Il presente file rappresenta la versione Post-print (post referaggio) della seguente pubblicazione:

The p66Shc Protein Mediates Insulin Resistance and Secretory Dysfunction in Pancreatic β-Cells Under Lipotoxic Conditions. *Diabetes. 2022 Aug 1;71(8):1763-1771. doi: 10.2337/db21-1066.*

American Diabetes Association. diabetes

The p66^{Shc} protein mediates insulin resistance and secretory dysfunction in pancreatic beta-cells under lipotoxic conditions

Journal:	*Diabetes*
Manuscript ID	DB21-1066.R1
Manuscript Type:	Brief Report: Pathophysiology
Date Submitted by the Author:	n/a
Complete List of Authors:	Biondi, Giuseppina; University of Bari, Department of Emergency and Organ Transplantation Marrano, Nicola; University of Bari, Department of Emergency and Organ Transplantation Dipaola, Lucia; University of Bari, Department of Emergency and Organ Transplantation Borrelli, Anna; University of Bari, Department of Emergency and Organ Transplantation Rella, Martina; University of Bari, Department of Emergency and Organ Transplantation D'Oria, Rossella; University of Bari, Department of Emergency and Organ Transplantation Genchi, Valentina A.; University of Bari, Department of Emergency and Organ Transplantation Caccioppoli, Cristina; University of Bari, Department of Emergency and Organ Transplantation Porreca, Immacolata; Genetic Research Centre "Gaetano Salvatore" (IRGS), Biogem Cignarelli, Angelo; University of Bari, Department of Emergency and Organ Transplantation Perrini, Sebastio; University of Bari, Department of Emergency and Organ Transplantation Marchetti, Piero; University of Pisa, Department of Clinical and Experimental Medicine Vincenti, Leonardo; University Hospital Polyclinic, Division of General Surgery Laviola, Luigi; University of Bari, Department of Emergency and Organ Transplantation Giorgino, Francesco; University of Bari, Department of Emergency and Organ Transplantation Natalicchio, Annalisa; University of Bari, Department of Emergency and Organ Transplantation

Title page

Full title:

The p66^{Shc} protein mediates insulin resistance and secretory dysfunction in pancreatic beta-cells under lipotoxic conditions

Short running title:

p66^{Shc} in beta-cell insulin resistance

Authors:

Giuseppina Biondi[1], Nicola Marrano[1], Lucia Dipaola[1], Anna Borrelli[1], Martina Rella[1], Rossella D'Oria[1], Valentina A. Genchi[1], Cristina Caccioppoli[1], Immacolata Porreca[2], Angelo Cignarelli[1], Sebastio Perrini[1], Piero Marchetti[3], Leonardo Vincenti[4], Luigi Laviola[1], Francesco Giorgino[1], Annalisa Natalicchio[1]

Affiliation:

[1]Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy.

[2]Genetic Research Centre "Gaetano Salvatore" (IRGS), Biogem, Ariano Irpino (AV), Italy.

[3]Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.

[4] University Hospital Polyclinic, Division of General Surgery, Bari, Italy.

Corresponding author:

Francesco Giorgino, M.D., Ph.D.; Section of Internal Medicine, Endocrinology, Andrology and Metabolic Diseases; Department of Emergency and Organ Transplantation; University of Bari Aldo Moro; ORCID iD: 0000-0001-7372-2678; Piazza Giulio Cesare, 11, I-70124 Bari, Italy; Phone +39 080.5593522 080.5478689 080.5478152; E-mail: francesco.giorgino@uniba.it.

Word count: ~~2000~~2234; **Number of tables or figures:** 4

Abstract

We evaluated the role of the p66^{Shc} redox adaptor protein in pancreatic beta-cell insulin resistance that develops under lipotoxic conditions and with excess body fat. Prolonged exposure to palmitate in vitro or the presence of overweight/obesity augmented p66^{Shc} expression levels and caused an impaired ability of exogenous insulin to increase cellular insulin content and secreted C-peptide levels in INS-1E cells and human and murine islets. ~~When compared with pancreatic islets from lean subjects, islets from overweight/obese subjects showed increased *p66^{Shc}* mRNA levels and an impaired insulin effect to increase insulin content and secreted C-peptide.~~ In ~~pancreatic beta~~ INS-1E cells, p66^{Shc} knockdown resulted in enhanced insulin-induced augmentation of insulin content and C-peptide secretion and prevented the ability of palmitate to impair these effects of insulin. Conversely, p66^{Shc} overexpression impaired insulin-induced augmentation of insulin content and C-peptide secretion both in the absence and presence of palmitate. Under lipotoxic condition, the effects of p66^{Shc} ~~appear to require~~ are mediated by p53-induced increase in p66^{Shc} protein levels and JNK-induced p66^{Shc} phosphorylation at Ser^{36} and appear to involve the phosphorylation of the ribosomal protein S6 kinase at Thr^{389} and of insulin receptor substrate-1 at Ser^{307}, resulting in the inhibition of insulin-stimulated protein kinase b phosphorylation at Ser^{473}. Thus, the p66^{Shc} protein mediates the impaired beta-cell function and insulin resistance induced by saturated fatty acids and excess body fat.

Introduction

The loss of pancreatic beta-cell functional mass is a necessary and early event during the development of type 2 diabetes, as well as a potential target for the treatment and potential cure of type 2 diabetes (**1**). Insulin-secreting beta-cells are targeted by insulin itself, which acts in an autocrine manner to promote beta-cell viability and function (reviewed in **2**). Constitutively secreted insulin is necessary to maintain beta-cell glucose sensitivity (**3**), and alterations in insulin receptor and insulin receptor substrate-1 (IRS-1) result in secretory dysfunction and glucose intolerance (reviewed in **2**). However, the physiological relevance of autocrine insulin activity remains somewhat controversial due to the different insulin doses and glucose levels used to assess beta-cell secretory function (**2**, **4**).

Several metabolic 'stressors' can result in impaired insulin activity or 'insulin resistance' in pancreatic beta-cells, as shown in other insulin-targeted tissues. The exposure of pancreatic beta-cells to high glucose levels induces the c-Jun N-terminal kinase (JNK)- and extracellular signal-regulated kinase (ERK)1/2-mediated inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1), resulting in inhibition of insulin signalling and consequent impairment of insulin's ability to regulate its own biosynthesis (**5**). Similarly, chronic exposure of beta-cells to elevated free fatty acid levels, particularly long-chain saturated fatty acids (SFAs), can induce the JNK-mediated phosphorylation of IRS-1/2, inhibiting insulin-induced insulin gene transcription (**6**).

The $p66^{Shc}$ protein is a redox enzyme, capable of sensing and generating reactive oxygen species (ROS), that also plays a role in metabolic dysfunction. In previous work (**7**), we described for the first time the pro-~~survival~~apoptotic role of $p66^{Shc}$ in ~~pancreatic beta-~~INS-1E cells, which is dependent upon its Ser^{36} phosphorylation. Moreover, $p66^{Shc}$ expression levels were found to be increased in ~~pancreatic beta-~~INS-1E cells exposed to SFAs, in pancreatic islets isolated from mice fed a high-fat diet, and in human pancreatic islets isolated from overweight/obese subjects (**7**). Previously, $p66^{Shc}$ has been implicated in the development of obesity-induced insulin resistance

66 through the S6K/IRS-1/protein kinase B (AKT) pathway in endothelial cells and adipose tissue (**8**,
67 **9**).

68 To date, the effects of SFAs and excess body fat on insulin action and signalling in pancreatic beta-
69 cells have not been explored, and the role of $p66^{Shc}$ in this process has not been investigated. In this
70 study, we provide evidence that $p66^{Shc}$ is crucially involved in lipotoxicity-induced beta-cell insulin
71 resistance.

Research Design and Methods

Pancreatic islet isolation and culture

Human islets were isolated from pancreata obtained from multi-organ donors at the Islet Cell Laboratory of the University of Pisa, Italy, or from pancreatic fragments obtained from patients undergoing pancreatectomy to treat tumors in the ampulla of Vater at the Division of General Surgery of the University Hospital Polyclinic of Bari, Italy. Human pancreatic tissues were processed with the approval of the local Ethics Committee after informed consent was obtained. Anonymised patient information is provided in the **Human Islets Checklist**. Twenty male, 4–8-week-old, C57BL/6 mice were purchased from Charles River Laboratories (Calco, Lecco, Italy). Animal experiments were conducted after obtaining approval from the Ethics Committee of the Genetic Research Centre "Gaetano Salvatore", Biogem, Ariano Irpino, Italy, in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (2011) and the regulations established in Italy and the EU for animal experiments. Mouse and human pancreatic islets were isolated and cultured as ~~previously~~described in ~~7,~~ **10**, **11**, and **Supplemental Methods**.

~~Pancreatic beta-cell~~ INS-E cells culture

Rat insulin-secreting INS-1E cells (passage 15–30) were a kind gift from C. B. Wollheim, University of Geneva, Switzerland. INS-1E cells were cultured as ~~previously~~ described in **Supplemental Methods** ~~(7)~~.

Pancreatic islets and ~~beta-cell~~ INS-1E cells treatment

Human (preparation 13-17 in **Human Islets Checklist**) and mouse islets and INS-1E cells were treated with 0.5 mmol/l palmitate or oleate solution (Sigma-Aldrich Inc., St Louis, MO, USA), ~~prepared as previously reported (7),~~ or with the same volume of a 10% w/v ~~fatty acid~~FA-free BSA solution, as a control. After 24 h, islet and cells were stimulated with 10–100 nmol/l human recombinant insulin (Roche Diagnostics, Mannheim, Germany) for different times. To achieve

p66^{Shc} knockdown, INS-1E cells were transfected with 100 nmol/l *p66*^{Shc}-targeted short interfering RNA (siRNA, Qiagen, Hilden, Germany) using Lipofectamine® RNAiMAX ~~Transfection~~ Reagent and Opti-MEM® medium (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 48 h (**7**). The overexpression of *p66*^{Shc} was achieved by transducing INS-1E cells with recombinant adenoviruses carrying a construct encoding the *p66*^{Shc} protein (**7**). ~~In addition~~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). See **Supplemental Methods** for further details.

Measurement of insulin content and secreted C-peptide levels

After a wash with PBS 1X, murine and human pancreatic islets and INS-1E cells were incubated in Krebs-Ringer bicarbonate HEPES buffer (KRBH, 0.1% w/v BSA, 3 mmol/l glucose, 114 mmol/l NaCl, 4.4 mmol/l KCl, 1 mmol/l $MgSO_4$, 29.5 mmol/l $NaHCO_3$, 1.28 mmol/l $CaCl_2$, and 10 mmol/l HEPES, all from Sigma-Aldrich, pH 7.4.) (**12**) for 100 min, followed by stimulation with insulin (10–100 mol/l) diluted in fresh KRBH solution for 40 min. To evaluate insulin content, cells were washed twice with PBS 1X, then mechanically lysed in a non-denaturing lysis buffer (**13**). The level of C-peptide released in the medium, as a measure of endogenously produced insulin, and insulin contents were measured using enzyme-linked immunosorbent assays (ELISAs) specific for mouse (Shibayagi Co., Ltd, Ishihara, Japan), humans, and rats (Mercodia AB, Sylveniusgatan, Uppsala, Sweden).

Immunoblotting

Cells lysates were obtained and analysed by immunoblotting assays~~, as previously described~~ (**13** and **Supplemental Methods**) ~~(7)~~. A list of the antibodies used is shown in **Supplemental** **Table 1**.

Quantitative Real-Time PCR

RNA isolation, cDNA synthesis and quantitative Real-Time PCR were conducted as ~~previously~~ reported in **13** and **Supplemental Methods** (~~**7**~~, ~~**13**~~). Primers sequences are available in **Supplemental** **Table 2**.

Statistical analysis

Data are presented as the mean ± SEM. Statistical analysis was performed using the two-tailed unpaired Student's t-test. Significance was set at $p < 0.05$.

~~Data and Resource Availability~~

~~The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.~~

Results

Insulin increases its own content and C-peptide release in INS-1E cells and human and murine islets

Insulin stimulation increased insulin content and C-peptide secreted levels in INS-1E cells (**Fig. 1A** and **B**), and human (**Fig. 1C** and **D**) and murine (**Fig. 1E** and **F**) pancreatic islets. This was associated with increased gene expression levels of *Insulin 1*, but not *Insulin 2*, in INS-1E cells (**Supplemental Fig. 1**).

Palmitate impairs insulin ~~augmentation of insulin content and stimulation of C-peptide secretion in pancreatic beta-cells and islets~~ effects in INS-1E cells and human and murine islets

~~Insulin stimulation resulted in increased insulin content and C-peptide secretion in INS-1E cells (**Fig. 1A** and **B**), and in human (**Fig. 1I** and **J**) and murine (**Supplemental Figure 1A** and **B**) pancreatic islets. In contrast,~~ Prior exposure of cells and islets to the SFA palmitate impaired the stimulatory effects of insulin on its own cellular content (**Fig. 1A**, **C, E**) ~~**I**, and **Supplemental Figure 1A**)~~ and C-peptide secretion (**Fig. 1B**, **D, F**) ~~**J**, and **Supplemental Figure 1B**)~~. This occurred also in INS-E cells exposed to the unsaturated FA oleate (**Supplemental Fig. 2A** and **B**).

Palmitate-induced impairment of insulin effects in INS-1E cells is mediated by p66^{Shc}

The *p66^{Shc}* silencing approach resulted in a 40%–50% reduction of p66^{Shc} protein levels in all experimental conditions compared with control cells treated with vehicle only (**Fig. ~~1C~~2A**). Under these conditions, the palmitate-induced impairment of the insulin stimulatory effect on its own cellular content was largely prevented (**Fig. ~~1D~~2B**). In addition, *p66^{Shc}* knockdown resulted in increased insulin-induced C-peptide secretion both in the absence and presence of palmitate, compared with control cells (**Fig. ~~1E~~2C**).

