Free Radical Research, January 2005; 39(1): 81–88

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A Manganese porphyrin suppresses oxidative stress and extends the life span of streptozotocin-diabetic rats

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Accepted by Professor J. Vina

(Received 29 September 2004; in revised form 2 November 2004)

Abstract

Enhanced oxidative stress due to hyperglycemia has been implicated in diabetic complications and is considered a major cause of cell and tissue damage. The aim of the present study was to investigate whether synthetic manganese porphyrin, Mn(III) 5,10,15,20-tetrakis(*N*-methylpyridinium-2-yl)porphyrin (MnTM-2-PyP^{5+}) can ameliorate diabetes-induced oxidative stress and affect life span of diabetic rats.

Diabetes was induced by a single (60 mg/kg) intraperitoneal injection of streptozotocin in male Wistar rats. Oxidative stress was monitored by measuring malondialdehyde levels (MDA) in blood plasma and erythrocytes using HPLC. The antioxidant status was assessed by measuring the total radical-trapping potential (TRAP) of blood plasma. Life span of the animals was used as an indication of the overall effect of MnTM-2-PyP^{5+}. MnTM-2-PyP^{5+} was administered subcutaneously at 1 mg/kg for the duration of the experiment, five times/week followed by one week of rest.

Diabetes increased plasma and erythrocyte levels of MDA and decreased TRAP. MnTM-2-PyP^{5+} had no effect on blood glucose and glycosylated hemoglobin, but significantly increased TRAP and lowered MDA. This Mn porphyrin decreased mortality and markedly extended the life span of the diabetic animals.

MnTM-2-PyP^{5+} suppressed diabetes-induced oxidative stress, which presumably accounts for its beneficial effect on the life span of the diabetic rats. The results indicate that Mn(III) *N*-alkylpyridylporphyrins can be used as potent therapeutic agents in diabetes.

Keywords: *Diabetes, oxidative stress, manganese porphyrin, SOD mimic, antioxidant, diabetic*

Introduction

Enhanced oxidative stress due to hyperglycemia has been documented in diabetes and has been implicated in diabetic complications.[1,2] Hyperglycemia may lead to the production of free radicals derived from the direct autoxidation of glucose,[3] from glycation of proteins with the consequent increased formation of the glucose-derived advanced glycosylation end products (AGE),[4] from the activation of the NAD(P)H oxidases[5,6] NOS,[7] and from xanthine oxidase.[8] Studies of the animal models of diabetes revealed that some of the functional and morphological abnormalities caused by hyperglycemia could be prevented by antioxidants.[9–11] However, in general the findings are inconclusive because other reports indicated absence of improvement of diabetic complications with antioxidant treatment.[12,13] This might result from insufficient understanding of the mechanisms underlying oxidative damage, which hampers the selection of specific targets for antioxidant therapy as well as proper antioxidants.

Metalloporphyrins are synthetic antioxidants whose advantages are that they are stable, of low-molecular weight, water soluble, cell permeable,[14] lack

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760400022368

antigenicity and demonstrate broad antioxidant capacity including catalytic scavenging of superoxide,[15,16] peroxynitrite,[17,18] carbonate radical,[17] nitric oxide,[19] and hydrogen peroxide.[20] They are also potent inhibitors of lipid peroxidation.[21–23] We have previously demonstrated that both *ortho* isomers, manganese(III) 5,10,15,20-tetrakis(*N*-methylpyridinium-2-yl)porphyrin (MnTM-2-PyP^{5+}, AEOL-10112) and its *N*-ethyl derivative (MnTE-2-PyP^{5+}, AEOL-10113) have nearly identical O_2^- dismuting and $ONOO^-$ reducing ability and exhibited similar efficacy in alleviating superoxide-dependent damage of SOD-deficient *Escherichia coli*.[15,16,24] Both porphyrins were also reported to be effective radioprotectors.[25,26] Ethyl derivative was tested and found protective in other rodent models of oxidative stress injury such as sickle cell disease,[27] cancer,[28,29] stroke,[30] and spinal cord injury.[31]

Recently, Piganelli et al. showed that MnTE-2-PyP^{5+} can prevent or delay the onset of autoimmune diabetes by protecting β-cell destruction,[32] and by protecting human pancreatic islets against oxidative stress *in vitro*.[33]

The aim of the present study was to investigate the ability of MnTM-2-PyP^{5+} to protect against diabetes-induced oxidative stress. We herewith demonstrate that this Mn porphyrin is efficient in suppressing oxidative damage and can extend the life span of streptozotocin-diabetic rats.

