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Sestrin2 suppression aggravates oxidative stress and apoptosis in endothelial cells subjected to pharmacologically induced endoplasmic reticulum stress

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ABSTRACT

Endoplasmic reticulum (ER) stress is an inflammatory response that contributes to endothelial cell dysfunction, a hallmark of cardiovascular diseases, in close interplay with oxidative stress. Recently, Sestrin2 (SESN2) emerged as a novel stress-inducible protein protecting cells from oxidative stress. We investigated here, for the first time, the impact of *SESN2* suppression on oxidative stress and cell survival in human endothelial cells subjected to pharmacologically (thapsigargin)-induced ER stress and studied the underlying cellular pathways. We found that *SESN2* silencing, though did not specifically induce ER stress, it aggravated the effects of thapsigargin-induced ER stress on oxidative stress and cell survival. This was associated with a dysregulation of Nrf-2, AMPK and mTORC1 signaling pathways. Furthermore, *SESN2* silencing aggravated, in an additive manner, apoptosis caused by thapsigargin. Importantly, *SESN2* silencing, unlike thapsigargin, caused a dramatic decrease in protein expression and phosphorylation of Akt, a critical pro-survival hub and component of the AMPK/Akt/mTORC1 axis. Our findings suggest that patients with conditions characterized by ER stress activation, such as diabetes, may be at higher risk for cardiovascular complications if their endogenous ability to stimulate and/or maintain expression levels of SESN2 is disturbed or impaired. Therefore, identifying novel or repurposing existing pharmacotherapies to enhance and/or maintain SESN2 expression levels would be beneficial in these conditions.

1. Introduction

Cardiovascular diseases driven by the epidemic prevalence of obesity and diabetes are placed at the pinnacle of the global mortality statistics, contributing to enormous social and financial burden. Reactive oxygen species contribute to both physiological functions and the pathogenesis of multiple cardiovascular disorders such as cardiomyopathies, atherosclerosis, stroke, and hypertension (Panth et al., 2016). Excessive reactive oxygen species release can imbalance the antioxidant defense system leading to a state of oxidative stress that can affect the normal function of various key tissues and cells. Oxidative stress was shown to be particularly deleterious to the functions of the endothelium, a thin monolayer composed of endothelial cells lining the whole vasculature (Maamoun et al., 2019b). The endothelium plays major roles in maintaining vascular homeostasis through the secretion of a variety of factors and substances, such as nitric oxide (NO) and endothelin 1, that regulate key processes including vasodilation and vasoconstriction, cell growth, angiogenesis, and fibrinolysis. Loss of proper functioning of endothelial cells is known as endothelial dysfunction which marks the initiating step towards vascular damage and atherosclerosis. It is characterized by

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reduced NO bioavailability, weakened vascular tone, impaired angiogenic capacity and other phenotypic changes in the endothelial cell lining (Abdelsalam et al., 2019; Incalza et al., 2018; Osman et al., 2020).

Conditions leading to excessive demand for protein production inside the endoplasmic reticulum (ER), such as diabetes and obesity, affect the folding capacity of chaperone molecules residing within the ER. This causes the buildup of unfolded and misfolded proteins inside the ER lumen, causing rapidly the activation of the unfolded protein response (UPR). The UPR aims at restoring ER homeostasis and bringing it back to a resting state; however, extended activation of UPR leads to a condition of ER stress which promotes selective expression of stress inducible proteins including pro-apoptotic signaling molecules, activating transcription factor (ATF)-4 and CCAAT/enhancer-binding protein (CHOP) (Masuda et al., 2013), and the activation of several pro-inflammatory signaling pathways such as c-Jun NH (2)-terminal kinase (JNK)/c-Jun response (Masuda et al., 2013; Panda et al., 2018).

ER stress is closely connected to oxidative stress and dysfunction of endothelial cells (Maamoun et al.,2019a, 2019b; Osman et al., 2020). Enhanced protein synthesis within the ER causes the formation of excessive non-native disulfide bonds, leading to exhaustion of glutathione (an antioxidant utilized to scavenge reactive oxygen species) which in turn increases oxidative stress (Maamoun et al., 2019a). ER stress also causes a loss in Ca^{2+} homeostasis which enhances reactive oxygen species production, followed by endothelial cell dysfunction (Carreras-Sureda et al., 2018; Gorlach et al., 2006). Conversely, an increase in protein expression and enzymatic activity of ER oxidoreductase (ERO)-1α leads to excess release of hydrogen peroxide (H_2O_2), followed by ER stress activation and dysfunction of endothelial cells (Wu et al., 2019). Sustained ER stress increases the risk of cardiovascular complications by inducing an imbalance in endothelial cell functions (Maamoun et al.,2019a, 2019b). Reversal of ER stress was reported to protect against endothelial cell dysfunction by increasing endothelial NO synthase (eNOS) activity and improving endothelium-dependent vascular relaxation (Galen et al., 2012, 2014; Kassan et al., 2012).

