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An SOD mimic protects $NADP^+$-dependent isocitrate dehydrogenase against oxidative inactivation

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Abstract
The isocitrate dehydrogenases (ICDs) catalyse the oxidative decarboxylation of isocitrate to alpha-ketoglutarate and can use either NAD^+ or $NADP^+$ as a cofactor. Recent studies demonstrate that the $NADP^+$-dependent isocitrate dehydrogenase, as a source of electrons for cellular antioxidants, is important for protection against oxidative damage. ICD, however, is susceptible to oxidative inactivation, which in turn compromises cellular antioxidant defense. This study investigates the effect of a superoxide dismutase (SOD) mimic, MnTM-2-PyP^{5+}, on the inactivation of $NADP^+$-dependent ICD in SOD-deficient *Escherichia coli* and in diabetic rats. The findings show that *E. coli* ICD is inactivated by superoxide, but the inactivated enzyme is replaced by *de novo* protein synthesis. Statistically significant decrease of ICD activity was found in the hearts of diabetic rats. MnTM-2-PyP^{5+} protected ICD in both models.

Keywords: *Isocitrate dehydrogenase inactivation, manganese porphyrin, MnTM-2-PyP, SOD mimic, diabetes*

Abbreviations: *ROS, reactive oxygen species; RNS, reactive nitrogen species; ICD, isocitrate dehydrogenase; SOD, superoxide dismutase; XO, xanthine oxidase; MnTM-2-PyP, manganese(III) 5,10,15,20-tetrakis(N-methylpyridinium-2-yl)porphyrin (MnTM-2-PyP^{5+} (charges omitted throughout text for clarity), AEOL-10112); LB, Luria-Bertoni; PQ^{++}, paraquat; DETAPAC, diethylenetriaminepentaacetic acid.*

Introduction

Isocitrate dehydrogenases (ICDs) encompass a family of enzymes that catalyse the conversion of isocitrate to alpha-ketoglutarate in the Krebs cycle [1]. Eukaryotic cells contain two different types of ICDs that depend on NAD^+ or $NADP^+$ as cofactors and serve different biological functions. $NADP^+$-linked ICD is found both in mitochondria and cytosol [2,3] and as a source of NADPH for the regeneration of cellular antioxidants is shown to protect against oxidative damage [4–6], against singlet oxygen- [7] and heat shock-induced apoptosis [8,9] and against cadmium toxicity [10]. Recent studies demonstrated that the antioxidant function of ICD is crucial for defense against ionizing radiation [11–13]. At the same time the $NADP^+$-linked ICD itself is susceptible to oxidative inactivation. Nitric oxide [14], peroxynitrite [15], ROS [16] and lipid peroxidation products [17,18] inactivate the enzyme, presumably by modifying essential thiol groups. *In vitro* experiments with purified enzyme demonstrate that it loses activity if exposed to H_2O_2, superoxide radical, hydroxyl radical and to photochemically generated singlet oxygen [16]. These findings suggest that *in vivo*, under

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conditions of oxidative stress, ICD would be a sensitive ROS/RNS target. Inactivation of ICD would further contribute to perturbation of the balance between oxidants and antioxidants and therefore will exacerbate oxidative/nitrosative stress. This assumption, however, is based mainly on *in vitro* experiments where ICD has been exposed to concentrations of ROS exceeding biologically relevant values.

Our previous experiments have shown that SOD-deficient *E. coli* is a useful tool for identifying ROS-sensitive targets [19–21]. It has been determined that the electron transport chain of exponentially growing *E. coli* generates $\sim 3\,\mu M$ of superoxide per second [22]. In the absence of SOD, $O_2^{\bullet -}$ produced at such a rate is enough to inactivate critical metabolic enzymes [23]. In contrast to eukaryotes, *E. coli* contains only one ICD, which is NADP$^+$-dependent and is regulated by phosphorylation/dephosphorylation [24]. Reportedly, *E. coli* ICD can be oxidatively inactivated by iron/ascorbate [25].