Materials and methods

The 5,10,15,20-tetrakis(2-pyridyl)porphyrin (H_2T-2-PyP) was supplied by MidCentury Chemicals (Chicago, IL). The *N*-methylation and metal incorporation was accomplished as previously described.[15] All other chemicals were obtained from standard sources.

Diabetes in male Wistar rats was induced by a single (60 mg/kg) intraperitoneal injection of streptozotocin (STZ). Induction of diabetes was confirmed by the presence of glucosuria within 24 h. Animals that maintained blood glucose concentrations above 15 mmol/l in the first week of diabetes were randomly divided into two groups designated as "Diabetic" and "Diabetic + MnTM-2-PyP^{5+}". The animals in the second group received subcutaneous injection of sterile MnTM-2-PyP^{5+} solution in isotonic saline, 1 mg/kg for the duration of the experiment, five times/week for four weeks and then had one-week resting period, while the animals in the first group were injected with isotonic saline only.

Blood samples were taken from the tail vein for the determination of the glucose and glycosylated hemoglobin (HbA1C). The effect of MnTM-2-PyP^{5+} on the status of antioxidants in the blood was assessed by measuring the total radical-trapping potential (TRAP). TRAP was assayed as described by Tsai et al.[34] In brief, 50 μl of plasma, to which 4% (v/v) linoleic acid was added, was diluted in PBS in an oxygen electrode apparatus. Oxidation was initiated with 4 mM 2,2′-azobis(2-amidinopropane) (AAPH), and oxygen consumption was monitored. Under such conditions the oxygen content decreased slowly while plasma antioxidants were being consumed, but rapidly increased once they were depleted. The time interval from the addition of AAPH to the onset of the rapid O_2 consumption was t_{plasma}. The antioxidant Trolox (4 mM) was then added, leading to a second slow phase of O_2 consumption followed by a second rapid phase. The time interval from the addition of Trolox to the onset of the second rapid phase was t_{Trolox}. TRAP was calculated as TRAP = $(t_{plasma}/t_{Trolox}) \times k$, where k was a constant that accounts for Trolox concentration and plasma dilution.

Lipid peroxidation was assessed by measuring the malondialdehyde levels (MDA). MDA levels of plasma and erythrocytes were determined using high-performance liquid chromatography (HPLC) according to a modified technique of Carbonneau et al.[35] Briefly, 250 μl of packed erythrocytes were suspended in 250 μl of 0.9% NaCl, and sample was taken for hemoglobin analysis. The erythrocyte suspension was then frozen at −80°C. After thawing at 37°C for 10 min, MDA assay was immediately performed as follows. An aliquot of 0.5 ml of hemolyzed erythrocytes was incubated with NaOH at 60°C for 30 min. The hydrolyzed sample was then acidified with 10% trichloroacetic acid to precipitate proteins. After centrifugation at 1,000*g* for 10 min, 300 μl of supernatant was treated with 50 μl of 0.5% thiobarbituric acid (TBA), and the color reaction was activated by heating for 30 min at 100°C. The MDA–TBA complex was isolated by HPLC using a C18 micro Bondapack column (Waters, 125 Å, 10 μm particle size [4.6 × 250 mm] HPLC cartridge column) with a 60/40 (v/v) mixture of 10 mmol/l phosphate buffer, pH 5.8, and methanol as a mobile phase. The flow rate of the solvent was 1.0 ml/min. The excitation and the emission of the fluorometric detector were set at 515 nm and at 532 nm, respectively. The 1,1,3,3-tetraethoxypropane was used as a standard.

