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ORIGINAL CONTRIBUTIONS

Sera of Obese Type 2 Diabetic Patients Undergoing Metabolic Surgery Instead of Conventional Treatment Exert Beneficial Effects on Beta Cell Survival and Function: Results of a Randomized Clinical Study

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Abstract

Background Pancreatic beta cells are highly sensitive to oxidative and endoplasmic reticulum (ER) stress, commonly occurring in type 2 diabetes (T2D) and obesity.

Objective We aimed at investigating cellular responses of human beta cells exposed to sera from obese T2D patients treated differently, namely by conventional therapy or laparoscopic sleeve gastrectomy (LSG).

Methods Serum samples from obese T2D men randomized to conventional treatment or LSG were taken at baseline and 6 months later. After exposing 1.1B4 cells to study patients' sera, the following were assessed: cellular viability and proliferation (by MTT and xCELLigence assays), reactive oxygen species (ROS) production (with DCFH-DA), and expression of ER stress markers, oxidative- or autophagy-related proteins and insulin (by real-time PCR and Western blot).

Results At 6-month follow-up, patients undergoing LSG achieved an adequate glycemic control, whereas conventionally treated patients did not. As compared to 1.1B4 cells incubated with baseline sera (control), cells exposed to sera from LSG-treated participants exhibited (i) increased viability and proliferation ($p < 0.05$); (ii) diminished levels of ROS and p53 ($p < 0.05$); (iii) enhanced protein expression of autophagy-related SIRT1 and p62/SQSTM1 ($p < 0.05$); (iv) significantly decreased transcript levels of ER stress markers ($p < 0.05$); and (v) augmented insulin expression ($p < 0.05$). Conversely, the 6-month conventional therapy appeared not to impact on circulating redox status. Moreover, 1.1B4 cells exposed to sera from conventionally treated patients experienced mild ER stress.

Conclusion Circulating factors in patients with improved diabetes after metabolic surgery exerted favorable effects on beta cell function and survival.

Keywords Human 1.1B4 beta cells · Obesity · Type 2 diabetes · Oxidative stress · ER stress · Laparoscopic sleeve gastrectomy

Introduction

Pancreatic beta cell dysfunction, a salient feature of type 2 diabetes (T2D), ensues long before the clinical diagnosis of diabetes and worsens steadily over years, irrespective of treatment regimens [1]. Most patients with T2D are overweight or obese, having additional metabolic stressors that affect beta cell function [2]. Several randomized controlled trials in obese T2D individuals have consistently reported bariatric/metabolic surgery to bring T2D into remission, and to be more efficient in improving glycemic control than conventional medical treatment based on lifestyle adjustments and pharmacotherapy [3, 4]. Regarding beta cell functional changes after metabolic surgery, experimental and clinical studies have provided mixed results [5, 6].

In diabetes and obesity, beta cells have to adapt to insulin-dependent increases in secretory demand, which compels cells to intensify protein flux through endoplasmic reticulum (ER) [7]. As unfolded and misfolded proteins accumulate in the ER

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lumen, a condition termed ER stress, cells activate highly specific signaling pathways, collectively named the unfolded protein response (UPR), to re-establish the ER homeostasis [8]. Upon persistent or severe ER stress, the UPR becomes cytotoxic rather than cytoprotective, upregulating pro-apoptotic factors, such as CHOP (C/EBP homologous protein) [9]. Three ER membrane-resident ER stress-sensing proteins, namely inositol-requiring enzyme 1 alpha/beta (IRE1 alpha/beta), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK), initiate different branches of the UPR. Normally, these stress sensors are kept inactive by the ER chaperone, BiP (immunoglobulin heavy chain-binding protein, also known as glucose-regulated protein 78, GRP78). During ER stress, the BiP-sensor complexes dissociate, allowing downstream signaling cascades to be activated and to dictate the cell's fate [8, 9].

The recovery of beta cell functional state demands mitigation of oxidative and ER stress [10]. We assumed that the reduction of metabolic stress imposed by high glucose and certain circulating factors in obese T2D patients can positively impact on beta cells' survival, expression of stress-related proteins, and insulin production. Thus, in this study, human 1.1B4 pancreatic beta cells were exposed for 72 h to serum samples taken from obese T2D adults at baseline and at 6-month follow-up, subsequent to receiving conventional therapy for diabetes or laparoscopic sleeve gastrectomy (LSG), the most performed bariatric procedure nowadays. Our findings showed that sera from patients who attained optimal glycemic control, mainly by metabolic surgery, had favorable effects on pancreatic beta cell survival and function.

Materials and Methods

Participants

Forty male subjects (aged 35–65 years) having body mass indexes (BMI) of 30–50 kg/m^2 and T2D (duration of diabetes between 1 and 15 years) were enrolled in the study. All individuals had glycated hemoglobin (HbA_{1c}) levels between 7.0 and 9%, and C-peptide levels > 1.0 ng/ml; none of them had poorly controlled medical problems or underwent previous abdominal surgery. Sample size was calculated using G*power 3.1.9.2 software. Assuming a HbA1c reduction of − 1.9% 1 year after sleeve gastrectomy, compared with conventional medical treatment [11], 18 patients per group were required for 95% power at a two-sided with significance level of 0.025, and a standard deviation (SD) of 1.82%. Allowing for a 10% dropout rate, 40 patients were enrolled into the study.

The study was conducted in accordance with the Declaration of Helsinki, and approved by the ethics committees of the National Institute of Diabetes, Nutrition and Metabolic Diseases "N. Paulescu", Ponderas Academic Hospital, and the Institute of Cellular Biology and Pathology "N. Simionescu" (Bucharest, Romania). The obese T2D patients had a screening visit at the National Institute of Diabetes, Nutrition and Metabolic Diseases "N. Paulescu" to ensure that they met the inclusion and exclusion criteria for the study. After receiving thorough explanations about the purpose, risks, and procedures of the research, each person provided written informed consent for being included in the study. The participants were randomly assigned in a 1:1 ratio to receive *conventional medical treatment*, based on intensive lifestyle interventions and optimal pharmacotherapy, or *metabolic surgery* by LSG; the computer-generated randomization was performed by an independent statistician. At baseline and 6 months after the beginning of the study, the participants underwent extensive medical evaluation at the National Institute of Diabetes, Nutrition and Metabolic Diseases "N. Paulescu," including physical examination, measurements of anthropometric characteristics, and routine blood tests. Patients assigned to LSG underwent additional investigations (such as ECG, abdominal ultrasound, upper endoscopy) at Ponderas Academic Hospital. The LSG operations were performed by the same surgical team using standard techniques. More information about the clinical study, including patient flow diagram, eligible patients, and actual numbers, is available in a recently published paper by Stefan and co-workers [12]. The primary outcome of the clinical study was the rate of diabetes remission (defined as HbA1c below 6.0% without pharmacological therapy). Secondary endpoints included measures of glycemic control, weight loss, markers of insulin resistance and beta cell function, and several appetite-controlling hormones.

