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# Stress of endoplasmic reticulum modulates differentiation and lipogenesis of human adipocytes

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ABSTRACT

*Background:* Adipocytes are cells specialized for storage of neutral lipids. This storage capacity is dependent on lipogenesis and is diminished in obesity. The reason for the decline in lipogenic activity of adipocytes in obesity remains unknown. Recent data show that lipogenesis in liver is regulated by pathways initiated by endoplasmic reticulum stress (ERS). Thus, we aimed at investigating the effect of ERS on lipogenesis in adipose cells.
*Methods:* Preadipocytes were isolated from subcutaneous abdominal adipose tissue from obese volunteers and *in vitro* differentiated into adipocytes. ERS was induced pharmacologically by thapsigargin (TG) or tunicamycin (TM). Activation of Unfolded Protein Response pathway (UPR) was monitored on the level of eIF2α phosphorylation and mRNA expression of downstream targets of UPR sensors. Adipogenic and lipogenic capacity was evaluated by Oil Red O staining, measurement of incorporation of radio-labelled glucose or acetic acid into lipids and mRNA analysis of adipogenic/lipogenic markers.
*Results:* Exposition of adipocytes to high doses of TG (100 nM) and TM (1 μg/ml) for 1–24 h enhanced expression of several UPR markers (HSPA5, EDEM1, ATF4, XBP1s) and phosphorylation of eIF2α. This acute ERS substantially inhibited expression of lipogenic genes (DGAT2, FASN, SCD1) and glucose incorporation into lipids. Moreover, chronic exposure of preadipocytes to low dose of TG (2.5 nM) during the early phases of adipogenic conversion of preadipocytes impaired both, lipogenesis and adipogenesis. On the other hand, chronic low ERS had no apparent effect on lipogenesis in mature adipocytes.
*Conclusions:* Acute ERS weakened a capacity of mature adipocytes to store lipids and chronic ERS diminished adipogenic potential of preadipocytes.



## 1. Introduction

Adipocytes are cells highly specialized for storage of neutral lipids. They are equipped with dedicated receptors and transporters necessary for an uptake and transport of nonesterified fatty acids (NEFA) and with enzymatic cascade enabling NEFA incorporation into triglycerides. Moreover, adipocytes are able to synthetize lipids de novo, from glucose [1]. Glucose is necessary also for the synthesis of glycerol phosphate, the backbone of triglycerides. Thus lipogenic activity of adipocytes directly influence fatty acid and glucose plasma levels and this homeostatic effect is regulated by many factors [2,3]. Paradoxically, obesity impairs

*Abbreviations:* ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; NEFA, nonesterified fatty acids; TG, thapsigargin; TM, tunicamycin; UPR, unfolded protein response.

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capacity of adipocytes to synthetize and store lipids [4,5], which further contributes to high plasma NEFA levels, a putative cause of obesity-related hepatic and muscle insulin resistance [6]. The reason for the deterioration of lipogenic activity of adipocytes remains unclear. Notably, several enzymatic steps of lipogenesis and the formation of lipid droplets take place in the endoplasmic reticulum (ER), an organelle also essential for calcium homeostasis and protein folding [7,8]. The situation when the folding and other metabolic capacities of ER are overwhelmed is referred to as endoplasmic reticulum stress (ERS). ERS activates a defense mechanism called unfolded protein response (UPR) in order to enhance ER capacity and restore ER homeostasis [9]. The signs of chronic ERS have recently been found in obese and insulin resistant subjects [10,11]. The significance of ERS for metabolic health was confirmed by experiments on rodents corroborating ERS as a trigger of insulin resistance and other metabolic disturbances caused by obesity [12]. Importantly, ERS and consequently UPR were found to be important regulators of lipogenesis in liver [13]. But there is a lack of comprehensive studies that would investigate whether metabolic stress sensed through ER controls lipogenesis also in human adipose tissue. Thus, we aimed at investigating the effect of ERS on lipogenesis in human adipose cells.

