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THE NITRIC OXIDE DONOR, V-PYRRO/NO, PROTECTS AGAINST CADMIUM-INDUCED HEPATOTOXICITY IN MICE

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Running Title: Protection against Cd hepatotoxicity by V-PYRRO/NO

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Abbreviations:

V-PYRRO/NO, O^2-Vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate; NO, nitric oxide; ALT, alanine aminotransferase.

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ABSTRACT

The nitric oxide (NO) donor, V-PYRRO/NO, is metabolized by P-450 enzymes to release NO within the liver and is effective in protecting against hepatotoxicity of endotoxin and acetaminophen. This study examined the effects of V-PYRRO/NO on cadmium (Cd) hepatotoxicity in mice. Mice were given multiple injections of V-PYRRO/NO (10 mg/kg, sc at 2-hr intervals) before and after a hepatotoxic dose of Cd (3.7 mg Cd/kg as $CdCl_2$, ip). V-PYRRO/NO administration reduced Cd-induced hepatotoxicity, as evidenced by reduced serum alanine aminotransferase activity, improved pathology, and reduced hepatic lipid peroxidation. The protection by V-PYRRO/NO was not mediated by altered Cd distribution to the liver or within hepatic subcellular fractions. Similar inductions of metallothionein, a metal-binding protein, were observed in mice receiving Cd alone or Cd plus V-PYRRO/NO. Real-time RT-PCR analysis revealed that V-PYRRO/NO administration suppressed the expression of inflammation-related genes such as macrophage inflammatory protein-2, CXC chemokine, thrombospondin-1, intracellular adhesion molecular-1 and interleukin-6. V-PYRRO/NO also suppressed the expression of acute phase protein genes and genes related to cell-death pathways, such as c-jun/AP-1, nuclear factor-κB, early response growth factor-1, heme oxygenase-1, caspase-3, growth arrest and DNA damaging protein-153. In summary, the liver-selective NO donor, V-PYRRO/NO, protects against Cd hepatotoxicity in mice. This protection is not mediated through altered distribution of Cd, but may be related to reduced hepatic inflammation, reduced acute phase responses and the suppression of cell-death related components.

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Keywords: V-PYRRO/NO, cadmium hepatotoxicity, metallothionein, gene expression, inflammation, acute phase response.

INTRODUCTION

O^2-Vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO), a nitric oxide (NO) prodrug that targets the liver, is created by adding a vinyl functional group to the terminal oxygen of pyrrolidine diazeniumdiolate (Saavedra et al., 1997). V-PYRRO/NO is a stable diazeniumdiolate which can circulate freely throughout the body until it is metabolized to release NO by enzymes, presumably cytochromes P450s, in the liver (Saavedra et al., 1997; Stinson et al., 2002) and elsewhere. The release of NO from V-PYRRO/NO within hepatocytes has been confirmed by the presence of the NO metabolites, nitrite/nitrate (Saavedra et al., 1997; Qu et al., 2002, Kim et al., 2000; Liu et al., 2002a), and by the stimulation of hepatic cyclic guanosine 3′,5′-monophosphate (cGMP) production (Saavedra et al., 1997; Ou et al., 1997).

V-PYRRO/NO has been shown to protect against endotoxin/TNF-α-induced liver injury and hepatocellular apoptosis (Saavedra et al., 1997; Ou et al., 1997; Kim et al., 2000; Liu et al., 2002a), and to protect against acetaminophen hepatotoxicity (Liu et al., 2003). V-PYRRO/NO is also effective in protecting against monocrotaline-induced hepatic sinusoid injury (Deleve et al., 2003), liver damage from ischemia/reperfusion (Ricciardi et al., 2001), and bile-duct ligation-induced portal hypertension and fibrosis (Moal et al., 2002). Whether the protective effects of V-PYRRO/NO against chemically induced hepatotoxicity can be extended to inorganics such as cadmium (Cd) is unknown.

Cd produces extensive liver injury after acute (Dudley et al., 1982) and chronic exposure in animals (Dudley et al., 1984). Unlike most hepatotoxicants, Cd produces liver damage

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without biotransformation as it does not undergo enzymatic conjugation and there is no possibility of degradation. Endothelial cells are thought to be the initial target of Cd in the liver (Nolan and Shaikh, 1986; Liu et al., 1992). Following acute Cd administration, hepatic congestion, ischemia and hypoxia occur very rapidly (Dudley et al., 1982; 1984; Habeebu et al., 1998). The resultant ischemic hypoxia leads to neutrophil infiltration, Kupffer cell activation, and inflammation, which could potentially contribute to the wide-spread hepatocellular apoptosis and necrosis observed with Cd (Dudley et al., 1984; Habeebu et al., 1998; Rikans and Yamano, 2000).

Inflammatory mediators, such as TNF-α, IL-1, IL-6, IL-8 and NO, are thought to play diverse roles in acute Cd-induced hepatotoxicity (Kayama et al., 1996; Yamano et al., 2000; Horiguchi et al., 2000). The same work indicates these inflammatory mediators may be the actual basis of Cd hepatotoxicity (Yamano et al., 2000), while other data point toward production of these mediators as an adaptive mechanism for Cd tolerance (Min et al., 2002). In this regard, knockout mice, TNF-α-/- or iNOS-/-, are not resistant to Cd toxicity (Harstad and Klaassen, 2002a, b). Thus, NO, like other mediators of inflammation, could have dual effects on progression of chemically induced hepatic pathology depending on experimental conditions and type of toxicants used (Kim and Billiar, 2001; Liu et al., 2003).

