Manganese porphyrins are a potent class of catalytic antioxidants whose activity was recently shown to be partially dependent upon flavin-containing enzymes (R. Kachadorian et al., 2004, Biochem. Pharmacol. 67, 77–85). We tested whether manganese porphyrins could redox cycle with the flavin-containing enzyme, cytochrome P450 reductase, and whether this results in the inhibition of xenobiotic metabolism. The effect of manganese porphyrins on xenobiotic metabolism in rat and human liver microsomes was assessed spectrophotometrically by following the O-dealkylation of benzyloxy- (BROD) and methoxyresorufin (MROD). Redox cycling of manganese porphyrins with human cytochrome P450 reductase was assessed both spectrophotometrically and polarographically by following the consumption of NADPH and oxygen, respectively. The tetraakis(N-pyridinium-2-yl) meso-substituted manganoporphyrin, MnTEPyP5+, and the tetraakis(1,3-dimethyl imidazolium-2-yl) meso-substituted manganoporphyrin, MnTDMIP5+, were 40 to 100 times more potent inhibitors of rat and human liver microsomal BROD metabolism than the tetraakis(1,3-diethyl imidazolium-2-yl) meso-substituted manganoporphyrin, MnTDEIP5+, or the tetraakis(4-benzoic acid) meso-substituted manganoporphyrin, MnTBAP. A similar pattern of inhibition was also seen in β-naphthoflavone-induced rat liver microsomal MROD metabolism. This pattern of xenobiotic metabolism inhibition correlated with the compound’s ability to microsomal MROD metabolism. This pattern of xenobiotic metabolism inhibition was also seen in human rat.

Key Words: xenobiotic metabolism; catalytic antioxidants; oxidoreductase; human; rat.

Received December 9, 2004; accepted February 1, 2005

A number of new metal complexes are currently under investigation as catalytic antioxidant therapeutic agents and include manganese-containing porphyrins, salens, and cyclic polyamines (Day, 2004). Manganese porphyrins possess a number of antioxidant capacities such as superoxide (Pasternack and Skowronek, 1979) and hydrogen peroxide dismutation (Day et al., 1997), inhibition of lipid peroxidation (Day et al., 1999a), and scavenging peroxynitrite (Szabo et al., 1996). These activities have been linked to their protective effects in a wide variety of in vitro and in vivo models of oxidative stress (Day, 2004). Much of the compound development of manganese porphyrins has focused on changing their redox potentials towards those of the endogenous superoxide dismutase (SOD) enzymes (Batinic-Haberle et al., 1998). Some of the newer manganese porphyrins also have similar redox potentials and fast second order rate constants for their dismutation of superoxide, >10⁷ M⁻¹ s⁻¹ (Batinic-Haberle et al., 1998). The endogenous SODs have the advantage that their protein structures provide a selective channel that limits electron transfers to small molecules like superoxide (Djinovic et al., 1992). The newly developed classes of metal complexes lack this degree of selectivity and thus are likely to give and accept electrons from a wide variety of biological molecules and potentially oxidoreductase enzymes.

Cytochrome P450 reductase is an important oxidoreductase that provides electrons for xenobiotic metabolism to the cytochrome P450 enzymes and heme oxygenase (Schacter et al., 1972). NADPH supplies reducing equivalents to the flavin-domain of cytochrome P450 reductase (Vermilion and Coon, 1978). Cytochrome P450s are a superfamily of enzymes involved in the synthesis and degradation of drugs and xenobiotics and in the formation of a number of important endogenous compounds, including hormones and neurotransmitters (Gonzalez, 1992). Inhibition of cytochrome P450 reductase has the potential to disrupt a number of pathways essential to normal cellular function, such as heme oxygenase (Choi and Alam, 1996).

A number of drugs and other xenobiotics are known to redox cycle with oxidoreductases, however, many of these reactions are associated with oxidative stress and cellular toxicity.
Cycling with cytochrome P450 reductase. Xenobiotic metabolism and whether this may be through redox nates and that this activity partially contributes to their ability has recently reported that manganoporphyrins can redox cycle ically, a few of these types of reactions can also lead to enhanced antioxidant activity, particularly if the xenobiotic can detoxify any generated reactive oxygen species. Our laboratory has recently reported that manganoporphyrins can redox cycle with flavin-dependent cellular enzymes in rat brain homogenates and that this activity partially contributes to their ability to inhibit lipid peroxidation (Kachadorian et al., 2004). In this report, we investigated whether manganoporphyrins affect xenobiotic metabolism and whether this may be through redox cycling with cytochrome P450 reductase.

