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Antidiabetic Activity of *Nigella sativa* Seed Extract in Cultured Pancreatic β-cells, Skeletal Muscle Cells, and Adipocytes*

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Abstract

The seeds of *Nigella sativa* L. (NS), a plant of the Runanculaceae family, are used in traditional medicine in North Africa and the Middle East for the treatment of diabetes. Despite widespread use and a number of scientific studies, the target tissues and cellular mechanisms of action of this plant product are not well understood. This study evaluated the effects of NS seed crude ethanol extract on insulin secretion in INS832/13 and βTC-tet lines of pancreatic β-cells and on glucose disposal by C2C12 skeletal muscle cells and 3T3-L1 adipocytes. An 18-h treatment with NS amplified glucose-stimulated insulin secretion by more than 35% without affecting sensitivity to glucose. NS treatment also accelerated β-cell proliferation. An 18-h treatment with NS increased basal glucose uptake by 55% (equivalent to approximately two-fold the effect of 100 nM insulin) in muscle cells and approximately by 400% (equal to the effect of 100 nM insulin) in adipocytes; this effect was perfectly additive to that of insulin in adipocytes. Finally, NS treatment of pre-adipocytes undergoing differentiation accelerated triglyceride accumulation comparably with treatment with 10 μM rosiglitazone. It is concluded that the well-documented *in vivo* antihyperglycemic effects of NS seed extract are attributable to a combination of therapeutically relevant insulinotropic and insulin-like properties.

Keywords: Glucose uptake, insulin secretion, natural health products, traditional medicine, type 2 diabetes mellitus.

Introduction

Type 2 diabetes mellitus is a disorder characterized by defects in insulin action and insulin secretion resulting in glucose intolerance and chronically elevated glycemia (Bianchi et al, 2007; Meece, 2007). More than 150 million people worldwide are afflicted by this condition, and this figure is expected to double in the next 20 years (Boutayeb et al., 2004). Current medications for the treatment of type 2 diabetes mellitus are limited, and no one medication can enhance insulin secretion and sensitivity simultaneously (Cohen et al., 2007); there is a clear need for new and more active therapeutic agents. Products from the plant realm hold tremendous potential for meeting this growing need. Indeed, thousands of plant species are documented to produce antihyperglycemic effects when ingested (Alarcon-Aguilara et al., 1998; Marles et al., 1995; Yeh et al., 2003), and metformin, the most widely prescribed insulin-sensitizer, is derived from a metabolite isolated from *Galega officinalis* L., a plant consumed for centuries as a treatment for diabetes (Bailey, 2000).

*Dedicated to Professor John Thor Arnason of the University of Ottawa, Department of Biology, on the occasion of his sixtieth birthday.

†The authors, Benhaddou-Andaloussi and Martineau, contributed equally to this work.

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Nigella sativa L. (NS) is a herbaceous plant of the Ranunculaceae family that grows spontaneously and widely in several southern Mediterranean and Middle Eastern countries (Haddad et al., 2006). The seeds of NS, also known as black seed or black cumin, are often used as a spice but are also used extensively in the traditional medicine of many of these countries, including Morocco, to treat diabetes and several other disorders (Bellakhdar et al., 1991; Ziyyat et al., 1997; Haddad et al., 2001). The antihyperglycemic effects of NS have been the object of numerous *in vivo* scientific studies using various animal models (Al-Awadi et al., 1985, 1987, 1991; Al Hader et al., 1993; Labhal et al., 1997, 1999; Meral et al., 2001; El-Dakhakhny et al., 2002; Fararh et al., 2002; Kanter et al., 2003; Le et al., 2004; Rchid et al., 2004; Haddad et al., 2006). Whereas *in vivo* effects are well-known, few studies have addressed the mechanisms of action of NS extract, and there remains controversy regarding its site(s) of action (Haddad et al., 2006). The aim of this study was to elucidate target tissues through which NS extract exerts its antihyperglycemic action by using cell-based assays of antidiabetic activity. This study demonstrates that NS seed extract possesses the unusual and therapeutically relevant properties of increasing insulin secretion while simultaneously exerting insulin-like effects in peripheral tissues.

Materials and Methods

Plant material

Seeds of NS were obtained from a herbalist in Rabat, Morocco, in August 2003 and were authenticated by an experienced botanist (Prof. Oulyahya, Institut Scientifique, Rabat, Morocco). A voucher specimen has been deposited in the herbarium of the Institut Scientifique of Rabat. Seeds were washed and dried and then powdered with an electric micronizer. Powder was extracted 3 times with 80% ethanol, and the solvent was evaporated at 40°C under reduced pressure. This procedure resulted in a two-phase extract. The oily and the solid phases were recombined in proportion to their yield (typically 55% and 45%, respectively) and solubilized freshly in DMSO prior to treatment of cells. The seeds of *Trigonella foenum-graecum* L. (fenugreek) were purchased from Lone Wolf Herb (Phippen, SK, Canada) for use as a positive control and were extracted similarly to NS. All extracts were conserved at 4°C in a dessicator and protected from light and humidity.

