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LETTER

Manganese Corroles Prevent Intracellular Nitration and Subsequent Death of Insulin-Producing Cells

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ABSTRACT Reactive oxygen species are heavily involved in the pathogenesis of diabetes mellitus (DM) because the insulin-producing beta cells are particularly vulnerable to free-radical-mediated cytotoxicity. Catalytic anti-oxidants have been successfully applied for attenuation of DM and its consequences, but most recent research revealed that preventing the nitration of vital proteins/enzymes might be an even more powerful strategy. We now report an unprecedented efficiency of manganese(III) corroles regarding the protection of rat pancreatic beta cells against intracellular nitration by peroxynitrite and subsequent cell death. A comparison between analogous corroles and porphyrin metal complexes reveals significant superiority of the former in all examined aspects. This is particularly true for the positively-charged manganese(III) corrole, which decomposes peroxynitrite fast enough and through a unique catalytic mechanism that is devoid of potentially nitrating reaction intermediates.

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Diabetes mellitus (DM) is a metabolic disorder resulting from either low levels of insulin or from abnormal resistance to insulin's effects coupled with inadequate levels of insulin secretion (*1*). Both kinds of DM (types 1 and 2) are intimately correlated with the proper function and mass of the insulin-producing beta cells, controlled by a combination of beta cell replication, neogenesis (the emergence of new beta cells), and apoptosis (*2*). Reactive oxygen species are heavily involved in the pathogenesis of DM, because beta cells express low levels of antioxidant genes and are hence particularly vulnerable to free radical-mediated cytotoxicity (*3*–*5*). Consistent with this hypothesis, experimental studies show that beta cells engineered to overexpress antioxidant enzymes are significantly more protected against reactive oxygen species and cytokine-mediated toxicity (*6*). Highly significant progress has been made regarding the utilization of catalytic antioxidants for attenuation of DM and its consequences (*7*–*9*), but most recent research revealed that preventing the nitration of vital proteins and enzymes might be an even more powerful strategy (*10*). The main cytokine in the pathogenesis of type 2 diabetes is interleukin-1b (*11*), which is a primary mediator of inducible nitric oxide synthase (iNOS), responsible for excessive production of nitric oxide (NO) in beta cells (*12*). Hyperglycemia is further known to cause a rise in the concentration of both superoxide anion radical ($O_2^{-\cdot}$) and NO (*13*), whose combination leads to peroxynitrite (HOONO) and its subsequent transformation into the most toxic reactive oxygen and nitrogen species (ROS/RNS), ·OH and ·NO_2, respectively (Figure 1, pathway I) (*14*). Both radicals are responsible for modifications of lipoproteins and cellular proteins, but the nitration of tyrosine, cysteine, and tryptophan residues and subsequent inactivation of numerous vital enzymes that eventually may cause cell dysfunction and death is considered the main biomarker for the involvement of peroxynitrite (*15*).

The particularly severe cytotoxicity of peroxynitrite (HOONO) may safely be attributed to the fact that there is no biological defense system against it, as all natural antioxidants (including diet-supplied and enzymes, such as superoxide dismutases and catalases) do not react with it faster than the vital biomolecules (*16*). Regarding diabetes, protein tyrosine nitration is considered to be responsive to changes in glucose concentrations in islets of Langerhans and insulinoma beta cells and is an important factor in the pathogenesis of the disease (*17*). This calls for the development of synthetic reagents that may either prohibit the formation of peroxynitrite (by neutralizing its precursors) or decompose it efficiently to biologically benign species. The largest advances regarding this aspect is with metalloporphyrins that either display SOD activity (*i.e.*, neutralize $O_2^{-\cdot}$) or decompose peroxynitrite in a catalytic fashion. The latter approach has been found to be quite efficient for treating various diseases that are related to oxidative and nitrosative stress, after sub-