Several enzymatic and non-enzymatic antioxidants are known to scavenge free radicals and reduce oxidative stress. Among these antioxidant effectors, sestrins (SESN) have attracted much interest in recent years. The family of SESN consists of three highly conserved member proteins, SESN1, SESN2 and SESN3. SESN function as stress-inducible metabolic regulators, protecting cells from genotoxic and oxidative trauma arising from DNA damage, mutations, hypoxia, starvation, and ER stress (Lee et al., 2012). SESN inhibit reactive oxygen species both directly through their enzymatic activity (redox-regulating actions) and by regulating specific cellular metabolic networks (Pasha et al., 2017). SESN regulate and contribute to the homeostasis of metabolic functions through the upstream regulation of the mammalian target of rapamycin complex 1 (mTORC1) and 5′-AMP-activated protein kinase (AMPK) pathways, that are crucial for nutrient sensing and energy in cells (Budanov and Karin, 2008; Sun et al., 2020). Of note, under stress conditions, SESN2 upregulation was found to enhance the degradation of Kelch-like ECH-associated protein 1 (Keap-1) via autophagy. In normal conditions, nuclear erythroid-related factor-2 (Nrf-2), an important transcription factor which upregulates the expression of multiple antioxidant genes, remains in an inactive state through binding to Keap-1, and hence SESN2-facilated degradation of Keap-1 leaves Nrf-2 active (Shin et al., 2012).

Disruption of the SESN crosstalk networking or inactivation of SESN genes have major pathological implications including oxidative stress injury, dysfunction of mitochondria, lipid buildup, muscle decline, and increased risk for complications of diabetes (Lee et al., 2012; Masuda et al., 2013; Pasha et al., 2017). *SESN2* knockdown reduced AMPK phosphorylation and the expression of antioxidant genes such as catalase and superoxide dismutase (SOD)-2, enhanced reactive oxygen species release, and caused cell death in cardiomyocytes following treatment with lipopolysaccharide (LPS) both *in vitro* and in mice (Hwang et al., 2018). *SESN2* knockdown aggravated cardiomyocyte hypertrophy induced by phenylephrine. However, when SESN2 protein was overexpressed, cardiomyocytes became protected from hypertrophy mediated by phenylephrine and extracellular signal-regulated kinases (ERK)1/2 signaling was suppressed correspondingly (Dong et al., 2017). The expression of all SESN family members is found to be increased in mouse hearts during the early stages of cardiac hypertrophy, while their expression significantly decreases in the late stages of cardiac hypertrophy and in failing rodent hearts (Liao et al., 2016). SESN2 was found to be induced via JNK/c-Jun pathway and offered a compensatory survival response to angiotensin II injury in human umbilical vein endothelial cells (HUVEC) (Yi et al., 2014). Knockdown of *SESN2* exacerbated angiotensin II-induced oxidative stress and apoptosis (Yi et al., 2014). *SESN2* knockdown enhanced the expression of several ER stress markers in LPS-exposed HUVECs (Hwang et al., 2017). The expression of CHOP and protein kinase RNA-like ER kinase (PERK), and the phosphorylation of inositol-requiring enzyme (IRE)-1α, were significantly increased, suggesting a role for SESN2 in endothelial cell survival and ER stress response (Hwang et al., 2017). Nonetheless, the exact role of SESN2 in regulating reactive oxygen species generation, cell survival and the underlying cellular mechanisms in the context of ER stress activation in endothelial cells, is yet to be fully understood.

In the current study, we investigated, the impact of *SESN2* suppression on oxidative stress and cell survival in human endothelial cells subjected to pharmacologically (thapsigargin)-induced ER stress and studied the underlying cellular pathways with specific focus on oxidative stress and cell survival. We found that *SESN2* deletion aggravated ER stress-induced oxidative stress and cell death through a differential action on autophagy, Nrf-2, AMPK/Akt/mTOR and mitogen-activated protein kinase (MAPK) signaling responses.

2. Materials & methods

2.1. Cell maintenance and treatments

EA.hy926 endothelial cell line was acquired from the American Type Culture Collection (ATCC, Virginia, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing low glucose (1 g/L) and supplemented with fetal bovine serum (FBS, 10%), L-glutamine (2 mM), penicillin (10,000 units) and streptomycin (10 mg/L). All reagents were obtained from Gibco (ThermoScientific, Waltham, USA). Cells were incubated at 37 °C in a 95% humidified atmosphere saturated with 5% CO_2. Only cells up to passage 20 were used in the experiments.

