A novel Gymnema sylvestre extract protects pancreatic beta-cells from cytokine-induced apoptosis

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Inflammatory cytokines such as interleukin-1β, TNF-α, and interferon-γ are known to be involved in mediating β-cells death in diabetes mellitus (DM). Thus, protecting from β-cells death in patients with DM may be a useful target in alleviating symptoms of hyperglycemia. Traditional plant-based remedies have been used to treat DM for many centuries and may play a role in protecting β-cell from death. An example of these remedies is Gymnema sylvestre (GS) extract. In this study, we investigated the effect of this plant extract on β-cells apoptosis. Om Santal Adivasi (OSA®) maintained cell membrane integrity in MIN6 cells and mouse islets. Om Santal Adivasi significantly protected MIN6 cells and mouse islets from cytokine-induced apoptosis. In the presence of cytokines, OSA® significantly reduced the expression and activity of caspase-3. The antiapoptotic effect of OSA® as shown by microarray analysis is largely mediated by activating pathways involved in cell survival (mainly casein kinase II pathway) and the free radical scavenger system (specifically superoxide dismutase and catalase). This study indicates that the GS isolate OSA® protects against cytokine-induced apoptosis of β-cells by increasing the expression of cell survival pathways and free radical scavenger system.

KEYWORDS
apoptosis, diabetes, plant-derived therapies

1 INTRODUCTION

Diabetes mellitus (DM) has been associated with reductions in the functional β-cell mass, mainly due to excessive induction of apoptotic cell death (Donath & Halban, 2004; Johnson & Luciani, 2010; Lupi & Del Prato, 2008). In Type 1 DM, for example, the death of β-cells can be initiated by the release of proinflammatory cytokines from activated macrophages and T cells. Cytokines such as interleukin-1β (IL-1β), TNF-α, and interferon-γ (IFN-γ), acting through their cell-surface receptors on β-cells, activate several signaling pathways, which are involved in apoptosis and β-cell survival (Cnop et al., 2005). Examples of these pathways include nuclear factor kappa B (NF-κB)-dependent pathways, mitogen-activated protein kinases, Janus kinase, signal transducer and activator of transcription, granzyme B, AKT (also known as protein kinase B), cAMP responsive element binding protein (CREB), Bcl-2 family, and endoplasmic reticulum (ER) stress pathways (Cnop et al., 2005; Eizirik & Mandrup-Poulsen, 2001).
Endoplasmic reticulum stress can be involved in the death of β-cells following exposure to chronic insult. The ER contains three major ER stress signaling molecules, namely, ER to nucleus signaling 1, protein kinase RNA-like ER kinase, and activating transcription factor 6 (Kadowaki & Nishitoh, 2013). Initially, these molecules act as pro-survival signals by activating ER chaperone molecules such as CREB4, OASIS (also known as CAMP responsive element binding protein 3-like 1), CREBH, and antioxidant mediators. During chronic ER stress, these molecules, mainly ER to nucleus signaling 1 and protein kinase RNA-like ER kinase, shift to proapoptotic actions and signal cell death by upregulating the NF-κB pathway and activating CHOP (also known as CCAAT/enhancer binding protein, beta). CHOP is responsible for the transcription of proapoptotic genes (Götz & Montenarh, 2013; Saito, 2014).

Prevention of the death of β-cells has great potential as a therapeutic target for DM. However, most of the currently used therapies do not protect the functional β-cell mass during the development of DM, with the exception of exendin-4, a glucagon-like peptide 1 agonist. Exendin-4 has been suggested to protect β-cells from apoptosis in studies using insulin-secreting cells and primary islets in in vitro settings (Ferdaoussi et al., 2008). However, the cost and route of administration have limited the use of exendin-4 as a first-line therapy (S. Persaud & Jones, 2008). Therefore, the search for an affordable, orally administered, and safe therapy that protects the β-cell mass continues.