Conversely, p66^{Shc} overexpression in INS-1E cells resulted in a marked increase of p66^{Shc} protein levels (**Fig. ~~1F~~2D**), and in the inhibition of the ability of insulin to augment insulin content (**Fig. ~~1G~~2E**) and secreted C-peptide levels (**Fig. ~~1H~~2F**) in cells not exposed to palmitate; in the presence of palmitate, these effects of insulin were impaired further (**Fig. ~~1G and H~~2E and F**).

Following overexpression of the p66^{Shc}Ala^{36} mutant protein (**Fig. ~~1F~~2D**), which is unable to undergo phosphorylation at the key Ser^{36} site (**7**), the palmitate-induced impairment of the effects of insulin to enhance its own content (**Fig. ~~1G~~2E**) and C-peptide secretion (**Fig. ~~1H~~2F**), respectively, were no longer observed.

Islets from overweight and obese subjects show elevated p66^{Shc} mRNA expression and reduced response to insulin

In islets (preparation 1-12 in **Human Islets Checklist**) obtained from overweight/obese subjects, *p66^{Shc}* mRNA levels were higher than in islets from lean subjects (**Fig. ~~1K~~2G**). In addition, the effects of insulin to increase insulin content (**Fig. ~~1L~~2H**) and secreted C-peptide (**Fig. ~~1M~~2I**) were blunted.

Role of JNK, p53 protein, and oxidative stress in SFAs-induced insulin resistance in INS-1E cells

The inhibition of JNK (**Fig. 3A**) or p53 protein activity (**Fig. 3E**) reduced palmitate-induced p66^{Shc} phosphorylation at Ser^{36} (**Fig. 3B**) and p66^{Shc} protein expression (**Fig. 3F**), respectively, and prevented the palmitate effects on insulin-induced insulin content (**Fig. 3C** and **G**) and C-peptide secretion (**Fig. 3D** and **H**). Of note, the reduction in ROS levels impaired insulin effects both under basal and palmitate-stimulated conditions (**Supplemental Fig. 3**).

p66^{Shc} mediates the palmitate-induced alterations of insulin signalling via S6K/IRS-1/AKT in ~~pancreatic beta~~ INS-1E cells

Palmitate treatment increased S6K Thr^{389} phosphorylation (**Fig. ~~2A~~4A**) and IRS-1 Ser^{307} phosphorylation, resulting both in reduced IRS-1 protein levels (**Fig. ~~2B~~4B**) and impaired ability of insulin to stimulate AKT Ser^{473} phosphorylation (**Fig. ~~2C~~4C**). However, in beta-cells with *p66^{Shc}* knockdown, the effects of palmitate on phosphorylation of S6K (**Fig. ~~2A~~4A**) and IRS-1 (**Fig. ~~2B~~4B**) proteins were abrogated, and this enhanced insulin-stimulated AKT Ser^{473} phosphorylation (**Fig. ~~2C~~4C**). Conversely, p66^{Shc} overexpression did not alter S6K Thr^{389} phosphorylation (**Fig. ~~2D~~4D**) and exacerbated the palmitate-mediated IRS-1 Ser^{307} phosphorylation and reduction of IRS-1 protein levels (**Fig. ~~2E~~4E**), leading to complete abrogation of insulin-stimulated AKT Ser^{473} phosphorylation (**Fig. ~~2F~~4F**). Of note, p66^{Shc} overexpression increased IRS-1 Ser^{307} phosphorylation, reduced IRS-1 protein levels (**Fig. ~~2E~~4E**), and impaired insulin-stimulated AKT Ser^{473} phosphorylation (**Fig. ~~2F~~4F**), also in cells not exposed to palmitate.

Discussion

~~In this study, w~~We show that acute insulin stimulation increased the cellular insulin content and ~~extent of secreted~~ C-peptide release ~~in multiple cellular models of pancreatic beta-cells, including rat INS-1E cells and human and murine pancreatic islets. Notably, t~~ in rat INS-1E cells and human and murine pancreatic islets. This occurred independently of glucose interference, as all experiments were conducted under low-glucose conditions. Moreover, both exposure to palmitate *in vitro* and presence of excess of body fat *in vivo* resulted in the impaired ability of insulin to stimulate its biosynthesis and secretion in beta-cells and murine and human pancreatic islets *in vitro* and in human pancreatic islets *ex vivo*, respectively. These results mirror and corroborate the previous observation of blunted insulin-induced insulin gene transcription following exposure of mouse pancreatic islets to palmitate (**6**). Our results in INS-1E cells indicate that palmitate alters the insulin signalling pathway by activating the phosphorylation of Thr^{389} in the S6K protein and the inhibitory phosphorylation of Ser^{307} in IRS-1, which are known to result in reduced IRS-1 protein levels and impairment of insulin-induced AKT activation (**9**, **14**). These results support the emerging concept that lipotoxicity is capable to induce an insulin-resistant state in pancreatic beta-cells, similarly to other classical insulin target tissues.

Importantly, we show for the first time that $p66^{Shc}$ plays a key role in this lipotoxicity-mediated insulin resistance in pancreatic beta-cells, since *p66*^{Shc} knockdown or overexpression respectively prevented or worsened palmitate-induced insulin resistance by reducing or boosting palmitate-induced activation of the S6K/IRS-1/AKT pathway (**Fig. ~~2~~4G**). These results resemble the effects of lipotoxicity in endothelial and adipose cells (**8**, **9**). ~~Moreover, we observed that $p66^{Shc}$ phosphorylation at Ser^{36} is necessary to mediate the detrimental effects of palmitate, as divergent effects were found following overexpression of the wild-type and mutant (Ala^{36}) forms of $p66^{Shc}$, respectively. Phosphorylation at Ser^{36} was previously demonstrated to be necessary for the harmful effects of $p66^{Shc}$ under various stressful conditions, including lipotoxicity-induced beta-cell~~

~~apoptosis (7).~~ Experiments with overexpression of the wild-type and mutant (Ala^{36}) forms of $p66^{Shc}$ suggested that $p66^{Shc}$ phosphorylation at Ser^{36} is required to mediate the effects of palmitate on insulin action. Furthermore, we found that palmitate-induced insulin-resistance in INS-1E cells is mediated by p53 and JNK proteins. These results resemble the mechanisms already demonstrated for palmitate-induced beta-cell apoptosis (**7**).

The *ex vivo* experiments performed in human pancreatic islets isolated from donors without diabetes support the involvement of $p66^{Shc}$ in lipotoxicity-induced beta-cell insulin resistance in human obesity, as both elevated *p66^Shc^* mRNA expression and impaired insulin effects were observed in islets obtained from overweight/obese subjects, in comparison with islets obtained from lean subjects. ~~SFAs are known to increase $p66^{Shc}$ levels and phosphorylation of Ser^{36}, through the activation of the tumor suppressor p53 protein and the kinase JNK, respectively (7), and this represents a key event for SFAs-induced apoptosis in beta-cells (**Fig. 2G**).~~ Overall, ~~this evidence identifies~~ $p66^{Shc}$ can be identified as a crucial mediator of lipotoxicity-promoted beta-cell failure in the context of human obesity. Interestingly, elevated $p66^{Shc}$ levels have also been found in the liver of patients with alcoholic and non-alcoholic steatohepatitis (**15**, **16**), in the heart in response to cardiac dysfunction (**17**) and hyperglycemia (**18**), and in endothelial cells exposed to dyslipidemia (**19**), hyperglycemia (**20**), and pro-inflammatory cytokines (**21**). Taken together, this evidence suggests that $p66^{Shc}$ is ubiquitously involved in multi-organ damage in response to metabolic stressors.

The physiological significance of the effects of autocrine basal insulin *in vivo* are still debated (**2**, **4**, **22-24**), even though under low-glucose conditions insulin could regulate its own content and release, representing a physiological mechanism to replenish insulin stores and make beta-cells prone to rapidly satisfy insulin demand in response to secretagogues (**3**, **22**, **25-27**). Of note, insulin signaling in beta-cells can be triggered by insulin binding at high concentrations to insulin receptor (IR), insulin-like growth factor-1 receptor (IGF-1R), or IR/IGF-1R hybrids (**28**). It has been

suggested that insulin promotes its own biosynthesis and secretion mainly through IR/IRS-1 signaling (**29-31**). On the other hand, the insulin binding to IGF-1R mainly leads to the activation of IRS-2, which could play a major role in the regulation of beta-cell mass (**32-33**).

In conclusion, ~~this study identified~~ p66^{Shc} ~~as a mediator of~~ mediates lipotoxicity-induced beta-cell insulin resistance, an emerging mechanism for beta-cell failure in obesity and type 2 diabetes. Of interest, glucagon-like peptide 1 receptor agonists, which reportedly inhibit palmitate-induced apoptosis by preventing JNK phosphorylation (~~**22**~~**34**), also inhibited p66^{Shc} phosphorylation on Ser^{36} (**7**), and may thus use this mechanism as well to enhance insulin secretion. Since the loss of pancreatic beta-cell functional mass is a necessary and early event in type 2 diabetes (**1**), the combined effects of p66^{Shc} on beta-cell secretory-function and survival suggest that this protein may represent a potential ~~promising pharmacological~~ target for the prevention or treatment type 2 diabetes onset or progression.

Acknowledgments

Author Contributions

All of authors contributed to conception and design, acquisition of data, analysis, and interpretation of data; they drafted the final article or revised it critically for important intellectual content and finally approved the version to be published.

Conceptualization, F.G. and A.N.; data curation, G.B., N.M., L.D., A.N., and F.G.; formal analysis and investigation G.B., N.M., L.D., A.B., M.R., R.D., V.A.G., C.C.; project administration, F.G. and A.N.; resources, I.P., P.M. and L.V.; supervision, L.L., F.G., and A.N.; visualization, A.C., S.P. and L.L.; writing—original draft, A.N.; writing—review and editing, F.G.

Guarantor Statement

F.G. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest Statement

No potential conflicts of interest relevant to this article were reported.

Funding

This work was supported by AGER 2 Project (grant n° 2016-0174, COMPETITIVE e Claims of Olive oil to iMProvE The market ValuE of the product), Ministero dell'Università e della Ricerca of Italy (Progetti di Rilevante Interesse Nazionale, 2015), the European Union (European Social Fund, PON R&I 2014–2020, AIM 1810057), and the "Fondazione per la Ricerca Biomedica Saverio e Isabella Cianciola".

Prior Presentation Information

Data have been previously presented at scientific meetings (79th ADA Scientific Session, 2019; 40th National Meeting of the Italian Society of Endocrinology, 2019; 27th National Meeting of the Italian Society of Diabetology, 2018; 53th EASD Annual Meeting, 2017; International Symposium on Insulin Receptor and Insulin Action, 2017; 39th National Meeting of the Italian Society of Endocrinology, 2017).

Reference to prior publication of the study in abstract form:

The p66Shc protein mediates saturated fatty acid-induced insulin resistance in pancreatic beta cells. G. Biondi, A. Natalicchio, N. Marrano, R. Spagnuolo, R. Labarbuta, L. Dipaola, A. Cignarelli, P. Marchetti, S. Perrini, L. Laviola, F. Giorgino. Diabetologia. 2017 Sep;60 Suppl 1:S194.

195-OR: The p66Shc protein mediates insulin resistance in pancreatic ß cells under lipotoxic conditions. L. Dipaola, A. Natalicchio, G. Biondi, N. Marrano, M. Bugliani, A. Cignarelli, S. Perrini, P. Marchetti, L. Laviola, F. Giorgino. Diabetes 2019 Jun; 68(Supplement 1).

Data and Resource Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

References

1. Marrano N, Biondi G, Cignarelli A, Perrini S, Laviola L, Giorgino F, Natalicchio A. Functional loss of pancreatic islets in type 2 diabetes: How can we halt it? Metabolism 2020;110:154304.

2. Leibiger IB, Leibiger B, Berggren PO. Insulin signaling in the pancreatic beta-cell. Annu Rev Nutr 2008;28:233-251.

3. Srivastava S, Goren HJ. Insulin constitutively secreted by beta-cells is necessary for glucose-stimulated insulin secretion. Diabetes 2003;52(8):2049-2056.

4. Rhodes CJ, White MF, Leahy JL, Kahn SE. Direct autocrine action of insulin on β-cells: does it make physiological sense? Diabetes 2013;62(7):2157-2163.

5. Andreozzi F, D'Alessandris C, Federici M, Laratta E, Del Guerra S, Del Prato S, Marchetti P, Lauro R, Perticone F, Sesti G. Activation of the hexosamine pathway leads to phosphorylation of insulin receptor substrate-1 on Ser307 and Ser612 and impairs the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin insulin biosynthetic pathway in RIN pancreatic beta-cells. Endocrinology 2004;145(6):2845-57.

6. Solinas G, Naugler W, Galimi F, Lee MS, Karin M. Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. Proc Natl Acad Sci U S A 2006;103(44):16454-16459.

7. Natalicchio A, Tortosa F, Labarbuta R, Biondi G, Marrano N, Carchia E, Leonardini A, Cignarelli A, Bugliani M, Marchetti P, Fadini GP, Giorgio M, Avogaro A, Perrini S, Laviola L, Giorgino F. The p66(Shc) redox adaptor protein is induced by saturated fatty acids and mediates lipotoxicity-induced apoptosis in pancreatic beta cells. Diabetologia 2015; 58(6):1260-71. Erratum in: Diabetologia 2015;58(11):2682.

8. Paneni F, Costantino S, Cosentino F. p66(Shc)-induced redox changes drive endothelial insulin resistance. Atherosclerosis 2014;236(2):426-429.

9. Ranieri SC, Fusco S, Panieri E. Mammalian life-span determinant p66ShcA mediates obesity-induced insulin resistance. Proc Natl Acad Sci U S A 2010;107(30):13420-13425.

10. Li DS, Yuan YH, Tu HJ, Liang QL, Dai LJ. A protocol for islet isolation from mouse pancreas. Nat Protoc 2009;4:1649-1652.