## 2. Materials and methods

### 2.1. Cells and chemicals

Cells were derived from needle biopsies of subcutaneous adipose tissue from obese volunteers that were recruited at the Third Faculty of Medicine of Charles University, University Hospital Kralovske Vinohrady, Czech Republic, and Toulouse University Hospitals, France. Isolation, expansion and differentiation of cells was described previously [14]. The study was performed according to the Declaration of Helsinki and was approved by the respective Ethical Committees. Volunteers were informed on the study, and written informed consent was obtained before participation in the study.

Thapsigargin was supplied by Alexis (Lauzen, Switzerland), tunicamycin by LKT Laboratories, Inc. (St. Paul, Maine, USA) and Rosiglitazone by Cayman (Tallin, Estonia). Culture media were from Lonza Std. (Basel, Switzerland). FBS (qualified for MSC) was from ThermoFisher (Carlsbad, California, USA), FGFβ and EGF from Immunotools (Friesoythe, Germany). Other chemicals were from Sigma Aldrich (St. Louis, Missouri, USA).

### 2.2. Gene expression analysis

Isolation of RNA, cDNA synthesis and qRT-PCR was described previously [14]. TaqMan Gene expression assay for PPARγ, SCD1, FASN, DGAT2, SREBP1C, HSPA5, ATF4, EDEM1, PLIN1 were from Applied Biosystems (Carlsbad, California, USA). aP2 and XBP1 full-length and spliced were detected by specific primers (aP2-forward 5′-GCATGGCCAAACCTAACATGA-3′, aP2-reverse 5′ CCTGG CCCAGTATGAAGGAAA-3′, XBP1-total-forward 5′- CGCTGAGGAG-GAAACTGAA-3′, XBP1-total-reverse 5′- CACTTGCTGTTCCAGCT-CACTCAT/3′, XBP1-spliced-forward 5′- GAGTCCGCAGCAGGTGCA-3′, XBP1-spliced reverse 5′- ACTGGGTCCAAGTTGTCCAG-3′) by Sybr Green technology (Power Sybr Green Master Mix). Gene expression of target genes was normalized to expression of GUSB or RPS13 and fold change of expression was calculated using ΔΔ Ct method.

For western blotting, cells were harvested and lysates processed as described previously [15]. Antibodies against total and phosphorylated eIF2α were from Cell Signaling (Danvers, MA, USA).

### 2.3. Apoptosis assay

Cells were exposed to 0, 2.5 and 100 nM TG for 24 h. Then they were trypsinized and fixed in 70% ethanol at 4 °C overnight. After two washes with PBS, cells were stained with 50 μg/ml Propidium Iodide and treated with 0.1 mg/ml RNAse I diluted in PBS for 30 min at 37 °C. DNA content analysis was performed on FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo 8.2 (Tree Star Inc, Ashland, OR, USA).

### 2.4. Oil red O (ORO) staining

12 days differentiated cells were fixed and stained as described previously [14].

### 2.5. Glucose and acetic acid incorporation into lipids

Cells were incubated for 3 h in Krebs Ringer buffer or DMEM-no glucose medium supplemented with 2% BSA, 10 mM HEPES, 66 nM insulin, 2 mM glucose and 2 μCi D-[$^{14}$C(U)]glucose (PerkinElmer) or 5 mM acetic acid and 2 μCi [1−$^{14}$C]-Acetic Acid (PerkinElmer). Neutral lipids were extracted and analyzed as described [16]. Distribution of de novo incorporated $^{14}$C among major lipid species was analyzed after lipid separation by thin-layer chromatography on Silica Gel plates developed in Heptane:Isopropylether:Acetic acid mixture (60:40:4) for 1 h, visualized by iodine vapor, quantitatively scraped from plate and analyzed by liquid scintillation counting. Results from metabolic measurements were normalized to total protein content of cell extracts.

### 2.6. Analysis of mitochondrial respiration

Oxygen consumption rates (OCR) was measured using an extracellular flux analyzer XF24 (Seahorse Bioscience, Copenhagen, Denmark). Preadipocytes were seeded at a density of 6000 cells per well (XF24 Cell Culture Microplate) and allowed to reach confluence when the differentiation was started. At day 11 cells were treated with ER stressors for 24 h. The culture medium was replaced with the XF Assay medium supplemented with 4 mM L-glutamine, 1 mM pyruvate and 5.5 mM glucose 1 h prior to measurement. OCR measurements were obtained before and after sequential additions of 1 μM oligomycin, 0.5 μM FCCP and 2 μM rotenone/antimycin A to the medium.

