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Osteocalcin protects pancreatic beta cell function and survival under high glucose conditions

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ABSTRACT

Diabetes is characterized by progressive beta cell dysfunction and loss due in part to oxidative stress that occurs from gluco/lipotoxicity. Treatments that directly protect beta cell function and survival in the diabetic milieu are of particular interest. A growing body of evidence suggests that osteocalcin, an abundant non-collagenous protein of bone, supports beta cell function and proliferation. Based on previous gene expression data by microarray, we hypothesized that osteocalcin protects beta cells from glucose-induced oxidative stress. To test our hypothesis we cultured isolated rat islets and INS-1E cells in the presence of normal, high, or high glucose ± osteocalcin for up to 72 h. Oxidative stress and viability/mitochondrial function were measured by H_2O_2 assay and Alamar Blue assay, respectively. Caspase 3/7 activity was also measured as a marker of apoptosis. A functional test, glucose stimulated insulin release, was conducted and expression of genes/protein was measured by qRT-PCR/western blot/ELISA. Osteocalcin treatment significantly reduced high glucose-induced H_2O_2 levels while maintaining viability/mitochondrial function. Osteocalcin also significantly improved glucose stimulated insulin secretion and insulin content in rat islets after 48 h of high glucose exposure compared to untreated islets. As expected sustained high glucose down-regulated gene/protein expression of INS1 and BCL2 while increasing TXNIP expression. Interestingly, osteocalcin treatment reversed the effects of high glucose on gene/protein expression. We conclude that osteocalcin can protect beta cells from the negative effects of glucose-induced oxidative stress, in part, by reducing TXNIP expression, thereby preserving beta cell function and survival.

1. Introduction

Diabetes is characterized by progressive beta cell dysfunction and loss due in part to oxidative stress that occurs from gluco- and lipo-toxicity [1–5]. It is widely accepted that contributors to beta cell dysfunction and loss include but are not limited to, chronic hyperglycemia, hyperlipidemia, insulin resistance, and proinflammatory cytokines. Persistent hyperglycemia can lead to beta cell adaptation initially, followed by de-compensation and the accumulation of reactive oxygen and nitrogen species which impairs function and viability [6–9]. Since beta cells have low intrinsic antioxidant mechanisms and are inefficient at repairing oxidative DNA damage compared to other tissues, they are more vulnerable to these stressors [7,10]. Treatments that directly protect beta cell function and survival in the diabetic milieu are of particular interest.

Recent studies describe the bone derived peptide, osteocalcin (OCN), as having direct effects on islet physiology. Knockout Esp −/− mice, lacking protein tyrosine phosphatase had high circulating levels of uncarboxylated OCN (Glu-OCN) resulting in increased beta cell mass and increased insulin sensitivity and were protected from induced obesity and diabetes [11]. Chronic administration of Glu-OCN either by injection or subcutaneous osmotic pumps significantly improved glucose tolerance and insulin sensitivity, increased energy expenditure, and prevented type 2 diabetes in mice fed either a high fat or normal diet [12,13].

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Recently we have shown that OCN, secreted from a human osteoblast cell line, has beneficial effects on isolated human islets in culture [14]. Specifically we observed that OCN 1) increased insulin secretion and content, and 2) up- or down-regulated genes to enhance the protective mechanisms against cellular stress. This important finding suggests that OCN may be a potential treatment for diabetes by preventing progressive beta cell failure due to oxidative and endoplasmic reticulum (ER) stress associated with metabolic derangements of hyperglycemia and/or dyslipdemia. Since preservation of a functional beta cell mass is critical for prevention of diabetes, the effectiveness of OCN in protecting islets from glucotoxicity is worthy of additional study. The goal of this project is to determine the effectiveness of OCN in attenuating glucose induced oxidative stress and subsequent cell damage.

2. Methods and materials

2.1. Rat islet isolation

Islets were isolated from the pancreas of 250–300 g Sprague Dawley male rats (Harlan Laboratories, Indianapolis, IN), using a modified method of collagenase distension that has been previously described [15]. Rat islets were allowed to recover in Ham's F12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and 10 mM Hepes 24 h prior to the start of an experiment.