This study examined the effect of V-PYRRO/NO on acute Cd-induced hepatotoxicity in mice, using pharmacological doses of the NO donor prodrug. The results demonstrate that V-PYRRO/NO protected against Cd-induced hepatotoxicity including congestion, apoptosis, and necrosis. This protection is not due to altered distribution of Cd, but potentially may be related to the vasodilatory and anti-apoptotic properties of V-PYRRO/NO.

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MATERIALS AND METHODS

Chemicals. V-PYRRO/NO was synthesized as previously described (Saavedra et al., 1997). The structure of V-PYRRO/NO and the mode of NO release have been demonstrated in previous publications (Saavedra et al., 1997; Liu et al., 2002a; Stinson et al., 2002). Cadmium chloride ($cdCl_2$) was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were commercially available and of reagent grade.

Animals. Male CD-1 mice, weighing 25-30 g, were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care at the National Institute of Environmental Health Sciences at 20-22°C with a 12-h light/dark cycle for at least one week before treatment. Animals were allowed free access to Rodent Laboratory Chow (#5002, Ralston Purina Co., St. Louis, MO) and tap water. All procedures involving the use of laboratory animals were reviewed and approved by the Institutional IACUC.

Experimental design. Mice were given repeated s.c. injections with 10 mg V-PYRRO/NO/kg body weight in saline at 1 hr before and again at 1, 3, and 5 hrs after Cd (3.7 mg Cd/kg as $CdCl_2$, ip, in saline). Controls received vehicle injections at the same time points. Hepatotoxicity was evaluated at 1, 4, and 8 hrs after cadmium injection. The use of multiple s.c. injections of V-PYRRO/NO was based on data showing that this compound has a short plasma half-life in mice (Stinson et al., 2002), and our previous work using the same multiple injection protocol to protect against D-galactosamine/endotoxin-induced hepatotoxicity in mice without producing toxicity (Liu et al., 2002a). Thus, these doses are considered “pharmacologically-relevant”.

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*Evaluation of hepatotoxicity***.** Serum alanine aminotransferase (ALT) activity was assayed as a marker of hepatocellular death using a commercially available kit (Infinite ALT, Sigma Chemical Co). A portion of the liver was fixed in 10% neutral formalin, processed by standard histological techniques, stained with hematoxylin and eosin, and examined for morphological evidence of liver injury.

Lipid peroxidation assay. The concentrations of 4-hydroxyalkenals, 4-dydroxy-2(E)-nonenal (4-HNE) and malonialdehyde (MDA) were measured in liver homogenates using a lipid peroxidation assay kit (#43674) from CalBiochem (San Diego, CA). Approximately 200 mg of liver tissue was homogenize in 9 vol of ice-cold 20 mM Tris-HCl buffer (pH 7.4), and then centrifuged at 3000 x *g* for 10 min at 4°C. Supernatants (200 μl) were mixed with 650 μl of methanol:acetonitrile (1:3, v/v), and 150 μl of the chromogenic agent, *N*-methyl-2-pheylindole to yield a purple chromophore, which is measured spectrophotometrically at the absorbance of 586 nm using 4-hydroxynonenal as standard. The level of lipid peroxidation was expressed as the amount of 4-HNE + MDA (nmol) per gram of tissue.

Subcellular distribution of cadmium. Four hrs after Cd (3.7 mg/kg, ip) treatment, livers were removed and homogenized in 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) with a Teflon pestle glass homogenizer. Various fractions were prepared by differential centrifugation at 4°C. The resultant pellets were defined as nuclei (600 x g, 10 min), mitochondria (10,000 x g, 10 min), microsome (22,000 x g, 30 min), and cytosol (final supernatant). The Cd content in the various fractions was analyzed by atomic absorption spectrophotometer (Perkin-Elmer AAnalst 100, Norwalk, CT) after digestion with nitric acid.

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Hepatic metallothionein determination. A portion of liver was homogenized in 10 mM Tris-HCl buffer (1:5. W:V), followed by centrifugation at 20,000 g for 10 min. Metallothionein (MT) concentrations in the hepatic cytosol were determined by the Cd/hemoglobin assay (Eaton and Toal, 1982).

Real-time RT-PCR Analysis. Expression of the selected genes was quantified using real-time RT-PCR analysis as described by Walker (2001). Briefly, total RNA was reverse transcribed with MuLV reverse transcriptase and oligo-dT primers. The forward and reverse primers for selected genes were designed using Primer Express software and are listed in Table 1. The SYBR green DNA PCR kit (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values and the relative differences between groups were expressed as relative increases setting control as 100%. Assuming that the Ct value is reflective of the initial starting copy and that there is 100% efficacy, a difference of one cycle is equivalent to a two-fold difference in starting copy.

Statistics. Data represent means and standard errors of (n = 5 to 20). For comparisons between two groups, a Students' t test was performed. For multiple comparisons data were analyzed using a one-way analysis of variance, followed by Duncan's multiple range test. The level of significance was set at $p < 0.05$.

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RESULTS

Protection by V-PYRRO/NO against Cd-induced hepatotoxicity.