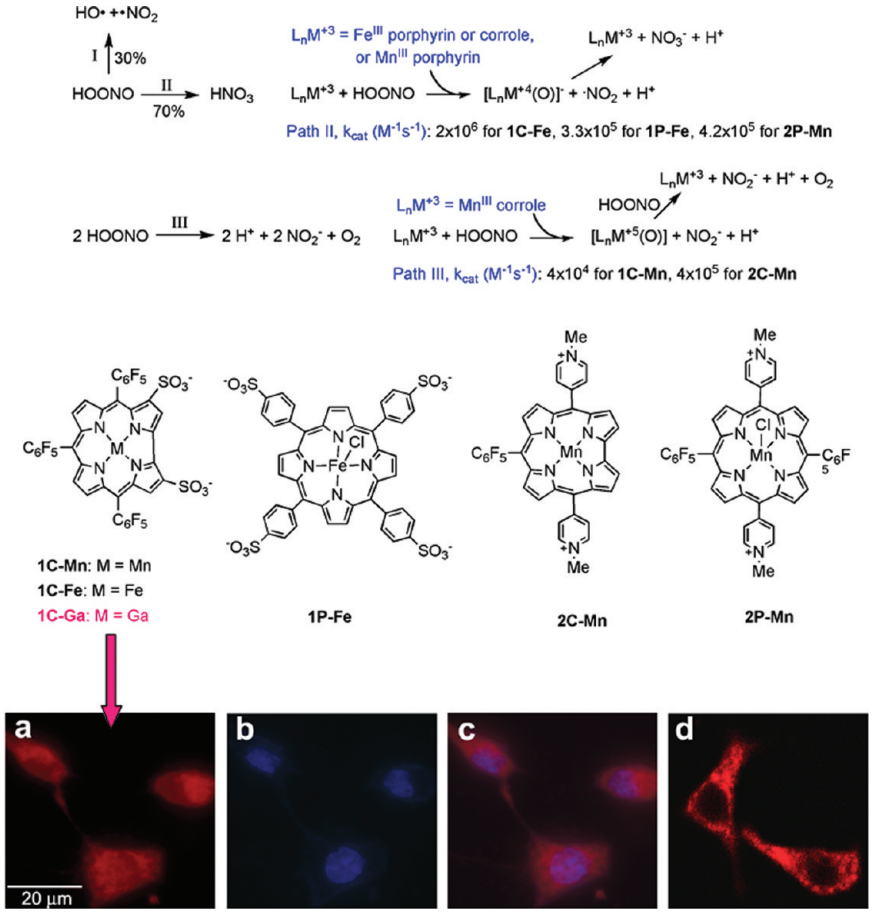

Figure 1. Decomposition modes of peroxynitrite, structures of the applied catalysts, and cellular uptake images of a fluorescent analogue. Top: decomposition of peroxynitrite (the indicated percentages refer to the noncatalyzed process) *via* (I) homolysis, (II) isomerization, and (III) disproportionation, together with a brief outline of the mechanistic pathways catalyzed by the various complexes and the catalytic rate constants at pH 7.4 and 25 °C (data from refs 30, 24, and 33, where that for 2P-Mn **is in the presence of ascorbate). Middle: chemical structures of the corrole and porphyrin metal complexes tested in this study. Bottom: cellular uptake of the fluorescent corrole** 1C-Ga **by RIN-m cells: a) corrole detection by fluorescence; b) nuclei staining by DAPI; c) merged images of panels a and b; and d) confocal fluorescence image.**

stantial optimization of the porphyrin structures (*18–21*).