2.2. INS-1E cell line

Prior to experiments, INS-IE cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM Na-Pyruvate, 55 uM 2-mercaptoethanol, and 10 mM Hepes.

2.3. Experimental treatment design

Unless otherwise stated, INS-1E cells were counted and dispersed into 60 mm dishes or 96 well plate with DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM Na-Pyruvate, 55 uM 2-mercaptoethanol, and 10 mM Hepes. INS-1E cells were cultured up to 72 h in 11 mM or 33 mM glucose and in the absence or presence of purified native human OCN (3 or 5 ng/ml) (Fitzgerald Industries International, Acton, MA). Medium was changed every 48 h and OCN was added to cultures every 24 h. Cells were collected at 48 or 72 h after treatment and assayed for various biomarkers as described in results.

Rat islets were allowed to rest for 24 h after isolation prior to the experiment. Unless otherwise stated, islets were dispersed into 60 mM dishes (200IEQ) and cultured in CMRL medium up to 72 h in 11 mm or 25 mM glucose and in the absence or presence of OCN (0.3 or 1 ng/ml) (Fitzgerald Industries International, Acton, MA). Medium was changed every 48 h and OCN was added to cultures every 24 h. Islets were collected at 48 or 72 h after treatment and assayed for various biomarkers as described in results.

2.4. Hydrogen peroxide (H_2O_2) assay

At the end of 48 and 72 h of treatment, INS-1E cells and islets were collected and protein extracted using Micro BCA Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer's instructions. Hydrogen peroxide content was determined in 1 ug of protein by using Bioxytech® H_2O_2-560 (Percipio Biosciences, Manhattan Beach, CA) according to the manufacturer's instructions.

2.5. Viability assay

INS-1E cells were dispersed into 96 well plates at 2×10^4 cells/well. Islets were dispersed into 96 well plates at 10 islets/well in the 100–150 um size range. Alamar blue assay (Invitrogen, Carlsbad, CA) was performed according to the manufacturer's instructions. Alamar blue was added to islets 18 h and INS-1E cells 4 h prior to reading fluorescence (560/590) activity.

2.6. Apoptosis assay

INS-1E cells were plated at 1×10^4/well in 96 well plates. At the end of the 48 and 72 h treatment, Apo-ONE® Homogeneous Caspase-3/7 buffer/substrate (Promega, Madison, WI) was added to each well and processed according to the manufacturer's instructions. Islets were collected from culture dishes at the end of 48 and 72 h of treatment and spun down in a microcentrifuge tube (~300IEQ/tube). Apo-ONE® Homogeneous Caspase-3/7 buffer/substrate (250 ul) was added to the islet pellet and remaining 250 ul of supernatant. Tubes were covered in foil and placed on a platform rocker, rocking for 50 min at room temperature. 100 ul of the homogenate was transferred to each well of a white-walled 96 well luminometer plate. Bioluminescence in each sample was measured using a microplate luminometer.

2.7. Glucose stimulated insulin secretion assay (GSIS) and insulin content

The GSIS was conducted after 48 h of treatment as previously described [16]. Islets were collected at the end of the GSIS and insulin content was determined as previously described [14].

2.8. Gene expression

Islets or INS-1E cells were collected and total RNA was isolated and analyzed as previously described [16]. Gene specific primers for B-cell lymphoma 2 (*Bcl2*) and beta actin (*Atcb*) were from Qiagen and Insulin 1 (*Ins*) (sense 5′-GAT CTT CA ACC TTG GCA CTG-3′; antisense 5′-CCA GTT GGT AGA GGG AGC AG-3′) and thioredoxin-interacting protein (*Txnip*) (sense 5′-CGA GTC AAA GCC GTC AGG AT-3′; antisense 5′-TTC ATA GCG CAA GTA GTC CAA GGT-3′) were from Integrated DNA Technologies (Coralville, IA). All samples were normalized to internal standard, *Atcb*. Comparisons of relative gene expression were made between islets cultured in normal glucose, high glucose and high glucose with or without osteocalcin. All samples were run in duplicate.

