of DEA/NO (0.1 mM) protects yeast from copper toxicity. However, a higher concentration of DEA/NO (1 mM) increases the susceptibility of the yeast to copper toxicity at concentrations of copper as low as 0.01 mM. An intermediate concentration of DEA/NO (0.5 mM) exhibits a slight increase in yeast susceptibility to low levels of copper but protection at high copper levels. Further investigation of the protective effect of NO at low concentrations indicates that 0.1 mM DEA/NO represents the optimum concentration for protection (at least among the DEA/NO concentrations examined) since 0.05 and 0.01 mM

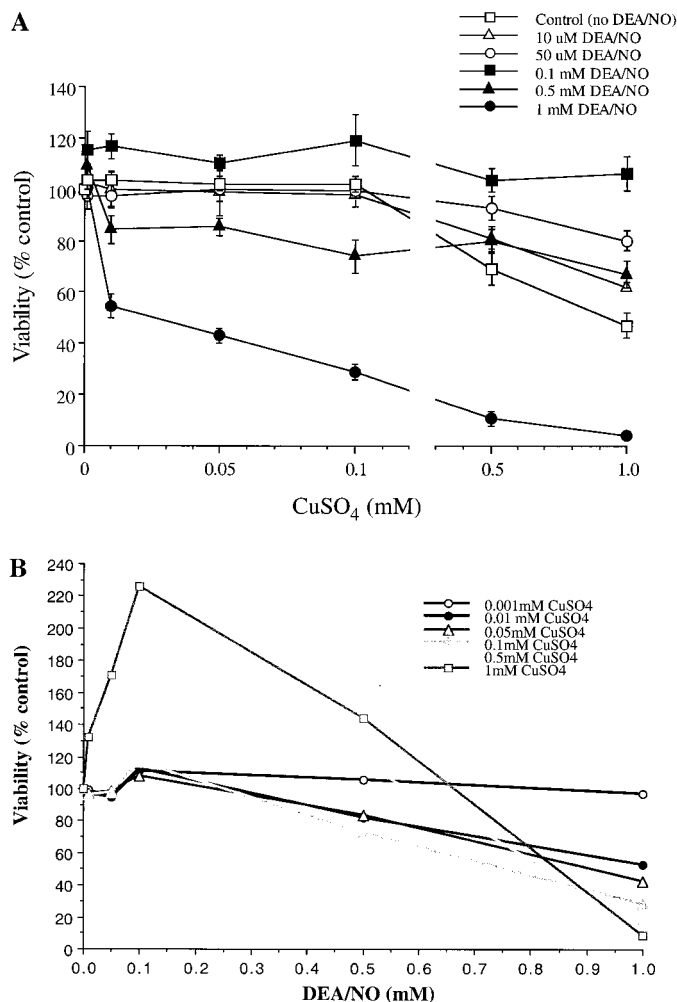


FIG. 3. Effect of DEA/NO on copper toxicity in wild-type EG217 yeast. Wild-type EG217 yeast cells were grown to late-log phase in synthetic dextrose medium (SDC). (A) Cells were exposed to 0 mM (open squares), 10 μM (open triangles), 50 μM (open circles), 0.1 mM (closed squares), 0.5 mM (closed triangles), or 1 mM (closed circles) DEA/NO immediately following addition of 0–1 mM CuSO₄. Cells were incubated for 4 h at 30°C and assayed for viability. Viability is expressed as the percentage of colonies formed relative to control (no CuSO₄). The values are the means ($n \geq 3$) derived from separate experiments ($\pm$ SE). (B) Different representation of the same data to show the relative effects of 0–1 mM DEA/NO on yeast viability in 0–1 mM CuSO₄. Viability is expressed as percentage of colonies formed relative to control (no DEA/NO).

DEA/NO exhibited less protection (Fig. 3B). Furthermore, the degree of protection against copper toxicity by 0.1 mM DEA/NO increased with increasing levels of copper exposure. That is, 0.1 mM DEA/NO protected against 0.1 mM copper by about 15–20% whereas over 200% protection was observed at 1 mM copper (versus controls with no DEA/NO present).

