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SR-135, a Peroxynitrite Decomposing Catalyst, Enhances β -cell Function and Survival in B6D2F1 Mice Fed a High Fat Diet

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Abstract

Peroxynitrite has been implicated in β -cell dysfunction and insulin resistance in obesity. Chemical catalysts that destroy peroxynitrite, therefore, may have therapeutic value for treating type 2 diabetes. To this end, we have recently demonstrated that Mn(III) bis(hydroxyphenyl)-dipyrromethene complexes, SR-135 and its analogues, can effectively catalyze the decomposition of peroxynitrite *in vitro* and *in vivo* through a 2-electron mechanism (Rausaria et al. 2011). To study the effects of SR-135 on glucose homeostasis in obesity, B6D2F1 mice were fed with a high fat-diet (HFD) for 12 weeks and treated with vehicle, SR-135 (5 mg/kg), or a control drug SRB for 2 weeks. SR-135 significantly reduced fasting blood glucose and insulin levels, and enhanced glucose tolerance as compared to HFD control, vehicle or SRB. SR-135 also enhanced glucose-stimulated insulin secretion based on *ex vivo* studies. Moreover, SR-135 increased insulin content, restored islet architecture, decreased islet size, and reduced tyrosine nitration and apoptosis. These results suggest that a peroxynitrite decomposing catalyst enhances β -cell function and survival under nutrient overload.

Keywords

diabetes; peroxynitrite; pancreatic β -cell; insulin; nitrotyrosine; apoptosis

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Introduction

Recent evidence indicates that obesity is associated with a chronic low-grade inflammation (1–3). The primary causes of obesity-induced inflammatory responses are endoplasmic reticulum (ER) stress and oxidative stress (1, 4, 5). ER stress caused by chronic stimuli for protein and lipid synthesis in expanding adipocytes leads to the activation of inflammatory signaling pathways (6, 7). Reactive oxygen species (ROS) generation is elevated in obesity due to sustained high levels of mitochondrial metabolism of excess nutrients and elevated expression and activity of NADPH oxidases in adipocytes and macrophages accumulated within various tissues. (1, 5, 8).

Generation of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and other constitutive isoforms and production of ROS concomitant with reduced antioxidant enzymes (4, 9, 10) under the setting of chronic low grade inflammation in obesity, promotes formation of peroxynitrite ($^{\cdot-}\text{OONO}$). Peroxynitrite, and the secondary free radical species formed upon its uncatalyzed decomposition in vivo, are powerful oxidizing and nitrating species that cause DNA damage, lipid peroxidation, and post-translational modification of proteins (11–13). Nitro-oxidative stress, driven by peroxynitrite, has been implicated in the pathophysiology of a variety of diseases including diabetes and its complications (14–16). Increased peroxynitrite formation has been observed in obese rodents (17, 18) and diabetic patients (19, 20). Peroxynitrite-mediated nitration of certain tyrosine residues in key insulin signaling proteins has been shown to interfere with insulin signaling (21–23). The role of peroxynitrite in the destruction of β -cells in autoimmune type 1 diabetes has also been reported (24, 25). Taken together, these studies suggest that peroxynitrite may play a pivotal role in insulin resistance and development of β -cell defects associated with obesity-induced type 2 diabetes.

Evidence from animal studies indicates that peroxynitrite is a viable therapeutic target for type 2 diabetes and diabetic complications. The peroxynitrite decomposing catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) (26) improved insulin sensitivity by restoring insulin signaling and insulin-stimulated glucose uptake in high fat diet (HFD)-fed mice (17, 23). Peroxynitrite decomposition with 5,10,15,20-tetrakis (N-methyl-4'-pyridyl)porphyrinato iron(III) (FeTMPyP) showed renoprotective effects (27) and improved stroke outcome (28) in diabetic rats. These highly charged metalloporphyrins were primarily designed as research tools with high water solubility for determining the mechanisms of catalysis. As such, they are not orally active. Considering the potential therapeutic value of these compounds for diabetes and other diseases driven by the overproduction of peroxynitrite, orally active catalysts with selectivity for the decomposition of peroxynitrite over superoxide were developed (29). These new compounds provide two significant advantages over previously reported highly charged metalloporphyrins; 1) more drug-like properties that enable oral treatment, 2) selectivity for peroxynitrite over superoxide allows normal cellular signaling by superoxide to proceed. In this paper, we show that SR-135 (log P = 4.05), one of the new class of Mn(III)-bis(hydroxyphenyl)-dipyrromethene peroxynitrite decomposition catalysts, was effective in lowering fasting blood glucose and insulin levels and enhancing glucose

tolerance. Moreover, SR-135 enhanced β -cell function, restored islet architecture, and reduced apoptosis under nutrient overload.