### 2.7. Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software using Wilcoxon matched-pair signed rank or Mann Whitney test, as appropriate. The level of significance was set at $p < 0.05$.

## 3. Results

### 3.1. Acute high intensity ERS reduces adipogenesis and lipogenesis of human preadipocytes and adipocytes

To evaluate the effect of acute ERS on lipogenic capacity of adipocytes, we exposed *in vitro* differentiated adipocytes from 15 donors to two commonly used ER stressors, thapsigargin (TG) and tunicamycin (TM), for 24 h. Both, 100 nM TG and 1 μg/ml TM [17], dramatically enhanced expression of major ER chaperone HSPA5 (heat shock 70 kDa protein 5), a marker of ERS (Fig. 1A). The same treatment decreased mRNA levels of genes involved in lipogenesis, i.e. fatty acid synthase (FASN), stearoyl desaturase (SCD1), and diacylglycerol O-acyltransferase 2 (DGAT2) (Fig. 1A). The suppressive effect of ERS on lipogenesis was confirmed by a decreased

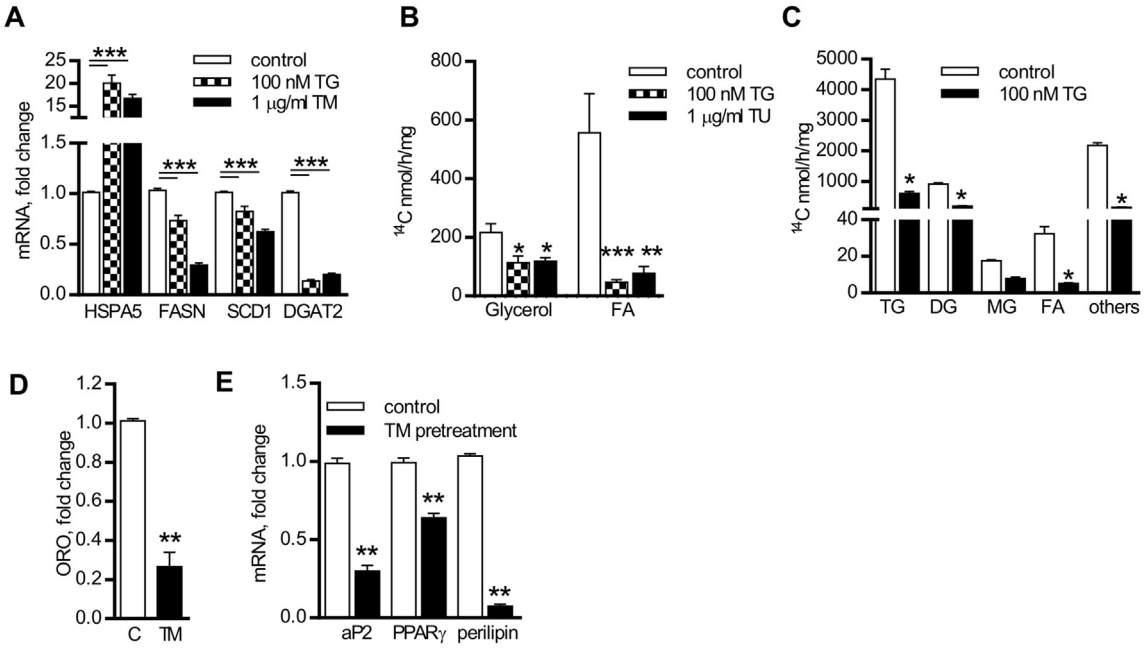


**Fig. 1.** Acute ERS lowers lipogenesis in human adipocytes and adipogenesis of preadipocytes. Preadipocytes were differentiated for 12 days and then incubated for 24 h with DMSO or 100 nM TG or 1 μg/ml TM. **A**. mRNA expression of HSPA5, FASN, SCD1 and DGAT2 was measured by qRT-PCR and normalized to GUSB expression (n = 15). **B**. Glucose carbon ($^{14}$C) incorporation into lipids (hydrolyzed into fatty acids (FA) and glycerol) during 3 h incubation was determined by liquid scintillation and normalized to protein content (n = 3). **C**. Distribution of de novo incorporated $^{14}$C in lipid species was analyzed after TLC separation of extracted lipids (n = 2). **D**. Preadipocytes were exposed to 1 μg/ml TM for 4 h and then differentiated in the absence of TM for 12 days. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n = 5). **E**. Preadipocytes were exposed to 1 μg/ml TM for 4 h and then differentiated in the absence of TM for 3 days. mRNA expression of adipogenic markers was analyzed by qRT-PCR and normalized to RPS13 (n = 5). Data are means ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

capacity of adipocytes treated with TG to incorporate glucose carbon into lipids (Fig. 1B, C). Thus, in adipocytes, acute high intensity ERS lowers lipogenic capacity of adipocytes on both transcriptional and enzymatic level.

As lipogenesis in adipocytes was found to be dependent on mitochondrial activity [18,19] that could be impaired by calcium accumulation induced by ER stressors, we analyzed respiration capacity of adipocytes treated with 100 nM TG or 1 μg/ml TM for 24 h. Acute ERS had no impact on the proton leak by mitochondria nor the spare oxidative capacity (proton leak: control-116.4 ± 11.16, TG-106.6 ± 10.89, TM-94.94 ± 10.18 pmol/min; spare oxidative capacity: control- 631.1 ± 39.11, TG-555.1 ± 54.85, TM-677 ± 54.01 pmol/min).

