Supplemental Materials

Supplemental Methods

Pancreatic islet isolation and culture

Human pancreatic islets were cultured in Medium 199 with Earle's salts (Sigma-Aldrich Inc.) containing 5 mmol/l glucose and supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% v/v penicillin and streptomycin, 50 µg/ml gentamicin (all from ThermoFisher Scientific), and 0.25 µg/ml amphotericin (Aurogene s.r.l., Rome, Italy).

Mouse pancreatic islets were cultured in RPMI 1640 medium containing 11 mmol/l glucose and supplemented with 10% v/v heat-inactivated FBS and 1% v/v penicillin and streptomycin.

INS-1E cells culture

Rat insulin-secreting INS-1E cells were cultured in RPMI 1640 medium containing 11 mmol/l glucose and supplemented with 10% v/v heat-inactivated FBS, 1% v/v penicillin and streptomycin, 1% v/v non-essential amino acids (ThermoFisher Scientific), 10 mmol/l HEPES pH 7.4, 1 mmol/l pyruvic acid, and 50 µmol/l beta-mercaptoethanol (all from Sigma-Aldrich) in a 5% CO_2 atmosphere at 37 °C.

Pancreatic islets and INS-1E cells treatments

Palmitate was prepared by dissolving the powder in 0.1 mol/l NaOH at 70 °C to obtain a 5 mmol/l palmitate solution, then complexing it with 10% FA-free BSA (FA to BSA molar ratio of 3.3:1). Oleate was prepared by complexing a solution of 0.5 mol/l oleate in ethanol with 10% FA-free BSA at 37 °C.

In INS-1E cells, the insulin concentration was 9 nmol/l (± 1 nmol/l, n=3) in the KRBH buffer (after the wash out and prior to stimulation with 10 nmol/l insulin), and 71 nmol/l (± 9 nmol/l, n=3) in the culture medium (after stimulation with BSA as control for palmitate stimulation and prior to stimulation with 100 nmol/l insulin). Therefore, the doses of insulin used for the execution of the experiments can be considered consistent with the insulin physiological levels in INS-1E cells.

To achieve $p66^{Shc}$ knockdown, INS-1E cells were seeded in 6-well dishes with 2 ml of complete medium until a confluence of 70%, then transfected with 100 nmol/l $p66^{Shc}$ siRNA using 2.5 µl/well Lipofectamine® RNAiMAX Reagent and 0.5 ml/well Opti-MEM® medium (ThermoFisher Scientific) for 48 h. During the last 24 h, the cells were incubated with palmitate or BSA as control, then stimulated with insulin. Control cells were treated with Lipofectamine only.

To achieve $p66^{Shc}$ hyperexpression, INS-1E cells were grown in 6-well dishes until they reached 60% confluency. The cells were infected with 1 µl of the adenovirus constructs in 400 µl of medium and incubated at 37 °C and 5% CO_2 for 90 min, after which 1600 µl of the medium was added. After 24 hours, the cells were treated according to the different experimental conditions. Where indicated, INS-1E cells were transfected with an adenoviral $p66^{Shc}$ construct harboring a Ser^{36} to Ala^{36} mutation. An empty adenovirus vector was used as control (mock). Viral titers used

were: $5.2*10^7$ PFU/ml for Ad/mock, $2.5*10^5$ PFU/ml for Ad/p66^{Shc}, and $1.2*10^5$ PFU/ml for Ad/p66^{Shc} (Ala^{36}).

Where indicated, INS-1E cells were treated with 30 µmol/l SP600125 for 2 h or 30 µmol/ pifithrin-alpha for 1 h (both Sigma-Aldrich) prior to stimulation with palmitate/BSA to achieve the inhibition of JNK or p53 protein activity, respectively.

Immunoblotting

Equal amounts of proteins were resolved by electrophoresis on 6% or 10% w/v sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene difluoride or nitrocellulose membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories). Proteins were visualized by a chemiluminescence reaction, and images were captured using a Versadoc or a ChemiDoc Imaging System, and quantified by densitometric analysis using Quantity One image analysis software or the Image Lab Software 6.1, respectively (all from Bio-Rad Laboratories).