In CD-1 mice, Cd (3.7 mg/kg, ip) treatment produced serological evidence of liver injury starting 4 hr after administration (Fig. 1), as evidenced by increased serum activity of alanine aminotransferase (ALT). Serum ALT increased ~ 25-fold over control values by 8 hrs after Cd exposure. V-PYRRO/NO administration alone did not alter ALT activity (Liu et al., 2002a), but significantly decreased Cd-induced release of hepatic ALT, as evidenced by a 59% decrease in serum ALT activity at 8 hr after Cd (220 U/L for V-PYRRO/NO + Cd vs 540 U/L of Cd alone). Protective effects of V-PYRRO/NO on Cd-induced ALT release were not evident at earlier time points.

Cd-induced hepatotoxicity is characterized by congestion and widespread hepatocellular degeneration, apoptosis, and necrosis (Klaassen and Liu 1997). Consistent with serology, Cd-induced liver pathology was evident 8 hrs after injection of the metal, at which point widespread hepatic congestion occurred (Fig. 2A, arrows). In addition, Cd-induced foci of hepatocelluar damage were also evident. These pathological alterations were dramatically ameliorated in the liver of animals receiving V-PYRRO/NO treatments (Fig. 2B). In the Cd plus V-PYRRO/NO-treated animals, hepatic congestion and hepatocellular necrosis were rare events.

Oxidative stress and lipid peroxidation are thought to be mechanisms for Cd-induced hepatotoxicity (Liu et al., 2002b). Thus, the concentrations of 4-hydroxy-2(E)-nonenal (4-HNE) and malondialdehyde (MDA) as products of lipid peroxidation, were measured in liver homogenates. As expected, Cd administration markedly increased hepatic lipid peroxidation by 80% over control. The V-PYRRO/NO treatments significantly reduced these increases in Cd-

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induced lipid peroxidation at 8 h after Cd treatment (Fig. 3). This is clear evidence that V-PYRRO/NO has decreased the oxidative stress induced by hepatotoxic doses of Cd.

Metallothionein and subcellular Cd distribution

Metallothionein (MT), a metal-binding protein important for protecting against Cd toxicity (Klaassen et al., 1999), was assessed in mouse livers after Cd alone or Cd plus V-PYRRO/NO treatment. Cd alone greatly increased hepatic MT concentrations (~5-fold) over controls (Fig. 4). In mice given V-PYRRO/NO and Cd, hepatic MT levels were not different from Cd alone. V-PYRRO/NO when given alone moderately (~2 fold) increased liver MT at 8 h (data not shown).

Altered subcellular distribution of Cd has been observed as an important mechanism for reduction of toxicity. The amount of Cd reaching critical organelles is thought to be a key factor in Cd-induced hepatotoxicity. Thus, subcellular distribution of Cd was determined at 4 hr after Cd administration. As shown in Fig. 5, V-PYRRO/NO treatment did not alter the distribution of Cd to various subcellular fractions (nuclear, mitochondrial, and cytosolic). Similar patterns for the subcellular distribution of Cd were also observed at 1 and 8 h of Cd exposure, and these were similarly not altered by the V-PYRRO/NO treatments (data not shown).

Real-time RT-PCR analysis of expression of genes of interest

To examine the potential mechanism by which V-PYRRO/NO protects the liver from Cd-induced injury, pertinent gene expression changes were examined with real-time RT-PCR (Table 2). Cd toxicity is initially associated with endothelial cell damage, resulting in

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congestion, hypoxia, ischemia and inflammation (Dudley et al., 1984; Nolan and Shaikh, 1986; Habeebu et al., 1998). Thus, the expression of genes associated with hypoxia, ischemia and inflammation was assessed. As shown in Table 2, 8 hrs after a hepatotxic dose of Cd, there were dramatic increases in the expression of mouse macrophage inflammatory protein (MIP-2, 45-fold), thrombospondin-1 (TSP1, 55-fold), mouse chemokine (mKC, 11-fold), intracellular adhension molecule-1 (ICAM-1, 2-fold), interleukin-6 (IL-6, 58-fold), and tumor necrosis factor-α (TNF-α, 16-fold) genes. V-PYRRO/NO significantly diminished the Cd-induced enhanced expression for the MIP-2, TSP1, and ICAM-1 genes. However, V-PYRRO/NO had no significant effects on Cd-increased expression of mKC, IL-6, TNF-α and iNOS.

Cd is thought to produce toxicity by the activation of transcription factors, such as the AP-1 complex (Liu et al., 2002b), NF-κB (Hart et al., 1999), and activation of caspase-3 (Habbebu et al., 1998). As a result of Cd toxicity, the DNA damage responsible proteins such as GADD45 and GADD153 are also increased (Liu et al., 2002b). Consistent with previous observations, acute Cd hepatotoxicity greatly enhanced the expression of c-jun/AP-1 (187-fold) and NF-κB (36-fold), while the expression of both genes was diminished to 17- and 13-fold, respectively, with co-treatment with V-PYRRO/NO. There was also a significant suppression of Cd-induced acute phase protein genes by V-PYRRO/NO, including heme oxygenase-1 (HO-1, 19-fold vs 82 fold), and early response growth factor-1 (ERG1, 60-fold vs 106-fold). V-PYRRO/NO administration also suppressed the expression of Cd-induced caspase-3 (1.5-fold vs 2.5-fold) and growth arrest and DNA damage responsible protein-153 (GADD153, 3-fold vs 6-fold). However, V-PYRRO/NO had no effect on the induction of MT-1 mRNA (45-fold vs 50-fold), consistent with the MT protein assay (see Fig. 4).