Reagents and cell lines

Cell culture reagents were purchased from Invitrogen (Burlington, ON, Canada) and Wisent (Saint Bruno, QC, Canada). Other reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA), unless otherwise noted. INS832/13 rat β pancreatic cells and βTC-tet murine β pancreatic cells were generous gifts from B. Newgard (Duke University) and S. Efrat (Tel-Aviv University), respectively. C2C12 murine myoblasts and 3T3-L1 murine pre-adipocytes were purchased from ATCC (Manassas, VA, USA).

Cell culture

C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovin serum (FBS), 10% horse serum (HS), and antibiotics (penicillin 100 IU/mL, streptomycin 100 μg/mL) at 37°C and 5% CO_2. Upon 75–80% confluence, serum content was reduced to 2% HS in order to induce differentiation into myotubes. Medium was changed every 48 h. Experiments were performed on well-differentiated multinucleated cells 6 to 7 days later. 3T3-L1 cells were grown in DMEM supplemented with 10% FBS and antibiotics until 75–80% confluent. 3-Isobutyl-1-methylxanthine (IBMX; 500 μM), insulin (500 nM), and dexamethasone (10 μM) were then added to the medium for 2 days to induce adipogenesis. Cells were allowed to differentiate for an additional 10 to 14 days in insulin-containing (500 nM) medium until more than 90% of cells contained lipid droplets visible by phase-contrast microscopy. INS832/13 cells were grown in RPMI 1640 medium containing 11 mM glucose and supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and antibiotics until 80% confluent. βTC-tet cells were grown in DMEM supplemented with 15% HS and 2.5% FBS until 80% confluent. All cells were treated with 200 μg/mL total ethanol extract in culture medium with a final DMSO concentration of 0.1%, with the exception of INS832/13 treated at a dose of 100 μg/mL. This concentration was based on a maximal solubility of 200 mg/mL in DMSO. The concentrations tested did not induce any morphologic changes following 18-h treatment in any of the four cell types employed.

Insulin secretion assay

The INS832/13 and βTC-tet cell lines were used to test for effects of NS on potentiation of glucose-stimulated insulin secretion (GSIS). These pancreatic β-cell lines release insulin in response to physiologic concentrations of glucose in a dose-dependent manner (Knaack et al., 1994; Efrat et al., 1995; Fleischer et al., 1998; Hohmeier et al., 1998). INS832/13 cells were seeded in 12-well plates at a density of 2×10^5 to 4×10^5 cells per well and incubated in growth medium for 24 h in complete RPMI until 80% confluence. Cells were then treated for 18 h with or without NS and with glucose adjusted to 3 mM to confer glucose sensivity to the cells. βTC-tet cells were seeded in 12-well plates at a density of 2.5×10^5. Upon reaching 80% confluence, growth medium was supplemented with 1 μg/mL tetracycline for 48 h to arrest proliferation and to remove any influence of potential proliferative effects on measurement of insulin secretion.

The βTC-tet cell line is developed from transgenic mice expressing the SV40 T antigen (Tag) under the control of the insulin II promoter and regulated by the tetracycline conditional gene expression system (Efrat et al., 1995; Fleischer et al., 1998). This cell line is dependent on Tag expression for its proliferation: in the absence of tetracycline, the Tag transgene is expressed and the cells proliferate normally, whereas in the presence of tetracycline, Tag expression is shut off and cell replication is arrested. Growth-arrested βTC-tet cells were then treated for 18 h with or without NS extract. β pancreatic cell lines were rinsed and preincubated with Krebs-Ringer HEPES buffer (KRHB) (10 mM HEPES, 2 mM $NaHCO_3$,0.5 mM NaH_2PO_4, pH 7.4, 135 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl, 0.5 mM $MgCl_2$, and 0.07% fatty-acid-free BSA) for 1 h at 37°C in presence or absence of the extract. For INS832/13 cells, the medium was then replaced with KRHB containing 2 mM glucose in the presence or absence of the extract (basal insulin secretion) or KRHB containing 4 mM or 11 mM glucose, or 30 mM KCl in the presence or absence of extract for an additional period of 1 h. βTC-tet cells were treated with 2 mM (basal insulin secretion), 6, 8, 10, or 16 mM glucose, or 30 mM KCl for 1 h in the presence or absence of extract in buffer supplemented with 0.5% IBMX. Samples of culture medium were taken at the end of this period. Samples were centrifuged 3 min at 4°C at 3000 $\times$ *g*, and supernatants were stored at −20°C until assayed by radioimmunoassay (RIA). Insulin secretion in INS832/13 was normalized to the total cellular protein content by the bicinchoninic acid method (Pierce, Biolynx, Brockville, ON, Canada). Insulin secretion was expressed per well for βTC-tet cells as all wells contained approximately the same number of cells. Total cellular insulin content was also measured in unstimulated (basal state) cells. Insulin was extracted in 1.25% HCl–75% ethanol, and samples were kept overnight at 4°C. These samples were sonicated and centrifuged at 30,000 $\times g$ for 5 min before measurement of insulin in the supernatant by RIA.