Blood samples were taken after an overnight fast, and spun at 3000 g, 4 °C, for 10 min. Serum or plasma samples were separated and stored at − 80 °C for later analyses. Insulin resistance and beta cell function were evaluated by the homeostatic model assessment (HOMA-IR and HOMA-%B, respectively) [13].

Cell Culture

The insulin-secreting human pancreatic beta cell line 1.1B4 was from the European Collection of Authenticated Cell Culture (ECACC, Salisbury, UK). Cells were cultured in RPMI-1640 medium containing 11 mM glucose supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37 °C, in a 5% CO_2 incubator. Cells were grown to ~ 80% confluency, serum-starved for 24 h, and then incubated with 10% serum samples taken from study patients at baseline and at 6-month follow-up. Based on the sera added in the culture media, 1.1B4 cells were divided into four groups: (i) and (ii) cells exposed to sera from conventionally treated patients, taken at baseline (CTB group), or at 6-month follow-up (CT6 group); and (iii) and (iv) cells incubated with sera from

metabolic surgery-treated patients, collected at baseline (MSB group), or 6 months post-surgery (MS6 group).

Cell Viability Assay

Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, based on the MTT reduction by mitochondrial enzymes, followed by the formation of deep purple–colored formazan. Briefly, cells seeded in a 96-well plate at a density of 5×10^3 cells per well were allowed to adhere overnight, and starved for 24 h. Then, medium was supplemented with 10% sera from obese T2D participants. After 72 h, 0.5 mg/ml MTT solution was added to each well, and the culture plate was maintained for 4 h at 37 °C and 5% CO_2. The MTT-containing medium was aspirated, and the formazan crystals were dissolved using acidified isopropanol (0.04 M HCl in isopropanol). Thirty minutes after crystal solubilization, the absorbance was measured in a plate reader (Tecan Infinite 200 Pro, Tecan, Mannedorf, Switzerland) at 570 nm (reference wavelength of 690 nm). Viability of cells exposed to baseline serum samples was referred to as control (100%), and the results were expressed as mean percentage of viable cells relative to control ± standard deviation (SD).

Real-Time Cell Proliferation Assay

Cell proliferation experiments were performed using an xCELLigence Real-Time Cell Analyzer (RTCA DP version; Roche, Mannheim, Germany), which provides a quantitative readout of cell number and proliferation rate by recording the impedance changes after attachments/detachments of the cells from the electrodes. In brief, the RTCA DP device was placed in a humidified incubator at 37 °C and 5% CO_2, and a 96-well plate (E-Plate®) containing cell-free RPMI medium, supplemented with 10% FBS, was connected to the system and checked for proper electrical contacts; background impedance of each well was measured for 3 min. Next, 1.1B4 cells were seeded in E-plate at a density of 17.5×10^3 cells per well, and 1 h later, the culture media were replaced with the media containing 10% sera of participants. The E-plate was locked in the RTCA DP device, and cells were monitored every 15 min for up to 90 h. Experiments were run in duplicate. The impedance values were acquired automatically with the RTCA software (version 1.2.1) and expressed as *Cell index* values.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from 1.1B4 cells with TRI Reagent (Zymo Research, Irvine, CA, USA). Samples of 1-μg RNA were reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Quantitative PCR reactions were performed on a ViiA7 real-time PCR system (Applied Biosystems, UK) using 0.2-μg cDNA, SYBR Select Master Mix (Applied Biosystems, Foster City, CA), and the primer pairs listed in Table 1. Each sample was run in

Table 1 Primer list for quantitative real-time PCR analysis

Primer		Sequence 5′-3′	Amplicon length (bp)	GenBank accession no.
BIP	FW	TAA TGA TGC CCA ACG CCA AGC	305	AF188611.1
	RW	CAT CTT TGC CCG TCT TCT TTT TG		
CHOP	FW	GTC ATT GCC TTT CTC CTT CGG	216	NM_001195057
	RW	GTG CTT GTG ACC TCT GCT GG		
ATF6	FW	GAA TCC GCT TGT CAG TCT CGC	338	AB015856.1
	RW	GAT GTG TCC TGT GCC TCT TTA GC		
sXBP1	FW	GCA GGT GCA GGC CCA GT	100	NM_005080.3
	RW	GAA TGC CCA ACA GGA TAT CAG ACT		NM_001079539.1
uXBP1	FW	TGG AAC AGC AAG TGG TAG ATT TAG AA	125	NM_005080.3
	RW	CAT CCC CAA GCG CTG TCT T		NM_001079539.1
EDEM3	FW	TGA GTA GGG AGG AGA AAC AGA AGC	207	NM_001319960.1
	RW	TGT CCA AAG AAT CAA TCA GTG TCA G		
DNAJC3	FW	TAC GAA GGT GCT GAA TGT GGA G	265	NM_006260.4
	RW	AGT GAC CTC TCT GTA ATC TTG CTG C		
Insulin	FW	ACC CAG CCG CAG CCT TTG TGA AC	144	NM_000207
	RW	GCT CCA CCT GCC CCA CCT GC		
ACTB	FW	GTC TTC CCC TCC ATC GT	82	NM_001101.3
	RW	CGT CGC CCA CAT AGG AAT		

Table 2 Anthropometric and clinical characteristics of participants at baseline and 6-month follow-up