2.9. Western blot

Islets were washed in phosphate-buffered saline (PBS) and homogenized by RIPA protein extraction buffer (50 mM TrisHCL pH7.4, 150 mM NaCl, 2 mM EDTA, 1%NP-40, 0.1% SDS). Protein concentrations were measured using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Western blot assay was performed as previously described, loading 15–20 ug total protein per lane [16]. Primary antibodies, BCL2, TXNIP, and ATCB were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Image/data analysis of protein bands was performed using Image Studio™ Lite (LI-COR, Lincoln, NE).

2.10. Statistics

Experiments were repeated 3–4 times with 3 replicates for each treatment and control samples unless otherwise stated. Treatment and controls groups were compared using student T test, one-way

ANOVA combined with a Tukey post hoc test for multiple comparisons. Data are presented as mean + S.E.M. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Osteocalcin attenuates glucose-induced H_2O_2 levels and maintains viability/metabolic activity

To determine if OCN can reduce a known biomarker of glucose-induced oxidative stress, we measured H_2O_2 in INS-1E cells and isolated rat islets exposed to normal glucose, high glucose or high glucose plus OCN treatment for 48 and 72 h.

In INS-1E cells and isolated rat islets, H_2O_2 content was significantly higher in high glucose treated cells compared to normal glucose treated cells (Fig. 1A,B). Osteocalcin treatment significantly reduced glucose induced H_2O_2 accumulation at 48 h with a further reduction at 72 h.

To determine if OCN preserves mitochondria function/viability in beta cells under high glucose conditions, we used Alamar Blue as a redox indicator to measure metabolic activity/viability.

Cell viability was significantly reduced in INS-1E cells and isolated rat islets after exposure to high glucose while OCN treatment maintained viability under high glucose conditions at 48 and 72 h (Fig. 1C,D).

3.2. Caspase 3/7 activity is reduced by OCN under high glucose conditions

To determine the effects of OCN on oxidative stress-induced apoptosis, we measured caspase 3/7 activity using Capase-Glo 3/7 assay in INS-1E cells and rat islets exposed to normal glucose, high glucose or high glucose plus OCN treatment for 48 and 72 h.

INS-1E cells exposed to high glucose had significantly higher caspase activity then cells exposed to normal glucose. OCN treatment significantly reduced caspase activity at 48 h and 72 h (Fig. 2A).

Isolated rat islets exposed to high glucose had significantly higher caspase activity then islets exposed to normal glucose. Osteocalcin treatment significantly reduced caspase activity under high glucose conditions to similar activity measured in normal glucose cultured islets at 48 and 72 h (Fig. 2B).

3.3. Osteocalcin preserves beta cell function under conditions of oxidative stress

A static incubation GSIS test was performed on isolated rat islets after 48 h of treatment. As expected, chronic exposure to high glucose resulted in impaired glucose stimulated insulin release compared to control islets (normal glucose). However, OCN treatment maintained beta cell responsiveness to glucose under high glucose conditions with insulin release similar to normal glucose controls (Fig. 2C). At the end of the experiment islets were collected for insulin content measurements. Insulin content in islets cultured in high glucose had significantly lower insulin than islets culture in normal glucose. However, OCN treatment maintained insulin content, similar to normal glucose controls (Fig. 2D).

3.4. The effects of osteocalcin on gene/protein expression

The effects of OCN on gene/protein expression were determined in rat islets after 48 h of treatment. It is known that hyperglycemia