Quantitative Real-Time PCR

Pancreatic islets RNA was prepared using 1 ml of TRIzol reagent (ThermoFisher Scientific) for each pellet. Islets were homogenized using IKA T10 basic homogenizer (Sigma-Aldrich) for 1 min, 200 µl chloroform (Merck KGaA, Darmstadt, Germany) was added to each sample, and the tubes were centrifuged at 1200 × g for 15 min at 4 °C. The upper aqueous phase was collected.

To obtain RNA from INS-1E cells, they were lysed in RLT buffer, and the lysate was homogenized by using QIAshredder columns (Qiagen).

Total RNA from pancreatic islets or beta-cells lysates was purified using the RNeasy Mini Kit (Qiagen); genomic DNA contamination was eliminated by DNase digestion (Qiagen). RNA concentrations were determined by Qubit Fluorometric Quantification (ThermoFisher Scientific). cDNA synthesis was performed on 500 ng total RNA using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). mRNA reverse transcription was performed using an Eppendorf Thermal Cycler (Eppendorf, AG, Hamburg, Germany). Real-time PCR reactions were performed using a 2X ready-to-use master mix (iTaq Universal SYBR Green Supermix purchased by Bio-Rad Laboratories) in 96-well hard-shell PCR plates covered with optically clear Microseal 'B' PCR plate sealing film (all from Bio-Rad Laboratories). Real-time PCR was performed in a Cfx Connect Real-Time System (Bio-Rad Laboratories) under the following conditions: 95 °C for 3 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Relative RNA levels were determined by analyzing changes in SYBR green fluorescence during PCR using the $2^{-\Delta\Delta Ct}$ method. *p66^{Shc}* mRNA levels were normalized using *18S* mRNA as the reference gene. No template controls were included for each analysed gene.

Reactive oxygen species (ROS) production measurements

Cells were stimulated with 1 mmol/l N-Acetyl-L-cysteine (NAC, Sigma-Aldrich) for 2 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h or 100 µmol/l H_2O_2 for 1 h. Intracellular ROS were detected through the evaluation of dihydroethidium (DHE) oxidation. INS-1E cells were seeded on glass coverslips, and incubated with 15 µmol/l DHE (ThermoFisher Scientific) in the dark at 37 °C for 15 min. The ROS-dependent oxidation of the

fluorescent probe (excitation 488 nm/emission 585 nm) was measured by acquiring fluorescent images on a Nikon ECLIPSE Ti-S fluorescence microscope (Nikon, Minato, Tokyo, Japan).

79 **Supplemental Table 1.** Characteristics of antibodies used for immunoblotting.

Specificity	Antibody	Dilution used
Phospho-AKT (Ser^{473})	Cell Signaling Technology Inc., #9271	1:1000
Total AKT	Cell Signaling Technology Inc., #4691	1:1000
Beta-actin	Santa Cruz Biotechnology, sc-47778	1:1000
Phospho-IRS 1 (Ser^{307})	Cell Signaling Technology Inc., #2381	1:500
Total IRS-1	Millipore, #06-248	1:500
Phospho-p70 S6K (Thr^{389})	Cell Signaling Technology Inc., #9205	1:1000
Total Shc	Merck Millipore, #06-203	1:1000
Phospho-p66^{Shc} (Ser^{36})	Invitrogen, #44828M	1:500
Phospho-c-Jun (Ser^{63}) II	Cell Signaling Technology Inc., #9261	1:1000
Acetyl p53 (Lys^{382})	Cell Signaling Technology Inc., #2525	1:500