Variable	Conventional medical therapy ($n = 17$)			Metabolic surgery ($n = 19$)		
	At baseline	After 6 months	Change from baseline	At baseline	After 6 months	Change from baseline
Anthropometric parameters						
BMI (kg/m2)	40.5 (36.6–45.4)	39. 1(35.1–43.9)	− 1.4 (− 1.5 to − 1.45)*	39.6 (37.4–43.1)	29.5 (28.6–31.4)	− 10.5 (− 8.9 to − 11.7)**
WC (cm)	139.0 (127.0–142.0)	133.0 (119.0–149.8)	− 6.0 (− 1.0 to − 4.5)*	135.0 (128.0–144.5)	105.0 (100.0–113.0)	− 30.0 (− 28.0 to − 31.5)**
Glucose metabolism parameters						
FPG (mg/dL)	190.1 (147.2–250.1)	145.2 (128.9–247.4)	− 44.9 (− 18.3 to − 21.7)*	197.0 (143.2–266.6)	98.6 (85.2–117.8)	− 98.4 (− 58.0 to − 148.8)**
FPI (μU/mL)	22.3 (13.9–42.4)	17.4 (13.9–31.9)	− 4.9 (0.0 to − 10.6)	17.2 (8.9–35.9)	7.4 (4.0–10.7)	− 9.8 (− 5.0 to − 25.2)**
C-peptide (ng/mL)	9.9 (8.1–11.1)	7.7 (6.6–9.5)	− 2.2 (− 1.4 to − 1.6)	9.7 (9.1–10.3)	7.5 (6.7–8.9)	− 2.2 (1.5 to − 2.5)*
HbA1c (%)	7.96 (7.2–8.0)	7.91 (6.7–9.9)	− 0.05 (− 0.5 to − 0.4)	8.4 (7.8–10.0)	6.0 (4.7–6.5)	− 2.4 (− 2.2 to − 3.6)**
Lipid profile						
Total-C (mg/dL)	203.2 ± 31.6	194.2 ± 42.3	− 9.0 ± 9.7$^{\#}$	184.7 ± 36.0	184.6 ± 29.8	− 0.1 ± 5.3
HDL-C (mg/dL)	35.7 (33.8–42.4)	35.3 (30.7–41.8)	− 0.4 (− 3.1 to − 0.7)	35.2 (29.6–40.2)	39.2 (35.1–46.3)	4.1 (− 5.5 to − 6.1)*
LDL-C (mg/dL)	120.5 (101.3–136.4)	119.9 (98.0–144.7)	− 0.6 (− 4.3 to − 8.2)	112.5 (99.9–129.6)	125.1 (111.4–131.4)	12.6 (11.4 to 1.7)*
Triglycerides (mg/dL)	201.1 (136.7–266.2)	167.4 (125.7–234.9)	− 33.8 (− 11.0 to − 31.3)*	162.6 (129.1–269.1)	88.5 (72.9–120.0)	− 74.2 (− 54.4 to − 176.1)**
F-FFA (μmol/L)	276.3 (266.3–343.8)	242.6 (198.2–302.8)	− 33.7 (− 68.1 to − 41.0)*	241.9 (216.9–296.5)	212.0 (194.1–224.8)	− 29.9 (− 122.8 to − 71.7)*
Homeostasis model assessments						
HOMA-IR	10.3 (7.1–22.1)	7.4 (5.4–12.8)	− 2.9 (− 1.7 to − 9.4)	8.2 (4.9–16.6)	1.8 (1.0–3.1)	− 6.4 (− 3.9 to − 13.5)**
HOMA-%B	90.7 (37.9–119.2)	86.8 (33.4–168.0)	− 3.9 (− 4.5 to − 48.8)	56.0 (29.9–130.3)	72.0 (34.2–142.3)	16.0 (4.3 to 12.0)*
Gastrointestinal and adipose-derived hormones						
GLP-1 (ng/mL)	49.6 (45.9–52.6)	50.6 (45.3–52.9)	− 0.9 (− 0.6 to − 0.4)	47.3 (41.3–55.6)	15.2 (9.3–28.4)	− 3 2.1 (− 32.0 to − 27.2)**
Ghrelin (pg/mL)	100.5 (87.2–133.7)	136.8 (110.2–195.4)	36.3 (22.9 to 61.7)	118.6 (95.6–151.0)	93.8 (87.1–100.2)	− 24.8 (− 17.5 to − 50.8)**
Adiponectin (μg/mL)	4.3 (1.8–11.2)	4.0 (2.2–8.9)	− 0.3 (− 0.3 to − 2.36)	2.2 (1.7–3.0)	3.7 (2.3–8.4)	1.5 (0.6 to 5.4)**
Leptin (ng/mL)	15.5 (10.5–19.3)	10.2 (6.1–19.3)	− 5.4 (− 4.5 to − 0.0)	7.7 (15.4–12.1)	2.6 (2.2–3.5)	− 5.1 (− 3.1 to − 8.6)**

Data are presented as median and interquartile range (in parentheses) or mean ± standard deviation. *BMI* body mass index, *WC* waist circumference, *FPG* fasting plasma glucose, *FPI* fasting plasma insulin, *HbA1c* glycosylated hemoglobin, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *F-FFA* fasting free fatty acids, *HOMA-IR* homeostasis model assessment of insulin resistance, *HOMA-%B* homeostasis model assessment of β-cell function, *GLP-1* glucagon-like peptide-1. *$p < 0.05$ and **$p < 0.01$ as determined through Wilcoxon signed ranks test; $^{\#}p < 0.01$ as determined through paired *t* test