For *SESN2* gene silencing experiments, *SENS2* siRNA and scrambled control duplexes were designed and manufactured by Integrated DNA Technologies (IDT, Coralville, Iowa, USA), and were used according to the instructions of the manufacturer. Cells were seeded into 6-well plates (50,000 cells/well). The second day, cells were either left untreated (control) or treated with scrambled or *SESN2* silencing duplexes. Briefly, lipofectamine RNAiMAX (ThermoScientific, Waltham, USA) and duplexes were dissolved separately into 250 μl of OptiMEM solution each (ThermoScientific), and then, carefully mixed and incubated at room temperature for 20 min. Then, mixture was added to the wells containing 2 ml of culture medium without FBS. The final concentration of duplexes was 10 nM. Cell culture medium was then changed after 24 h and cells incubated for a further 24 h (a total of 48 h post-transfection). Cells were then incubated in the presence or absence of thapsigargin (100 nM) (Invitrogen, Waltham, USA) for either 4 h or 18 h. Because scrambled control siRNA duplexes had no effect on SESN2 protein expression or cell viability (data not shown) in comparison to *SESN2* siRNA duplexes which reduced both SESN2 expression and cell viability, the rest of experiments were conducted using "untreated cells" as negative controls.

2.2. Western blot analysis

After treatments, cells were carefully washed in Phosphate Buffer Saline (PBS) to remove excess culture medium (ThermoScientific, Waltham, USA), and then total proteins were extracted using cold Radioimmunoprecipitation Assay (RIPA) lysis buffer [0.5 M Tris pH 6.8, 20% Sodium dodecyl sulfate (SDS)] supplemented with a cocktail of protease and phosphatase inhibitors (ThermoScientific). Concentrations of proteins were assessed using the bicinchoninic acid assay (ThermoScientific). Equal quantities of proteins (10–20 μg) were loaded and separated on SDS-PAGE gels (8, 10 or 12% as appropriate for target molecular weights) and transferred to polyvinylidene difluoride (PVDF) membranes (ThermoScientific). After blocking for 1 h in tris-buffered saline containing 0.1% of Tween 20 (Sigma-Aldrich, Hamburg, Germany) (T-TBS) supplemented with either 5% of Bovine Serum Albumin (BSA) (phosphorylated targets) or 5% dry milk (non-phosphorylated targets), the blot membranes were then washed in T-TBS and left to incubate with primary antibodies overnight (Pasha et al., 2019; Ravindran et al., 2019). Blots were probed with the following primary antibodies raised against: Binding immunoglobulin protein (BiP), CHOP, SESN2, p-AMPK-α (Thr172), p-mTOR (Ser2448), p-Akt (Ser473), Akt, p-p42/44 MAPK (Thr202/204), p42/44 MAPK, macroautophagy markers, light chain 3 (LC-3 I/II), p-Unc-51-like kinase (ULK)-1 (Ser555), ULK-1 (1:1000 dilution) (Cell Signaling Technology, Danvers, USA), or AMPK-α1/2, Keap-1, Nrf-2 (1:1000 dilution) (Abcam, Cambridge, UK) or mouse anti-β-actin (1:5000) (Santa Cruz Biotechnology, Dallas, USA). Blots were then washed thrice in T-TBS and incubated at room temperature for 1 h with appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000). When reprobing membranes with a different antibody was required, old primary and secondary antibodies were stripped off using restore western blot stripping buffer (ThermoScientific) following the recommendations of the manufacturer. Briefly, PVDF membranes were incubated for 15–30 min at 37 °C with stripping buffer. To visualize protein bands in a Biorad ChemiDoc MP imager (Biorad, Hercules, USA), enhanced chemiluminescence (ECL) reagent (Abcam, Cambridge, UK) was used. Quantification of protein band intensities was performed using image studio lite software (LI-COR Biosciences, Lincoln, USA).

2.3. Total RNA isolation and gene expression analysis

After treatments, cells were carefully washed in PBS to remove excess culture medium (ThermoScientific, Waltham, USA), before total RNA isolation and cDNA synthesis were conducted for gene expression studies by quantitative PCR. The innuPREP RNA kit (Analytikjena, Berlin, Germany) was used to extract total RNA by following the instructions of the manufacturer. The concentration and quality of RNA samples were assessed using a NanoDrop 2000 (ThermoScientific). Samples were then quickly transferred to −80 °C until further use. The synthesis of cDNA was performed from total RNA (500 ng) using the RevertAid reverse transcription kit (ThermoScientific) and an oligo $(dT)_{12\text{-}18}$ primer following the manufacturer's recommendations. Using GoTaq qPCR Master Mix (Promega, Madison, USA), target genes were amplified in an Applied Biosystems QuantStudio™ 5 Real-Time PCR System (ThermoScientific). Melting curves were determined for all samples to ensure specificity of amplicons. Reference gene stability was determined using BestKeeper algorithm (Pfaffl et al., 2004). All experiments were performed independently with five biological repeats and 3 technical replicates, with five different passage numbers of EA.hy926 cells. Analysis of data was performed using the ΔΔCt comparative method. Relative mRNA expression of target genes was normalized to housekeeping gene expression (*β-actin*) and presented as fold-change of negative (untreated) control group. Human primer pairs were obtained from Primer bank and manufactured by Integrated DNA Technologies (IDT, Coralville, Iowa, USA) or Sigma-Aldrich (Hamburg, Germany). Primer pair sequences that were used here are summarized in Table 1.

