MnTMPyP, a metalloporphyrin-based superoxide dismutase/catalase mimetic, protects INS-1 cells and human pancreatic islets from an in vitro oxidative challenge

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Received 28 March 2006; accepted 2 September 2006
Available online 26 January 2007

Abstract

Aims. – Pancreatic islets can be lost early following allotransplantation from oxidative stress. Antioxidant enzyme overexpression could confer a beneficial effect on islets exposed to reactive oxygen species (ROS) and nitrogen species. Here, we tested the effect of MnTMPyP, a superoxide dismutase/catalase mimetic.

Methods. – INS-1 insulin-secreting cells or human islets were cultured with MnTMPyP and exposed to a superoxide donor (the hypoxanthine/xanthine oxidase (HX/XO) system), a nitric oxide donor [3-morpholinosydnonimine (SIN-1)] or menadione. Viability of INS-1 cells was assessed by WST-1 colorimetric assay and FACS analysis (Live/Dead® test). ROS production was determined using fluorescent probes. Islet viability was estimated by WST-1 assay and endocrine function by static incubation.

Results. – Following MnTMPyP treatment, ROS production in INS-1 cells was reduced by 4- to 20-fold upon HX/XO challenge and up to 2-fold upon SIN-1 stress. This phenomenon correlated with higher viability measured by WST-1 or Live/Dead\textsuperscript{®} test. MnTMPyP preserved islet viability upon exposure to SIN-1 or menadione but not upon an HX/XO challenge. Similarly, decrease in insulin secretion tended to be less pronounced in MnTMPyP-treated islets than in control islet when exposed to SIN-1, but no changes were noticed during an HX/XO stress.

Conclusions. – MnTMPyP was able to improve the viability of INS-1 cells and human islets exposed to oxidative challenges in vitro. Protection of INS-1 cells could be as high as 90%. This agent is therefore potentially attractive in situations involving the overproduction of ROS, such as islet transplantation.

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Résumé

Protection des cellules INS-1 et des îlots pancréatiques humains contre un stress oxydant in vitro par la MnTMPyP, métalloporphyrine àactivité superoxyde dismutase et catalase.

Objectifs. – Le stress oxydant contribue à une perte précoce des îlots pancréatiques greffés. La surexpression d’enzymes anti-oxydantes protège les îlots des effets délétères des espèces radicales de l’oxygène (ROS) et du monoxyde d’azote (NO). Nous avons étudié ici les effets deMnTMPyP, qui possède une activité superoxyde dismutase et catalase.

Méthodes. – Des cellules insulinosécrétrices INS-1 et des îlots humains ont été cultivés en présence de MnTMPyP et exposés à un donneur d’anions superoxyde [hypoxanthine/xanthine oxydase (HX/XO)], de NO [3-morpholinosydnonimine (SIN-1)] ou à la ménadione. La viabilité des cellules INS-1 a été évaluée par le test colorimétrique WST-1 et par cytométrie de flux (test Live/Dead\textsuperscript{®}), la production de ROS par sondes fluorescentes, la viabilité des îlots par le test WST-1 et la fonction endocrine par incubation statique.

Abbreviations: IEQ, equivalent number of islets; MnTMPyP, Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin; NO, nitric oxide; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine.HCl; SOD, superoxide dismutase.

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**1. Introduction**

Despite improvements in clinical islet transplantation outcome, several obstacles remain to be solved before this procedure can stand as a reliable therapeutic procedure for type 1 diabetes mellitus. Current protocols usually require two pancreatic organs per recipient to restore a normal glucose metabolism. A large proportion of the graft is lost in the early days following transplantation, from non immune, non specific mechanisms. Among these mechanisms including thrombosis, apoptosis and ischemic injury, oxidative stress is very important [1]. The mediators of beta-cell oxidative damage include nitric oxide (NO) and peroxynitrite (ONOO\(^{-}\)). Their deleterious effects are reinforced by the low intracellular antioxidant status of pancreatic islets.

In previous works, adenoviral-mediated gene transfer of SOD or catalase was shown to prevent NO-induced beta-cells damage and HX/XO injury [2,3] but techniques using adenovirus are not clinically used because of their potential toxicity. Moreover, the large size and short in vivo life-span of these enzymes as proteins limit their clinical use.