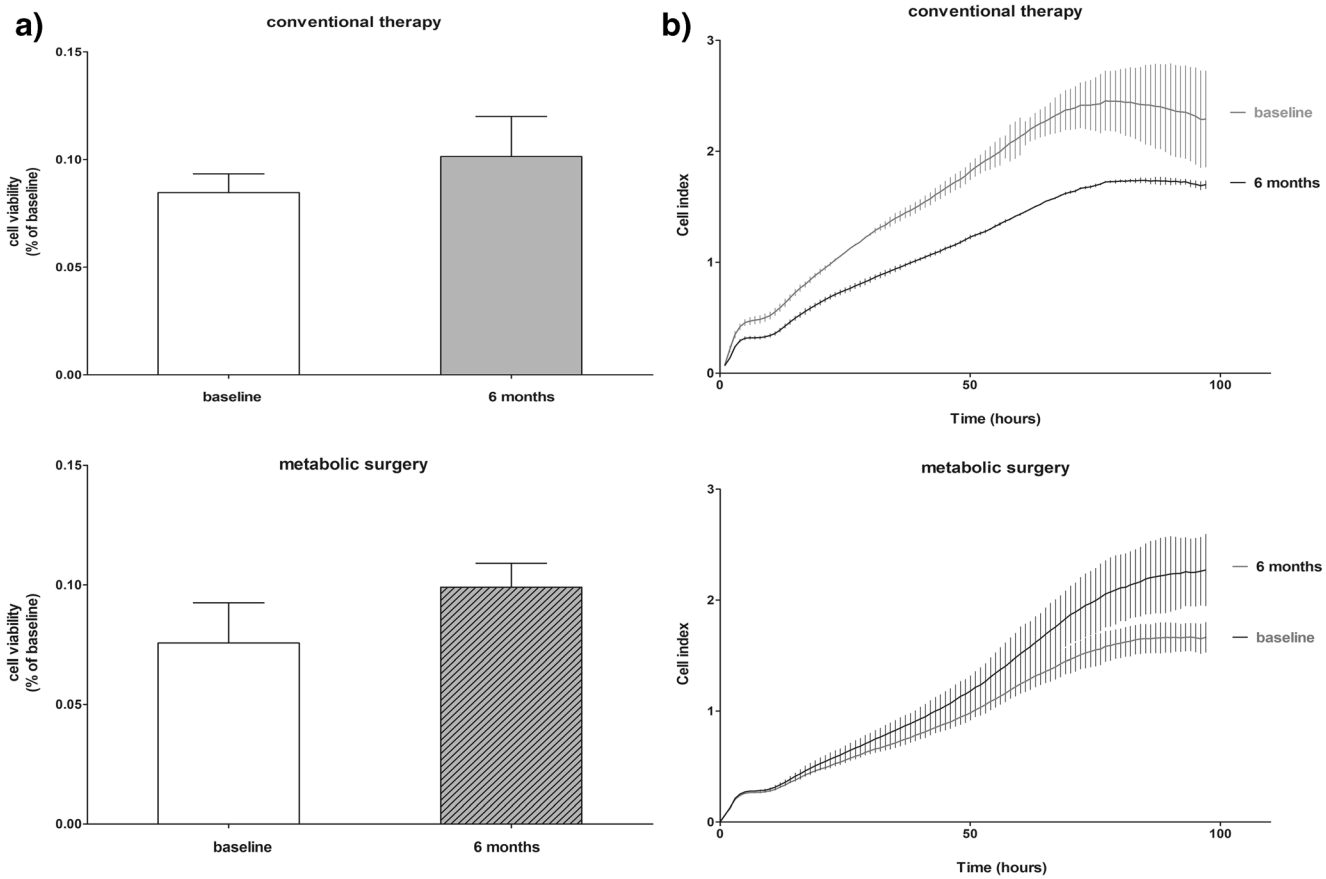


Fig. 1 Viability and proliferation of 1.1B4 beta cells exposed to sera from obese type 2 diabetic patients receiving conventional treatment or metabolic surgery. **a** Cellular viability assessed by MTT assay. Data are presented as mean ± standard deviation (SD) and are representative of four independent experiments. *$p < 0.05$ vs. cells treated with baseline serum samples (as determined by paired Student's *t* test). **b** The proliferation rates of beta cells determined with xCELLigence Real-Time Cell Analyzer. Cell proliferation profiles are representative of three independent experiments. Each data point represents the average of 2 wells ± SEM. *$p < 0.05$ vs. cells exposed to baseline sera (as determined by paired Student's *t* test)

triplicate. The levels of tested genes were normalized to beta actin gene. The relative expression of each gene was calculated using the ddCt method.

Western Blotting

Cells were seeded at 5×10^5 cells per dish in 60-mm dishes. At ~80% confluency, cells were exposed to participants' sera for 72 h and lysed in 2× Laemmli sample buffer (Serva, Heidelberg, Germany) containing 4% 2-mercaptoethanol. Equal amounts of protein (~40 μg per lane) were resolved by SDS polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane (Bio-Rad Laboratories, CA, USA). Membranes were blocked with 5% non-fat dry milk, or 3% bovine serum albumin (BSA), in Tris-buffered saline containing 0.05% Tween-20 for 1 h, and incubated overnight at 4 °C with specific primary antibodies against p53, phospho-p38 MAPK, MnSOD, sirtuin1 (SIRT1), sequestosome 1 (SQSTM1/ p62), BiP, insulin, and beta actin (all from Santa Cruz Biotechnology, Dallas, TX, USA; dilution 1:200). Immunoreactive proteins were detected with appropriate horseradish peroxidase-conjugated secondary antibodies (rabbit or mouse; 1:1000 dilution; Santa Cruz Biotechnology, Heidelberg, Germany). Protein bands were developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, UK, Ltd., Little Chalfont, Buckinghamshire, UK), and visualized with LAS-4000 luminescent image analyzer (FUJIFILM, Tokyo, Japan). Bands intensities were quantified with TotalLab Quant software. Each protein expression level was normalized to beta actin.

Quantification of Intracellular Reactive Oxygen Species

Reactive oxygen species (ROS) production was determined with 2′,-7′-dichlorofluorescein diacetate (DCFH-

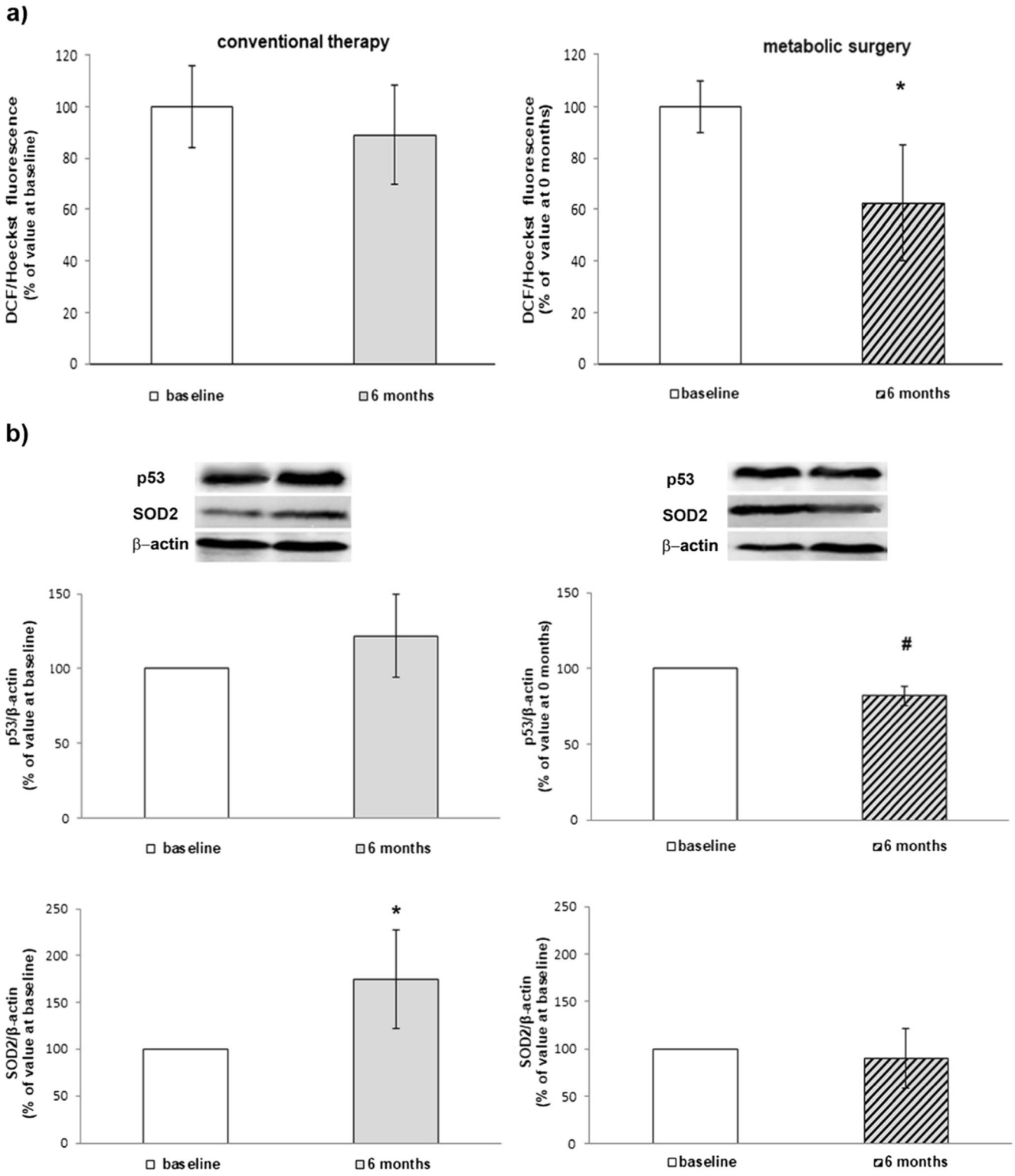


Fig. 2 Reactive oxygen species (ROS) production (**a**) and expression of ROS-related proteins p53 and SOD2 (**b**) in 1.1B4 beta cells exposed for 72 h to sera from conventional or metabolic surgery-treated patients, taken at baseline or at 6-month follow-up. **a** The relative amount of intracellular ROS was determined with DCFH-DA. Data are representative of four independent experiments. ROS levels in cells exposed to baseline sera were referred to as 100% (control), and results were expressed as mean percentage relative to control ± standard deviation (SD). $^{*}p < 0.05$ vs. cells treated with baseline sera (as determined by paired Student's *t* test). **b** Protein expression levels of p53 and SOD2. Top: representative immunoblots. Bottom: corresponding bar graphs showing the densitometry values; beta actin served as loading control. $^{*}p < 0.05$, $^{\#}p < 0.01$ vs. cells exposed to baseline sera (as determined by paired Student's *t* test)

