Diabetologia (2015) 58:1260–1271
DOI 10.1007/s00125-015-3563-2

ARTICLE

The p66^{Shc} redox adaptor protein is induced by saturated fatty acids and mediates lipotoxicity-induced apoptosis in pancreatic beta cells

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Received: 3 February 2015 / Accepted: 27 February 2015 / Published online: 26 March 2015

Abstract

Aims/hypothesis The role of the redox adaptor protein p66^{Shc} as a potential mediator of saturated fatty acid (FA)-induced beta cell death was investigated.

Methods The effects of the FA palmitate on p66^{Shc} expression were evaluated in human and murine islets and in rat insulin-secreting INS-1E cells. p66^{Shc} expression was also measured in islets from mice fed a high-fat diet (HFD) and from human donors with different BMIs. Cell apoptosis was quantified by two independent assays. The role of p66^{Shc} was investigated using pancreatic islets from *p66$^{Shc-/-}$* mice and in INS-1E cells with knockdown of p66^{Shc} or overexpression of wild-type and phosphorylation-defective p66^{Shc}. Production of reactive oxygen species (ROS) was evaluated by the dihydroethidium oxidation method.

Results Palmitate induced a selective increase in p66^{Shc} protein expression and phosphorylation on Ser^{36} and augmented apoptosis in human and mouse islets and in INS-1E cells. Inhibiting the tumour suppressor protein p53 prevented both the palmitate-induced increase in p66^{Shc} expression and beta cell apoptosis. Palmitate-induced apoptosis was abrogated in islets from *p66$^{Shc-/-}$* mice and following *p66^{Shc}* knockdown in INS-1E cells; by contrast, overexpression of p66^{Shc}, but not that of the phosphorylation-defective p66^{Shc} mutant, enhanced palmitate-induced apoptosis. The pro-apoptotic effects of p66^{Shc} were dependent upon its c-Jun N-terminal kinase-mediated phosphorylation on Ser^{36} and associated with generation of ROS. p66^{Shc} protein expression and function were also elevated in islets from HFD-fed mice and from obese/overweight cadaveric human donors.

Conclusions/interpretation p53-dependent augmentation of p66^{Shc} expression and function represents a key signalling response contributing to beta cell apoptosis under conditions of lipotoxicity.

Keywords Apoptosis · Beta cell · Exendin-4 · JNK · p53 · p66^{Shc} · Palmitic acid · Pancreatic islet

Annalisa Natalicchio and Federica Tortosa contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-015-3563-2) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Abbreviations

APC	Allophycocyanin
DHE	Dihydroethidium
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
GLP-1	Glucagon-like peptide 1

HFD	High-fat diet
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
NAC	*N*-Acetyl-L-cysteine
PFU	Plaque-forming unit
PKC	Protein kinase C
ROS	Reactive oxygen species
siRNA	Short, interfering RNA
WT	Wild-type

Introduction

Saturated fatty acid (FA)-induced apoptosis of pancreatic beta cells has long been recognised as a major mechanism linking excess dietary fat and beta cell damage, leading to impaired insulin secretion and hyperglycaemia in type 2 diabetes, particularly when associated with visceral obesity [1–3].

FA-induced beta cell apoptosis involves a variety of signalling mechanisms, including endoplasmic reticulum (ER) stress induction [4], mitochondrial dysfunction [5], activation of specific intracellular kinases such as the members of the mitogen-activated protein kinase (MAPK) family c-Jun N-terminal kinase (JNK) and p38 MAPK [6, 7] and protein kinase C (PKC)δ [8], and peroxisome-generated reactive oxygen species (ROS) [7, 9]. The tumour suppressor protein p53 is also implicated in FA-induced beta cell apoptosis [7, 10], since both palmitate and oleate were shown to stimulate apoptosis of NIT-1 beta cells through p53 [7], and p53 inhibition was found to be involved in growth factor-dependent promotion of beta cell survival via Akt/protein kinase B [10]. However, p53 signalling in the context of beta cell lipotoxicity is still poorly defined.

$p66^{Shc}$, a 66 kDa proto-oncogene *Src* collagen homologue (Shc) adaptor protein, is the largest of three protein isoforms ($p66^{Shc}$, $p52^{Shc}$ and $p46^{Shc}$) encoded by the *Shc* gene [11, 12]. $p66^{Shc}$ possesses specific functions, such as modulation of $p46/52^{Shc}$ complex activation and downstream signalling via MAPK kinase (MEK)–extracellular signal-regulated kinase (ERK) [13–16] and control of actin cytoskeleton turnover and glucose transport [16, 17]. Importantly, $p66^{Shc}$ is implicated in both sensing and activation of cellular oxidative stress and consequent induction of apoptosis [18]. $p66^{Shc}$ signalling is strictly dependent upon phosphorylation of Ser^{36} in the protein CH2 domain, triggered by cell exposure to oxidative stress-inducing agents [18]. Recently, $p66^{Shc}$ knockout mice were found to exhibit protection from hyperglycaemia-induced microvascular disease [19] and from development of diabetic autonomic neuropathy [20]. Levels of $p66^{Shc}$ mRNA and $p66^{Shc}$ protein were found to be increased in the kidney cortex of diabetic mice [19] and in circulating leucocytes from diabetic patients [21], suggesting that $p66^{Shc}$ could 'sense' the impaired metabolic milieu in diabetes and promote cellular dysfunction. In support of this concept, the $p66^{Shc}$ protein was found to act as a downstream effector of the tumour suppressor *p53* gene in oxidative stress-induced apoptosis [18, 22], and a p53 response element in the gene promoter of $p66^{Shc}$ has also been identified [23]. On the other hand, FAs increase the level of p53 acetylation in human monocytes [24]. Therefore, we investigated the involvement of p53 and $p66^{Shc}$ in FA-dependent apoptosis in pancreatic beta cells.

Methods

Animals

Animals were kept in an animal house under controlled temperature, humidity and lighting. Animal experimentation respecting the regulations of Italy and the EU were conducted with the approval of the ethics committee (CESA) of the Gaetano Salvatore Research Institute (IRGS), Biogem, Italy (internal ID 0907), in accordance with the National Institutes of Health (NIH) Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). Wild-type (WT) C57Bl/6 mice were purchased from Charles River Laboratories (Calco, Italy). $p66^{Shc-/-}$ mice were on matched C57Bl/6 genetic backgrounds. Generation of $p66^{Shc-/-}$ mice has been previously described [18]. From weaning at the age of 3 weeks onwards, mice received a standard diet. At the age of 3 weeks, mice were randomised to a high-fat diet (HFD; purchased from Mucedola (Settimio Milanese, Milan, Italy) and consisting of 60% fat from palm oil) or continued on a standard diet for an additional 21 days. Blood samples were collected from the tail vein of fed mice.

Pancreatic islets

Mouse islets were isolated by bile duct perfusion and collagenase digestion and maintained in culture, as previously described [25]. After isolation, islets were studied within 3 days. Human pancreatic islets were isolated from nine lean and 13 overweight/obese cadaveric donors (electronic supplementary material [ESM] Table 1). Pancreases were excised and processed with the approval of the regional ethics committee. Islets were obtained and cultured, as previously reported [26, 27], and studied within 3 days from isolation. Cell viability in islets, measured by Trypan Blue exclusion, was higher than 90% after 3 days in culture.