Gymnema sylvestre (GS) is a large woody climber plant, from the Asclepiadaceae family, and is native to central and southern India, tropical Africa, and Australia. Gymnema sylvestre leaves have been shown to have antidiabetic properties. Several studies have found that the leaves of GS had the ability to reduce blood glucose levels in multiple animal models of diabetes (Gupta, 1961, 1963; Gupta & Varyiar, 1961; Okabayashi et al., 1990; K. R. Shanmugasundaram, Panneerelvam, Samudram, & Shanmugasundaram, 1983; Srivastava, Nigam, Bhatt, Verma, & Prem, 1985). Most of these GS effects were attributed to low molecular weight GS extracts. In this study, we used a high molecular weight GS extract (A. K. Chatterji, 2005a, 2005b) that was subsequently designated as the Om Santal Adivasi (OSA®) extract, after the Santal tribe who first used GS leaves in Ayurvedic medicine. We have shown that this OSA® is effective in improving β-cell secretory function without any deleterious effect on cell membranes (B. Liu et al., 2009). We have also shown that this extract improved glucose tolerance in an animal model of diabetes and in patients with Type 2 (T2) DM in vivo and stimulated insulin secretion from a β-cell line and primary islets: effects that were partially due to calcium influx and to protein kinase activation in vitro (Al-Romaiyan et al., 2010; Al-Romaiyan et al., 2012; Al-Romaiyan, King, Persaud, & Jones, 2013). Other GS extracts have been reported to preserve β-cell mass in streptozotocin- or alloxan-treated rodents (Ahmed, Rao, & Rao, 2010; E. R. Shanmugasundaram, Gopinath, Radha Shanmugasundaram, & Rajendran, 1990). However, the molecular signaling underlying this effect is unknown. Therefore, the aim of this study was to determine whether OSA® has a protective effect in β-cells that have been challenged with cytokines and, if so, to identify the possible pathways that may contribute to the mechanism(s) by which OSA® exerts its action.

## METHODS

### 2.1 Plant material and preparation

The GS extract used in this study (OSA®) is commercially available. The collection, identification, preparation, and extraction of the plant is detailed in protocols described in the U.S. Patents 6949261 and 6946151 (A. K. Chatterji, 2005a, 2005b). The OSA® extract used in this study was a gift from Ayurvedic-Life International LLC, Wisconsin, USA. Om Santal Adivasi solutions were freshly prepared as 200-mg/ml stock in water and diluted in tissue culture media for use in the in vitro experiments.

### 2.2 Maintenance of MIN6 cells

The MIN6 cells used in this study were a gift from Prof. J. I. Miyazaki (University of Tokyo, Japan). MIN6 cells (Passages 40–44) were maintained as monolayers in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2-mM glutamine, and 100-U/ml penicillin with 0.1-mg/ml streptomycin under standard tissue culture conditions. The cells were harvested and used in experiments when the cell confluence reached 70–80%.

### 2.3 Isolation of mouse islets

The pancreata of outbred white albino Institute for Cancer Research (Harlan, UK) mice were digested by collagenase as described previously (Papadimitriou, King, Jones, & Persaud, 2007). The mouse was killed by neck dislocation and the abdominal area was opened. The common bile duct was exposed and clamped. Once collagenase (1 mg/ml) was injected, the pancreas was removed and digested at 37°C. The digested pancreas was washed with minimum essential medium supplemented with 10% neonatal calf serum and 100-U/ml penicillin with 0.1-mg/ml streptomycin and sieved into a sterile tube. The islets were purified on Histopaque density gradients and cultured in 35-mm bacterial dishes in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum and 100-U/ml penicillin/0.1-mg/ml streptomycin.