Material and methods

Animals

Male B6D2F1 mice (the F1 hybrids of C57BL/6 and DBA/2, 4–6 weeks) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were maintained in our animal facility under controlled conditions (temperature 68–73°F and 12 h light-dark cycle). Male B62DF1 mice have been shown to develop severe diabetes characterized by hyperglycemia, glucosuria, and elevated hemoglobin A1C levels, with progressive structural and functional defect in islets after 3 to 4 months of high fat diet feeding (30). After a one week acclimation period, the mice (4 per cage) were fed for 12 weeks with a commercial lean chow diet (El-Mel, St. Louis, MO) or a high fat diet (45% kcal fat diet, Harlan laboratories, Madison, WI) and water *ad libitum*. After a 12 week diet period, three groups (n=12 per group) of the mice fed the HFD were treated intra-peritoneally with 5 mg/kg SR-135, 5 mg/kg SRB, or vehicle (10% EtOH, 10% DMSO, 30% PEG-400, 48% 0.2% methylcellulose, 2% Tween 80) every other day for two weeks. The dosage and frequency of drug administration were determined based on preliminary studies that assessed toxicity and clearance of the compounds from the peritoneal cavity (unpublished observation). The mice were continuously fed the HFD and bodyweight and food intake were determined every other day during the 2 weeks of drug treatment. Intraperitoneal glucose tolerance test (IPGTT) was performed before and after drug treatment. After the 2 weeks of drug treatment, the mice were fasted for 12 h prior to sacrifice, followed by blood collection through retro-orbital bleeding and isolation of the pancreas. Whole pancreases were snap frozen in liquid nitrogen and stored at –70°C for immunohistochemistry studies (n=8) or immediately processed to isolate islets (n=4). All animal maintenance and treatment protocols complied with the Guide for Care and Use of Laboratory Animals as adopted by the National Institute of Health and approved by the SIUE Institutional Animal Care and Use Committee (IACUC).

Chemicals

SR-135 and its control compound SRB were synthesized as previously reported (31). Collagenase type XI, Hanks' balanced salt solution, and monoclonal mouse anti-glucagon antibody were obtained from Sigma (St. Louis, MO). Total cholesterol, HDL-cholesterol, and triglyceride liquid reagents and their respective standards, fetal bovine serum, and penicillin-streptomycin were from Fisher Scientific (Pittsburgh, PA) and CMRL-1066 was from Invitrogen (Grand Island, NY). Insulin rabbit mAb and cleaved caspase 3 rabbit mAb were obtained from Cell Signaling (Danvers, MA). The secondary antibodies, DyLight 649-conjugated donkey anti-rabbit or anti-mouse IgG and Alexa 488-conjugated donkey anti-rabbit or anti-mouse antibody were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and 4'-6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen (Grand Island, NY). Immunocruz rabbit ABC staining system was from Santa Cruz Biotechnology (Dallas, Texas) and 3-nitrotyrosine mouse mAb was from Abcam (Cambridge, MA). Insulin mouse mAb and rat insulin RIA kit was from Millipore (St. Louis, MO). All other chemicals were from commercially available sources.

Determination of peroxynitrite decomposition activity of SR-135 and SRB

4-acetylphenylboronic acid (9.5×10^{-7} moles in 24.0 μ L DMSO) was dispensed into a small vial equipped with a magnetic stir bar. 2 mL of 100 mM phosphate buffer (pH = 7.2) containing 0.7% sodium dodecyl sulphate and 100 μ M diethylene triamine pentaacetic acid (DTPA) was added, followed by 9.5×10^{-7} moles of SR-135 or SRB. To this rapidly stirred mixture 9.5×10^{-7} moles peroxynitrite was added by rapid injection. The mixture was stirred for one minute and analyzed by liquid chromatography-mass spectrometry (LCMS) as described previously (31). Reactions were run in multiplets ($n = 5$) and compared to controls (also $n=5$) which contained everything except the catalyst. The peak areas for phenol oxidation products were compared for catalyst vs control runs to determine percent inhibition.

Prevention of Nitration Assay

The ability of SR135 and SRB to prevent the nitration of Leu-enkephalin (LENK, Tyr-Gly-GlyPhe-Leu) was determined. Solutions of 0 or 1 μ mol of the LENK in 3 mL of 100 mM phosphate buffer containing 0.7% SDS and 100 μ M DTPA were prepared. To these mixtures 1, 0.2, or 0.1 μ mol of the compound (SRB or SR135) was added. Freshly prepared peroxynitrite (1 μ mol) was then rapidly injected into these stirred solutions. The reactions were aged for 1 minute and the amounts of unreacted LENK and 3-NT-LENK were quantified by LCMS analysis.

SR-135 and SRB uptake into isolated rat islets

Isolated rat islets (30) were incubated for 2 days in complete CMRL-1066 containing 10 mM glucose in the presence and absence of 10 μ M SR-135 or 10 μ M SRB. Color and fluorescent images of islets were captured using a 20X objective in a Leica DMI inverted fluorescent microscope.

Energy intake

Food consumed by 4 mice/cage were weighed and recorded every other day. Gross energy for lean chow diet (protein; 29.8%, carbohydrate; 56.7%, Fat; 13.4% by weight) and HFD (protein; 17.3%, carbohydrate; 47.6%, Fat; 23.2% by weight) are 4.09 kcal/g and 4.7 kcal/g, respectively. Food intake was converted to energy intake/g body weight by multiplying food intake (g) with respective energy density (kcal/g) and conversion factor (4.18 kJ/kcal) and then divided by average body weight.

Fasting blood glucose levels

Mice were fasted for 5h and blood samples were collected from the tip of the tail. Blood glucose levels were determined using a Contour glucose meter.

Intra-peritoneal glucose tolerance tests

Mice were fasted for 5 h and administered 1g/kg glucose by intraperitoneal injection. Blood samples were collected from the tip of the tail at 0, 30, 60, 90, and 120 min and blood glucose levels were determined using a Contour glucose meter. Area under the blood glucose versus time curve was calculated using GraphPad Prism 6.0.