Cell cultures

Rat insulin-secreting INS-1E cells (passage 15-30; a kind gift from C. B. Wollheim, University of Geneva, Switzerland)

were grown and treated with or without 0.5 mmol/l palmitic acid (Sigma-Aldrich, St Louis, MO, USA), as previously reported [6, 28]. All chemical inhibitors were provided by Calbiochem (Darmstadt, Germany). Exendin-4 (exenatide) was obtained from Ely Lilly (Indianapolis, IN, USA).

Cell transfection

Adenoviral transfection studies Generation of an adenoviral construct encoding for the p66^{Shc} protein and transfection of INS-1E cells with recombinant adenoviruses were carried out according to previously reported procedures [17]. INS-1E cells were also transfected with an adenoviral p66^{Shc} construct carrying a Ser^{36} to Ala^{36} mutation, as indicated. An empty adenovirus was used as control for the infection ('mock').

Short, interfering RNA transfection studies INS-1E cells grown to 70% confluence were transiently transfected with *p66^{Shc}* short, interfering RNA (siRNA) 1 or *p66^{Shc}* siRNA 2 (Qiagen, Hilden, Germany) or with *p53* siRNA (s128540; Life Technologies, Carlsbad, CA, USA). See ESM Methods for further details.

Gene expression analysis by quantitative RT-PCR

RNA isolation and cDNA synthesis were conducted as previously described [6, 27]. Primers were designed using Primer Express version 3.0 (Life Technologies, Carlsbad, CA, USA) and purchased from Life Technologies. Primer sequences are reported in ESM Table 2. See ESM Methods for further details.

Immunoblotting

Cells lysates were obtained and analysed by immunoblotting, as previously described [6]. A list of the antibodies used is shown in ESM Table 3. See ESM Methods for further details.

Measurements of apoptosis

Apoptosis was measured by evaluating mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates, by annexin V allophycocyanin (APC) labelling or by TUNEL assay. See ESM Methods for further details.

ROS production measurements

Intracellular ROS were detected through the evaluation of dihydroethidium (DHE) oxidation. See ESM Methods for further details.

Statistical analysis

All data are presented as means±SEM. Statistical analysis was performed using the two-tailed unpaired Student's *t* test or ANOVA, followed by the Tukey multiple comparison test, as appropriate. Statistical significance was set at $p<0.05$.

Results

Saturated FAs increase p66^{Shc} protein expression in pancreatic islets and beta cells in vitro

Exposure of isolated murine islets to palmitic acid (palmitate, 0.5 mmol/l) for up 24 h resulted in a ~2.5-fold increase in *p66^{Shc}* mRNA and protein at 16 and 24 h ($p<0.05$; Fig. 1a, c) without changing the expression of p46^{Shc} and p52^{Shc}, the other Shc protein isoforms (ESM Fig. 1a). Similarly, challenge with palmitate for 24 h increased p66^{Shc} protein content in human pancreatic islets ($p<0.05$; Fig. 1d). Palmitate selectively augmented *p66^{Shc}* mRNA and protein expression approximately threefold also in rat insulinoma INS-1E cells ($p<0.05$; Fig. 1b and ESM Fig. 1b), confirming the ability of this saturated FA to increase p66^{Shc} expression in pancreatic beta cells. p66^{Shc} protein content in murine islets and rat INS-1E cells was not influenced by BSA in the culture medium (ESM Fig. 2a, b). Furthermore, an increase in p66^{Shc} phosphorylation on Ser^{36} was found in all experimental cell systems exposed to palmitate ($p<0.05$; Fig. 1c–e), largely in consequence of augmented protein content, since the ratio of phosphorylated to total p66^{Shc} was unchanged (ESM Fig. 1c). Exposure of INS-1E cells to the unsaturated FA oleate (0.5 mmol/l) for up 48 h also resulted in an approximately twofold increase in p66^{Shc} protein and Ser^{36} phosphorylation ($p<0.05$; ESM Fig. 3a).

Palmitate-induced apoptosis involves p66^{Shc}

Exposure of INS-1E cells to palmitate raised the proportion of apoptotic cells from 1.7% to 13.4% ($p<0.05$; ESM Fig. 4). Transfection of INS-1E cells with two independent siRNAs resulted in a 55–80% reduction in p66^{Shc} protein levels compared with control ($p<0.05$; Fig. 2a), and prevented the palmitate-induced increase in p66^{Shc} expression/phosphorylation on Ser^{36} and apoptosis ($p<0.05$; Fig. 2b, c). Furthermore, the increased apoptosis in response to excess FAs was completely abrogated in islets from *p66$^{Shc-/-}$* C57Bl/6 mice (Fig. 2e), in which p66^{Shc} protein levels were reduced by 90% and did not change following palmitate treatment ($p<0.05$; Fig. 2d). On the other hand, INS-1E cells infected with recombinant adenovirus overexpressing p66^{Shc} (Ad/*p66^{Shc}*) showed a fourfold augmentation of p66^{Shc} protein levels ($p<0.05$; Fig. 3a) and enhancement of both basal and palmitate-stimulated p66^{Shc} Ser^{36} phosphorylation and