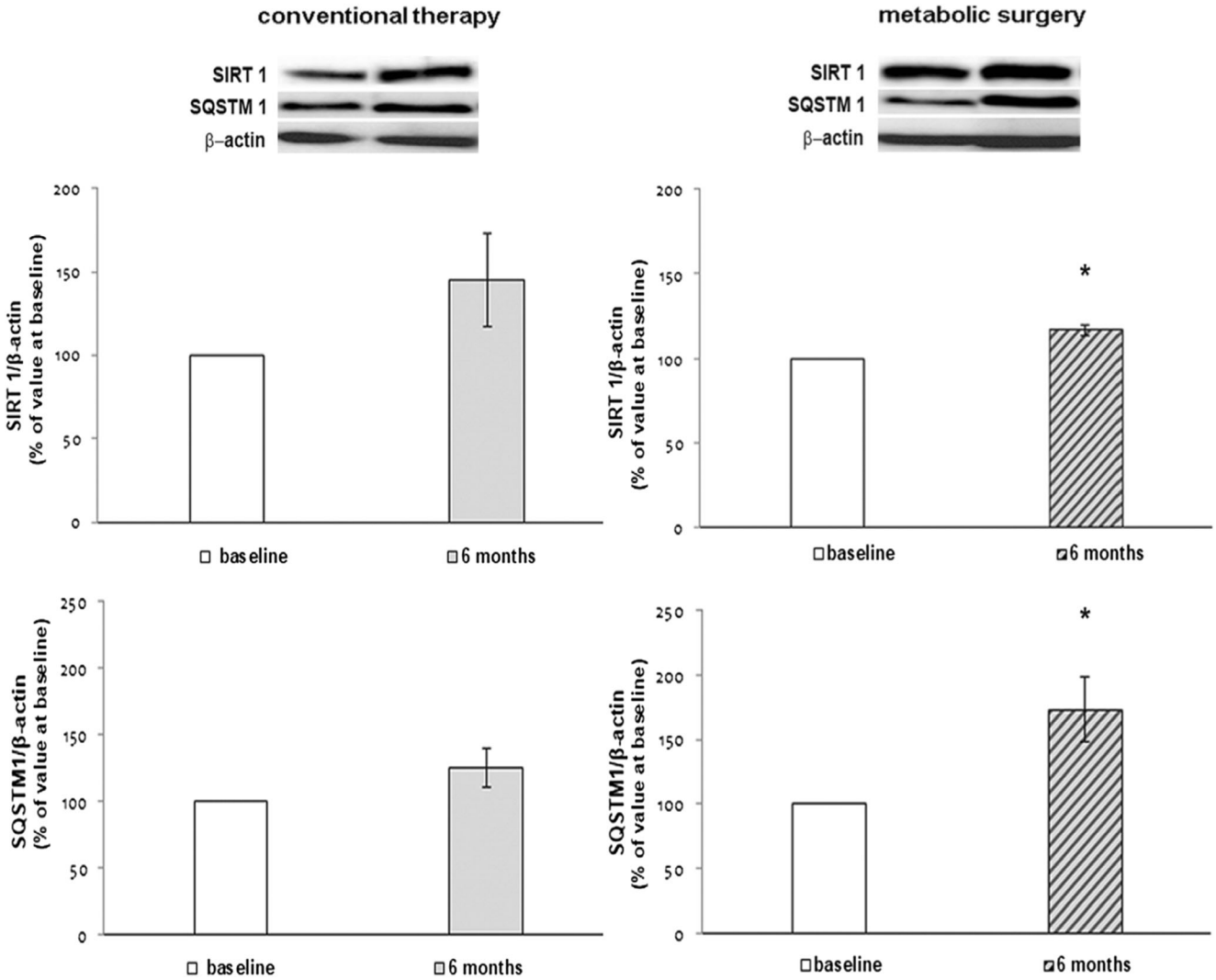


Fig. 3 Protein expression of autophagy-related proteins SIRT1 and SQSTM1/p62 in 1.1B4 beta cells exposed for 72 h to sera from study patients at baseline and at 6-month follow-up. Top panels: representative immunoblots. Bottom panels: corresponding bar graphs showing the densitometric values of proteins normalized to beta actin. Data are representative of at least three independent experiments. Results at 6 months of follow-up are expressed as percentages of values of baseline conditions, i.e., cells exposed to baseline sera from conventional or metabolic surgery treated patients. $*p < 0.05$ vs. cells exposed to baseline sera (as determined by paired Student's *t* test)

DA), which yields a fluorescent compound, 2′,7′-dichlorofluorescein (DCF) after being oxidized by ROS. The 1.1B4 cells were seeded in a 96-well black plate at a density of 5×10^3 cells per well, starved by serum deprivation for 24 h, and incubated for 72 h with 10% sera taken from study patients at baseline and 6 months later. Subsequently, the medium was changed to Hanks' Balanced Salt Solution (HBSS) buffer containing 10 μM DCFH-DA (Sigma-Aldrich), and cells were kept in the dark, at 37 °C, for 1 h. The DCF signal at 530 nm (excitation wavelength of 480 nm) was measured in a microplate reader (Tecan Infinite 200 Pro, Tecan, Mannedorf, Switzerland). The average DCF fluorescence intensity was normalized to the mean fluorescence intensity of DNA dye Hoechst 33342 measured at 485 nm (excitation wavelength of 355 nm). ROS levels in cells exposed to baseline serum samples were considered 100% (control), and the results were expressed as mean percentage ROS level relative to control ± standard deviation (SD).

Statistical Analysis

Statistical analyses were performed using SPSS 21.0 software (SPSS Inc. Chicago, IL). Normally distributed data are mean ± standard deviation (SD), unless otherwise specified, and data with skewed distribution are median values with interquartile range (IQR). Normality of data was assessed with Kolmogorov-Smirnov test, and non-normal variables were log-transformed before analysis. Differences between measures at baseline and at 6-month time point were assessed by

Student's *t* test or Wilcoxon signed-rank test, as appropriate. The level for statistical significance was set at $p < 0.05$.