For analysis of basal and stimulated insulin secretion and total cellular insulin content, samples were placed on ice and appropriate dilutions were made with phosphate buffer (50 mM Na_2HPO_4, pH 7.5, 25 mM EDTA, 1% BSA RIA-grade, 0.01% thimerosal). Diluted samples were incubated in 12 $\times$ 75 mm polypropylene RIA tubes with rat ^{125}I-insulin (Linco Research, Saint Charles, MO, USA) and a primary antibody against rat insulin (Linco, no. 1013) at 4°C overnight in the dark. The tubes were then incubated with the precipitating reagent (Linco, no. 2020) for 20 min at 4°C, and tubes were then centrifuged at 5350 $\times$ *g* for 15 min. Radioactivity in the pellet was measured by gamma counter (Wallac Wizard 1470; Perkin Elmer, Woodbridge, ON, Canada). Human insulin was used as a standard. Four to six replicates were performed for each experimental condition.

βTC-tet proliferation assay

To test for a proliferative effect of NS on β cells, extract was applied to replicating (non–growth arrested) βTC-tet cells, and incorporation of ^{3}H-thymidine was evaluated. Cells were seeded in 24-well plates at a density of 1.0×10^5 and incubated in growth medium for 24 h. Incubation was continued for another 48 h in growth medium while one group was treated with tetracycline (1 μg/mL) in order to arrest growth, as above. Replicating cells were then incubated 18 h in presence or absence of NS. Methyl ^{3}H-thymidine (1 μCi/mL; 4 Ci/mmol; no. 2404105, MP Biomedicals, Irvine, CA, USA) was added over the last 6 h of treatment to all cells. Cells were then rinsed 3 times in PBS, lysed with 0.1 M NaOH for 30 min, and scraped. The lysates were added to 4 mL liquid scintillation cocktail (Ready-Gel 586601; Beckman Coulter, Fullerton, CA, USA), and incorporated radioactivity was measured in a scintillation counter (LKB Wallac, model 1219; Perkin Elmer, Woodbridge, ON, Canada). Four replicates were performed for each experimental condition.

Glucose transport assay

Differentiated C2C12 and 3T3-L1 cells grown in 12-well culture plates were treated with 200 μg/mL NS extract for 18 h or 1 h. A vehicle control (DMSO), and positive controls [400 μg/mL metformin or 75 μg/mL fenugreek (Vats et al., 2002; Spoor et al., 2006)] were included in each experiment. Three hours prior to the experiment, the medium was replaced with serum-free DMEM containing extract to establish a baseline insulin-free state. After the treatment, cells were incubated for 30 min with 0, 1, or 100 nM insulin in Krebs-phosphate buffer (20 mM HEPES, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, pH 7.4, 120 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgSO_4$) containing 5 mM glucose and NS extract or appropriate positive and vehicle controls at 37°C. Cells were then washed twice in warm glucose-free Krebs-phosphate buffer before incubation for exactly 10 min at 37°C in glucose-free Krebs-phosphate buffer containing 0.5 μCi/mL 2-deoxy-D-[1–^{3}H]glucose (TRK383; Amersham Biosciences, Buckinghamshire, UK). Cells were then placed on ice and immediately washed 3-times with ice-cold Krebs-phosphate buffer. Cells were lysed and scraped in 0.1 N NaOH. Lysates were transferred to 4 mL Ready-Gel, and radioactivity was counted in a liquid scintillation counter, as above. Two separate experiments of three to four replicates were performed.

Rate of adipogenesis assay

PPARγ agonists, such as the commonly prescribed rosiglitazone and other members of the thiazolidinedione (glitazone) family, are known to increase insulin sensitivity in muscle and adipose tissue (Konrad et al., 2005). One of the hallmarks of glitazones is their ability to increase rate of adipogenesis. To test NS for glitazone-like activity, we performed a rate of adipogenesis assay, as performed elsewhere (Martineau et al., 2005; Spoor et al., 2006). Briefly, 3T3-L1 pre-adipocytes were seeded in 12-well plates and allowed

Table 1. Effect of NS on insulin secretion and insulin content in INS832/13 cells.