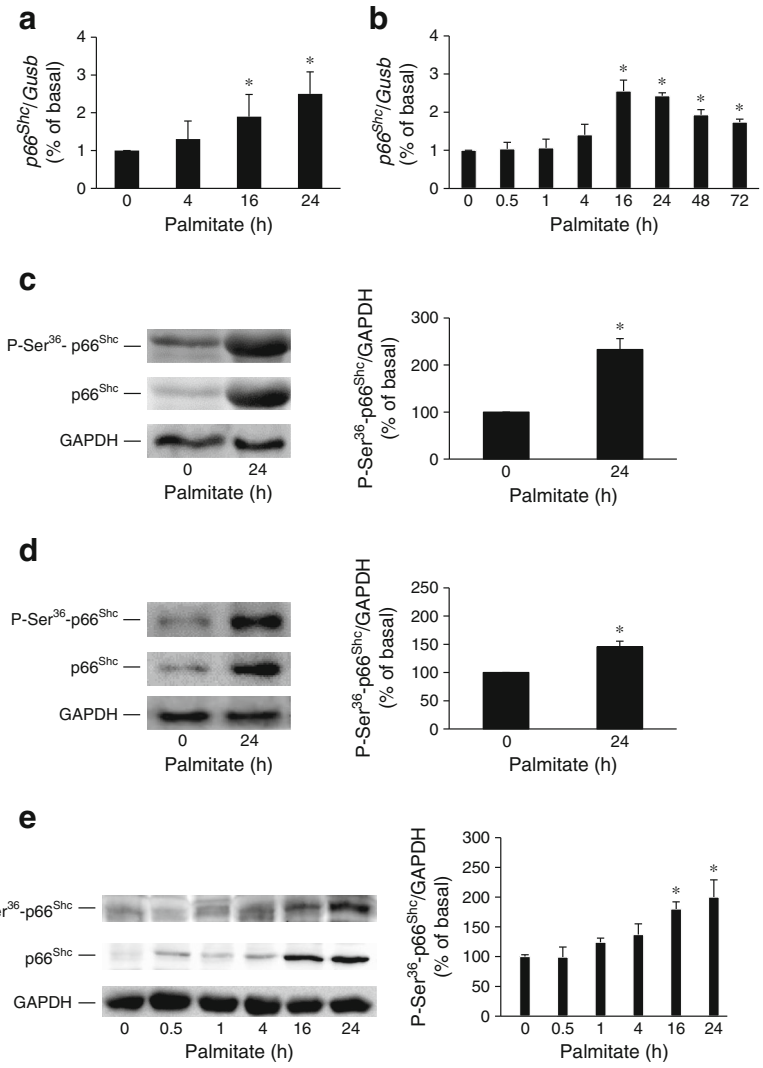

Fig. 1 Saturated FAs induce $p66^{Shc}$ expression and phosphorylation on Ser^{36} in murine and human pancreatic islets and in rat INS-1E cells. (**a**, **b**) *p66*^{Shc} mRNA levels in (**a**) murine islets ($n=5$ independent experiments) and (**b**) INS-1E cells ($n=9$) incubated with 0.5 mmol/l palmitate for the indicated times or left untreated. (**c**–**e**) Immunoblot analysis and relative quantification of $p66^{Shc}$ protein levels in (**c**) murine islets, (**d**) human islets and (**e**) INS-1E cells incubated with 0.5 mmol/l palmitate for the indicated times or left untreated ($n=4$–5). *$p<0.05$ vs basal (Student's *t* test or ANOVA, as appropriate). GAPDH, glyceraldehyde-3-phosphate dehydrogenase

apoptosis compared with non-infected (WT) and mock-infected (Ad/mock) control cells ($p<0.05$; Fig. 3b–d). The lowest adenovirus dose was used (i.e. 40 plaque-forming units [PFU]/ml), which resulted in a fourfold increase in $p66^{Shc}$ protein content ($p<0.05$; Fig. 3a) while maintaining normal cellular morphology (data not shown). Increased $p66^{Shc}$ protein expression, even in the absence of palmitate, resulted in dose-dependent augmentation of cell apoptosis ($p<0.05$; ESM Fig. 5). Altogether, these results demonstrate that the $p66^{Shc}$ protein plays an essential role in FA-mediated apoptotic beta cell death.

Role of $p66^{Shc}$ in high glucose-induced apoptotic beta cell death

The effects of other apoptotic agents, such as high glucose, were also investigated. INS-1E cells challenged with 25 mmol/l glucose levels for up 24 h showed selective augmentation of $p66^{Shc}$ protein levels and Ser^{36} phosphorylation by ~2.5-fold, as well as increased apoptosis ($p<0.05$; ESM Fig. 3c, d). Overexpression of $p66^{Shc}$ enhanced both basal and glucose-stimulated apoptosis compared with control ($p<0.05$; ESM Fig. 6a). On the other hand, siRNA-mediated reduction of $p66^{Shc}$ protein levels resulted in partial inhibition of glucose-induced apoptosis by approximately 50% ($p<0.05$; ESM Fig. 6b). These results show that $p66^{Shc}$ contributes to beta cell apoptosis induced by high glucose.

The tumour suppressor p53 protein mediates augmentation of $p66^{Shc}$ expression and function in response to FAs

$p66^{Shc}$ has reportedly been shown to act as a downstream effector of p53 in stress-induced apoptosis [22]. Treatment of both INS-1E cells and isolated murine islets with palmitate induced a 2.5-fold increase in *p53* mRNA and protein levels ($p<0.05$; Fig. 4a, b and data not shown). When INS-1E cells were pretreated with pifithrin-α, an inhibitor of the p53

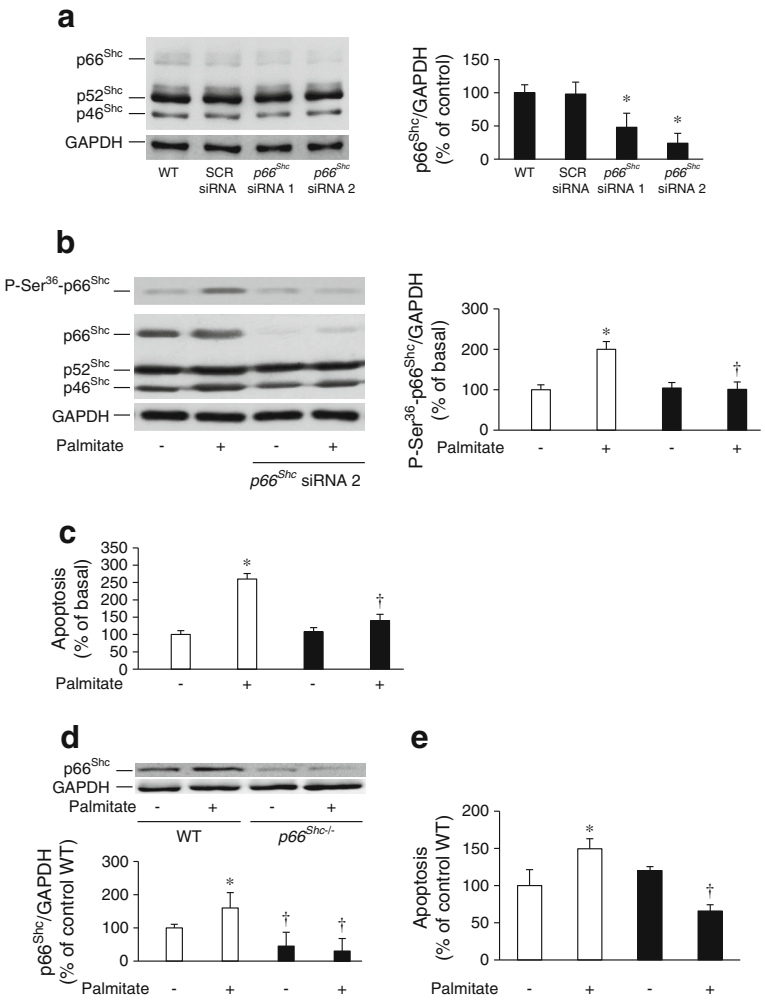

Fig. 2 Palmitate-induced apoptosis is impaired in murine islets and rat INS-1E cells with reduced p66^{Shc} protein expression. (**a**) Immunoblot analysis and quantification ($n=4$) of p66^{Shc} protein levels in INS-1E cells transfected with two independent siRNAs targeting *p66^{Shc}* or with a scrambled control siRNA (SCR siRNA). *$p<0.05$ vs control (ANOVA). (**b**) Immunoblot analysis of p66^{Shc} Ser^{36} phosphorylation ($n=4$) and (**c**) apoptosis ($n=3$) in INS-1E cells transfected with siRNA 2 (black bars) or not transfected (white bars) and then incubated with 0.5 mmol/l palmitate for 24 h or left untreated. *$p<0.05$ vs basal; †$p<0.05$ vs palmitate-treated cells (Student's *t* test). (**d**) Immunoblot analysis of p66^{Shc} Ser^{36} phosphorylation ($n=4$) and (**e**) apoptosis ($n=4$) in murine islets from WT (white bars) and *p66^{Shc}*-null (*p66$^{Shc-/-}$*, black bars) mice incubated with 0.5 mmol/l palmitate for 24 h or left untreated. *$p<0.05$ vs basal; †$p<0.05$ vs WT (Student's *t* test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase

protein, the palmitate-induced increase in p53 and p66^{Shc} expression was completely abrogated ($p<0.05$; Fig. 4c) and so was palmitate-induced apoptosis ($p<0.05$; Fig. 4d). Reduced palmitate-induced p66^{Shc} expression and apoptosis were similarly observed when p53 protein expression was inhibited using a specific siRNA ($p<0.05$; Fig. 4e, f). Thus, p53 mediates the palmitate-induced upregulation of p66^{Shc} and the resulting pro-apoptotic response in insulin-secreting cells.