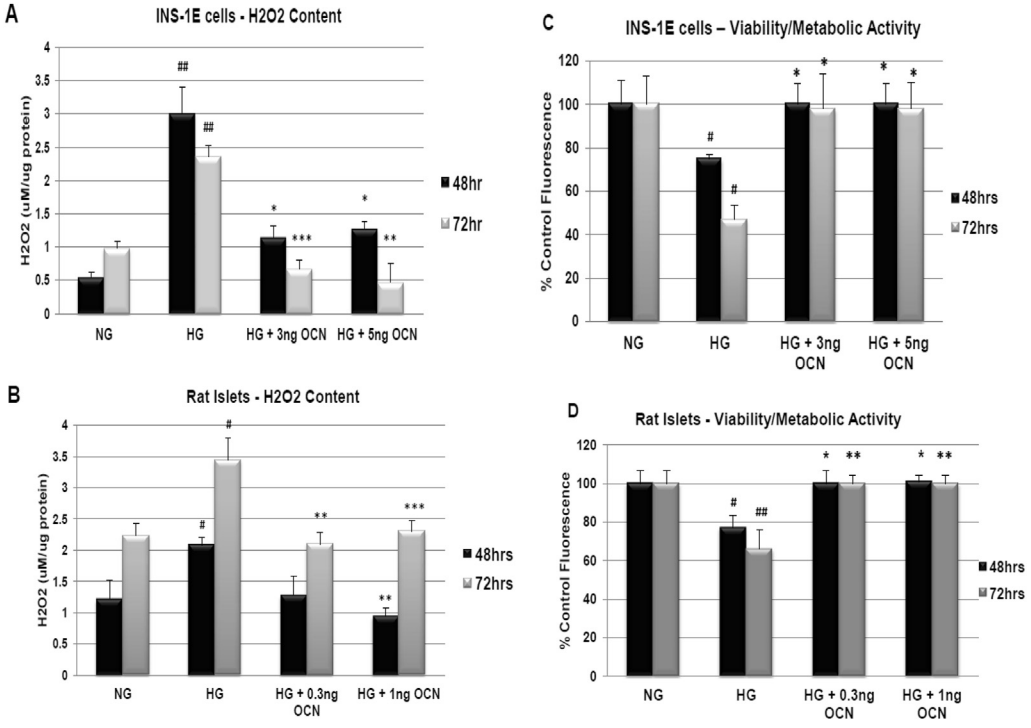

Fig. 1. OCN reduces H_2O_2 content and maintains viability/metabolic activity under high glucose conditions. Total protein was extracted from INS-1E cells and rat islets at 48 and 72 h after exposure to normal glucose (NG) or high glucose (HG) or high glucose with OCN treatment. A significant reduction in H_2O_2 content was observed with both concentrations of OCN under HG conditions in (A) INS-1E cells and (B) isolated rat islets. INS-1E cells and isolated rat islets were exposed to normal glucose (NG) or high glucose (HG) or HG with OCN treatment. Alamar Blue redox reagent was added to each well and fluorescence was measured at 48 and 72 h in the same cells. OCN treatment maintained mitochondrial function/viability in (C) INS-1E cells and (D) isolated rat islets at 48 and 72 h of high glucose exposure ($n = 3$–4) $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ compared to NG controls; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to HG controls.

