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Research Paper

Late administration of Mn porphyrin-based SOD mimic enhances diabetic complications ☆

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ABSTRACT

Mn(III) *N*-alkylpyridylporphyrins (MnPs) have demonstrated protection in various conditions where increased production of reactive oxygen/reactive nitrogen species (ROS/RNS), is a key pathological factors. MnPs can produce both pro-oxidative and antioxidative effects depending upon the cellular redox environment that they encounter. Previously we reported (*Free Radic. Res.* 39: 81–8, 2005) that when the treatment started at the onset of diabetes, Mn(III) *meso*-tetrakis(*N*-methylpyridinium-2-yl) porphyrin, MnTM-2-PyP^{5+} suppressed diabetes-induced oxidative stress. Diabetes, however, is rarely diagnosed at its onset. The aim of this study was to investigate if MnTM-2-PyP^{5+} can suppress oxidative damage and prevent diabetic complications when administered more than a week after the onset of diabetes. Diabetes was induced by streptozotocin. The MnP-based treatment started 8 days after the onset of diabetes and continued for 2 months. The effect of the treatment on activities of glutathione peroxidase, superoxide dismutase, catalase, glutathione reductase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and glyoxalases I and II as well as malondialdehyde and GSH/GSSG ratio were determined in kidneys. Kidney function was assessed by measuring lysozyme and total protein in urine and blood urea nitrogen. Vascular damage was evaluated by assessing vascular reactivity. Our data showed that delayed administration of MnTM-2-PyP^{5+} did not protect against oxidative damage and did not prevent diabetic complications. Moreover, MnTM-2-PyP^{5+} contributed to the kidney damage, which seems to be a consequence of its pro-oxidative action. Such outcome can be explained by advanced oxidative damage which already existed at the moment the therapy with MnP started. The data support the concept that the overall biological effect of a redox-active MnP is determined by (i) the relative concentrations of oxidants and reductants, *i.e.* the cellular redox environment and (ii) MnP biodistribution.

Introduction

It is generally accepted that hyperglycemia is the major cause of oxidative stress in diabetes [1], and that oxidative stress is a main contributor to diabetic complications [2]. Hyperglycemia-induced increased mitochondrial production of superoxide is regarded as the event triggering all other mechanisms underlying diabetic complications [2–6]. Those findings suggest that catalytic scavenging of superoxide at the site of mitochondrial electron transport chain could prevent or delay complications of diabetes [4,5,7]. Such removal of superoxide could be achieved by Mn porphyrin-based SOD mimics [8–15].

Mn(III) *N*-alkylpyridylporphyrins (MnPs) were originally designed as SOD mimics [16]. The values of $k_{cat}(O_2^{\cdot-})$ for some of those compounds are similar or nearly identical to those of SOD enzymes; thus these Mn porphyrins are among the most potent synthetic SOD mimics [12,13]. Substantial knowledge of the biology of oxidative stress injuries and on the chemistry and biochemistry of Mn porphyrins, including their effects on redox-based signaling pathways, has accumulated over the last two decades. Researchers became aware that the *in vivo* actions of MnPs are much more complex than initially anticipated and that

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the scientific community is far away from fully comprehending MnPs' *in vivo* behavior [13]. It has been established that cellular redox environment (levels of reactive species and levels and activities of endogenous antioxidant defenses), biodistribution of Mn porphyrins and their co-localization with redox-active species (low-molecular weight species and proteins) would impact the type and the magnitude of therapeutic outcome [12,13,17]. Possible *in vivo* reactions of MnPs with reactive species, including signaling molecules, have been discussed in details recently [13]. Cysteines of signaling proteins are likely involved in the mechanism of action of MnPs, as are cellular reductants and reactive species, in particular H_2O_2 [13]. The ability of MnP to mimic SOD enzyme, *i.e.* to reduce and oxidize $O_2^{\cdot -}$ with nearly identical rate constants, suggests that MnPs may act as either anti- or pro-oxidants; both types of actions may be beneficial, the former in normal tissue and the latter in tumor. However, the pro-oxidative action may result in both (i) antioxidative; and (ii) prooxidative effects/therapeutic outcomes. Several reports substantiate the pro-oxidative action of MnP which resulted in antioxidative effects. The adaptive response to mild pro-oxidative action of MnP (either given alone or combined with *N*-acetylcysteine) resulted in upregulation of endogenous antioxidants, *i.e.* in antioxidative therapeutic outcome [18,19]. The inhibition of NF-κB activation in diabetes and stroke models, which was assigned to the oxidation of NF-κB cysteines by MnP, resulted in a suppression of inflammation and downregulation of inflammatory cytokines, *i.e.* in antioxidative therapeutic outcome [9,20–23]. In lymphoma study, though, the pro-oxidative action of MnP led to apoptosis [20].

Most of the works published so far report high therapeutic efficacy of Mn porphyrins [13,15,24,25]. Data from only few studies are available where administration of MnP did not have therapeutic effect. While MnP was efficacious in reducing infarct volume size if administered at all tested times up to 6 h, no beneficial outcome of MnP was demonstrated when the therapy started 12 h after the middle cerebral artery occlusion [15,26]; at such late time, the inflammatory processes have already damaged biological targets beyond repair. Delayed administration of MnP had no effect on tumor growth suppression: tumor radiosensitization by MnP was observed only if the treatment of mice bearing sc 245-GM glioblastoma multiforme xenografts, started when the tumor size was $\sim$80 mm^3, but not when it averaged $\sim$300 mm^3 (Tovmasyan et al., unpublished). The rate of tumor growth also determined the outcome of MnP therapy: the faster the tumor growth the smaller the effect of the MnP (Tovmasyan et al., unpublished). Further, in a mouse xenograft prostate tumor model the tumor growth delay was observed only if the therapy with MnP started immediately upon the implantations of tumor cells [27]. Similar result was recently shown in a prostate mouse sc xenograft model: the tumor growth suppression was achieved when administration of H_2O_2-producing MnP/ascorbate system started when tumors size averaged $\sim$30 mm^3 [28]. In a 4T1 mouse breast tumor xenograft study, where administration of MnP and ascorbate started when tumors were $\sim$200 mm^3, only trend towards tumor growth suppression was observed [29].

In a previous study, the treatment with MnTM-2-PyP started at the onset of diabetes at 24 h post-STZ injection and continued throughout the duration of the study [14]. Under such conditions the Mn porphyrin suppressed the diabetes-induced oxidative stress, decreased the mortality and markedly increased the life span of a diabetic rat. Diabetes, however, is rarely diagnosed at its onset. Thus we were left wondering what the therapeutic outcome of MnP may be if the treatment does not start early, at the onset of diabetes. To answer that question we performed a 2nd study where administration of MnTM-2-PyP started eight days post-onset of diabetes. Under such conditions no suppression of diabetic complications was detected; moreover substantial evidence is provided that MnP acted as a pro-oxidant amplifying diabetes-induced kidney damage.

Materials and methods

MnTM-2-PyP

The 5,10,15,20-tetrakis(2-pyridyl)porphyrin (H_2T-2-PyP^{4+}) was supplied by MidCentury Chemicals (Chicago, IL, USA). The *N*-methylation and metal incorporation was accomplished as already described [30]. For simplicity, charges are omitted throughout the text from MnTM-2-PyP^{5+} and its metal-free non-quaternized ligand H_2T-2-PyP^{4+}.

Diabetes

Male Wistar rats weighing 150–200 g were used in this study. The rats were maintained and cared for as outlined by the 'Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985) and by Kuwait University guidelines for care and use of laboratory animals. They were divided into four groups (each consisting of eight randomly selected rats) as follows: (1) Control; (2) Control+MnTM-2-PyP; (3) Diabetic; and (4) Diabetic+MnTM-2-PyP. Diabetes was induced by a single (60 mg/kg) intraperitoneal injection of streptozotocin. Induction of diabetes was confirmed by the presence of glucosuria within 24 h. Rats which maintained blood glucose concentrations above 15 mM during the first week of diabetes were randomly divided into two groups designated as "Diabetic" (group 3) and "Diabetic+MnTM-2-PyP" (group 4). Eight days after the diabetes was established the animals in the second group, as well as a matching group of non-diabetic control rats (group 2), started to receive subcutaneous injections of a sterile saline solution of MnTM-2-PyP, 1 mg/kg/day for five days per week, with two days rest after each five-day cycle, as previously described [14]. The whole treatment lasted for two months. The animals in groups (1) and (3) were injected with isotonic saline only. Blood samples were collected from the tail vein for determination of glucose and glycosylated hemoglobin (HbA_{1C}). At the end of the two-month period the animals were transferred to metabolic cages for collection of urine. The urine collection was carried out during 24 h; food and water were provided *ad libitum*. During this period, the tubes used for the urine collection were immersed into an ice-cold water bath to avoid the loss of enzyme activity. After the 24 h-period of urine collection, the animals were fasted for 12 h and sacrificed under sodium pentobarbital anesthesia. The kidneys were excised, perfused with ice-cold saline, snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Unless otherwise indicated, for analyses, kidneys were cut into small pieces and homogenized using ice-cold 0.2 M phosphate buffer pH.7.6 (5× volume/kidney weight). The homogenates were centrifuged at 2500 rpm for 10 min at 4 °C and the supernatants were used for the specified assays. Protein was determined by the method of Lowry [31].