Table 1
List of human primer pairs used in the study.

Target	Forward	Reverse
ATF-4	5′-CCCTTCACCTTCTTACAACCTC-3′	5′-TGCCCAGCTCTAAACTAAAGGA-3′
β-actin	5′-CATGTACGTTGCTATCCAGG-3′	5′-CTCCTTAATGTCACGCACGAT-3′
Beclin-1	5′-TGAGGGATGGAAGGGTCTAAG-3′	5′-GCCTGGGCTGTGGTAAGTAATC-3′
BiP	5′-CATCACGCCGTCCTATGTCG-3′	5′-CGTCAAAGACCGTGTTCTCG-3′
CHOP	5′-GAACGGCTCAAGCAGGAAATC-3′	5′-TTCACCATTCGGTCAATCAGAG-3′
GRP94	5′-GCTGACGATGAAGTTGATGTGG-3′	5′-CATCCGTCCTTGATCCTTCTCTA-3′
HO-1	5′-AAGACTGCGTTCCTGCTCAAC-3′	5′-AAAGCCCTACAGCAACTGTCG-3′
Keap-1	5′-GGCTGTCCTCAATCGTCTCC-3′	5′-TCTGTTTCCACATCGTAGCG-3′
NOX-2	5′-ACCGGGTTTATGATATTCCACCT-3′	5′-GATTTCGACAGACTGGCAAGA-3′
NQO-1	5′-AGCCCAGATATTGTGGCC G-3′	5′-CCTTTCAGAATGGCTGGCAC-3′
p47^{phox}	5′-CAAGAGTACCGCGACAGACAT-3′	5′-AGGTCTTCTCGTAGTTGGCAAT-3′
Pdg	5′-GTTCCAAGACACGATGGCAAAC-3′	5′-CACCGAGCAAAGACAGCT TCTC-3′
SOD-1	5′-GGTGGGCCAAAGGATGAAGAG-3′	5′-CCACAAGCCAAACGACTTCC-3′
SOD-2	5′-GCTCCGGTTTTGGGGTATCTG-3′	5′-GCGTTGATGTGAGGTTCCAG-3′
SOD-3	5′-ATGCTGGCGCTACTGTGTTC-3′	5′-CTCCGCCGAGTCAGAGTTG-3′
TRIB3	5′-AAGCGGTTGGAGTTGGATGAC-3′	5′-CACGATCTGGAGCAGTAGGTG-3′

2.4. X-box binding protein 1 (XBP-1) splicing assay

The expression of *uXBP-1* (unspliced) and *sXBP-1* (spliced) transcripts of *XBP-1* was determined as previously described (Agouni et al., 2011). Splicing of *XBP-1* and expression of *β-actin* housekeeping gene were analyzed in cDNA from all experimental groups using innuMIX standard PCR masterMix (Analytikjena, Berlin, Germany). The PCR amplification was conducted in an Applied Biosystems ProFlex thermocycler (ThermoScientific, Waltham, USA) under the following cycling conditions: 94 °C (3 min); followed by 35 cycles (94 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s); and finally, 10 min at 72 °C. Primer pairs used for the amplification of spliced and unspliced *XBP-1* are as follow:

Forward: 5′-AAACAGAGTAGCAGCTCAGACTGC-3'.

Reverse: 5′-TCCTTCGGGTAGACCTCTGGGAG-3'.

To distinguish between *uXBP-1* and *sXBP-1*, the products of PCR amplification were separated on a carefully prepared agarose gel (2.5%). Image studio lite software (LI-COR Biosciences, Lincoln, USA) was used to for the semi-quantitative analysis of band intensities. Results were expressed as the ratio between *sXBP-1* over *uXBP-1* normalized to the expression of *β-actin* housekeeping gene and presented as percentage (%) of untreated control.

2.5. Cell death assessment by flow cytometry

Cell death was quantified by flow cytometry imaging using an annexin V-based apoptosis kit (BD Biosciences, San Jose, USA). After cell transfections and treatments, as described above, supernatants were collected, and cells were collected with trypsin (ThermoScientific, Waltham, USA) and suspended in 1X annexin V binding buffer (BD Biosciences) (one million cells/ml). After this, cell suspensions (100 μl containing 100,000 cells) were mixed with annexin V (5 μl) and

propidium iodide (PI) (2 μl) (BD Biosciences) and left to incubate for 30 min while protected from light at room temperature. Cells were then immediately analyzed by flow cytometry using a BD LSR Fortessa system (BD Biosciences). Live, early apoptotic, late apoptotic and total apoptotic cells were expressed as a percentage of total cells counted (Zachariah et al., 2020). The analysis allows differentiation of 4 cell populations based on the staining profile with PI and annexin V: Annexin V negative/PI negative cells are live; Annexin V positive/PI negative indicate that cells are in early apoptosis stage; Annexin V negative/PI positive cells staining indicate cells are necrotic; Annexin V positive/PI positive staining indicate that cells are in late apoptosis stage.

