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Decreased hepatic ischemia-reperfusion injury by manganese–porphyrin complexes

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Abstract

Reactive oxygen and nitrogen species have been implicated in ischemia-reperfusion (I/R) injury. Metalloporphyrins (MP) are stable catalytic antioxidants that can scavenge superoxide, hydrogen peroxide, peroxynitrite and lipid peroxyl radicals. Studies were conducted with three manganese–porphyrin (MnP) complexes with varying superoxide dimutase (SOD) and catalase catalytic activity to determine if the MnP attenuates I/R injury in isolated perfused rat livers. The release of the hepatocellular enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) was maximal at 1 min reperfusion, decreased rapidly and increased gradually by 90 min. Manganese tetrakis-(*N*-ethyl-2 pyridyl) porphyrin (MnTE-2-PyP) decreased ALT, AST, LDH at 1–90 min reperfusion, while manganese tetrakis-(*N*-methyl-2 pyridyl) porphyrin (MnTM-2-PyP) and manganese tetrakis-(ethoxycarbonyl) porphyrin (MnTECP) decreased ALT and LDH from 5 to 90 min reperfusion. The release of thiobarbituric acid-reacting substances (TBARS) was diminished by MnTE-2-PyP and MnTM-2-PyP at 90 min. The extent of protein nitration (nitrotyrosine, NT) was decreased in all three MnPs treated livers. These results demonstrate that MnP complexes can attenuate hepatic I/R injury and may have therapeutic implications in disease states involving oxidants.

Keywords: *Ischemia, liver, metallophoryrin, free radical, nitric oxide*

Introduction

Warm ischemia-repurfusion (I/R) injury of the liver is a common complication of hepatic surgery, liver transplantation and shock with fluid resuscitation. Significant morbidity and mortality may result from consequent liver dysfunction and in more severe cases, remote organ failure may result. The mechanisms of hepatic I/R injury have been extensively reviewed [1–4]. Reactive oxygen species (ROS), including superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) and reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), have been implicated. ROS can inflict direct tissue damage and initiate a cascade of deleterious cellular responses, leading to inflammation, cell death and organ failure. Moreover, NO can affect cellular enzymes via nitrosylation of thiol residues and can react with $O_2^{\cdot -}$ to form $ONOO^-$, a potent oxidizing and nitrating agent. In hepatic I/R

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injury, the mitochondria, Kupffer cells, neutrophils and xanthine oxidase are potential sources of ROS, while there are a variety of cellular and tissue sources of NO in the liver. In hepatic I/R injury, protein carbonyls, a marker of protein oxidation and malondialdehyde (MDA), a marker of lipid peroxidation, have been shown to be elevated. In addition, 3-nitrotyrosine (NT), a marker of protein nitration, is strongly expressed in the centrilobular region following I/R [5].

Free radical scavengers have been utilized, alone or in combination with other agents, to protect against hepatic I/R injury [6–16]. Superoxide dimutase (SOD), a metalloprotein, defends against oxidative stress by dismutating $O_2^{\cdot-}$ into H_2O_2, which in turn is converted to H_2O and O_2 by catalase. Overexpression of SOD in cell cultures and in whole animals can protect against I/R injury in the brain, heart, liver and kidneys [17–21]. However, exogenous SOD is limited not only by its large size and restricted cell permeability, but also a short circulating half-life, antigenicity and instability. Clinical trials using exogenous SOD, have met with limited success leading to the development of many low-molecular-weight SOD mimetics [22]. Metalloporphyrin (MP) compounds are a unique class of catalytic antioxidants that are water-soluble, cell-permeable, stable and can be derivatized to facilitate binding to cell surfaces [22]. MPs can scavenge $O_2^{\cdot-}$, H_2O_2, $ONOO^-$ and lipid peroxyl radicals and their antioxidant potency appears to be determined by the type of metal center, redox potential and electrostatic charge. Various MP compounds have been demonstrated to block oxidant stress in *in vitro* and *in vivo* models [23]. Several human diseases involving ROS and/or RNS are likely to benefit from MP therapy, including organ transplantation, inflammatory diseases, myocardial infarction, stroke and trauma.