Hyperglycemia

The blood glucose and glycosylated hemoglobin (HbA1C) were measured as described before [32] and used as markers of glycemic stress.

Determination of blood urea nitrogen

Blood samples were taken before autopsy and centrifuged at 3000 rpm for 10 min. Blood urea nitrogen (BUN) was measured in plasma samples using Sigma reagents (Sigma Technical Bulletin No. 640, Sigma, St. Louis, MO, USA).

Determination of electrolyte, total protein, and lysozyme excretion in the urine

The total urine volume was determined gravimetrically and the collected urine was centrifuged at 5000 rpm and 4 °C for 15 min. Urine samples from each animal were aliquoted and stored at −20 °C until electrolytes, protein and lysozyme determinations were carried out. The electrolytes (Na^+ and K^+) were determined in the urine using an EasyLyte Plus Analyser (Medica Corporation, 5 Oak Park Drive, Bedford, MA 01730-1413, USA). Total protein concentration in the urine was determined by the method of Schacterle and Pollack [33]. The lysozyme activity in the urine was assayed as described by Cojocel and Baumann [34]. The total protein excretion was expressed as mg/24 h and total lysozyme excretion as μg/24 h.

Glucose 6-phosphate dehydrogenase (G6PD)

The activity of G6PD in kidneys was measured by following the increase in the absorbance at 340 nm due to the formation of NADPH as described by Lee [35]. One ml of a reaction mixture contained 0.1 M Tris–HCl buffer (pH 8.0), 0.01 M G6P and 0.01 M $NADP^+$. One unit of G6PD is defined as the activity of G6PD that reduces 1 μmole/min of $NADP^+$ to NADPH at 25 °C.

Glyceraldehyde-3-phosphate dehydrogenase (GA3PD)

The protocol of Dagher [36] was followed to determine the activity of GA3PD in kidneys. One ml of the assay mixture contained 0.05 M of Tris–HCl buffer (pH 8.8), 0.01 M NAD^+ and (2:1) ratio of 0.02 M GA3PD and 0.01 M disodium arsenate. The formation of NADH, due to the reduction of NAD^+ by GA3PD, was measured spectrophotometrically at 340 nm. One unit is defined as the activity of GA3PD that is required to reduce 1 μmole/min of NAD^+ to NADH at 25 °C.

Superoxide dismutase (SOD)

The SOD activity of kidneys was determined following the protocol of McCord and Fridovich [37]. The assay mixture contained 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 10 μM cytochrome c, 50 μM xanthine and 2 nM xanthine oxidase. The reduced cytochrome c was measured at 550 nm. One unit of SOD activity was expressed as the amount of SOD that inhibits the rate of cytochrome *c* reduction by 50%.

Glutathione peroxidase (GPx)

The GPx activity in kidney was measured by a coupled enzyme assay using BIOXYTECH® GPx-340™ kit according to manufacturer's instructions. One unit of GPx is defined as the amount of enzyme that catalyzes the oxidation of 1.0 μmole/min of NADPH at pH 7.0 at 25 °C.

Glutathione reductase (GR)

GR activity of kidneys was determined as described by Smith et al. [38]. The assay is based on the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) by GSH resulting in increase in the absorbance at 412 nm. One ml of assay mixture contained 0.05 M of potassium phosphate buffer (pH 8.0), 0.06 M of DTNB, 0.024 M of GSSG and 0.035 M NADPH. One unit of glutathione reductase activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmole/min of DTNB. The assay is more sensitive and less subject to interference than the widely used assay where NADPH oxidation is monitored.

Catalase (CAT)

CAT activity of kidneys was assayed as described by Visick and Clarke [39] by measuring the decomposition of H_2O_2 at 240 nm. The reaction mixture consisted of 12.5 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0). One unit of catalase is defined as the activity of catalase that decomposes 1.0 μmole/min of H_2O_2 to O_2 and H_2O at 25 °C. The H_2O_2 concentration was determined by using the molar extinction coefficient of hydrogen peroxide at 240 nm, $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$.

GSH/GSSG ratio

The BIOXYTECH® GSH/GSSG-412TM Colorimetric Determination of the Reduced and Oxidized Glutathione kit was used to measure the oxidized glutathione and the total glutathione in kidneys.

Lipid peroxidation

Lipid peroxidation in kidneys was assessed by measuring malondialdehyde (MDA) content. Since the thiobarbituric acid (TBA) assay lacks specificity, HPLC separation was performed. In brief, MDA was measured in a 0.5 ml assay mixture following the protocol of Buege and Aust [40]. The separation of MDA was performed using GBC HPLC system: LC1650 Autosampler, LC1150 pump, LC1205 UV/VIS detector (Winchrom detector control) with Thermo hypersil keystore (C18) ODS hypersil (Octadecylsilane) column (250 × 4 mm in diameter, 5 μm particle size) with acetonitrile: TRIS buffer (1:10) as a mobile phase. Twenty μl of samples were loaded and the separation was carried out at a flow rate of 1 ml/min. The MDA peaks were monitored at 270 nm.

Methylglyoxal (MG) and glyoxalases activities assays

For those assays the kidney samples were homogenized following the procedure described by Phillips et al. [41], and homogenates were used for the determination of glyoxalases activities and the content of methylglyoxal [41].

Vascular reactivity

The descending aorta was removed and carefully cleaned of any adhering connective tissues. Care was taken to preserve the integrity of the endothelium. Endothelial function was confirmed by the ability of acetylcholine (10^{-6} M) to relax artery segments contracted with noradrenaline (NA) (10^{-7} M). The artery ring segments (3–4 mm in length) were set up in 25.0 ml tissue baths containing Krebs' solution of the following composition: NaCl, 119; KCl, 4.7; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5 and glucose, 11 mmol/l. The temperature was maintained at 37 °C. The solution was continuously gassed with a 5% CO_2/95% O_2 mixture at pH ~ 7.4. The samples were allowed to equilibrate under a resting tension of 1.0 g for up to 60 min during which time the bath fluid was changed at least once. Isometric contractions were recorded through the dynamometer UF1 transducers on a Lectromed 4-channel polygraph (MultiTrace 4 P). After the period of equilibration, KCl (80 mM) was added to the bath to test for tissue viability. The addition of KCl was repeated after 30 min. The tissues were then washed repeatedly over the next 30-min period. Thereafter, increasing concentrations (1 log unit increments) of noradrenaline were added to the bath to generate a concentration–response curve. The contractile response to each aliquot of NA was allowed to reach a peak before the addition of the next aliquot. After obtaining the maximum response, the samples were washed several times and allowed to rest for 60 min. The potency of NA

was expressed as pD_2 value where pD_2 is the negative logarithm of the agonist concentration producing 50% of the maximum response. Next, the aorta segments were contracted with NA (10^{-7} M). After a stable contraction had been achieved, increasing concentrations of carbachol (1 log unit increments) were added to the bath and the relaxation response was recorded. Once maximal response was achieved, the samples were washed several times and allowed to rest for 60 min. The response to each concentration of carbachol was expressed as a percentage of NA-induced contraction. The potency of carbachol was expressed as the pD_2 value. Similar experiments were carried out using sodium nitroprusside as agonist.

Statistical analysis

Mean values and standard deviation (S.D.) were shown. One-way analysis of variance and the Student–Neumann–Keuls test were used for statistical analysis of the data. A 0.05 level of probability was accepted as a criterion of significance. The significance is indicated as $^{*}P<0.05$ compared to control and $^{**}P<0.05$ compared to diabetic, non-treated group.

Results

Hyperglycemia

Blood glucose concentrations were significantly higher in the STZ rats, when compared to the controls. As reported before [42], the MnTM-2-PyP treatment had no effect on the blood glucose (Table 1). Prolonged hyperglycemia was assessed by the content of glycosylated hemoglobin (HbA_{1C}). Accordingly, HbA_{1C} level was elevated in the STZ rats and remained unaffected by the MnP treatment (Table 1).

Table 1
Blood glucose concentrations and the levels of glycosylated hemoglobin.

Experimental group	Glucose (mM)	HbA_{1C} (μM)
Control	3.7 ± 0.5	45 ± 5.8
Control + MnP	4.1 ± 0.5	42 ± 7.1
Diabetic	18.4 ± 2.4*	99.5 ± 9.0*
Diabetic + MnP	19.6 ± 3.1*	107.2 ± 19.1*

* $P<0.05$ compared to control.

GSH/GSSG ratio

Under physiological conditions the cells maintain high levels of reduced glutathione (GSH) and low level of its oxidized form (GSSG). Increased reactive oxygen species (ROS) production, as well as decreased glutathione reductase and glucose-6-phosphate dehydrogenase activities, are among the factors leading to the decrease in GSH with concomitant increase in GSSG levels. Therefore, the shift in the GSH/GSSG ratio is considered a sensitive and early indicator of oxidative stress. As demonstrated in Fig. 1 the GSH/GSSG ratio dropped dramatically in the kidneys of the diabetic animals. Fig. 1 also shows that MnTM-2-PyP treatment affected the GSH/GSSG ratio neither in normal nor in diabetic rats.