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DISCUSSION

This study demonstrates that V-PYRRO/NO is effective in protecting against the hepatoxicity of Cd in mice, as evidenced biochemically by decreased serum ALT activity and histologically by diminished liver pathology. In addition, Cd-induced lipid peroxidation in the liver was reduced by the NO-releasing prodrug. Furthermore, Cd-induced aberrant expression of genes related to inflammation and cell death was greatly ameliorated by V-PYRRO/NO. Thus, this liver-selective NO donor clearly blocks the hepatotoxicity induced by inorganic Cd. This is consistent with V-PYRRO/NO-induced reductions in hepatotoxicity by a variety of organic hepatotoxic chemicals (Liu et al., 2002a; 2003). This observation with Cd is of particular importance since the metallic element, unlike many organic hepatotoxicants, is not metabolized in the liver and there is currently no effective therapy for Cd intoxication.

Oxidative stress has been implicated as a key event in Cd toxicity (Liu et al., 2002b). Indeed, acute Cd hepatotoxicity is associated with the production of free radicals in the liver, as evidenced by production of free radicals and increased lipid peroxidation (Liu et al., 2002b). In the present study, Cd-induced lipid peroxidation was suppressed by V-PYRRO/NO, suggesting that the reduced oxidative stress and/or lipid peroxidation could be an important aspect of the hepatoprotective effects of V-PYRRO/NO. Various other NONOates can act as antioxidants against chemically induced oxidative stress (Fitzhugh and Keefer, 2000). Thus, antioxidant capacity of V-PYRRO/NO likely contributes to reduction of Cd-induced hepatotoxicity.

One mechanism by which Cd induces radical production is mediated through inflammatory cells (Rikans and Yamano, 2000). In this regard, the Kupffer cell inhibitor gadolinium chloride blocks Cd-induced hepatotoxicity (Sauer et al., 1998; Harstad and

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Klaassen, 2002c), and diminishes Cd-induced hepatic radical formation (Liu et al., unpublished data). Cd-induced activation of Kupffer cells could result from endothelial cell damage, leading to subsequent ischemia/congestion, hypoxia, and inflammation (Nolan and Shaikh, 1986; Liu et al., 1992). In the present study, Cd-induced hepatic congestion and inflammation were greatly attenuated by V-PYRRO/NO. Accordingly, Cd induced over-expression of thrombospondin-1, ICAM-1, mKC and MIP-2, IL-6 and TNF-α, all mediators of inflammatory response, are greatly suppressed by V-PYRRO/NO. This effect may be related to vasodilatory effects of V-PYRRO/NO (Ricciaridi et al., 2001; Moal et al., 2002), which potentially reduce hepatic congestion/ischemia. In addition, exogenous NO has also shown to suppress the expression of thrombospondin-1 (Wang et al., 2002), ICAM-1 (Liu et al., 1998), and to suppress the expression of pro-inflammatory cytokines such as IL-6 and TNF-α (Menger et al., 1999; Liu et al., 2002a). Thus, V-PYRRO/NO treatment appeared to maintain vascular integrity in the face of Cd insult by delivering NO to the liver (Ricciardi et al., 2001). Thus, initial protection against Cd-induced hepatic vascular damage by the NO-releasing pro-drug appears to sharply limit the subsequent inflammatory response and ensuing cascade of ischemic hypoxia and hepatocellular death.

Cd induced endothelial cell damage, hypoxia and inflammation can activate transcription factors such as AP-1, NF-κB and hypoxia-inducible transcription factor-1 (HIF-1) (Hart et al., 1999; Faller, 1999; Liu et al., 2002b). These in turn increase the expression of acute phase proteins such as HO-1 and ERG1 (Liu et al., 2002b). NO has been proposed to suppress prooxidant transcription factor activation (Buzard and Kasprzak, 2000). In the present study, Cd-induced expression of c-jun/AP-1, NF-κB and the acute phase protein gene EGR1 and HO-1

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was significantly suppressed by V-PYRRO/NO, suggesting that NO may also exert its protective effects through the inhibition of the signal transduction pathways leading to cell death.

Cd produces both apoptosis and necrosis in the mouse liver (Habeebu et al., 1998). In the present study, Cd-induced hepatocellular death, regardless of specific type, was clearly ameliorated by V-PYRRO/NO. Similarly, Cd increased expressions of caspase-3 (a key enzyme in the execution of apoptosis) and GADD 153 (an indicator of DNA damage) were also suppressed by V-PYRRO/NO. NO is thought to play a role in directly reducing liver apoptosis (Kim and Billiar, 2001). Indeed, V-PYRRO/NO protection against the hepatotoxicity of D-galactosamine/endotoxin and acetaminophen appears to be related to suppression of apoptotic pathways (Liu et al., 2002, 2003). Thus, the protective effect of V-PYRRO/NO against Cd hepatotoxicity could also be due, at least in part, to direct suppression of cell death pathways leading to apoptosis and necrosis. Again this would block progression of liver pathology despite the occurrence of initial Cd lesions. Indeed, V-PYRRO/NO was effective in decreasing Cd-induced apoptosis and suppressed Cd-induced JNK activation in cultured rat liver cells (Qu et al., 2002).