### 2.4 Viability assay

The effect of OSA® on cell viability was assessed using Promega CellTiter-Glo® assay (in MIN6 cells and mouse islets) or trypan blue exclusion test (in mouse islets). MIN6 or mouse islets were seeded at a density of 10,000 cells per well or 5 islets per well, respectively, in a white 96-well plate overnight under standard tissue culture conditions. MIN6 cells were treated with OSA® (0.06–1.0 mg/ml) and mouse islets were treated with OSA® (0.03–0.125 mg/ml) for 24 hr. CellTiter-Glo® reagent was added to each well, and the mixture was incubated for 15 min. At the end of the incubation period, luminescence was measured using Veritas luminometer (Promega UK). In the trypan blue exclusion test, mouse islets were treated with OSA® at a concentration of either 0.25 or 1.0 mg/ml for 24 hr and were then
stained and incubated with 0.1% (weight per volume) trypan blue dye in phosphate-buffered saline (PBS) for 15 min at 37°C. The cells were then washed with PBS and visualized under the light microscope. Images were captured using a Nikon Coolpix 4500 digital camera (Surrey, UK).

2.5 | Apoptosis assay

2.5.1 | Measurement of caspase-3 and -7 activity in MIN6 cells and mouse islets following exposure to cytokines

The effect of mixed cytokines on apoptosis in MIN6 cells and mouse islets was assessed using Promega Caspase-Glo® 3/7 apoptosis assay, following the manufacturer's protocol. MIN6 cells (10,000 cells per well) or mouse islets (5 islets per well) were seeded in white 96 wells (10,000 cells per well) or mouse islets (5 islets per well) were seeded in a white 96 well plate overnight under standard tissue culture conditions. Due to the vulnerability of MIN6 cells to cytokines, MIN6 cells were treated with either IL-1β (50 U/ml), TNF-α (1,000 U/ml) or IFN-γ (1,000 U/ml) in combination with two or more cytokines, for 16–18 hr. Mouse islets were treated with a mixture of cytokines altogether (IL-1β, TNF-α and IFN-γ) for 16–18 hr. Caspase-Glo® reagent was added to each well. Following 1-hr incubation, luminescence was measured using a Veritas luminometer (Promega, UK). The choice of cytokines was based on the accumulating data that these inflammatory mediators are responsible for β-cell death in animal models of diabetes and in patients with diabetes (reviewed in detail in Cnop et al., 2005; Donath, Storling, Maedler, & Mandrup-Poulsen, 2003b). The concentrations and incubation time of cytokines used in this study were also based on previously published reports (Cardozo et al., 2001b; Eizirik, Kutlu, Rasschaert, Darville, & Cardozo, 2003; Eizirik & Mandrup-Poulsen, 2001; Kutlu et al., 2003; Sarkar et al., 2009).

2.5.2 | Measurement of cytokine-induced caspase-3 and -7 activity in MIN6 cells and mouse islets following exposure to OSA®

MIN6 cells (10,000 cells per well) or mouse islets (5 islets per well) were seeded in white 96-well plates and incubated overnight under standard tissue culture conditions. MIN6 cells or mouse islets were incubated with OSA® (at a range of concentration of 0.03–0.125 mg/ml in MIN6 cells and 0.06–0.25 mg/ml in mouse islets) for 8 hr before being exposed to cytokines mixture in the absence or continued presence of OSA®. In MIN6 cells, two (IL-1β + TNF-α or TNF-α + IFN-γ) or more cytokines were used, whereas in mouse islets, a mixture of all three cytokines was used. Luminescence from each well was measured using a Veritas luminometer after 1-hr incubation with Caspase-Glo® reagent. In these experiments, exendin-4 (1 μM) was used as a positive control because it was reported to protect β-cells from apoptosis in vitro (Ferdousi et al., 2008). MIN6 cells or mouse islets were treated with exendin-4 for 8 hr before being challenged with cytokines in the absence or continued presence of exendin-4.