In this study we used SOD-deficient *E. coli* to investigate the effect of endogenously generated superoxide on the activity of the NADP$^+$-linked ICD and its protection by a Mn-porphyrin SOD mimic, MnTM-2-PyP [26]. We then used a streptozotocin-diabetic rat model to test if MnTM-2-PyP can prevent the inactivation of the NADP$^+$-dependent ICD *in vivo*. Mn porphyrins are potent catalytic $O_2^{\bullet -}$ scavengers. The most efficacious of them are those that bear positively charged *N*-alkylpyridyl groups that allow both thermodynamic and electrostatic facilitation for the approach of superoxide. Such a compound is MnTM-2-PyP, with $\log k_{cat} = 7.79$ ($\log k_{cat}$ for SOD enzymes is 8.84–9.30 [27–29]). We have previously shown that MnTM-2-PyP was able to substitute for SOD in SOD-deficient *E. coli* [26] and extended the life-span of streptozotocin diabetic rats [30].

Materials and methods

The 5,10,15,20-tetrakis(2-pyridyl)porphyrin (H_2T-2-PyP^{4+}) was supplied by MidCentury Chemicals (Chicago, IL). The *N*-methylation and metal incorporation to obtain MnTM-2-PyP was accomplished as previously described [26]. The structure of the SOD mimic is shown in Figure 1.

All other chemicals were obtained from standard sources.

The strains of *E. coli* used were: GC4468 = parental; QC1799 = GC4468 △ sodA3 △ sodB-kan [31,32]. These were prepared by D. Touati (Institute Jacques Monod, CNRS, University Paris, France). Additional strains were: AB1157 parental; JI132 = AB1157 plus (sodA:: MmdPR13) 25 (sodB-kan) 1-Δ 2 [33]; These were provided by J. Imlay (University of Illinois, Champaign-Urbana, Urbana, IL). Although results

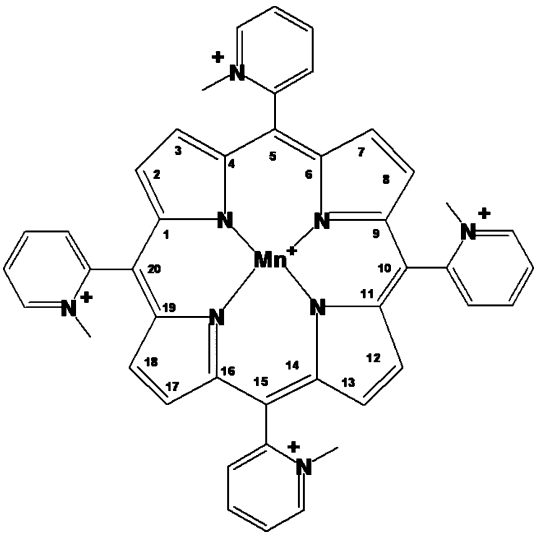

Figure 1. MnTM-2-PyP^{5+}, Mn(III) tetrakis(N-methylpyridinium-2-yl)porphyrin.

are presented for GC4468 and QC1799, similar results were also obtained with AB1157 and JI132. The *sodA sodB* strains are referred to as SOD-nulls even though they retain the periplasmic Cu, ZnSOD, which is present in only very small amounts.

Starter cultures were grown overnight at 37°C in Luria-Bertoni (LB) medium and were then diluted 200-fold into LB or M9CA medium. M9CA medium consisted of minimal A salts [34], 0.2% casamino acids, 0.2% glucose, 3 mg pantothenate and 5 mg of thiamine per litre. Growth was followed at 600 nm. Unless otherwise indicated, cultures intended for enzyme assays were harvested when $A_{600nm} = 0.6$–1.0. The cells were thrice washed and then lysed by sonication in 50 mM TRIS (pH 7.4), containing 1.0 mM cysteine, 1.0 mM citrate and 0.5 mM $MnCl_2$ [35] for assay of aconitase. Debris were removed by centrifugation at 16 000 × g for 10 min. The cell extracts thus obtained were assayed for aconitase [36] and ICD [37]. The aconitase activity was assayed spectrophotometrically at 340 nm in a mixture containing 50 mM Tris-Cl buffer, pH 7.4, 30 mM sodium citrate, 0.5 mM $MnCl_2$, 0.2 mM NADP$^+$, 2.0 units/ml of isocitrate dehydrogenase, and cell extract, in a total volume of 1.0 ml. To obtain 100% aconitase activity the enzyme was reactivated by anaerobic incubation with 0.5 mM dithiothreitol, 20 mM ferrous ammonium sulphate and 20 mM Na_2S [35].