	Glucose concentration (mM)				
Treatment	2	4	11	2 + 30 mM KCl	Total insulin content
DMSO 0,1%	221 ± 28	331 ± 13	737 ± 33	411 ± 23	8113 ± 514
Nigella 200 μg/mL	606 ± 40*	638 ± 51*	1213 ± 110*	821 ± 43*	7678 ± 650

Data expressed as ng of insulin mg of protein^{-1} h^{-1}.
Result are mean ± SEM for n = 6.
*Significantly different ($p \leq 0.05$) from vehicle control group.

to proliferate until fully confluent. One day after confluence, proliferation medium was replaced with differentiation medium containing the hormone cocktail, as above, for 2 days and with insulin-supplemented medium, as above, for an addition 4 days, resulting in incomplete differentiation and visible intracellular triglyceride droplets in a small percentage of control cells at the end of this period. During this entire differentiation period, cells were treated with NS extract or 0.1% DMSO vehicle in differentiation medium. Vehicle in proliferation medium was used as a negative control. Rosiglitazone (Alexis Biochemicals, San Diego, CA, USA), solubilized in DMSO, was used as a positive control at 10 μM. Medium was changed every 24 h during differentiation. At the end of the sixth day, cell morphology was evaluated by phase-contrast microscopy, and cellular triglyceride content was determined as a marker of differentiation and used to compare the rate of differentiation between conditions. Triglyceride content was quantified using the AdipoRed dye assay (Cambrex Bioscience Inc, Walkersville, MD, USA). 3T3-L1 adipocytes were washed gently with phosphate-buffered saline before the addition of 60 μl AdipoRed reagent per well. Fluorescence was measured in a plate reader (Wallac Victor2, Perkin Elmer, Woodbridge, ON, Canada) at $\lambda_{ex} = 485$ nm, $\lambda_{em} = 572$ nm. Four replicates were performed for each experimental condition. The mean value obtained from the negative control condition was considered as background and subtracted from all other readings.

Statistical analysis

All data are reported as the mean ± SEM of the indicated number of experiments. Results were analysed by one-way analyzis of variance (ANOVA) and Fisher *post hoc* test using StatView software (SAS Institute Inc, Cary, NC, USA). Statistical significance was set at $p \leq 0.05$.

Results

NS treatment potentiates pancreatic insulin secretion

NS extract was tested for insulinotropic activity in insulin-secreting INS832/13 and βTC-tet cells. After an 18-h treatment with NS extract, insulin secretion over a period of 1 h was assessed in response to various concentrations of glucose and in response to potassium. In both cell types, insulin secretion was significantly increased under all conditions; in INS832/13 cells, secretion was increased by approximately 400 ng mg protein^{-1} h^{-1} (Table 1), and in growth-arrested βTC-tet cells, secretion was increased proportionally by 39–49%. The dose-response relationship between glucose and insulin secretion was shifted upward (Fig. 1a), indicating no change in the triggering mechanisms of insulin release but an increase in the stimulus amplification mechanisms (Henquin, 2000). The insulinotropic effects were not accompanied by a significant change in total cellular insulin content (Table 1 and Fig. 1c).

NS treatment accelerates pancreatic β cell proliferation

The insulinotropic effect of NS treatment was smaller in growth-arrested βTC-tet cells than in proliferating INS832/13 cells, suggesting that NS may exert a proliferative effect. Indeed, such an effect has been proposed to explain the protective effect of NS treatment against streptozotocin-destruction of β-cells *in vivo* (Kanter et al., 2003). In order to test whether NS can stimulate β-cell proliferation, replicating insulin-secreting βTC-tet cells were incubated 18 h with NS or vehicle, and ^{3}H-thymidine incorporation over the last 6 h was assessed. NS treatment significantly increased DNA synthesis over this period by 13% compared with the DMSO vehicle control (Fig. 2).

NS treatment enhances glucose uptake in muscle cells and adipocytes

NS extract was tested for insulin-like properties by assessing basal and insulin-stimulated glucose uptake in differentiated C2C12 myotubes and 3T3-L1 adipocytes, two insulin-responsive and GLUT-4–containing cell lines (He et al., 2007). After an 18-h treatment with NS extract, basal glucose uptake was markedly increased in both muscle (Fig. 3) and fat (Fig. 4) cells. In muscle cells, basal uptake was increased by 55%, corresponding to 2.1-times the effect of 100 nM insulin (a supraphysiologic dose). The effect of NS treatment could not be further increased by acute stimulation with insulin (1 or 100 nM); indeed, NS treatment combined with 100 nM insulin for 30 min resulted in