Glyceraldehyde-3-phosphate dehydrogenase

GAPDH is ubiquitous enzyme displaying a variety of functions [43]. Since the enzyme contains a highly reactive thiol at its active site (cysteine 149) [44], it is sensitive to inactivation by sugars [45], and reactive oxygen and nitrogen species [46]. Inhibition of GAPDH has been reported to cause accumulation of dicarbonyls like methylglyoxal (MG), derived from triosephosphates [46,47]. Fig. 1 shows that GAPDH activity in the kidneys of the diabetic rats was lower than in the normoglycemic controls, which is in agreement with previous reports [48]. The MnT-2-PyP treatment of healthy and diabetic animals had no effect on the activity of GAPDH.

Malondialdehyde

MDA was significantly elevated in the diabetic groups (Fig. 1) in agreement with earlier reports [14]. This confirms the hyperglycemia-induced oxidative damage. However, no decrease of MDA was observed in the group of diabetic animals treated with MnTM-2-PyP. Moreover, a trend towards increase of MDA by MnTM-2-PyP was observed in the diabetic group.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is reportedly inactivated by glycation [49]. Thus, in diabetes, increased production of superoxide combined with suppressed SOD activity, resulting in increased superoxide steady state concentrations, might be the leading causes of the oxidative damage. Fig. 2, however, shows that the total SOD activity in the kidneys of diabetic animals was higher than in the normoglycemic controls. Treatment with MnTM-2-PyP further increased the SOD activity. The increase is statistically significant compared to the diabetic, non-treated group.

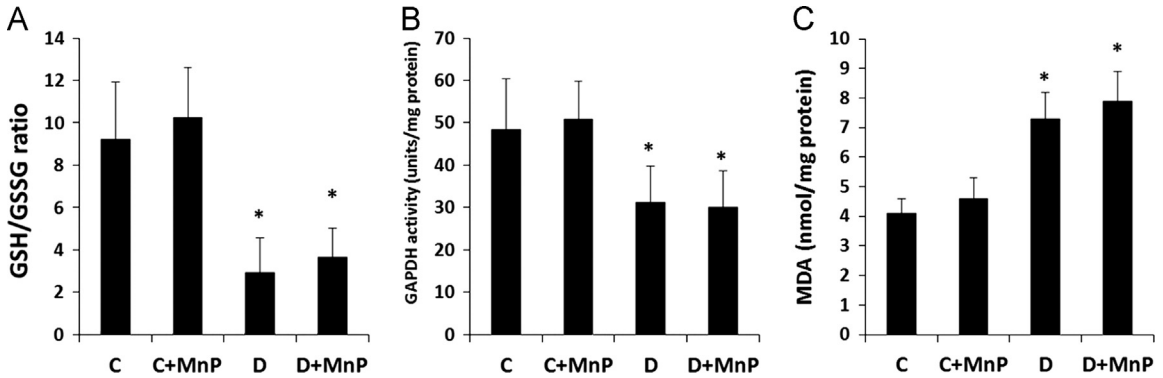


Fig. 1. Effect of Mn porphyrin-based SOD mimic on markers of oxidative stress: glutathione levels, lipid peroxidation and GAPDH. (A) GSH/GSSG ratio; (B) activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and (C) malondialdehyde (MDA) content. Groups are: C – Control; C+MnP – Control+MnTM-2-PyP; D – Diabetic; D+MnP – Diabetic+MnTM-2-PyP. $^{*}P<0.05$ compared to control.

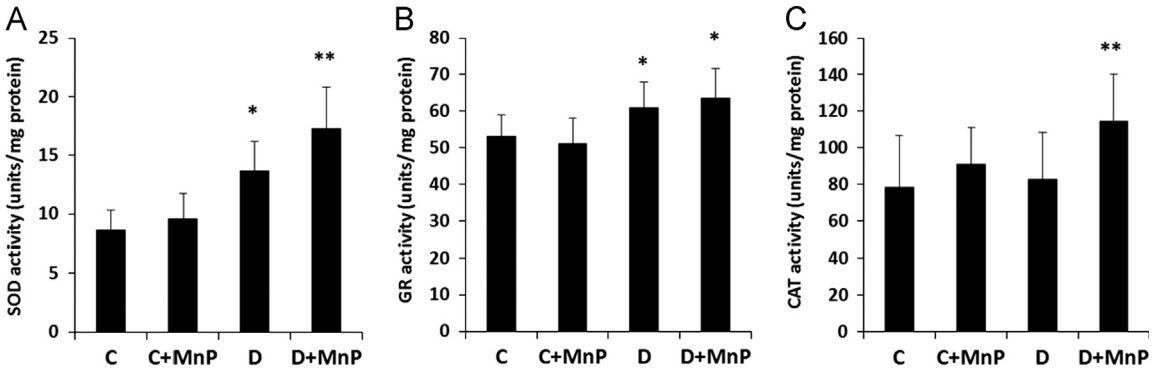


Fig. 2. Effect of Mn porphyrin-based SOD mimic on activities of antioxidative enzymes. (A) SOD; (B) glutathione reductase (GR); and (C) Catalase. Groups are: C – Control; C+MnP – Control+MnTM-2-PyP; D – Diabetic; D+MnP – Diabetic+MnTM-2-PyP. $^{*}P<0.05$ compared to control, $^{**}P<0.05$ compared to Diabetic.

Table 2
Kidney content of methylglyoxal and activities of GPx, G6PD and glyoxalases.

	Control	Control+MnP	Diabetic	Diabetic+MnP
GPx (U/mg protein)	1842 ± 170	1730 ± 187	3011 ± 624*	2803 ± 733*
G6PD (U/mg protein)	6.2 ± 2.2	5.4 ± 1.7	2.4 ± 0.9*	2.8 ± 1.3*
MG (pmol/mg protein)	4.92 ± 0.38	4.31 ± 0.43	6.75 ± 0.62*	6.28 ± 0.61*
Glyoxalase I (U/mg protein)	0.64 ± 0.04	0.67 ± 0.04	0.53 ± 0.03*	0.55 ± 0.03*
Glyoxalase II (U/mg protein)	0.27 ± 0.02	0.25 ± 0.03	0.18 ± 0.01*	0.21 ± 0.03*

GPx, glutathione peroxidase; G6PD, glucose-6-phosphate dehydrogenase; MG, methylglyoxal.

* $P<0.05$ compared to control.

Glutathione peroxidase

Similarly to SOD, the GPx activity in kidneys was significantly elevated after two months of diabetes (Table 2) and remained higher in the diabetic+MnTM-2-PyP group.

Glutathione reductase

Glutathione reductase activity was increased by 15% when compared to the control group (statistically significant, $p<0.05$). The MnP treatment had no effect on GR activity (Fig. 2).

Catalase activity

Diabetes alone did not induce any changes in the catalase activity in the kidney (Fig. 2). Treatment of non-diabetic rats with MnTM-2-PyP also did not cause statistically significant changes, even though a tendency towards increase was noticed. Treatment of diabetic animals with MnP, however, produced statistically significant elevation of catalase activity in the kidney (Fig. 2C).

Glucose-6-phosphate dehydrogenase

G6PD has a unique role in cell survival as it is the principle source of NADPH [50]. Diabetes was reported to cause inhibition of G6PD *via* activation of protein kinase A, which contributes to the oxidative stress in rat kidney cortex [51]. Decrease in the G6PD activity, and consequently, decrease in the levels of NADPH, makes cells sensitive to oxidative damage. Table 2 shows that the activity of G6PD in the kidneys of the diabetic animals was significantly decreased. Similar results were reported for the kidney cortex of STZ diabetic rats at 4, 8 and 16 weeks [51]. In both, diabetic and non-diabetic MnTM-2-PyP-treated animals, the G6PD activity was not statistically different compared to the non-treated groups.

Methylglyoxal content

Table 2 shows that the concentration of methylglyoxal in the kidneys of the STZ rats was significantly increased, when compared to the control animals. MnTM-2-PyP did not prevent the accumulation of MG in the diabetic rats, and had no effect on the level of MG in the non-diabetic animals (Table 2). The extent of accumulation of MG depends on the relative rates of its formation and degradation. As mentioned above, low activity of GAPDH is considered among the reasons for accelerated MG production. Under normal conditions MG is removed mainly by the action of the glyoxalases.

Glyoxalases activity

The glyoxalases are responsible for detoxification of methylglyoxal and other reactive aldehydes, which are by-products of metabolism. The detoxification is accomplished by the sequential action of two thiol-dependent enzymes: glyoxalase I, which catalyzes the isomerization of the spontaneously formed hemithioacetal adduct between GSH and 2-oxoaldehydes (such as methylglyoxal) into S-2-hydroxyacylglutathione; and glyoxalase II, which hydrolyzes these thiolesters, and in the case of methylglyoxal catabolism produces D-lactate and GSH from S-D-lactoylglutathione.