The observed bilateral concentration-dependent effect of NO on copper toxicity toward yeast is similar to that observed previously with DEA/NO on Ace1 activ-

TABLE I

Effects of NO on Copper-Dependent Ace1 Activation and Resistance to Copper Toxicity

Conditions	Ace1 activity (% control) ^a	Viability (% control) ^a
0.1 mM DEA/NO	108 ^b	116
0.1 mM CuSO ₄		
0.1 mM DEA/NO	154	276
1 mM CuSO ₄		
1 mM DEA/NO	0	9
1 mM CuSO ₄		

^a Control experiments were carried out in the absence of DEA/NO but at the copper sulfate levels listed (see Experimental Procedures for details). Values represent the average of duplicate samples and are representative of several experiments.

^b This value was taken from Fig. 2B.

ity. That is, as hypothesized above, the effect of NO on the copper-mediated activation of Ace1 is mirrored by the effect of NO on yeast susceptibility toward copper toxicity. Consistent with the modest protection by 0.1 mM DEA/NO against low (0.1 mM) copper, a slight increase in Ace1 activity was also observed under similar conditions (0.1 mM DEA/NO and 0.1 mM copper, Fig. 2B). However, the ability of low 0.1 mM DEA/NO to protect yeast from copper toxicity was significantly more pronounced at high (1 mM) copper. Thus, in order to further examine the apparent parallel between the effect of NO on copper-mediated Ace1 activity and susceptibility to copper toxicity, the effect of 0.1 mM DEA/NO on Ace1 activity in the presence of high (1 mM) copper was determined. It was found that copper-dependent Ace1 activity was greatly enhanced in the presence of 0.1 mM DEA/NO at 1 mM copper. The similarity between the effects of NO on Ace1 activity and yeast viability in response to copper is shown in Table I. Thus, both the effect of NO on copper-dependent Ace1 activation and that on the resistance to copper toxicity are highly correlative; both are slightly enhanced at low NO (0.1 mM DEA/NO) and low copper (0.1 mM copper sulfate), both are dramatically enhanced at low NO (0.1 mM) and high copper (1 mM copper sulfate), and finally, both are inhibited by high NO (>0.5 mM DEA/NO) at any copper concentration (0.1–1 mM copper sulfate).

If indeed the effect of NO on copper-mediated Ace1 activation were primarily responsible for the effects of NO on yeast copper sensitivity, it would be expected that *ace1Δ* mutants would not exhibit a dramatic NO-dependent increase in susceptibility toward copper toxicity at high NO levels and no increase in resistance at low NO levels. Thus, *ace1Δ* mutants were exposed simultaneously to 0, 0.1, and 1 mM DEA/NO along with 0–1 mM copper sulfate for 4 h. Viability was assayed,

as before, by counting colonies formed after replating the exposed cultures onto YPD plates.

Yeast *ace1Δ* mutants exhibited the expected increase in susceptibility toward copper toxicity (Fig. 4A). Similar to wild-type yeast, exposure of *ace1Δ* mutants to 1 mM DEA/NO also exhibited an increase in susceptibility to copper toxicity. However, unlike the effect exhibited by wild-type yeast, low levels of NO did not protect against copper toxicity in the *ace1Δ* mutants. Finally, in yeast mutants overexpressing *ACE1*, the ability of NO to enhance copper toxicity was significantly blunted (compare Figs. 3A and 4B).

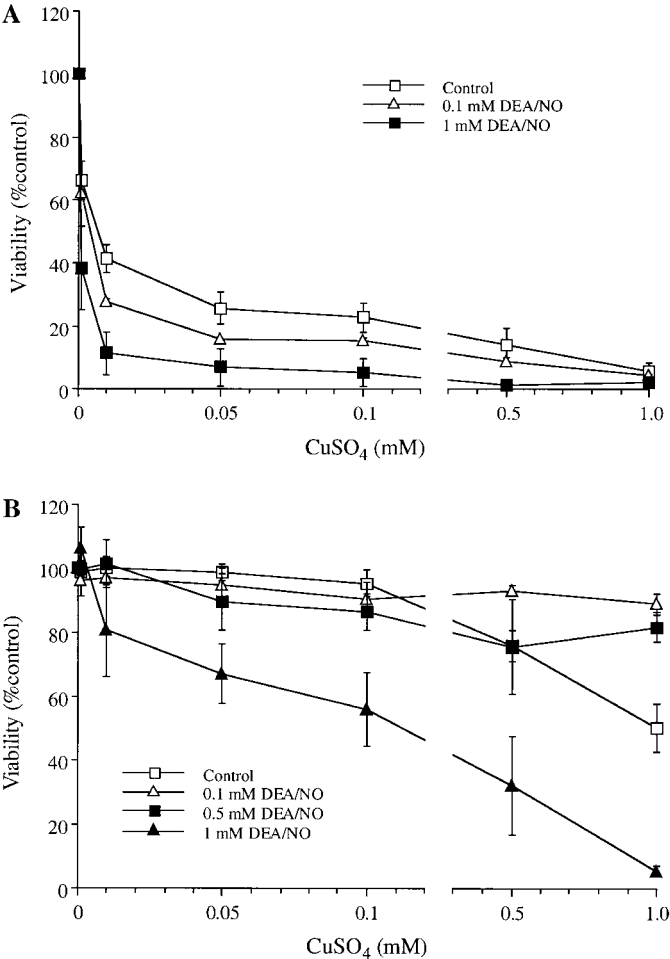
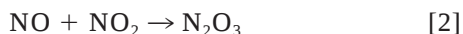