2.5.3 | Measurement of caspase-3 mRNA expression levels in mouse islets following exposure to OSA®

Mouse islets (150 islets) were pretreated with vehicle or 0.125-mg/ml OSA® for 8 hr first. The mouse islets were then incubated with or without a combination of IL-1β (50 U/ml), TNF-α (1,000 U/ml), and IFN-γ (1,000 U/ml) in the presence or absence of OSA® for 16–18 hr. RNA was extracted using a Qiagen RNeasy mini kit according to the manufacturer’s protocol. We converted 0.5 µg of RNA to complementary DNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen 18080-044). The RNA expression of caspase-3 (forward primer: 5′-GGGCCTGTGACTGAAAAA-3′ and reverse primer: 5′-AGCCTCACCAGTTACCTCT-3′) was measured using a Roche® thermocycler (LightCycler 480) and LightCycler® FastStart DNA masterPLUS SYBR Green I (Roche®, UK) using the absolute quantification method (S. J. Persaud, Burns, Belin, & Jones, 2004; S. J. Persaud et al., 2002). The reaction setting was as follows: preincubation at 95°C for 10 min followed by amplification cycles (denaturation step at 95°C for for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 10 s, cycles = 36). Actin (forward primer: 5′-ATGAAAGTGGACGT TGACATCGT-3′ and reverse primer: 5′-CCTAGAACATTGCG GTGCACGATG-3′) was used as a housekeeping gene.

2.6 | Microarray analysis of islet gene expression

2.6.1 | Experimental design

Mouse islets (200 per group) were exposed to the following conditions: control (no cytokines); control (no cytokines) + 0.125-mg/ml OSA®; cytokines (50-U/ml IL-1β, 1,000-U/ml TNF-α, and 1,000-U/ml IFN-γ); and cytokines + 0.125-mg/ml OSA®. In OSA®-treated groups, the islets were preincubated with OSA® for 8 hr before being exposed to the cytokines for 16–18 hr. The islets were washed with ice-cold PBS, and RNA was extracted using a Qiagen RNeasy mini kit according to the manufacturer’s protocol.

2.6.2 | Gene expression profiling

RNA of each sample was amplified, labeled, and purified using the MessageAmp™ Premier RNA amplification kit according to the manufacturer’s protocol (Ambion, UK). The protocol involves complementary DNA synthesis, in vitro transcription, and amplified RNA (aRNA) purification. We fragmented 15.5 µg of biotinylated aRNA by incubating the reaction with 5× array fragmentation buffer containing 200 mM Tris acetate (pH 8.2), 500-mM potassium acetate, and 150-mM magnesium acetate at 94°C for 35 min. We removed 1 µl of fragmented aRNA to test the quality of the fragmentation reaction. The reaction yielded a profile of 35–200 nucleotides of aRNA fragments with the peak at approximately 200 nucleotides. The fragmented aRNA was used in the hybridization step. Each fragmented sample was mixed with hybridization cocktail, and the mixture was hybridized to a Mouse Genome 430 2.0 array chip (Affymetrix, UK). The chips were washed and stained using Fluidics Station 450 (Affymetrix, UK) and were
scanned at 570 nm using GeneChip® Scanner 3000 7G. Image acquisition was obtained using an Affymetrix GeneChip® Command Console. The signal intensity was normalized and analyzed using Affymetrix Expression Console software and Affymetrix Microarray Suite (MAS 5.0). MAS 5.0 applies an algorithm of the average of the differences in perfect match and mismatch probes. Mismatch probes are used to measure the background noise levels for each probe pair.

2.7 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences between treatment groups were assessed using one-way analysis of variance (ANOVA), Student’s t test, or Bonferroni’s multiple comparison test as appropriate, and differences between treatments were considered significant at \( P < .05 \). The Student’s t test was used if there were only two treatment groups to compare, and Bonferroni’s multiple comparison was used as post hoc analysis following ANOVA. Analysis of variance was used if there were more than two treatment groups analyzed.

The signal intensities, in microarray analysis, were calculated using MAS 5.0. The Qlucore software was used to determine and to calculate the ratio and fold changes of genes between two different treatment groups in which one group acted as basal. A false discovery rate of at least 30% and a \( P < .05 \) were used to filter genes that were differently expressed. The possible signaling pathways and networks were generated using GeneGo® software, a pathway analysis tool.