In this study, three manganese-containing metalloporphyrin (MnP) complexes with varying levels of SOD and catalase catalytic activity (Figure1, Table I) were evaluated in isolated perfused rat livers to determine their ability to attenuate the effects of I/R on hepatic injury. These studies indicted that the MnPs with the highest SOD activity (manganese tetrakis-(*N*-ethyl-2 pyridyl) porphyrin (MnTE-2-PyP) and manganese tetrakis-(*N*-methyl-2 pyridyl) porphyrin (MnTM-2-PyP)), were the most effective at attenuating I/R injury while the MnP with minimal SOD activity but catalase similar to MnTE-2-PyP (manganese tetrakis-(ethoxycarbonyl) porphyrin (MnTECP)) was markedly less effective at attenuating liver injury.

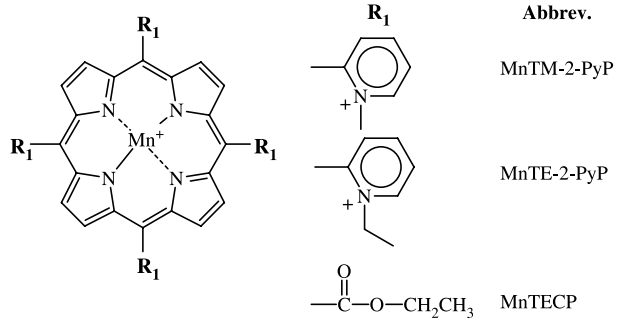

Figure 1. The chemical structures of several manganese containing meso-porphyrins: manganese (III) meso-tetrakis(*N*-methylpyridinium-2-yl) porphyrin (MnTM-2-PyP), manganese (III) meso-tetrakis(*N*-ethylpyridinium-2-yl) porphyrin (MnTE-2-PyP), manganese (III) meso-tetrakis(ethoxycarbonyl) porphyrin (MnTECP) are illustrated.

Table I. Comparison of *in vitro* antioxidant activities.

Compounds	SOD activity⋆ (U/mg)	Catalase activity† (k, min^{-1})	Lipid peroxidation‡ (IC_{50}, μM)
MnTM-2-PyP	9500	5.4	1.1
MnTE-2-PyP	10,650	2.4	1.2
MnTECP	9	1.9	1.5

⋆Units of SOD activity defined as the amount of compound that inhibits the reduction of cytochrome c by 50%.† Pseudo first order rate constant for hydrogen peroxide decay.‡ The concentration of a compound that inhibits iron/ascorbate-mediated lipid peroxidation in tissue homogenate by 50%.

Methods

Biochemicals

All chemicals unless otherwise specified were obtained from Sigma Chemical Co. (St Louis, MO). MnTE-2-PyP, MnTM-2-PyP and MnTECP were supplied by Aeolus Pharmaceuticals, Inc. (Research Triangle, NC) as their chloride salts. Polyclonal anti-NT antibody (clone 4709) was kindly provided by Dr Joseph S. Beckman [24].

Animal protocols

All animal protocols were approved by the Animal Review Board of the University of Alabama at Birmingham. Male C57BL/6 mice (25–30 g) were purchased from Charles River Laboratories (Boston, MA). Under anesthesia with intraperitoneal pentobarbital (50 mg/kg), the mice underwent laparotomy. Portal vein was identified and cannulated with a 24-gauge catheter, secured by a 2–0 silk ligature and then connected to an isolated tissue perfusion system (Figure 2). The inferior vena cava to minimize elevated intrahepatic pressure and the liver perfused with oxygenated Krebs–Henseleit solution (37°C) at 1 ml/min/gm to remove blood. The liver was then extirpated, placed into a temperature-controlled perfusion chamber and perfusion rate increased to 2.5 ml/min/gm. After 15 min of perfusion, MnTE-2-PyP, MnTM-2-PyP and MnTECP were added into