Ser^{36} phosphorylation of p66^{Shc} is necessary for palmitate-induced apoptosis

To investigate the role of p66^{Shc} Ser^{36} phosphorylation, a key event in p66^{Shc} signalling [18], in FA-triggered beta cell apoptosis, a mutant p66^{Shc} protein, in which Ser^{36} was replaced by Ala, was overexpressed approximately threefold in INS-1E cells (Ad/*p66^{Shc}Ala^{36}*), reaching levels similar to those obtained after palmitate treatment (Fig. 5a). In Ad/*p66^{Shc}Ala^{36}* cells, basal p66^{Shc} phosphorylation was similar to control Ad/mock cells and reduced compared with Ad/*p66^{Shc}* cells, whereas palmitate-stimulated phosphorylation was reduced compared with both Ad/*p66^{Shc}* and Ad/mock cells ($p<0.05$; Fig. 5a), consistent with the phosphorylation-defective trait of the overexpressed p66^{Shc} mutant likely acting as a dominant-negative protein. Accordingly, the rates of cellular apoptosis were reduced in Ad/*p66^{Shc}Ala^{36}* compared with Ad/*p66^{Shc}* cells under basal conditions ($p<0.05$; Fig. 5b, c), and reduced compared with both Ad/*p66^{Shc}* and Ad/mock cells following exposure to palmitate ($p<0.05$; Fig. 5b, c), strictly correlating with the level of Ser^{36} phosphorylation of p66^{Shc}. Thus, Ser^{36} phosphorylation of p66^{Shc} is essential for its ability to mediate the pro-apoptotic effects of palmitate. Similar results were observed in INS-1E cells challenged with high glucose (ESM Fig. 6a).

Studies with specific JNK, ERK and p38 MAPK inhibitors demonstrated that the stress-activated kinase JNK, but not ERK or p38 MAPK, is involved in the ability of palmitate to stimulate p66^{Shc} phosphorylation on Ser^{36} and the resulting pro-apoptotic response in INS-1E cells. In both Ad/mock and Ad/*p66^{Shc}* INS-1E cells, pretreatment with the JNK inhibitor SP600125, but not with the MEK-1/2 inhibitor PD98059 or the p38 MAPK inhibitor SB203680 (ESM Fig. 7a, b), effectively prevented palmitate-stimulated JNK phosphorylation

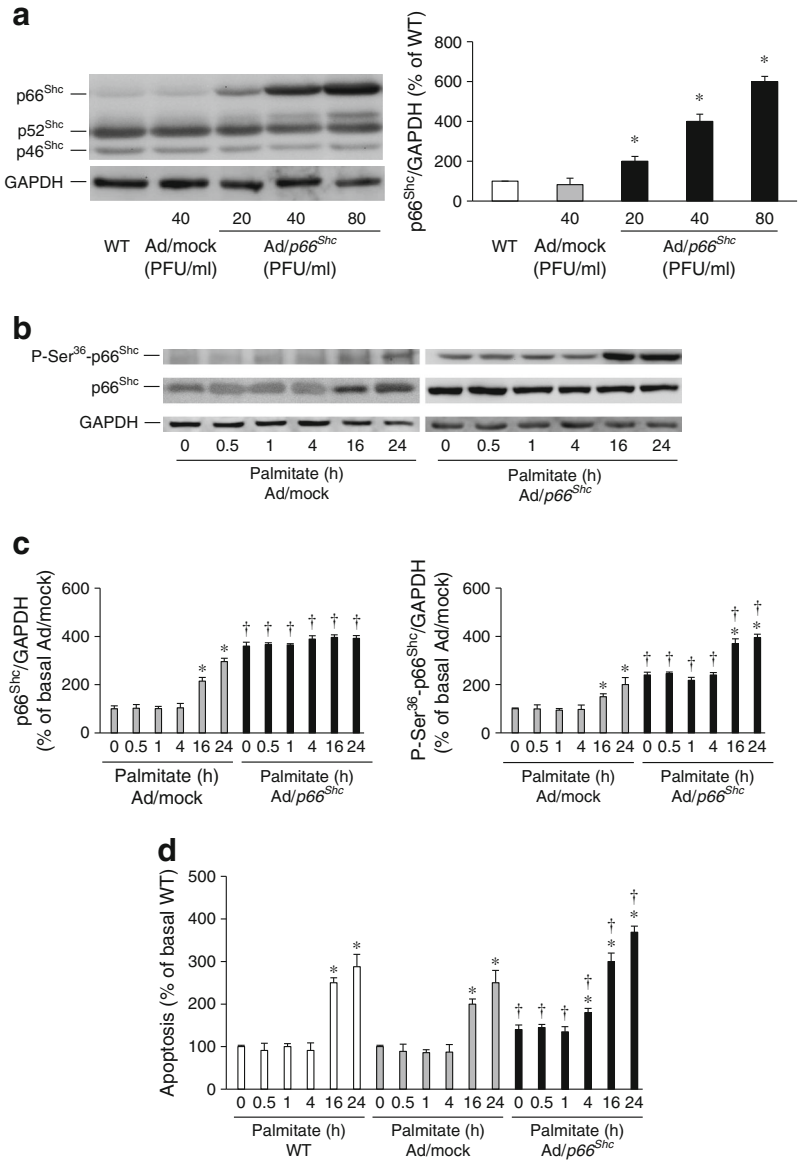

Fig. 3 p66^{Shc} overexpression by adenovirus-mediated gene transfer enhances basal and palmitate-induced apoptosis in INS-1E cells. (**a**) Immunoblot analysis of p66^{Shc} protein levels in INS-1E cells infected with different doses of a recombinant adenoviral vector encoding *p66^{Shc}* (Ad/*p66^{Shc}*), infected with empty adenovirus (Ad/mock) or left untreated (WT) ($n=4$). *$p<0.05$ vs non-infected WT cells (ANOVA). (**b**, **c**) Immunoblot analysis and quantification of Ser^{36}-phosphorylated p66^{Shc} and (**d**) apoptosis ($n=3$) in Ad/mock cells (grey bars) and Ad/*p66^{Shc}* INS-1E cells (black bars), and relative quantification ($n=3$) following challenge with 0.5 mmol/l palmitate for the indicated times. *$p<0.05$ vs basal; $^{\dagger}p<0.05$ vs control Ad/mock and WT (Student's *t* test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase

(data not shown) as well as p66^{Shc} phosphorylation on Ser^{36} ($p<0.05$; Fig. 6a) and apoptosis ($p<0.05$; Fig. 6b). Interestingly, glucagon-like peptide 1 (GLP-1) receptor agonists, which reportedly inhibit palmitate-induced apoptosis by preventing JNK phosphorylation [6], also inhibited p66^{Shc} phosphorylation on Ser^{36}. Pretreatment of Ad/mock cells with 10 nmol/l exendin-4 for 16 h resulted in abrogation of palmitate-induced Ser^{36} phosphorylation of p66^{Shc} ($p<0.05$; Fig. 6c). Thus, JNK-mediated-p66^{Shc} phosphorylation on Ser^{36} is a key signalling node in the effects of palmitate on beta cell apoptosis.