Extensive studies have been carried out to find the suitable SOD-mimics to substitute superoxide dismutase. Several low-molecular-weight molecules have been developed and characterized. There are four main classes of SOD mimics: desferrioxamine, macrocycles, salen compounds and mesoporphyrins. Like their native counterpart, the metal-dependent SOD-mimics could contain Zn, Fe or Mn but have relatively long metabolic half-life and are able to penetrate into the cells. The Mn porphyrins have a broad antioxidant specificity which includes scavenging superoxide anion (O\(_{2}^{-}\)) [4], hydrogen peroxide (H\(_{2}\)O\(_{2}\)) [5], ONOO\(^{-}\) [6], NO [7] and diminishing lipid peroxidation [8].

In this study, we examined the effects of Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) on INS-1 cells and human islets exposed to cytotoxic challenges. MnTMPyP is a SOD/catalase mimetic that possesses the catalytic activity of both enzymes and is permeable to cells. Thus this molecule can potentially exhibit the beneficial and synergistic effects of both SOD and catalase towards an excess of superoxide and hydrogen peroxide. We found that MnTMPyP conferred a significant protection against HX/XO or SIN-1 stress and induced a reduction of ROS production. The addition of MnTMPyP to menadione-treated INS-1 cells resulted in protection from zero to mild depending on the menadione concentrations used.

Regarding human islets, MnTMPyP addition before and during the stress period showed quite an efficient protection against SIN-1 or menadione but not against XO. Likewise, preliminary data on endocrine function suggest that it was preserved in case of SIN-1 exposure but not with XO.

**2. Material and methods**

**2.1. Materials**

Antioxidant treatment. MnTMPyP (BioMol, TEBU, Le Perray-en-Yvelines, France), resuspended in water to obtain a 10 mmol/l stock solution was added at a final concentration of 25 μmol/l to INS-1 cells or human islets 30 min to 24 hours before stress application and during the period of stress. In each experiment, treatment and control conditions were compared.

INS-1 cells and human pancreatic islets. Rat insulinoma cell line INS-1 were cultured in RPMI 1640 medium as previously described [9]. Human pancreatic islets were isolated in the Cell Culture Core Laboratory of the University Hospital of Lille (France) or in UMTCT Grenoble Cell Therapy Unit (Saint-Ismer, France) and cultured in CMRL medium supplemented with 10% FCS as previously described [3].

**2.2. Methods**

**2.2.1. Cytotoxic challenges**

**2.2.1.1. HX/XO challenge.** Xanthine oxidase (XO) and hypoxanthine (HX) (Sigma, Saint Quentin Fallavier, France) were dissolved before use in RPMI medium, then added to INS-1 cells (96-wells plates, 5.10^4 cells per well) in 100 μl at 0.5 mmol/l HX final concentration, while concentrations of XO varied from 0 to 50 mU/ml. This XO/HX system produces superoxide anions, hydroxyl radicals and H\(_{2}\)O\(_{2}\). Control cells received 0.5 mmol/l HX alone. HX/XO was left for 1.5 h, then removed and cells were incubated in 100 μl fresh medium for 16 h before viability determination.

**2.2.1.2. SIN-1 challenge.** We used SIN-1 ((3-Morpholino sydnonimine.HCl), Sigma, Saint Quentin Fallavier, France) as source of nitric oxide in our study. The nitrovasodilator SIN-1 slowly decomposes to release both nitric oxide and superoxide in an equimolar manner and thereby produces peroxynitrite, a powerful oxidant [10]. A 4 mmol/l stock solution dissolved in
PBS was diluted in RPMI medium to obtain final concentrations from 0 to 2000 μmol/l and added to INS-1 cells in 100 μl (5.10⁴ cells per well). Control wells received RPMI/PBS medium. Cells were cultured for 16 h and cytotoxic medium replaced on the next day, just before viability tests.

2.2.1.3. Menadione treatment. INS-1 cells were treated for 6 h with concentrations of menadione sodium bisulfite (Sigma, Saint Quentin Fallavier, France) from 0 to 100 μmol/l diluted in medium from a 100 mmol/l menadione stock solution. After the incubation period, menadione was replaced by fresh medium and the cells left overnight at 37 °C, 5% CO₂, before viability determination. The intracellular metabolism of menadione results in the generation of ROS including H₂O₂, O₂⁻, and when in the presence of metal ions, the highly deleterious hydroxyl radical, OH°.

2.2.2. In vitro cell viability

2.2.2.1. WST1 test. INS-1 cells mitochondrial activity was assessed by WST-1 assay (Roche Diagnostics, Meylan, France). This colorimetric assay is derived from the more commonly known MTT test previously established as a valid method for islet viability assessment [11]. Each experiment was carried out with 6 wells per experimental group.