We have recently introduced corroles, highly promising molecules in many fields (*22–25*), as new catalysts for decomposition of ROS and RNS. It started with the discovery that lipophilic corroles may be easily converted into amphiphilic derivatives of unique bipolarity (*26*) and the utilization of the corresponding gallium(III) complex (**1C-Ga** of Figure 1) for noncovalent association with proteins and cellular imaging (*27*). This approach was most recently used for the detection of breast cancer tumors in whole animals (**1C-Ga** is highly fluorescent), as well as for the elimination of its growth (*28*). In parallel, we have noticed a highly significant catalase-like activity (catalytic disproportionation of hydrogen peroxide) of the manganese(III) and iron(III) complexes of the same corrole (**1C-Mn** and **1C-Fe**, respectively, of Figure 1) (*29*). This was followed by investigations that focused on catalytic decomposition of peroxynitrite in purely chemical systems. The catalytic rate of the iron(III) complex **1C-Fe** was found to be exceptionally large, with indications that it acts as an isomerization catalyst (accelerating pathway II in Figure 1) (*30*). The manganese(III) complex of the same corrole (**1C-Mn**) and that of a corrole with two *meso*-pyridinium groups (**2C-Mn**) were identified as the first (and so far only) manganese complexes to display catalytic capability in the absence of reducing agents, as well as the only catalysts for disproportionation of peroxynitrite to HNO_2 and O_2 *via* a mechanism that does not involves any nitrating species (pathway III in Figure 1) (*24*). Biochemical investigations revealed **1C-Fe** as a very efficient antioxidant regarding the reaction between low density lipoproteins (LDL) and peroxynitrite, while **1C-Mn** acted as a pro-oxidant under identical conditions. Oral administration of these complexes to mice engineered to develop atherosclerosis revealed that **1C-Fe** attenuated the development of atherosclerotic lesions more efficiently than any other compound reported to date (*31*). Despite the discouraging *in vitro* effect of **1C-Mn**, that complex still displayed a beneficial *in vivo* effect, which raised the possibility that it is due to its effect on preventing nitration rather than oxidation.

These findings triggered the current study, where the protective properties of both negatively and positively charged corrole metal complexes against peroxynitrite-induced cytotoxicity were tested in insulin-producing beta cells. The first issue was to examine whether the corroles with sulfonic acid head groups (**1C-Fe** and **1C-Mn**) possess the capacity to penetrate live cells and accumulate therein, as they might actually be rejected by the negatively charged cell membrane. This concern was addressed with **1C-Ga**, the structural analogue of **1C-Fe**

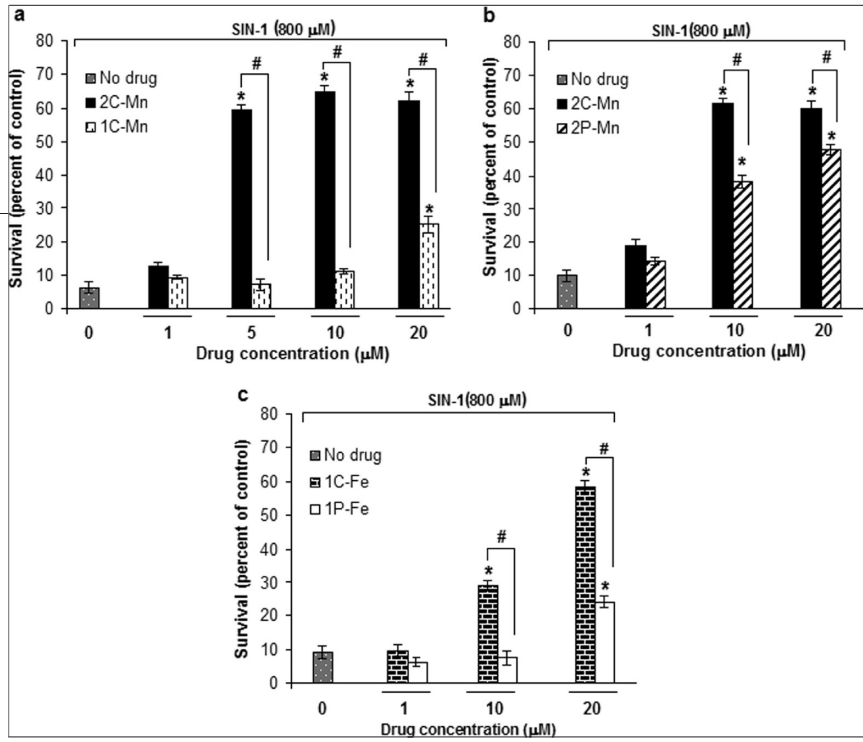

Figure 2. Survival based on the MTT test of SIN-1-treated RIN-m cells as a function of the various peroxynitrite decomposition catalysts. a) Manganese(III) corroles 2C-Mn **and** 1C-Mn**, b) the structurally analogous manganese(III) corrole** 2C-Mn **and porphyrin** 2P-Mn**, and (c) the structurally related iron(III) corrole** 1C-Fe **and porphyrin** 1P-Fe**. *$p < 0.001$ *versus* vehicle-treated cells; #$p < 0.005$ *versus* corresponding corrole/porphyrin.**