Blood collection

Terminal blood collection (~500 microliter) was performed by retro-orbital bleeding. Blood was collected into a microfuge tube containing 1.5 mg EDTA. The blood samples were centrifuged in a microfuge for 20 min at 6,000 rpm. The plasma was separated and frozen at -70°C until use.

Determination of plasma TAG, total-cholesterol, and HDL levels

Blood plasma (50 μl) was mixed with HemogloBind (50 μl) to remove hemoglobin from lysed red blood cells. Standard curves and triplicate of unknown samples (2 μl) were mixed with an appropriate liquid reagent (200 μl) in a 96 well plate. The samples were incubated at 37°C for 5 min, followed by absorbance reading at 500 nm using a Multiskan plate reader. For HDL cholesterol determination, the HDL fraction was first isolated by precipitating all beta-lipoproteins (LDL and VLDL) using 20% w/v polyethylene glycol, according to the manufacturer's instructions. The plasma concentrations of TAG, total-cholesterol, and HDL were determined using GraphPad Prism 6 statistics software.

Insulin secretion

Freshly isolated islets (5 islets) per treatment group ($n=7-10$) were preincubated in complete CMRL-1066 containing 5.6 mM glucose for 30 min. Culture medium was replaced with CMRL-1066 containing either 5.6mM or 20 mM glucose and further incubated for 1h. Supernatants were assayed for insulin content by radioimmunoassay following the manufacturer's instructions. A 1:10 dilution of plasma was used in the assay for plasma insulin levels.

Frozen sectioning of whole pancreas and immunohistochemistry

Freshly isolated pancreases were placed in microfuge tubes, snap-frozen in liquid nitrogen, and stored at -70°C until use. Every 10th section of 10 μm thickness was collected and fixed in 4% paraformaldehyde and 1% Triton-X 100 in PBS. Pancreas sections were then washed in PBS to remove the residual paraformaldehyde, blocked in 2% BSA in PBS, and treated with appropriate primary and secondary antibodies. DAPI was used for nuclear staining. Fluorescent images were obtained using a 40X objective in an Olympus FluoView confocal microscope.

Quantitation of β -cell area

Pancreas sections were washed, blocked for 20 min in 2% BSA in PBS, and incubated for 30 min each with primary and secondary antibodies, insulin rabbit mAb and biotinylated secondary antibody, respectively. Following the manufacturer's instructions, tissues were stained with AB enzyme reagent, followed by peroxidase substrate containing DAB chromagen. Tissues were counterstained with Gill's formulation #2 hematoxylin and mounted on microscope slides. Images of pancreas sections were captured using a 2.5X objective in a Leica DMI inverted fluorescent microscope. The ratio of β -cell area to exocrine tissue area per section was calculated using the ImageJ image processing and analysis program.

Quantitation of tyrosine nitration

Pancreas sections were washed, blocked, and immunostained with 3-nitrotyrosine mouse mAb and Alexa 488-conjugated donkey anti-mouse antibody as primary and secondary antibodies, respectively, and DAPI for nuclear staining. For the co-localization study, pancreas sections from HFD-fed mice were incubated with 3-nitrotyrosine mouse mAb and insulin rabbit mAb as primary antibodies, followed by Alexa 488-conjugated donkey anti-mouse antibody and Dylight 649-conjugated donkey anti-rabbit antibody as secondary antibodies. Fluorescent images were obtained using a 40X objective in an Olympus FluoView confocal microscope. Integrated fluorescent intensity over number of nuclei for each islet was determined using ImageJ image processing and analysis program. The levels of nitrotyrosine under different conditions were normalized to that of the control condition.

Determination of apoptotic cells

Pancreas sections were washed, blocked, and immunostained with cleaved caspase 3 rabbit mAb and Alexa 488-conjugated donkey anti-rabbit IgG as primary and secondary antibodies, respectively. DAPI was used for nuclear staining. For the co-localization study, pancreas sections from HFD-fed mice were incubated with cleaved caspase 3 rabbit mAb and insulin mouse mAb as primary antibodies, followed by Dylight 488-conjugated donkey anti-rabbit antibody and Dylight 649-conjugated donkey anti-mouse antibody as secondary antibodies. Fluorescent images were obtained using a 40X objective in an Olympus FluoView confocal microscope. Percent of caspase 3 positive cells per islet was determined using ImageJ image processing and analysis program.

Statistics

Results are expressed as mean $\pm$ SEM. Differences between means were evaluated using ANOVA or the Student *t* test as appropriate. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Figure 1 shows the structures of SR-135 and SRB. SR-135 and its analogues have been strategically designed and synthesized to decompose peroxynitrite in a catalytic fashion through a two-electron cycle and exhibit more drug-like properties with a highly lipophilic structure (LogP ~ 4.0). A control compound for SR-135, SRB, was synthesized by replacing manganese with boron to abolish the catalytic activity but retaining the same organic scaffold structure. Lack of the catalytic activity of SRB was confirmed using boronate oxidation assay (Table 1). SR-135 showed $31.5 \pm 2.50\%$ inhibition of peroxynitrite-mediated boronate oxidation, which is translated to the catalytic activity with the second-order rate constant, $7.3 \pm 0.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. SRB, however, had no inhibitory action, confirming that SRB has no peroxynitrite decomposing catalytic activity. Next, the ability of SR-135 and SRB to prevent peroxynitrite-mediated nitration of LENK was studied. The 2-electron catalyst SR-135 afforded 100%, 98% and 52% inhibition of nitration with 1, 0.2 and 0.1 equivalents catalyst, respectively (Table 2). Thus, catalytic activity is clearly demonstrated at the lower concentrations of catalyst, with sub-stoichiometric concentration of SR-135 (0.2 equivalent) affording high levels of inhibition (98%). The control analogue,