11. Lupi R, Del Guerra S, Fierabracci V, Marselli L, Novelli M, Patanè G, Boggi U, Mosca F, Piro S, Del Prato S, Marchetti P. Lipotoxicity in human pancreatic islets and the protective effect of metformin (Abstract). Diabetes 2002;51 Suppl 1:S134-7.

12. Park SH, Kim SY, Baek WK, Lim B, Park JH, Sung HY, Kim YK, Bae KC, Bae JH, Song DK. Regulation of glucose-dependent insulin secretion by insulin: possible role of AMP-activated protein kinase. Life Sci 2009;85(3-4):178-83.

13. Marrano N, Spagnuolo R, Biondi G, Cignarelli A, Perrini S, Vincenti L, Laviola L, Giorgino F, Natalicchio A. Effects of Extra Virgin Olive Oil Polyphenols on Beta-Cell Function and Survival. Plants (Basel). 2021;10(2):286.

14. Zhang J, Gao Z, Yin J, Quon MJ, Ye J. S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2. J Biol Chem 2008;283(51):35375-82.

15. Perrini S, Tortosa F, Natalicchio A, Pacelli C, Cignarelli A, Palmieri VO, Caccioppoli C, De Stefano F, Porro S, Leonardini A, Ficarella R, De Fazio M, Cocco T, Puglisi F, Laviola L, Palasciano G, Giorgino F. The p66Shc protein controls redox signaling and oxidation-dependent DNA damage in human liver cells. Am J Physiol Gastrointest Liver Physiol 2015;309(10):G826-40.

16. Tomita K, Teratani T, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, Nishiyama K, Mataki N, Irie R, Minamino T, Okada Y, Kurihara C, Ebinuma H, Saito H, Shimizu I, Yoshida Y, Hokari R, Sugiyama K, Hatsuse K, Yamamoto J, Kanai T, Miura S, Hibi T. p53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. J Hepatol 2012;57(4):837-43.

17. Graiani G, Lagrasta C, Migliaccio E, Spillmann F, Meloni M, Madeddu P, Quaini F, Padura IM, Lanfrancone L, Pelicci P, Emanueli C. Genetic deletion of the p66Shc adaptor protein protects from angiotensin II-induced myocardial damage. Hypertension 2005;46(2):433-40.

18. Costantino S, Paneni F, Mitchell K, Mohammed SA, Hussain S, Gkolfos C, Berrino L, Volpe M, Schwarzwald C, Lüscher TF, Cosentino F. Hyperglycaemia-induced epigenetic changes drive persistent cardiac dysfunction via the adaptor p66Shc. Int J Cardiol 2018;268:179-186.

19. Kim YR, Kim CS, Naqvi A, Kumar A, Kumar S, Hoffman TA, Irani K. Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction. Am J Physiol Heart Circ Physiol 2012;303(2):H189-96.

20. Zhang M, Lin L, Xu C, Chai D, Peng F, Lin J. VDR Agonist Prevents Diabetic Endothelial Dysfunction through Inhibition of Prolyl Isomerase-1-Mediated Mitochondrial Oxidative Stress and Inflammation. Oxid Med Cell Longev 2018;2018:1714896.

21. Laviola L, Orlando MR, Incalza MA, Caccioppoli C, Melchiorre M, Leonardini A, Cignarelli A, Tortosa F, Labarbuta R, Martemucci S, Pacelli C, Cocco T, Perrini S, Natalicchio A, Giorgino F. TNFα signals via p66(Shc) to induce E-Selectin, promote leukocyte transmigration and enhance permeability in human endothelial cells. PLoS One 2013;8(12):e81930.

22. Braun M, Ramracheya R, Rorsman P. Autocrine regulation of insulin secretion. Diabetes Obes Metab 2012;14 Suppl 3:143-151.

23. Skovsø S, Panzhinskiy E, Kolic J, Cen HH, Dionne DA, Dai XQ, Sharma RB, Elghazi L, Ellis CE, Faulkner K, Marcil SAM, Overby P, Noursadeghi N, Hutchinson D, Hu X, Li H, Modi H, Wildi JS, Botezelli JD, Noh HL, Suk S, Gablaski B, Bautista A, Kim R, Cras-Méneur C, Flibotte S, Sinha S, Luciani DS, Nislow C, Rideout EJ, Cytrynbaum EN, Kim JK, Bernal-Mizrachi E, Alonso LC, MacDonald PE, Johnson JD. Beta-cell specific Insr deletion promotes insulin hypersecretion and improves glucose tolerance prior to global insulin resistance. Nat Commun 2022;13(1):735.

24. Mari A, Tura A, Natali A, Anderwald C, Balkau B, Lalic N, Walker M, Ferrannini E; RISC Investigators. Influence of hyperinsulinemia and insulin resistance on in vivo β-cell function: their role in human β-cell dysfunction. Diabetes 2011 Dec;60(12):3141-7.

25. Rachdaoui N. Insulin: The Friend and the Foe in the Development of Type 2 Diabetes Mellitus. Int J Mol Sci 2020;21(5):1770.

26. Ohsugi M, Cras-Méneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA. Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. J Biol Chem 2005;280(6):4992-5003.

27. Wang J, Gu W, Chen C. Knocking down Insulin Receptor in Pancreatic Beta Cell lines with Lentiviral-Small Hairpin RNA Reduces Glucose-Stimulated Insulin Secretion via Decreasing the Gene Expression of Insulin, GLUT2 and Pdx1. Int J Mol Sci 2018;19(4):985.

28. Nagao H, Cai W, Wewer Albrechtsen NJ, Steger M, Batista TM, Pan H, Dreyfuss JM, Mann M, Kahn CR. Distinct signaling by insulin and IGF-1 receptors and their extra- and intracellular domains. Proc Natl Acad Sci U S A 2021;118(17):e2019474118.

29. Marchetti P, Lupi R, Federici M, Marselli L, Masini M, Boggi U, Del Guerra S, Patanè G, Piro S, Anello M, Bergamini E, Purrello F, Lauro R, Mosca F, Sesti G, Del Prato S. Insulin secretory function is impaired in isolated human islets carrying the Gly(972)-->Arg IRS-1 polymorphism. Diabetes 2002;51(5):1419-24.

30. Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 1999;96(3):329-39.

31. Goldfine AB, Kulkarni RN. Modulation of β-cell function: a translational journey from the bench to the bedside. Diabetes Obes Metab 2012;14 Suppl 3:152-60.

32. Hakuno F, Takahashi SI. IGF1 receptor signaling pathways. J Mol Endocrinol 2018;61(1):T69-T86.

33. Hennige AM, Ozcan U, Okada T, Jhala US, Schubert M, White MF, Kulkarni RN. Alterations in growth and apoptosis of insulin receptor substrate-1-deficient beta-cells. Am J Physiol Endocrinol Metab 2005;289(2):E337-46.

34.~~22.~~ Natalicchio A, Labarbuta R, Tortosa F, Biondi G, Marrano N, Peschechera A, Carchia E, Orlando MR, Leonardini A, Cignarelli A, Marchetti P, Perrini S, Laviola L, Giorgino F. Exendin-4 protects pancreatic beta cells from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway. Diabetologia 2013;56(11):2456-66.

Tables

Table 1. Characteristics of antibodies used for immunoblotting.

Specificity	Antibody	Dilution used
Phospho-AKT (Ser473)	Cell Signaling Technology Inc., #9271S	1:1000
Total AKT	Cell Signaling Technology Inc., #4691S	1:1000
Beta-actin	Santa Cruz Biotechnology, sc-47778	1:1000
Phospho-IRS-1 (Ser307)	Cell Signaling Technology Inc., #2381S	1:500
Total IRS-1	Millipore, #06-248	1:500
Phospho-p70 S6K (Thr389)	Cell Signaling Technology Inc., #9205	1:1000
Total Shc	Merck Millipore, #06-203	1:1000

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

Gene	Species	Primer sequences
RNA18S5	Homo sapiens	FOR: 5'-CGAACGTCTGCCCTATCAACTT-3' REV: 5'-ACCCGTGGTCACCATGGTA-3'
p66^{Shc}	Homo sapiens	FOR: 5'-CCCCCAAGCCCAAGTACAA-3' REV: 5'-GACCCAGAAGCCCCTTCCT-3'

Figure Legends

Figure 1. Role of p66^{Shc} protein in lipotoxicity-induced beta-cell insulin resistance. **A**, **B**, **I**, **J**: INS-1E cells (**A** and **B**) and human pancreatic islets (**I** and **J**) were cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, followed by stimulation with 10 (**A**, **B**) or 100 (**I**, **J**) nmol/l insulin for 40 minutes. **A** and **I**: Insulin content was measured by enzyme-linked immunosorbent assay (ELISA), normalised to protein concentration, and expressed as a percentage of untreated control (n = 4 independent experiments); the fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) is also shown. **B** and **J**: Secreted C-peptide levels were measured by ELISA, normalised against total protein concentration, and expressed as a percentage of the untreated control (n = 4 independent experiments); the fold-increase of insulin-stimulated secreted C-peptide levels over control (not treated with palmitate) is also shown. *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate. **C**– **E**: INS-1E cells were transfected with *p66^{Shc}* siRNA for 24 h, then cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for an additional 24 h, and finally stimulated with 10 nmol/l insulin for 40 minutes. The transfection reagent only was used as control. **C**: Representative immunoblot of p66^{Shc} and beta-actin protein levels with the densitometric analysis of the bands, expressed as relative optical density. Values were corrected using total beta-actin levels as a loading control and normalised against untreated control (n = 4 independent experiments). **D**: Insulin content was measured with a specific ELISA, normalised to protein concentration, and shown as fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) (n = 5 independent experiments). **E**: Secreted C-peptide levels were measured by ELISA, normalised to total protein concentration, and expressed as a percentage of the untreated control (n = 4 independent experiments). *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent. **F**–**H**: INS-1E cells were infected with a recombinant adenoviral vector encoding p66^{Shc} (Ad/*p66^{Shc}*), a phosphorylation-defective

~~p66^{Shc} protein containing a Ser^{36} to Ala mutation (Ad/Ala^{36}), or with empty adenovirus (Ad/mock) for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, and then stimulated with 10 nmol/l insulin for 40 minutes. **F:** Representative immunoblot of p66^{Shc} and beta-actin protein levels with the densitometric analysis of bands, expressed as the relative optical density, corrected using total beta-actin levels as a loading control, and normalised against control (n = 8 independent experiments). **G**: Insulin content was measured by ELISA, normalised to total protein concentration, and shown as fold-increase over control (not treated with palmitate) (n = 5 independent experiments). **H**: Secreted C-peptide levels were measured by ELISA, normalised to total protein concentration, and expressed as a percentage of the control (n = 8 independent experiments). *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs Ad/mock; §p < 0.05 vs Ad/*p66^{Shc}*. **K**: *p66^{Shc}* mRNA levels in pancreatic islets from lean (BMI < 25 kg/m^2, white bars) and overweight/obese (BMI ≥ 25 kg/m^2, grey bars) subjects. †p < 0.05 vs BMI < 25 kg/m^2 (n = 4 independent experiments). **L** and **M**: Pancreatic islets were isolated from lean or overweight/obese subjects and then stimulated with 100 nmol/l insulin for 40 min. **L:** Insulin content was measured by ELISA, normalised to total protein concentration, and expressed as a percentage of the untreated control (n = 4 independent experiments). **M:** 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; †p < 0.05 vs BMI < 25. Data are expressed as the mean ± SEM. Palm, palmitate.~~

Figure 1. Effects of insulin and palmitate on insulin content and C-peptide release in INS-1E cells and human and murine islets. INS-1E cells (**A**, **B**), human islets (**C**, **D**) and murine islets (**E**, **F**) were cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, then incubated in KRBH buffer for 100 min, and finally stimulated with 10 (**A**, **B**) or 100 (**C**-**F**) nmol/l insulin in fresh KRBH buffer for 40 minutes. **A**, **C**, **E**: Insulin content was measured

by enzyme-linked immunosorbent assay (ELISA), normalized to protein concentration, and expressed as a percentage of untreated control (at least n = 4 independent experiments); the fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) is also shown. **B**, **D**, **F**: Secreted C-peptide levels were measured by ELISA, normalized against total protein concentration, and expressed as a percentage of the untreated control (at least n = 3 independent experiments); the fold-increase of insulin-stimulated secreted C-peptide levels over control (not treated with palmitate) is also shown. *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate. Data are expressed as the mean ± SEM. Palm, palmitate.

Figure 2. Role of $p66^{Shc}$ protein in lipotoxicity-induced beta-cell insulin resistance. **A**– **C**: INS-1E cells were transfected with *p66*^{Shc} siRNA for 24 h, then cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for an additional 24 h. Successively, cells were incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40 minutes. The transfection reagent only was used as control. **A**: Representative immunoblot of $p66^{Shc}$ and beta-actin protein levels with the densitometric analysis of the bands, expressed as relative optical density. Values were corrected using total beta-actin levels as a loading control and normalized against untreated control (n = 4 independent experiments). **B**: Insulin content was measured with a specific ELISA, normalized to protein concentration, and shown as fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) (n = 5 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). **D**–**F**: INS-1E cells were infected with a recombinant adenoviral vector encoding $p66^{Shc}$ (Ad/*p66*^{Shc}), a phosphorylation-defective $p66^{Shc}$ protein containing a Ser^{36} to Ala mutation (Ad/Ala^{36}), or with empty adenovirus (Ad/mock) for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, then incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh

KRBH buffer for 40 minutes. **D:** Representative immunoblot of $p66^{Shc}$ and beta-actin protein levels with the densitometric analysis of bands, expressed as the relative optical density, corrected using total beta-actin levels as a loading control, and normalized against control (n = 8 independent experiments). **E**: Insulin content was measured by ELISA, normalized to total protein concentration, and shown as fold-increase over control (not treated with palmitate) (n = 5 independent experiments). **F**: Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the control (n = 8 independent experiments). **G**: *$p66^{Shc}$* mRNA levels in pancreatic islets from lean (BMI < 25 kg/m^2, white bars) and overweight/obese (BMI ≥ 25 kg/m^2, grey bars) subjects (n = 4 independent experiments). **H** and **I**: Pancreatic islets were isolated from lean or overweight/obese subjects, then incubated in KRBH buffer for 100 min, and finally stimulated with 100 nmol/l insulin in fresh KRBH medium for 40 min. **H:** Insulin content was measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (n = 4 independent experiments). **I:** 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 without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent; §p < 0.05 vs Ad/mock; || p < 0.05 vs Ad/*$p66^{Shc}$*; ¶ p < 0.05 vs BMI < 25 kg/m^2. Data are expressed as the mean ± SEM. Palm, palmitate.