and **1C-Mn** that has no catalytic antioxidative ability but can be easily traced as a result of its intense fluorescence. Nuclei staining of insulinoma RIN-m cells by DAPI (4′,6-diamidino-2-phenylindole) and detection of **1C-Ga** by fluorescence microscopy of the same cells (Figure 1, panels a and b, as well as the overlaid panel of these results in Figure 1, panel c) clearly show that the corrole easily penetrates cell membranes, accumulates in the cytoplasm, and remains excluded from the nucleus as exemplified by confocal microscopy (Figure 1, panel d). These examinations clearly indicate that even corroles with negatively charged head groups are eligible agents for intracellular cytoprotection, most likely *via* protein-induced internalization (*27*). The positively charged complexes could not be checked by this method because we have not yet developed a fluorescent analogue of these corroles.

The potency of corroles regarding the protection of pancreatic beta RIN-m cells against induced oxidative/nitrosative stress was tested by the MTT cell viability test that reflects mitochondrial function. Addition of 800 μM SIN-1, a molecule that is known to create a flux of peroxynitrite in physiological fluid (*32*), led to less than 10% cell survival (Figure 2). Comparing the cytoprotective properties of the positively and negatively charged manganese(III) corroles (Figure 2, panel a) revealed that **2C-Mn** is highly potent at ≥5 μM, whereas **1C-Mn** displays only limited potency even at 20 μM. The manganese(III) corrole **2C-Mn** was also more effective than the corresponding porphyrin complex **2P-Mn** (Figure 2, panel b), and within the iron(III) complexes, the corrole derivative **1C-Fe** was much more active than the porphyrin complex **1P-Fe** (Figure 2, panel c). These results are supported by DAPI staining of nuclei, showing most significant preservation in the number of live cells that were treated with **1C-Fe** and **2C-Mn** prior to exposure to 800 μM SIN-1 (Figure S1, Supporting Information).

Since the most important goal of this study was to determine the effect of the compounds on peroxynitrite-induced cellular protein nitration, insulinoma RIN-m cells were treated with 600 μM SIN-1 in the absence or presence of 20 μM catalysts. Protein nitration was detected by immunofluorescence analysis, using a monoclonal antibody against nitrotyrosine (a red response), and the cells were also stained for nuclei by DAPI (blue color). Images a and b of Figure 3, panel A illustrate that tyrosine nitration was markedly induced by exposure to SIN-1 compared with control cells, and images c−g reflect the effect of the various catalysts. Quantification of the results (Figure 3, panel B) shows that administration of the positively charged manganese corrole **2C-Mn** almost completely abolished SIN-1-induced tyrosine nitration, whereas the iron and manganese complexes **1C-Fe** and **1C-Mn** provided only partial reduction. On the contrary, both porphyrin complexes (**2P-Mn** and **1P-Fe**) not only were ineffective in this regard but even catalyzed the process (increased nitration). Another test for nitration was performed by Western blot analysis using antinitrotyrosine antibodies. The results revealed minimal amounts of nitrotyrosine in the proteins of control cells, dramatically increased nitrotyrosine signals in those derived from cells incubated with 400 μM SIN-1 for 24 h, and highly significant differences in homogenates from cells treated with the various catalysts (Figure 4). Densitometric analysis clearly shows that only **2C-Mn** provided almost total attenuation of SIN-1-induced tyrosine nitration of the main band (about 93% in the particular gel shown and similar results in repeated experiments), most probably attributable to nitrated serum albumin. The other complexes were much less efficient in preventing nitration, including that of several higher and lower molecular weight proteins.