MATERIALS AND METHODS

Reagents. Chemicals and biologicals include manganese (III) meso-tetra-kis(1,3-dimethylimidazolium-2-yl) porphyrin pentachloride (MnTDMIP\(^3\)), manganese (III) meso-tetra-kis(1,3-dimethylimidazolium-2-yl) porphyrin pentachloride (MnTDEIP\(^3\)), manganese (III) meso-tetra-kis(4-benzoic acid) porphyrin sodium chloride salt (MnTBAP) (kind gifts from Aeolus Pharmaceuticals, Greenwood Village, CO); sterile phosphate buffered saline pH 7.4 (PBS, Invitrogen Corporation, Carlsbad, CA); 7-methoxyresorufin, 7-benzyloxyresorufin, and resorufin (Molecular Probes, Eugene, OR). Human recombinant cytochrome P450 reductase was purchased from Panvera (Madison, WI). Control and induced rat liver microsomes and pooled human liver microsomes were purchased from In Vitro Technologies (Baltimore, MD). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Xenobiotic metabolism assays. Microsomal cytochrome P450 activity was assessed using two different substrates, benzyloxyresorufin (BR) and methoxyresorufin (MR) that are metabolized to resorufin by a broad range of microsomes were incubated with increasing concentrations of manganoporphyrin, MnTDMIP\(^5\), and the tetra-kis(1,3-dimethyl imidazolium-2-yl) meso-substituted manganoporphyrin, MnTEPyP\(^5\)\(^+,\) and the tetra-kis(1,3-dimethyl imidazolium-2-yl) meso-substituted manganoporphyrin, MnTDMIP\(^5\)\(^+,\). 40 to 100 times more potent inhibitors of rat liver microsomal BROD metabolism than the tetra-kis(1,3-dimethyl imidazolium-2-yl) meso-substituted manganoporphyrin, MnTDEIP\(^5\)\(^+,\) or the tetra-kis(4-benzoic acid) meso-substituted manganoporphyrin, MnTBAP (Fig. 2). To test whether there was species specificity; the reaction was also examined in pooled human liver microsomes. A similar pattern of liver microsomal BROD inhibition by manganoporphyrins was also observed in the human samples (Fig. 3). The only difference between rat and human microsomal BROD inhibition was that the manganoporphyrins were about three-fold less potent. The apparent mechanism of the

RESULTS

In Vitro Inhibition of Liver Microsomal Metabolism by Manganoporphyrins

Cationic porphyrins are being pursued as catalytic antioxidants for the treatment of a variety of human diseases that involve the overproduction of reactive oxygen and nitrogen species (Fig. 1). A common concern during drug development involves the inhibition of xenobiotic metabolism that may result in drug-drug interactions. A series of manganoporphyrins were tested for their ability to inhibit liver microsomal xenobiotic metabolism. Resorufin analogs provide a convenient and sensitive fluorometric method to assess changes in xenobi otic metabolism in vitro (Lubet et al., 1990). Control rat liver microsomes were incubated with increasing concentrations of manganoporphyrins and inhibition of benzyloxyresorufin O-dealkylase activity (BROD) metabolism was measured spectrofluorometrically. The inhibitory concentrations of manganoporphyrins that decreased xenobiotic metabolism by 50% (IC\(_{50}\)) were determined by fitting a sigmoidal curve with variable slope to the data. The tetra-kis(N-4-pyridinium-2-yl) meso-substituted manganoporphyrin, MnTDEIP\(^5\)\(^+,\) and the tetra-kis(1,3-dimethyl imidazolium-2-yl) meso-substituted manganoporphyrin, MnTDMIP\(^5\)\(^+,\) were 40 to 100 times more potent inhibitors of rat liver microsomal BROD metabolism than the tetra-kis(1,3-dimethyl imidazolium-2-yl) meso-substituted manganoporphyrin, MnTDEIP\(^5\)\(^+,\) or the tetra-kis(4-benzoic acid) meso-substituted manganoporphyrin, MnTBAP (Fig. 2). To test whether there was species specificity; the reaction was also examined in pooled human liver microsomes. A similar pattern of liver microsomal BROD inhibition by manganoporphyrins was also observed in the human samples (Fig. 3). The only difference between rat and human microsomal BROD inhibition was that the manganoporphyrins were about three-fold less potent. The apparent mechanism of the
Manganoporphyrin inhibition of xenobiotic metabolism was further investigated using \( \beta \)-naphthoflavone-induced rat liver microsomes and following methoxyresorufin O-dealkylase (MROD) activity spectrofluorometrically. \( \beta \)-Naphthoflavone induces the expression of CYP1A family of cytochrome \( \text{P}450 \) enzymes that prefers shorter alkyl chain substituted resorufin analogs (Novak and Qualls, 1989). Again the pattern of MROD inhibition by manganoporphyrins was remarkably similar to pattern observed for BROD inhibition (Fig. 4). These data suggested that the manganoporphyrins may share a common mechanism for the inhibition of xenobiotic metabolism and further studies focused on the commonly shared cytochrome \( \text{P}450 \) reductase that supplies electrons to the different cytochrome \( \text{P}450 \) isozymes.