Figure 3. Role of JNK kinase and p53 in palmitate effects on insulin-induced insulin content and C-peptide release in INS-1E cells. **A**, **B**: Cells were stimulated with 10 or 30 µmol/l JNK inhibitor (SP600125) or dimethyl sulfoxide (DMSO) as control for 2 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h. **A**: Representative immunoblot of c-Jun (Ser^{63}) phosphorylation and beta-actin protein levels, quantified by densitometric analysis of the bands, expressed as relative optical density. C-Jun (Ser^{63}) phosphorylation values were corrected using total beta-actin levels as a loading control and normalized against untreated control (n = 3

independent experiments). **B**: Representative immunoblot of $p66^{Shc}$ (Ser^{36}) phosphorylation and protein levels, quantified by densitometric analysis of the bands, expressed as relative optical density. $p66^{Shc}$ (Ser^{36}) phosphorylation values were normalized against $p66^{Shc}$ protein, then against untreated control (n = 3 independent experiments). **C**, **D**: Cells were stimulated with 30 µmol/l JNK inhibitor (SP600125) or dimethyl sulfoxide (DMSO) as control 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. **E**, **F**: Cells were stimulated with 30 µmol/l pifithrin-alpha (PFT-α) or dimethyl sulfoxide (DMSO) as control for 1 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h. **E**: Representative immunoblot of p53 (Lys^{382}) acetylation and beta-actin protein levels, quantified by densitometric analysis of the bands, expressed as relative optical density. P53 (Lys^{382}) acetylation values were corrected using total beta-actin levels as a loading control and normalised against untreated control (n = 6 independent experiments). **F**: Representative immunoblot of $p66^{Shc}$ protein levels, quantified by densitometric analysis of the bands, and expressed as relative optical density. $P66^{Shc}$ values were normalized against beta-actin levels, then against untreated control (at least n = 5 independent experiments). **G**, **H**: Cells were stimulated with 30 µmol/l pifithrin-alpha (PFT-α) or dimethyl sulfoxide (DMSO) as control for 1 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. **C**, **G:** Insulin content was measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (at least n = 3 independent experiments). **D**, **H:** Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (at least n = 3 independent experiments). *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs control without inhibitor. Data are expressed as the mean ± SEM. Palm, palmitate; JNKi, JNK inhibitor (SP600125); PFT-α, pifithrin-alpha.

Figure ~~2~~4. Molecular mechanisms by which p66^{Shc} mediates palmitate-induced insulin resistance in beta-cells. **A–C:** INS-1E cells were transiently transfected with *p66^{Shc}* siRNA for 24 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for an additional 24 h, and then stimulated with 100 nmol/l insulin for 10 min. The transfection reagent only was used as a control. S6K (Thr^{389}) (**A**), IRS-1 (Ser^{307}) (**B**), and AKT (Ser^{473}) (**C**) phosphorylation was measured by immunoblotting and quantified by densitometric analysis of the related bands, normalized against beta-actin (**A**), total IRS-1 (**B**), and total AKT (**C**), respectively. The resulting relative optical density values are expressed as percentages of the untreated control. **D–F:** INS-1E cells were infected with recombinant adenoviral vector encoding p66^{Shc} (Ad/*p66^{Shc}*) or with empty adenovirus (Ad/mock) for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h and stimulated with 100 nmol/l insulin for 10 minutes. S6K (Thr^{389}) (**D**), IRS-1 (Ser^{307}) (**E**) and AKT (Ser^{473}) (**F**) phosphorylation was measured by immunoblotting and quantified by densitometric analysis of the related bands, normalized against beta-actin (**D**), total IRS-1 (**E**), and total AKT (**F**), respectively. The resulting relative optical density values are expressed as percentages of the untreated control. **A** and **C**: n = 5 independent experiments; **B** and **E:** n = 3 independent experiments; **D**: n = 13 independent experiments; **F**: n = 8 independent experiments. Data are expressed as the mean ± SEM. *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent; §p < 0.05 vs Ad/mock. Palm, palmitate. **G:** Saturated fatty acids increase p66^{Shc} levels and its phosphorylation in Ser^{36}, via the p53 protein and JNK kinase, respectively, thus inducing apoptosis and insulin resistance in pancreatic beta-cells (**7**). In addition, p66^{Shc} activation induces the phosphorylation of Thr^{389} in the S6K protein and the inhibitory phosphorylation of Ser^{307} in IRS-1, which in turn reduce IRS-1 protein levels and impair insulin-induced AKT activation, thus impairing the ability of insulin to increase its own cellular content and C-peptide secretion in pancreatic beta-cells. AKT, protein kinase b; IRS-1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinase; p53, tumor suppressor

protein 53; p66^{Shc}, SHC (Src homology 2 domain-containing) transforming protein 1, 66 kDa isoform; S6K, ribosomal protein S6 kinase.

Title page

Full title:

The p66^{Shc} protein mediates insulin resistance and secretory dysfunction in pancreatic beta-cells under lipotoxic conditions

Short running title:

p66^{Shc} in beta-cell insulin resistance

Authors:

Giuseppina Biondi[1], Nicola Marrano[1], Lucia Dipaola[1], Anna Borrelli[1], Martina Rella[1], Rossella D'Oria[1], Valentina A. Genchi[1], Cristina Caccioppoli[1], Immacolata Porreca[2], Angelo Cignarelli[1], Sebastio Perrini[1], Piero Marchetti[3], Leonardo Vincenti[4], Luigi Laviola[1], Francesco Giorgino[1], Annalisa Natalicchio[1]

Affiliation:

[1]Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy.

[2]Genetic Research Centre "Gaetano Salvatore" (IRGS), Biogem, Ariano Irpino (AV), Italy.

[3]Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.

[4] University Hospital Polyclinic, Division of General Surgery, Bari, Italy.

Corresponding author:

Francesco Giorgino, M.D., Ph.D.; Section of Internal Medicine, Endocrinology, Andrology and Metabolic Diseases; Department of Emergency and Organ Transplantation; University of Bari Aldo Moro; ORCID iD: 0000-0001-7372-2678; Piazza Giulio Cesare, 11, I-70124 Bari, Italy; Phone +39 080.5593522 080.5478689 080.5478152; E-mail: francesco.giorgino@uniba.it.

Word count: 2234; **Number of tables or figures:** 4

Abstract

We evaluated the role of the $p66^{Shc}$ redox adaptor protein in pancreatic beta-cell insulin resistance that develops under lipotoxic conditions and with excess body fat. Prolonged exposure to palmitate in vitro or the presence of overweight/obesity augmented $p66^{Shc}$ expression levels and caused an impaired ability of exogenous insulin to increase cellular insulin content and secreted C-peptide levels in INS-1E cells and human and murine islets. In INS-1E cells, $p66^{Shc}$ knockdown resulted in enhanced insulin-induced augmentation of insulin content and C-peptide secretion and prevented the ability of palmitate to impair these effects of insulin. Conversely, $p66^{Shc}$ overexpression impaired insulin-induced augmentation of insulin content and C-peptide secretion both in the absence and presence of palmitate. Under lipotoxic condition, the effects of $p66^{Shc}$ are mediated by p53-induced increase in $p66^{Shc}$ protein levels and JNK-induced $p66^{Shc}$ phosphorylation at Ser^{36} and appear to involve the phosphorylation of the ribosomal protein S6 kinase at Thr^{389} and of insulin receptor substrate-1 at Ser^{307}, resulting in the inhibition of insulin-stimulated protein kinase b phosphorylation at Ser^{473}. Thus, the $p66^{Shc}$ protein mediates the impaired beta-cell function and insulin resistance induced by saturated fatty acids and excess body fat.

Introduction

The loss of pancreatic beta-cell functional mass is a necessary and early event during the development of type 2 diabetes, as well as a potential target for the treatment and potential cure of type 2 diabetes (**1**). Insulin-secreting beta-cells are targeted by insulin itself, which acts in an autocrine manner to promote beta-cell viability and function (reviewed in **2**). Constitutively secreted insulin is necessary to maintain beta-cell glucose sensitivity (**3**), and alterations in insulin receptor and insulin receptor substrate-1 (IRS-1) result in secretory dysfunction and glucose intolerance (reviewed in **2**). However, the physiological relevance of autocrine insulin activity remains somewhat controversial due to the different insulin doses and glucose levels used to assess beta-cell secretory function (**2**, **4**).

Several metabolic 'stressors' can result in impaired insulin activity or 'insulin resistance' in pancreatic beta-cells, as shown in other insulin-targeted tissues. The exposure of pancreatic beta-cells to high glucose levels induces the c-Jun N-terminal kinase (JNK)- and extracellular signal-regulated kinase (ERK)1/2-mediated inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1), resulting in inhibition of insulin signalling and consequent impairment of insulin's ability to regulate its own biosynthesis (**5**). Similarly, chronic exposure of beta-cells to elevated free fatty acid levels, particularly long-chain saturated fatty acids (SFAs), can induce the JNK-mediated phosphorylation of IRS-1/2, inhibiting insulin-induced insulin gene transcription (**6**).

The $p66^{Shc}$ protein is a redox enzyme, capable of sensing and generating reactive oxygen species (ROS), that also plays a role in metabolic dysfunction. In previous work (**7**), we described for the first time the pro-apoptotic role of $p66^{Shc}$ in INS-1E cells, which is dependent upon its Ser^{36} phosphorylation. Moreover, $p66^{Shc}$ expression levels were found to be increased in INS-1E cells exposed to SFAs, in pancreatic islets isolated from mice fed a high-fat diet, and in human pancreatic islets isolated from overweight/obese subjects (**7**). Previously, $p66^{Shc}$ has been implicated in the

development of obesity-induced insulin resistance through the S6K/IRS-1/protein kinase B (AKT) pathway in endothelial cells and adipose tissue (**8**, **9**).

To date, the effects of SFAs and excess body fat on insulin action and signalling in pancreatic beta-cells have not been explored, and the role of $p66^{Shc}$ in this process has not been investigated. In this study, we provide evidence that $p66^{Shc}$ is crucially involved in lipotoxicity-induced beta-cell insulin resistance.

Research Design and Methods

Pancreatic islet isolation and culture

Human islets were isolated from pancreata obtained from multi-organ donors at the Islet Cell Laboratory of the University of Pisa, Italy, or from pancreatic fragments obtained from patients undergoing pancreatectomy to treat tumors in the ampulla of Vater at the Division of General Surgery of the University Hospital Polyclinic of Bari, Italy. Human pancreatic tissues were processed with the approval of the local Ethics Committee after informed consent was obtained. Anonymised patient information is provided in the **Human Islets Checklist**. Twenty male, 4–8-week-old, C57BL/6 mice were purchased from Charles River Laboratories (Calco, Lecco, Italy). Animal experiments were conducted after obtaining approval from the Ethics Committee of the Genetic Research Centre "Gaetano Salvatore", Biogem, Ariano Irpino, Italy, in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (2011) and the regulations established in Italy and the EU for animal experiments. Mouse and human pancreatic islets were isolated and cultured as described in **10**, **11**, and **Supplemental Methods**.

INS-E cells culture

Rat insulin-secreting INS-1E cells (passage 15–30) were a kind gift from C. B. Wollheim, University of Geneva, Switzerland. INS-1E cells were cultured as described in **Supplemental Methods**.

Pancreatic islets and INS-1E cells treatment

Human (preparation 13-17 in **Human Islets Checklist**) and mouse islets and INS-1E cells were treated with 0.5 mmol/l palmitate or oleate solution (Sigma-Aldrich Inc., St Louis, MO, USA), or with the same volume of a 10% w/v FA-free BSA solution, as a control. After 24 h, islet and cells were stimulated with 10–100 nmol/l human recombinant insulin (Roche Diagnostics, Mannheim, Germany) for different times. To achieve $p66^{Shc}$ knockdown, INS-1E cells were transfected with

100 nmol/l *p66*^{Shc}-targeted short interfering RNA (siRNA, Qiagen, Hilden, Germany) using Lipofectamine® RNAiMAX Reagent and Opti-MEM® medium (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 48 h (**7**). The overexpression of *p66*^{Shc} was achieved by transducing INS-1E cells with recombinant adenoviruses carrying a construct encoding the *p66*^{Shc} protein (**7**). 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). See **Supplemental Methods** for further details.

Measurement of insulin content and secreted C-peptide levels

After a wash with PBS 1X, murine and human pancreatic islets and INS-1E cells were incubated in Krebs-Ringer bicarbonate HEPES buffer (KRBH, 0.1% w/v BSA, 3 mmol/l glucose, 114 mmol/l NaCl, 4.4 mmol/l KCl, 1 mmol/l $MgSO_4$, 29.5 mmol/l $NaHCO_3$, 1.28 mmol/l $CaCl_2$, and 10 mmol/l HEPES, all from Sigma-Aldrich, pH 7.4.) (**12**) for 100 min, followed by stimulation with insulin (10–100 mol/l) diluted in fresh KRBH solution for 40 min. To evaluate insulin content, cells were washed twice with PBS 1X, then mechanically lysed in a non-denaturing lysis buffer (**13**). The level of C-peptide released in the medium, as a measure of endogenously produced insulin, and insulin contents were measured using enzyme-linked immunosorbent assays (ELISAs) specific for mouse (Shibayagi Co., Ltd, Ishihara, Japan), humans, and rats (Mercodia AB, Sylveniusgatan, Uppsala, Sweden).