The activities of both *glyoxalase I and glyoxalase II* were slightly lower in the diabetic rats (Table 2), which is in agreement with earlier reports [41]. The MnP treatment had practically no effect on glyoxalase I and its activity remained lower than in the controls. The same result was observed for glyoxalase II, even though a slight, statistically insignificant tendency for the increase in its activity was observed in the MnTM-2-PyP-treated animals (Table 2).

Overall kidney damage

The effect of MnTM-2-PyP on hyperglycemia-induced kidney damage was determined by assessing the total protein and

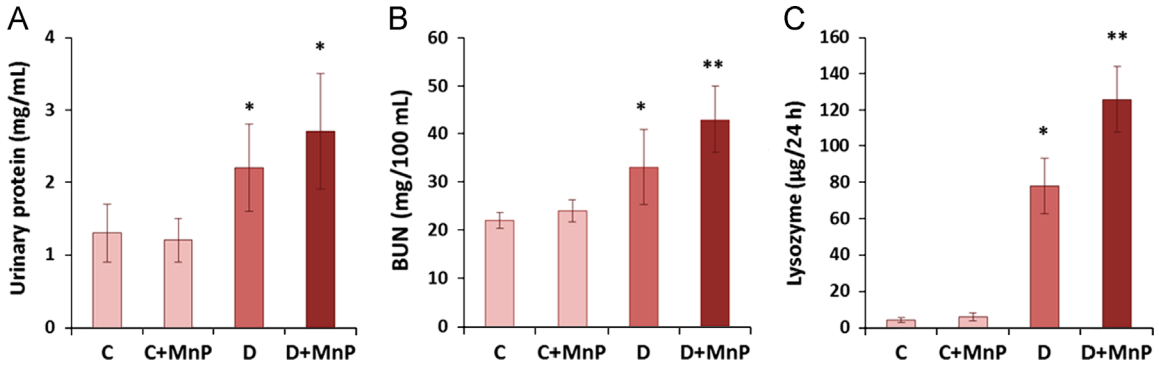


Fig. 3. Mn porphyrin-based SOD mimic enhances diabetes-induced kidney damage. The effect of MnTM-2-PyP treatment on hyperglycemia-induced kidney damage was evaluated by measuring (A) urinary protein, (B) blood urea nitrogen and (C) lysozyme content in urine. Groups are: C – Control; C+MnP – Control+MnTM-2-PyP; D – Diabetic; D+MnP – Diabetic+MnTM-2-PyP. *$P < 0.05$ compared to control, **$P < 0.05$ compared to Diabetic.

lysozyme content in urine. Similarly to the above listed findings, no positive effect of the MnP treatment on diabetes-induced proteinuria was observed (Fig. 3). The MnP treatment actually caused an increase in urinary protein, which, however was statistically insignificant. The treatment increased the excretion of lysozyme, which is a marker of impaired tubular reabsorption of low molecular weight proteins (Fig. 3). This coincided with increased blood urea nitrogen levels in the diabetic rats, which was further significantly augmented by the treatment (Fig. 3).

Vascular (Aortic) reactivity

Stimulation with noradrenaline (10^{-9}–10^{-4} M) induced reproducible and concentration-dependent contractions of aorta segments from the control and diabetic rats (Fig. 4A). The concentration–response curve of the aorta segments from diabetic rats was displaced to the left but there was no change in the response maximum. Treatment of control rats with MnTM-2PyP did not affect the response to NA (not shown) and failed to reverse the leftward displacement of NA concentration–response curve in diabetic rats (Fig. 4A).

Carbachol

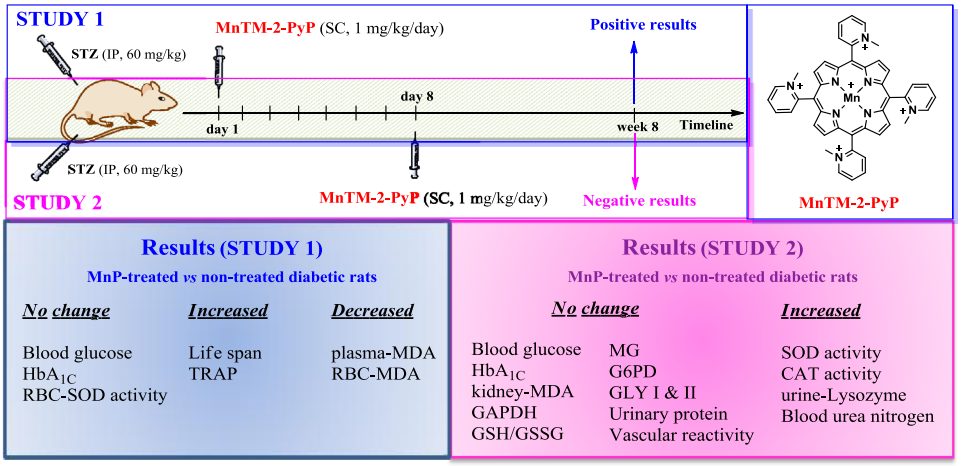
Carbachol (10^{-9} M–10^{-4} M) produced reproducible and concentration-dependent relaxation of precontracted aorta segments from control and diabetic rats (Fig. 4B). Carbachol-induced relaxation was significantly reduced in aorta segments from diabetic rats. Treatment of control rats with MnTM-2PyP did not affect the response to carbachol (not shown) and also failed to reverse the attenuated response aorta segments from diabetic rats to carbachol (Fig. 4B). The relaxant effect of sodium nitroprusside was not affected by diabetes (not shown).

Discussion

Cationic Mn(III) *ortho N*-substituted pyridylporphyrins (MnPs) are considered among the most efficacious SOD mimics, based on their log $k_{cat}(O_{2^-})$ approaching that of the SOD enzyme [12]. The experimental evidence thus far collected demonstrate that while they may not act *in vivo* predominantly as SOD mimics [13], their properties which favor O_{2^-}-dismutation (electron-deficiency and cationic charge) make them favor reactions with anionic deprotonated reactive species [anionic thiolates, $ONOO^-$, $HClO^-$, monodeprotonated ascorbic acid (HA^-)] [13]. In turn, the therapeutic efficacy of MnPs parallels their $k_{cat}(O_{2^-})$ values: the higher the k_{cat} values, the higher is the efficacy of MnPs as therapeutics [12,13]. Due to the key impact of mitochondria in pathological conditions, it is important to note that all cationic MnPs accumulate in mitochondria; the degree of accumulation depends upon their lipophilicities [13,17,52]. The SOD-like activity of MnP, *i.e.* ability to reduce and oxidize, O_{2^-} (a mild pro- and antioxidant), indicates that MnP can operate as mild pro- and antioxidant also, as confirmed by experimental evidence. The pro-oxidative action, though, can result in anti- and pro-oxidative effects (see Introduction and Refs. [13,20,53,54] for details).

The ability of MnTM-2-PyP to suppress oxidative damage and diabetic complications was investigated using the same standard STZ diabetes model while varying the time of the initiation of treatment. STZ is a potent alkylating agent that directly kills the beta cells of the pancreas and thus completely abolishes insulin secretion. Irrespective of the fact that STZ diabetes is the most often used chemically-induced diabetes model, it has certain limitations which should be kept in mind when data based on this model are interpreted. Among the drawbacks are generalized STZ toxicity and genotoxicity, producing DNA strand breaks, alkali-labile sites, unscheduled DNA synthesis, DNA adducts, chromosomal aberrations, micronuclei, and sister chromatid exchanges [55]. Even though alkylation is considered the main mechanism of STZ-induced cell damage, several lines of evidence indicate that STZ produces free radicals [56–60], and that beta cell killing is radical-mediated [61]. This fact should be taken into consideration particularly when data from experiments with redox-active compounds are interpreted. In addition, the STZ diabetes model differs substantially from the diabetes in humans by its duration and time of manifestation of diabetic complications. In most of the cases, the diabetic complications in humans are observed relatively long time after the onset of diabetes. In STZ-treated animals complications appear within few weeks of inducing diabetes [62]. While humans receive insulin and other glucose-lowering agents, diabetic animals are normally not subjected to glucose control, which results in early display of severe diabetic consequences including reduced growth, dehydration, and metabolic derailment. Poor general health and suppressed immune responses dramatically reduce the lifespan of the diabetic animals. Therefore, it is difficult to extrapolate from the accelerated, short term STZ diabetes model to the chronic, long lasting human diabetes, where glucose level, hygiene and general health are well controlled [62].

This study provides evidence which supports the pro-oxidative action of MnP in enhancing the kidney damage. Such outcome contradicts an earlier study where, under similar conditions, MnP suppressed oxidative stress and protected the STZ diabetic rats [14]. The differences in the experimental design of this and earlier STZ rat diabetes study are shown in Scheme 1. In the 1st study the therapy with MnP started at the onset of diabetes, while in the 2nd study the sc injections of MnP started at day 8 after the onset of diabetes (Scheme 1).