ICD activity was assayed as described by Fatania et al. [37], by monitoring the production of NADPH at 340 nm. The 1.0 ml reaction mixture contained 33 mM Tris-EDTA buffer (pH 7.4), 1.33 mM $MnCl_2$, 1.3 mM DL-Isocitrate and 0.1 mM NADP$^+$. One unit of the enzyme activity is defined as production of 1 μmol of NADPH per minute.

In vitro, superoxide was generated using xanthine oxidase (XO) with hypoxanthine as a substrate.

Hypoxanthine (500 μM) and XO were added to cell extracts to give an initial superoxide generation rate of 5 μM/min.

$NADP^+$/NADPH ratio was determined as described by Micheli et al. [38].

All *E. coli* experiments were repeated three times with 3–5 replicates.

Diabetes in male Wistar rats was induced by a single (60 mg/kg) intraperitoneal injection of STZ. Induction of diabetes was confirmed by the presence of glucosuria within 24 h. Rats which maintained blood glucose concentrations above 15 mM were randomly divided into two groups designated as 'Diabetic' and 'Diabetic treated'. The animals in the second group received subcutaneous injection of sterile MnTM-2-PyP solution, 1 mg/kg/day for 2 months, for 5 days per week, with 2 days rest after each 5-day cycle. This drug administration scheme was adopted to avoid side-effects of MnTM-2-PyP administration, probably due to blood pressure drop [39].

Sixty days after beginning of the treatment, the animals were anaesthetized after 12 h of fasting and killed by decapitation. Hearts were perfused with cold saline, snap-frozen in liquid nitrogen and stored at -80°C until analysis. The samples were homogenized immediately before assays and homogenates were used for determination of ICD [40], aconitase [41] and fumarase [42,43] activities. One unit of fumarase was taken to be the activity that converted 1 μmol/min of L-malate to fumarate using $\varepsilon_{250nm} = 1.62\ mM^{-1}\ cm^{-1}$. The initial concentration of L-malate was 50 mM and the assay buffer was 50 mM sodium phosphate, pH 7.3 at 25°C.

Diabetes experiments were repeated twice with 7–12 animals per group.

Mean value and standard error, one-way analysis of variance and the Student-Neumann-Keuls test were used for the statistical analysis of the data. The 0.05 level of probability was used as the criterion of significance. In the figures significance is indicated as $^\star p < 0.05$ compared to control or non-treated group.

Results and discussion

The aim of our initial experiments was to check if *E. coli* ICD is susceptible to inactivation by superoxide *in vitro*. A system containing xanthine oxidase with hypoxanthine as a substrate was used as a source of $O_2^{\bullet -}$. Xanthine oxidase alone did not have any appreciable effect on ICD, while the incubation with hypoxanthine alone caused a slight decrease in ICD activity (not shown). Results presented in Figure 2 demonstrate that the complete system, xanthine oxidase, plus hypoxanthine, inactivated ICD in the SOD-deficient cell extracts faster (line 4) than in cell extracts of parental cells (line 1). It is interesting to note that when SOD-deficient cells were grown in the presence of 20 μM of the SOD mimic, MnTM-2-PyP, and then disrupted, such cell extracts demonstrated low inactivation rates similar to those in the parental cell extracts (Figure 2, line 2). However, when the SOD-deficient cells were grown in the absence of the SOD mimic and the mimic was added at final concentrations of 20 μM to the homogenate after the disruption of the cells, the effect was negligible (Figure 2, line 3).