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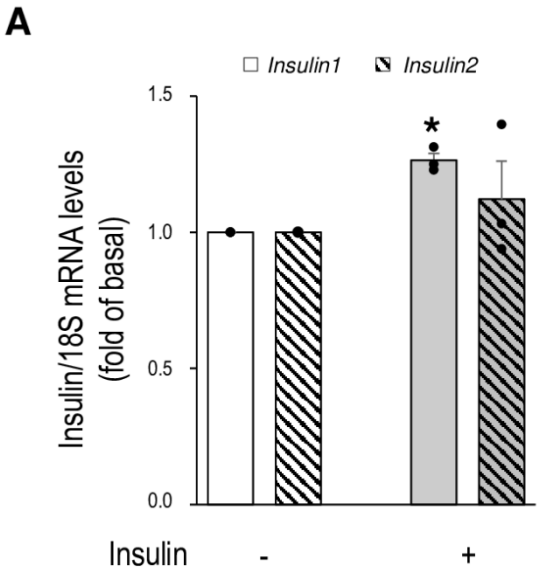
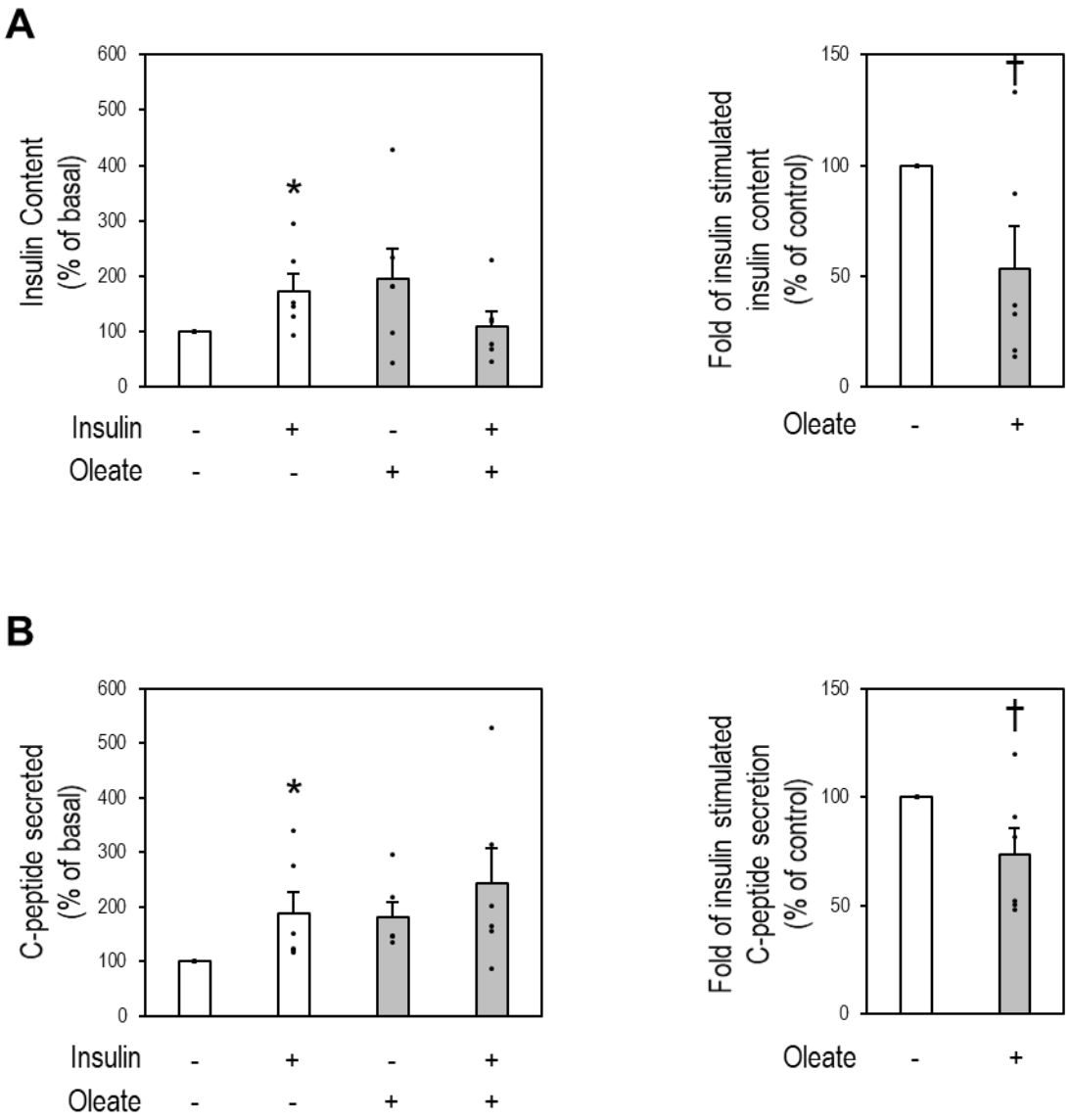
Supplemental Table 2. Primers used for quantitative real-time PCR analysis.