Scheme 1. Summary of the differences between the current (Study 2) and the previous (Study 1) investigations. The Mn porphyrin-based SOD mimic suppresses oxidative damage and extends the lifespan of STZ diabetic rats if administered at the onset of diabetes, but acts as prooxidant if the therapy starts at day 8 days post-onset of diabetes. In the 1st study, the treatment with MnTM-2-PyP started at 24 h after streptozotocin injection, continued for the duration of study (5 days/week) with one week rest after 4 weeks of sc injections. In the 2nd study, the treatments with MnTM-2-PyP followed the same scheme, but started 8 days post-STZ injection and continued for two months without a week rest after four weeks of treatment.

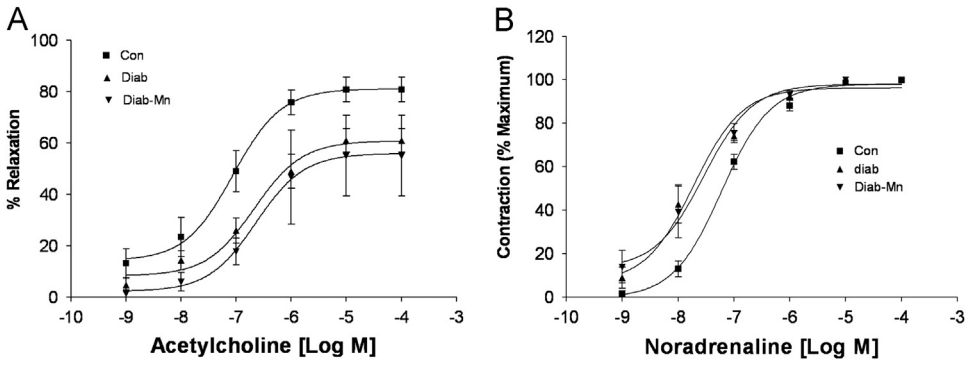
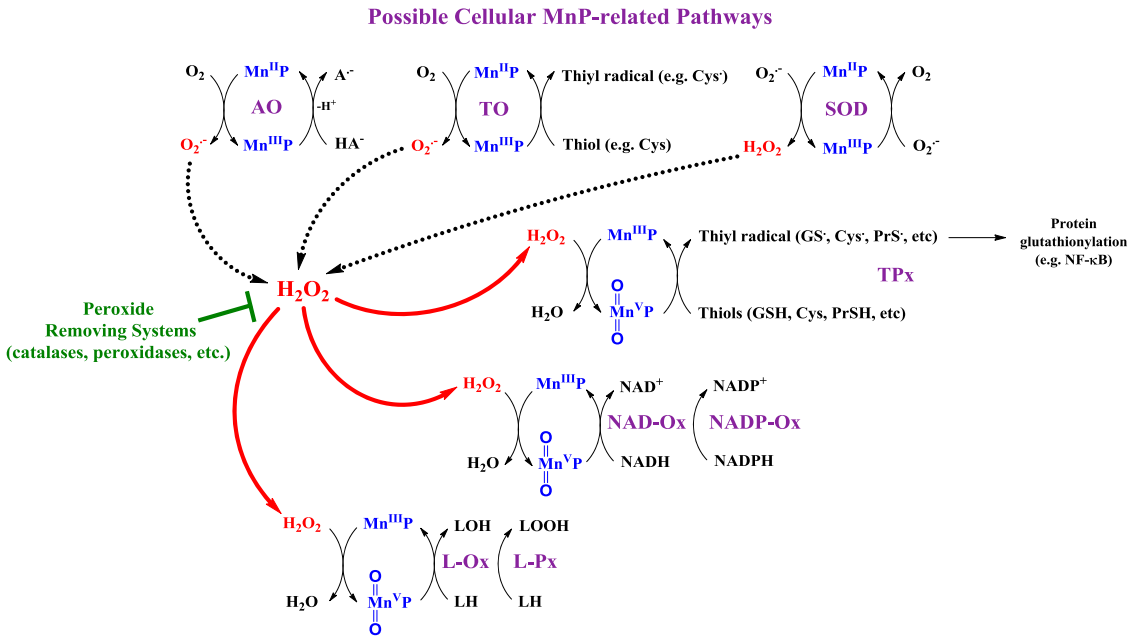


Fig. 4. Vascular responsiveness to vasoconstrictor and vasodilator. (A) Carbachol-induced relaxation of aorta ring segments from control and diabetic rats. Artery segments were pre-contracted with noradrenaline (10^{-7} M). ■ represents responses to carbachol in aorta segments from control rats while ▾ and ▴ represent responses in aorta segments from streptozotocin-treated rats with and without treatment with MnTM-2-PyP. Each point on the graph is the mean ± S.E. of five experiments. (B) Reactivity of thoracic aorta segments to noradrenaline in control and diabeticrats. ■ Represents responses to noradrenaline in aorta segments from control rats while ▾ and ▴ represent responses in aorta segments from streptozotocin-treated rats with and without treatment with MnTM-2-PyP. Each point on the graph is the mean ± S.E. of five experiments.

The GSH depletion and lipid peroxidation are early events in the STZ diabetic model, taking place as early as at the third day after STZ injection [63] and are accompanied with increase of urinary albumin [64]. These findings suggest that in the current (2nd) study the MnP therapy started at a stage of advanced oxidative stress, while in the 1st study the treatment began before oxidative damage could be detected. Our current results confirmed previous data, indicating that diabetic nephropathy is accompanied with oxidative damage, accumulation of reactive oxoaldehydes, and changes in the activities of important metabolic and antioxidant enzymes. The delayed treatment did not show beneficial effect and did not ameliorate the overall kidney damage (Figs. 1–4). Moreover, judging by the urinary lysozyme, urinary protein and blood urea nitrogen, MnTM-2-PyP enhanced diabetic kidney damage (Fig. 3). Further, MnTM-2-PyP did not reverse the effect of hyperglycemia on vascular reactivity (Fig. 4). G6PD is a critical enzyme, which provides reducing equivalents in the form of NADPH for regeneration of GSH. Suppressed G6PD activity in diabetes is among the reasons for low GSH/GSSG ratio (Fig. 1). Our data further show that the MDA content, reflecting lipid peroxidation, was elevated in the kidneys of the diabetic rats and was not decreased by MnP (Fig. 1). The SOD activity was higher in diabetic kidneys compared to controls and further increased by MnP treatment of the diabetic rats, but did not change when healthy rats received MnP (Fig. 2). The only plausible explanation for such increase in SOD activity is the upregulation of the enzyme as an adaptive response to oxidative stress. Therefore, in this model of diabetes, the MnP did not act as an SOD mimic, but likely as a pro-oxidant. Eventually, by redox-cycling indicated in Scheme 2, MnP contributed to the increase of superoxide and consequently H_2O_2, which in turn contributed to kidney damage (Fig. 3). Such an effect has been observed in a rat kidney ischemia/reperfusion model [18], where MnP was given as a part of mixture containing growth factors and amino acids [18]. Upregulation of extracellular and mitochondrial SODs, GR, GPx, and several peroxiredoxins was observed [18]. Addition of a simple thiol, *N*-acetylcysteine, to the mixture, which acted as a reductant of Mn porphyrin, thus stimulating redox-cycling, resulted in stronger upregulation of the antioxidant enzymes [65]. Again, had MnP acted as an SOD mimic it would have not upregulated SOD enzymes. Further, in the current study, MnTM-2-PyP did not protect the oxidation-sensitive enzymes like GAPDH from inactivation (Table 2). Low GAPDH activity leads to the accumulation of methylglyoxal, which is a potent contributor to the production of Advanced Glycation Endproducts (AGE). Catalase activity was also increased by the action of MnP (Fig. 2) suggesting further the adaptive responses to the increased production of H_2O_2.

Scheme 2 summarizes possible reactions involving MnPs, which might have contributed to the production of H_2O_2. Production of H_2O_2 has been proven in various systems were MnP was administered with ascorbate [28,29,66,67]. Such scenario is relevant even without



Scheme 2. Possible MnP-based pro-oxidative pathways, which produce H_2O_2 and subsequently utilize it to amplify the diabetes-induced oxidative damage to kidneys. Under pathological conditions and especially if the levels of peroxide-removing enzymes are insufficient, H_2O_2 may rise above tolerable levels. MnPs can contribute to such an increase in H_2O_2 through pathways displayed in the scheme. Once levels of H_2O_2 are increased, MnP may use H_2O_2, along with GSH, to inhibit NF-κB transcription and thus stimulate apoptotic pathways. It can further act as GPx or TO, and contribute to lipid peroxidation and NADPH and NADH oxidation, decreasing cellular supply of reducing equivalents. *Abbreviations*: AO – Ascorbate oxidase activity, TO – Thiol oxidase activity, SOD – Superoxide dismutase activity, TPx – Thiol peroxidase activity, NAD-Ox – NADPH/NADH oxidation ability, L-Ox – Lipid oxidation ability. The scheme is based on data reported in [29,72,73] and unpublished results from aqueous chemistry experiments (Tovmasyan et al., unpublished).

exogenous ascorbate, due to high, mM levels of endogenous ascorbate. We have demonstrated adaptive responses (upregulation of catalases and peroxidases) when *E. coli* grew in the presence of MnP/ascorbate [66]. Our aqueous chemistry data demonstrate that Mn porphyrin utilizes H_2O_2 to oxidize NADPH, NADH or thiols (Tovmasyan et al., unpublished). MnP can also oxidize thiols directly [13]. *In vivo* depletion of NADPH could further deplete GSH [53,67]. MnP was shown to induce lipid peroxidation in the absence of cellular reductants [68–70]. When combined with GSH and H_2O_2, MnP catalyzes glutathionylation of the p65 subunit of NF-κB thus preventing its DNA binding [20,53,71]. Moreover, it was found that MnP glutathionylates complexes I, III and IV of the mitochondrial electron transport chain, with subsequent inactivation of complexes I and II and suppression of ATP production [53].