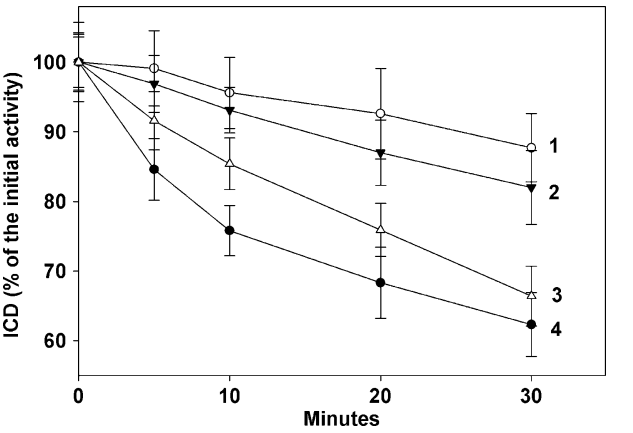

Figure 2. Inactivation of ICD by enzymatically generated superoxide. *E. coli* cell extracts (~1.0 mg protein/ml) were incubated at 37°C in the presence of 500 μM hypoxanthine and xanthine oxidase to give an initial superoxide generation rate of 5 μM/min. Experiments were repeated three times with 3–5 replicates. Means ± SE are presented. Line 1, parental; Line 2, cell extract of *sodAsodB* cells grown with 20 μM of MnTM-2-PyP; Line 3, *sodAsodB* cell extracts plus 20 μM MnTM-2-PyP; Line 4, *sodAsodB* cell extracts.

Near its substrate-binding site ICD contains -SH groups whose modification renders the enzyme inactive [37,44–49]. It has been shown that modification of these -SH groups is responsible for the inactivation of the enzyme by nitric oxide [14], $ONOO^-$ [15] and 4-hydroxynonenal [17]. Superoxide radical, however, has a relatively low reactivity. The estimated rate constant for $O_2^{\bullet -}$ reaction with thiols is in the range of 30–1000 $M^{-1}\ s^{-1}$ [50] and superoxide practically does not react with other amino acid residues of proteins. Therefore, the inactivation of ICD will not result from direct enzyme–superoxide interaction, but from interactions with secondary, highly reactive ROS derived from $O_2^{\bullet -}$, e.g. $HO^\bullet$. Production of $HO^\bullet$ by the Fenton reaction requires catalytic 'free' Fe^{2+} and it has been shown that in SOD-deficient *E. coli* superoxide liberates Fe^{2+} by attacking Fe-S clusters [51–54]. It has been estimated that in aerobically grown SOD-deficient *E. coli* the content of 'free' Fe increases ~8-fold, reaching 80 μM [52]. In *in vitro* experiments, treatment of *E. coli* cell extracts with 10 μM $FeSO_4$ plus ascorbate inactivated ICD [25]. Affinity cleavage experiments revealed that iron coordinated to Asp283 and Asp307, catalysing site

specific HO$^\bullet$ production, was responsible for the inactivation [25]. Judging by the inhibition of the $O_2^{\bullet -}$ driven aconitase inactivation (Figure 3, bars 1 and 2), when the SOD-null cells were grown in the presence of MnTM-2-PyP, the SOD mimic protected the Fe-S clusters and hence prevented the release of Fe^{2+}. Addition of MnTM-2-PyP after disruption of the cells did not protect aconitase (Figure 3, third bar) and, therefore, did not prevent the release of 'free' iron. We further tried to prove that liberation of iron is responsible for the inactivation of ICD in SOD-deficient extracts by using metal chelators. ICD, however, requires metal ions for its activity [55] and chelators that bind Fe (desferroxamine, DETAPAC) inactivated the enzyme.