FIG. 1. The chemical structures of several manganoporphyrins: manganese (III) meso-tetrakis(1,3-dimethylimidazolium-2-yl) porphyrin (MnTDMIP\(^{5+}\)), manganese (III) meso-tetrakis(1,3-diethylimidazolium-2-yl) porphyrin (MnTDEIP\(^{5+}\)), manganese (III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin (MnTEPyP\(^{5+}\)), and manganese (III) meso-tetrakis(4-benzoic acid) porphyrin (MnTBAP) are illustrated.

FIG. 2. In vitro inhibition of rat liver microsomal metabolism by a series of manganoporphyrins. Control rat liver microsomes are incubated with increasing concentrations of manganoporphyrins and inhibition of benzoyloxresorufin O-dealkylase (BROD) activity is measured spectrofluorometrically. Data is presented as the percent of BROD activity in the absence of manganoporphyrins. Control BROD activity is 120 ± 10 pmol/min/mg. Each value is performed in triplicate and data are presented as their mean ± SEM. The inhibitory concentrations of manganoporphyrins that decreased xenobiotic metabolism by 50% (IC\(_{50}\)) are determined by fitting a sigmoidal curve with variable slope to the data. Both the MnTEPyP\(^{5+}\) and MnTDMIP\(^{5+}\) cationic manganoporphyrins are potent inhibitors of rat liver microsomal BROD metabolism while the cationic MnTDEIP\(^{5+}\), and the non-cationic MnTBAP, manganoporphyrins are weak inhibitors.

FIG. 3. In vitro inhibition of human liver microsomal metabolism by a series of manganoporphyrins. Pooled human liver microsomes are incubated with increasing concentrations of manganoporphyrins and inhibition of methoxyresorufin O-dealkylase (BROD) activity is measured spectrofluorometrically. Data is presented as percent of BROD activity in the absence of manganoporphyrins. Control BROD activity is 60 ± 12 pmol/min/mg. Each value is performed in triplicate and data are presented as their mean ± SEM. The inhibitory concentrations of manganoporphyrins that decreased xenobiotic metabolism by 50% (IC\(_{50}\)) are determined by fitting a sigmoidal curve with variable slope to the data. Both the MnTEPyP\(^{5+}\) and MnTDMIP\(^{5+}\) cationic manganoporphyrins are potent inhibitors of human liver microsomal BROD metabolism while the cationic MnTDEIP\(^{5+}\), and the non-cationic MnTBAP, manganoporphyrins are weak inhibitors.