FIG. 4. Effect of DEA/NO on copper toxicity in *ace1Δ* and Ace1-overexpressing yeast. *ace1Δ* (KKY7) or Ace1-overexpressing (217ACE) yeast cells were grown to late-log phase in synthetic dextrose medium with uracil omitted (SD-ura) or synthetic dextrose medium with leucine omitted (SD-leu), respectively. (A) KKY7 yeast cells were exposed to 0, 0.1, or 1 mM DEA/NO immediately following addition of 0–1 mM CuSO₄. (B) 217ACE yeast cells were exposed to 0, 0.1, 0.5, or 1 mM DEA/NO immediately following addition of 0–1 mM CuSO₄. Viability is expressed as the percentage of colonies formed relative to control (no CuSO₄). The values are the means ($n \geq 2$) derived from separate experiments ($\pm$ SE).

DISCUSSION

There exists a close correlation between the actions of NO on the copper-dependent activation of the yeast transcription factor Ace1 and the effect of NO on the susceptibility to copper toxicity in *S. cerevisiae* since (1) NO at high concentrations inhibits copper-dependent Ace1 activation (Fig. 2) and enhances copper toxicity (Fig. 3), (2) NO at low concentrations enhances copper-dependent Ace1 activation (Fig. 2) and increases the resistance of yeast to copper toxicity (Fig. 3), (3) unlike wild-type yeast, *ace1Δ* mutants exhibit no protection from copper toxicity at low NO levels (Fig. 4A), and (4) *ACE1*-overexpressing mutants are less susceptible to the enhancement of copper toxicity by NO (Fig. 4B). The close correlation between Ace1 activity and copper toxicity is not unreasonable since the *CUP1* locus (the Ace1 binding site) is known to be responsible for copper resistance in yeast (19, 20). Thus, high levels of NO inhibit copper-dependent Ace1 activation, which would result in a decreased copper-dependent expression of yeast metallothioneins and Sod1. Presumably, the NO-mediated loss or decrease of expression of these stress-protective proteins leads to an increased susceptibility to copper toxicity. To be sure, the inhibition of Ace1 activity by high-level exposure is not likely to be the only mechanism by which NO can increase the toxicity of copper. This is evidenced by the observation that yeast mutants delete in *ACE1* still exhibit a slight increase in susceptibility to copper toxicity in the presence of NO (Fig. 4A). However, it is clear that NO inhibition of Ace1 is a major, though not exclusive, mechanism by which NO increases yeast susceptibility to copper toxicity.

The mechanism by which NO inhibits copper-dependent Ace1 activation is unclear. However, due to the similarity in metal binding of Ace1 and metallothioneins, the interaction of NO with metallothionein should provide mechanistic clues into the nature of the NO–Ace1 interaction. Previous studies of the chemistry of NO with metallothionein indicate that metal loss via thiol modification occurs (8, 10). By analogy, it is likely that NO-mediated thiol ligand oxidation or modification (possibly via nitrosothiol formation) causes metal release and/or renders Ace1 incapable of copper binding, therefore precluding binding to the *CUP1* promoter. For example, oxidation of NO by O₂ generates N₂O₃, which is capable of nitrosating thiols (reactions [1]–[3]).



Furthermore, it is well established that metal-binding proteins such as metallothionein and Ace1 are highly susceptible to proteolytic degradation when demetalated (for example, 16, 32–35). Thus, NO-mediated demetalation of Ace1 may cause it to be degraded proteolytically, therefore not allowing the cell to transcriptionally respond to copper stress. Alternatively, the metal-binding thiols of Ace1 may be converted to oxidized species by NO (or NO-derived species) which cannot be physiologically reduced back to the active thiols. For example, reaction of thiols with HNO, the one-electron reduction product of NO, results in the formation of the stable sulfinamide species (36).