3 RESULTS

3.1 Effect of cytokines on apoptosis in MIN6 cells and mouse islets

Chronic exposure of MIN6 cells to a combination of two or more cytokines induced apoptosis as assessed by significant elevations in caspase-3 and -7 activities compared with MIN6 cells that were not exposed to cytokines. A combination of IL-1β, TNF-α, and IFN-γ caused the highest elevations in caspase-3 and -7 levels (Figure 1a), although combinations of two cytokines were also effective. Similarly, chronic exposure of mouse islets to a combination of the three cytokines resulted in significant increases in caspase-3 and -7 activities (Figure 1b) as compared with islets not treated with cytokines.

3.2 Effect of OSA® on MIN6 cell and mouse islets viability

The effect of OSA® on cell viability was examined to determine the range of OSA® concentrations that was appropriate to use in the apoptosis and microarray experiments. Chronic exposure of MIN6 cells to OSA® at concentrations ≤0.125 mg/ml did not compromise cell viability (Figure 2a,b), whereas higher concentrations resulted in reduced cell viability when compared with MIN6 cells that were not treated with OSA®. Om Santal Adivasi at concentrations of 0.06 and 0.125 mg/ml maintained cell viability at 91 ± 4% and 88 ± 3%, respectively. The IC50 value was calculated as 164.9 ± 1.0 μg/ml (Figure 2b). Therefore, a concentration range between 0.03 and 0.125-mg/ml OSA® was used in the subsequent experiments with MIN6 cells. Our adenosine triphosphate (ATP) viability assay of mouse islets showed similar results to that of MIN6 cells. Incubation of mouse islets with OSA® (0.03–0.125 mg/ml) had no deleterious effect on islet cell viability (Figure 2c). In the trypan blue staining test, mouse islets were exposed to OSA® at a concentration of 0.25 or 1 mg/ml. Incubating mouse islets with OSA® (0.25 mg/ml but not 1 mg/ml) did not alter plasma membrane integrity of mouse islets as evident by the lack of nuclear trypan blue intake (Figure 2d), indicating that OSA® had no deleterious effect on cell viability of mouse islets at this concentration. Therefore, in subsequent experiments with mouse islets, OSA® was used at concentrations of 0.25 mg/ml or less.
3.3 | Effect of OSA® on cytokine-induced apoptosis in MIN6 cells and mouse islets

In MIN6 cells, OSA® (0.03–0.125 mg/ml) or exendin (1 μM) alone did not have any significant effect on cell apoptosis (Figure 3). Addition of two or more cytokines significantly increased caspase activities, indicative of increased apoptosis. Preincubation with OSA® attenuated apoptosis induced by IL-1β + TNF-α and IL-1β + TNF-α + IFN-γ combinations but not by the combination of TNF-α + IFN-γ (Figure 3). Exendin decreased cytokine-induced caspase-3 and -7 levels in the same fashion as OSA®. Pretreatment of MIN6 cells with exendin for 8 hr also protected them from apoptosis induced by the cocktail of three cytokines (Figure 3).

The protective effects of OSA® or exendin on cytokine-induced apoptosis in MIN6 cells were also observed in experiments using primary mouse islets. Om Santal Adivasi (0.06–0.25 mg/ml) or exendin alone did not cause any detectable changes in apoptosis in mouse islets, as assessed by changes in caspase-3 and -7 levels shown in Figure 4. However, pretreating mouse islets with either OSA® or exendin for 8 hr caused a reduction in cytokine-induced caspase-3 and -7 levels (Figure 4).