In addition, we tested an effect of acute ER stress on adipogenic capacity of preadipocytes. In order to limit ERS only to preadipocytes we employed reversibly acting TM in high-dose (1 μg/ml). Confluent preadipocytes were treated with TM for 4 h, then washed by PBS several times and subjected to standard 12 day adipogenic procedure using media free of ERS inducer. This treatment resulted in approximately 60% reduction of neutral lipid content compared to control conditions (Fig. 1D) without apparent effect on the viability of cells. Moreover, the effect of TM-pretreatment of preadipocytes on adipogenesis was detectable already after 3 days of differentiation when mRNA levels of aP2, PPARγ and perilipin were reduced compared to control conditions (Fig. 1E).

### 3.2. Chronic low ERS impairs adipogenesis and associated lipogenesis

Obesity leads to chronic low intensity rather than acute high intensity ERS [10,20]. Therefore, we aimed at imitating chronic ERS in adipose cells by the use of TG dose capable of activating UPR without acute induction of downstream effectors [21]. To determine such a dose, we exposed both preadipocytes and mature adipocytes to 1, 2.5, 5 and 100 nM TG for 1, 4 and 24 h and then analyzed expression of genes representing early and late markers of unfolded protein response (UPR). Neither dose of TG caused appearance of hypodiploid apoptotic preadipocytes within 24 h (not shown). Early marker of UPR activation, i.e. phosphorylated eIF2α (PERK arm of UPR), was induced already by 2.5 nM TG (Fig. 2A) within 1 h, while an induction of expression of downstream ERS effectors (ATF6 arm- HSPA5 [22], PERK arm- ATF4 [23], IRE1 arm-EDEM1, XBP1 splicing [24]) within 4 and 24 h required higher TG concentrations (5 or 100 nM TG) (Fig. 2B–D). Therefore, 2.5 nM TG was selected for chronic treatments of cells.

We investigated whether low intensity but chronic ERS reduces adipogenic conversion of preadipocytes similarly as acute high intensity ERS. Preadipocytes were differentiated in the absence or presence of 2.5 nM TG. Chronic treatment of cells with TG led to a mild increase of mRNA levels of HSPA5, ATF4 and EDEM1 during the whole time course of differentiation (Fig. 3A). Capacity to accumulate neutral lipids was lowered by more than 50% in TG-treated adipocytes as detected by Oil Red O staining (Fig. 3B, C). This was accompanied by diminished mRNA levels of differentiation markers (i.e. a key adipogenic factor, PPARγ, transcription factor SREBP-1c and late adipogenic markers, aP2 and perilipin) (Fig. 3D, E). mRNA expression of the lipogenic genes SCD1, DGAT2 and FASN was also lowered (Fig. 3F).