It was also found that low levels of NO (represented by 0.1 mM DEA/NO) slightly increase the copper-dependent activation of Ace1 and exhibit dramatic protection against copper toxicity. The increased resistance to copper toxicity exhibited by low levels of NO is particularly evident at the most toxic levels of copper (Fig. 3B). The inability of low-level NO to offer protection from copper toxicity in the Ace1-delete yeast mutant (Fig. 4A) further supports the idea that Ace1 activity is vital to protection from copper and that the effect of NO on yeast susceptibility to copper toxicity is intimately linked with its actions on Ace1. Currently, there is no obvious biochemical, physiological, or chemical precedence to serve as a reasonable rationale for the phenomena observed at low NO levels, but several hypotheses may be advanced. One possibility is that low NO levels elicit the release of copper from a site more labile than the copper binding site of Ace1. For example, copper stored in Cup1 or vacuolar copper may be released by NO levels not capable of inactivating Ace1. This would lead to an activation of Ace1, increased expression of Sod1 and metallothionein, and, therefore, a protection against copper toxicity. However, considering the known similarity between Ace1 and Cup1, this may not be likely (although vacuolar copper may still be a pool of copper with particular sensitivity to release by NO). It is more likely that NO may be protecting cells from high copper exposure by inhibiting the uptake of copper into the cell. For example, NO may inhibit the actions of the copper-responsive transcription factor Mac1 (37, 38), which would lead to a decrease in expression of the high-affinity copper uptake transporter Ctr1 (39). This would preclude high-affinity copper uptake and, therefore, protect the yeast from exposure to high levels of copper. Since only cuprous ion is a substrate for the high-affinity uptake transporter Ctr1, yeast contain a membrane-bound reductase, Fre1, for reducing extracellular cupric ion to transportable cuprous ion (40). Thus, inhibition of Fre1 would also be expected to provide some protection from copper toxicity by inhibiting high-affinity copper uptake. In partial support of the idea that low levels of NO protect cells from copper-

mediated toxicity by inhibiting copper uptake, we have observed that low levels of NO dramatically inhibit Fre1 activity (unpublished results).

The ability of NO to regulate and/or alter metal metabolism may represent a vital function in its normal physiology and pathophysiology. For example, high-level NO exposure may render cells susceptible to metal stress by blocking the expression of otherwise protective proteins such as metallothionein or Sod1. Interestingly, it has been reported that increased levels of metallothionein in rat hepatocytes protect them from NO toxicity (41).

The concentration ranges of DEA/NO utilized in this study should generate NO concentrations which are physiologically accessible. Concentrations of NO which may act pathophysiologically, such as in the vicinity of activated macrophages, have been reported to be in the low-micromolar range (i.e., 10 μ M) (for example, 42). The "high-level" DEA/NO concentration (1 mM) used herein has been reported to transiently generate similar (10–30 μ M) levels of NO (43). The low-level DEA/NO exposure (0.1 mM) utilized in this study should generate transient low maximum concentrations of NO (1–2 μ M) (44) and represent normal physiological concentration ranges, which have been reported to be in the range of 1 μ M maximum concentrations (i.e., at the surface of endothelial cells) (45). Thus, the effects observed at the high levels of DEA/NO may represent a mechanism by which NO can be cytotoxic in conjunction with other toxic species such as copper, whereas the effects of low-level DEA/NO exposure indicate that NO can also serve as a regulator of metal metabolism under normal physiological conditions.

These studies on the effect of NO on Ace1 and copper toxicity were initiated as a result of the great degree of understanding of the role of Ace1 in yeast metal metabolism and copper toxicity. That is, the yeast–Ace1 system represents a mechanistically defined and biologically relevant model for the examination of NO–thiol–metal interactions. However, the results herein may have relevance beyond simple yeast–NO interactions. It is becoming increasingly evident that significant homology between metal metabolism in yeast and mammalian cells exists. In fact, *S. cerevisiae* serves as a reasonable template for the discovery of analogous mammalian cell processes. Compared to mammalian systems, the copper regulatory system in *S. cerevisiae* is clearly defined and surprisingly well conserved. Human homologues to many of the proteins involved in copper regulation in yeast have been identified, including two plasma membrane transporters (yeast Ctr1/human CTR1 and yeast Ccc2/human Wilson disease protein) and three cytosolic chaperone proteins (yeast Atx1/human HAH1, yeast Lys7/human CCS, and yeast Cox17/human COX17) (for a review, see 46). In addition, iron transport and copper transport are inti-