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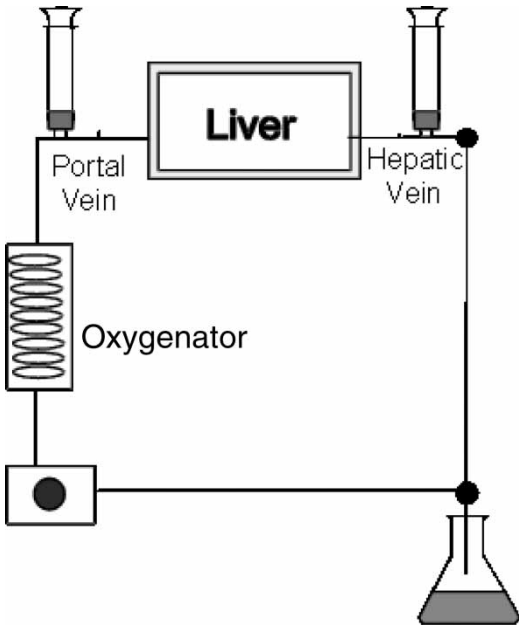

Figure 2. The effects of pretreatment with MnPs on tissue injury was evaluated in the isolated perfused mouse lives.

the perfusate (10 μM final concentration) and allowed to recirculate for 15 min to evaluate for any acute hepatotoxicity effects. Another two groups of livers were perfused with 0.1 and 1 μM of MnTE-2-PyP. Then, the livers were rendered globally ischemic via total cessation of the perfusion pump under normothermic condition for 60 min, followed by reperfusion with MnP-supplemented solutions for 90 min (recirculating system). Aliquots of perfusate were obtained prior to perfusion with MnP compounds, prior to ischemia and at 1, 2.5, 5, 10, 15, 30, 45, 60 and 90 min of reperfusion for analysis of release of intracellular hepatic enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Samples at 1 and 90 min of reperfusion were also analysed for thiobarbituric acid-reacting species (TBARS). At termination, livers were fixed in paraformaldehyde for histopathology and immunohistochemistry of NT.

SOD assay

Superoxide dismutase activity was measured by cytochrome c reductase as previously described [25]. Briefly, buffer consisted of 50 mM potassium phosphate containing 0.1 mM EDTA at pH 7.8. Cytochrome c 9(10 μM of the oxidized form) and xanthine were added (50 μM). Xanthine oxidase (2 nM) was added to initiate formation of superoxide. Rates of reduction of cytochrome c were followed at 550 nm for 1 min. The concentration of MP that inhibited the rate of cytochrome reduction by 50% was defined as 1 SOD unit of activity.

Catalase assay

The disputation of H_2O_2 was measured by following the formation of O_2 with a Clark-type electrode as we have previously described [26].

Lipid peroxidation assay

The ability of mimetics to inhibit lipid peroxidation (TBARS, IC_{50} μM) in aliquots of the perfusate were assessed as previously described [27]. Iron and ascorbate were used to initiate lipid peroxidation in tissue homogenates and the formation of TBARS was measured as an index of lipid peroxidation. The molar extinction coefficient at 532 nm (1.4×10^5/mol/cm) was used to calculate TBARS concentration using known amounts of MDA as standards. To prevent further peroxidation during assay procedures, TBA reagent was made with 0.025% BHT. Sample absorbance was converted to MDA equivalencies (μM) by extrapolation from the MDA standard curve.

AST, ALT, and LDH assay

Aliquots of the perfusate for each time period was collected and kept at 4°C before analysis using a centrifugal chemical analyser (Fara II, Roche Biochemicals). The ALT, AST and LDH levels were determined within 2 h of collection using standard assays optimized for a clinical analyser [28].

Histopathology and immunohistochemistry

Mouse livers were fixed by infusion with 4% paraformaldehyde followed by embedding in paraffin wax. Thin slices, 10 μm, were prepared and examined under light microscope with appropriate magnification. Nitrated proteins were identified by polyclonal anti-3-NT antibody (1:1000). The anti-NT antibody was diluted in PBS containing bovine serum albumin for stabilization thereby preventing denaturation of the antibody sample. Powdered milk has been associated with non-specific binding of the primary antibody and an associated overall high background staining due to endogenously nitrated proteins due to mastitis in milk cows. Specific binding of anti-NT antibody to tyrosine residues was demonstrated by incubating (16 h at 4°C) the antibody with 3-NT (10 mM in PBS) which was adjusted to pH 7.4 following addition of NT as previously described by Beckman [24,29]

Statistics

All data were compared using a one-way analysis of variance (ANOVA) with *post hoc* pairwise testing of significant differences using a Student–Newman–Keuls

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(SNK) test. A *p*-value less than 0.05 was considered statistically significant.