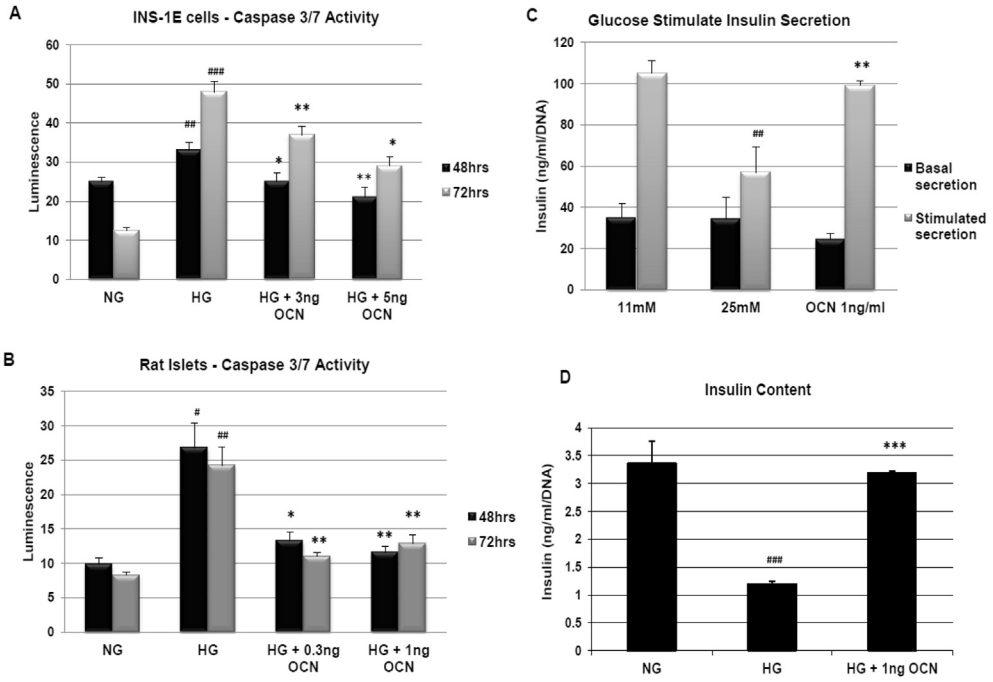

Fig. 2. OCN treatment reduces caspase 3/7 activity and preserves beta cell function under conditions of oxidative stress. Caspase 3/7 activity was measured in INS-1E cells and rat islets at 48 and 72 h after exposure to normal glucose (NG) or high glucose (HG) or HG with OCN treatment. OCN treatment reduced Caspase 3/7 activity in (A) INS-1 cells and (B) isolated rat islets. A GSIS test was performed on isolated rat islets collected after 48 h of exposure to normal glucose or high glucose with or without OCN treatment. (C) Islets cultured in HG had significantly lower insulin secretion in response to glucose compared to NG cultured islets. OCN treatment preserved glucose responsiveness under oxidative stress conditions. (D) Islets were collected after the GSIS test and insulin content was determined. Significantly reduced insulin content was seen in HG treated islets compared to NG and HG/OCN treated islets. Islet insulin content was normalized to islet DNA (ng) ($n = 3$–4) $^{\#}P < 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ compared to NG controls; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to HG controls.

can reduce INS and BCL2 expression in islets. Results from our experiments show that sustained high glucose exposure resulted in an approximately 2 fold decrease in *Ins1* and *Bcl2* gene expressions compared to normal glucose controls. Interestingly, OCN treatment significantly reversed down-regulation of *Ins1* and *Bcl2* expressions under high glucose conditions (Fig. 3A,B). TXNIP has been recently described as a regulator of the cellular redox state and has been described as one of the most up-regulated genes in islets under oxidative stress conditions. We found that islets cultured for 48 h in high glucose had a significant increase in *Txnip* expression by 17 fold compared to normal glucose controls while OCN treatment significantly reduced *Txnip* expression in islets from the same treatment group (Fig. 3C).

Protein levels of BCL2 were significantly reduced by high glucose while OCN treatment reversed the decrease at 0.3 ng (not significant) and at 1 ng (significant) similar to normal controls (Fig. 4A). Protein expression of TXNIP was significantly increased by high glucose conditions compared to normal glucose controls. Osteocalcin treatment reduced TXNIP protein expression at 0.3 ng (not significant; $P = 0.058$) and 1 ng (significant) compared to high glucose controls at 48 h (Fig. 4B). Insulin protein expression (ELISA) was significantly reduced under high glucose conditions compared to normal glucose controls. Osteocalcin treatment increased INS protein expression at 0.3 ng (not significant) and 1 ng (significant) under high glucose conditions compared to high glucose controls (Fig. 4C).