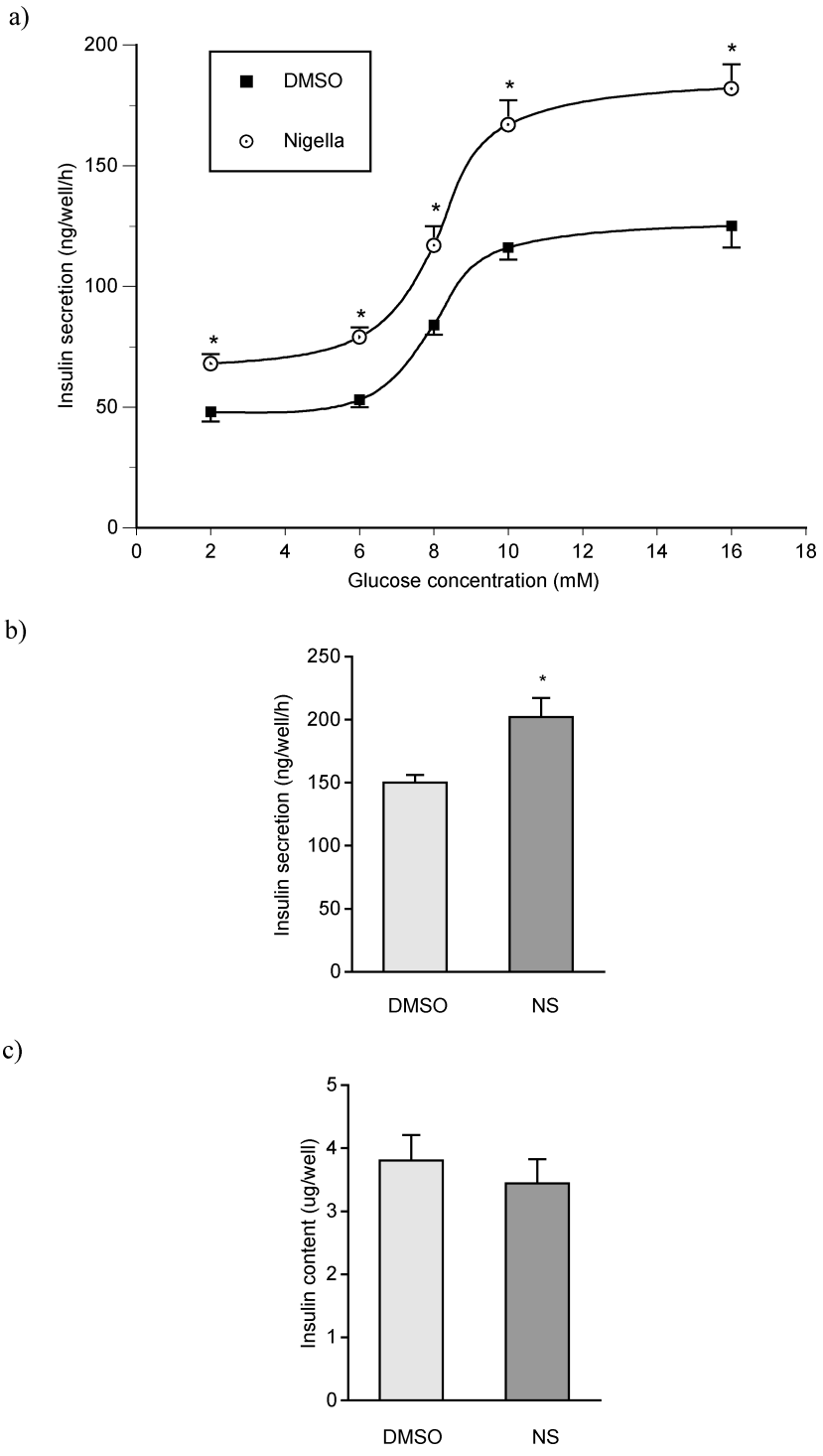

Figure 1. The effect of NS seed extract on glucose-stimulated insulin secretion, potassium-stimulated insulin secretion, and total insulin content was assessed in growth-arrested βTC-tet pancreatic β-cells. Cells were treated with 200 μg/mL extract or with vehicle only (0.1% DMSO) for 18 h. Insulin secretion was measured over a 1-h period in response to (a) 2, 6, 8, 10, or 16 mM glucose, or (b) 2 mM glucose and 30 mM KCl. Results are expressed as micrograms of insulin per well per hour. (c) Intracellular insulin content was measured in cells under basal (2 mM glucose) condition. Results are expressed as micrograms insulin per well. Mean $\pm$ SEM for n = 4. * Significantly different ($p \leq 0.05$) from the corresponding control (DMSO).