To determine a critical period of time for ERS to exert inhibitory effect on adipogenesis, preadipocytes were differentiated in the presence of 2.5 nM TG for various days (0–6, 1–12, 3–12, 6–12, 9–12). Capacity to store neutral lipids evaluated by ORO staining was strongly impaired in adipocytes exposed to TG between days 0–6 and 1–12, mildly between days 3–12 and not between days 6–12 and 9–12 of differentiation (Fig. 3G, H). Lipogenesis measured

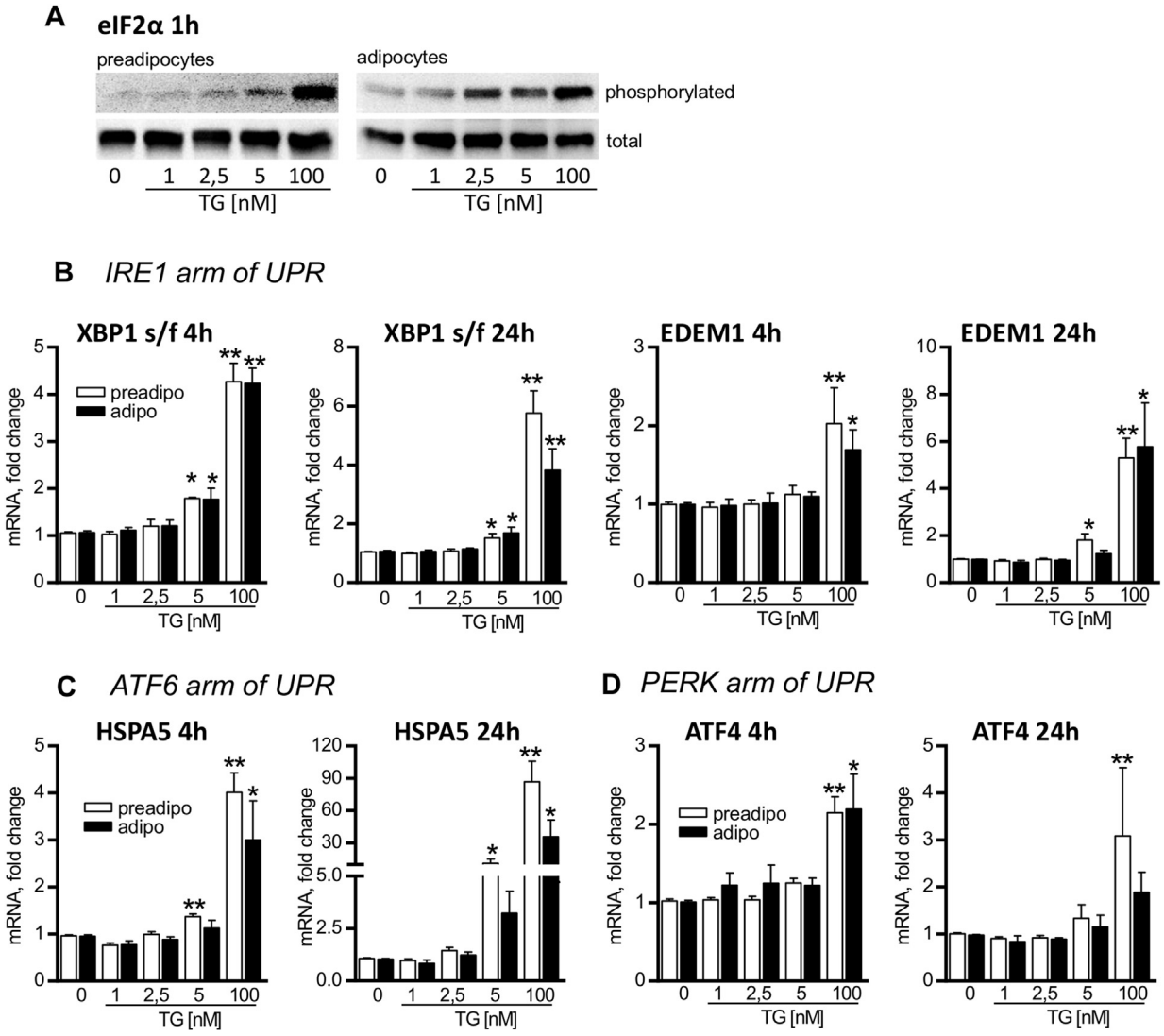


**Fig. 2.** Determination of TG dose appropriate for experiments with chronic low ERS. Cells were incubated with DMSO or 1, 2.5, 5, 100 nM TG for indicated time. **A**. Western blotting analysis of eIF2α activation (n = 3, the representative image is shown). **B**–**D**. mRNA expression of HSPA5, EDEM1 and ATF4 was measured by qRT-PCR and normalized to GUSB expression. mRNA expression of XBP1-spliced was normalized to XBP1-total expression (n = 4). Data are means ± SE, *p < 0.05, **p < 0.01.

as $^{14}$C-glucose carbon incorporation into lipids was also not altered when cells were exposed to 2.5 nM TG at day 6–12 of differentiation (not shown).

### 3.3. *Lipogenic capacity of mature adipocytes is not influenced by chronic low ERS*

Next, we analyzed the effect of chronic (6 days) low ERS on adipocytes differentiated for 12 days. Accumulation of neutral lipids ($^{14}$C-glucose carbon incorporation) was not affected by 2.5 nM TG (Fig. 4A), similarly as seen when TG was applied between day 6 and 12 of adipogenesis (not shown). Only expression of perilipin was decreased while other lipogenic genes remained unaffected by this treatment (Fig. 4B).