Tolerance to acute Cd hepatotoxicity has been attributed to the induction of MT (Goering and Klaassen, 1984). MT is a low-molecular weight, cysteine-rich, metal-binding protein that can bind Cd in the cytosol and renders it inert and thereby reduces the amount of Cd at critical cellular targets (Klaassen et al., 1999). We examined the effect of V-PYRRO/NO on hepatic MT induction and subcellular distribution of Cd. V-PYRRO/NO had no effect on Cd induction of hepatic MT, both at the transcript and the protein levels. Thus, MT is not likely a major factor in V-PYRRO/NO-induced resistance to Cd. NO released from V-PYRRO/NO

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likely blocked Cd-induced hepatic endothelial damage and preserved microvascular circulation. The presumed circulation improvement by V-PYRRO/NO (Ricciardi et al., 2001; Moal et al., 2002, Deleve et al., 2003) may have reduced Cd distribution to the liver, however, V-PYRRO/NO administration had no effects on distribution of Cd to the liver, nor the altered subcellular distribution of Cd within the liver. Thus, induction of MT and altered toxicokinetics of Cd are not the primary mechanisms of V-PYRRO/NO-mediated protection. However, this does not exclude the potential role of presynthesized MT in Cd tolerance following V-PYRRO/NO pre-treatment in cultured rat liver cells (Qu et al., 2002).

In summary, this study demonstrates that the liver-selective NO donor, V-PYRRO/NO, is effective in protecting against Cd-induced liver injury in mice. This protective effect does not appear to be due to altered subcellular Cd distribution or induction of MT, but does involve the inhibition of oxidative stress and resulting apoptosis, probably through the maintenance of hepatic vasculature to prevent ischemia and hypoxia.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Effect of V-PYRRO/NO on Cd-induced liver injury in mice. Mice were given Cd (3.7 mg/kg, ip) with or without the administration of V-PYRRO/NO (10 mg/kg, s.c. 1 h before and 1, 3, and 5 h after Cd. Liver injury was evaluated by serum alanine aminotransferase (ALT) activity. Data are mean ± SEM of 6-20 mice. *, Significantly different from controls, $p < 0.05$. #, Significantly different from Cd alone, $p < 0.05$.

Fig. 2. Photomicrographs of the mouse liver. Sections were stained with hematoxylin and eosin, 100 x magnification. A. Cd alone. Foci of hepatocellular damage are evident and a large number of erythrocytes in the sinusoid indicative of congestion (arrows). B. Cd plus V-PYRRO/NO, hepatic congestion and cell death are mild, while the cell swelling is the predominant lesion.

Fig. 3. Hepatic lipid peroxidation in Cd-treated mice. Mice were given Cd (3.7 mg/kg, ip) with or without the administration of V-PYRRO/NO (10 mg/kg, s.c. 1 h before and 1, 3, and 5 h after Cd. Liver lipid peroxidation levels were assessed by the concentration of 4-HNE + MDA at 1, 4, and 8 h after Cd administration. Data are mean ± SEM of 6-8 mice. *, Significantly different from controls, $p < 0.05$; #, Significantly different from Cd alone, $p < 0.05$.

Fig. 4. Effect of V-PYRRO/NO on hepatic metallothionein. Mice were given Cd (3.7 mg/kg, ip) with or without the administration of V-PYRRO/NO (10 mg/kg, s.c. 1 h before and 1, 3, and

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5 h after Cd. Liver metallothionein levels were assessed by the Cd/haemoglobin assay. Data are mean ± SEM of 6-8 mice. *, Significantly different from controls, $p < 0.05$.

Fig. 5. Effect of V-PYRRO/NO on subcellular distribution of Cd. Mice were given Cd (3.7 mg/kg, ip) with or without the administration of V-PYRRO/NO (10 mg/kg, s.c. 1 h before and 1, 3, h after Cd). Four hours after Cd administration, livers were removed and various subcellular fractions were prepared for Cd analysis by atomic absorption spectrometry. Data are mean ± SEM of 5 mice.

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Table 1: Primer sequences used for quantitative real-time RT-PCR analysis of liver tissue.

Gene	Accession Number	Forward Primer	Reverse Primer
MIP-2	NM_009140	CCTCAACGGAAGAACCAAAGAG	CTCAGACAGCGAGGCACATC
Thrombospondin1	M87276	GCCGGATGACAAGTTCCAA	GCCTCAAGGAAGCCAAGAAGA
mKC	NM_008176	TGGCTGGGATTCACCTCAAG	GTGGCTATGACTTCGGTTTGG
ICAM-1	NM_010493	GTCTCGGAAGGGAGCCAAGTA	CGACGCCGCTCAGAAGAA
IL-6	J03783	GCCCACCAAGAACGATAGTCA	GAAGGCAACTGGATGGAAGTCT
TNF-α	XM_110221	GACCCTCACACTCAGATCATCTTCT	CCTCCACTTGGTGGTTTGCT
c-jun/AP-1	J04115	ACTCCGAGCTGGCATCCA	CCCACTGTTAACGTGGTTCATG
iNOS	M87039	ACATCAGGTCGGCCATCACT	CGTACCGGATGAGCTGTGAATT
NF-κB	AF069542	GGCGGCACGTTTTACTCTTT	CCGTCTCCAGGAGGTTAATGC
Heme oxygenase-1	M33203	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA
EGR1	M20157	AGGTTCCCATGATCCCTGACT	GGTACGGTTCTCCAGACCCTG
GADD153	X67083	CTCCTGTCTGTCTCTCCGGAA	TACCCTCAGTCCCCTCCTCA
Caspase-3	U19522	TCCTGGTCTTTGTACGCTACCA	CCTGATGTCGAAGTTGAGGTAGCT
Metallothionein-1	BC027262	AATGTGCCCAGGGCTGTGT	GCTGGGTTGGTCCGATACTATT
G3PDH	M32599	AGTATGACTCCACTCACGGCAAAT	GTCTCGCTCCTGGAAGATGGT

MIP-2, Macrophage Inflammatory Protein-2; mKC, a mouse CXC chemokine; ICAM-1, Intercellular Cell Adhesion Molecule-1; IL-6, Interleukin-6; TNF-α, Tumor Necrosis Factor-α; iNOS, inducible nitric oxide synthase; EGR1, Early Growth Response Protein-1; GADD153, Growth Arrest and DNA Damage inducible protein 153; G3PDH, glyceraldehydes-3-phosphate dehydrogenase.