3.4 | Effect of OSA® on cytokine-induced caspase-3 mRNA expression in mouse islets

Treating mouse islets with 0.125-mg/ml OSA® for 8 hr before 16–18 hr exposure to the three cytokine mixture reduced caspase-3 mRNA expression (Figure 5), although the basal caspase-3 mRNA expression did not change when mouse islets were treated with OSA® alone.
3.5  Microarray analysis of the effect of cytokines and OSA® on apoptosis-induced gene expression in mouse islets

Microarray analysis was used to investigate the genes responsible for the protective effect of OSA® against cytokine-induced apoptosis. Chronic (16–18 hr) incubation of islets in the presence of cytokines modified the mRNA expression of approximately 200 genes related to apoptosis and cell survival, and the full data set for the microarray analysis is shown in Table S1. Selected genes whose expressions were modified by cytokines are listed in Table S2. Preincubation in the presence of OSA® before exposing the islets to cytokines was associated with a downregulation in caspase-3 expression, consistent with our Promega Caspase-Glo® 3/7 activity measurements (Figure 4) and mRNA expression data (Figure 5). Furthermore, OSA® caused the upregulation of mRNA transcripts of protective antiapoptotic genes and downregulation of apoptotic genes that are mainly involved in the ER stress (Table 1).

4  DISCUSSION

It is well known that reduction in β-cell mass is a major contributor to the pathogenesis of DM. In principle, a decrease in the β-cell mass...
OSA® (0.125 mg/ml) in the presence or absence of cytokines (interleukin-1β + TNF-α + interferon-γ) for 16–18 hr. Caspase-3 mRNA expression was measured. OSA® alone had no effect on caspase-3 mRNA levels but significantly reduces the cytokine-induced increase in caspase-3 mRNA. Data are mean ± standard error of the mean, n = 3, *P < 0.05 (by analysis of variance followed by Bonferroni multiple comparison test).

could be caused by either a decrease in the rate of β-cell proliferation or an increase in β-cell loss through apoptosis. Inappropriate activation of apoptosis is thought to be the main mode of β-cell loss in DM (Donath & Halban, 2004; Johnson & Luciani, 2010; Lupi & Del Prato, 2008). Proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) have been reported to play important roles in the death of β-cells in DM (Cnop et al., 2005), especially in Type 1 DM, although there is now accumulating evidence that T2DM is an inflammatory condition associated with inappropriate levels of circulating cytokines (Donath, Storling, Maedler, & Mandrup-Poulsen, 2003a). Cytokine-induced NF-κB-dependent and -independent pathways lead to apoptosis by acting on various steps in both intrinsic and extrinsic pathways of apoptosis.

In our experiments, chronic exposure of MIN6 cells to cytokines induced apoptosis as assessed by measurements of caspase-3 and -7 activities. The incubation of MIN6 cells with cytokines for 16–18 hr was defined as “chronic” to differentiate it from acute incubations that usually last for no more than a few hours. As expected, elevated levels of caspase-3 and -7 were evident in MIN6 cells treated with a mixture of two or more cytokines. These observations were consistent with published data that showed activation of caspase-3 was obtained 18 to 24 hr after cytokine treatment in MIN6 cells (Sarkar et al., 2009). In addition, mouse islets chronically exposed to cytokines also exhibited 2.5-fold increase in the activities of caspase-3 and -7.

Our microarray data, in accordance with other published microarray data (Cardozo et al., 2001a; Cardozo, Kruhoffer, Leeman, Orntoft, & Eizirik, 2001; Sarkar et al., 2009), showed that exposure of mouse islets to cytokines caused general modifications in the expression of genes involved in cell metabolism, cell adhesion, antigen presentation, cell cycle, ion channel formation, cytokine processing, protein folding, DNA repair, apoptosis, and cell defense. Changes in mRNA levels of some of these genes may precipitate the death of β-cells. The microarray data revealed complex changes in the expression of genes involved in apoptosis, with cytokines inducing the upregulation of both proapoptotic and antiapoptotic genes.