p66^{Shc} signals via generation of ROS in FA-mediated apoptosis

ROS-dependent oxidation of DHE was then measured in INS-1E cells overexpressing the WT or mutant p66^{Shc} protein, both under basal conditions and following palmitate exposure. ROS levels were found to correlate with the level of p66^{Shc} phosphorylation on Ser^{36}, since they were increased in Ad/*p66^{Shc}* but not in Ad/*p66^{Shc}Ala^{36}* cells, compared with control cells, and showed a further increase in response to palmitate ($p<0.05$; Fig. 7a, b). In addition, pretreatment of INS-1E cells with the antioxidant *N*-acetyl-L-cysteine (NAC) resulted in significant inhibition of palmitate-induced augmentation of ROS levels ($p<0.05$; Fig. 7c) and apoptosis ($p<0.05$; Fig. 7d). Furthermore, NAC could largely inhibit the increased levels of apoptosis found in Ad/*p66^{Shc}* cells, whereas it was without effect in cells with p66^{Shc} knockdown (Fig. 7e, f). Altogether, these findings show that ROS generation contributes to p66^{Shc}-induced apoptosis in the context of lipotoxicity.

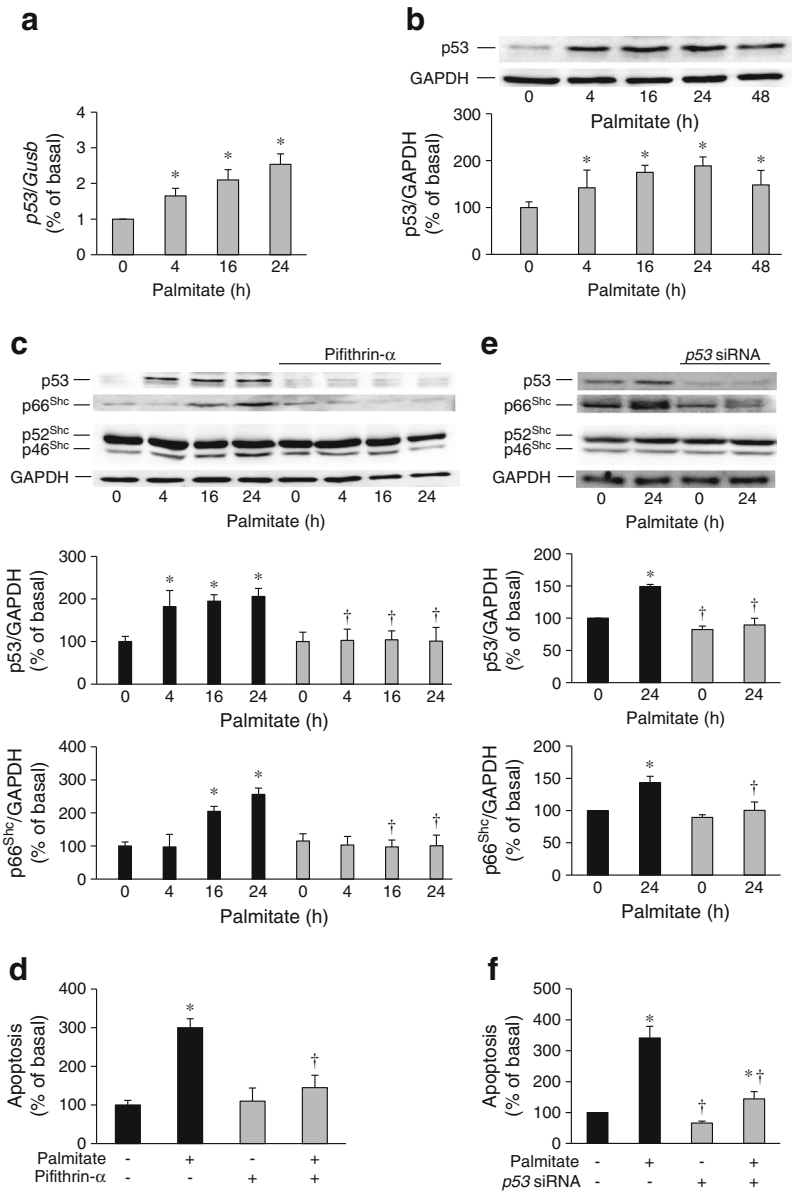

Fig. 4 p53 mediates palmitate-induced augmentation of $p66^{Shc}$ expression and cellular apoptosis. (**a**) *p53* mRNA ($n=9$) and (**b**) protein ($n=5$) levels in INS-1E cells exposed to 0.5 mmol/l palmitate for the indicated times. *$p<0.05$ vs basal (ANOVA). (**c**, **e**) Immunoblot analysis of p53 and $p66^{Shc}$ protein levels ($n=3$) and (**d**, **f**) apoptosis ($n=3$) in INS-1E cells pretreated with 30 μmol/l pifithrin-α for 1 h (grey bars) or left untreated (black bars), or transfected with siRNA targeting *p53* (grey bars) or left untreated (black bars), and then exposed to 0.5 mmol/l palmitate for the indicated times. *$p<0.05$ vs basal; †$p<0.05$ vs palmitate-treated cells (Student's *t* test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Lipotoxic conditions are associated with increased expression of $p66^{Shc}$ in pancreatic islets in vivo

Finally, islet expression of $p66^{Shc}$ was investigated in vivo. In HFD-fed mice, serum triacylglycerol levels were higher than those in mice fed a standard diet (1.64±0.17 vs 1.06±0.29 mmol/l; $p<0.05$), and $p66^{Shc}$ mRNA levels in the pancreatic islets were augmented approximately twofold compared with controls ($p<0.05$; Fig. 8a).

The mRNA levels of $p66^{Shc}$ were also increased several-fold in pancreatic islets from human donors with BMI >24.9 kg/m^2 compared with donors with BMI ≤24.9 kg/m^2 ($p<0.05$; Fig. 8c), and this was associated with an elevation in *p53* mRNA levels ($p<0.05$; Fig. 8b). Intriguingly, islets from obese donors showed twofold higher mRNA levels of *BAX* (encoding BCL2-associated X protein), 2.4-fold higher mRNA levels of *CASP3* (encoding caspase 3) and 2.9-fold higher mRNA levels of *CYCS* (encoding cytochrome c somatic) compared with non-obese donors ($p<0.05$; Fig. 8d), suggesting greater activation of the pro-apoptotic response. Thus, conditions with high mobilisation of FAs are associated with augmentation of $p66^{Shc}$ expression in pancreatic islets in vivo.