Results

Participant Characteristics

Anthropometric and clinical data of the study subjects are given in Table 2. Thirty-six out of 40 participants (90%) completed the study. At baseline, median BMI, fasting plasma glucose, and HbA_{1c} did not differ significantly between patients assigned to conventional treatment or metabolic surgery (Table 2). At 6-month follow-up, the conventionally treated patients succeeded to significantly lower their BMI and waist circumference, but achieved only modest improvements of fasting plasma glucose, insulin, C-peptide, triglycerides, cholesterol, fatty acids, and leptin levels, together with a minor decrease of the HOMA-IR index ($p > 0.05$ vs. baseline) (Table 2). As compared to baseline, at 6-month post-operative follow up, the participants attained a marked reduction of BMI ($p < 0.01$), and a prominent improvement of metabolic status, i.e., HbA_{1c} levels below 6.5%, increased insulin sensitivity ($p < 0.01$ for HOMA-IR and $p < 0.05$ for HOMA-%B, respectively), enhanced plasma adiponectin levels, and diminished circulating levels of fatty acids, leptin, GLP-1, and ghrelin ($p < 0.05$ for all mentioned parameters) (Table 2).

Beta Cell Viability and Proliferation after Exposure to Sera from Obese T2D Patients with Conventional Therapy or Metabolic Surgery

According to MTT assay, there was no significant differences between viability of beta cells exposed to 10% sera obtained from conventionally treated patients at 6-month follow-up (CT6 cells) or at baseline (CTB cells) (Fig. 1a). Conversely, there were much more viable cells in MS6 group than in MSB group ($p < 0.05$). Real-time analysis of cell proliferation with xCELLigence system indicated a lower proliferation rate of 1.1B4 cells in CT6 group compared to cells in CTB group ($p < 0.05$), and a significantly higher proliferation of MS6 cells compared to MSB cells ($p < 0.05$) (Fig. 1b). Altogether, these results reflected an increased potential of circulating factors present in patients attaining a good glycemic control within 6 months of follow-up (mainly by metabolic surgery) to promote beta cell survival.

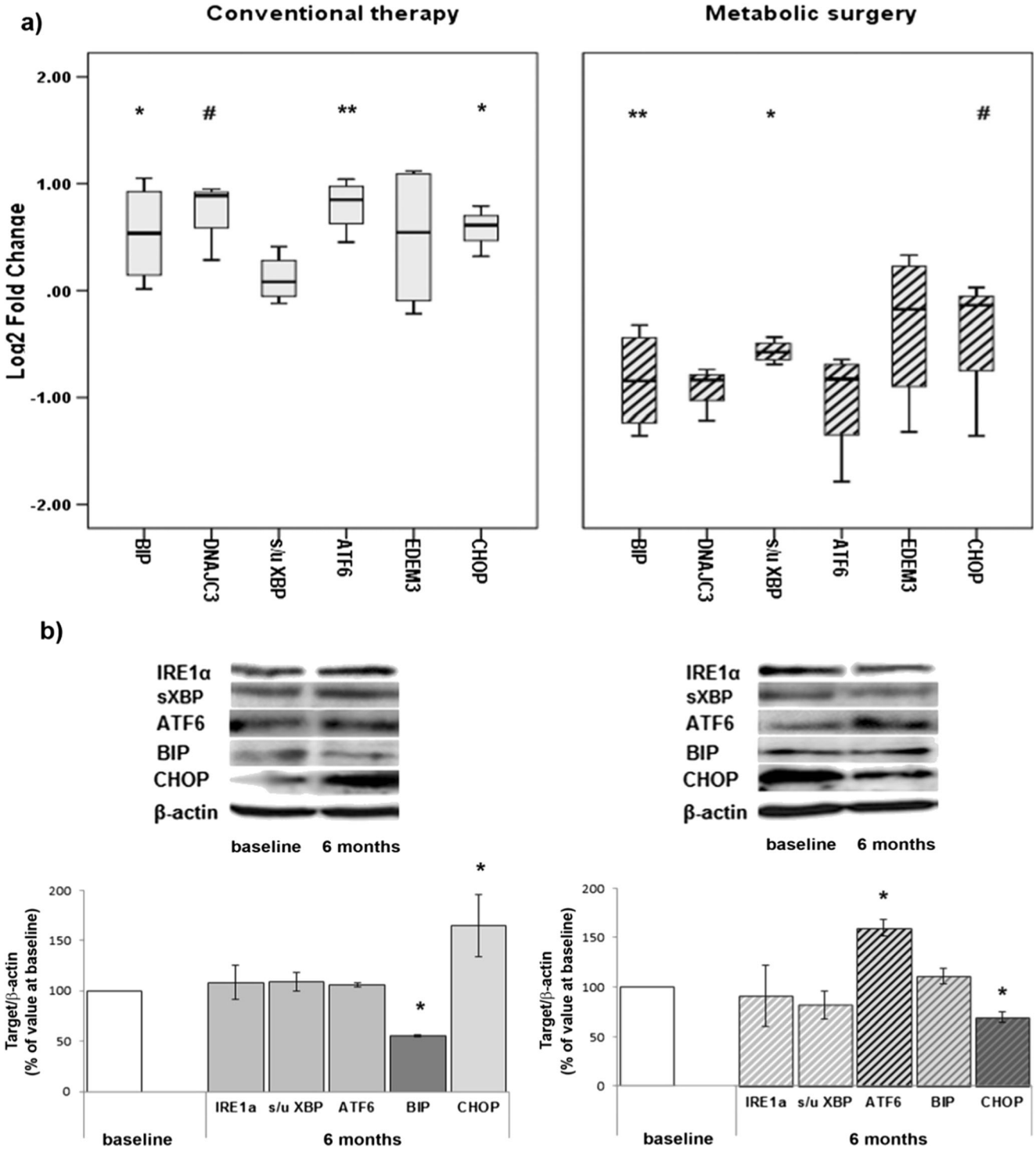
Reactive Oxygen Species Production and ROS-Related Proteins in Beta Cells Exposed to Study Patients' Sera

Sera from obese T2D people could induce a glucolipotoxic situation for 1.1B4 beta cells, promoting the formation of ROS. Therefore, we examined the levels of ROS and the protein expression levels of p53, phospho-p38 MAPK, and MnSOD (or SOD2) in 1.1B4 cells after a 72-h exposure to sera from the study patients. Based on DCF fluorescence intensity, ROS levels were comparable in CT6 and CTB cells, whereas the production of ROS in MS6 cells was substantially diminished in comparison with MSB cells ($p < 0.05$; Fig. 2a). Immunoblot analysis of redox-sensitive transcription factor p53 showed that p53 expression was similar in CT6 and CTB cells, while it was significantly decreased in MS6 cells vs. MSB cells (Fig. 2b). Regarding the p38 MAPK activation, there were no significant differences among CT6 and CTB groups and MS6 and MSB groups, respectively (data not shown). The protein expression of the ROS-neutralizing enzyme SOD2 was higher in CT6 cells ($p < 0.05$ vs. CTB cells) probably enhancing CT6 cells ability to clear ROS. On the contrary, the protein expression of SOD2 was comparable in MS6 and MSB cells (Fig. 2b). Together, these data denoted a protective effect of circulating factors in patients having an adequate glycemic control at 6 months of follow-up, attained mostly by metabolic surgery, against ROS accumulation in beta cells.