## 4. Discussion

Pathways activated by ERS represent primarily an adaptive homeostatic process that aims at protecting cellular metabolism disturbed by various insults. Extreme severity or chronicity of ERS is however linked with poor cellular survival and suboptimal metabolic performance [25]. This study brings evidence that severe ERS substantially reduces lipogenic capacity of adipocytes while chronic low ERS impairs lipogenesis through inhibition of adipogenic conversion of preadipocytes. As adipocytes are cells primarily dedicated to synthesis of lipids, the inhibition of lipogenesis represents a major disturbance of their metabolic function. Indeed, lipogenesis is repressed in obese adipose tissue [26], whose demands for the synthetic and secretory activity are enhanced and result in persistent deficiency of ER capacity. Notably, adipocytes used in this study were differentiated and exposed to ER stressors in fatty acid free medium, so the described effects of ERS on lipogenesis actually represents effects on lipogenesis de novo (DNL). In vivo significance of DNL in adipose tissue was considered negligible until recent discovery that in lean subjects, 20% of triglycerides were synthetized in adipose tissue de novo [1]. As we did not observe any effect of ERS on mitochondria OXPHOS capacity, the impact of ERS on lipogenesis in adipocytes appears to be direct and not exerted through suppression of mitochondrial function.

Lipogenesis has been previously found to be regulated by activity of UPR components in liver [13,27]. While UPR activation by