Results

Hepatic enzymes

The ALT, AST and LDH levels did not increase significantly after infusion of the MnP compounds, suggesting an absence of acute hepatotoxicity from the MnPs. The post-ischemic release of hepatocellular enzymes peaked at 1 min of reperfusion, reflecting washout of accumulated enzymes and immediate reperfusion injury. This was designated as maximum injury. The hepatocellular enzymes in the effluent decreased rapidly, followed by a steady increase after 15–90 min of reperfusion, reflecting cumulative injury in the closed, recirculating system. The infusion of MnTE-2-PyP at a concentration of 10 μM lowered the level of ALT release significantly compared to the untreated control at all time points (1–90 min) during reperfusion (Figure 3a top). The infusion of 10 μM MnTM-2-PyP, or MnTECP, decreased the level of ALT release significantly compared to the untreated control from 5 to 90 min of reperfusion (Figure 3a top). The AST levels were also decreased following the infusion of the MnP compounds. All of the MnP compounds tested decreased the AST levels with MnTE-2-PyP significantly reducing the AST levels at all of the time points between 1 and 90 min of reperfusion. The release of AST generally paralleled the ALT release with MnTE-2-PyP being the most effective (Figure 3b middle). The LDH levels were significantly decreased compared to the untreated controls with MnTE-2-PyP from 1 to 90 min, MnTM-2-PyP from 2.5 to 90 min and MnTECP from 5 to 90 min of reperfusion, respectively (Figure 3c bottom).

Figure 5 emphasizes the effects of MnP on ALT release since it is a more specific marker for liver injury than AST and LDH. In addition, the figure depicts the effects of the MnP on the initial (1 min) and cumulative (90 min) release of ALT. The infusion of MnTE-2-PyP significantly decreased ALT at 1 and 90 min following reperfusion (Figure 4). The MnTM-2-PyP and MnTECP significantly decreased ALT release significantly compared to the untreated control at 90 min (Figure 4 bottom).

To further characterize the dose response of MnTE-2-PyP on I/R injury, concentrations from 0.1 to 10 μM were evaluated for the effect on post-ischemic enzyme release. It was demonstrated that 10 μM MnTE-2-PyP significantly decreased I/R injury at both 1 min (Figure 5 top) and 90 min (Figure 5 bottom). Cumulative ALT release (90 min) was also significantly decreased by 1 μM concentration of MnTE-2-PyP (Figure 5 bottom). The release of AST and LDH at 1 min reperfusion was also significantly