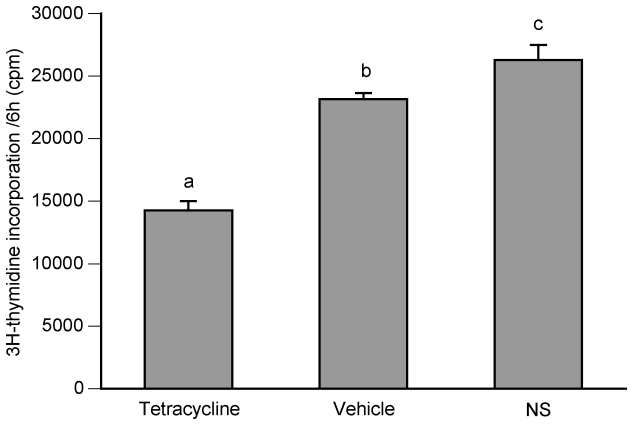

Figure 2. The effect of NS extract on pancreatic β-cell proliferation was assessed in βTC-tet cells. Replicating cells were treated with 200 μg/mL NS extract or with vehicle only (0.1% DMSO) for 18 h, whereas growth-arrested cells were treated with vehicle only. During the last 6 h of treatment, cells were exposed to ^{3}H-thymidine. Cells were washed and lysed, and incorporated radioactivity was then assessed. Result are expressed as counts per minute (cpm) per well per 6 h. Mean $\pm$ SEM for n $=$ 4. Different letters denote significant differences ($p \leq 0.05$) between conditions.

2.3-fold more uptake than 100 nM insulin in vehicle-treated cells (Fig. 3a). In adipocytes, basal uptake was increased four fold, which is equal to the effect of 100 nM insulin. In contrast with muscle cells, the effect of acute insulin stimulation (1 or 100 nM) was perfectly additive to that of NS treatment; consequently, 100 nM insulin in NS-treated cells resulted in an increase of 2.3-fold more than 100 nM insulin in vehicle control cells (Fig. 4a). A 1-h treatment with NS extract resulted in smaller increases in basal uptake, corresponding with approximately one-half of the effect of 100 nM insulin in either cell type. Furthermore, the effect of acute insulin stimulation (1 or 100 nM) was not additive to that of NS treatment in either cell type (Fig. 3b and 4b). Taken together, these data suggest that NS and insulin elicit their acute responses through a common pathway and that a longer NS treatment may increase maximal glucose uptake through an upregulation of the content of key proteins involved in uptake.

NS treatment accelerates adipogenesis

To test for glitazone-like activity, triglyceride accumulation was measured in differentiating 3T3-L1 adipocytes treated with NS extract throughout their differentiation period. At day 6 of differentiation, a time when differentiation is incomplete and lipid droplets are observable in a small percentage of vehicle control cells, chronic NS treatment resulted in 3.5-fold higher triglyceride content than in control cells, indicating a higher rate of adipogenesis (Fig. 5). This effect was comparable with the effect of 10 μM rosiglitazone.

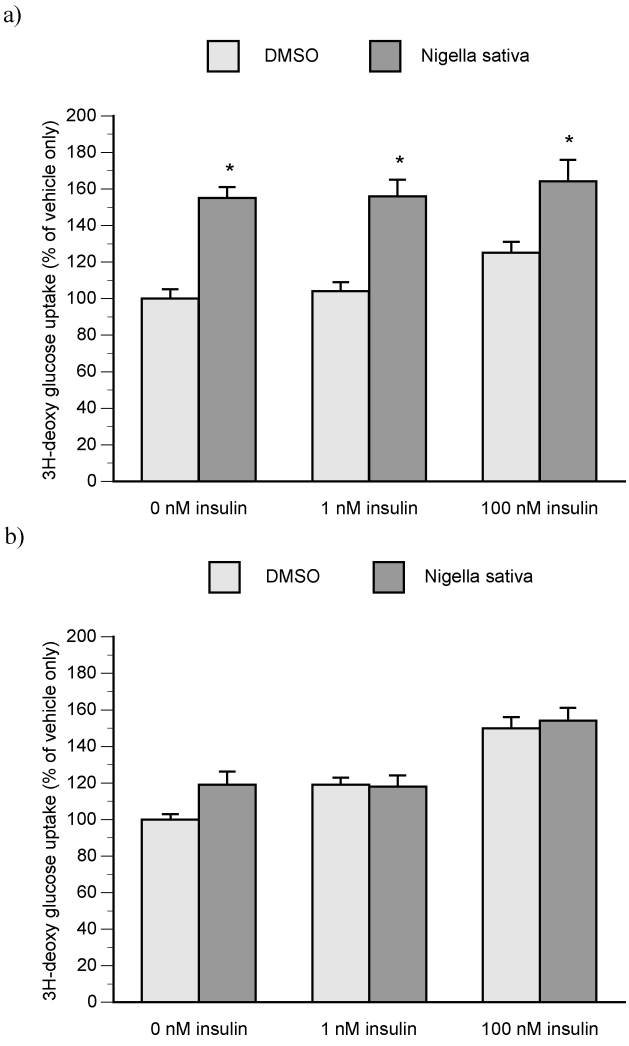

Figure 3. Basal and insulin-stimulated ^{3}H-deoxyglucose uptake were assessed in C2C12 myotubes treated with 200 μg/mL NS extract or vehicle (0.1% DMSO) for (a) 18 h or (b) 1 h. Data are expressed normalized to basal uptake in vehicle control condition. Mean $\pm$ SEM for n $=$ 5 to 6. * Significantly different ($p \leq 0.05$) from vehicle control corresponding with same insulin condition.