mately linked in both humans and yeast (for review, see 47, 48), as seen in the homology between yeast Fet3 and human ceruloplasmin, both multicopper ferroxidases involved in the transport of iron (49–51). Thus, the use of *S. cerevisiae* to study the effects of NO on metal metabolism may have implications to mammalian systems. To be sure, there has not yet been identified a human homologue for Ace1 and it remains to be seen whether NO can behave similarly toward metal–thiol complexes in mammalian cells. However, the use of thiols to bind, transport/chaperone, and sequester copper is well established in both yeast and mammalian systems (i.e., yeast and human CTR1, Ccc2 and Wilson's disease protein, Lys7 and CCS, yeast and human COX17), indicating potential physiological and pathophysiological roles for NO in various aspects of copper metabolism. Moreover, our results regarding the effects of NO on the copper regulatory system of yeast have implications to the metabolism of other metals as well. Since iron metabolism is intimately linked to copper metabolism, the effect of NO on copper-binding proteins can potentially have a significant influence on the iron transport/sequestration/utilization. Also, the interaction of NO with mammalian metallothioneins has implications to zinc and copper metabolism since it has been speculated that mammalian metallothioneins are involved in the metabolism of the two metals. Regardless, the utility of the yeast model system to examine basic NO physiology/pathophysiology is clear and it is likely that the effect on metal metabolism, possibly via NO–protein thiol interactions, will be an important and fundamental aspect of NO biology.

REFERENCES

1. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.* **43**(2), 109–141.
2. Hibbs, J. B., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. (1988) *Biochem. Biophys. Res. Commun.* **157**(1), 87–94.
3. Lipinski, P., and Drapier, J.-C. (1997) *J. Biol. Inorg. Chem.* **2**, 559–566.
4. Bouton, C., Raveau, M., and Drapier, J.-C. (1996) *J. Biol. Chem.* **271**(4), 2300–2306.
5. Drapier, J.-C. (1997) *Methods* **11**, 319–329.
6. Drapier, J.-C., Hirling, H., Wietzerbin, J., Kaldy, P., and Kuhn, L. C. (1993) *EMBO J.* **12**(9), 3643–3649.
7. Berendji, D., Kolb-Bachofen, V., Meyer, K. L., Grapenthin, O., Weber, H., Wahn, V., and Kroncke, K.-D. (1997) *FEBS Lett.* **405**, 37–41.
8. Kroncke, K. D., Fehsel, K., Schmidt, T., Zenke, F. T., Dasting, I., Wesener, J. R., Bettermann, H., Breunig, K. D., and Kolb-Bachofen, V. (1994) *Biochem. Biophys. Res. Commun.* **200**(2), 1105–1110.
9. Wink, D. A., and Laval, J. (1994) *Carcinogenesis* **15**(10), 2125–2129.
10. Misra, R. R., Hochadel, J. F., Smith, G. T., Cook, J. C., Waalkes, M. P., and Wink, D. A. (1996) *Chem. Res. Toxicol.* **9**, 326–332.