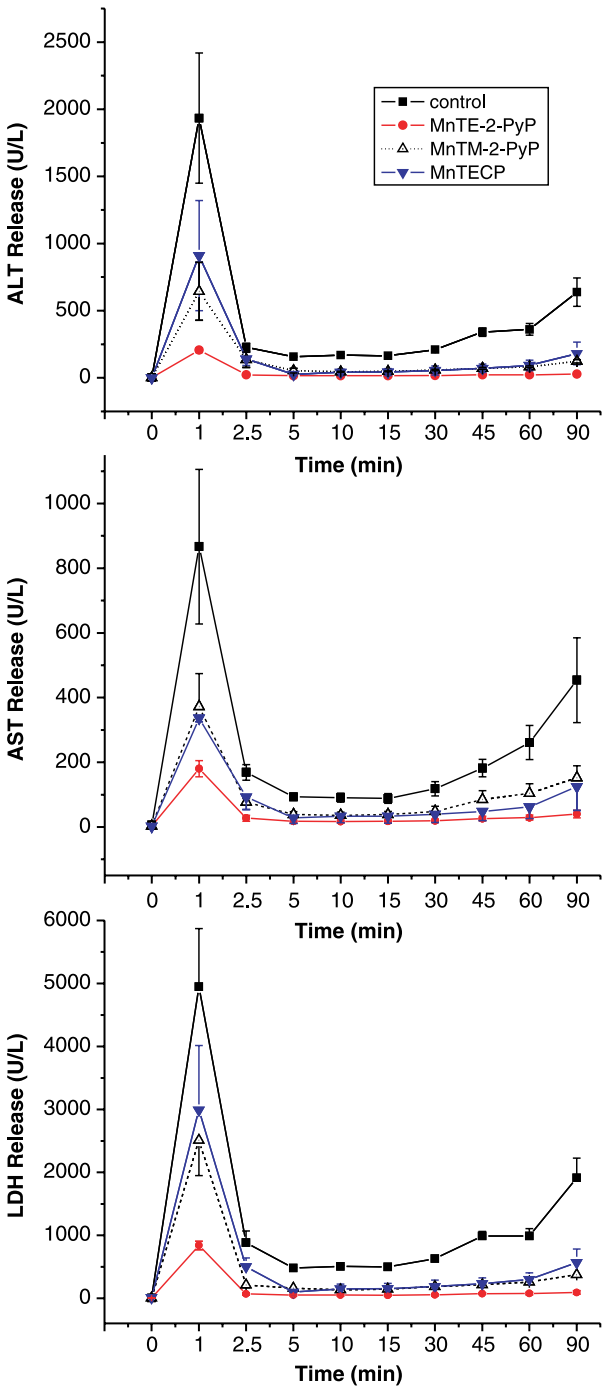

Figure 3. The effects of MnPs on the time course of release of hepatocellular enzymes after I/R. The release of hepatocellular enzymes- ALT (top), AST (middle) and LDH (bottom) during baseline and over 90 min reperfusion ($n = 6$ per treatment, $p < 0.05$).

attenuated with 10 μM MnTE-2-PyP. The cumulative ischemic injury (90 min), as assessed by AST and LDH release, was significantly decreased by both 1 and 10 μM of MnTE-2-PyP (data not shown).

To measure the effects of MnPs on lipid peroxidation, TBARS were determined. Both MnTE-2-PyP and MnTM-2-PyP significantly decreased TBARS

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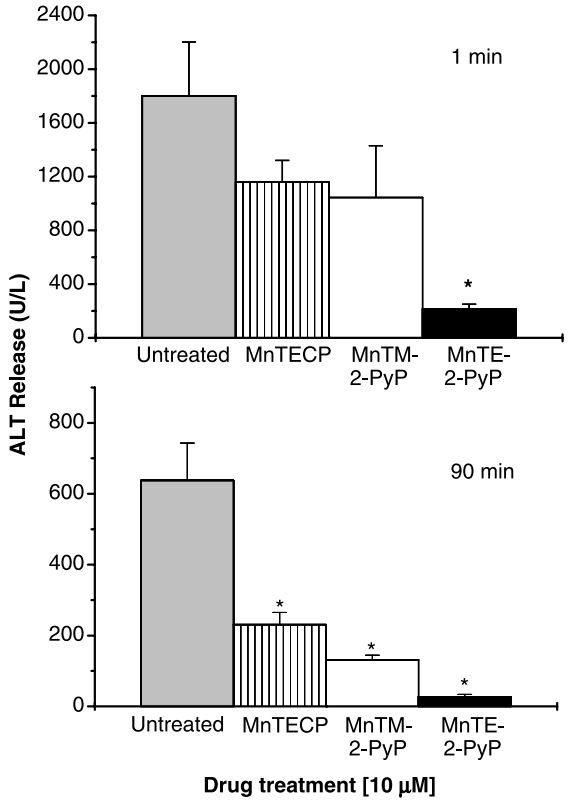

Figure 4. Effect of MnPs on hepatic I/R. The release of ALT was partially attenuated by MnPs at 1 and 90 min. MnTE-2-PyP significant blocked ALT release at 1 and 90 min reperfusion ($n = 6$ per treatment, $p < 0.05$).