We demonstrate that corroles act as potent peroxynitrite decomposition catalysts in rat pancreatic beta cells: they efficiently penetrate cell membranes, accumulate in the cell interior and provide cell survival under conditions of induced oxidative/nitrosative stress. In particular, the manganese(III) corrole **2C-Mn** is capable of abolishing protein modifications and dysfunction caused by nitration much more efficiently than all other complexes. These findings are very much consistent with the mechanism elucidated for peroxynitrite de-

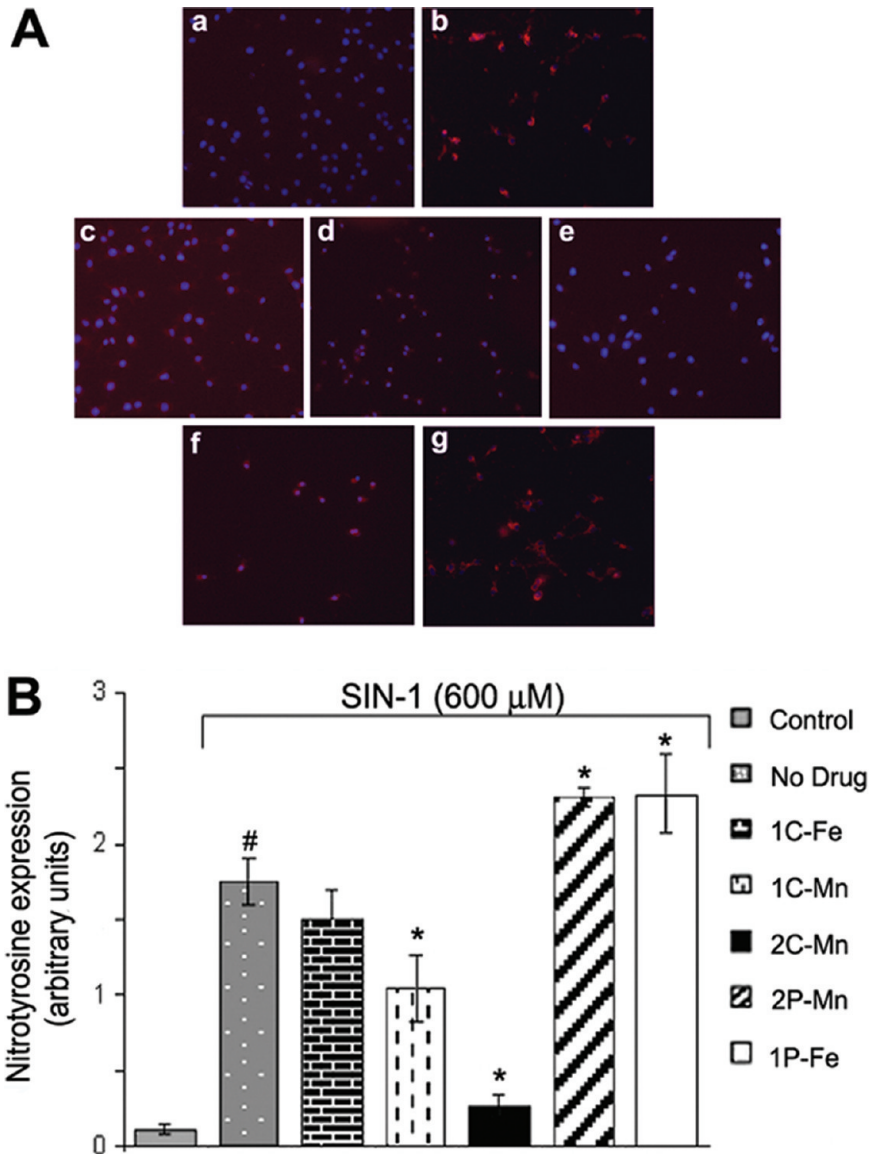

Figure 3. Proteins nitration levels in (a) untreated RIN-m cells, (b) cells treated with 600 μM SIN-1, and cells treated with 600 μM SIN-1 in the presence of 20 μM of (c) 1C-Fe**, (d)** 1C-Mn**, (e)** 2C-Mn**, (f)** 1P-Fe**, or (g)** 2P-Mn**. The images (A) are representative fields, and the graph (B) represents absolute values of 8 to 14 separate fields, expressed as mean ± SEM. #$p < 0.001$ *versus* control; *$p < 0.001$ *versus* no drug.**