SOD activity in erythrocytes was measured by the xanthine oxidase/cytochrome c assay.[36] The data are given as mean ± SD. Differences are calculated with Student *t* test. Significance was assumed at $P < 0.05$.

Results

Blood glucose concentrations were increased about five fold in diabetic rats compared with those in control rats. The MnTM-2-PyP^{5+} treatment had no effect on the blood glucose concentrations in diabetic rats (Table I). In addition to hyperglycemia the diabetic animals showed an elevated hemoglobin glycosylation (Table I), which was not prevented by

Table I. Blood glucose concentrations and glycosylated hemoglobin levels in control, diabetic, and diabetic—MnTM-2-PyP^{5+} treated groups.

Experimental group	Duration (months)	Glucose (mmol/l)	HbA_{1C} (%)
Control	2	3.7 ± 0.5	3.8 ± 0.8
	5	4.1 ± 0.7	3.9 ± 0.9
Diabetic	2	18.4 ± 2.4⋆	8.2 ± 1.2⋆
	5	17.4 ± 1.7⋆	9.6 ± 1.5⋆
MnTM-2-PyP^{5+}	2	19.6 ± 3.1	7.6 ± 1.1
	5	18.7 ± 2.1	8.4 ± 1.6

⋆$P < 0.05$ compared to control.

the MnTM-2-PyP^{5+} treatment. No significant differences between 2 months and 5 months of diabetes were found.

Within the first week of diabetes, the mortality rate was ~15% (9 out of 60 animals died). Six animals from the STZ-treated group showed neither glucosuria nor hyperglycemia on periodic testing. These animals were housed separately and were excluded from the analysis. The remaining 45 diabetic rats were randomly divided into two groups: diabetic, untreated (23 animals), and diabetic, MnTM-2-PyP^{5+}-treated (22 animals).

It was noticed that on a longer run the blood sugar of the STZ rats started to fall,[37] presumably as a result of regeneration of beta cells and development of islet cell adenomas, which can secrete enough insulin to reverse the diabetes.[38] For the duration of experiments (12 months), 10 animals from the diabetic untreated group (~43%) and 8 animals from the MnTM-2-PyP^{5+}-treated group (~36%) reverted to normal blood glucose. These animals were not counted as surviving the diabetes period and were excluded from the analysis.

All diabetic animals, treated (13) and untreated (14), that survived the first week, survived the next 5 months of diabetes (Figure 1). After five months of diabetes, however, mortality in the diabetic untreated group started to increase, and at the end of the 12 months observation period, only 2 animals were still alive (Figure 1). In the MnTM-2-PyP^{5+}-treated group animals started to die later (compare curves 1 and 2 in Figure 1), and at the end of the observation period 6 animals were still alive. Thus, the mortality in the untreated group was 85%, while it was 57% in the MnTM-2-PyP^{5+}-treated group.

It is generally accepted that oxidative stress causes irreversible cell injury leading to diabetic complications. The susceptibility to the oxidative stress is a function of the overall balance between the factors that induce oxidative stress and those that prevent it. The TRAP in plasma is considered a more reliable estimation of plasma antioxidant capability than the measurement of each antioxidant in particular.[39] Figure 2 shows that TRAP in the STZ diabetic rats was lower than in the controls (compare bars C and D). The MnTM-2-PyP^{5+} treatment restored TRAP almost to the control levels. No significant difference between the TRAP values at 2 and 5 months of diabetes were found.

We also used oxidative damage of lipids (lipid peroxidation) as an indication of the severity of the oxidative stress. Lipid peroxidation was quantified by measuring malondialdehyde, which is an end product of the peroxidation of unsaturated fatty acids. After two months of diabetes, plasma MDA concentrations were ~1.5 times higher in the diabetic group, compared to the non-diabetic controls (Figure 3A).