4. Discussion

Osteocalcin has been reported to have beneficial effects on pancreatic beta cells by increasing insulin content/secretion and stimulating proliferation. Here we describe a role for OCN, as an

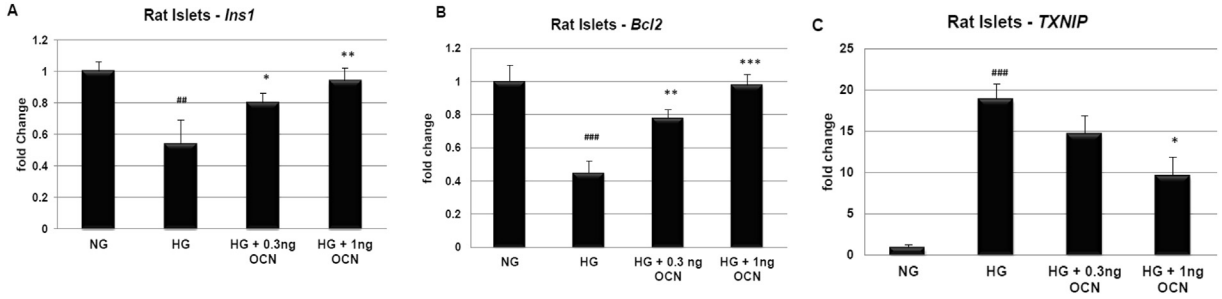

Fig. 3. The effects of OCN on gene expression under high glucose conditions. Total RNA was isolated from rat islets at 48 h after exposure to normal glucose (NG) or high glucose (HG) or HG with OCN treatment. The mRNA levels were determined by qRT-PCR and normalized to an internal control (*Actb*). Relative expression levels to NG control samples are expressed as fold change. The following genes were probed: (A) *Ins1*, (B) *Bcl2*, (C) *Txnip*. $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ compared to NG controls; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to HG control.

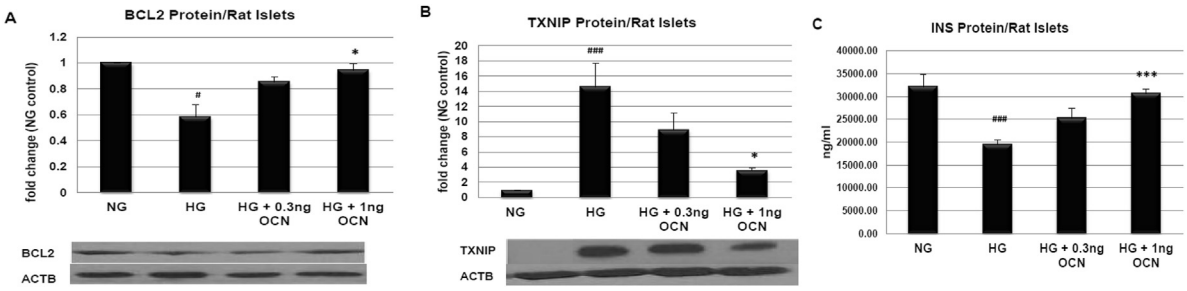

Fig. 4. The effects of OCN on protein expression under high glucose conditions. Total protein was isolated from rat islets at 48 h after exposure to normal glucose (NG) or high glucose (HG) or HG with OCN treatment. (A) BCL2 and (B) TXNIP protein was measured by western blot and (C) INS levels were measured by ELISA. The intensity of the bands generated in 3 independent experiments was normalized to ACTB. Representative blot panel is shown. $^{\#}P \leq 0.05$, $^{\#\#\#}P \leq 0.001$ compared to NG controls; $^{*}P \leq 0.05$, $^{***}P \leq 0.001$ compared to HG control.

anti-oxidant, reducing glucose-induced oxidative stress and subsequent cell damage in isolated rat islets and INS-1E beta cell line. Our results expand on the ever evolving role of OCN in supporting beta cell function and survival.

The negative effects of sustained hyperglycemia on beta cell function and survival are well documented [2,7–9]. Along with a reduction in H_2O_2, OCN preserved viability and reduced apoptosis in INS-1E cells and isolated rat islets under sustained high glucose conditions. Osteocalcin was not as robust in reducing caspase activity in INS-1E cells compared to islets. Despite the higher caspase activity detected in INS-1E cells, we did not detect a significant reduction of viability/mitochondria function at the same time points tested.