The fact that an external source of $O_2^{\bullet -}$ inactivated ICD much faster in the SOD$^-$ cell extracts suggests that ICD activity would be lower in the SOD-deficient cells, where the steady state [$O_2^{\bullet -}$] is higher than in the parental cells. Comparison of the ICD activity in exponentially growing SOD$^-$ and SOD$^+$ cells revealed, however, that contrary to the expectations, ICD activity was not lower in the SOD$^-$ cells. Since ICD expression and activity depend on the availability of nutrients and growth conditions [56,57], the strains were grown in two different media, LB and M9CA, and ICD activity was assayed at different time intervals. No loss of ICD activity was observed in the exponentially growing SOD-null cultures. For the entire period of growth in LB medium, the specific activity of ICD in the SOD$^-$ remained slightly higher than in the SOD$^+$ strain (Figure 4). Similar results

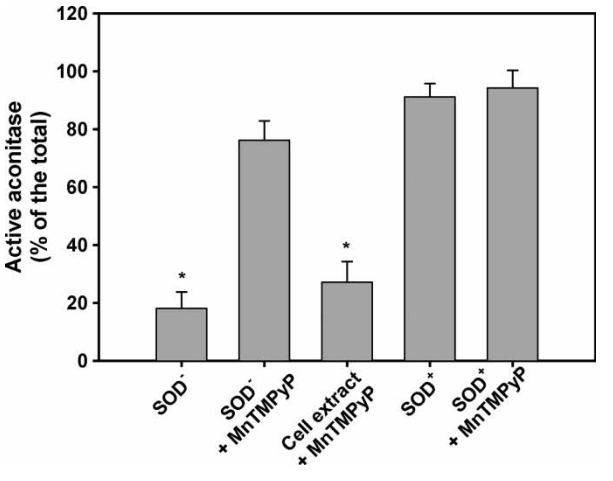

Figure 3. Aconitase activity in parental and SOD-deficient cells grown with or without MnTM-2-PyP. *E. coli* cultures were grown to a density of A_{600nm} 0.7–0.8 with or without 20 μM of MnTM-2-PyP. The cells were washed, disrupted by sonication and aconitase was assayed. The assay mixture contained 50 mM Tris-Cl buffer, pH 7.4, 30 mM sodium citrate, 0.5 mM $MnCl_2$, 0.2 mM $NADP^+$, 2.0 units/ml of isocitrate dehydrogenase and cell extract. Results are presented as a percentage of the total aconitase activity obtained after reactivation of the enzyme. Experiments were repeated three times with 3–5 replicates. Bars represent means ± SE.

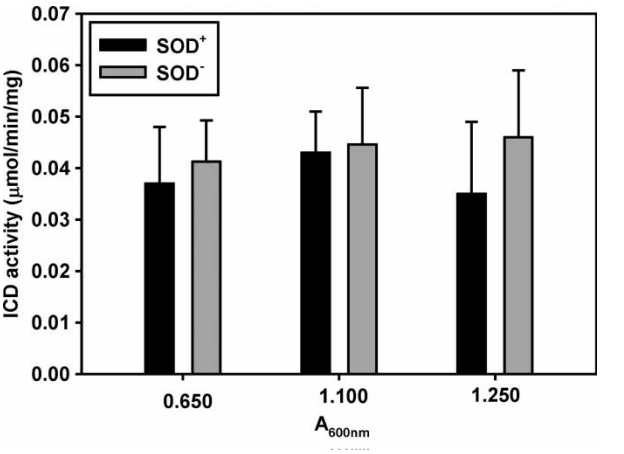

Figure 4. Isocitrate dehydrogenase activity in exponentially growing *E. coli* cultures. *E. coli* parental and SOD-deficient strains were grown in LB medium to the indicated densities. The cells were washed, disrupted by sonication and ICD activity was measured in 1.0 ml reaction mixture contained 33 mM Tris-EDTA buffer (pH 7.4), 1.33 mM $MnCl_2$, 1.3 mM DL-Isocitrate and 0.1 mM $NADP^+$. Experiments were repeated three times with 3–5 replicates. Bars represent means ± SE.

were obtained when cultures were grown in M9CA medium (not shown), but in that case the activity of ICD in the SOD$^-$ cells remained slightly lower than in the parental cells. This result was unexpected and indicated that either ICD is not inactivated or that the enzyme is inactivated, but the inactive protein is reactivated or continuously replaced by *de novo* protein synthesis.