SRB, showed 14% inhibition using 1 equivalent of the compound. This modest inhibition is most likely due to the stoichiometric scavenging of secondary decomposition products of peroxynitrite formed under the conditions of the assay (i.e. hydroxyl radical and nitrogen dioxide radical) (32). No inhibition was observed at lower concentrations of SRB indicating the compound is not a catalyst. The modest effects of SRB in reducing nitration and apoptosis described below may be explained by this mild stoichiometric scavenging activity.

Membrane permeability of SR-135 and SRB was assessed by incubating isolated rat islets with 10 μ M SR-135 or 10 μ M SRB for 2 days. SR-135 absorbs visible light at 654 nm with quenched fluorescence due to its paramagnetism. SRB, on the other hand, is highly fluorescent with excitation and emission wavelengths at $\sim$ 620 nm and $\sim$ 650 nm, respectively. The upper panels of Figure 2 show color images of islets. Islets treated with SR-135 (panel b) show darker colors due to accumulation of SR-135 molecules inside islet cells as compared to control (panel a) and SRB-treated islets (panel c). The lower panels of Figure 2 show fluorescent images of islets. Islets treated with SRB (panel f) show strong fluorescence signals. Taken together, these results provide evidence that both SR-135 and SRB permeate the lipid bilayer and are readily taken up by islet cells.

Mice on a HFD gained weight at a faster rate than those on a lean diet (Figure 3A). After 12 weeks, average weights of lean diet- and HFD-fed mice were 35.1 ± 1.23 g and 43.0 ± 0.66 g, respectively (1st and 2nd white bars of Figure 3A). During the 2 weeks of the drug treatment, the bodyweights of the mice treated with vehicle, SRB, and SR-135 were slightly decreased, but not significantly. SR-135 had no significant effects on food intake (Figure 3B). Mice fed a lean diet consumed significantly higher calories than those fed a high fat diet.

Fasting blood glucose levels were determined before and after the drug treatment (Figure 3C). SR-135 significantly reduced fasting blood glucose levels (204.9 ± 8.3 mg/dl vs. 154.3 ± 6.8 mg/dl) of HFD-fed mice, whereas SRB had no effect, suggesting that peroxynitrite decomposing activity of SR-135 was responsible for the reduction. Intra-peritoneal glucose tolerance tests were performed before and after the drug treatment to study the effects of SR-135 on whole-body glucose homeostasis. All HFD-fed mice exhibited impaired glucose tolerance before drug treatment was initiated (Figures 3D and 3E). After the drug treatment phase, HFD-fed mice showed impaired glucose tolerance compared to lean diet-fed mice (AUC $32,833 \pm 2961$ vs. $20,170 \pm 1033$) as shown in Figures 3F and 3G. Vehicle- and SRB-treated HFD-mice showed intermediate values for AUC between those of HFD- and lean diet-fed mice, suggesting that vehicle itself had some effect. DMSO, a minor (10%) component of the vehicle is postulated to have some antioxidant effects, which might have contributed to the vehicle effect. Vehicle, however, did not show significant effects on β -cell function or survival, suggesting that its effects are probably due to its actions on peripheral tissues. SR-135-treated HFD- mice exhibited significantly enhanced glucose tolerance similar to lean diet-fed mice.

After the drug treatment, the mice were fasted for 12 h prior to asphyxiation by CO₂, followed by terminal blood collection. The relatively large quantity of blood ($\sim$ 500–700 μ l) enabled us to determine the fasting plasma levels of insulin (Figure 3H) and lipids (Table 3).

The plasma insulin levels of the HFD-fed mice were significantly higher than those of lean diet-fed mice (1.60 ± 0.09 ng/ml vs. 0.28 ± 0.06 ng/ml), supporting the IPGTT results and indicating that HFD-fed mice display elevated insulin resistance. The mice treated with SR-135 showed markedly reduced plasma insulin levels, statistically significant from vehicle- or SRB-treated group. The plasma levels of TAG, cholesterol, and HDL were also determined (Table 3). All three plasma lipid panels were significantly higher in mice fed HFD than lean diet. SR-135-treated HFD-fed mice exhibited significantly lower levels of total cholesterol level compared to HFD-, vehicle-, and SRB-treated HFD-fed mice.

To assess the effect of SR-135 on β -cell function, pancreatic islets were isolated from the mice under different conditions and glucose-stimulated insulin secretion was determined by radioimmunoassay. Islets isolated from HFD-, vehicle-, and SRB-treated HFD-mice showed markedly lower glucose-stimulated insulin secretion (Figure 4, 2nd, 3rd, 4th black bars) than those from lean diet-fed mice (Figure 4, 1st black bar). This suggests that β -cell defects developed concurrently with insulin resistance in the periphery as shown by glucose intolerance (Figure 3D and 3E) and elevated plasma insulin levels (Figure 3H). Islets isolated from SR-135-treated HFD-mice, on the other hand, showed significantly higher glucose-stimulated insulin secretion (Figure 4, 5th black bar) than the HFD control groups, suggesting that SR-135 enhanced β -cell function under nutrient overload. The levels of insulin secretion by SR-135-treated HFD-mice, however, were significantly lower than those from lean diet-fed mice. Basal insulin secretion was similar under all conditions.