FIG. 4. In vitro inhibition of rat liver \( \beta \)-naphthoflavone-induced microsomal metabolism by a series of manganoporphyrins. \( \beta \)-Naphthoflavone-induced rat liver microsomes are incubated with increasing concentrations of manganoporphyrins and inhibition of methoxyresorufin O-dealkylase (MROD) activity is measured spectrofluorometrically. Data is presented as percent of MROD activity in the absence of manganoporphyrins. Control MROD activity is 55 ± 6 pmol/min/mg. Each value is performed in triplicate and data are presented as their mean ± SEM. The inhibitory concentrations of manganoporphyrins that decreased xenobiotic metabolism by 50% (IC\(_{50}\)) are determined by fitting a sigmoidal curve with variable slope to the data. The MnTEPyP\(^{5+}\) and MnTDMIP\(^{5+}\) cationic manganoporphyrins are potent inhibitors of rat liver \( \beta \)-naphthoflavone-induced microsomal metabolism while the cationic MnTDEIP\(^{5+}\) and the non-cationic MnTBAP manganoporphyrins are much less potent inhibitors.
One potential explanation for manganoporphyrins’ inhibition of microsomal xenobiotic metabolism would be their ability to compete with cytochrome P450s for electrons from cytochrome P450 reductase. To test for this possibility, two manganoporphyrins with similar charge and redox potentials were assayed for their ability to stimulate NADPH utilization by human recombinant cytochrome P450 reductase. Neither compound stimulated NADPH oxidation in the absence of cytochrome P450 reductase. Both MnTEPyP$^{5+}$ and MnTDEIP$^{5+}$ stimulated cytochrome P450 reductase oxidation of NADPH. (A) Lineweaver-Burke plot of data reveals a lower Km and higher Vmax for MnTEPyP$^{5+}$ than MnTDEIP$^{5+}$.

**FIG. 5.** Cationic manganoporphyrins stimulate NADPH oxidation by human cytochrome P450 reductase. Human recombinant cytochrome P450 reductase and NADPH (250 µM) is incubated in the presence of increasing concentrations of either MnTDEIP$^{5+}$ or MnTEPyP$^{5+}$ and NADPH oxidation is followed spectrophotometrically at 340 nm for 1 min. Each value is performed in triplicate and data are presented as their mean ± SEM. (A) Both MnTEPyP$^{5+}$ and MnTDEIP$^{5+}$ stimulated cytochrome P450 reductase oxidation of NADPH. (B) Lineweaver-Burke plot of data reveals a lower Km and higher Vmax for MnTEPyP$^{5+}$ than MnTDEIP$^{5+}$.

**Manganoporphyrins Redox Cycle with Cytochrome P450 Reductase**

Most compounds that redox cycle with flavin-containing proteins, such as cytochrome P450 reductase, often can utilize oxygen as a terminal electron acceptor (Hochstein, 1983). To test for this possibility, oxygen utilization was assessed using an oxygen electrode in the presence of NADPH, MnTEPyP$^{5+}$, and human recombinant cytochrome P450 reductase (Fig. 6A). MnTEPyP$^{5+}$’s ability to redox cycle with human recombinant cytochrome P450 reductase in a dose-dependent manner. MnTEPyP$^{5+}$’s ability to redox cycle with human recombinant cytochrome P450 reductase was compared with another well-known redox cycler, paraquat (Fig. 6B). MnTEPyP$^{5+}$ was a much more active redox cycling agent with human recombinant cytochrome P450 than paraquat. The mechanism of MnTEPyP$^{5+}$ redox cycling was further investigated using a non-specific flavin domain inhibitor, diphenyleneiodonium (DPI) (McGuire et al., 1994). DPI was an effective inhibitor of MnTEPyP$^{5+}$ redox cycling with human recombinant cytochrome P450 reductase with an IC$_{50}$ of 4 µM (Fig. 7). The rank order potency of the manganoporphyrins to redox cycle with human recombinant cytochrome P450 reductase was similar to...
inhibit microsomal xenobiotic metabolism. Structure activity relationships revealed that the cationic manganoporphyrins, MnTEPyP$_{5}^{+}$ and MnTDMIP$_{5}^{+}$, were potent inhibitors of microsomal BROD and MROD activities. However, this potent inhibition was lost by chain extension of the alkyl groups of MnTDMIP$_{5}^{+}$ to form MnTDEIP$_{5}^{+}$. These data suggested that bulky alkyl substitutions on the imidazolium could be used to limit the inhibition of xenobiotic metabolism. Interestingly, both MnTDMIP$_{5}^{+}$ and MnTDEIP$_{5}^{+}$ have similar redox potentials and catalytic antioxidant activities (Kachadourian et al., 2004), so that the chain extension did not appear to affect their in vitro therapeutic activities. However, recent studies from our laboratory suggest that in vivo catalytic antioxidant activity of manganoporphyrins is partially due to redox cycling with flavin containing enzymes (Kachadourian et al., 2004). It is also possible that each flavin containing oxidoreductase has different structural requirements for efficient electron transfers and one may be able to selectively utilize some oxidoreductases while avoiding others. Therefore, it remains to be determined whether the in vivo activity of manganoporphyrins will be severely affected by bulky alkyl substitutions. It is also likely that other metal complexes besides porphyrins also share this ability to compete for electrons from endogenous flavin containing oxidoreductases. Many of these complexes also share similar redox potentials, can accept one to two electrons, and are smaller and more hydrophobic than most metalloporphyrins. However, to date, most characterized antioxidant activities for the manganosalens (Eukarion series) and polycyclic amines (Metaphore series) have been restricted to in vitro reactions devoid of flavin containing oxidoreductases (Baudry et al., 1993; Salvemini et al., 1999).