2.6. Reactive oxygen species production measurement

Reactive oxygen species production was quantified using MitoSOX™ Red reagent (ThermoScientific, Waltham, USA). MitoSOX™ Red live-cell permeable reagent that is quickly and specifically targeted to the mitochondria (Dikalov and Harrison, 2014). Inside the mitochondria, superoxide, but not other reactive oxygen species or reactive nitrogen species, oxidizes MitoSOX™ Red which then emits red fluorescence. Following treatments, cells were suspended at a density of 1 million cells/ml in DMEM supplemented with MitoSOX red (5 μM) and incubated for 20 min at 37 °C in dark. Then, cells were washed 3 times in warm PBS to remove excess MitoSOX™ Red. Cells were then immediately analyzed by flow cytometry using a BD LSR Fortessa system (BD Biosciences, San Jose, USA) for recording mean fluorescence intensity (MFI) and the proportion of positive cells. Results are represented as bar graphs representing MFI normalized to signal from control (untreated) cells expressed as fold change.

2.7. Statistical analysis

Results are expressed as mean ± standard error of mean (S.E.M.), and *n* represents the number of biological repeats. The normality of data was tested each time using Shapiro-Wilk normality test. Statistical analyses were performed with GraphPad Prism® 7.01e software for Mac using one-way ANOVA followed by Tukey's multiple comparison *post hoc* test (normally distributed data) or non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test (not normally distributed data). A two-tailed $P \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Pharmacologically induced ER stress enhanced SESN2 protein expression

As depicted in Fig. 1, exposure of cells to *SESN2* siRNA duplexes successfully reduced SESN2 protein expression compared to untreated controls (Fig. 1A and B). Incubation of cells with thapsigargin (100 nM) for both 4 h (Fig. 1A) and 18 h (Fig. 1B) significantly increased SESN2 protein expression, which was prevented in the presence of *SESN2* siRNA duplexes (Fig. 1A and B).

3.2. Impact of SESN2 suppression on pharmacologically induced ER stress in endothelial cells

SESN2 plays a crucial role in modulating stress conditions by activating various stress markers and metabolic pathways to achieve cellular homeostasis (Pasha et al., 2017). We investigated the impact of *SESN2* silencing in EA.hy926 endothelial cells upon their exposure to pharmacologically-induced ER stress by thapsigargin. Thapsigargin is a selective inhibitor of sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase, which blocks entry of calcium into SR/ER leading to ER stress and eventual cell death. As shown in Fig. 2, and as expected, exposure of EA.hy926 cells to thapsigargin (100 nM; 4 h) caused a significant increase in mRNA expression of all ER stress markers studied, namely, *ATF-4*, *BiP*, *GRP94*, *CHOP* and tribbles-related protein 3 (*TRIB3*), indicating a strong activation of ER stress response. The silencing of *SESN2* in endothelial cells caused a significant increase in mRNA expression of *CHOP* (Fig. 2D) and *TRIB3* (Fig. 2E), without