As discussed earlier, cytokines have been well documented to have detrimental effects on β-cell survival, so the identification of an agent that can prevent or counteract some or all of the cytokine-induced damages may prevent the progressive loss of β-cell mass seen in patients with diabetes. In our experiments, we used a novel GS extract named OSA® to investigate whether it can protect β-cells from cytokine-induced apoptosis. In the present study, when MIN6 cells were treated with two or more cytokines, the increase in caspase-3 and caspase-7 activities were markedly reduced in cells pretreated with OSA®. This effect of OSA® was comparable with that seen with exendin, which was reported to protect β-cells from apoptosis in vitro (Ferdoussi et al., 2008). The reduction in caspase-3 and -7 activities seen with OSA® was evident if cells were treated with a mixture of IL-1β + TNF-α + IFN-γ and IL-1β + TNF-α but not with TNF-α + IFN-γ, suggesting that OSA® may affect signaling pathways or downstream effectors that are activated by the combination of IL-1β + TNF-α but not by TNF-α + IFN-γ.

Similarly, prolonged incubation of mouse islets with cytokines also stimulated caspase-3 and -7 activities, which again was significantly inhibited by pretreatment with OSA®. This effect of OSA® was again comparable with that seen with exendin. The increase in caspase-3 levels in mouse islets following cytokine treatment was also confirmed using microarray analysis. Consistent with previously published data (Sarkar et al., 2009), cytokines induced increase in caspase-3 mRNA levels that was reduced by approximately 50% by OSA® pretreatment. Similarly, the reduction in caspase-3 mRNA levels by OSA® seen in the microarray experiments was further validated by our real-time polymerase chain reaction data that showed that pretreating mouse islets with OSA® in the presence of cytokines significantly decreased caspase-3 mRNA expression levels. Therefore, OSA® protects β-cells from cytokine-induced apoptosis.

To explore the possible molecular pathways by which OSA® inhibited caspase-3 and -7, we used microarray analysis of cytokine-treated mouse islets in the presence or absence of OSA®. Enrichment analysis of pathways using GeneGo® software indicated that the
Abbreviation: ER, endoplasmic reticulum.

antiapoptotic activity of OSA® may be mediated through activation of pathways involved in cell survival, most notably the casin kinase signaling pathway. Our observations showed that islets treated with OSA® in the presence of cytokines showed increased casin kinase II (CK-II) mRNA levels when compared with islets treated with cytokines alone. The mRNA expression of downstream effectors of CK-II resulted in upregulation of ER stress (Malhotra & Kaufman, 2007). Furthermore, CK-II also has been shown to inhibit CHOP and thus prevent cell apoptosis (Götz & Montenarh, 2013; Kadowaki & Nishitoh, 2013). In our experiments, treating mouse islets with OSA® in the presence of cytokines resulted in upregulation of ER stress protectors such as Eif2, CREB4, OASIS, and antioxidant genes while there was downregulation of ER stress intensifiers such as CHOP (Table 1), indicating that part of the antiapoptotic effect of OSA® seen in our experiments with MIN6 cells and mouse islets may be due to activation of CK-II, which in turn modifies the ER stress signals.

Another mechanism of the CK-II antiapoptotic effect is the direct effect of CK-II on caspase activation. Several studies have shown that the activity of caspases can be directly regulated by CK-II. In mice, CK-II phosphorylation protected caspase-9 from caspase-8 cleavage, and induction of apoptosis occurred with loss of the phosphorylation site (McDonnell et al., 2008). Casein kinase II has also been reported to prevent caspase-2 activation by phosphorylating caspase-2 (Shin et al., 2005). Similarly, inhibition of caspase-8 activation has been documented following phosphorylation of the caspase inhibiting protein apoptosis repressor with caspase recruitment domain by CK-II (Li et al., 2002). Again, the OSA®-induced reductions in caspase expression and activity observed in our experiments may demonstrate that these effects may also be due to CK-I-mediated direct inhibition of caspase activity.

It has been demonstrated that islets are equipped with free radical scavenging systems (Acharya & Ghaskadbi, 2010). During normal oxidative phosphorylation, a small percentage of reactive oxygen radicals is produced, which is quickly detoxified by manganese superoxide dismutase to hydrogen peroxide (H₂O₂) then converted to H₂O and O₂ by catalase in the cytosol (Evans, 2002). Again, the OSA®-induced reductions in caspase expression and activity observed in our experiments may demonstrate that these effects may also be due to CK-I-mediated direct inhibition of caspase activity.