Discussion

Nigella sativa seed extract is used in the traditional medicine of numerous North African countries as a treatment for diabetes (Bellakhdar, 1991; Ziyyat et al., 1997; Haddad et al., 2001, 2006). The *in vivo* antihyperglycemic activity of this extract is well-known (Al-Awadi et al., 1985, 1987, 1991; Al Hader et al., 1993; Labhal et al., 1999; Meral et al., 2001; El-Dakhakhny., 2002; Farah et al., 2002; Kanter et al., 2003). Although this action has been mainly attributed to insulinotropic effects, observed in animal studies as well as in isolated pancreatic islets (Rchid et al., 2004), the contribution of peripheral sites of action has been suggested (Al-Awadi et al., 1991; Fararh et al., 2004). The purpose of this study was to use cell-based assays of

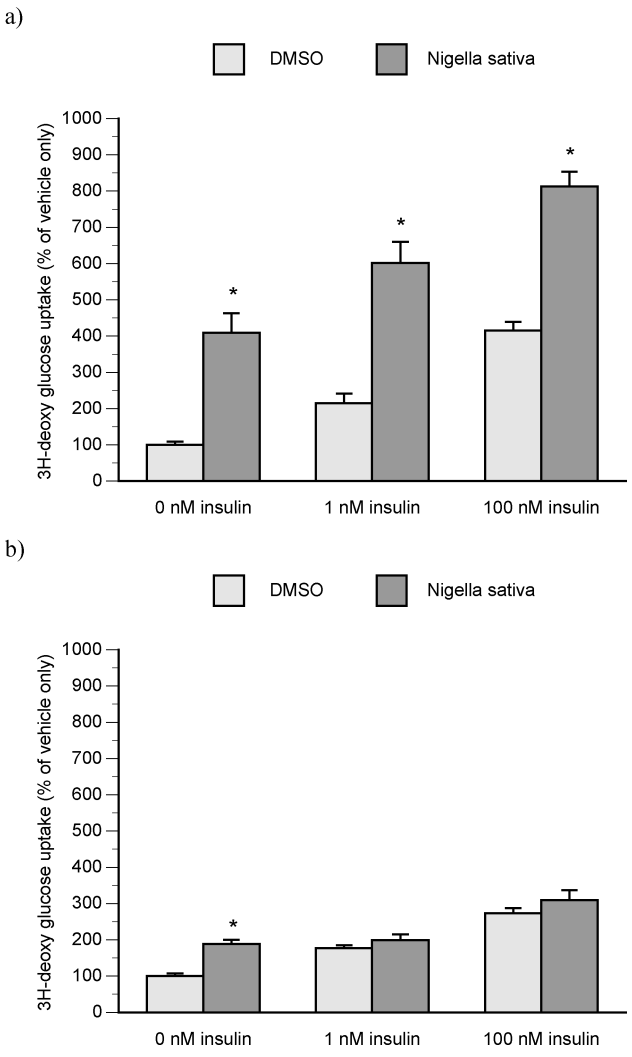

Figure 4. Basal and insulin-stimulated ^{3}H-deoxyglucose uptake were assessed in 3T3-L1 adipocytes treated with 200 μg/mL NS extract or vehicle (0.1% DMSO) for (a) 18 h or (b) 1 h. Data are expressed normalized to basal uptake in vehicle control condition. Mean $\pm$ SEM for n = 6 to 8. * Significantly different ($p \leq 0.05$) from vehicle control corresponding with same insulin condition.

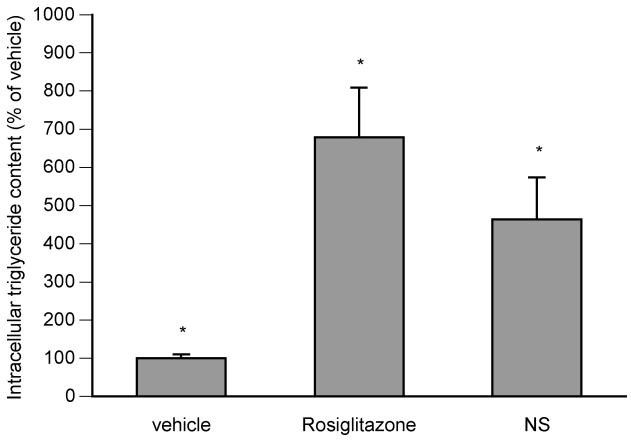

Figure 5. The rate of accumulation of triglycerides was assessed in differentiating 3T3 pre-adipocytes treated for 6 days with 200 μg/mL of NS extract, vehicle (0.1% DMSO), or 10 μM rosiglitazone in differentiation medium. At the end of the treatment period, triglyceride content was assessed by AdipoRed fluorescent dye. Results are expressed normalized to vehicle control in differentiation minus vehicle control in proliferation medium. Mean $\pm$ SEM for n = 4. * Significantly different ($p \leq 0.05$) from vehicle control.

antidiabetic activity to elucidate the cellular site(s) of action of NS biological activity. NS was confirmed to exert an important insulinotropic effect on pancreatic β-cells and was also found to have significant insulin-like effects in peripheral tissues, namely the stimulation of basal glucose uptake in skeletal muscle cells and adipocytes, in addition to the rosiglitazone-like enhancement of adipogenesis.