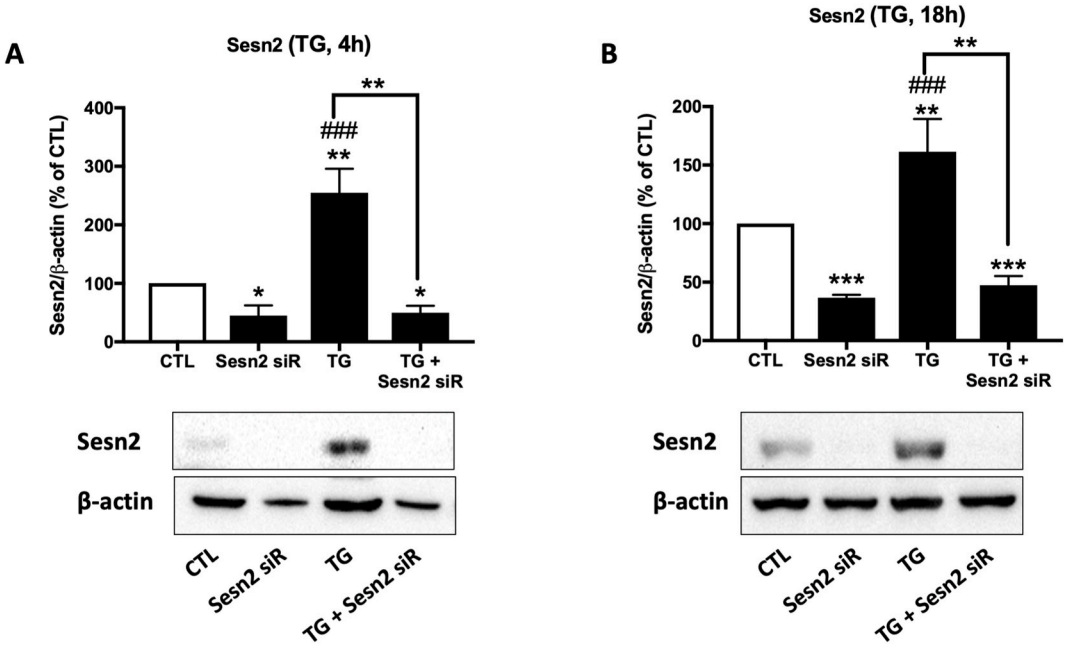

Fig. 1. Effects of *SESN2* silencing and thapsigargin (TG)-mediated ER stress on SESN2 protein expression. (A, B), Western bot analysis of SESN2 protein expression, normalized against β-actin used as a loading control protein. Cells were left untreated or incubated with *SESN2* siRNA duplexes for 48 h, and then exposed to thapsigargin (100 nM) for either 4 h (A) or 18 h (B). Data are presented as mean ± S.E.M. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{**}P < 0.01$ versus CTL or *versus* indicated groups; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus *SESN2* siR. Treatment groups include CTL, control; *SESN2* siR, *SESN2* silenced; TG, thapsigargin; *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