Cytotoxicity of NO donors or prooxidant agents and percentage of protection of MnTMPyP were defined according to the following formula:

- Cytotoxicity on control cells C = (ODC − OD₅₀/OD₅₀) × 100, where ODC = OD of control cells and OD₅₀ = OD of control cells exposed to cytotoxic stress;
- Cytotoxicity on MnTMPyP-treated cells T = (ODₐ − OD₅₀/ODₐ) × 100, where ODₐ = OD of MnTMPyP-treated cells and OD₅₀ = OD of MnTMPyP-treated cells exposed to cytotoxic stress;
- Percentage of protection = (C-T/C) × 100.

The same method was applied to human islets.

2.2.2.2. Live/Dead test. Cell viability and cytotoxicity were determined by simultaneous staining live and dead cells using a two-color fluorescence assay, the Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes, Montluçon, France). The membrane-impermeant DNA dye ethidium homodimer was used to identify dead cells whose plasma membrane integrity was disrupted. The membrane-permeant dye calcine-AM was used to label live cells. It penetrates into the cells where it is metabolized by cytoplasmic esterases and becomes a fluorescent but membrane-impermeant probe which is retained in viable cells. The cells were detached with trypsin, combined with cells floating in the medium, washed with PBS, and then the dyes were added in PBS to final concentrations of 0.5 μmol/l for ethidium homodimer and 0.2 μmol/l for calcine-AM. After a 15 minutes incubation period, the cells were analyzed by a 4-color Becton Dickinson FACScalibur. The percentage of viable and dead cells was estimated in both control and MnTMPyP-treated groups.

In vitro human pancreatic islet function. Glucose-stimulated insulin secretion was determined by static incubation. 80 IEQ were plated in duplicate on 3 μm Millicell inserts (Dominique Dutscher, Brumath, France) in a 24-well plate and consecutively stimulated for 3 different 1-h periods in 0.5% BSA Krebs buffer containing 3.3 mmol/l glucose (period 1 and 3), 27.5 mmol/l glucose + 10 mmol/l theophylline (period 2). Medium samples were frozen at −20 °C for further insulin determination by RIA. Results of insulin release were expressed as absolute value upon period 2.

Loading of cells with fluorescent probes and determination of intracellular ROS levels. To study the effect of oxidative stress and cytotoxic challenge on intracellular oxidant generation, we used two probes, 5-(and 6-) chloromethyl-2′, 7′- dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) and dihydroethidium (DHE), for intracellular H₂O₂ and superoxide anion (O₂⁻) measurement respectively. The probes were from Molecular Probes (Interchim, Montluçon, France). In fact, CM-H₂DCFDA fluorescence informs on the global oxidative activity of the cells because of its capacity to detect other reactive species than H₂O₂ such as ONOO⁻, HOO° and OH°. CM-H₂DCFDA passively diffuses into the cells where its acetate groups are cleaved by intracellular esterases. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell. DHE permeates the cell membrane easily and in the presence of O₂⁻ it can be directly oxidized to red fluorescent ethidium bromide which is intercalated into DNA to enhance red fluorescence of the nucleus. Thus, cellular fluorescence intensity indicates the levels of intracellular ROS and can be monitored by flow cytometry.

A 1.73 mmol/l stock solution of CM-H₂DCFDA was prepared daily in DMSO, kept on ice, and diluted to 5 μmol/l just before the experiment. INS-1 cells were loaded with CM-H₂DCFDA for 1 hour at 37 °C, at the end of the stress period. Next, the cells were detached with trypsin, washed with PBS and analyzed on a Becton Dickinson FACScalibur. For DHE loading, a 5 mmol/l stock solution was prepared in DMSO, then diluted to 2 μmol/l final in culture medium. The cells were incubated for 30 minutes at 37 °C and harvested as described above. Results were calculated as the fold difference between non-stressed cells and stressed-cells in the control group and the MnTMPyP-treated group.

Statistical analysis. Cytotoxicity levels were compared by analysis of variance using ANOVA followed by a Scheffe’s test between the 2 groups (control cells and cells receiving MnTMPyP). Significance was set at P < 0.05. Data are shown as means ± SEM.