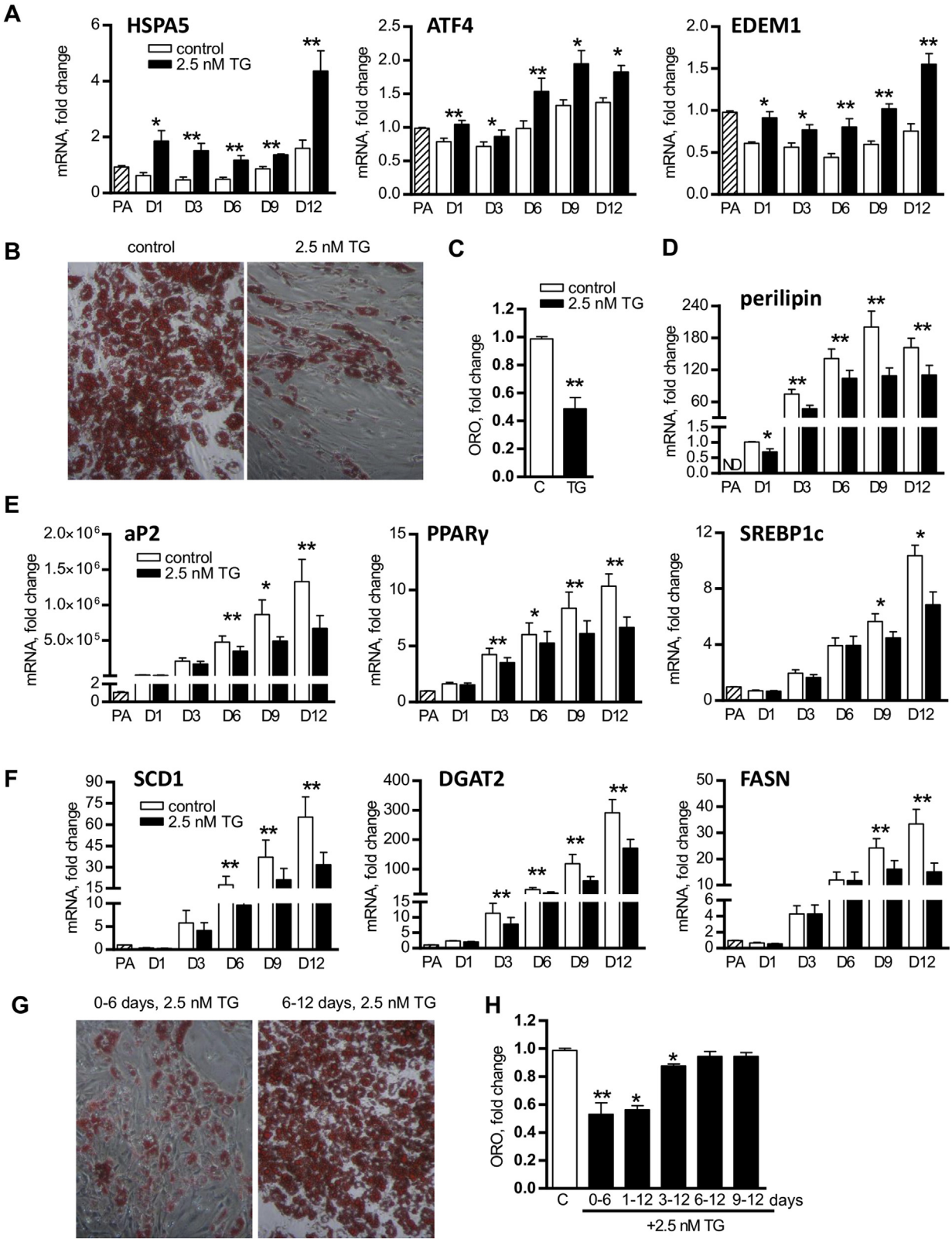


**Fig. 3.** Chronic low ERS impairs adipogenesis and associated lipogenesis. Preadipocytes (PA) were differentiated for 12 days in the absence or presence of 2.5 nM TG. Cells were harvested upon indicated days for mRNA analysis or at day 12 for ORO staining. mRNA expression of UPR effectors (**A**), adipogenic (**D, E**) and lipogenic markers (**F**) was measured by qRT-PCR and normalized to RPS13 expression (n = 4, ND-not detectable). **B**. Representative image of cells staining with ORO at day 12. **C**. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n = 5). **G**. Preadipocytes were differentiated and 2.5 nM TG was added to the differentiation media for indicated days. Representative image of cells staining with ORO at day 12. **H**. Preadipocytes were differentiated and 2.5 nM TG was added to the differentiation media for indicated days. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n = 5). Data are means ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$.