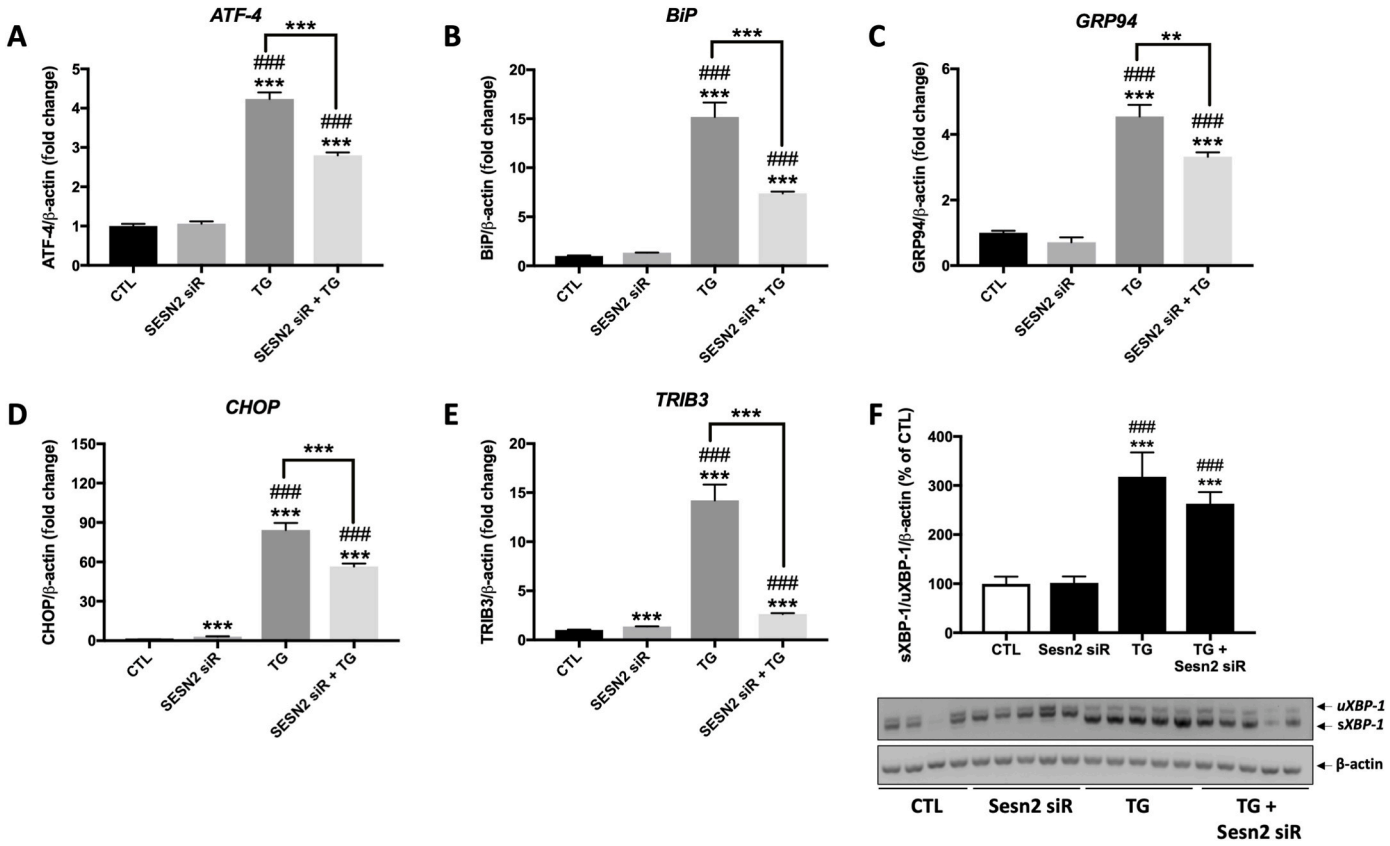

Fig. 2. Effects of *SESN2* suppression and thapsigargin (TG) on mRNA expression of various ER stress markers. Relative mRNA expression levels (fold change) of ER stress target markers, *ATF-4* (A), *BiP* (B), *GRP94* (C), *CHOP* (D), and *TRIB 3* (E), normalized against *β-actin* housekeeping gene (n = 5 in each group). (F) Images represent semi-quantitative PCR quantification of *sXBP-1, uXBP-1* and *β-actin* housekeeping gene resolved on 2.5% agarose gel. Bars represent pooled densitometry data normalized to *β-actin* and expressed as percentage (%) of untreated group (CTL) (n = 4–5 in each group). Cells were treated with *SESN2* siRNA for 48 h and then exposed or not to thapsigargin (100 nM) for 4 h. Data are presented as mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* CTL or *versus* indicated groups; $^{\#\#\#}P < 0.001$ *versus SESN2* siR. Treatment groups include CTL, control; *SESN2* siR, *SESN2* silenced; TG, thapsigargin; *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

affecting other markers studied. Interestingly, when cells were challenged with thapsigargin in the presence of *SESN2* siRNA duplexes, although an increase in mRNA expression of all the markers of ER stress studied was observed compared to untreated control condition, this increase was significantly smaller compared to thapsigargin alone (Fig. 2A–2E). The analysis of *XBP-1* splicing revealed that the ratio of *sXBP-1* over *u-XBP-1* was higher in EA.hy926 cells exposed to thapsigargin (100 nM; 4 h) compared to untreated cells and those silenced for *SESN2* (Fig. 2F).

As depicted in Fig. 3, the exposure of endothelial cells to thapsigargin for either 4 h or 18 h enhanced protein expression of ER stress molecules, BiP (Fig. 3A) and CHOP (Fig. 3B). Protein expression of BiP was significantly lower in cells silenced for *SESN2* (Fig. 3A, right panel). Cells challenged with thapsigargin for 18 h in the presence of *SESN2* siRNA also showed a decrease in BiP protein expression compared to controls and thapsigargin alone group (Fig. 3A, right panel). In the presence of *SESN2* siRNA duplexes, protein expression of CHOP in cells treated with thapsigargin for 4 h or 18 h was lower compared to cells only treated with thapsigargin; however, CHOP expression levels were still significantly higher compared to control condition and *SESN2* siRNA alone group (Fig. 3B).

3.3. Effect of SESN2 suppression on reactive oxygen species production in endothelial cells challenged with thapsigargin

SESN inhibit reactive oxygen species production and increase levels of antioxidants in cells under stress conditions (Pasha et al., 2017). The effect of *SESN2* silencing on thapsigargin-induced ER stress with special emphasis on reactive oxygen species production and mRNA expression of NADPH oxidase subunits and key antioxidant enzymes was thus studied here. As shown in Fig. 4, reactive oxygen species production detected by MitoSOX red dye, increased by 1.6-fold in thapsigargin group, and 1.9-fold in *SESN2* siRNA group. Interestingly, reactive oxygen species production increased by 5.5-folds compared to controls in the concomitant presence of thapsigargin and *SESN2* siRNA duplexes, indicating a drastic and more than additive increase in oxidative stress in endothelial cells deficient for *SESN2* (Fig. 4). Both *SESN2* deficiency and exposure of cells to thapsigargin increased, to the same extent, mRNA expression of *NOX-2* (Fig. 5A), the catalytic and membrane-bound subunit of NADPH oxidase, and $p47^{phox}$, a major regulatory subunit of the enzyme (Perisic et al., 2004) (Fig. 5B).

With regards to antioxidant enzymes, *SESN2* deficiency caused an increase in mRNA of *SOD-1* compared to control and thapsigargin groups (Fig. 5C). Deficiency in *SESN2* and exposure to thapsigargin caused a significant increase in mRNA expression of *SOD-2* (Fig. 5D) and *SOD-3* (Fig. 5E) compared to controls with mRNA expression of both *SODs* being higher in the presence of thapsigargin compared to *SESN2* siRNA alone group. Of note, the increase in mRNA expression of both *SOD-1* and *SOD-2* caused by thapsigargin was not affected by *SESN2* silencing (Fig. 5C and 5D). Finally, exposure of cells to thapsigargin did not affect mRNA expression of heme oxygenase-1 (*HO-1*), whereas *SESN2* silencing caused a significant increase in its expression levels (Fig. 5F).