Table 1: Effect of Om Santal Adivasi (OSA®) on selected genes involved in apoptosis/survival in mouse islets.

| Probe set ID | Gene symbol | Gene title | Fold change | Function |
|-------------|-------------|------------|-------------|----------|
| 1419295_at  | Cebp31      | cAMP responsive element binding protein 3-like 1/OASIS | 4.5         | Implicated in ER stress response; antiapoptotic |
| 1453099_at  | Csnk2a2     | casein kinase 2, alpha prime polypeptide | 2.2         | Implicated in cell survival signal and inhibits apoptosis |
| 1424218_a_at| Cebp31      | cAMP responsive element binding protein 3-like 4/CREB4 | 2.1         | Implicated in ER stress response; antiapoptotic |
| 1448820_a_at| Eif2s2      | eukaryotic translation initiation factor 2 | 1.8         | Implicated in ER stress response; antiapoptotic |
| 1416429_a_at| Cat         | catalase   | 1.6         | Implicated in protecting the cells against oxidative damage |
| 1417193_at  | Sod2        | superoxide dismutase 2, mitochondrial | 1.4         | Implicated in protecting the cells against oxidative damage |
| 1427844_a_at| Cebp        | CCAAT/enhancer binding protein (C/EBP), beta/CHOP | -2          | Implicated in ER stress response; proapoptotic |

Abbreviation: ER, endoplasmic reticulum.
compounds. It would be intriguing to isolate and characterize these high molecular weight components and to identify those with antiapoptotic properties.

In conclusion, a heightened state of oxidative and ER stresses and apoptotic-based cell death contribute to β-cell deterioration and death seen in DM. In addition to its insulin-releasing effects (Al-Romaiany et al., 2013; Al-Romaiany et al., 2010; B. Liu et al., 2009), this study has shown for the first time that OSA® has protective effect on β-cells islets of Langerhans in vitro. Chronic OSA® treatment partially protected a β-cell line and primary islets from cytokine-induced apoptotic cell death, most likely mediated through the activation of CK-II and subsequent downstream effectors (Figure 6). Together, the results of the current study may suggest the possible use of OSA® as an important therapeutic option in the treatment of T2DM.

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CONFLICT OF INTEREST
The authors show no conflict of interests.

AUTHOR CONTRIBUTIONS
A. A. designed and conducted the experiments and also analyzed and interpreted the data from functional and microarray experiments. B. L. performed the RNA expression experiments. S. P. and P. J. contributed to designing some of the experiments and also to reading and correcting the manuscript. All authors read and approved the final manuscript.

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FIGURE 6 Proposed mechanisms of the antiapoptotic effect of Om Santal Adivasi (OSA®). The data from our microarray measurements of mRNA expression in cytokine- and/or OSA®-treated islets are consistent with a model in which OSA® activates casein kinase II (CK-II), which in turn modulates cell survival by either modifying caspase activities or the endoplasmic reticulum stress response or both. Activation of CK-II results in the stimulation of eukaryotic translation initiation factor 2/activating transcription factor 4/antioxidants and activating transcription factor 4/CAMP responsive element binding protein 4–CAMP responsive element binding protein 3-like 1 pathways leading to cell protection against endoplasmic reticulum stress. Furthermore, CK-II also has been shown to inhibit CCAAT/enhancer binding protein, beta and thus prevent cell apoptosis. ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; CHOP, CCAAT/enhancer binding protein, beta; CREB4, cAMP responsive element binding protein 3-like 4; Eif2, eukaryotic translation initiation factor 2; OASIS, cAMP responsive element binding protein 3-like 1; SOD, superoxide dismutase. A pointed arrow means activation; a blunted arrow means inhibition.
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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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