The effects of SR-135 on the architecture of islets and insulin content are shown in Figure 5. Pancreas sections were immunostained for insulin and glucagon to localize β - and α -cells. Normal healthy rodent islets contain β -cells in the center and α -cells in the periphery of the islet. Excess nutrients alter the islet morphology *in vitro* (33) and *in vivo* (34), causing α -cells to migrate towards the center of the islet. Figure 5A demonstrates that islets embedded in the pancreases isolated from lean diet-fed mice showed localization of β -cells in the center (panels a) and α -cells in the periphery (panels c) as anticipated. Islets from HFD-, vehicle, and SRB-treated HFD-mice show α -cells migrated to the center of the islets (panels g, k, o). In the islet from SR-135-treated HFD-mice, α -cells are localized in the periphery (panel s) similar to those found in the lean diet-fed control islets (panel c). Furthermore, insulin content was significantly diminished in islets from HFD-, vehicle, and SRB-treated HFD-mice (panels c, i, m) as compared to that from lean diet-fed control islet (panel a). Insulin content in islet from SR-135-treated HFD-mice (panel q) was similar to that from lean diet-fed control islet (panel a). Figure 5B shows the quantitation of insulin content in islets under various conditions.

Rodent β -cells increase in size and number in response to an increased metabolic demand (33, 35). To assess the size of islets, pancreas sections were stained with insulin rabbit mAb and avidin biotinylated chromogen reagent as described in the Methods section. Islets of different size and shape are observed in a pancreas. To normalize these differences across different conditions, every 10th of the 10 μ m pancreas sections was collected and more than 100 islet areas per condition were determined. Figure 6A shows representative islets embedded in exocrine tissues under different conditions. The islets isolated from HFD-, vehicle-, and SRB-treated HFD-mice (panels b, c, and d, respectively) are significantly

larger than that from lean-diet-fed mice (panel a). SR-135 treatment (panel e) significantly reduced the size of islets as compared to those from the HFD-fed control mice. Figure 6B shows the quantitation of % β -cell area under various conditions.

We hypothesize that peroxynitrite-induced oxidative and nitrative stress contributes to insulin resistance and β -cell dysfunction under conditions of nutrient overload. To determine the effect of SR-135 on peroxynitrite activity, nitration of tyrosine residues in islets was studied using 3-nitrotyrosine mouse mAb. Figure 7A shows representative islets embedded in exocrine tissues under different conditions. As anticipated, the levels of 3-nitrotyrosine were significantly higher in islets from HFD-fed or vehicle-treated mice (panels b and c) as compared to those from lean diet-fed mice (panel a). Unexpectedly, the levels of 3-nitrotyrosine were significantly reduced in islets from SRB-treated mice (panel d). The stoichiometric scavenger action of SRB is postulated to reduce the levels of peroxynitrite, nitrogen dioxide radical and hydroxyl radical (32). SR-135 (panel e), as anticipated, reduced the levels of 3-nitrotyrosine to the baseline levels of the lean diet-fed control (as compared to the intermediate reduction afforded by SRB; panel d and Figure 7B). Co-staining of insulin and 3-nitrotyrosine (Figure 7A, panel f) of an islet embedded in a pancreas section from HFD-fed mice suggests that peroxynitrite is generated within β -cells shown by yellow punctate staining (inset). Figure 7B shows the quantitation of nitrotyrosine under various experimental conditions.

The effects of SR-135 on apoptosis was studied using cleaved caspase 3 rabbit mAb. The percent of caspase 3 positive cells (shown in green) per islet was significantly increased in islets from HFD-fed or vehicle-treated mice (Figure 8A, panels b and c) as compared to those from lean diet-fed mice (Figure 8A, panel a). The control drug SRB afforded a significant but modest reduction in the number of apoptotic cells (Figure 8B, 4th bar), consistent with its actions as a stoichiometric scavenger as noted above. In comparison to SRB, SR-135 showed a markedly greater reduction in the number of apoptotic cells, to the level of lean diet-fed control. To study whether caspase 3 positive cells are β -cells, co-staining of insulin and cleaved caspase 3 were performed. Only a small fraction of cleaved caspase 3 staining overlapped with insulin staining (Figure 8A, panel f and inset). At the stage where caspase 3 is active, insulin may have already been degraded.

DISCUSSION

The major finding of this study is that peroxynitrite decomposing catalysts may have therapeutic value in enhancing β -cell function and survival under nutrient overload. SR-135, strategically designed to be a more drug-like molecule compared to other existing catalysts, was highly effective in enhancing glucose homeostasis in B62DF1 mice fed a HFD. SR-135 enhanced glucose-stimulated insulin secretion and insulin content, reversed the distortion of islet architecture, and reduced the percent apoptotic cells in islets. SR-135 also decreased islet size in HFD-fed mice comparable to those of lean diet-fed mice. As anticipated, SR-135 significantly reduced the levels of tyrosine nitration in islets.