3. Results

3.1. Formation of intracellular and mitochondrial ROS

ROS generated endogenously inside cells could be detected by flow cytometry using CM-H₂DCFDA or DHE. Fig. 1A shows intracellular ROS formation using CM-H₂DCFDA. INS-1 cells exposed to HX/XO showed an increase in the cytosolic ROS level. In cells without MnTMPyP (Control group
panel), while concentration of XO rose, the mean of fluorescence increased by 5.8 fold for a 12 mU/ml dose and 105 fold for 20 mU/ml versus 0 mU/ml. When 25 μmol/l MnTMPyP was added (MnTMPyP group panel), the values of fluorescence changed less radically, with 1.04 fold for 12 mU/ml XO and 4.39 fold for 20 mU/ml XO versus non-stressed MnTMPyP cells. Generation of intracellular ROS using DHE is shown in Fig. 1B. When no MnTMPyP was added (Control group panel), the fluorescence values showed a moderate increase proportionally with the increasing concentration of XO. A 12 mU/ml concentration induced a 2.46 fold increase compared to the non-stressed cells (0 mU/ml XO), whereas with 20 mU/ml XO, a 3.15 fold increase was measured. In INS-1 cells cultured with 25 μmol/l MnTMPyP, no increase was observed whatever the concentration of XO used. The mean value of fluorescence even showed a reduction in the production of ROS inside the cells, with about 75% fluorescence of the non-stressed cells (MnTMPyP group panel).

Using the same method, we followed the formation of ROS after the addition of SIN-1. On Fig. 2A, we showed the mean fluorescence increase in INS-1 measured by CM-H2DCFDA cells without MnTMPyP (Control group panel) when concentrations of SIN-1 increased. Fluorescence increases by 4.80 fold and 13.40 fold for 1200 and 2000 μmol/l SIN-1 respectively versus non-stressed cells. Addition of MnTMPyP decreased by 2.65 fold the fluorescence value for a 1200 μmol/l dose of SIN-1 but for 2000 μmol/l of SIN-1, MnTMPyP did not reduce the formation of ROS (MnTMPyP group panel). The use of the DHE probe (Fig. 2B) exhibits a completely different profile with no increase in the fluorescence mean value. When doses of SIN-1 raised, the mean fluorescence decreased to 72 and 68% of the non-stressed cells for 1200 and 2000 μmol/l respectively in cells cultured without MnTMPyP (Control group panel), while for the cells treated with 25 μmol/l MnTMPyP, the values were 82 and 62% of the non-stressed cells (MnTMPyP group panel).

3.2. Effect of MnTMPyP addition on cellular viability

After exposure of INS-1 cells to increasing doses of XO in the presence of HX, a dose-dependent cytotoxic effect was shown by WST-1 assay (Table 1) and completed by FACS analysis using the Live/Dead® kit (Fig. 3). As shown in Table 1, doses of XO of 2 mU/ml to 16 mU/ml resulted in a cytotoxicity of 21.6 ± 14.4% to 94.5 ± 2.8% respectively in cells without MnTMPyP. When MnTMPyP was added, values of cytotoxicity decreased and were comprised between 4.1 ± 2.3 and 19.5 ± 9.7% for XO doses ranging from 2 mU/ml to 16 mU/ml. Thus, a 25 μM MnTMPyP concentration conferred a cytoprotection of around 82%.

Using the Live/Dead® kit (Fig. 3), percentages of ethidium homodimer-positive cells (dead cells) increased while percen-
tages of positive cells for calcein (live cells) decreased when stress is applied. FACS data showing cellular repartition between live and dead cells did not give percentages of cytotoxicity in the same order of magnitude than those obtained in WST-1 assay. Indeed, from 0 mU/ml to 20 mU/ml XO, percentage of dead cells ranged from 2.7 to 79.9% in control cells and from 4.3 to 19.9% in cells cultured with MnTMPyP. Moreover, for a 16 mU/ml XO stress, even though in WST-1 assay the value of cytotoxicity was 94.5% we found only 45.7% of dead cells in INS-1 cells without MnTMPyP using the Live/Dead® kit. When we looked at the cytotoxicity measures in INS-1 cells cultured with MnTMPyP, the values were 19.5% in the WST-1 method and 8.1% with the FACS technique.

Exposure of INS-1 cells to SIN-1 resulted in a dose-dependent cytotoxicity, measured by WST-1 assay (Table 2).