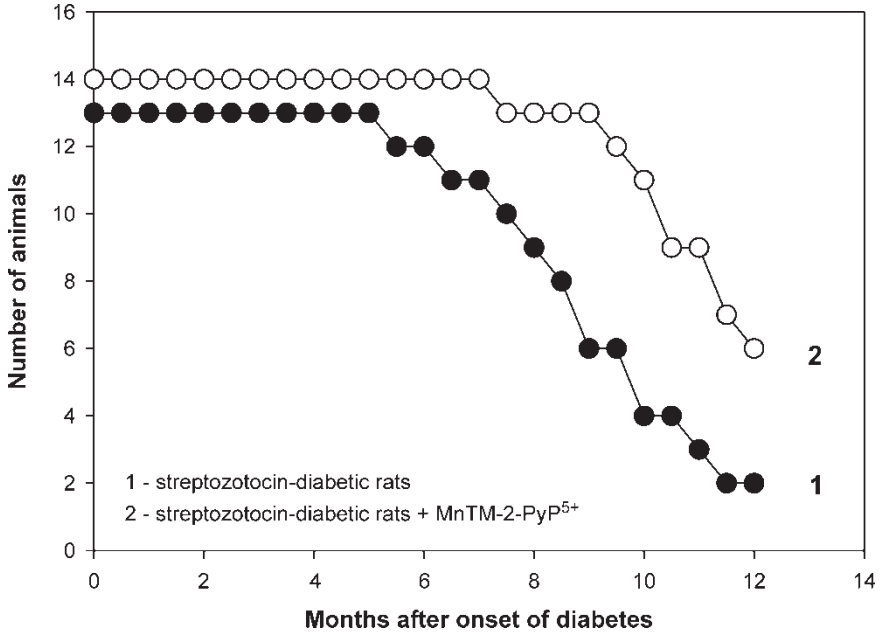

Figure 1. Life span of diabetic (**1**), and diabetic MnTM-2-PyP^{5+}-treated rats (**2**).

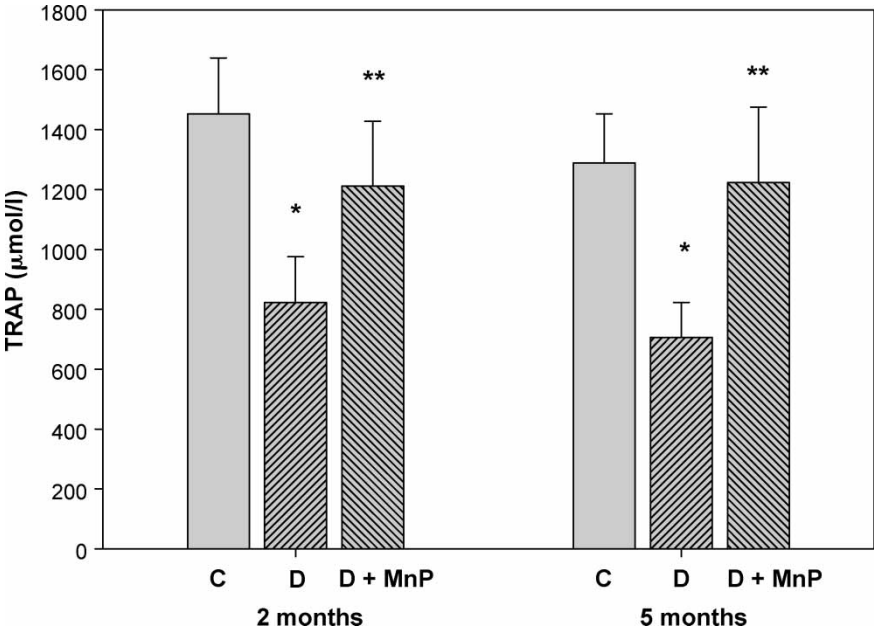

Figure 2. TRAP in plasma at 2 and 5 months of diabetes. Bar C, control; bar D, diabetic rats; bar D + MnP, diabetic MnTM-2-PyP^{5+}-treated rats. $*P < 0.05$ compared to control. $**P < 0.05$ compared to diabetic.