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Table 2: Real Time RT-PCR analysis of liver tissue in control, Cd- and Cd + PYRRO/NO-treated mice.

	Control	Cd alone	Cd+NO
Inflammatory Markers			
MIP-2	1.0 ± 0.3	44.6 ± 12.0*	9.3 ± 0.9*#
Thrombospondin-1	1.0 ± 0.2	55.4 ± 7.2*****	17.6 ± 8.5***#**
MKC	1.0 ± 0.2	10.8 ± 2.2*	6.8 ± 4.4*
ICAM-1	1.0 ± 0.1	2.1 ± 0.3*	1.0 ± 0.1#
IL-6	1.0 ± 0.4	58.4 ± 17.0*	38.1 ± 7.0*
TNF-α	1.0 ± 0.2	15.7 ± 4.5*	11.7 ± 5.5*
iNOS	1.0 ± 0.2	12.1 ± 0.7*	11.8 ± 1.7*
AcutePhase Protein genes & Cell-Death Markers			
c-jun/AP-1	1.0 ± 0.3	187 ± 14.2*	17.7 ± 5.4*#
NF-κB	1.0 ± 0.2	36.0 ± 9.7*	13.3 ± 3.1*#
Hemeoxygenase-1	1.0 ± 0.3	82.0 ± 9.9*****	18.8 ± 6.8***#**
EGR1	1.0 ± 0.3	106 ± 14.7*****	60.6 ± 16.8*#
GADD153	1.0 ± 0.4	6.4 ± 1.6*****	2.7 ± 0.9***#**
Caspase-3	1.0 ± 0.3	2.4 ± 0.3*****	1.5 ± 0.3***#**
Metallothionein-1	1.0 ± 0.2	50.2 ± 14*****	44.6 ± 13*****

Mice were given CdCl2 (3.7 mg Cd/kg, ip), or Cd + V-PYRRO/NO (10 mg/kg, sc, x4). Liver samples were taken at 8 hr after Cd administration, and total RNA was isolated for real-time RT-PCR analysis. In each individual sample, the expression level of each gene was first normalized with that of G3PDH and then the relative differences between groups were expressed as relative increases setting controls as 1.0. Data represent means ± SE of n = 4-6 animals per group. *P<0.05 (compared to control), #P<0.05 (compared to Cd alone). Gene abbreviations are listed in Table 1.