Table 1

<table>
<thead>
<tr>
<th>XO (mU/ml)</th>
<th>Percentage of cytotoxicity</th>
<th>Percentage of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MnTMPyP</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21.6 ± 14.4</td>
<td>4.1 ± 2.3</td>
</tr>
<tr>
<td>6</td>
<td>66.0 ± 14.6</td>
<td>9.7 ± 6.4*</td>
</tr>
<tr>
<td>16</td>
<td>94.5 ± 2.8</td>
<td>19.5 ± 9.7**</td>
</tr>
</tbody>
</table>

INS-1 cells were cultured without (control) or with 25 µmol/l MnTMPyP and exposed to 2, 6 or 16 mU/ml XO in the presence of 0.5 mmol/l HX for 1.5 hour. Cytotoxicity was calculated and results were given as mean ± sem (n = 6). Percentages of protection were also shown. Cytotoxicity levels were compared by analysis of variance and Scheffe’s test between the 2 groups (control and MnTMPyP; *P = 0.009, **P < 0.0001).

Table 2

<table>
<thead>
<tr>
<th>SIN-1 (µmol/l)</th>
<th>Percentage of cytotoxicity</th>
<th>Percentage of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MnTMPyP</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.8 ± 0.8</td>
<td>1.3 ± 1.3</td>
</tr>
<tr>
<td>1400</td>
<td>17.4 ± 13.1</td>
<td>4.7 ± 3.1</td>
</tr>
<tr>
<td>1800</td>
<td>67.4 ± 11.4</td>
<td>10.4 ± 3.1*</td>
</tr>
<tr>
<td>2000</td>
<td>81.6 ± 4.9</td>
<td>31.6 ± 11.8**</td>
</tr>
</tbody>
</table>

INS-1 cells were cultured without (control) or with 25 µmol/l MnTMPyP and exposed to 1000, 1400, 1800 or 2000 µmol/l of SIN-1 for 16 hours. Cytotoxicity was calculated and results were given as mean ± sem (n = 7). Percentages of protection were also shown. Cytotoxicity levels were compared by analysis of variance and Scheffe’s test between the 2 groups (control and MnTMPyP; *P = 0.0009, **P = 0.001).

Fig. 2. Effects of SIN-1 on ROS formation measured by CM-H$_{2}$DCFDA (A) or DHE (B) fluorescence in INS-1 (control and MnTMPyP). Cells were exposed to 0, 1200 or 2000 µmol/l of SIN-1. At the end of the period of stress, cells were loaded with 5 µmol/l CM-H$_{2}$DCFDA or 2 µmol/l DHE for 1 hour and 30 minutes respectively. The cells were then analyzed by flow cytometry. The experiment was repeated three times and a set of representative figures is shown.
only of 25.8% at 1000 μmol/l of SIN-1 in cells without MnTMPyP and still of 90.2% in MnTMPyP-treated cells. When doses of SIN-1 increased from 1400 to 2000 μmol/l, percentages of live cells decreased to 9.3% in the group without MnTMPyP treatment and 11.8% in MnTMPyP-treated group. Protection calculated in these conditions showed a value of 87.7% for 1000 μmol/l SIN-1 and dramatically fell to 10.1% at 1400 μmol/l, then to 3.9 and 3.1 for 1800 and 2000 μmol/l respectively. This cytoprotective effect of MnTMPyP evidenced by the Live/Dead® test was consistently observed up to a concentration of 1000 μmol/l SIN-1 during our three experiments, but was inconsistent beyond.

Menadione treatment resulted in a rapid decrease in viability, with 17.9 ± 7.3% toxicity for a concentration of 20 μmol/l, 79 ± 10.6% for a 25 μmol/l dose and 90.6 ± 0.7% for 50 μmol/l dose of menadione in control cells (Table 3). At 20 μmol/l, MnTMPyP-treated cells showed 68.7 ± 4.9% toxicity thus allowing no protection to INS-1 cells. Nevertheless, when menadione concentrations raised to 25 and 50 μmol/l, cytotoxicity in MnTMPyP-treated cells was 72.3 ± 5.3% and 75.4 ± 13.1% respectively, a little bit lower than in control cells.