Gene	Species	Forward primer sequence	Reverse primer sequence
RNA18S5	Homo sapiens	5'-CGAACGTCTGCCCTATCAACTT-3'	5'-ACCCGTGGTCACCATGGTA-3'
p66^{Shc}	Homo sapiens	5'-CCCCCAAGCCCAAGTACAA-3'	5'-GACCCAGAAGCCCCTTCCT-3'
RNA18S	Rattus Norvegicus	5'-TGATTAAGTCCCTGCCCTTTGT-3'	5'-GATCCGAGGGCCTCACTAAA-3'
INS1	Rattus Norvegicus	5'-CTGCCCAGGCTTTTGTCAA-3'	5'-TCCCCACACACCAGGTACAGA-3'
INS2	Rattus Norvegicus	5'-GCAAGCAGGTCATTGTTCCA-3'	5'-GGTGCTGTTTGACAAAAGCC-3'

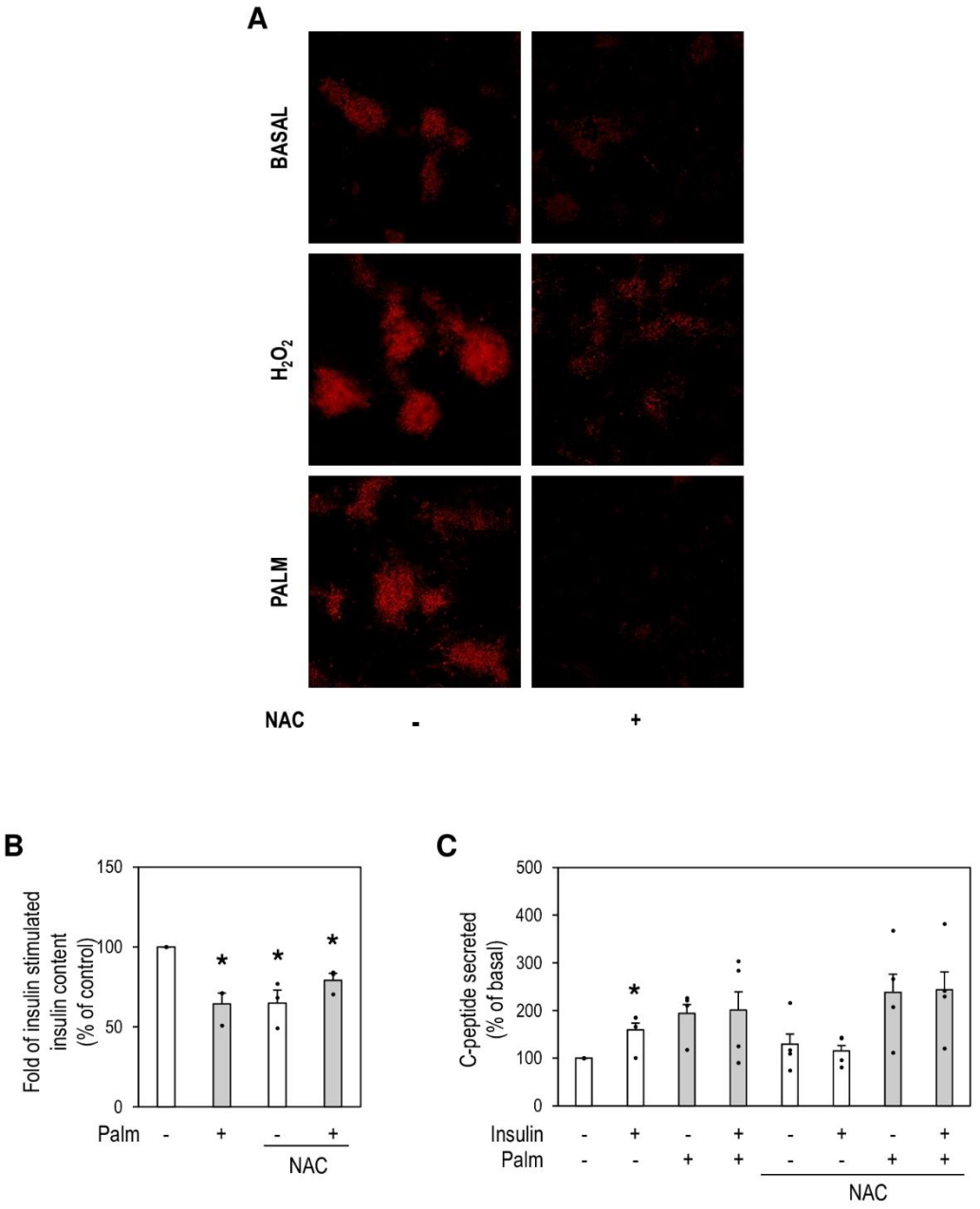
Supplemental Figures



Supplemental Figure 1. Insulin increases *Insulin 1* gene expression levels in INS-1E cells. **A**: Cells were incubated in KRBH buffer for 100 min, then stimulated with 10 nM insulin in fresh KRBH buffer for 40 min. *Insulin 1* and *Insulin 2* gene expression was evaluated by quantitative RT-PCR analysis normalized to 18S gene expression (n = 3 independent experiments). *p < 0.05 vs control. Data are expressed as the mean ± SEM.



Supplemental Figure 2. Effects of oleate on insulin-induced insulin content and C-peptide release in INS-1E cells. **A**, **B**: Cells were cultured in the presence of 0.5 mmol/l oleate (grey bars) or BSA (white bars), as a control, for 24 h, followed by incubation in KRBH buffer for 100 min, and stimulation with 10 nmol/l insulin in fresh KRBH buffer for 40 minutes. **A**: Insulin content was measured by enzyme-linked immunosorbent assay (ELISA), normalized to protein concentration, and expressed as a percentage of untreated control (n = 6 independent experiments); the fold-increase of insulin-stimulated insulin content over control (not treated with oleate) is also shown. **B**: Secreted C-peptide levels were measured by ELISA, normalized against total protein concentration, and expressed as a percentage of the untreated control (n = 6 independent experiments); the fold-increase of insulin-stimulated secreted C-peptide levels over control (not treated with oleate) is also shown. *$p < 0.05$ vs control without insulin; †$p < 0.05$ vs control without oleate. Data are expressed as the mean ± SEM.



Supplemental Figure 3. Effects of reactive oxygen species (ROS) on insulin activity in INS-1E cells. **A**: The treatment with NAC reduces dihydroethidium (DHE) fluorescence (red) under basal condition and after stimulation with H_2O_2 or palmitate in INS-1E cells. **B**, **C**: INS-1E cells were stimulated with 1 mmol/L NAC for 2 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h prior to be incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40 minutes. **B**: Insulin content was measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (n = 3 independent experiments). **C**: Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (n = 4 independent experiments). *$p < 0.05$ vs control. Data are expressed as the mean ± SEM. Palm, palmitate; NAC, N-Acetyl-L-cysteine; H_2O_2, hydrogen peroxide.