Immunoblotting

Cells lysates were obtained and analysed by immunoblotting assays (**13** and **Supplemental Methods**). A list of the antibodies used is shown in **Supplemental Table 1**.

Quantitative Real-Time PCR

RNA isolation, cDNA synthesis and quantitative Real-Time PCR were conducted as reported in **13** and **Supplemental Methods**. Primers sequences are available in **Supplemental Table 2**.

116 **Statistical analysis**

117 Data are presented as the mean ± SEM. Statistical analysis was performed using the two-tailed
118 unpaired Student's t-test. Significance was set at $p < 0.05$.

Results

Insulin increases its own content and C-peptide release in INS-1E cells and human and murine islets

Insulin stimulation increased insulin content and C-peptide secreted levels in INS-1E cells (**Fig. 1A** and **B**), and human (**Fig. 1C** and **D**) and murine (**Fig. 1E** and **F**) pancreatic islets. This was associated with increased gene expression levels of *Insulin 1*, but not *Insulin 2*, in INS-1E cells (**Supplemental Fig. 1**).

Palmitate impairs insulin effects in INS-1E cells and human and murine islets

Prior exposure of cells and islets to the SFA palmitate impaired the stimulatory effects of insulin on its own cellular content (**Fig. 1A**, **C, E)** and C-peptide secretion (**Fig. 1B**, **D, F)**. This occurred also in INS-E cells exposed to the unsaturated FA oleate (**Supplemental Fig. 2A** and **B**).

Palmitate-induced impairment of insulin effects in INS-1E cells is mediated by $p66^{Shc}$

The *p66*^{Shc} silencing approach resulted in a 40%–50% reduction of $p66^{Shc}$ protein levels in all experimental conditions compared with control cells treated with vehicle only (**Fig. 2A**). Under these conditions, the palmitate-induced impairment of the insulin stimulatory effect on its own cellular content was largely prevented (**Fig. 2B**). In addition, *p66*^{Shc} knockdown resulted in increased insulin-induced C-peptide secretion both in the absence and presence of palmitate, compared with control cells (**Fig. 2C**).

Conversely, $p66^{Shc}$ overexpression in INS-1E cells resulted in a marked increase of $p66^{Shc}$ protein levels (**Fig. 2D**), and in the inhibition of the ability of insulin to augment insulin content (**Fig. 2E**) and secreted C-peptide levels (**Fig. 2F**) in cells not exposed to palmitate; in the presence of palmitate, these effects of insulin were impaired further (**Fig. 2E** and **F**).

Following overexpression of the p66^{Shc}Ala^{36} mutant protein (**Fig. 2D**), which is unable to undergo phosphorylation at the key Ser^{36} site (**7**), the palmitate-induced impairment of the effects of insulin to enhance its own content (**Fig. 2E**) and C-peptide secretion (**Fig. 2F**), respectively, were no longer observed.

Islets from overweight and obese subjects show elevated p66^{Shc} mRNA expression and reduced response to insulin

In islets (preparation 1-12 in **Human Islets Checklist**) obtained from overweight/obese subjects, *p66^{Shc}* mRNA levels were higher than in islets from lean subjects (**Fig. 2G**). In addition, the effects of insulin to increase insulin content (**Fig. 2H**) and secreted C-peptide (**Fig. 2I**) were blunted.

Role of JNK, p53 protein, and oxidative stress in SFAs-induced insulin resistance in INS-1E cells

The inhibition of JNK (**Fig. 3A**) or p53 protein activity (**Fig. 3E**) reduced palmitate-induced p66^{Shc} phosphorylation at Ser^{36} (**Fig. 3B**) and p66^{Shc} protein expression (**Fig. 3F**), respectively, and prevented the palmitate effects on insulin-induced insulin content (**Fig. 3C** and **G**) and C-peptide secretion (**Fig. 3D** and **H**). Of note, the reduction in ROS levels impaired insulin effects both under basal and palmitate-stimulated conditions (**Supplemental Fig. 3**).

p66^{Shc} mediates the palmitate-induced alterations of insulin signalling via S6K/IRS-1/AKT in INS-1E cells

Palmitate treatment increased S6K Thr^{389} phosphorylation (**Fig. 4A**) and IRS-1 Ser^{307} phosphorylation, resulting both in reduced IRS-1 protein levels (**Fig. 4B**) and impaired ability of insulin to stimulate AKT Ser^{473} phosphorylation (**Fig. 4C**). However, in beta-cells with *p66^{Shc}* knockdown, the effects of palmitate on phosphorylation of S6K (**Fig. 4A**) and IRS-1 (**Fig. 4B**) proteins were abrogated, and this enhanced insulin-stimulated AKT Ser^{473} phosphorylation (**Fig. 4C**). Conversely, p66^{Shc} overexpression did not alter S6K Thr^{389} phosphorylation (**Fig. 4D**) and

exacerbated the palmitate-mediated IRS-1 Ser^{307} phosphorylation and reduction of IRS-1 protein levels (**Fig. 4E**), leading to complete abrogation of insulin-stimulated AKT Ser^{473} phosphorylation (**Fig. 4F**). Of note, $p66^{Shc}$ overexpression increased IRS-1 Ser^{307} phosphorylation, reduced IRS-1 protein levels (**Fig. 4E**), and impaired insulin-stimulated AKT Ser^{473} phosphorylation (**Fig. 4F**), also in cells not exposed to palmitate.

Discussion

We show that acute insulin stimulation increased the cellular insulin content and C-peptide release in rat INS-1E cells and human and murine pancreatic islets. This occurred independently of glucose interference, as all experiments were conducted under low-glucose conditions. Moreover, both exposure to palmitate *in vitro* and presence of excess of body fat *in vivo* resulted in the impaired ability of insulin to stimulate its biosynthesis and secretion in beta-cells and murine and human pancreatic islets *in vitro* and in human pancreatic islets *ex vivo*, respectively. These results mirror and corroborate the previous observation of blunted insulin-induced insulin gene transcription following exposure of mouse pancreatic islets to palmitate (**6**). Our results in INS-1E cells indicate that palmitate alters the insulin signalling pathway by activating the phosphorylation of Thr^{389} in the S6K protein and the inhibitory phosphorylation of Ser^{307} in IRS-1, which are known to result in reduced IRS-1 protein levels and impairment of insulin-induced AKT activation (**9**, **14**). These results support the emerging concept that lipotoxicity is capable to induce an insulin-resistant state in pancreatic beta-cells, similarly to other classical insulin target tissues.

Importantly, we show for the first time that $p66^{Shc}$ plays a key role in this lipotoxicity-mediated insulin resistance in pancreatic beta-cells, since *p66^{Shc}* knockdown or overexpression respectively prevented or worsened palmitate-induced insulin resistance by reducing or boosting palmitate-induced activation of the S6K/IRS-1/AKT pathway (**Fig. 4G**). These results resemble the effects of lipotoxicity in endothelial and adipose cells (**8**, **9**). Experiments with overexpression of the wild-type and mutant (Ala^{36}) forms of $p66^{Shc}$ suggested that $p66^{Shc}$ phosphorylation at Ser^{36} is required to mediate the effects of palmitate on insulin action. Furthermore, we found that palmitate-induced insulin-resistance in INS-1E cells is mediated by p53 and JNK proteins. These results resemble the mechanisms already demonstrated for palmitate-induced beta-cell apoptosis (**7**).

The *ex vivo* experiments performed in human pancreatic islets isolated from donors without diabetes support the involvement of $p66^{Shc}$ in lipotoxicity-induced beta-cell insulin resistance in

human obesity, as both elevated *p66^{Shc}* mRNA expression and impaired insulin effects were observed in islets obtained from overweight/obese subjects, in comparison with islets obtained from lean subjects. Overall, p66^{Shc} can be identified as a crucial mediator of lipotoxicity-promoted beta-cell failure in the context of human obesity. Interestingly, elevated p66^{Shc} levels have also been found in the liver of patients with alcoholic and non-alcoholic steatohepatitis (**15**, **16**), in the heart in response to cardiac dysfunction (**17**) and hyperglycemia (**18**), and in endothelial cells exposed to dyslipidemia (**19**), hyperglycemia (**20**), and pro-inflammatory cytokines (**21**). Taken together, this evidence suggests that p66^{Shc} is ubiquitously involved in multi-organ damage in response to metabolic stressors.

The physiological significance of the effects of autocrine basal insulin *in vivo* are still debated (**2**, **4**, **22-24**), even though under low-glucose conditions insulin could regulate its own content and release, representing a physiological mechanism to replenish insulin stores and make beta-cells prone to rapidly satisfy insulin demand in response to secretagogues (**3**, **22**, **25-27**). Of note, insulin signaling in beta-cells can be triggered by insulin binding at high concentrations to insulin receptor (IR), insulin-like growth factor-1 receptor (IGF-1R), or IR/IGF-1R hybrids (**28**). It has been suggested that insulin promotes its own biosynthesis and secretion mainly through IR/IRS-1 signaling (**29-31**). On the other hand, insulin binding to IGF-1R mainly leads to the activation of IRS-2, which could play a major role in the regulation of beta-cell mass (**32-33**).

In conclusion, p66^{Shc} mediates lipotoxicity-induced beta-cell insulin resistance, an emerging mechanism for beta-cell failure in obesity and type 2 diabetes. Of interest, glucagon-like peptide 1 receptor agonists, which reportedly inhibit palmitate-induced apoptosis by preventing JNK phosphorylation (**34**), also inhibited p66^{Shc} phosphorylation on Ser^{36} (**7**), and may thus use this mechanism as well to enhance insulin secretion. Since the loss of pancreatic beta-cell functional mass is a necessary and early event in type 2 diabetes (**1**), the combined effects of p66^{Shc} on beta-

219 cell secretory-function and survival suggest that this protein may represent a potential target for the
220 prevention or treatment type 2 diabetes onset or progression.

Acknowledgments

Author Contributions

All of authors contributed to conception and design, acquisition of data, analysis, and interpretation of data; they drafted the final article or revised it critically for important intellectual content and finally approved the version to be published.

Conceptualization, F.G. and A.N.; data curation, G.B., N.M., L.D., A.N., and F.G.; formal analysis and investigation G.B., N.M., L.D., A.B., M.R., R.D., V.A.G., C.C.; project administration, F.G. and A.N.; resources, I.P., P.M. and L.V.; supervision, L.L., F.G., and A.N.; visualization, A.C., S.P. and L.L.; writing—original draft, A.N.; writing—review and editing, F.G.

Guarantor Statement

F.G. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest Statement

No potential conflicts of interest relevant to this article were reported.

Funding

This work was supported by AGER 2 Project (grant n° 2016-0174, COMPETITIVE e Claims of Olive oil to iMProvE The market ValuE of the product), Ministero dell'Università e della Ricerca of Italy (Progetti di Rilevante Interesse Nazionale, 2015), the European Union (European Social Fund, PON R&I 2014–2020, AIM 1810057), and the "Fondazione per la Ricerca Biomedica Saverio e Isabella Cianciola".

Prior Presentation Information

Data have been previously presented at scientific meetings (79th ADA Scientific Session, 2019; 40th National Meeting of the Italian Society of Endocrinology, 2019; 27th National Meeting of the Italian Society of Diabetology, 2018; 53th EASD Annual Meeting, 2017; International Symposium on Insulin Receptor and Insulin Action, 2017; 39th National Meeting of the Italian Society of Endocrinology, 2017).

Reference to prior publication of the study in abstract form:

The p66Shc protein mediates saturated fatty acid-induced insulin resistance in pancreatic beta cells. G. Biondi, A. Natalicchio, N. Marrano, R. Spagnuolo, R. Labarbuta, L. Dipaola, A. Cignarelli, P. Marchetti, S. Perrini, L. Laviola, F. Giorgino. Diabetologia. 2017 Sep;60 Suppl 1:S194.

195-OR: The p66Shc protein mediates insulin resistance in pancreatic ß cells under lipotoxic conditions. L. Dipaola, A. Natalicchio, G. Biondi, N. Marrano, M. Bugliani, A. Cignarelli, S. Perrini, P. Marchetti, L. Laviola, F. Giorgino. Diabetes 2019 Jun; 68(Supplement 1).

Data and Resource Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

References

1. Marrano N, Biondi G, Cignarelli A, Perrini S, Laviola L, Giorgino F, Natalicchio A. Functional loss of pancreatic islets in type 2 diabetes: How can we halt it? Metabolism 2020;110:154304.

2. Leibiger IB, Leibiger B, Berggren PO. Insulin signaling in the pancreatic beta-cell. Annu Rev Nutr 2008;28:233-251.

3. Srivastava S, Goren HJ. Insulin constitutively secreted by beta-cells is necessary for glucose-stimulated insulin secretion. Diabetes 2003;52(8):2049-2056.

4. Rhodes CJ, White MF, Leahy JL, Kahn SE. Direct autocrine action of insulin on β-cells: does it make physiological sense? Diabetes 2013;62(7):2157-2163.

5. Andreozzi F, D'Alessandris C, Federici M, Laratta E, Del Guerra S, Del Prato S, Marchetti P, Lauro R, Perticone F, Sesti G. Activation of the hexosamine pathway leads to phosphorylation of insulin receptor substrate-1 on Ser307 and Ser612 and impairs the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin insulin biosynthetic pathway in RIN pancreatic beta-cells. Endocrinology 2004;145(6):2845-57.

6. Solinas G, Naugler W, Galimi F, Lee MS, Karin M. Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. Proc Natl Acad Sci U S A 2006;103(44):16454-16459.