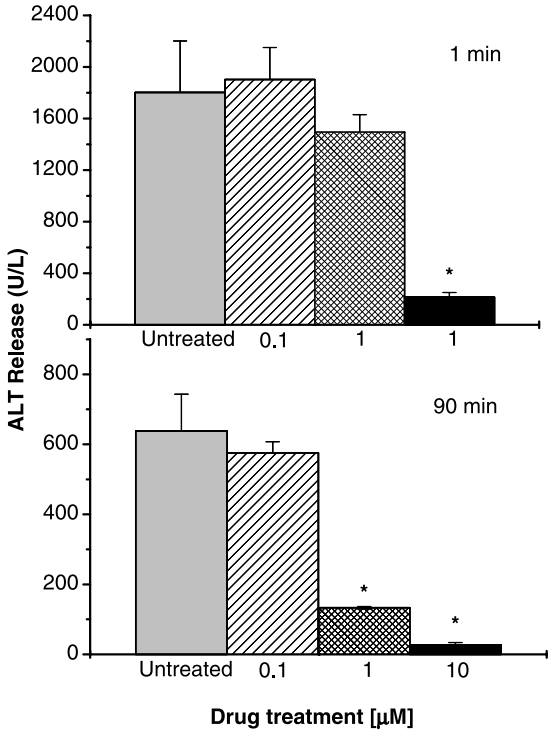

Figure 5. Dose-dependent response of MnTE-2-PyP on liver injury. The release of ALT was significantly ($p < 0.05$) attenuated by MnTE-2-PyP at concentrations of 1 and 10 μM at 90 min reperfusion ($n = 6$ per treatment, $p < 0.05$).

at 90 min of reperfusion compared to the untreated I/R group (Figure 6 bottom).

There was little or no NT staining in the control livers (Figure 7). I/R caused significant tissue injury and extensive nitration that was most intense adjacent to the central vein branches (zone 3), intermediate levels in the midzonal region and lowest levels immediately adjacent to the portal vein. The extent of nitration was most markedly attenuated in the MnTE-2-PyP treated livers. The infusion of MnTM-2-PyP and MnTECP also markedly diminshed the extent of NT staining, although to a lesser extent (Figure 7). Blocking of the anti-3-NT antibody with excess 3-NT (10 mM) blocked essentially all of the immunoreactive staining indicating specificity of the 3-NT staining (data not shown)

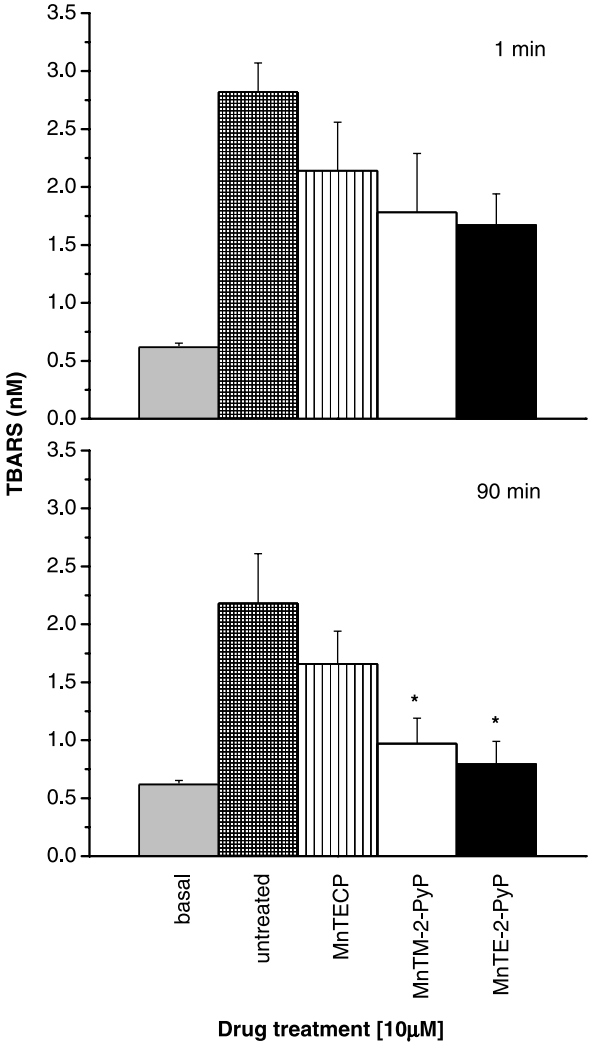

Figure 6. Effect of MnP on release of lipid oxidation products. MnPs significantly decreased TBARS at 1 and 90 min of reperfusion. Both MnTE-2-PyP and MnTM-2-PyP significantly decreased TBARS 90 min reperfusion ($n = 6$ per treatment, $p < 0.05$).