Discussion

Dysfunction of beta cells in type 2 diabetes may be further exacerbated in states with elevated plasma FAs [29, 30]. In this study, we show for the first time that $p66^{Shc}$, an important

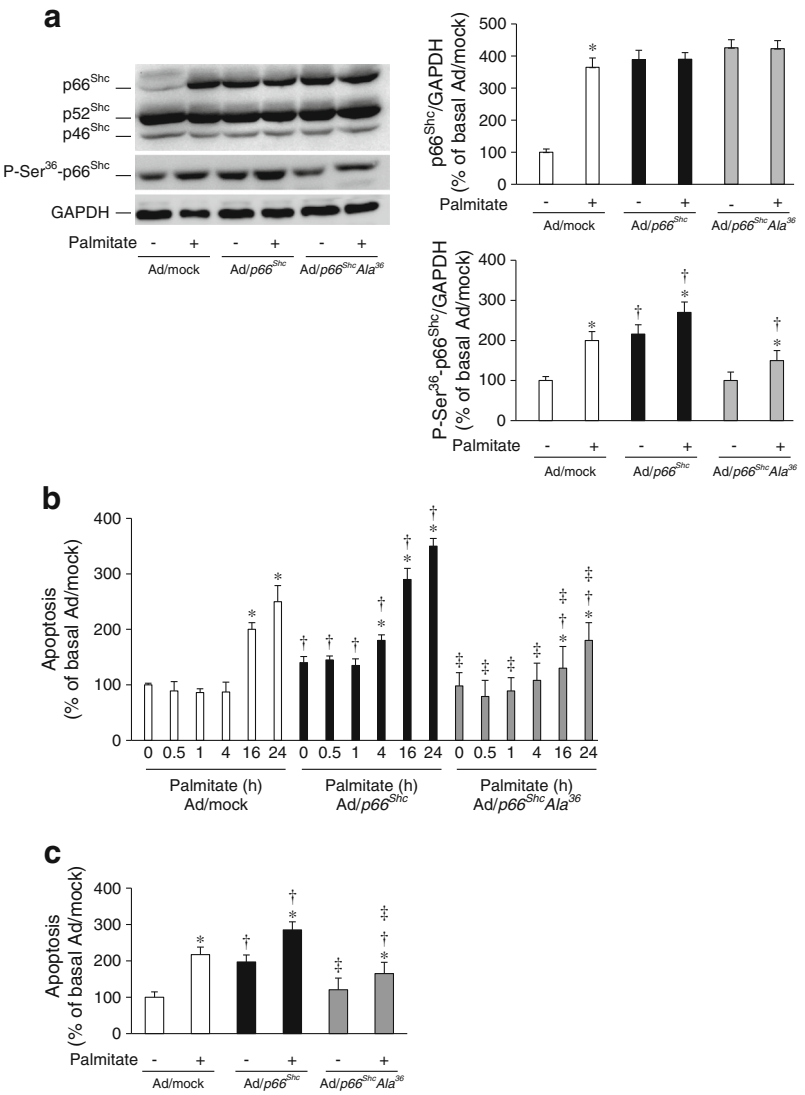

Fig. 5 Ser^{36} phosphorylation of p66^{Shc} is essential for palmitate-induced apoptosis. INS-1E cells were infected with recombinant adenoviral vectors encoding p66^{Shc} (Ad/*p66^{Shc}*), a phosphorylation-defective p66^{Shc} protein with mutation of Ser^{36} to Ala (Ad/*p66^{Shc}Ala^{36}*), or with empty adenovirus (Ad/mock). (**a**) Immunoblot analysis of Ser^{36}-phosphorylated p66^{Shc} ($n=4$) and (**b**, **c**) apoptosis in Ad/mock, Ad/*p66^{Shc}* and Ad/*p66^{Shc}Ala^{36}* INS-1E cells incubated with 0.5 mmol/l palmitate for 24 h or left untreated. Apoptosis was evaluated by measuring (**b**) cytoplasmic oligosomes ($n=4$) and (**c**) the proportion of annexin V APC-positive cells ($n=4$). *$p<0.05$ vs basal; †$p<0.05$ vs control Ad/mock; ‡$p<0.05$ vs Ad/*p66^{Shc}* (Student's *t* test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase

redox sensor and pro-apoptotic member of the Shc protein family of molecular adaptors, is implicated in the negative effects of FAs on pancreatic beta cells.

Accumulating evidence suggests that palmitic acid, a saturated FA, plays an important role in beta cell death [31, 32]. Prolonged exposure of beta cells to elevated palmitate concentrations has been shown to cause downstream JNK activation and increased p53 expression, events which are in part mediated by enhanced oxidative stress and typically linked to cellular damage [7, 24, 33, this study]. The identification of a p53 response/binding element in the promoter region of *p66^{Shc}* indicated that *p66^{Shc}* is a p53 target gene and that *p66^{Shc}* is indispensable for p53-induced apoptosis [22, 23, 34]. In this study, murine and human islets, as well as INS-1E cells, showed increased levels of p66^{Shc} when chronically exposed to elevated palmitate concentrations. Moreover, pancreatic islets isolated from HFD-fed mice displayed robust increases in *p66^{Shc}* mRNA expression, establishing a link between in vivo lipotoxicity and *p66^{Shc}* regulation. Both the use of pifithrin-α, an inhibitor of p53 function, and siRNA-mediated p53 knockdown allowed us to prove that the ability of saturated FAs to promote p66^{Shc} expression is mediated by p53, in line with similar results in other cell systems [23]. We found that p53 expression was increased in response to palmitate in both mouse islets and INS-1E beta cells, as well as in human islets from overweight/obese compared with normal-weight donors.

Beta cell challenge with palmitate resulted in enhanced *p66^{Shc}* mRNA and protein expression as well as phosphorylation of this protein on Ser^{36}, a well-characterised pro-apoptotic event [18, 35, 36]. By using multiple approaches, i.e. siRNA-mediated *p66^{Shc}* gene silencing, use of islets from *p66$^{Shc-/-}$* mice and forced expression of p66^{Shc} in INS-1E cells, the palmitate-triggered apoptosis was shown to require p66^{Shc}. In mouse embryo fibroblasts [22] and vascular cells [37], targeted *p66^{Shc}* gene deletion conferred protection against apoptosis in response to oxidative stress and HFD,