composition by the different catalysts. The catalytic cycles of manganese(III) and iron(III) porphyrins, as well as of iron(III) corroles, proceed through a high valent metal(IV) intermediate and $\cdot NO_2$, which could indeed lead to increased nitration (Figure 1, pathway II) (*33*). On the other hand, the manganese(III) corroles proceed through quite a nonreactive (oxo)manganese(V) intermediate and release relatively harmless NO_2^- rather than $\cdot NO_2$ (Figure 1, pathway III) (*24, 30*), which explains the apparent inhibition of nitration. The larger potency of **2C-Mn** relative to that of **1C-Mn** nicely reflects the rate of peroxynitrite decomposition catalysis (Figure 1), as the slow-acting **1C-Mn** is apparently less capable of inverting the spontaneous decay of peroxynitrite to $\cdot OH$ and $\cdot NO_2$ that leads to tyrosine nitration. The strongest support for the importance of the decomposition reaction mechanism, as well as for the harmful consequences of nitration, comes from the data compiled in Figure 4. As the reaction rate of **2C-Mn** with peroxynitrite is about the same as for the porphyrin complexes and quite slower than that of the iron(III) corrole **1C-Fe**, the outstanding potency of **2C-Mn** in cell culture regarding the prevention of intracellular nitration and consequential cellular death (Figures 2−4) clearly indicates that the main factor underlying its superiority resides in its unique mechanism of action on peroxynitrite. Differences in cellular uptake and/or intracellular distribution of the positively and negatively charged metallocorroles may also be important and will be studied in the near future. The results of the current study, together with the observed attenuation of atherosclerosis by metallocorroles (*27*), highlight the large potential of catalysts that may efficiently *prevent both oxidative and nitrosative damage* to biomolecules that are vital for the survival of beta cells and the consequential effects of DM.

METHODS

1C-Fe and **1C-Mn** (*29*), **2C-Mn** and **2P-Mn** (*24*) were prepared as previously described; **1P-Fe** (iron(III) *meso*-tetra(4-sulfonatophenyl)porphine chloride) was purchased from Frontier Scientific.

Cell Culture. RIN-m cells were cultured in RPMI l640 medium, supplemented with 2% fetal calf serum and grown in a humidified cell incubator at 37 °C under a 5% CO_2 atmosphere. In all experiments, the cells were pretreated with corroles/porphyrins 30 min prior to the addition of SIN-1. Western blots, immunohistochemistry, cellular uptake, and MTT viability tests were performed under standard conditions. A more complete description is provided in Supporting Information.

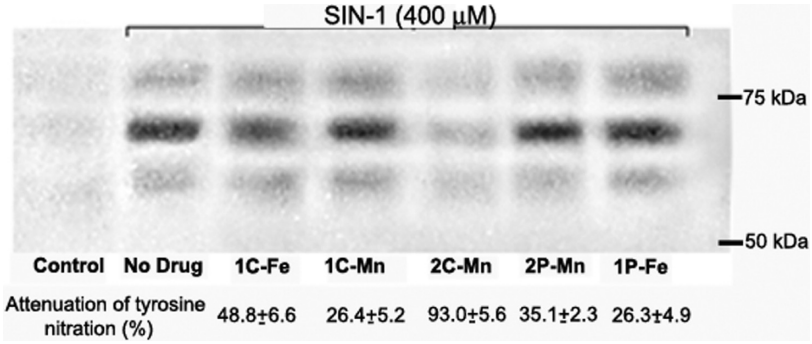

Figure 4. Western blot analysis revealing effects of corroles 1C-Fe**,** 1C-Mn**,** 2C-Mn **and porphyrins** 2P-Mn**,** 1P-Fe **(20 μM) on proteins nitration levels by SIN-1 (400 μM) induced damage.**

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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