7. Natalicchio A, Tortosa F, Labarbuta R, Biondi G, Marrano N, Carchia E, Leonardini A, Cignarelli A, Bugliani M, Marchetti P, Fadini GP, Giorgio M, Avogaro A, Perrini S, Laviola L, Giorgino F. The p66(Shc) redox adaptor protein is induced by saturated fatty acids and mediates lipotoxicity-induced apoptosis in pancreatic beta cells. Diabetologia 2015; 58(6):1260-71. Erratum in: Diabetologia 2015;58(11):2682.

8. Paneni F, Costantino S, Cosentino F. p66(Shc)-induced redox changes drive endothelial insulin resistance. Atherosclerosis 2014;236(2):426-429.

9. Ranieri SC, Fusco S, Panieri E. Mammalian life-span determinant p66ShcA mediates obesity-induced insulin resistance. Proc Natl Acad Sci U S A 2010;107(30):13420-13425.

10. Li DS, Yuan YH, Tu HJ, Liang QL, Dai LJ. A protocol for islet isolation from mouse pancreas. Nat Protoc 2009;4:1649-1652.

11. Lupi R, Del Guerra S, Fierabracci V, Marselli L, Novelli M, Patanè G, Boggi U, Mosca F, Piro S, Del Prato S, Marchetti P. Lipotoxicity in human pancreatic islets and the protective effect of metformin (Abstract). Diabetes 2002;51 Suppl 1:S134-7.

12. Park SH, Kim SY, Baek WK, Lim B, Park JH, Sung HY, Kim YK, Bae KC, Bae JH, Song DK. Regulation of glucose-dependent insulin secretion by insulin: possible role of AMP-activated protein kinase. Life Sci 2009;85(3-4):178-83.

13. Marrano N, Spagnuolo R, Biondi G, Cignarelli A, Perrini S, Vincenti L, Laviola L, Giorgino F, Natalicchio A. Effects of Extra Virgin Olive Oil Polyphenols on Beta-Cell Function and Survival. Plants (Basel). 2021;10(2):286.

14. Zhang J, Gao Z, Yin J, Quon MJ, Ye J. S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2. J Biol Chem 2008;283(51):35375-82.

15. Perrini S, Tortosa F, Natalicchio A, Pacelli C, Cignarelli A, Palmieri VO, Caccioppoli C, De Stefano F, Porro S, Leonardini A, Ficarella R, De Fazio M, Cocco T, Puglisi F, Laviola L, Palasciano G, Giorgino F. The p66Shc protein controls redox signaling and oxidation-dependent DNA damage in human liver cells. Am J Physiol Gastrointest Liver Physiol 2015;309(10):G826-40.

16. Tomita K, Teratani T, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, Nishiyama K, Mataki N, Irie R, Minamino T, Okada Y, Kurihara C, Ebinuma H, Saito H, Shimizu I, Yoshida Y, Hokari R, Sugiyama K, Hatsuse K, Yamamoto J, Kanai T, Miura S, Hibi T. p53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. J Hepatol 2012;57(4):837-43.

17. Graiani G, Lagrasta C, Migliaccio E, Spillmann F, Meloni M, Madeddu P, Quaini F, Padura IM, Lanfrancone L, Pelicci P, Emanueli C. Genetic deletion of the p66Shc adaptor protein protects from angiotensin II-induced myocardial damage. Hypertension 2005;46(2):433-40.

18. Costantino S, Paneni F, Mitchell K, Mohammed SA, Hussain S, Gkolfos C, Berrino L, Volpe M, Schwarzwald C, Lüscher TF, Cosentino F. Hyperglycaemia-induced epigenetic changes drive persistent cardiac dysfunction via the adaptor p66Shc. Int J Cardiol 2018;268:179-186.

19. Kim YR, Kim CS, Naqvi A, Kumar A, Kumar S, Hoffman TA, Irani K. Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction. Am J Physiol Heart Circ Physiol 2012;303(2):H189-96.

20. Zhang M, Lin L, Xu C, Chai D, Peng F, Lin J. VDR Agonist Prevents Diabetic Endothelial Dysfunction through Inhibition of Prolyl Isomerase-1-Mediated Mitochondrial Oxidative Stress and Inflammation. Oxid Med Cell Longev 2018;2018:1714896.

21. Laviola L, Orlando MR, Incalza MA, Caccioppoli C, Melchiorre M, Leonardini A, Cignarelli A, Tortosa F, Labarbuta R, Martemucci S, Pacelli C, Cocco T, Perrini S, Natalicchio A, Giorgino F. TNFα signals via p66(Shc) to induce E-Selectin, promote leukocyte transmigration and enhance permeability in human endothelial cells. PLoS One 2013;8(12):e81930.

22. Braun M, Ramracheya R, Rorsman P. Autocrine regulation of insulin secretion. Diabetes Obes Metab 2012;14 Suppl 3:143-151.

23. Skovsø S, Panzhinskiy E, Kolic J, Cen HH, Dionne DA, Dai XQ, Sharma RB, Elghazi L, Ellis CE, Faulkner K, Marcil SAM, Overby P, Noursadeghi N, Hutchinson D, Hu X, Li H, Modi H, Wildi JS, Botezelli JD, Noh HL, Suk S, Gablaski B, Bautista A, Kim R, Cras-Méneur C, Flibotte S, Sinha S, Luciani DS, Nislow C, Rideout EJ, Cytrynbaum EN, Kim JK, Bernal-Mizrachi E, Alonso LC, MacDonald PE, Johnson JD. Beta-cell specific Insr deletion promotes insulin hypersecretion and improves glucose tolerance prior to global insulin resistance. Nat Commun 2022;13(1):735.

24. Mari A, Tura A, Natali A, Anderwald C, Balkau B, Lalic N, Walker M, Ferrannini E; RISC Investigators. Influence of hyperinsulinemia and insulin resistance on in vivo β-cell function: their role in human β-cell dysfunction. Diabetes. 2011 Dec;60(12):3141-7.

25. Rachdaoui N. Insulin: The Friend and the Foe in the Development of Type 2 Diabetes Mellitus. Int J Mol Sci 2020;21(5):1770.

26. Ohsugi M, Cras-Méneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA. Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. J Biol Chem 2005;280(6):4992-5003.

27. Wang J, Gu W, Chen C. Knocking down Insulin Receptor in Pancreatic Beta Cell lines with Lentiviral-Small Hairpin RNA Reduces Glucose-Stimulated Insulin Secretion via Decreasing the Gene Expression of Insulin, GLUT2 and Pdx1. Int J Mol Sci 2018;19(4):985.

28. Nagao H, Cai W, Wewer Albrechtsen NJ, Steger M, Batista TM, Pan H, Dreyfuss JM, Mann M, Kahn CR. Distinct signaling by insulin and IGF-1 receptors and their extra- and intracellular domains. Proc Natl Acad Sci U S A. 2021;118(17):e2019474118.

29. Marchetti P, Lupi R, Federici M, Marselli L, Masini M, Boggi U, Del Guerra S, Patanè G, Piro S, Anello M, Bergamini E, Purrello F, Lauro R, Mosca F, Sesti G, Del Prato S. Insulin secretory function is impaired in isolated human islets carrying the Gly(972)-->Arg IRS-1 polymorphism. Diabetes 2002;51(5):1419-24.

30. Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 1999;96(3):329-39.

31. Goldfine AB, Kulkarni RN. Modulation of β-cell function: a translational journey from the bench to the bedside. Diabetes Obes Metab 2012;14 Suppl 3:152-60.

32. Hakuno F, Takahashi SI. IGF1 receptor signaling pathways. J Mol Endocrinol 2018;61(1):T69-T86.

33. Hennige AM, Ozcan U, Okada T, Jhala US, Schubert M, White MF, Kulkarni RN. Alterations in growth and apoptosis of insulin receptor substrate-1-deficient beta-cells. Am J Physiol Endocrinol Metab 2005;289(2):E337-46.

34. Natalicchio A, Labarbuta R, Tortosa F, Biondi G, Marrano N, Peschechera A, Carchia E, Orlando MR, Leonardini A, Cignarelli A, Marchetti P, Perrini S, Laviola L, Giorgino F. Exendin-4 protects pancreatic beta cells from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway. Diabetologia 2013;56(11):2456-66.

Figure Legends

Figure 1. Effects of insulin and palmitate on insulin content and C-peptide release in INS-1E cells and human and murine islets. INS-1E cells (**A**, **B**), human islets (**C**, **D**) and murine islets (**E**, **F**) were cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, then incubated in KRBH buffer for 100 min, and finally stimulated with 10 (**A**, **B**) or 100 (**C**-**F**) nmol/l insulin in fresh KRBH buffer for 40 minutes. **A**, **C**, **E**: Insulin content was measured by enzyme-linked immunosorbent assay (ELISA), normalized to protein concentration, and expressed as a percentage of untreated control (at least n = 4 independent experiments); the fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) is also shown. **B**, **D**, **F**: Secreted C-peptide levels were measured by ELISA, normalized against total protein concentration, and expressed as a percentage of the untreated control (at least n = 3 independent experiments); the fold-increase of insulin-stimulated secreted C-peptide levels over control (not treated with palmitate) is also shown. *$p < 0.05$ vs control without insulin; †$p < 0.05$ vs control without palmitate. Data are expressed as the mean ± SEM. Palm, palmitate.

Figure 2. Role of $p66^{Shc}$ protein in lipotoxicity-induced beta-cell insulin resistance. **A**– **C**: INS-1E cells were transfected with *$p66^{Shc}$* siRNA for 24 h, then cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for an additional 24 h. Successively, cells were incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40 minutes. The transfection reagent only was used as control. **A**: Representative immunoblot of $p66^{Shc}$ and beta-actin protein levels with the densitometric analysis of the bands, expressed as relative optical density. Values were corrected using total beta-actin levels as a loading control and normalized against untreated control (n = 4 independent experiments). **B**: Insulin content was measured with a specific ELISA, normalized to protein concentration, and shown as fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) (n = 5 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). **D**–**F**: INS-1E cells were infected with a recombinant adenoviral vector encoding $p66^{Shc}$ (Ad/*p66*^{Shc}), a phosphorylation-defective $p66^{Shc}$ protein containing a Ser^{36} to Ala mutation (Ad/Ala^{36}), or with empty adenovirus (Ad/mock) for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, then incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40 minutes. **D:** Representative immunoblot of $p66^{Shc}$ and beta-actin protein levels with the densitometric analysis of bands, expressed as the relative optical density, corrected using total beta-actin levels as a loading control, and normalized against control (n = 8 independent experiments). **E**: Insulin content was measured by ELISA, normalized to total protein concentration, and shown as fold-increase over control (not treated with palmitate) (n = 5 independent experiments). **F**: Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the control (n = 8 independent experiments). **G**: *p66*^{Shc} mRNA levels in pancreatic islets from lean (BMI < 25 kg/m^2, white bars) and overweight/obese (BMI ≥ 25 kg/m^2, grey bars) subjects (n = 4 independent experiments). **H** and **I**: Pancreatic islets were isolated from lean or overweight/obese subjects, then incubated in KRBH buffer for 100 min, and finally stimulated with 100 nmol/l insulin in fresh KRBH medium for 40 min. **H:** Insulin content was measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (n = 4 independent experiments). **I:** 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 without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent; §p < 0.05 vs Ad/mock; || p < 0.05 vs Ad/*p66*^{Shc}; ¶ p < 0.05 vs BMI < 25 kg/m^2. Data are expressed as the mean ± SEM. Palm, palmitate.

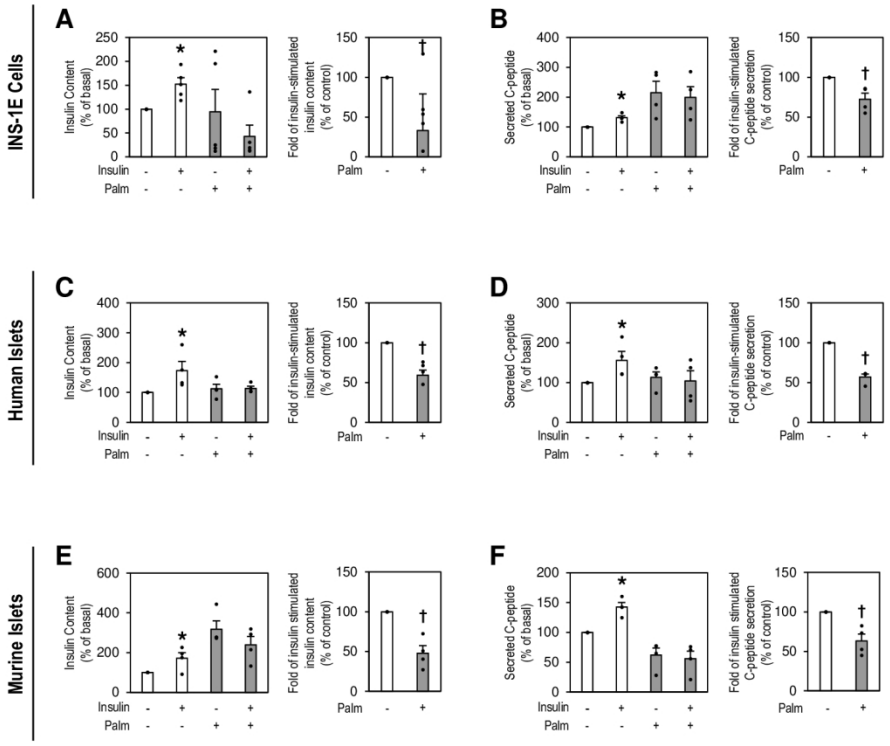
Figure 3. Role of JNK kinase and p53 in palmitate effects on insulin-induced insulin content and C-peptide release in INS-1E cells. **A**, **B**: Cells were stimulated with 10 or 30 µmol/l JNK inhibitor (SP600125) or dimethyl sulfoxide (DMSO) as control for 2 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h. **A**: Representative immunoblot of c-Jun (Ser^{63}) phosphorylation and beta-actin protein levels, quantified by densitometric analysis of the bands, expressed as relative optical density. C-Jun (Ser^{63}) phosphorylation values were corrected using total beta-actin levels as a loading control and normalized against untreated control (n = 3 independent experiments). **B**: Representative immunoblot of $p66^{Shc}$ (Ser^{36}) phosphorylation and protein levels, quantified by densitometric analysis of the bands, expressed as relative optical density. $p66^{Shc}$ (Ser^{36}) phosphorylation values were normalized against $p66^{Shc}$ protein, then against untreated control (n = 3 independent experiments). **C**, **D**: Cells were stimulated with 30 µmol/l JNK inhibitor (SP600125) or dimethyl sulfoxide (DMSO) as control 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. **E**, **F**: Cells were stimulated with 30 µmol/l pifithrin-alpha (PFT-α) or dimethyl sulfoxide (DMSO) as control for 1 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h. **E**: Representative immunoblot of p53 (Lys^{382}) acetylation and beta-actin protein levels, quantified by densitometric analysis of the bands, expressed as relative optical density. P53 (Lys^{382}) acetylation values were corrected using total beta-actin levels as a loading control and normalised against untreated control (n = 6 independent experiments). **F**: Representative immunoblot of $p66^{Shc}$ protein levels, quantified by densitometric analysis of the bands, and expressed as relative optical density. $P66^{Shc}$ values were normalized against beta-actin levels, then against untreated control (at least n = 5 independent experiments). **G**, **H**: Cells were stimulated with 30 µmol/l pifithrin-alpha (PFT-α) or dimethyl sulfoxide (DMSO) as control for 1 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. **C**, **G:** Insulin content was measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (at least n = 3 independent experiments). **D**, **H:** Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (at least n = 3 independent experiments). *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs control without inhibitor. Data are expressed as the mean ± SEM. Palm, palmitate; JNKi, JNK inhibitor (SP600125); PFT-α, pifithrin-alpha.