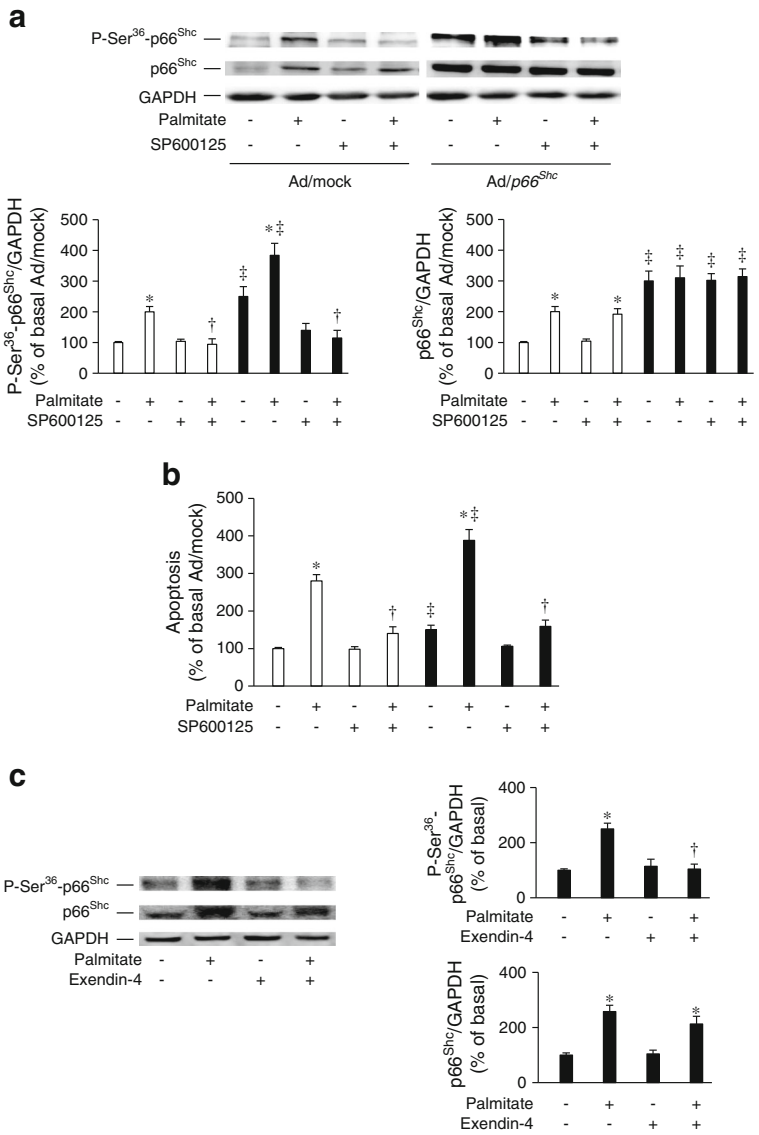

Fig. 6 The stress-activated kinase JNK mediates Ser^{36} phosphorylation of $p66^{Shc}$. (**a**) Immunoblot analysis of Ser^{36}-phosphorylated $p66^{Shc}$ ($n=4$) and (**b**) apoptosis, evaluated by measuring cytoplasmic oligosomes ($n=4$), in Ad/mock (white bars) and Ad/$p66^{Shc}$ (black bars) INS-1E cells pretreated with the JNK inhibitor SP600125 (10 μmol/l for 2 h) and then exposed to 0.5 mmol/l palmitate for 24 h or left untreated ($n=4$). *$p<0.05$ vs basal; †$p<0.05$ vs no SP600125; ‡$p<0.05$ vs Ad/mock (Student's *t* test). (**c**) Ser^{36} phosphorylation of $p66^{Shc}$ in Ad/mock INS-1E cells pretreated with exendin-4 (10 nmol/l for 16 h) and then incubated with 0.5 mmol/l palmitate for 24 h or left untreated ($n=4$). *$p<0.05$ vs basal; †$p<0.05$ vs palmitate-treated cells (Student's *t* test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase

respectively. Furthermore, our results show that $p66^{Shc}$ also has a role in beta cell glucotoxicity, since high glucose increased $p66^{Shc}$ protein expression and its Ser^{36} phosphorylation, and manipulations of $p66^{Shc}$ content affected high glucose-induced beta cell apoptosis. However, $p66^{Shc}$ knockdown resulted in apparently slightly greater inhibition of apoptosis induced by palmitate than by high glucose (compare Fig. 2 and ESM Fig. 6).

Ser^{36} phosphorylation of $p66^{Shc}$ is critical for inducing the apoptotic cascade in cells exposed to several toxic stimuli [18]. Accordingly, in this study, overexpression of a phosphorylation-defective $p66^{Shc}$ mutant protein in INS-1E cells did not affect basal and reduced palmitate-induced apoptosis, respectively. Depending on the cellular context and stimulus, Ser^{36} phosphorylation of $p66^{Shc}$ was found to be promoted by either the MAP kinases ERK-1/2 or the stress-activated kinases JNK and p38 MAPK [14, 15, 35, 38–42]. In specific cells, it was shown to be mediated by PKCβ activation [43–45]. We found that $p66^{Shc}$ phosphorylation was prevented by pretreatment of cells with the specific JNK inhibitor, as in other cell types [36, 39, 42, 46]. Interestingly, the GLP-1 analogue exendin-4, which reportedly prevents FA-induced apoptosis by inhibiting JNK phosphorylation [6], markedly inhibited $p66^{Shc}$ phosphorylation on Ser^{36}, linking GLP-1 receptor-dependent anti-apoptotic signalling to inhibition of JNK-mediated phosphorylation of $p66^{Shc}$. Within mitochondria, the $p66^{Shc}$ protein binds cytochrome c and acts as an oxidoreductase, shuttling electrons from cytochrome c to molecular oxygen [47]. This redox activity of $p66^{Shc}$ explains the increase in ROS levels caused by $p66^{Shc}$ overexpression, as well as their decrease in $p66^{Shc}$ knockout cells [48]. Similarly, in beta cells, ROS levels were increased in Ad/$p66^{Shc}$ cells and further augmented in response to palmitate, in close