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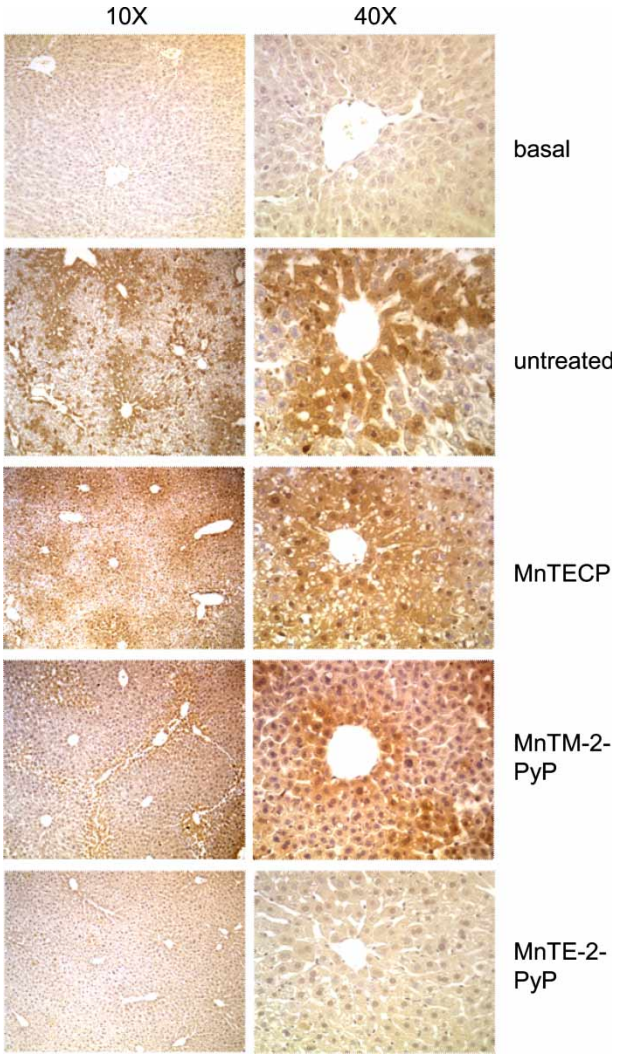

Figure 7. Immunohistochemistry by polyclonal anti-NT antibody. I/R resulted in extensive nitration of hepatic proteins, especially prominent in the centrilobular regions. Both MnTE-2-PyP and MnTECP treatment markedly reduced the extent of NT staining significantly (representative images).

Discussion

The present study demonstrated that reperfusion of the ischemic liver resulted in a biphasic release of intracellular enzymes (ALT, AST and LDH) with a quantitatively larger increase in enzyme release at 1 min after reperfusion representing "washout" of accumulated enzymes as well as immediate I/R. Post-ischemic hepatocellular enzymes returned toward baseline values and then increased throughout the 90 min reperfusion period in this recirculating system. All three MP SOD mimetics significantly decreased the hepatocellular injury associated with I/R. The MnP with the highest SOD activity (Table I), MnTE-2-PyP, was most effective at reducing the release of hepatocellular enzymes as well as TBAR formation and nitration of hepatic proteins. MnTM-2-PyP has less SOD activity but two times more catalase activity than MnTM-2-PyP and was intermediate in its ability to attenuate ischemia-induced hepatic injury and significantly decreased formation of TBARS at 90 min. MnTECP has minimal SOD activity but catalase activity similar to MnTE-2-PyP but was least effective in reducing hepatocellular enzyme release and did not significantly decrease the formation of TBARS.