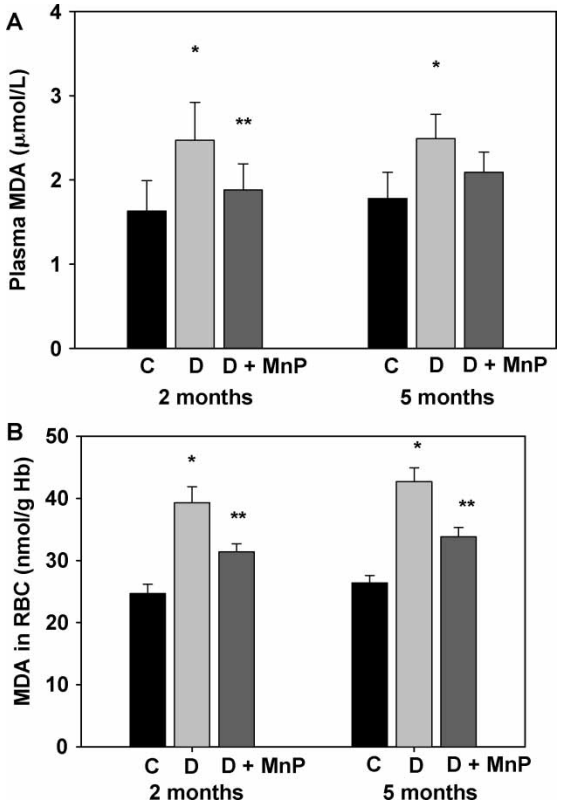

Figure 3. MDA levels in plasma and erythrocytes at 2 and 5 months of diabetes. Panel A, plasma MDA. Panel B, MDA levels in erythrocytes. Bar C, control; bar D, diabetic rats; bar D + MnP, diabetic MnTM-2-PyP^{5+}-treated rats. $*P < 0.05$ compared to control. $**P < 0.05$ compared to diabetic.

This difference became less pronounced after 5 months of diabetes. Treatment with MnTM-2-PyP^{5+} lowered MDA, but did not restore its level to that found in the controls (Figure 3A). Diabetes caused increased accumulation of MDA in the erythrocytes of the diabetic group, and MnTM-2-PyP^{5+} exerted significant protection (Figure 3B). The increased production of superoxide from glycated hemoglobin,[40] and the inactivation of the Cu,ZnSOD by glycation[41] seem to be the major cause of oxidative stress in diabetic erythrocytes. Table I shows that MnTM-2-PyP^{5+} did not prevent hemoglobin glycosylation. Results in Figure 4 demonstrate that MnTM-2-PyP^{5+} treatment neither protected Cu,ZnSOD against inactivation, nor

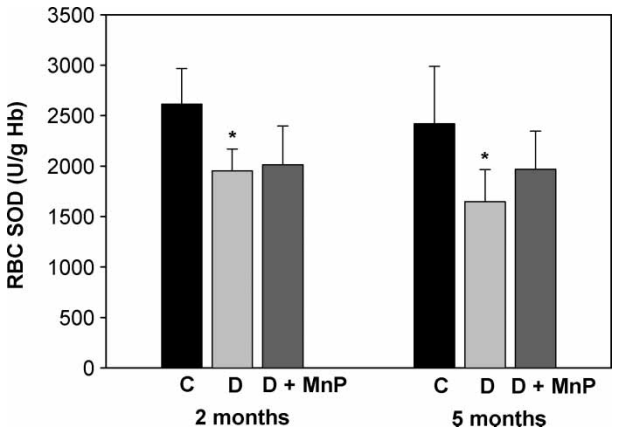

Figure 4. Erythrocyte superoxide dismutase. Bar C, control; bar D, diabetic rats; bar D + MnP, diabetic MnTM-2-PyP^{5+}-treated rats. $*P < 0.05$ compared to control.

significantly increased SOD activity within erythrocytes.