We observed that NS increases insulin secretion by pancreatic β-cells while preserving normal glucose sensitivity, thereby resulting in an upshifted relationship between insulin secretion and glucose concentration. Our study is thus in agreement with others that have observed that the basic and nonlipidic subfractions of NS seeds increase insulin secretion in isolated islets of Langerhans (Rchid et al., 2004). Others, using the volatile oil fraction, failed to see a pancreatic effect (El-Dakhakhny et al., 2001). This suggests that the secretayogue activity may be found in the nonlipidic components of our crude ethanol extract, and further studies will be necessary to elucidate this point.

Our study also shows that NS treatment does not affect the intracellular insulin content of the two lines of pancreatic β-cells used but exerts a proliferative effect on βTC-tet cells. A proliferative or regenerative activity has been suggested by others who have observed increased insulinemia after treatment of rats having undergone partial destruction of their β-cell mass with streptozotocin (Kanter et al., 2003).

We have also examined the biological activity of NS in extrapancreatic tissues, notably insulin-responsive skeletal muscle and adipose cells. In both cell types, treatment with NS extract significantly increased basal glucose uptake. In response to a 1-h treatment time, the relative increase in basal uptake corresponded with approximately one-half the effect of 100 nM insulin in either cell type, whereas in response to an 18-h treatment, the relative increase corresponded with double the effect of 100 nM in muscle cells or with the same effect as 100 nM insulin in adipocytes. The fact that the 18-h treatment resulted in a greater effect and that combined with acute insulin stimulation, it increased uptake above that which can be induced by a supraphysiologic dose of insulin in untreated cells (2.3-fold the effect of 100 nM insulin in either cell types), strongly suggest that NS affected gene expression of proteins involved in glucose transport. Such an effect would be compatible with stimulation of the AMP-activated protein kinase (AMPK) (Winder, 2000). Interestingly, the effect of the 1-h treatment was lost or masked by acute insulin stimulation; it

is unclear whether a similar phenomenon would have been observed after an 18-h treatment with a lower concentration of NS extract and whether this is indicative of NS and insulin acting through a common signaling pathway. Finally, whereas the relative magnitude of the effect of NS treatment on basal uptake was more important in muscle cells than in adipocytes, it is unclear whether NS exerts its effects through similar mechanisms in both cell types.

Similar insulinomimetic or insulin-sensitizing actitivites could also extend to hepatocytes, where reduced gluconeogenesis would further contribute to an overall antiglycemic effect of NS, as proposed by other investigators (Al-Awadi et al., 1991; Farah et al., 2004). In support of this, our laboratory has previously showed that NS exerts an insulin-sensitizing effect on hepatocytes isolated from normal rats after 4 weeks of *in vivo* treatment (Le et al., 2004).

The effect of NS treatment on rate of adipogenesis was evaluated in order to screen for glitazone-like properties. The glitazones, including the widely prescribed insulin-sensitizer rosiglitazone, are PPARγ agonists, and a consequence of their action in adipocytes is increased adipogenesis (Lazar, 2005). The antidiabetic effects of glitazones are attributable to PPARγ activation in adipocytes as well as to an activation of AMPK in muscle (Lazar, 2005). Chronic treatment with NS extract was found to significantly increase rate of adipogenesis, similar to rosiglitazone. It remains to be determined whether this is the result of PPARγ agonism or of some other mechanism and whether this activity is related to the observed enhancement of glucose uptake resulting from shorter treatment durations.

In summary, the results of our cell-based functional assays successfully identified sites of action that can explain the documented antihyperglycemic effects of NS seed extract. We have confirmed a proposed insulinotropic action of NS at the level of pancreatic β-cells and presented evidence of a proliferative effect of the plant extract on these cells as well. This holds promise for the maintenance of β-cell mass and a slowing of the progression of type 2 diabetes. Our study further provides novel and direct evidence for an insulin-like or insulin-sensitizing action of NS extract at the level of skeletal muscle and adipose tissue. Part of the action of NS extract may be related to stimulation of insulin-dependent and -independent intracellular signaling pathways, as well as to PPARγ agonism. In conclusion, NS seed ethanol extract exhibits the remarkable ability to concomitantly increase insulin secretion, induce proliferation of pancreatic β-cells, and stimulate glucose uptake in muscle and fat cells. Such beneficial properties warrant further work to elucidate molecular targets of NS seed extract and to isolate and identify the active compound(s).

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