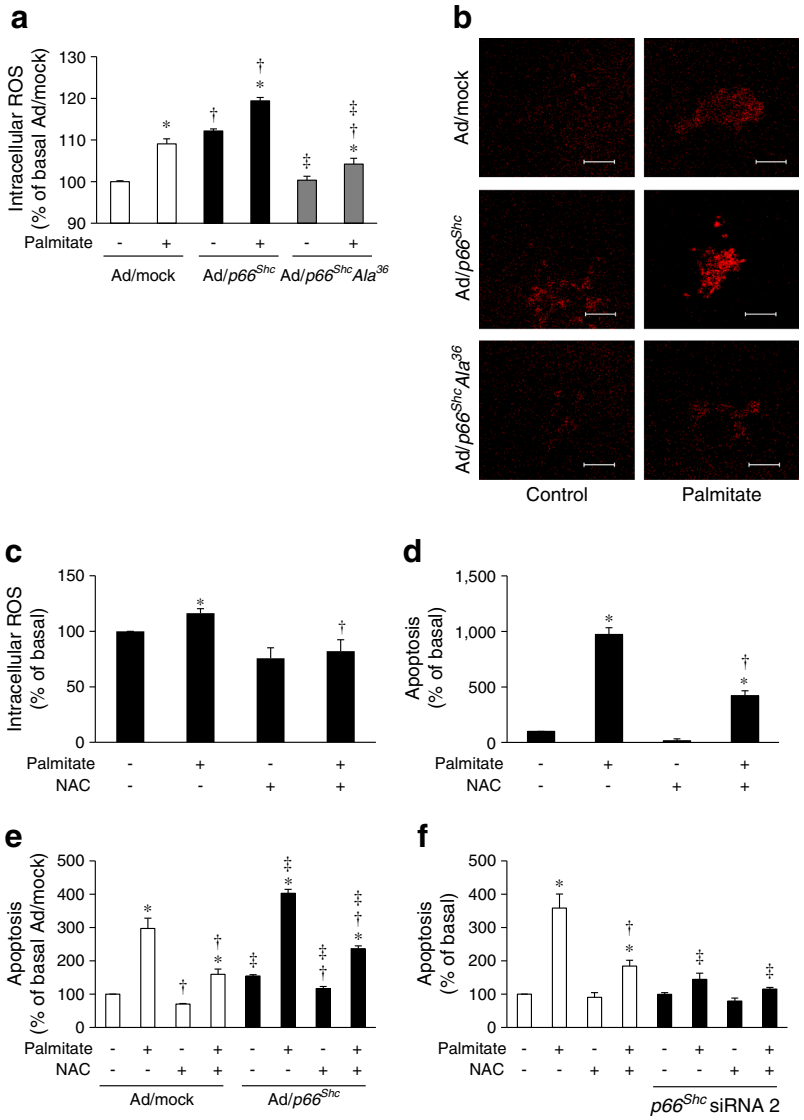

Fig. 7 Generation of intracellular ROS levels contributes to the pro-apoptotic effects of p66^{Shc}. (**a**) ROS levels in Ad/mock (white bars), Ad/*p66^{Shc}* (black bars) and Ad/*p66^{Shc}Ala^{36}* (grey bars) INS-1E cells exposed to 0.5 mmol/l palmitate for 24 h or left untreated ($n=4$). *$p<0.05$ vs basal; †$p<0.05$ vs Ad/mock; ‡$p<0.05$ vs Ad/*p66^{Shc}* (Student's *t* test). (**b**) ROS-dependent DHE oxidation (red) in the experimental cells. Scale bars, 47.62 μm. (**c**) ROS levels and (**d**) apoptosis evaluated by detection of cytoplasmic oligosomes ($n=4$) in Ad/mock INS-1E cells incubated in the presence or absence of NAC (1 mmol/l for 2 h) and then challenged with 0.5 mmol/l palmitate for 24 h. *$p<0.05$ vs basal; †$p<0.05$ vs palmitate-treated cells (Student's *t* test). (**e**) Apoptosis in Ad/mock (white bars) and Ad/*p66^{Shc}* (black bars) INS-1E cells incubated in the presence or absence of NAC (1 mmol/l for 2 h) and then challenged with 0.5 mmol/l palmitate for 24 h. *$p<0.05$ vs basal; †$p<0.05$ vs no NAC; ‡$p<0.05$ vs Ad/mock (Student's *t* test). (**f**) Apoptosis in control INS-1E cells (white bars) and INS-1E cells with siRNA-mediated p66^{Shc} knockdown (black bars) incubated in the presence or absence of NAC (1 mmol/l for 2 h) and then challenged with 0.5 mmol/l palmitate for 24 h. *$p<0.05$ vs basal; †$p<0.05$ vs no NAC; ‡$p<0.05$ vs not transfected cells (Student's *t* test)

association with the levels of p66^{Shc} phosphorylation on Ser^{36}; moreover, the antioxidant NAC reduced apoptosis both in control and Ad/*p66^{Shc}* cells, whereas it was without effect in cells with *p66^{Shc}* knockdown. Altogether, these results suggest that the p66^{Shc}-mediated ROS generation contributed to palmitate-induced apoptosis also in beta cells. Additional mechanisms could, however, be involved, including inhibition of beta cell survival signals. Indeed, overexpression of p66^{Shc} was found to reduce both basal and insulin-stimulated Akt phosphorylation in INS-1E cells (Natalicchio et al, data not shown).

We show that increased p66^{Shc} expression levels in the pancreatic islets can also be observed in vivo, in response to HFD in mice and in association with overweight and non-diabetic obesity in humans. In the islets from obese donors, elevated *p66^{Shc}* mRNA levels were associated with enhanced expression of *p53* and pro-apoptotic genes. Increased beta cell apoptosis has been reported in obese individuals, particularly

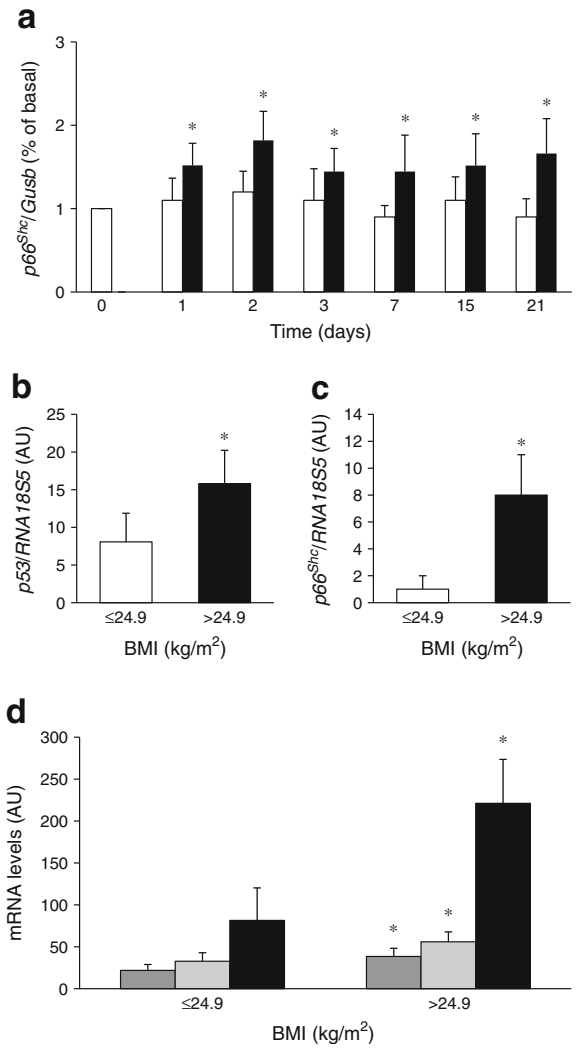

Fig. 8 (**a**) mRNA levels of $p66^{Shc}$ in islets from mice fed an HFD (black bars) or a standard diet (white bars) at multiple times ($n = 3$ each). Data are expressed relative to $p66^{Shc}$ mRNA levels at baseline. *$p<0.05$ vs baseline (ANOVA). (**b**) *p53* and (**c**) $p66^{Shc}$ mRNA levels in islets from normal-weight (BMI ≤24.9 kg/m^2, white bars) and overweight/obese (BMI >24.9 kg/m^2, black bars) human donors. *$p<0.05$ vs normal-weight donors (Student's *t* test). (**d**) mRNA levels of *BAX* (dark grey bars), *CASP3* (light grey bars) and *CYCS* (black bars) in human islets from normal-weight (BMI ≤24.9 kg/m^2) and overweight/obese (BMI >24.9 kg/m^2) donors. *$p<0.05$ vs normal-weight donors (Student's *t* test). AU, arbitrary units

those with type 2 diabetes [49]. Of note, increased p66^{Shc} levels have been reported in the aorta, kidneys and stem cells of experimental models of diabetes in vivo, as well as in circulating leucocytes from diabetic patients [19–21, 45, 50].

In summary, this study identifies p66^{Shc} as a novel signalling intermediate in FA-mediated apoptotic beta cell damage. Targeting p66^{Shc} in beta cells in vivo may potentially represent a novel strategy to prevent the deleterious effects of lipotoxicity on glucose control.

Acknowledgements We thank M. Gigante (University of Bari Aldo Moro, Bari, Italy) for assistance with the annexin V assays.

Funding This work was supported by Ministero dell'Università e della Ricerca, Italy, PRIN 2007 #200775N24E_004 (FG), PRIN 2010-2011 #2010JS3PMZ_010 (AN) and PRIN 2010-2011 #2010JS3PMZ_004 (PM).

Duality of interest FG has received grant support from Eli Lilly, AstraZeneca, Sanofi and Lifescan, and lecture fees from Eli Lilly, AstraZeneca, Sanofi, Lifescan, Novo Nordisk, Boehringer Ingelheim, Takeda and Janssen. AA is a consultant for and has received lecture fees and grant support from Eli Lilly, Novo Nordisk, Servier, Sanofi, AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Mediolanum, Takeda and Janssen. AN, FT, RL, GB, NM, EC, AL, AC, MB, PM, GPF, MG, SP, and LL have nothing to declare.

Contribution statement All authors contributed to the conception and design of the study or to the analysis and interpretation of data, and to drafting the article or revising it critically for intellectual content. All authors have given their final approval of the version to be published. FG is the guarantor of this work.

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