Diabetes is considered a main risk factor for vascular disease [74], leading to altered vascular responsiveness to vasoconstrictors and vasodilators [75,76]. Convincing data show that increased production of superoxide is the mechanism underlying vascular dysfunction [5,77]. Therefore, catalytic scavenging of superoxide by MnTM-2-PyP should have prevented the alterations in vascular responsiveness. Administration of MnTM-2-PyP, however, did not show any positive effect. The diabetes-related vascular dysfunction is characterized by altered vascular reactivity to vasoactive agents. Several studies have reported enhanced agonist-induced vasoconstriction in a variety of vascular smooth muscle preparations [78–81]. In addition, impaired endothelium-dependent relaxation to acetylcholine has been reported [78,79,82,83], while there was no change in endothelium-independent relaxation to sodium nitroprusside. Our data, which demonstrate the higher reactivity to noradrenaline and impaired relaxation to carbachol but not nitroprusside in aorta segments from diabetic rats, are in agreement with those reports. The delayed treatment with MnP failed to reverse both the enhanced response to noradrenaline and the impaired endothelium-dependent relaxation to carbachol (Fig. 4).

Results of this study differ from the previously reported data where MnTM-2-PyP [14,42,84] and analogs [22,85–90] ameliorated biochemical and physiological alterations triggered by oxidative stress. The reasons for such discrepancy are most probably related to differences in the timing of Mn porphyrin administration. In diabetes, different pathogenic mechanisms leading to diabetic complications are triggered by hyperglycemia-induced overproduction of superoxide by the mitochondrial electron transport chain [5]. It has been found that superoxide activates poly(ADP-ribose) polymerase, which in turn inhibits GAPDH. The inhibition of GAPDH activates four known pathways causing diabetic complications: the polyol pathway, the AGE production, the activation of protein kinase C, and the hexosamine pathway [5]. It seems reasonable to expect that once all those mechanisms are activated, the MnP could barely suppress or reverse such processes. The observed upregulation of SOD and catalase by MnP in diabetic tissue, together with augmented kidney damage in the MnP-treated diabetic animals suggest that MnTM-2-PyP has not acted as SOD mimic but as a pro-oxidant contributing to the diabetes-induced oxidative stress.

Concluding remarks

In summary, the overall action of a Mn porphyrin *in vivo* would depend upon its cellular accumulation and subcellular distribution, co-localization with reactive species, concentrations of ROS/RNS, levels of cellular reductants, *i.e.* cellular redox status, oxygen concentration and other, yet not fully understood factors. MnP would exert beneficial effects if introduced at early stages of oxidative stress. When the oxidative injury is already profound, the capability of the MnP to reduce reactive species and restore physiological redox environment is greatly diminished – *i.e. the therapeutic efficacy of a MnP would depend on the magnitude of the oxidative stress at the moment the MnP-based therapy is initiated.*