Table 3

<table>
<thead>
<tr>
<th>Menadione (μmol/l)</th>
<th>Percentage of cytotoxicity</th>
<th>Percentage of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MnTMPyP</td>
</tr>
<tr>
<td>20</td>
<td>17.9 ± 7.3</td>
<td>68.7 ± 4.9**</td>
</tr>
<tr>
<td>25</td>
<td>79.0 ± 10.6</td>
<td>72.3 ± 5.3</td>
</tr>
<tr>
<td>50</td>
<td>90.6 ± 0.7</td>
<td>75.4 ± 13.1</td>
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</tbody>
</table>

INS-1 cells were cultured without (control) or with 25 μmol/l MnTMPyP and exposed to 20, 25 or 50 μmol/l of menadione for 6 hours. Cytotoxicity was calculated and results were given as mean ± sem (n = 2). Percentages of protection were also shown. Cytotoxicity levels were compared by analysis of variance and Scheffe’s test between the 2 groups (control and MnTMPyP; *P = 0.03).
resulting in protection values of 8.5 and 16.8% respectively. Using the fluorescent probes, we noticed that the production of ROS increased proportionally with the menadione concentration but we did not observe any change when MnTMPyP was added. Elsewhere, with the Live/Dead® technique, we observed that the percentage of live cells diminished when the cells were exposed to menadione and we did not notice any improvement in the MnTMPyP group (data not shown).

3.3. Effect of MnTMPyP addition on human islet viability

Experiments were done on 4 islet preparations. The viability of human islets was tested by WST-1 assay 24 h after the exposure to a cytotoxic challenge.

XO/HX stress was performed on 2 batches of islets. Exposure of islets to 10 or 20 mU/ml XO resulted in a slight toxicity with values around 5% of the non-stressed islets value. Addition of 25 μmol/l MnTMPyP did not improve the viability of islets (data not shown).

Concerning menadione challenge (20 to 100 μmol/l), results of WST-1 assay performed on 2 islet batches showed relatively high cytotoxic effects with values varying from 17 to 87% compared to non-stressed islets. In a first experiment, the cytotoxicity observed in control islets with concentrations of menadione of 10, 20, 40 and 100 μmol/l was 29.3, 81.9, 87.6 and 81.9%, respectively, whereas cytotoxicity in MnTMPyP-treated islets was 53.5, 56.8, 44.4 and 66.3%, respectively. In a second experiment, with concentrations of menadione of 20, 25 and 45 μmol/l, cytotoxicity in the control group was 16.9, 33.8 and 64.8%, whereas cytotoxicity was absent in the MnTMPyP-treated group with the three concentrations.

Table 4 shows the results obtained for 3 different preparations of islets exposed to SIN-1. The values of cytotoxicity progressively increased proportionally to the dose of SIN-1.
Table 4: Effects of MnTMPyP treatment on human islet viability after a SIN-1 stress

<table>
<thead>
<tr>
<th>SIN-1 (μmol/l)</th>
<th>Groups</th>
<th>Percentage of cytotoxicity</th>
<th>Percentage of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MnTMPyP</td>
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</tr>
<tr>
<td>Batch 1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1000</td>
<td>42.7</td>
<td>28.4</td>
<td>90.4</td>
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<tr>
<td>1600</td>
<td>45.9</td>
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<td>2000</td>
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<tr>
<td>Batch 2</td>
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<tr>
<td>600</td>
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<tr>
<td>2000</td>
<td>5.5</td>
<td>3.3</td>
<td>40</td>
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</table>

Human pancreatic islets were cultured without (Control) or with 25 μmol/l MnTMPyP and exposed to 600, 1000, 1200, 1600 or 2000 μmol/l of SIN-1 for 16 hours. Cytotoxicity was calculated and results were given for 3 different islets batches. Percentages of protection were shown.

We can notice that the values changed a lot from one batch to the other. Meanwhile, MnTMPyP addition was able to protect islets in quite an efficient way with protection values ranging from 28 to 100%.

3.4. Effect of MnTMPyP addition on human islet endocrine function

The endocrine function of human islets was tested by static incubations 24 h after the induction of oxidative stress by XO or SIN-1. When XO was used to stress islets, stimulated insulin secretion values measured in the control group and in the MnTMPyP-treated group did not change when concentrations of XO rose from 0 to 40 μmol/ml (data not shown).

For SIN-1 stress, mean results of 3 experiments done on 3 different batches of islets are given in Table 5. Control islets normally responded to a glucose-theophylline challenge (baseline 2.84 ± 0.71, stimulated 21.4 ± 14.5 μU/h/islet). Insulin release was dramatically reduced after a SIN-1 stress. Insulin secretion during a glucose-theophylline stimulation decreased by 34.1, 53.3 and 81.3% when control islets were exposed to 600, 1200 or 2000 μmol/l of SIN-1, respectively. MnTMPyP-treated islets only showed 23.1, 11.5 and 0% reduction in insulin secretion level compared to non-stressed MnTMPyP-treated islets. When islets were not exposed to SIN-1, stimulated insulin secretion values in the MnTMPyP-treated islets group were much lower than in the control group (21.4 ± 14.5 versus 7.8 ± 5.2 μU/h/islet); however, MnTMPyP treated-islets retained a better stimulated insulin secretion value than control islets when a 2000 μmol/l dose of SIN-1 was used to stress them.