To test the later possibility, spectinomycin (500 μg/ml) was added to the *E. coli* culture to block protein synthesis [58]. No growth was observed after the addition of the antibiotic, as judged by A_{600nm}. Sixty minutes after the addition of the antibiotic, the cells were disrupted and ICD activity was assayed. Results are summarized in Figure 5. This shows that in SOD$^-$ cells where protein synthesis had been stopped, the ICD activity dropped almost 50%, while in the SOD$^+$ cells it remained practically unchanged. To prove that ROS/$O_2^{\bullet -}$ are responsible for the inactivation of the enzyme, in parallel experiments the antibiotic was added to cells grown with 20 μM of the SOD mimic MnTM-2-PyP. As seen (Figure 5), the SOD mimic protected ICD against inactivation.

In further experiments a redox cycling agent, paraquat (PQ^{++}), was added to increase superoxide production. Cells were pre-incubated for 15 min with spectinomycin before the addition of paraquat. It is interesting to note that PQ^{++} caused increase in the ICD activity in the parental strain, even though protein synthesis had been blocked (Figure 5). The ICD of *E. coli* is tightly regulated and is acting as a switch, directing substrates either to the Krebs cycle or to the glyoxylate cycle [24]. Inactivation and reactivation of ICD is achieved by phosphorylation and dephosphorylation of a critical Ser residue, which

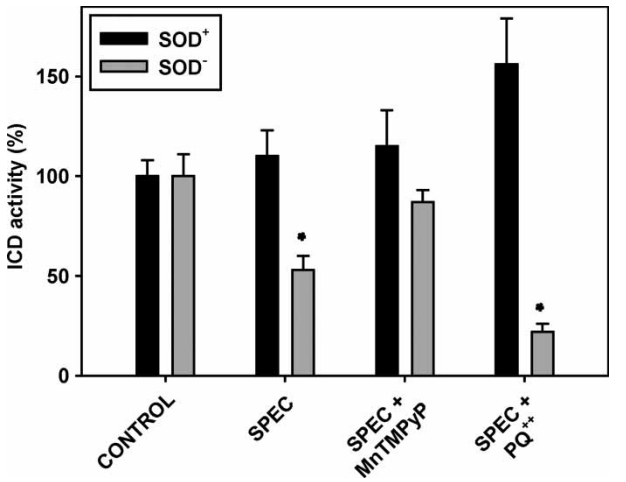

Figure 5. Effect of spectinomycin and paraquat on isocitrate dehydrogenase activity. *E. coli* parental and SOD-deficient strains were grown in LB medium to a density of A_{600nm} 0.6. Spectinomycin was added to 500 μM, followed 15 min later by addition of paraquat to 50 μM. One hour after the addition of paraquat the cells were washed, disrupted by sonication and ICD activity was measured. Experiments were repeated three times with 3–5 replicates. Bars represent means ± SE.

is catalysed by a bifunctional enzyme, ICD kinase/phosphatase [24]. A possible reason for the activation of ICD is dephosphorylation of the enzyme. Since PQ^{++} is a redox-cycling agent which uses NADPH, a decline of the NADPH/$NADP^+$ ratio could be a signal, which triggers the activation of the NADPH-producing enzymes in order to restore the NADPH/$NADP^+$ ratio [59]. When NADPH/$NADP^+$ ratio was measured, it was found that it decreased from 5.1 ± 1.6 (at zero time) to 1.3 ± 0.5 (at 60 min after the addition of PQ^{++}).

In contrast to the increase of ICD activity in the parental cells, addition of PQ^{++} to the SOD-deficient cells caused a sharp decrease of the ICD activity (Figure 5). The NADPH/$NADP^+$ ratio in the SOD-deficient cells decreased (from 3.8 ± 0.7 to 0.5 ± 0.4), but this did not lead to activation of ICD. It therefore appears that ICD is inactivated by endogenously produced superoxide. The damaged enzyme, however, is replaced by *de novo* protein synthesis. As mentioned before, in *E. coli* ICD activity determines the flux of isocitrate between the Krebs' cycle and the glyoxalate bypass [24]. The precise balance of the activities of the two pathways is so important that a specific, highly sophisticated mechanism of ICD regulation has evolved. In addition, ICD gene expression is a subject of complex regulation by *rpoS*, *arcA* and *fnr* [56,57]. It is tempting to speculate that uncontrolled drop of ICD activity triggers compensatory mechanisms inducing ICD gene expression. At the same time, the damaged protein is recognized and degraded by a special proteolytic system [60], both events contributing to faster ICD protein turnover.