Discussion

Hyperglycemia is shown to cause high production of reactive oxygen species in diabetes.[1,2] When combined with lower antioxidant capacity often reported in diabetes, hyperglycemia induces oxidative stress, which in turn appears to be a major contributory factor in the development of diabetic complications.[1,2] It is reasonable to expect that administration of antioxidants would prevent diabetic complications. Antioxidants such as α-lipoic acid, vitamin C, and vitamin E have shown some promise but not established therapeutic efficacy.[42] Brownlee[43,44] recently pointed out the key role of superoxide production in the pathogenesis of diabetic complications. If superoxide production is the initial cause of hyperglycemia-induced oxidative stress and cellular dysfunction, then scavenging of superoxide at its sites of formation seems to be appropriate strategy for the prevention of the hyperglycemia-induced deleterious effects. Thus, searching for superior antioxidative therapies remains essential for the prevention of diabetic complications. In this study, we explored whether synthetic Mn porphyrin may be effective in decreasing diabetic complications.

Manganese-based metalloporphyrins were initially developed as SOD mimics but besides dismuting superoxide, and much to their advantage, they also scavenge peroxynitrite, nitric oxide, carbonate radical, lipid peroxyl radicals, and hydrogen peroxide (see "Introduction Section" for references). Among them the *ortho* isomers of *N*-alkylpyridylporphyrins,[20,45] and *N*,*N*′-dialkylimidazolylporphyrins,[46,47] and their methoxyethyl derivatives[46] are the most potent catalysts of $O_2^{\cdot-}$ dismutation and are also very effective as $ONOO^-$ and $CO_3^{\cdot-}$ reductants.[17] Among those porphyrins, MnTM-2-PyP^{5+}, MnTE-2-PyP^{5+} and MnTDE-2-ImP^{5+} [Mn(III) 5,10,15,20-tetrakis(*N*,*N*′-diethylimidazolium-2-yl)porphyrin, AEOL-10150] are the compounds most often studied. They have nearly identical $O_2^{\cdot-}$ dismuting and $ONOO^-$ reducing ability, and offer protection in a variety of rodent models of oxidative stress (see "Introduction Section").

Recently, it was shown that MnTE-2-PyP^{5+} can delay or prevent the onset of autoimmune diabetes.[32] The porphyrin also significantly inhibited antigen-presenting cell-dependent T-cell proliferation and interferon-γ production *in vivo*, as well as lipopolysaccharide-dependent increase in TNF-α and NADPH oxidase-dependent release of superoxide.[32]

We demonstrated here that MnTM-2-PyP^{5+} suppresses the hyperglycemia-induced oxidative stress. The plasma antioxidant ability expressed through TRAP (Figure 2) was enhanced (after 2 months) or fully restored (after 5 months) upon administration of Mn porphyrin. Lipid peroxidation in plasma and erythrocytes of STZ diabetic rats has been well documented and our results, showing an increase in malondialdehyde levels in diabetic rats, support earlier reports.[48–50] MnTM-2-PyP^{5+} porphyrin, that was previously shown effective in preventing lipid peroxidation,[21–23] lowered the malondialdehyde content in both plasma and erythrocytes of diabetic rats as shown in Figures 3A and B. In addition to $OH^{\cdot}$, oxidative modifications of lipids may also be due to biologically relevant oxidizing and nitrating agent, peroxynitrite, a formation product between $O_2^{\cdot-}$ and $^{\cdot}NO$. Infusion of peroxynitrite into LDL preparations was reported to cause high yields of lipids and protein oxidation.[51] The reaction between NO and $O_2^{\cdot-}$ is fast enough ($1.6 \times 10^{10}\ M^{-1}\ s^{-1}$)[52] and can compete with the reaction of MnTM-2-PyP^{5+} with $O_2^{\cdot-}$ ($6.2 \times 10^{7}\ M^{-1}\ s^{-1}$).[15,16] It is thus likely that MnTM-2-PyP^{5+}, rather than directly scavenging $O_2^{\cdot-}$, would scavenge its reaction product with $^{\cdot}NO$, or $CO_3^{\cdot-}$ [17] arising from CO_2 adduct with $ONOO^-$ ($ONOOCO_2^-$), while redox cycling between $Mn^{III}P^{5+}$ and $O{=}Mn^{IV}P^{4+}$. The rate constant of MnTM-2-PyP^{5+} with $ONOO^-$ is $1.9 \times 10^{7}\ M^{-1}\ s^{-1}$,[17] and with $CO_3^{\cdot-}$ is $2.7 \times 10^{8}\ M^{-1}\ s^{-1}$.[17] Cellular reductants such as ascorbic acid, glutathione and uric acid will readily eliminate/reduce strongly oxidizing species $O{=}Mn^{IV}P^{4+}$.[22,23]