4. Discussion

Many factors contribute to islet loss and insufficient β-cell function after transplantation but islet function is already undermined by stressful events before transplantation. The non-physiological ambient conditions present during organ procurement and isolation require abrupt metabolic adaptation which may result in functional impairment and eventually cell death. Oxidative stress plays a major role in triggering the death of islets. Several reports explained the rationale of increasing antioxidant enzymes expression in beta cells to enhance their resistance to a cytotoxic challenge [1].

Here, we demonstrate that in vitro treatment by MnTMPyP globally reduced the levels of ROS produced by INS-1 cells and provided a significant protection towards two of the three cytotoxic challenges used (XO/HX and SIN-1), data obtained with menadione needing further investigations. For pancreatic islets, MnTMPyP addition showed more questionable results with values varying a lot from one islet batch to the other reflecting probably inter individual differences in their antioxidant status or due to deleterious effects of isolation procedure. Noteworthy, most studies published so far on the cytotoxicity of human islets have used rodent islets, and very few studies have used human pancreatic islets.

One interesting observation is that under standard culture conditions, INS-1 cells yet produced ROS suggesting that they will be extremely sensitive to additional cytotoxic oxidative stress. Elevated ROS levels affect the function and survival of β cells through direct oxidation of cellular macromolecules and activation of cellular stress-sensitive signalling pathways [12].

The reduction in ROS production measured with the CM-H2DCFDA and DHE probes during a XO/HX stress indicates that MnTMPyP seemed to be efficient in protecting the cells against all the deleterious species generated by this stress. This reduction in ROS production is coherent with the results obtained by either WST-1 assay or Live/Dead method. These findings demonstrate that MnTMPyP conferred a significant protection (above 80%) during this stress.

When SIN-1 was used to stress the cells a mild reduction in the ROS levels is noticed in MnTMPyP-treated cells using the CM-H2DCFDA probe. This would mean that, all types of ROS able to oxidize CM-H2DCFDA can not be completely scavenged by MnTMPyP. In addition, we were not able to detect any generation of ROS using the DHE probe despite its ability to detect intracellular O2 production. In our study, the absence of DHE oxidation would mean that all the superoxide produced by SIN-1 is eliminated: superoxide would either completely dismutate or would react with NO. If the first reaction takes place, NO not involved in the formation of peroxynitrite with superoxide could thereby potentiate the toxicity of H2O2 [13] despite the catalase activity of...
MnTMPyP. In the other case, ONOO\textsuperscript{−} could be the species responsible of CM-H\textsubscript{2}DCFDA oxidation without MnTMPyP scavenging. In this case, glutathione peroxidase which possesses a high affinity for ONOO\textsuperscript{−} and therefore has a greater efficacy against agents that simultaneously generate O\textsubscript{2}– and NO (like SIN-1) would be more appropriate [14]. But, MnTMPyP can also scavenge NO in the presence of glutathione [15]. So, future works will be needed to evaluate the effects of glutathione addition to MnTMPyP or to test combination with ebselen, a GPx mimic, in order to obtain a better protection [16].

The addition of MnTMPyP resulted in mild protection values from 8.5 to 16.8% when high concentrations of menadione were used (25 to 50 μmol/l) whereas no protection could be measured for lower concentration. A study on mouse myogenic cells showed that the extent of oxidative stress may determine the mode of cell death: at 10-40 μmol/l, menadione induced cell apoptosis, at 80 μmol/l, both apoptosis and necrosis and at 160 μmol/l, cell necrosis [17]. A 40 μmol/l menadione dose resulted in the depletion of cellular glutathione and increased in lipid peroxidation. These data could explain our observations of a biphasic effect of menadione on INS-1 cells with cytotoxic effects of MnTMPyP at low menadione concentrations and relative protection at higher concentrations.

The data obtained on human islets after a HX/XO challenge showed that MnTMPyP did not improve viability whereas results concerning endocrine function varied a lot depending on the islet batch and should be confirmed by additional experiments. SIN-1 toxicity can be attenuated by MnTMPyP treatment.