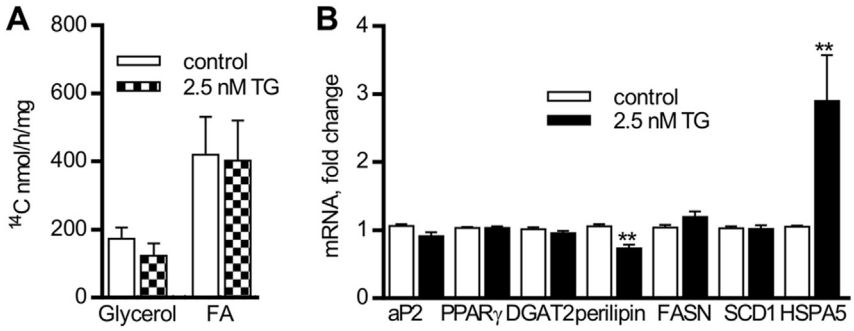


**Fig. 4.** Effect of low ERS stress on mature adipocytes. Mature adipocytes (12 days after onset of differentiation) were exposed for next 6 days to 2.5 nM TG. **A**. Glucose ($^{14}C$) incorporation into lipids (hydrolyzed into fatty acids FA and glycerol) during 3 h incubation of adipocytes treated as in Fig. 1B was determined by liquid scintillation and normalized to protein content (n = 3). **B**. mRNA expression of HSPA5, adipogenic and lipogenic markers was measured by qRT-PCR and normalized to RPS13 expression (n = 5). Data are means ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$.

glucose deprivation blocks lipogenesis in liver cells [28], under non-limiting glucose inflow in vivo ERS induced formation of lipid droplets leading to hepatic steatosis [29,30]. Thus, the negative effect of ERS on lipogenesis in the presence of glucose – as observed in this study – seems to be specific for adipose tissue. Despite this tissue specific effect of ERS, a pathophysiological outcome of unresolved ERS is the same in both adipose and liver cells, i.e. a deterioration of their primary metabolic specialization. Thus, eliciting ERS in both adipose tissue and liver at the same time may explain a vicious circle leading to profound disturbance of the whole body lipid metabolism in obese. Notably, opposing effects of ERS on lipogenesis in adipocytes versus liver cells fits well with fact that DNL seems to be regulated in AT and liver in an opposing manner. This disparity is probably based on differential activation of two key transcription factors SREPB-1c and ChREBP in both tissues, since expression of both of them has been shown to be lower in AT but higher in liver from obese compared to lean subjects [4,31]. Nevertheless, chronic ERS of low intensity that more closely imitates in vivo conditions was inefficient to diminish lipogenesis in mature adipocytes. This suggests that chronic ERS seen in obesity could have an impact rather on the newly recruited preadipocytes and thus could impair necessary renewal of adipose tissue. This hypothesis is supported by our observation that ERS activating all arms of UPR strongly inhibits adipogenic conversion of preadipocytes when present prior or in early stages of this process. Sensitivity of adipogenesis to low ERS was reported also by Kim et al. [32]. Early effect of ERS on adipogenesis suggests a regulation of a key adipogenic factor PPARγ or its upstream regulators. Indeed, cells pretreated with TM prior induction of adipogenesis were unable to enhance PPARγ expression as much as cells exposed to regular adipogenic medium for 3 days. Moreover, expression and transcriptional activity of PPARγ in 3T3-L1 cells was recently found to be inhibited by ATF3, a transcriptional inhibitor inducible by ERS [33]. Nevertheless, it remains unclear whether ATF3 plays a role in adipogenesis also under conditions of low chronic ERS. Interestingly, expression of ERS marker HSPA5 was increased 24 h after onset of differentiation by TG dose that does not have this effect in quiescent preadipocytes (compare Figs. 2C and 3A). These data suggest that hormonal stimulation to adipogenesis represents in fact additional ERS above the one induced by low doses of TG.

In conclusions, we found that acute ERS is a powerful inhibitor of lipogenesis in adipocytes, both at the level of mRNA expression and de novo triglyceride synthesis, while low intensity ERS blocked lipogenesis through an impairment of adipogenesis. These effects of ERS could therefore contribute to decreased lipogenic capacity of adipose tissue seen in obesity.

## Author contributions

M.K. performed experiments and data analysis and contributed to the writing of the manuscript, V.M, L.M, A.M. and J.K. performed experiments, V.S. performed adipose tissue biopsies and contributed to discussion and writing of the manuscript, D.L. contributed to discussion and writing of the manuscript. L.R. designed the study, performed experiments and data analysis and wrote the manuscript. L.R. is a guarantor of this work and, as such, 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.

## Conflicts of interest

The authors declare no conflict of interest

## Acknowledgments

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