The changes in post-ischemic hepatic injury correlate with the SOD activity of the MnPs – the more SOD activity, the less damage. The highest SOD activity among these MnPs was MnTE-2-PyP > MnTM-2-PyP ≫ MnTECP [27,30–32]. The *in vitro* SOD activity of the MnP compounds is determined by the metal-centered redox potentials, but additional *in vivo* factors also contribute to the actual efficacy [33]. The ability to increase SOD activity through administration of MnPs, could potentially remove $O_2^{\cdot-}$ and/or oxidative injury and may play a key role in protecting the liver from I/R damage. These findings are consistent with other findings showing that SOD overexpression attenuates I/R damage [17–21]. This pattern of MnP-mediated attenuation of hepatocellular injury does not seem to correlate with catalase activity or the ability to inhibit iron/ascorbate induced lipid peroxidation. MnTM-2-PyP has about two-times more catalase activity than MnTE-2-PyP and is similar to MnTECP.

There is considerable evidence that oxidants influence the development of liver I/R damage [34,35]. Moreover, a recent study demonstrated that SOD, catalase and glutathione peroxidase levels in the liver were significantly lower in rats following I/R [36], consistent with the hepatic protective effect of augmentation of antioxidant levels in I/R. Administration of MnTE-2-PyP has also been demonstrated to be cardioprotective reducing infarct size after myocardial I/R [37]. In addition, MnTE-2-PyP exhibits neural protective effects reducing both infarct size and the neurological deficit associated with experimental brain injury [38,39].

There is also considerable data that indicates that NO is produced by the liver [40]. There is also increasing recognition that NO plays an integral role in hepatic I/R injury [40–42]. Upon reperfusion NO can react with $O_2^{\cdot-}$ to form $ONOO^-$, leading to oxidation and nitration of tissue components [43]. In the present study, MnTE-2-PyP and MnTM-2-PyP markedly attenuated the extent of protein nitration in the centrilobular area of the liver following I/R (Figure 7), consistent with their putative role of MPs as peroxynitrite decomposition catalysts [44]. It has been demonstrated that these MP are Mn(III) porphyrins and can react quickly with $ONOO^-$ to form O=Mn(IV) and nitrogen dioxide ($NO_2^{\cdot}$) [45]. The differences in the ability of these MnPs to scavenge $ONOO^-$ could be due to how fast the MnPs react with $ONOO^-$, how well these MnPs recycle and how well these MnPs quench intermediate ROS and RNS [46,47].

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In addition to the ability of MPs to scavenge $O_2^{\cdot-}$, H_2O_2 and $ONOO^-$, MPs are also potent inhibitors of lipid oxidation. Studies have shown that MnPs can accept electrons from flavin-dependent endogenous enzymes and that this activity contributes to their antioxidant potency [48,49]. Biologic reductants such as ascorbate, urate and thiols can reduce and recycle the MnP [45,50]. Moreover, if reductants are limited, the MnPs may fail to scavenge radicals effectively thereby promoting lipid oxidation [51]. The calculated IC_{50} for MnTE-2-PyP, MnTM-2-PyP and MnTECP are similar (1.1–1.5 μM) and are 1–3 orders of magnitude more potent at inhibiting lipid peroxidation than CuZn SOD or the prototypical MnP, MnTBAP [27,31,32,52]. These findings are consistent with the observation that both MnTE-2-PyP and MnTM-2-PyP significantly attenuated the formation of TBARS at 90 min reperfusion (Figure 6).

The isolated perfused mouse liver model were designed to minimize the possible confounding effects of perfusion with blood components, such as circulating neutrophils, as well as the potential effects of the MP on hemodynamics [30]. In these studies, the reperfusion period was limited to 90 min *ex vivo* perfusion to minimize the effect of more sustained reperfusion periods on viability.

In conclusion, MnP complexes can attenuate hepatocellular damage, lipid peroxidation and protein nitration from I/R injury in isolated perfused mouse livers. In this model, MnTE-2-PyP was more effective than MnTM-2-PyP and MnTECP. The diverse antioxidant properties of the MP complexes may make them ideally suitable for therapeutic interventions in disease states involving I/R injury and free radicals, especially shock, trauma, stroke, myocardial infarction and organ transplantation.

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