According to data showing that SOD potentiates SIN-1 toxicity in neuronal cells [10] we could fear that MnTMPyP was unable to preserve INS-1 cells viability against a SIN-1 challenge. It has been shown that the effect of SOD on the toxicity of SIN-1 depends on the identity of the agent responsible for toxicity: if ONOO\textsuperscript{−} and/or O\textsubscript{2}– are the cause of toxicity, SOD will be protective since O\textsubscript{2}– dismutation will prevent ONOO\textsuperscript{−} formation. However, if either NO or the product of O\textsubscript{2}– dismutation (H\textsubscript{2}O\textsubscript{2}) are the toxic agents, SOD may potentiate toxicity [10]. In fact, albeit MnTMPyP appears to be more efficient in XO/HX challenge than in SIN-1 stress as shown by the measure of ROS production, these results are quite good since a protection around 70% against SIN-1 toxicity is obtained. But, WST-1 assay did not give coherent data with the Live/Dead\textsuperscript{®} method in the case of SIN-1 or menadione. Using this latter technique, addition of MnTMPyP did not potentiate SIN-1 toxicity but did not either improve cell viability. In a recent work, Barbu et al. [18] called for caution when using tetrazolium salt-based methods to assess viability. They showed a lack of correlation between MTT or XTT assays and a method using a propidium iodide staining and flow cytometry analysis in cytokine-induced beta cell death. It seems that MTT or XTT failed to reflect correctly beta cell death. An explanation could be that NO interacts with tetrazolium salts directly. This finding points out the need of complementary technique as Live/Dead\textsuperscript{®} method or others.

Discrepancies in the viability measured by colorimetric assay and cytometry implies that assessing the islet endocrine function represents a better way to validate their resistance to cytotoxic insults. Our study highlights the need of complementary methods to assess islet viability when SOD mimics or others antioxidants can interfere in the result. The measure of ROS production with the CM-H\textsubscript{2}DCFDA and DHE could be useful for assessing the overall oxidative stress. Indeed, it is not appropriate to think of these probes as detecting a specific species but rather as detectors of a broad range of oxidizing reactions. The precise ROS that mediate the oxidation can be therefore difficult to ascertain. The measure of CM-H\textsubscript{2}DCFDA fluorescence could also be a rapid and sensitive methodology in order to follow NO generation in β cells [19].

The isolation procedure subjected the islets to severe adverse conditions that impair survival and contribute to graft failure. Blocking oxidative stress can be beneficial in reducing islet vulnerability and can potentially have a significant impact on transplantation outcome. Recently the delivery of the native MnSOD to mouse islets by gene therapy approaches improved islet cell survival with a prolongation of graft function of about 50% longer than control [20]. A synthetic salen-manganese compound, EUK-8, can completely inhibit disease progression in NOD mice with established autoimmunity and can prolong islet allograft survival in diabetic NOD mice [21]. The addition of the SOD mimics AEOL 10113 and AEOL 10150 to the culture medium of islets after isolation significantly reduced the cell loss in the following days. In addition, exposing pancreatic tissue to SOD-mimics already during the digestion process, preserve a higher islet cell mass without impairment of islet functional performance. Moreover, it appears to enhance islet graft capacity in vivo [22]. Piganelli et al. report that a metalloporphyrin based antioxidant can prevent or delay the onset of autoimmune diabetes in mice [23].

Our findings are consistent with all these data and demonstrate that despite the low toxicity of MnTMPyP measured by WST-1 assay in non-stressed conditions, its addition results in a clear benefit in viability and function. MnTMPyP allows higher protection levels than adenoviral-based techniques without the drawbacks associated with adenoviruses [2,3]. The dosage of MnTMPyP could be more easily and accurately achieved than with an enzyme, and MnTMPyP could be synergistically combined with other synthetic substances such as epselen, a glutathione peroxidase mimic. Thus, the use of MnTMPyP would be easy in clinical settings. Note-worthy, several clinical trials have been recently reported using synthetic antioxidant compounds, especially in ischemic neurological diseases. Nevertheless, published works using other SOD mimics suggest that additional researches are necessary to determine which one will be the best candidate. Indeed, the metalloporphyrins have proven to be very effective compounds in a wide range of oxidative model, but their potency and efficacy can be quite variable. SOD mimics such as MnTMPyP can be attractive therapeutic agents in disease states involving the overproduction of...
ROS and represent a promising approach to improve viability of transplanted islets.

Acknowledgements

We are grateful to O. Vermeulen and L. Ydoux for technical assistance and M. Bayle for human insulin assay. This work was supported by grants from AGIRaDom and Direction de la Recherche Clinique.

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