Figure 4. Molecular mechanisms by which $p66^{Shc}$ mediates palmitate-induced insulin resistance in beta-cells. **A**–**C:** INS-1E cells were transiently transfected with *p66*^{Shc} siRNA for 24 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for an additional 24 h, and then stimulated with 100 nmol/l insulin for 10 min. The transfection reagent only was used as a control. S6K (Thr^{389}) (**A**), IRS-1 (Ser^{307}) (**B**), and AKT (Ser^{473}) (**C**) phosphorylation was measured by immunoblotting and quantified by densitometric analysis of the related bands, normalized against beta-actin (**A**), total IRS-1 (**B**), and total AKT (**C**), respectively. The resulting relative optical density values are expressed as percentages of the untreated control. **D**–**F:** INS-1E cells were infected with recombinant adenoviral vector encoding $p66^{Shc}$ (Ad/*p66*^{Shc}) or with empty adenovirus (Ad/mock) for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h and stimulated with 100 nmol/l insulin for 10 minutes. S6K (Thr^{389}) (**D**), IRS-1 (Ser^{307}) (**E**) and AKT (Ser^{473}) (**F**) phosphorylation was measured by immunoblotting and quantified by densitometric analysis of the related bands, normalized against beta-actin (**D**), total IRS-1 (**E**), and total AKT (**F**), respectively. The resulting relative optical density values are expressed as percentages of the untreated control. **A** and **C**: n = 5 independent experiments; **B** and **E:** n = 3 independent experiments; **D**: n = 13 independent experiments; **F**: n = 8 independent experiments. Data are expressed as the mean ± SEM. *p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent; §p < 0.05 vs Ad/mock. Palm,

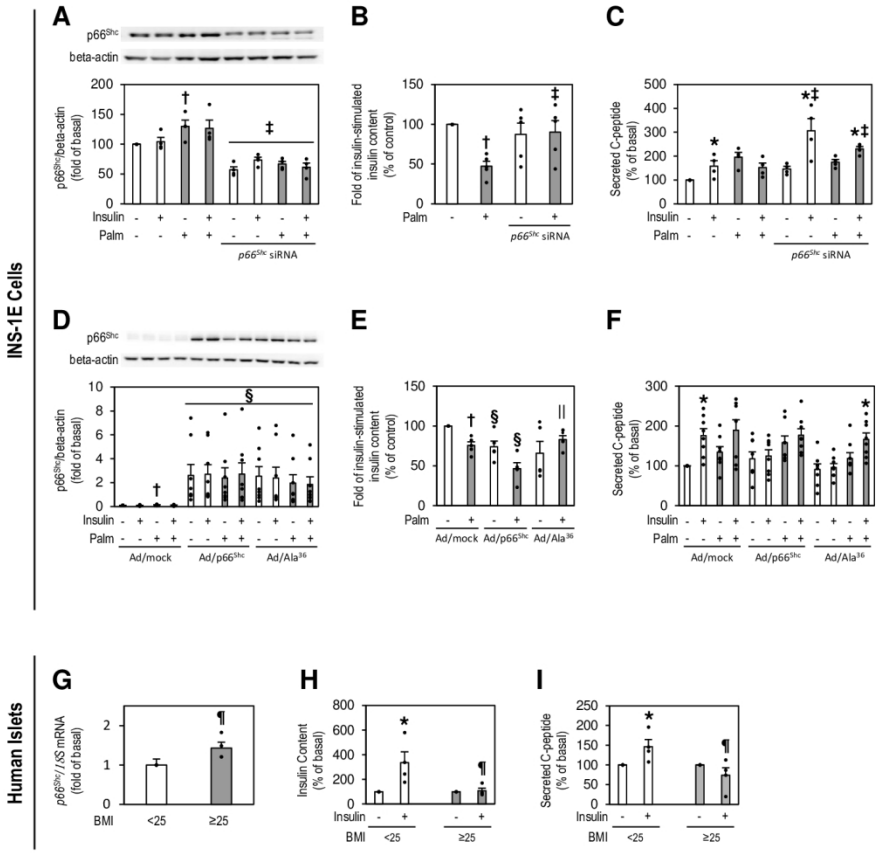
palmitate. **G:** Saturated fatty acids increase p66^{Shc} levels and its phosphorylation in Ser^{36}, via the p53 protein and JNK kinase, respectively, thus inducing apoptosis and insulin resistance in pancreatic beta-cells (**7**). In addition, p66^{Shc} activation induces the phosphorylation of Thr^{389} in the S6K protein and the inhibitory phosphorylation of Ser^{307} in IRS-1, which in turn reduce IRS-1 protein levels and impair insulin-induced AKT activation, thus impairing the ability of insulin to increase its own cellular content and C-peptide secretion in pancreatic beta-cells. AKT, protein kinase b; IRS-1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinase; p53, tumor suppressor protein 53; p66^{Shc}, SHC (Src homology 2 domain-containing) transforming protein 1, 66 kDa isoform; S6K, ribosomal protein S6 kinase.

Figure 1



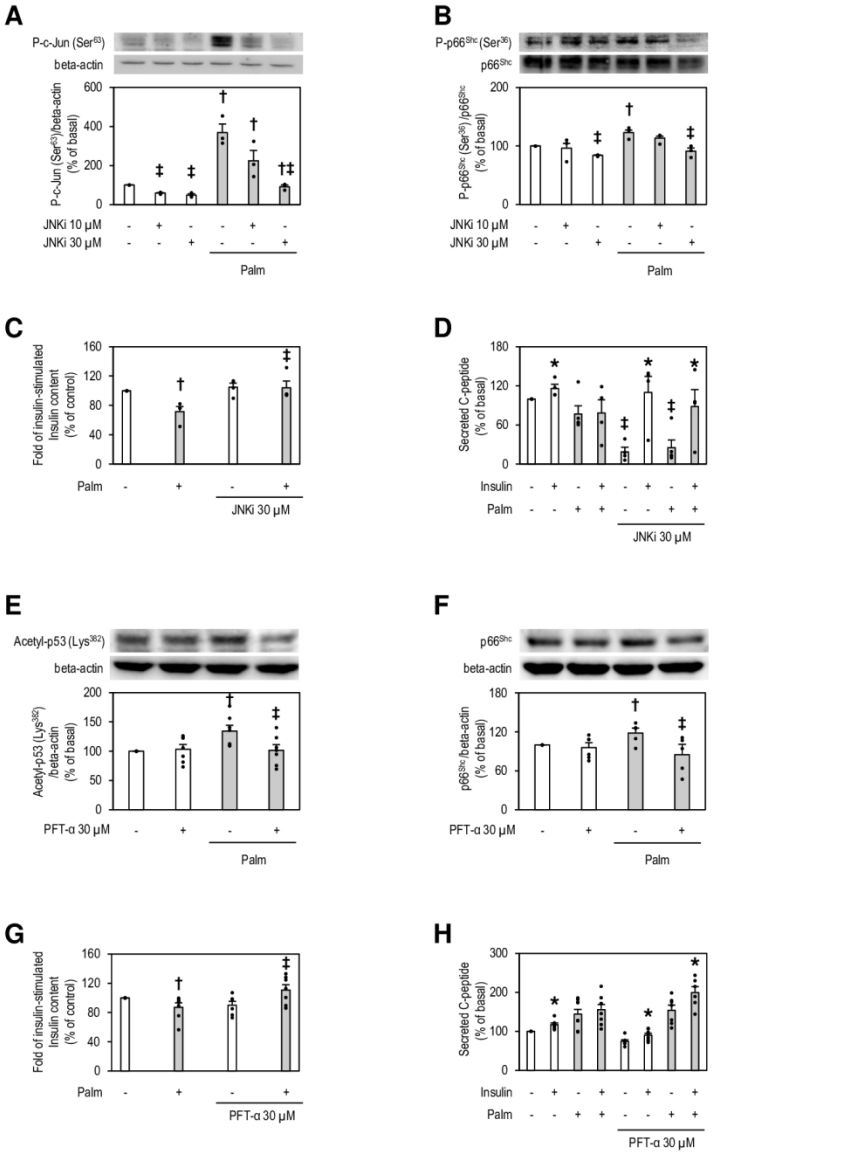
190x254mm (200 x 200 DPI)

Figure 2



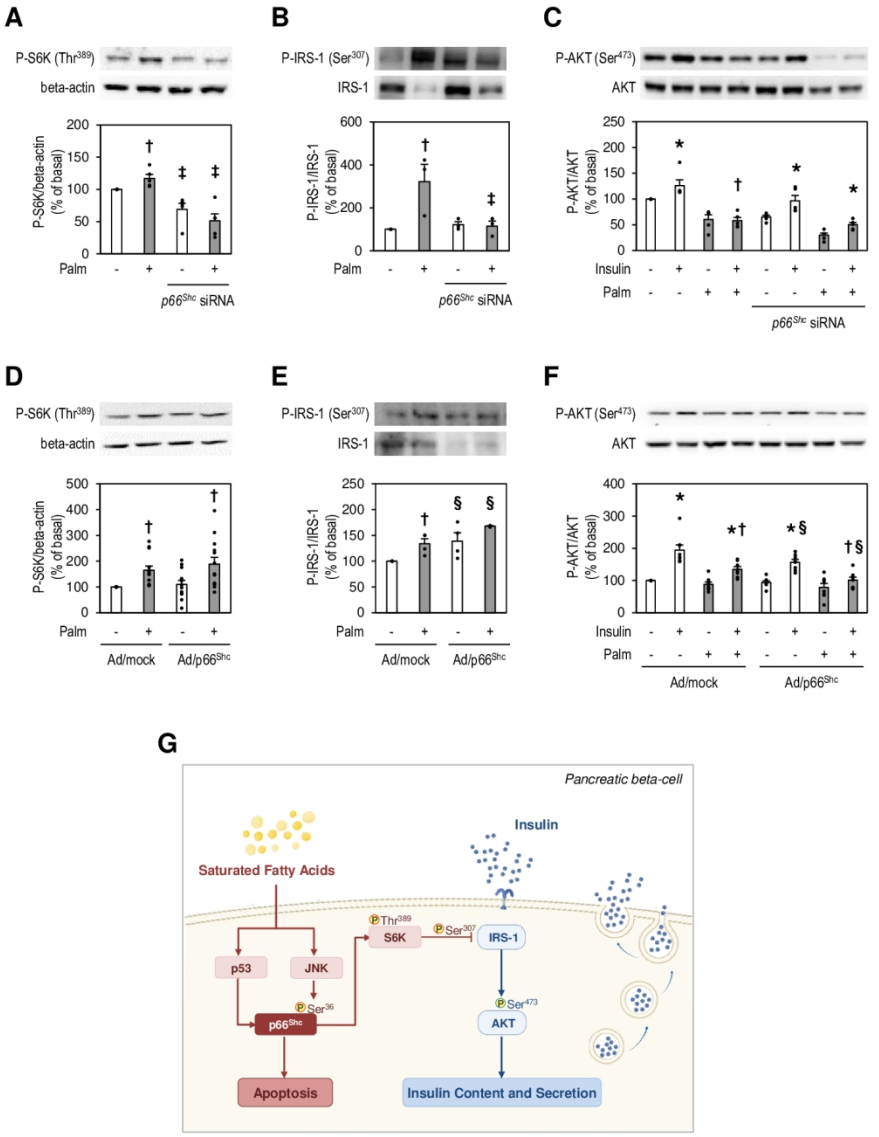
190x254mm (200 x 200 DPI)

Figure 3



190x254mm (200 x 200 DPI)

Figure 4



190x254mm (200 x 200 DPI)

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).