Fig. 4 The expression pattern of endoplasmic reticulum (ER) stress markers in 1.1B4 beta cells after a 72-h exposure to sera from conventional or metabolic surgery-treated participants. **a** Real-time PCR measurements of ER stress-related genes are presented in box plots as values of $\log_2$ fold change between treatment condition (i.e., cells incubated with sera taken at 6-month follow-up from patients receiving conventional treatment or metabolic surgery) and basal state (cells exposed to baseline sera); the expression of each gene was normalized to the expression of beta actin (as internal control gene). Boxes denote the interquartile ranges (IQR), horizontal lines within boxes represent medians, and whiskers show the full range of values. Data are from at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0$ .001, $^{\#}p < 0.001$ vs. cells exposed 72 h to baseline sera. **b** Protein expression levels of ER stress response markers. Top panel: representative Western blot bands. Bottom panel: bar graphs presenting the densitometric quantification of immunoblots; beta actin served as loading control. The results at 6-month time point are expressed as percentages of values at baseline conditions (i.e., cells treated with serum samples from conventional or metabolic surgery treated patients taken at baseline). Values are mean ± SD. $^{*}p < 0.05$ vs. cells treated with baseline sera (as determined by paired Student's *t* test)

Differential Expression of SIRT1 and SQSTM1/p62 Proteins in Beta Cells Exposed to Sera from Conventional or Metabolic Surgery-Treated Patients

Stress response signals might activate autophagy, which is important for beta cell survival [14]. Therefore, we examined the expression of SIRT1 and SQSTM1/p62, two autophagy-related proteins, also known to be associated with the insulin signaling pathway. The protein expression of SIRT1 in CT6 and CTB cells did not differ significantly, whereas SIRT1 protein levels were significantly higher in MS6 cells compared to MSB



cells (Fig. 3). As well, SQSTM1/p62 expression was similar among CT6 and CTB groups, and it was significantly greater in MS6 group compared to MSB group (Fig. 3). These findings broadened the idea that sera of patients reaching an adequate glycemic control in 6-month post-metabolic surgery contained factors with beneficial effects for beta cell survival.

Differential Expression of ER Stress-Related Proteins in Beta Cells Exposed to Sera of Obese T2D Patients Receiving Conventional or Metabolic Surgery Treatment

ER stress is causally involved in obesity and diabetes. Thus, we investigated the expression of the ER stress markers, BiP,

DNAJC3 (DnaJ (Hsp40) homolog C3, also known as p58IPK), ATF6, IRE1α, spliced (s) and unspliced (u) XBP1, CHOP (C/EBP homologous protein), and EDEM 3 (ER degradation-enhancing alpha-mannosidase-like protein 3), in 1.1B4 beta cells. As compared to CTB cells, in CT6 cells the transcript levels of BiP, DNAJC3, ATF6 and CHOP, and CHOP protein levels were markedly increased ($p < 0.05$) (Fig. 4a, b), which pointed to a moderate ER stress response in these cells. Intriguingly, in CT6 cells, BiP protein expression was decreased ($p < 0.05$ vs. CTB cells; Fig. 4b). Conversely, in MS6 cells, gene expression of BiP, DNAJC3 and CHOP, sXbp1 to uXbp1 mRNA ratio, and CHOP protein expression were significantly diminished compared to MSB cells (Fig. 4a, b), while the expression of ATF6 was markedly enhanced ($p < 0.05$ vs. MSB cells; Fig. 4b). The expression levels of all the other aforementioned genes and proteins were quite similar among CT6 and CTB groups and MS6 and MSB groups, respectively. Overall, these results implied that circulating factors in patients who underwent metabolic surgery proficiently ensured a favorable milieu for beta cell survival.

Increased Expression of Insulin in Beta Cells Exposed to Sera of Obese T2D Patients Treated by Metabolic Surgery

As shown in Fig. 5, gene and protein expression of insulin, as a marker of beta cell function, did not differ significantly in CT6 and CTB cells. However, at both mRNA and protein levels, insulin expression was significantly augmented in MS6 cells ($p < 0.05$ vs. MSB cells), suggesting that circulating factors in metabolic surgery-treated patients potently stimulated the insulin production in pancreatic beta cells.

Discussion

This study is, to the best of our knowledge, the first to report dissimilar responses of human pancreatic beta cells upon exposure to sera from obese T2D patients who received different treatment, namely conventional medical therapy or laparoscopic sleeve gastrectomy (LSG). Our results showed that serum samples from patients who attained an adequate glycemic control, mostly by metabolic surgery (LSG), exerted beneficial effects on the 1.1B4 human pancreatic beta cells' survival; expression of oxidative-, autophagy-, and ER stress-related proteins; and expression of insulin. The molecular mechanisms that underlie metabolic improvement after LSG have not yet been fully elucidated [15].

In this study, after 6 months of follow-up, metabolic surgery-treated patients exhibited robust decrease of HbA_{1c} and increase of insulin sensitivity (as reflected by HOMA indexes), but most patients with conventional treatment did not improve their diabetic condition (Table 2).

However, irrespective of the type of treatment followed by participants upon 6 months of study, their sera stimulated beta cells' viability (Fig. 1). Nevertheless, an increased rate of proliferation was noticed for 1.1B4 beta cells exposed to sera of surgically treated patients (MS6 cells; $p < 0.05$ vs. MSB cells), while CT6 cells exhibited a slower rate of proliferation, compared to CTB cells, a result that was thought to be linked up with increased expression of pro-apoptotic factor CHOP in CT6 cells. As suggested by similar levels of ROS in CT6 and CTB cells (Fig. 2a), the 6-month conventional therapy did not alter circulating redox status in conventionally treated patients. Conversely, decreased ROS levels in MS6 cells implied that certain circulating factors in surgically treated patients were protective against ROS formation. In MS6 cells, the survival rate was likely to be upheld by pro-survival factors SIRT1 and p62/SQSTM1 (p62), whose enhanced expression ($p < 0.05$ vs. MSB cells) was accompanied by a diminished expression of p53, pro-apoptotic factor, and one of the main targets of SIRT1 [16] (Figs. 2b and 3). SIRT1 plays important roles in regulating glucose homeostasis and insulin secretion in pancreatic beta cells [17]; regulates SOD2, which is critical for an efficient antioxidant defense [18]; and promotes autophagy [19] mandatory for beta cell survival under ER stress. As well, p62, an adapter protein in the autophagic process, newly identified as a partner of IRS-1 protein [20], might protect cells from the ER stress-induced cell death [21].

Pancreatic beta cells are highly sensitive to impaired UPR, being heavily engaged in the production and secretion of a large amount of insulin. Thus, we focused on the UPR branches that modulate induction of chaperones, folding enzymes, and components of the ER-associated degradation (ERAD) system, thus reducing the ER stress. It has been reported previously that, under mild hyperglycemia, human islets exhibited an adaptive UPR, aiding the cells to cope with the ER stress [22]. Consistently, in CT6 cells, enhanced transcript levels of the ER stress markers, BiP, DNAJC3, ATF6, and CHOP ($p < 0.05$ vs. CTB cells), stand for a mild ER stress activation, or maybe an ER stress relieve, considering that BiP and DNAJC3 overexpression helps restoring ER homeostasis. Interestingly, the homozygous deletion of DNAJC3, a stress-inducible co-chaperone for BiP involved in cell survival upon ER stress [23], could be accountable, in humans, for a monogenic, recessive form of diabetes [24]. As regards BiP expression in CT6 cells, our results revealed a discrepancy between its mRNA and protein levels. Purportedly, mRNA abundance is predictive of protein expression levels [25]. However, many studies have reported poor correlation between the transcript and protein levels, arising perhaps from complex regulatory steps implicated in converting mRNA to protein, and/or the differential half-lives of proteins and mRNAs [26, 27]. In a recent report, decreased expression of BiP in a model of neurological disorder was assumed to be due to enhanced BiP degradation rather than reduced BiP transcript levels [28].