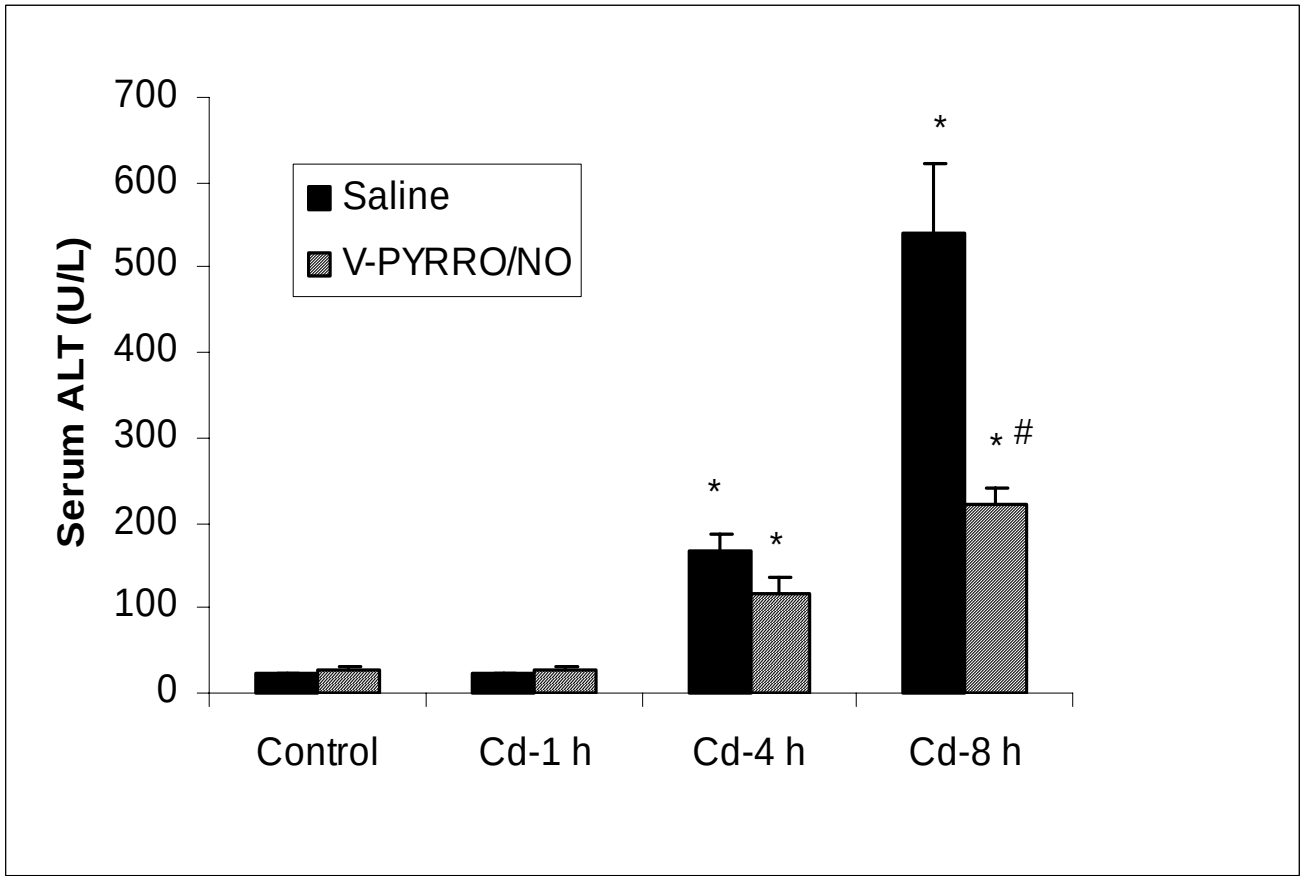

Fig. 1

Cd alone

V-PYRRO/NO + Cd

Fig. 2

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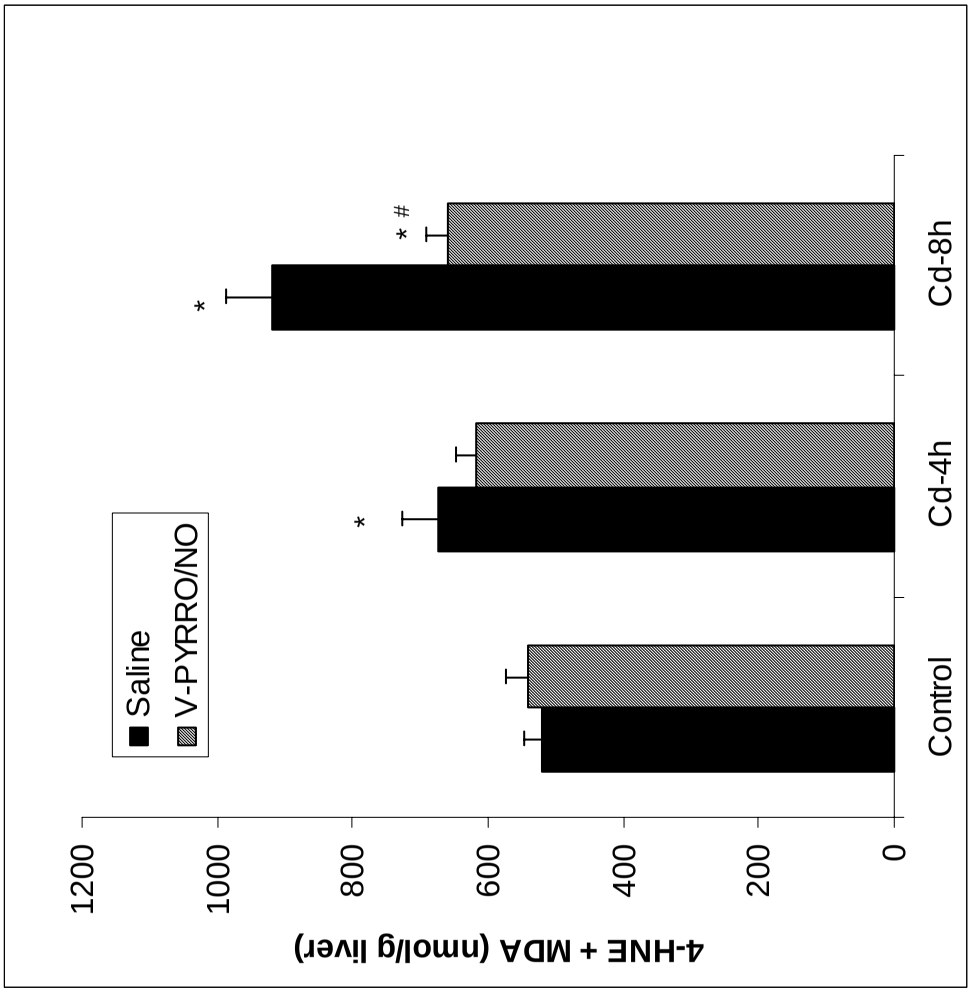