Oxidative stress plays a significant role in glucose-dependent beta cell dysfunction, reducing insulin secretion in response to glucose. There is considerable evidence that OCN treatment can enhance beta cell function, *in vivo* and *ex vivo*, in human and rodent islets under normal glucose conditions. Here we present evidence that OCN can preserve beta cell function and insulin content with a normal GSIS response after exposure to sustained high glucose conditions.

Exposure to sustained high glucose results in decreased/increased expression of various genes/proteins in beta cells related to differentiation, function and survival. The pro-survival or anti-apoptotic gene, BCL2, and insulin were down-regulated by exposure to sustained high glucose. Osteocalcin blocked down-regulation of both insulin and BCL2 gene and protein expression under high glucose conditions. These results correlate with the OCN preservation of beta cell function and viability seen under high glucose conditions.

The effects of OCN on TXNIP expression were of interest. Thioredoxin-interacting protein has been recently described as a regulator of the cellular redox state and over-expression decreases thioredoxin reductase activity, rendering cells more susceptible to oxidative stress and apoptosis. Thioredoxin-interacting protein has been identified as the most dramatically up-regulated gene in an oligonucleotide microarray on isolated human pancreatic islets treated with sustained high glucose concentrations [17]. In addition TXNIP is similarly upregulated in multiple other cell types in response to constant high glucose concentrations, including endothelial cells. TXNIP regulates peripheral glucose metabolism in humans [18] and its over expression has been shown to cause pancreatic beta cell apoptosis [19,20], endothelial cell dysfunction, and insulin resistance [21]. Lack of TXNIP has been shown to protect beta-cells against glucotoxicity [22]. Increased TXNIP expression is associated with decreased expression of the transcription factor, *MafA*, which has an important role in insulin gene expression. Our data confirm previous findings that sustained high glucose can dramatically increase TXNIP expression in islets. However, OCN treatment reduced TXNIP expression under high glucose conditions. Considering that elevated TXNIP expression is associated with beta cell apoptosis, diabetes and chronic complications, adjunct treatments such as OCN that reduce TXNIP expression, would be of therapeutic interest.

In addition to our report on the protective role of OCN in reducing oxidative stress in beta cells, there are also reports that OCN can reverse high fat diet-induced and chemically-induced endoplasmic reticulum (ER) stress in other tissues. It's been reported that high fat diet fed C57BL/6J and *ob*/*ob* mice had elevated levels of phosphorylation of PERK, eIF2alph, and IRE-1alph, and expression of ATF6beta, all indicating increased levels of ER stress in liver, adipose, and skeletal muscle [23]. Four weeks of OCN treatment resulted in a reduction of all biomarkers of ER stress in tissues studied.

The mechanisms by which OCN mediates its effects in beta cells are yet to be discovered. There is indirect evidence that OCN binds G protein coupled receptor, GPRC6A, in osteoblasts, small intestine epithelial and pancreatic beta cells. Quarles et al. reported that OCN induced ERK phosphorylation in HEK-293 cells transfected with GPRC6A receptor while no phosphorylation was present in non-transfected HEK-293 cells [24]. Also OCN induced phosphorylation of ERK in the mouse beta cell line, TC-6 cells, which express GPRC6A receptors. Genetically modified mice lacking GPRC6A receptors did not have an increase in insulin secretion or pancreas insulin expression with administered OCN compared to the wild type mice. It was also suggested through the use of biochemical inhibitors, that GPRC6A activates PLC, PKD1, and Ras/Raf/MEK/ERK pathways which have been implicated in cell survival, cell proliferation and insulin secretion. In other cell types, such as vascular endothelial cells, it was reported that IP3/AKT pathway is activated by OCN resulting in reduced linoleic acid-induced apoptosis and increased endothelial nitric oxide secretion [25].

In conclusion, the results of this study support the idea of OCN as potential adjunct therapy in the treatment of diabetes.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

KK and YY designed the project. PYT, DW, XL, JT, and MH performed the experiments. MC and WVM provided scientific support. KK wrote the manuscript; WVM and YY helped with manuscript revisions.

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