11. Stamler, J. S. (1994) *Cell* **78**, 931–936.
12. Stamler, J. S. (1995) *Curr. Top. Microbiol. Immunol.* **196**, 19–36.
13. Thiele, D. J. (1988) *Mol. Cell. Biol.* **8**(7), 2745–2752.
14. Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J., and Fogel, S. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 337–341.
15. Butt, T. R., Sternberg, E. J., Gorman, J. A., Clark, P., Hamer, D., Rosenberg, M., and Crooke, S. T. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3332–3336.
16. Winge, D. R., Nielson, K. B., Gray, W. R., and Hamer, D. H. (1985) *J. Biol. Chem.* **260**(27), 14464–14470.
17. Gralla, E. B., Thiele, D. J., Silar, P., and Valentine, J. S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8558–8562.
18. Culotta, V. C., Howard, W. R., and Liu, X. F. (1994) *J. Biol. Chem.* **269**(41), 25295–25302.
19. Fogel, S., and Welch, J. W. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5342–5346.
20. Fogel, S., Welch, J. W., Cathala, G., and Karin, M. (1983) *Curr. Genet.* **7**, 347–355.
21. George, G. N., Byrd, J., and Winge, D. R. (1988) *J. Biol. Chem.* **263**(17), 8199–8203.
22. Dameron, C. T., Winge, D. R., George, G. N., Sansonne, M., Hu, S., and Hamer, D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6127–6131.
23. Dameron, C. T., George, G. N., Arnold, P., Santhanagopalan, V., and Winge, D. R. (1993) *Biochemistry* **32**, 7294–7301.
24. Drago, R. S., and Paulik, F. E. (1960) *J. Am. Chem. Soc.* **82**, 96–98.
25. Huibregtse, J. M., Engelke, D. R., and Thiele, D. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 65–69.
26. Longo, V. D., Gralla, E. B., and Valentine, J. S. (1996) *J. Biol. Chem.* **271**(21), 12275–12280.
27. Elble, R. (1992) *Biotechniques* **13**, 18–20.
28. Holmquist, B. (1988) *Methods Enzymol.* **158**, 6–12.
29. Thorvaldsen, J. L., Sewell, A. K., McCowen, C. L., and Winge, D. R. (1993) *J. Biol. Chem.* **268**, 12512–12518.
30. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
31. Shinyashiki, M., Chiang, K. T., Switzer, C. H., Gralla, E. B., Valentine, J. S., Thiele, D. J., and Fukuto, J. M. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 2491–2496.
32. Winge, D. R., and Miklossy, K.-A. (1982) *J. Biol. Chem.* **257**(7), 3471–3476.
33. Furst, P., Hu, S., Hackett, R., and Hamer, D. (1988) *Cell* **55**, 705–717.
34. Furst, P., and Hamer, D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5267–5271.
35. Hu, S., Furst, P., and Hamer, D. (1990) *New Biologist* **2**(6), 544–555.
36. Wong, P. S.-Y., Hyun, J., Fukuto, J. M., Shiroda, F. N., DeMaster, E. G., and Nagasawa, H. T. (1998) *Biochemistry* **37**(16), 5362–5371.
37. Jungmann, J., Reins, H.-A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and Jentsch, S. (1993) *EMBO J.* **12**, 5051–5056.
38. Hassett, R., and Kosman, D. J. (1995) *J. Biol. Chem.* **270**, 128–134.
39. Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J., and Klausner, R. D. (1994) *Cell* **76**, 393–402.
40. Dancis, A., Klausner, R. D., Hinnebusch, A. G., and Barriocanal, J. G. (1990) *Mol. Cell. Biol.* **10**, 2294–2301.
41. Schwartz, M. A., Lazo, J. S., Yalowich, J. C., Allen, W. P., Whitmore, M., Bergonia, H. A., Tzeng, E., Billiar, T. R., Robbins, P. D., Lancaster, J. R., and Pitt, B. R. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 4452–4456.
42. Ishiropoulos, H., Zhu, L., and Beckman, J. S. (1992) *Arch. Biochem. Biophys.* **298**(2), 446–451.
43. Wink, D. A., Cook, J. A., Krishna, M. C., Hanbauer, I., DeGraft, W., Gamson, J., and Mitchell, J. B. (1995) *Arch. Biochem. Biophys.* **319**(2), 402–407.
44. Christodoulou, D., Kudo, S., Cook, J. A., Krishna, M. C., Miles, A., Grisham, M. B., Murugesan, R., Ford, P. C., and Wink, D. A. (1996) *Methods Enzymol.* **268**, 69–83.
45. Malinski, T., Taha, Z., Grunfeld, S., Patton, S., Kapturczak, M., and Tomboulis, P. (1993) *Biochem. Biophys. Res. Commun.* **193**(3), 1076–1082.
46. Valentine, J. S., and Gralla, E. B. (1997) *Science* **278**, 817–818.
47. Eide, D. (1997) *Curr. Opin. Cell Biol.* **9**, 573–577.
48. Winzerling, J. J., and Law, J. H. (1997) *Annu. Rev. Nutr.* **17**, 501–526.
49. Askwith, C., Eide, D., Ho, A. V., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994) *Cell* **76**, 403–410.
50. De Silva, D. M., Askwith, C. C., Eide, D., and Kaplan, J. (1995) *J. Biol. Chem.* **270**(3), 1098–1101.
51. De Silva, D., Davis-Kaplan, S., Fergestad, J., and Kaplan, J. (1997) *J. Biol. Chem.* **272**(22), 14208–14213.