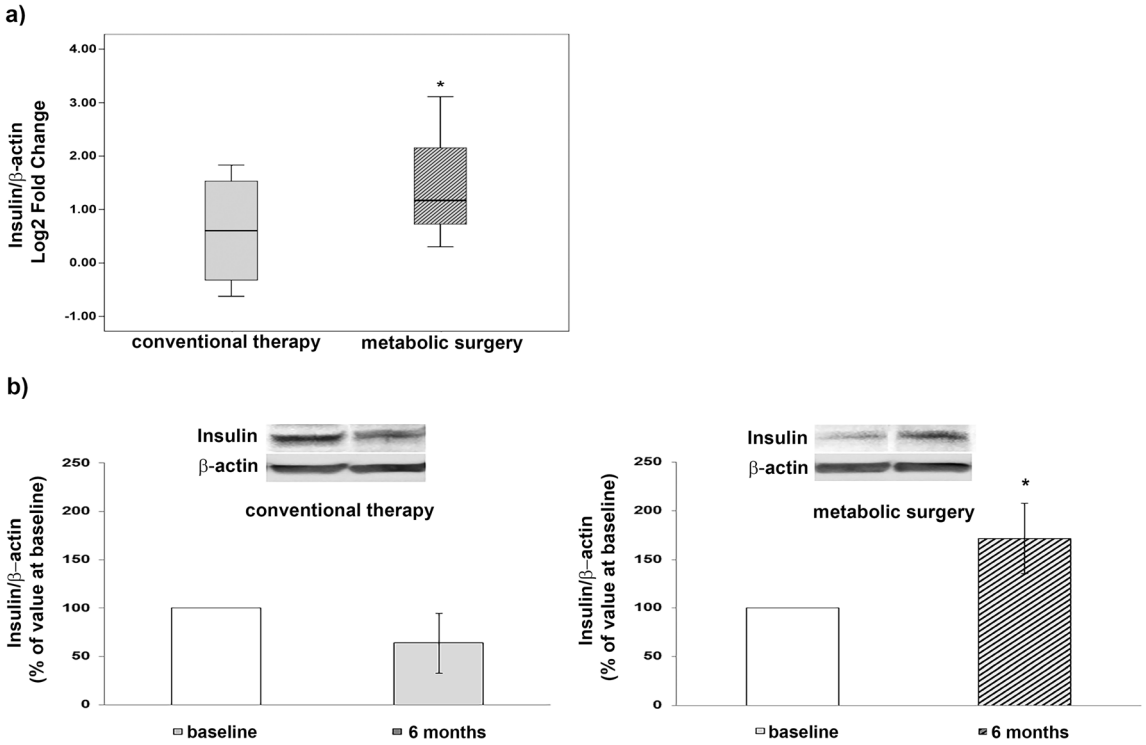


Fig. 5 Insulin protein and mRNA levels in 1.1B4 beta cells exposed to sera from the study patients. **a** Box plots of real-time PCR measurements expressed as $\log_2$ values of fold change between 6 months and baseline. The box borders represent the interquartile range, and the horizontal line in the box is the median. The expression values for insulin mRNA were normalized to those of the gene encoding beta actin. *$p < 0.05$ vs. cells treated with baseline sera. **b** Insulin protein expression. Top panel: representative Western blot bands. Bottom panel: bar graphs presenting the densitometric quantification of immunoblots; beta actin served as loading control. The results are expressed as percentage of values at baseline conditions (i.e., cells exposed to baseline sera from conventionally or surgically treated patients). Values are mean ± SD. *$p < 0.05$ vs. cells treated with baseline sera (as determined by paired Student's *t* test)

Likewise, in our study, in CT6 cells, the lack of correspondence between BiP mRNA and protein levels might be attributable to post-translational modifications that modulate BiP turnover and availability, in certain conditions [29]. As regards the CHOP expression in CT6 cells, the enhanced CHOP mRNA levels reflected indeed an augmented protein expression. Nonetheless, in CT6 cells, upregulation of CHOP was not associated with increased incidence of apoptotic cells, which was suggestive for the presence of a defensive UPR capable to subdue CHOP-mediated apoptosis, as reported by others in several cell types [30]. This might be in line with the observation that, in pancreases from lean and obese T2D subjects, staining for CHOP revealed that, although many cells are showing signs of stress, only small numbers of beta cells are dying at any one time [31]. Conversely, in MS6 cells, the transcript levels of BiP and CHOP were markedly lower ($p < 0.05$ vs. MSB cells; Fig. 4a), implying that the 1.1B4 beta cells encountered an adequate environment upon exposure to sera from metabolic surgery-treated patients and were not compelled to activate the ER stress response. In addition, in MS6 cells, the increased ATF6 protein expression might reflect the fact that beta cells' physiology depends on ATF6 even under non-stress conditions [32]. Again, the augmented protein expression of ATF6 did not reflect the mRNA levels. Conceivable, the translated protein might accumulate over time, leading to increased amount of protein, albeit low transcript levels. As to the markedly lowered CHOP expression in MS6 cells ($p < 0.05$ vs. MSB cells; Fig. 4b), this was supportive for the enhanced viability/proliferation of 1.1B4 beta cells exposed to sera from surgically treated patients taken at 6-month follow-up.

In our experimental system, the mRNA and protein levels of insulin were increased significantly in MS6 cells, which further suggested that sera taken from patients who underwent LSG 6 months ago favorable influenced beta cells' function and survival.

The current study has certain limitations. Two important limitations are the small number of participants and a small, limited amount of serum samples (about 1.5 ml) that we had obtained from each participant; the later limited the number of our experiments. As well, for more relevant results, the follow-up period should be longer than 6 months. Despite these limitations, we assume that our results will support future research regarding the molecular pathways leading to metabolic improvement and diabetes resolution after laparoscopic sleeve gastrectomy.

In conclusion, in this study, we reported that human pancreatic beta cells exhibited divergent cellular responses when exposed to sera from obese type 2 diabetes patients treated differently, namely by conventional therapy or by laparoscopic sleeve gastrectomy (metabolic surgery). This study adds to the currently available data that show the effectiveness of metabolic surgery in improving diabetes and reveals that circulating factors in patients who attained an adequate glycemic control after sleeve gastrectomy have beneficial effects on pancreatic beta cells function and survival. Understanding the molecular basis of beta cell adaptation to its metabolic environment is of great interest as it has the potential to open new avenues for developing non-invasive therapies for diabetes.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Statement of Human and Animal Rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and /or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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