Increased ROS/RNS production has been clearly documented in diabetes [61–63]. In addition to inactivation by ROS/RNS, the $NADP^+$-dependent ICD is highly susceptible to inactivation by glycation, both *in vitro* and *in vivo* [64]. Therefore, in the diabetic rats ICD is expected to be exposed simultaneously to ROS/RNS and high glucose, both causing inactivation of the enzyme. For example, in the kidney of streptozotocin diabetic rats the mitochondrial ICD activity was found to be ~30% of the activity found in healthy animals [64]. When we measured the total $NADP^+$-linked ICD in diabetic rat hearts, however, it was only 13% lower than the activity found in controls (Figure 6). Even though statistically significant, such a small decrease would barely have any effect on cardiomyocyte metabolism and NADPH production. Again we used aconitase inactivation to monitor superoxide production and Fe-S clusters disruption in the heart [41]. To verify that aconitase is selectively inactivated by superoxide, fumarase activity was measured in parallel. As seen (Figure 6), aconitase activity in the diabetic harts was ~30% lower compared to the normal animals, while fumarase remained unchanged. Treatment with the SOD mimic, MnTM-2-PyP, restored both aconitase and ICD activities to the control level (Figure 6). These results show that superoxide/$ONOO^-$ production is increased in diabetic hearts, yet ICD is almost unaffected. Such a finding either indicates that in the heart ICD is less accessible or Fe released from the Fe-S clusters is sequestered by other chelators or because of its importance the enzyme is a subject of replacement/reactivation. $NADP^+$-dependent ICD activity is considered crucial for maintaining cardiomyocyte energy and redox status [17] and inactivation of the enzyme might be life incompatible. It

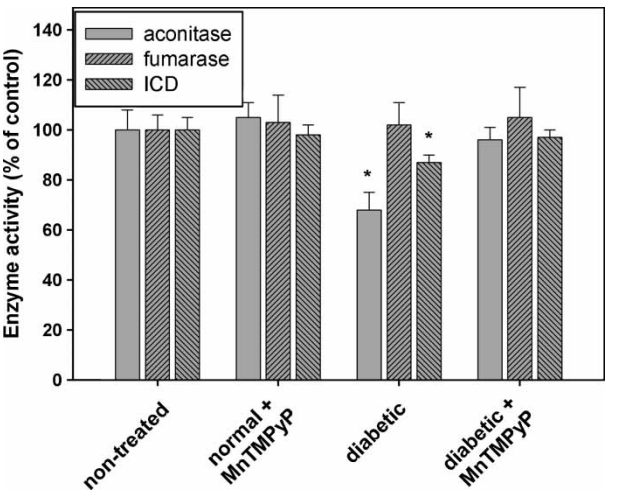

Figure 6. Activities of isocitrate dehydrogenase, aconitase and fumarase in rat hearts. The enzyme activities were measured in heart homogenates of control, non-diabetic MnTM-2-PyP-treated, diabetic and diabetic MnTM-2-PyP-treated rats. This experiment was repeated twice with 7–12 animals per group. Bars represent means ± SE. * $p < 0.05$ compared to non-treated group.

seems reasonable to expect that cardiomyocytes use various means to keep it active and our data reflect a steady-state ICD activity resulting from two opposing processes—inactivation by ROS/RNS/glycation and replacement/reactivation. The current study, however, does not provide sufficient data to clarify this point.

In conclusion, our experiments demonstrate that the SOD mimic, MnTM-2-PyP, protects ROS/RNS sensitive enzymes such as NADP$^+$-dependent ICD and aconitase against inactivation. This, together with effects on redox-based signalling pathways [65,66] and other, so far unidentified mechanisms, must be among the reasons for the beneficial effects of the SOD mimic in diabetes [30].

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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