Accumulating evidence suggests that peroxynitrite plays a pivotal role in the pathogenesis of obesity-induced diabetes (17, 19, 23, 36) and diabetic complications (27, 37, 38). Short-term

treatment (5 day) with FeTPPS decreased fasting blood glucose levels and improved glucose tolerance in HFD-induced insulin resistant C57/BL6 mice (17). The enhancement of glucose homeostasis by FeTPPS was due in part to restoring insulin signaling in skeletal muscle. FeTPPS abolished nitrotyrosine accumulation, increased glucose uptake, and restored insulin-induced Akt-phosphorylation in skeletal muscle of HFD-fed mice (17). Others (22, 23, 36, 39) have also shown that peroxynitrite impairs proteins involved in early steps of insulin signaling including IR β , IRS-1, and Akt. Our study supports these findings and demonstrates for the first time (to our knowledge) that a peroxynitrite decomposing catalyst restores β -cell function and islet morphology in obesity-induced insulin resistant mice.

Restoration of glucose-stimulated insulin secretion and content by SR-135 may be due to both direct and indirect effects of SR-135 on β -cells. Enhancement of insulin sensitivity of peripheral tissues such as skeletal muscle, liver, and adipocytes by SR-135 may have reduced metabolic demands on islets, thus restoring the islet architecture and β -cell function (17, 22, 23). Reduction of nitrotyrosine levels in islets (Figure 7A and 7B) suggests that SR-135 may have a direct protective role in preserving β -cell function and survival under nutrient overload. Our *in vitro* studies (unpublished observations) also indicated that SR-135 preserved insulin content and islet architecture in isolated rat and human islets exposed to excess nutrients (25 mM glucose and 500 μ M free fatty acids), suggesting direct effects of SR-135 on β -cells or islets. Specific mechanisms by which SR-135 restores β -cell function under nutrient overload are currently under investigation.

SR-135 is one of a new series of potent and orally active Mn(III) containing peroxynitrite catalysts (31). It decomposes peroxynitrite to nitrite through a two electron mechanism in a catalytic fashion and requires no biological co-reductant (31). In comparison to the polyanionic and polycationic peroxynitrite scavengers FeTPPS and FeTMPyP (log P = -4.54), respectively, SR-135 (log P = 4.05) and its analogs are lipophilic, and therefore more membrane-soluble, allowing for gastrointestinal absorption. The major route of peroxynitrite chemistry in aqueous compartments *in vivo* is through the reaction with carbon dioxide. The rate constant for the reaction of peroxynitrite with CO₂ is in the range of 10⁴ M⁻¹s⁻¹ (40), while the rate constant for the decomposition of peroxynitrite on its own is 0.9 M⁻¹s⁻¹ at 37 °C. We have developed complexes with high logPs to enhance not only gastrointestinal absorption but also their concentration in cells so that they can effectively compete with CO₂ for peroxynitrite. This is one main difference between SR-135 and previously reported polyanionic and polycationic peroxynitrite scavengers. The second order rate constant for the direct reaction of SR-series complexes with peroxynitrite ranges from 7.3 $\times$ 10⁵ M⁻¹s⁻¹ (31) to 2.4 $\times$ 10⁶ M⁻¹s⁻¹. These values are most likely underestimated due to solubility issues of the complexes in the activity assay media. Thus, *in vivo*, at concentrations in the low micromolar range, catalysts like SR-135 can effectively compete with CO₂ for peroxynitrite (41).

In this study, we treated mice with intraperitoneal injections to first study its effects in comparison to other peroxynitrite decomposing catalysts via the same route of administration. We are currently studying the effect of orally-administered SR-110, an analogue of SR-135, designed to be more orally active, in our diabetic mouse model.

SR-110 is being administered in a solvent-free vehicle to avoid the effects of the organic solvent shown in this study.

Tyrosine nitration of proteins involved in the early steps of the insulin signal transduction pathway in skeletal muscle has been shown to impair insulin action, possibly causing insulin resistance in obesity and inflammatory setting (17, 21, 23). Our present study further implicates peroxynitrite-mediated tyrosine nitration in the distortion of islet architecture and β -cell defects under nutrient overload.

Broniowska, et al. reported the interesting finding that β -cells do not produce peroxynitrite in response to cytokine treatment. It was therefore concluded that peroxynitrite does not contribute to cytokine-induced β -cell damage (42). This report seems to contradict results from numerous other studies that provided evidence for the role of peroxynitrite in cytokine-mediated and autoimmune β -cell damage (24, 25, 43–47). Broniowska et al. concluded that peroxynitrite does not contribute to cytokine-mediated β -cell damage based upon; 1) lack of protective effects of the peroxynitrite scavenger phenylalanine boronate in cytokine-mediated INS832/13 cell viability, and 2) their inability to detect peroxynitrite generated by these cells using coumarin-7 boronate under the cytotoxic conditions triggered by cytokines. As a positive control, they showed that these probes effectively quenched and detected extracellular peroxynitrite produced by macrophages. However, the extent to which phenylalanine boronate or coumarin-7 boronate can partition into cells is unknown and it is highly unlikely that phenylalanine boronate will penetrate the cell membrane. It is thus possible that these probes are unable to effectively quench or detect intracellular peroxynitrite. Macrophages, on the other hand, have evolved to generate extracellular peroxynitrite to kill invading organisms underscoring the ability of both probes to readily scavenge and detect the peroxynitrite produced by macrophages. It should not be inferred that β -cells are unable to generate peroxynitrite under all conditions based on this report. Furthermore, the identity of the molecular species, i.e. $\text{NO}\bullet$, peroxynitrite, or H_2O_2 , that is most responsible for β -cell damage is controversial. More studies are needed to resolve the long-standing debates regarding the contribution of peroxynitrite to β -cell damage.