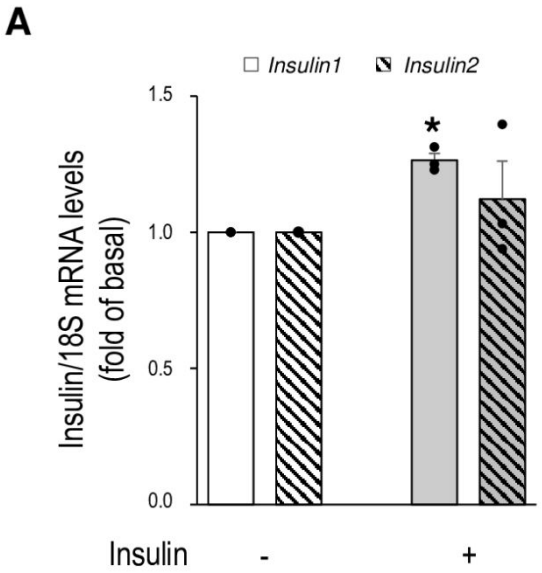
Supplemental Table 1. Characteristics of antibodies used for immunoblotting.

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

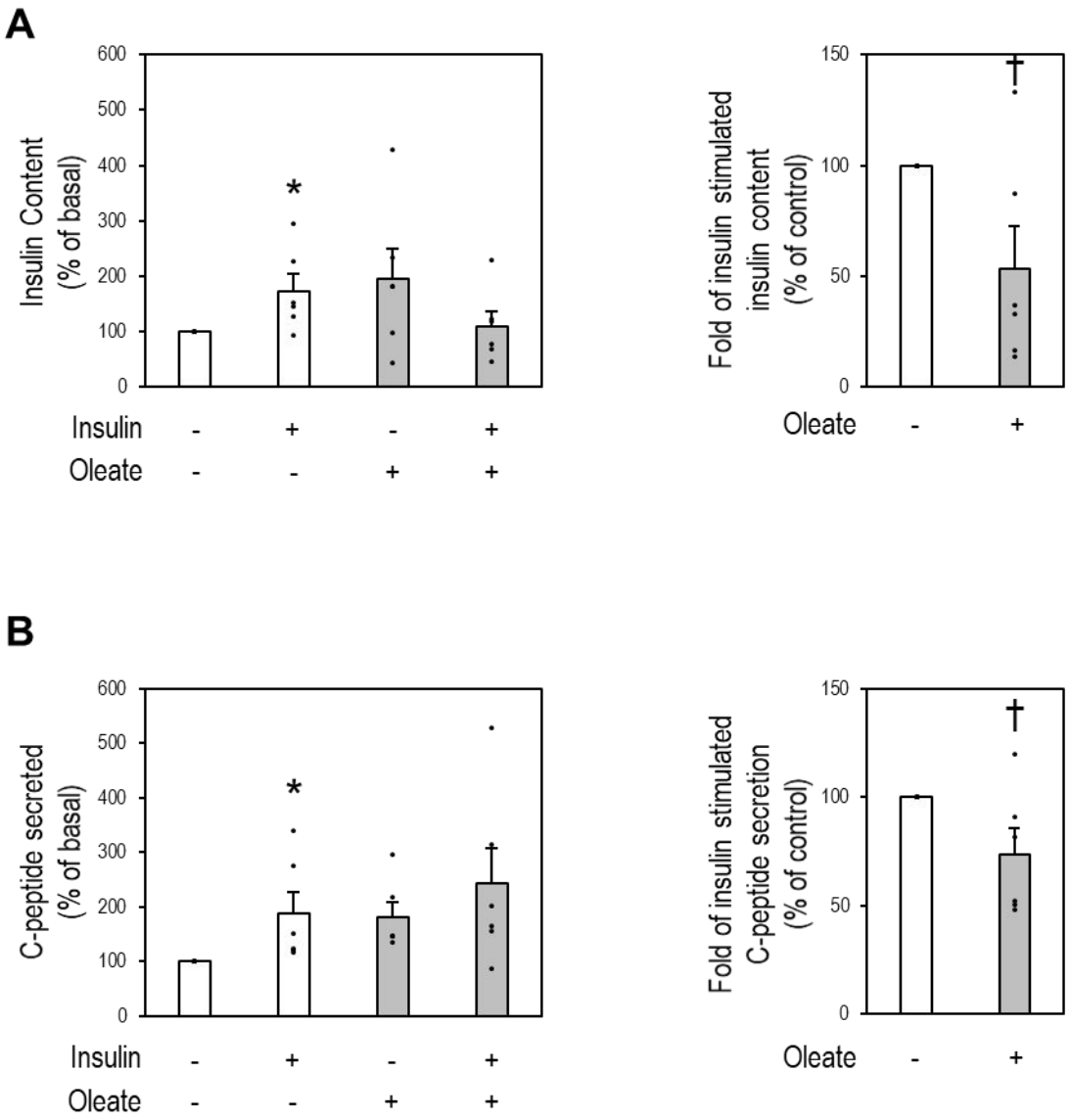
Supplemental Table 2. Primers used for quantitative real-time PCR analysis.

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'

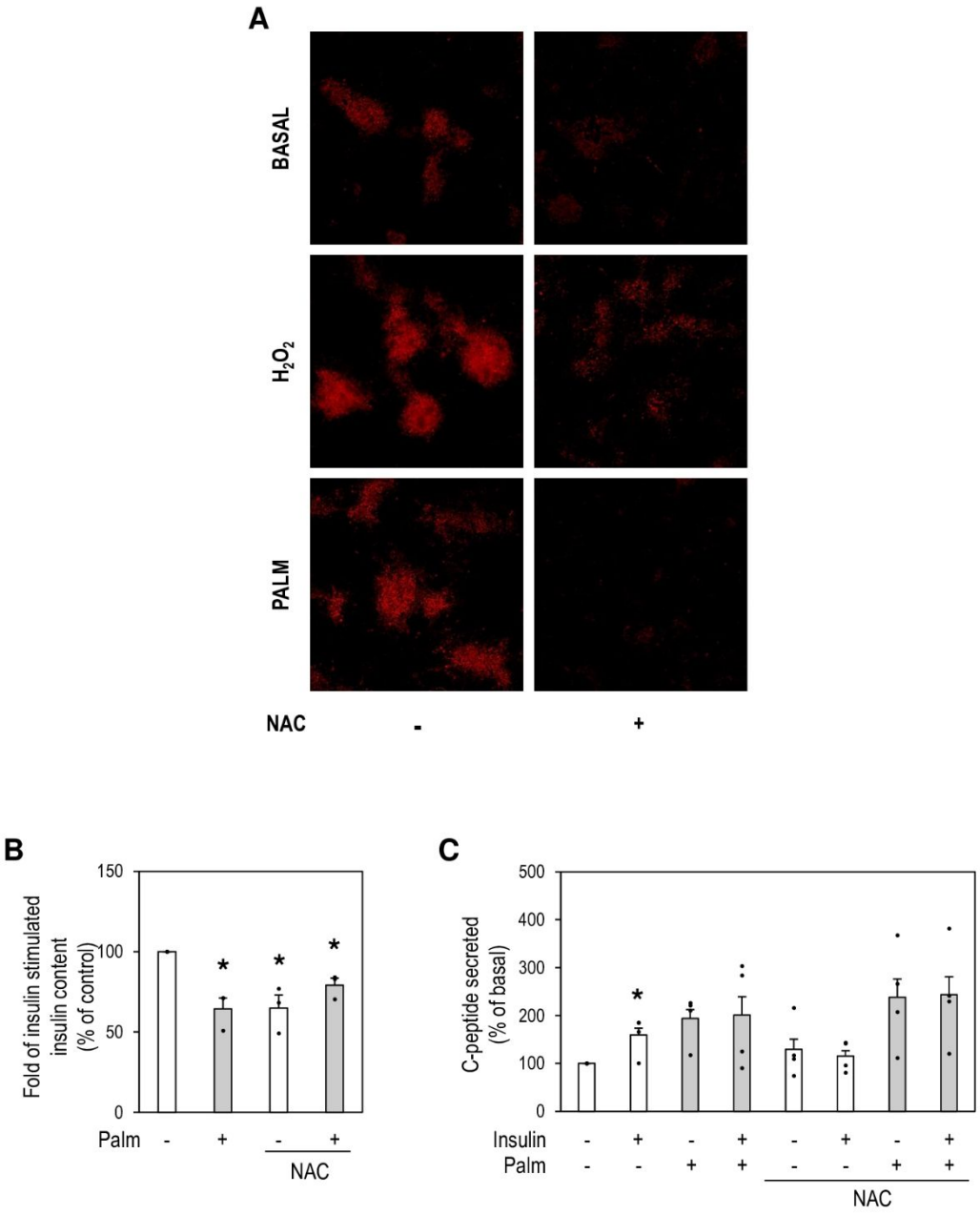
Supplemental Figures



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; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs control without NAC. Data are expressed as the mean ± SEM. Palm, palmitate; NAC, N-Acetyl-L-cysteine; H_2O_2, hydrogen peroxide.

American Diabetes Association. diabetes

Checklist for Reporting Human Islet Preparations Used in Research

Adapted from Hart NJ, Powers AC (2018) Progress, challenges, and suggestions for using human islets to understand islet biology and human diabetes. Diabetologia https://doi.org/10.1007/s00125-018-4772-2.

Manuscript DOI: https://doi.org/10.2337/db21-1066

Title: The p66^{Shc} protein mediates insulin resistance in pancreatic beta-cells under lipotoxic conditions

Author list: Giuseppina Biondi, Nicola Marrano, Lucia Dipaola, Anna Borrelli, Martina Rella, Rossella D'Oria, Valentina Annamaria Genchi, Cristina Caccioppoli, Immacolata Porreca, Angelo Cignarelli, Sebastio Perrini, Piero Marchetti, Leonardo Vincenti, Luigi Laviola, Francesco Giorgino, Annalisa Natalicchio

Corresponding author: Francesco Giorgino | **Email address: francesco.giorgino@uniba.it**

Islet preparation	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
MANDATORY INFORMATION								
Unique identifier	F65ND19.53	M70ND20.20	F55ND25.26	M71ND22.46	F67ND21.48	F61ND20.00	M77ND31.25	M78ND26.99
Donor age (years)	65	70	55	71	67	61	77	78
Donor sex (M/F)	F	M	F	M	F	F	M	M
Donor BMI (kg/m^2)	19.53	20.20	25.26	22.46	21.48	20.00	31.25	26.99

Fasting glycemia (mg/dl)	96	81	95	88	96	90	91	84
Origin/source of islets[b]	Bari	Bari	Bari	Bari	Bari	Bari	Bari	Bari
Islet isolation centre	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari
Donor history of diabetes? Yes/No	No	No	No	No	No	No	No	No
If Yes, complete the next two lines if this information is available								
Diabetes duration (years)								
Glucose-lowering therapy at time of death[c]								

RECOMMENDED INFORMATION								
Donor cause of death	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Warm ischaemia time (h)								
Cold ischaemia time (h)	<1	<1	<1	<1	<1	<1	<1	<1

Estimated purity (%)	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Estimated viability (%)								
Total culture time (h)[d]	24	24	24	24	24	24	24	24
Glucose-stimulated insulin secretion or other functional measurement[e]	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Handpicked to purity? Yes/No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Additional notes								

Islet preparation	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
MANDATORY INFORMATION								
Unique identifier	F71ND30.47	M57ND25.10	F63ND28.28	UPI_T2DSyst_304	UPI_T2DSyst_266	F66ND19.38	UPI_T2DSyst_235	M64ND21.80
Donor age (years)	71	57	63	80	58	66	52	64

Donor sex (M/F)	F	M	F	F	F	F	F	M
Donor BMI (kg/m^2)	30.47	25.10	28.28	24.80	25.80	19.38	25.40	21.80
Fasting glycemia (mg/dl)	126	116	86	122	104	82	85	162
Origin/source of islets[b]	Bari	Bari	Bari	Pisa	Pisa	Bari	Pisa	Bari
Islet isolation centre	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Department of Emergency and Organ Transplantation-University of Bari	Islet Laboratory – University of Pisa	Islet Laboratory – University of Pisa	Department of Emergency and Organ Transplantation-University of Bari	Islet Laboratory – University of Pisa	Department of Emergency and Organ Transplantation-University of Bari
Donor history of diabetes? Yes/No	No	No	No	No	No	No	No	No
If Yes, complete the next two lines if this information is available								
Diabetes duration (years)								
Glucose-lowering therapy at time of death[c]								

RECOMMENDED INFORMATION								
Donor cause of death	N/A	N/A	N/A	Cerebral hemorrhage	Post-anoxic encephalopathy	N/A	Ischaemic stroke	N/A

Warm ischaemia time (h)								
Cold ischaemia time (h)	<1	<1	<1	36	7	<1	15	<1
Estimated purity (%)	N/D	N/D	N/D	90	60	N/D	60	N/D
Estimated viability (%)								
Total culture time (h)[d]	24	24	24	72	72	24	72	24
Glucose-stimulated insulin secretion or other functional measurement[e]	N/D	N/D	N/D	Yes, evaluated by batch incubation	Yes, evaluated by batch incubation	N/D	Yes, evaluated by batch incubation	N/D
Handpicked to purity? Yes/No	Yes	Yes	Yes	No	No	Yes	No	Yes
Additional notes								

Islet preparation	**17**							
MANDATORY INFORMATION								

Unique identifier	M68ND24.71							
Donor age (years)	68							
Donor sex (M/F)	M							
Donor BMI (kg/m^2)	24.71							
Fasting glycemia (mg/dl)	81							
Origin/source of islets[b]	Bari							
Islet isolation centre	Department of Emergency and Organ Transplantation-University of Bari							
Donor history of diabetes? Yes/No	No							
If Yes, complete the next two lines if this information is available								
Diabetes duration (years)								
Glucose-lowering therapy at time of death[c]								

RECOMMENDED INFORMATION								
Donor cause of death	N/A							
Warm ischaemia time (h)								
Cold ischaemia time (h)	<1							
Estimated purity (%)	N/D							
Estimated viability (%)								
Total culture time (h)[d]	24							
Glucose-stimulated insulin secretion or other functional measurement[e]	N/D							
Handpicked to purity? Yes/No	Yes							
Additional notes								