Manganoporphyrins are potent antioxidants in a wide number of in vitro, ex vivo, and in vivo models of oxidative stress (Day, 2004). The present studies are paradoxical given that most compounds identified to date that undergo redox cycling are generally considered as pro-oxidants and not antioxidants. One possible explanation for these paradoxical activities is that the manganoporphyrins can not only generate reactive species but can also consume them, unlike compounds such as paraquat that only generate reactive species. Since manganoporphyrins have been shown to strongly protect against oxidative stress, it is very likely that their in vivo scavenging activities greatly exceed their ROS generating activities. In fact, the low steady-state levels of superoxide in biological systems suggest that the reduction of manganoporphyrins is likely to occur by either endogenous low molecular weight antioxidants, like glutathione and ascorbate, or flavin-dependent oxidoreductases both of which are much more abundant in cells than superoxide. This concept would support the manganoporphyrins acting as superoxide reductases that derive the first electron from a flavin-dependent oxidoreductase, which can use to convert superoxide to hydrogen peroxide (Liochev and Fridovich, 2000). In addition, manganoporphyrins can also dismute hydrogen peroxide to oxygen and water (Day et al., 1997). These properties probably...
contribute to the manganoporphyrin’s paradoxical activities of being both a potent antioxidant and redox cycling agent. However, under conditions such as elevated oxygen tensions the metalloporphyrins lose their antioxidant potency and are ineffective at preventing hyperoxia-induced lung injury (Day, unpublished data).

Flavin-dependent oxidoreductases comprise an abundant class of cellular enzymes with a wide range of important cellular functions. It is possible that inhibition of these enzymes could account for both adverse and possibly beneficial effects of the compounds. Many oxidoreductases, such as cytochrome P450 reductase, have been implicated either directly or indirectly in the endogenous production of reactive oxygen species (Bartoszek and Wolf, 1992; Rashba-Step and Cederbaum, 1994; Tindberg and Ingelman-Sundberg, 1989). Among this class of enzymes there are a number of enzymes well characterized to be involved in both oxidative and nitrosative stress. Nitric oxide synthases are well-characterized enzymes involved in both oxidative and nitrosative stress. Nitric oxide synthases are well-characterized enzymes involved in both oxidative and nitrosative stress.

In summary, manganoporphyrins were found to inhibit microsomal xenobiotic metabolism that demonstrated sensitivity to small changes in carbon length on the meso-substituents. The mechanism for this inhibition appears to involve the redox cycling with cytochrome P450 reductase. The rate of redox cycling with cytochrome P450 reductase correlated with the potency of microsomal xenobiotic metabolism.

ACKNOWLEDGMENTS

These studies were supported in part by a National Institutes of Health Grant HL-31992 and a research grant from Aeolus Pharmaceuticals, Inc.

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In summary, manganoporphyrins were found to inhibit microsomal xenobiotic metabolism that demonstrated sensitivity to small changes in carbon length on the meso-substituents. The mechanism for this inhibition appears to involve the redox cycling with cytochrome P450 reductase. The rate of redox cycling with cytochrome P450 reductase correlated with the potency of microsomal xenobiotic metabolism.