Under the conditions of nutrient overload, metabolic production and escape of superoxide from complex I and complex III of the mitochondrial electron transport chain is predicted to be much higher than superoxide production in β -cells treated with cytokines. Hyperglycemia (15, 48) and a chronic low inflammatory state of the animal due to obesity may increase intracellular levels of nitric oxide in β -cells through the upregulation of iNOS. Thus, high fluxes of superoxide and nitric oxide are anticipated to generate high levels of peroxynitrite in β cells under nutrient overload. The levels of nitrotyrosine, an indirect indicator of peroxynitrite activity and its decomposition products, were elevated in islets from HFD-treated control and vehicle groups (Figure 7A, panels b and c). The membrane permeable SR-135, in contrast to other highly charged and hydrophilic peroxynitrite scavengers, is predicted to partition into β -cells and decompose intracellular peroxynitrite. Figure 2 demonstrates that SR-135 is readily taken up into isolated islets. Current treatments for type 2 diabetes include several classes of hypoglycemic agents and/or insulin. These treatments, at best, delay the progression of the disease but are not effective enough to curb the epidemic of type 2 diabetes. Thus, novel classes of anti-diabetic agents are urgently needed.

Strategies to reduce oxidative and nitrate stress using peroxynitrite decomposing catalysts may provide unique advantages in; 1) targeting multiple organs and tissues, thus, enhancing insulin sensitivity and β -cell function and survival, 2) delaying or reversing the progression of the disease by halting cellular damage caused by oxidative stress associated with obesity.

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The abbreviations used are

HFD	high fat diet
ER	endoplasmic reticulum
ROS	reactive oxygen species
iNOS	inducible nitric oxide synthase
IPGTT	intra-peritoneal glucose tolerance test
mAB	monoclonal antibody
SR-135	Mn(III) complex of 3,5-bis(hydroxyphenyl)-1,2:6,7-bis-butanodipyrromethene
SRB	Boron complex of 3,5-bis(hydroxyphenyl)-1,2:6,7-bis-butano-dipyrromethene
FeTPPS	5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron (III) chloride
FeTMPyP	5,10,15,20-tetrakis (N-methyl-4'-pyridyl) porphyrinato iron (III)
DTPA	diethylene triamine pentaacetic acid
LCMS	liquid chromatography-mass spectrometry
LENK	Tyr-Gly-Gly-Phe-Leu
IRβ	insulin receptor β
IRS-1	insulin receptor substrate-1

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Highlights

- A peroxynitrite decomposing catalyst, SR-135, is readily taken up into β -cells.
- SR-135 treatment enhanced β -cell function and survival under nutrient overload.
- SR-135 treatment enhanced glucose tolerance and reduced plasma insulin levels.

Figure 1.

Structures of SR-135 and SRB. SR-135 has been strategically designed and synthesized to decompose peroxynitrite in a catalytic fashion through a two-electron cycle and contain more drug-like properties with highly lipophilic structure (LogP ~4.0). SRB is a control drug for SR-135. By replacing manganese with boron, the peroxynitrite decomposing catalytic activity has been abolished.

Figure 2. SR-135 and SRB uptake into isolated rat islets. Isolated rat islets (30) were incubated for 2 days in complete CMRL-1066 containing 10 mM glucose in the presence and absence of 10 μ M SR-135 or 10 μ M SRB. Color and fluorescent images of islets were captured using a 20X objective in a Leica DMI inverted fluorescent microscope. 10G; 10 mM glucose, 10G +SR-135; 10 mM glucose+10 μ M SR-135, 10G+SRB; 10 mM glucose+10 μ M SRB

Figure 3.

Mean body weights (A) and fasting blood glucose levels (C) of B6D2F1 mice (n=12) under various conditions before and after drug treatment. (B). Food intake during the drug treatment. Plasma glucose concentrations during the intra-peritoneal glucose tolerance test (1g/kg) and total area under the glucose curve (AUCglc) in mice under various conditions before (D and E) and after (F and G) the drug treatment. Mice were fasted for 5 h prior to the determination of the fasting blood glucose levels and IPGTT. H. Plasma insulin levels after drug treatment. Values are presented as means $\pm$ S.E.M. (n=12).

Figure 4.

SR-135 enhances glucose-stimulated insulin secretion. Five freshly isolated islets for each treatment group (n=7–10) were preincubated in complete CMRL-1066 containing 5.6 mM glucose for 30 min. Culture medium was replaced with CMRL-1066 containing either 5.6mM or 20 mM glucose and further incubated for 1h. Supernatants were assayed for insulin content by radioimmunoassay. Values are presented as means $\pm$ S.E.M.

A.

B.

Figure 5.

SR-135 restores islet architecture and increases insulin content. (A) Frozen pancreas sections (10 μ m) were processed for immunostaining (insulin (red), nuclei (blue), and glucagon (green)). Fluorescent images were obtained using a 40X objective in an Olympus FluoView confocal microscope. The figure shows representative images of islets under various conditions. (B) Integrated fluorescent intensity of insulin over number of nuclei for each islet was determined using ImageJ image processing and analysis program. Insulin content of islets under different conditions was normalized to that of the lean control condition. Data are the means $\pm$ SEM of ~18–20 islets obtained from 8–10 pancreas sections from 3 mice per condition.