Fig. 3

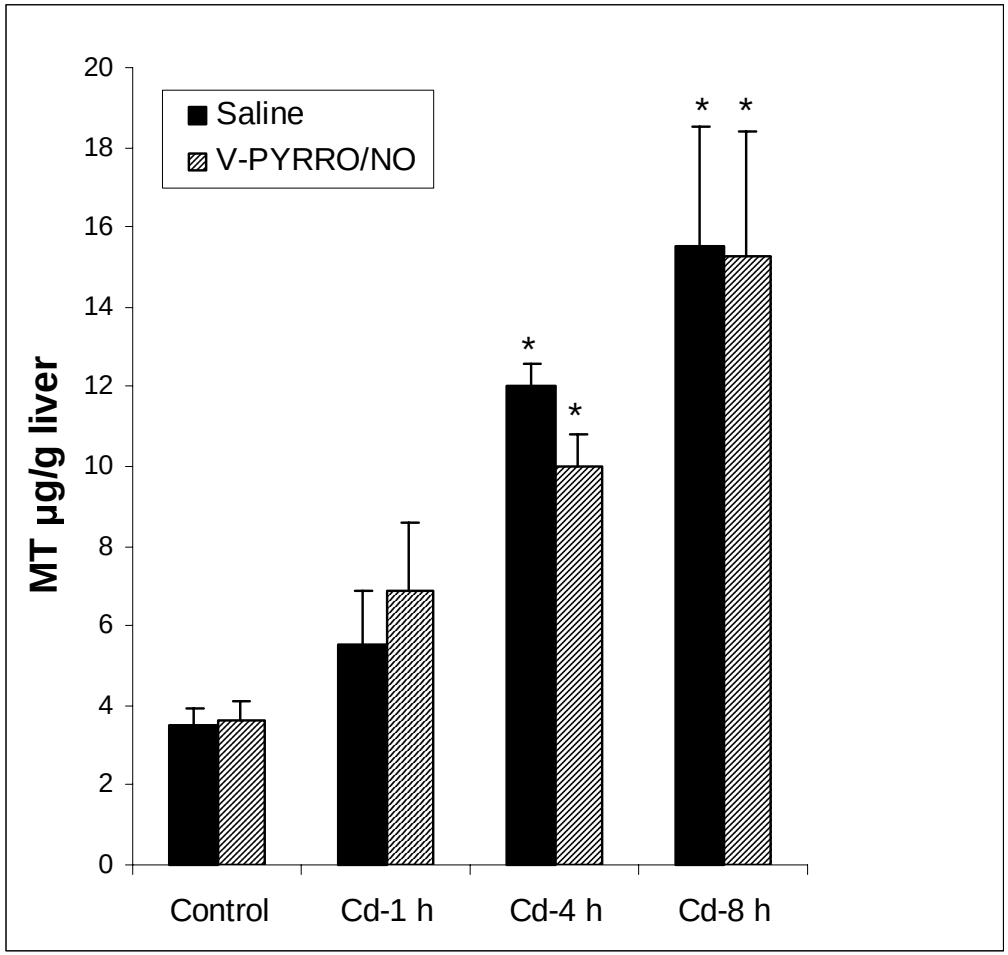

Fig. 4

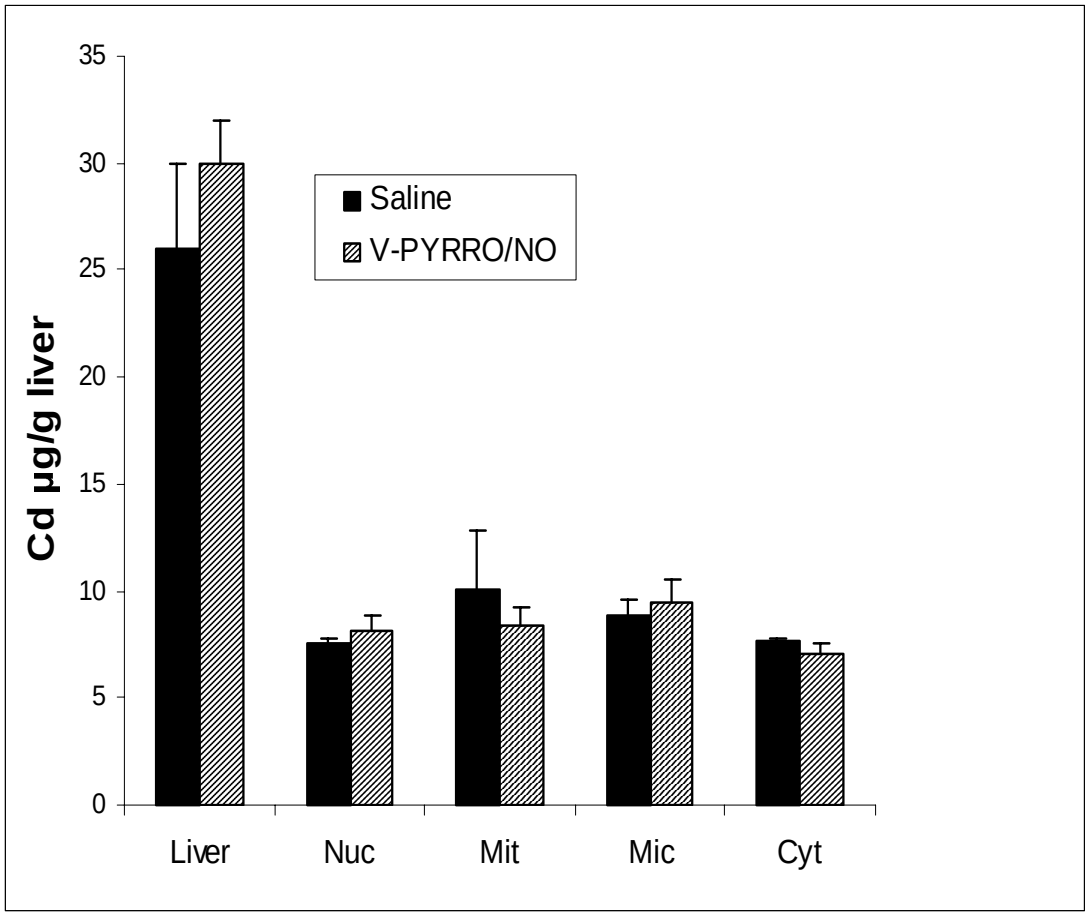

Fig. 5