Due to the facile *in vivo* reducibility of MnTM-2-PyP^{5+} (from $Mn^{III}P^{5+}$ to $Mn^{II}P^{4+}$[15,16] and from $O{=}Mn^{IV}P^{4+}$ to $Mn^{III}P^{5+}$),[18] it can act as oxidant also. Such an action, proposed by Tse et al.,[53] would inhibit NF-κB DNA binding (through oxidizing p50 subunit) and decrease the levels of iNOS expression that is otherwise able to produce $^{\cdot}NO$ up to micromolar levels.[54] Consequently, $^{\cdot}NO$ and $ONOO^-$ levels and lipid peroxidation would be decreased. The up-regulation of iNOS in diabetes has been reported; its inhibition was shown to prevent diabetic vascular dysfunction.[54] Moreover, α-lipoic acid, being (similar to Mn porphyrin) an antioxidant and modulator of NF-κB (reportedly through reducing oxidative stress-mediated NF-κB activation)[55] is able of decreasing iNOS expression.[54] Similar to our study, α-lipoic acid did not affect blood glucose levels. Based on the above said, the suppression of lipid peroxidation that we observed in this study may be due to the ability of MnTM-2-PyP^{5+} to reduce $^{\cdot}NO/ONOO^-$ levels and $ONOOCO_2^-$ degradation products and/or scavenge lipid peroxyl radicals.

Finally, we showed that MnTM-2-PyP^{5+} decreased mortality and significantly extended the life span of the diabetic rats (Figure 1). Thus, in untreated diabetic animals the mortality accounted for 85% as compared to 57% in animals treated with MnTM-2-PyP^{5+}. Oxidative stress has been considered a major determinant of life span.[56,57] It has been shown that superoxide-generating agents shortened the life

span of *Caenorhabditis elegans*, while elevation of SOD activity with Mn salen derivatives (EUK-8 and EUK-234) extended it.[58]

Both, studies of the autoimmune diabetes and of the streptozotocin-induced diabetes clearly indicate the therapeutic value of Mn(III) *N*-alkylpyridyl- and *N*,*N*′-dialkylimidazolylporphyrins. As already noted, pathways other than direct ROS scavenging, such as inhibition of transcription factors NF-κB,[32,33,53] and AP-1[59] DNA binding, may also be involved in the mechanism of action of Mn porphyrins. In support comes the recent study by Vulin and Stanley[60] where oxidative stress in diabetes led to the three-fold increase in plasminogen activator inhibitor 1 (PAI-1) transcription; the effect is additive with that of insulin. Such effect was ascribed to the effect on AP-1 site at −60/52 of the PAI-1 promoter.[60] Also, Itani et al. reported that insulin resistance is related to IKK/IκB/NF-κB pathways.[61]

Whether decreasing oxidative stress through transcription factors or directly scavenging ROS, the favorable redox ability of these Mn porphyrins (indicated by their high metal-centered redox potential of $\geq +220$ MV vs NHE)[47] is likely to be involved. Further work is needed to gain more insight into the possible diverse actions of this class of antioxidants. More lipophilic derivatives, even though of equal antioxidant potency when compared to their hydrophilic analogues, proved significantly more protective to SOD-deficient *E. coli* because they enter the cell more readily.[62] Therefore they may be even more effective in preventing diabetic complications than MnTM-2-PyP^{5+}.

Acknowledgements

This work was supported by grant MB 031 from Kuwait University. The authors thank Ms A.F. Beema, Ms Fatima Sequeira, and Dr T. Srikumar for their excellent technical assistance. Ines Batinic-Haberle is grateful for financial support from DOD CDMRP BC024326, Christopher Reeve Paralysis Foundation BA1-0103-1, and Aeolus/Incara Pharmaceuticals, RTP, NC.

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