Figure 6.

(A) SR-135 reduces islet size under nutrient overload. Frozen pancreas sections (10 μm) were treated with insulin rabbit mAb and biotinylated secondary antibody, followed by staining with AB enzyme reagent and peroxidase substrate containing DAB chromagen. Tissues were counterstained with hematoxylin. The figure shows representative images of islets under various conditions. (B) The bar graph shows the ratio of β-cell area over exocrine tissue determined using the ImageJ image processing and analysis program. Data are the means ± SEM of ~100 islets obtained from 15–20 pancreas sections from 3 mice per condition.

Figure 7.

(A) SR-135 reduces the levels of tyrosine nitration in islets. Frozen pancreas sections (10 μ m) were immunostained with 3-nitrotyrosine mouse mAb and Alexa 488-conjugated donkey anti-mouse antibody as primary and secondary antibodies, respectively, and DAPI for nuclear staining. Fluorescent images were obtained using a 40X objective in a confocal microscope. The figure shows representative images of islets under various conditions. Panel f shows co-staining of insulin (red) and 3-nitrotyrosine (green) of an islet from a HFD-fed mouse. Inset shows a 3x zoomed image of the area in white box. (B) The bar graph shows % control of integrated fluorescent intensity over number of nuclei for each islet determined using the ImageJ image processing and analysis program. Data are the means $\pm$ SEM of ~18–20 islets obtained from 8–10 pancreas sections from 3 mice per condition.

Figure 8.

(A) SR-135 reduces the number of apoptotic cells. Frozen pancreas sections (10 μ m) were immunostained with cleaved caspase 3 rabbit mAb and Alexa 488-conjugated donkey anti-rabbit IgG as primary and secondary antibodies, respectively. DAPI was used for nuclear staining. Fluorescent images were obtained using a 40X objective in a confocal microscope. The figure shows representative images of islets under various conditions. Panel f shows co-staining of insulin (red) and caspase 3 (green) of an islet from a HFD-fed mouse. Inset shows a 3x zoomed image of the area in white box. (B) The bar graph shows percent of caspase 3 positive cells per islet determined using the ImageJ image processing and analysis program. Data are the means $\pm$ SEM of ~18–20 islets obtained from 8–10 pancreas sections from 3 mice per condition.

Table 1

Inhibition of boronate oxidation by SR-135 and SRB. 4-acetylphenylboronic acid (9.5×10^{-7} moles in 24.0 μL DMSO) was mixed with 9.5×10^{-7} moles of SR-135 or SRB as described in the methods section. To this rapidly stirred mixture was added 9.5×10^{-7} moles peroxyxynitrite by rapid injection. The mixture was stirred for one minute and analyzed by LCMS. Reactions were run in multiplets ($n = 5$) and compared to controls (also $n=5$) which contained everything except the catalyst. The peak areas for phenol oxidation products were compared for catalyst vs control runs to determine percent inhibition.

Catalyst	% Inhibition (25 °C)	Second-order rate constant, k , $\text{M}^{-1}\text{s}^{-1}$ (25 °C) (calculated)
SR-135	31.5 ± 2.50	$^{a}7.3 \pm 0.6 \times 10^5$
SRB	0	---

^a Apparent second order rate constant for the oxidation of complex (1 equivalent) by PN (1 equivalent) estimated from % inhibition in 100 mM phosphate buffer (pH = 7.2), 0.7% SDS; no secondary antioxidants added; determined by LCMS after 1 min reaction time.

Table 2

Inhibition of tyrosine nitration by SR-135 and SRB. The ability of SR135 and SRB to prevent the nitration of Leu-enkephalin (LENK, Tyr-Gly-Gly-Phe-Leu) was determined. Solutions of 0 or 1 μmol of the LENK were mixed with 1, 0.2, or 0.1 μmol of SR-135 or SRB. Freshly prepared peroxynitrite (1 μmol) was then rapidly injected into these stirred solutions as described in the methods section. The reactions were aged for 1 minute and the amounts of unreacted LENK and 3-NT-LENK were quantified by LCMS analysis.

Catalyst	% Inhibition (25 °C)
SR-135 (1 eq)	100 $\pm$ 2.24
SR-135 (0.2 eq)	98.0 $\pm$ 9.39
SR-135 (0.1 eq)	52.0 $\pm$ 4.70
SRB (1 eq)	14.0 $\pm$ 0.30
SRB (0.2eq)	0 (not catalytic)

Table 3

Plasma TAG, total cholesterol, and HDL levels after drug treatment. Colorimetric assays were performed to determine TAG, total cholesterol, and HDL in the plasma of the mice under various conditions as stated in the Methods section.

Treatment group	TAG	Total Cholesterol	HDL
Lean	94.2 ± 5.9	93.5 ± 2.4	89.8 ± 10.1
HFD	123.4 ± 6.7**	157.0 ± 7.1****	196.3 ± 29.7**
HFD + Vehicle	110.9 ± 6.9	140.5 ± 6.6	213.9 ± 30.3
HFD + SRB	104.4 ± 4.6	140.8 ± 6.0	200.2 ± 43.7
HFD + SR-135	101.6 ± 9.9	132.7 ± 8.1 [†]	271.5 ± 28.5

* denotes a statistical significance between lean vs. HFD groups.

[†] denotes a statistical significance between HFD vs. HFD + SR-135 groups.