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References

[1] J.L. Rains, S.K. Jain, Oxidative stress, insulin signaling, and diabetes, Free Radical Biology and Medicine 50 (2011) 567–575.
[2] F. Giacco, M. Brownlee, Oxidative stress and diabetic complications, Circulation Research 107 (2010) 1058–1070.
[3] T. Nishikawa, D. Edelstein, X.L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M.A. Yorek, D. Beebe, P.J. Oates, H.P. Hammes, I. Giardino, M. Brownlee, Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage, Nature 404 (2000) 787–790.
[4] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications, Nature 414 (2001) 813–820.
[5] M. Brownlee, The pathobiology of diabetic complications: a unifying mechanism, Diabetes 54 (2005) 1615–1625.
[6] X.-L. Du, D. Edelstein, L. Rossetti, I.G. Fantus, H. Goldberg, F. Ziyadeh, J. Wu, M. Brownlee, Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation, Proceedings of the National Academy of Sciences of the United States of America 97 (2000) 12222–12226.
[7] A. Ceriello, Controlling oxidative stress as a novel molecular approach to protecting the vascular wall in diabetes, Current Opinion in Lipidology 17 (2006) 510–518.
[8] R. Bottino, A.N. Balamurugan, S. Bertera, M. Pietropaolo, M. Trucco, J.D. Piganelli, Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound, Diabetes 51 (2002) 2561–2567.
[9] R. Bottino, A.N. Balamurugan, H. Tse, C. Thirunavukkarasu, X. Ge, J. Profozich, M. Milton, A. Ziegenfuss, M. Trucco, J.D. Piganelli, Response of human islets to isolation stress and the effect of antioxidant treatment, Diabetes 53 (2004) 2559–2568.
[10] M.M. Delmastro-Greenwood, T. Votyakova, E. Goetzman, M.L. Marre, D. M. Previte A. Tovmasyan, I. Batinic-Haberle, M. Trucco, J.D. Piganelli, Mn porphyrin regulation of aerobicglycolysis: implications on the activation of diabetogenic immune cells, Antioxidants and Redox Signaling (2013) (ahead of print). http://dx.doi.org/10.1089/ars.2012.5167.
[11] M.M. Sklavos, S. Bertera, H.M. Tse, R. Bottino, J. He, J.N. Beilke, M.G. Coulombe, R.G. Gill, J.D. Crapo, M. Trucco, J.D. Piganelli, Redox modulation protects islets from transplant-related injury, Diabetes 59 (2010) 1731–1738.
[12] I. Batinić-Haberle, J.S. Rebouças, I. Spasojević, Superoxide dismutase mimics: chemistry, pharmacology, and therapeutic potential, Antioxidants and Redox Signaling 13 (2010) 877–918.
[13] I. Batinic-Haberle, A. Tovmasyan, E. Roberts, Z. Vujaskovic, K.W. Leong, I. Spasojevic, SOD therapeutics: latest insights into their structure-activity relationships and impact upon the cellular redox-based pathways. Antioxidants and Redox Signaling (2013) (ahead of print). http://dx.doi.org/10.1089/ars.2012.5147.
[14] L. Benov, I. Batinic-Haberle, A manganese porphyrin suppresses oxidative stress and extends the life span of streptozotocin-diabetic rats, Free Radical Research 39 (2005) 81–88.
[15] H. Sheng, R.E. Chaparro, T. Sasaki, M. Izutsu, R.D. Pearlstein, A. Tovmasyan, D.S. Warner, Metallo porphyrins as therapeutic catalytic oxido reductants in central nervous system disorders, Antioxidants and Redox Signaling (2013) (ahead of print). http://dx.doi.org/10.1089/ars.2013.5413.
[16] K.M. Faulkner, S.I. Liochev, I. Fridovich, Stable Mn(III) porphyrins mimic superoxide dismutase in vitro and substitute for it in vivo, Journal of Biological Chemistry 269 (1994) 23471–23476.
[17] S. Miriyala, I. Spasojevic, A. Tovmasyan, D. Salvemini, Z. Vujaskovic, D. St. Clair, I. Batinic-Haberle, Manganese superoxide dismutase, MnSOD and its mimics, Biochimica et Biophysica Acta 1822 (2012) 794814.
[18] J. Cohen, T. Dorai, C. Ding, I. Batinic-Haberle, M. Grasso, The administration of renoprotective agents extends warm ischemia in a rat model, Journal of Endourology 27 (2013) 343–348.
[19] T. Dorai, A.I. Fishman, C. Ding, I. Batinic-Haberle, D.S. Goldfarb, M. Grasso, Amelioration of renal ischemia-reperfusion injury with a novel protective cocktail, Journal of Urology 186 (2011) 2448–2454.
[20] M.C. Jaramillo, M.M. Briehl, J.D. Crapo, I. Batinic-Haberle, M.E. Tome, Manganese porphyrin, MnTE-2-PyP5+, acts as a pro-oxidant to potentiate glucocorticoid-induced apoptosis in lymphoma cells, Free Radical Biology and Medicine 52 (2012) 1272–1284.
[21] H. Sheng, I. Spasojevic, H.M. Tse, J.Y. Jung, J. Hong, Z. Zhang, J.D. Piganelli, I. Batinic-Haberle, D.S. Warner, Neuroprotective efficacy from a lipophilic redox-modulating Mn(III) N-Hexylpyridylporphyrin, MnTnHex-2-PyP: rodent models of ischemic stroke and subarachnoid hemorrhage, Journal of Pharmacology and Experimental Therapeutics 338 (2011) 906–916.
[22] H. Sheng, W. Yang, S. Fukuda, H.M. Tse, W. Paschen, K. Johnson, I. Batinic-Haberle, J.D. Crapo, R.D. Pearlstein, J. Piganelli, D.S. Warner, Long-term neuroprotection from a potent redox-modulating metalloporphyrin in the rat, Free Radical Biology and Medicine 47 (2009) 917–923.
[23] H.M. Tse, M.J. Milton, J.D. Piganelli, Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity, Free Radical Biology and Medicine 36 (2004) 233–247.
[24] J.D. Piganelli, M.M. Delmastro-Greenwood, H.M. Tse, Effects of metalloporphyrins on reducing inflammation and autoimmunity, Antioxidants and Redox Signaling (2013) (ahead of print). http://dx.doi.org/10.1089/ars.2013.5257.
[25] A. Tovmasyan, J.S. Reboucas, L.T. Benov, Simple biological systems for assessing the activity of SOD mimics, Antioxidants and Redox Signaling (2013) (ahead of print). http://dx.doi.org/10.1089/ars.2013.5576.
[26] G.B. Mackensen, M. Patel, H. Sheng, C.L. Calvi, I. Batinic-Haberle, B.J. Day, L. P. Liang, I. Fridovich, J.D. Crapo, R.D. Pearlstein, D.S. Warner, Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant, Journal of Neuroscience 21 (2001) 4582–4592.
[27] S. Mehrotra, M.J. Pecaut, T.L. Freeman, J.D. Crapo, A. Rizvi, X. Luo-Owen, J. M. Slater, D.S. Gridley, Analysis of a metalloporphyrin antioxidant mimetic (MnTE-2-PyP) as a radiomitigator: prostate tumor and immune status, Technology in Cancer Research and Treatment 11 (2012) 447–457.
[28] M. Rawal, S.R. Schroeder, B.A. Wagner, C.M. Cushing, J.L. Welsh, A.M. Button, J. Du, Z.A. Sibenaller, G.R. Buettner, J.J. Cullen, Manganoporphyrins increase ascorbate-induced cytotoxicity by enhancing H_2O_2 generation, Cancer Research 73 (2013) 5232–5241.
[29] X. Ye, D. Fels, A. Tovmasyan, K.M. Aird, C. Dedeugd, J.L. Allensworth, I. Kos, W. Park, I. Spasojevic, G.R. Devi, M.W. Dewhirst, K.W. Leong, I. Batinic-Haberle, Cytotoxic effects of Mn(III) N-alkylpyridylporphyrins in the presence of cellular reductant, ascorbate, Free Radical Research 45 (2011) 1289–1306.
[30] I. Batinić-Haberle, L. Benov, I. Spasojević, I. Fridovich, The ortho effect makes manganese(III) meso-tetrakis(N-methylpyridinium-2-yl)porphyrin a powerful and potentially useful superoxide dismutase mimic, Journal of Biological Chemistry 273 (1998) 24521–24528.
[31] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, Journal of Biological Chemistry 193 (1951) 265–275.
[32] J.C. Standefer, R.P. Eaton, Evaluation of a colorimetric method for determination of glycosylated hemoglobin, Clinical Chemistry and Laboratory Medicine 29 (1983) 135–140.
[33] G.R. Schacterle, R.L. Pollack, A simplified method for the quantitative assay of small amounts of protein in biologic material, Analytical Biochemistry 51 (1973) 654–655.
[34] C. Cojocel, K. Baumann, Renal handling of endogenous lysozyme in the rat, Renal Physiology 6 (1983) 258–265.
[35] C.Y. Lee, Glucose-6-phosphate dehydrogenase from mouse, Methods in Enzymology 89 (1982) 252–257.
[36] S.M. Dagher, W.C. Deal Jr., Glyceraldehyde-3-phosphate dehydrogenase from pig liver, Methods in Enzymology 89 (1982) 310–316.
[37] J.M. McCord, I. Fridovich, Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein), Journal of Biological Chemistry 244 (1969) 6049–6055.
[38] I.K. Smith, T.L. Vierheller, C.A. Thorne, Assay of glutathione reductase in crude tissue homogenates using 5,5′-dithiobis(2-nitrobenzoic acid), Analytical Biochemistry 175 (1988) 408–413.
[39] J.E. Visick, S. Clarke, RpoS- and OxyR-independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of rpoS mutations in common laboratory strains, Journal of Bacteriology 179 (1997) 4158–4163.
[40] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods in Enzymology 52 (C) (1978) 302–310.
[41] S.A. Phillips, D. Mirrlees, P.J. Thornalley, Modification of the glyoxalase system in streptozotocin-induced diabetic rats. Effect of the aldose reductase inhibitor Statil, Biochemical Pharmacology 46 (1993) 805–811.
[42] I. Khan, I. Batinic-Haberle, L.T. Benov, Effect of potent redox-modulating manganese porphyrin, MnTM-2-PyP, on the Na+/H+ exchangers NHE-1 and NHE-3 in the diabetic rat, Redox Report 14 (2009) 236–242.
[43] M.A. Sirover, New nuclear functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells, Journal of Cellular Biochemistry 95 (2005) 45–52.
[44] I. Schuppe-Koistinen, P. Moldeus, T. Bergman, I.A. Cotgreave, S-Thiolation of human endothelial cell glyceraldehyde-3-phosphate dehydrogenase after hydrogen peroxide treatment, European Journal of Biochemistry 221 (1994) 1033–1037.
[45] D.W. Hook, J.J. Harding, Inactivation of glyceraldehyde 3-phosphate dehydrogenase by sugars, prednisolone-21-hemisuccinate, cyanate and other small molecules, Biochimica et Biophysica Acta 1362 (1997) 232–242.
[46] P.J. Beisswenger, S.K. Howell, K. Smith, B.S. Szwergold, Glyceraldehyde-3-phosphate dehydrogenase activity as an independent modifier of methylglyoxal levels in diabetes, Biochimica et Biophysica Acta 1637 (2003) 98106.
[47] P.J. Beisswenger, K.S. Drummond, R.G. Nelson, S.K. Howell, B.S. Szwergold, M. Mauer, Susceptibility to diabetic nephropathy is related to dicarbonyl and oxidative stress, Diabetes 54 (2005) 3274–3281.
[48] P. Wentzel, A. Ejdesjo, U.J. Eriksson, Maternal diabetes in vivo and high glucose in vitro diminish GAPDH activity in rat embryos, Diabetes 52 (2003) 1222–1228.

[49] K. Arai, S. Maguchi, S. Fujii, H. Ishibashi, K. Oikawa, N. Taniguchi, Glycation and inactivation of human Cu–Zn-superoxide dismutase. Identification of the in vitro glycated sites, Journal of Biological Chemistry 262 (1987) 16969–16972.
[50] H.Y. Ho, M.L. Cheng, D.T. Chiu, G6PD—an old bottle with new wine, Chang Gung Medical Journal 28 (2005) 606–612.
[51] Y. Xu, B.W. Osborne, R.C. Stanton, Diabetes causes inhibition of glucose-6-phosphate dehydrogenase via activation of PKA, which contributes to oxidative stress in rat kidney cortex, American Journal of Physiology 289 (2005) F1040–F1047.
[52] I. Spasojevic, S. Miryala, A. Tovmasyan, D. Salvemini, Z. Vujaskovic, I. Batinic-Haberle, D. St. Clair, Lipophilicity of Mn(III) *N*-alkylpyridylporphyrins dominates their accumulation within mitochondria and therefore in vivo efficacy. A mouse study, Free Radical Biology and Medicine 51 (2011) S98.
[53] M.C. Jaramillo, M.M. Briehl, I. Batinic Haberle, M.E. Tome, Manganese porphyrin glutathionylates mitochondrial electron transport chain enzymes and sensitizes lymphoma cells to anti-lymphoma therapeutics. Antioxidants and Redox Signaling (in revision); 2013.
[54] M.C. Jaramillo, M.M. Briehl, M.E. Tome, Manganese porphyrin glutathionylates the p65 subunit of NF-κB to potentiate glucocorticoid-induced apoptosis in lymphoma, Free Radical Biology and Medicine 49 (2010) S63.
[55] A.D. Bolzan, M.S. Bianchi, Genotoxicity of streptozotocin, Mutation Research 512 (2002) 121–134.
[56] I. Flechner, K. Maruta, V. Burkart, K. Kawai, H. Kolb, U. Kiesel, Effects of radical scavengers on the development of experimental diabetes, Diabetes Research 13 (1990) 67–73.
[57] M. Nukatsuka, H. Sakurai, Y. Yoshimura, M. Nishida, J. Kawada, Enhancement by streptozotocin of O_2-radical generation by the xanthine oxidase system of pancreatic beta-cells, FEBS Letters 239 (1988) 295–298.
[58] O.M. Pitkanen, J.M. Martin, M. Hallman, H.K. Akerblom, H. Sariola, S.M. Andersson, Free radical activity during development of insulin-dependent diabetes mellitus in the rat, Life Sciences 50 (1992) 335–339.
[59] S. Sandler, A. Andersson, The partial protective effect of the hydroxyl radical scavenger dimethyl urea on streptozotocin-induced diabetes in the mouse in vivo and in vitro, Diabetologia 23 (1982) 374–378.
[60] S. Sandler, M. Welsh, A. Andersson, Streptozotocin-induced impairment of islet B-cell metabolism and its prevention by a hydroxyl radical scavenger and inhibitors of poly(ADP-ribose) synthetase, Acta Pharmacologica et Toxicologica (Copenhagen) 53 (1983) 392–400.
[61] L. Gille, P. Schott-Ohly, N. Friesen, S. Schulte im Walde, N. Udilova, H. Nowl, H. Gleichmann, Generation of hydroxyl radicals mediated by streptozotocin in pancreatic islets of mice in vitro, Pharmacology and Toxicology 90 (2002) 317–326.
[62] S.C. Apfel, Diabetic neuropathy models: are they relevant? Drug Discovery Today: Disease Models 3 (2006) 397–402.
[63] H. Braunlich, F. Marx, G. Stein, Glutathione status, lipid peroxidation and kidney function in streptozotocin diabetic rats, Experimental and Toxicologic Pathology 46 (1994) 143–147.
[64] B. Samnegard, S.H. Jacobson, G. Jaremko, B.L. Johansson, M. Sjoquist, Effects of C-peptide on glomerular and renal size and renal function in diabetic rats, Kidney International 60 (2001) 1258–1265.
[65] A. Tovmasyan, T. Weitner, E. Roberts, M. Jaramillo, I. Spasojevic, K. Leong, M. Tome, L. Benov, I. Batinic-Haberle, Understanding differences in mechanisms of action of Fe vs Mn porphyrins: comparison of their reactivities towards cellular reductants and reactive species, Free Radical Biology and Medicine 53 (2012) S120.
[66] I. Batinic-Haberle, Z. Rajic, L. Benov, A combination of two antioxidants (an SOD mimic and ascorbate) produces a pro-oxidative effect forcing *Escherichia coli* to adapt via induction of oxyR regulon, Anticancer Agents in Medicinal Chemistry 11 (2011) 329–340.
[67] Evans, M.K.; Tovmasyan, A.; Batinic-Haberle, I.; Devi, G.R. Mn porphyrin in combination with ascorbate acts as a pro-oxidant and mediates caspase-independent cancer cell death. Free Radical Biology and Medicine:(in revision); 2013.
[68] A. Bloodsworth, V.B. O'Donnell, I. Batinic-Haberle, P.H. Chumley, J.B. Hurt, B.J. Day, J.P. Crow, B.A. Freeman, Manganese-porphyrin reactions with lipids and lipoproteins, Free Radical Biology and Medicine 28 (2000) 1017–1029.
[69] G. Ferrer-Sueta, I. Batinic-Haberle, I. Spasojevic, I. Fridovich, R. Radi, Catalytic scavenging of peroxynitrite by isomeric Mn(III) N-methylpyridylporphyrins in the presence of reductants, Chemical Research in Toxicology 12 (1999) 442–449.
[70] A. Trostchansky, G. Ferrer-Sueta, C. Batthyany, H. Botti, I. Batinic-Haberle, R. Radi, H. Rubbo, Peroxynitrite flux-mediated LDL oxidation is inhibited by manganese porphyrins in the presence of uric acid, Free Radical Biology and Medicine 35 (2003) 1293–1300.
[71] M.C. Jaramillo, J.B. Frye, J.D. Crapo, M.M. Briehl, M.E. Tome, Increased manganese superoxide dismutase expression or treatment with manganese porphyrin potentiates dexamethasone-induced apoptosis in lymphoma cells, Cancer Research 69 (2009) 5450–5457.
[72] A. Tovmasyan, T. Weitner, H. Sheng, M. Lu, Z. Rajic, D.S. Warner, I. Spasojevic, J.S. Reboucas, L. Benov, I. Batinic-Haberle, Differential coordination demands in fe versus mn water-soluble cationic metalloporphyrins translate into remarkably different aqueous redox chemistry and biology, Inorganic Chemistry 52 (2013) 5677–5691.
[73] A.G. Tovmasyan, Z. Rajic, I. Spasojevic, J.S. Reboucas, X. Chen, D. Salvemini, H. Sheng, D.S. Warner, L. Benov, I. Batinic-Haberle, Methoxy-derivatization of alkyl chains increases the in vivo efficacy of cationic Mn porphyrins. Synthesis, characterization, SOD-like activity, and SOD-deficient *E. coli* study of meta Mn (III) N-methoxyalkylpyridylporphyrins, Dalton Transactions 40 (2011) 4111–4121.
[74] S. Uemura, H. Matsushita, W. Li, A.J. Glassford, T. Asagami, K.H. Lee, D.G. Harrison, P.S. Tsao, Diabetes mellitus enhances vascular matrix metalloproteinase activity: role of oxidative stress, Circulation Research 88 (2001) 1291–1298.
[75] T. Baluchnejadmojarad, M. Roghani, A. Imani, Protective effect of enalapril on vascular reactivity of the rat aorta, Vascular Pharmacology 40 (2004) 301–307.
[76] V. Senses, S. Ozyazgan, E. Ince, M. Tuncdemir, F. Kaya, M. Ozturk, G. Sultuybek, A.G. Akkan, Effect of 5-aminoimidazole-4-carboxamide riboside (AICA-r) on isolated thoracic aorta responses in streptozotocin-diabetic rats, Journal of Basic and Clinical Physiology and Pharmacology 12 (2001) 227–248.
[77] A. Ceriello, New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy, Diabetes Care 26 (2003) 1589–1596.
[78] W. Abebe, Effects of taurine on the reactivity of aortas from diabetic rats, Life Sciences 82 (2008) 279–289.
[79] T. Matsumoto, K. Wakabayashi, T. Kobayashi, K. Kamata, Alterations in vascular endothelial function in the aorta and mesenteric artery in type II diabetic rats, Canadian Journal of Physiology and Pharmacology 82 (2004) 175–182.
[80] W. Abebe, K.M. MacLeod, Protein kinase C-mediated contractile responses of arteries from diabetic rats, British Journal of Pharmacology 101 (1990) 465–471.
[81] R.E. White, G.O. Carrier, Enhanced vascular α-adrenergic neuroeffector system in diabetes: importance of calcium, American Journal of Physiology 255 (1988) H1036–H1042.
[82] P.D. Taylor, B.B. Oon, C.R. Thomas, L. Poston, Prevention by insulin treatment of endothelial dysfunction but not enhanced noradrenaline-induced contractility in mesenteric resistance arteries from streptozotocin-induced diabetic rats, British Journal of Pharmacology 111 (1994) 35–41.
[83] Y. Oyama, H. Kawasaki, Y. Hattori, M. Kanno, Attenuation of endothelium-dependent relaxation in aorta from diabetic rats, European Journal of Pharmacology 132 (1986) 75–78.
[84] I. Batinić-Haberle, L.T. Benov, An SOD mimic protects NADP+-dependent isocitrate dehydrogenase against oxidative inactivation, Free Radical Research 42 (2008) 618–624.
[85] A.S. Fernandes, J. Gaspar, M.F. Cabral, J. Rueff, M. Castro, I. Batinic-Haberle, J. Costa, N.G. Oliveira, Protective role of ortho-substituted Mn(III) N-alkylpyridylporphyrins against the oxidative injury induced by tert-butylhydroperoxide, Free Radical Research 44 (2010) 430–440.
[86] B. Gauter-Fleckenstein, K. Fleckenstein, K. Owzar, C. Jiang, I. Batinic-Haberle, Z. Vujaskovic, Comparison of two Mn porphyrin-based mimics of superoxide dismutase in pulmonary radioprotection, Free Radical Biology and Medicine 44 (2008) 982–989.
[87] B. Gauter-Fleckenstein, K. Fleckenstein, K. Owzar, C. Jiang, J.S. Rebouças, I. Batinic-Haberle, Z. Vujaskovic, Early and late administration of MnTE-2-PyP5+ in mitigation and treatment of radiation-induced lung damage, Free Radical Biology and Medicine 48 (2010) 1034–1043.
[88] Z.N. Rabbani, F.K. Salahuddin, P. Yarmolenko, I. Batinic-Haberle, B.A. Thrasher, B. Gauter-Fleckenstein, M.W. Dewhirst, M.S. Anscher, Z. Vujaskovic, Low molecular weight catalytic metalloporphyrin antioxidant AEOL 10150 protects lungs from fractionated radiation, Free Radical Research 41 (2007) 1273–1282.
[89] H. Saba, I. Batinic-Haberle, S. Munusamy, T. Mitchell, C. Lichti, J. Megyesi, L.A. MacMillan-Crow, Manganese porphyrin reduces renal injury and mitochondrial damage during ischemia/reperfusion, Free Radical Biology and Medicine 42 (2007) 1571–1578.
[90] G. Burkhard Mackensen, M. Patel, H. Sheng, C.L. Calvi, I. Batinić-Haberle, B.J. Day, L.P. Liang, I. Fridovich, J.D. Crapo, R.D. Pearlstein, D.S. Warner, Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant, Journal of Neuroscience 21 (2001) 4582–4592.