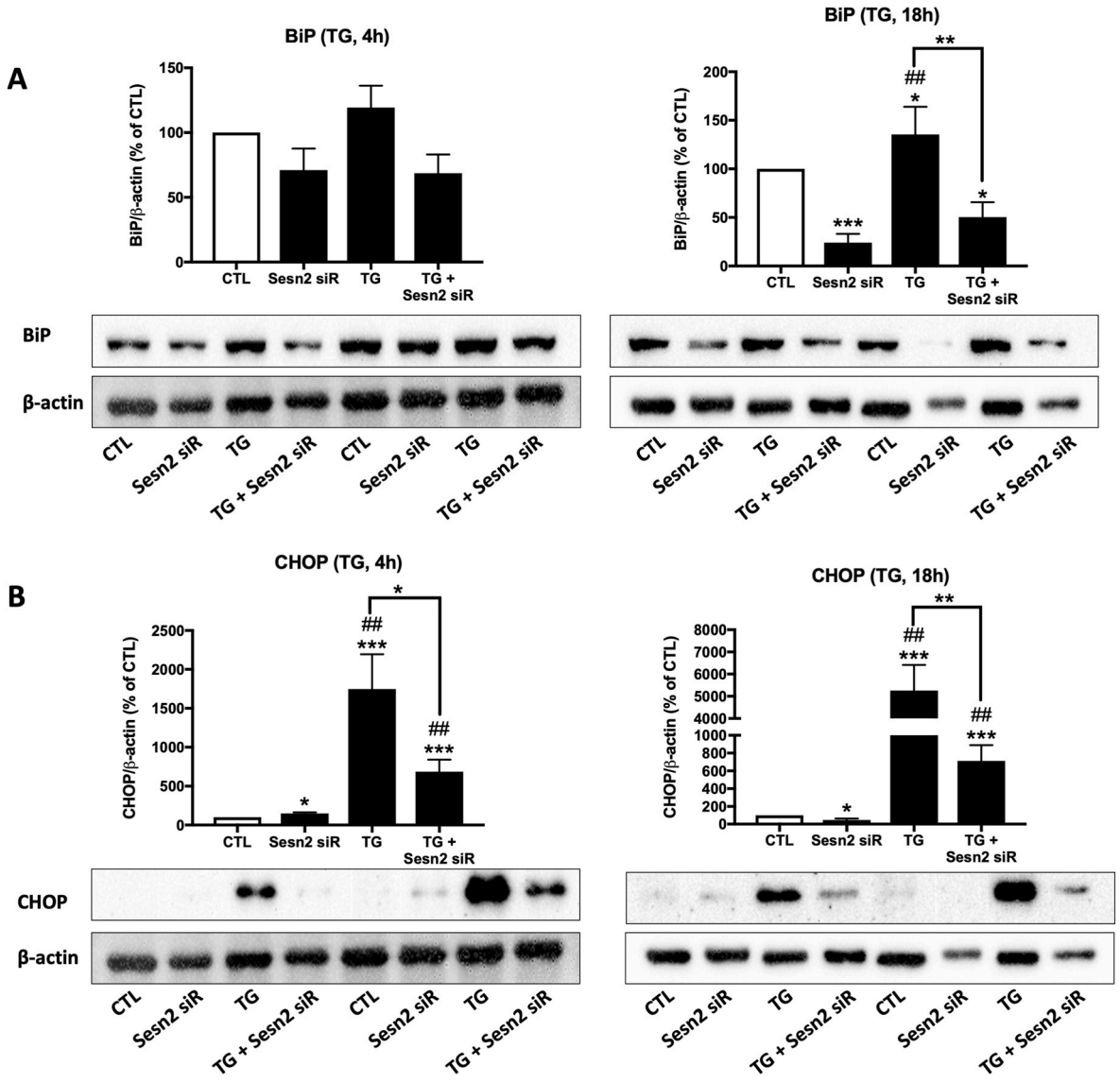

Fig. 3. Effects of *SESN2* suppression and thapsigargin (TG) on protein expression of ER stress markers. Western blot analysis of protein expression of BiP, normalized against β-actin, after treatment of cells with thapsigargin (TG) for 4 h (A, left panel) or 18 h (A, right panel) in the presence or absence of *SESN2* siRNA. (B), Western blot analysis of protein expression of CHOP, normalized against β-actin, after treatment of cells with thapsigargin (TG) for 4 h (B, left panel) or 18 h (B, right panel) in the presence of absence of *SESN2* siRNA. Cells were treated with *SESN2* siRNA for 48 h and then exposed to thapsigargin (100 nM), where specified. Representative images from 3 independent runs are presented. Bars represent pooled densitometry data normalized to control (CTL) (n = 3 per in each group). Data are presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* CTL or *versus* indicated groups; $^{\#\#}P < 0.01$ *versus SESN2* siR. Treatment groups include CTL, control; *SESN2* siR, *SESN2* silenced; TG, thapsigargin; *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

3.4. Effects of SESN2 suppression and thapsigargin-induced ER stress on the regulation of Nrf-2 signaling pathway

Nrf-2 is an important transcriptional factor that regulates the expression of various antioxidant genes. SESN2 mediates its antioxidant effects, at least partly, via the degradation of Keap-1 through p62-dependent autophagy and resulting activation of Nrf-2 (23). The effect of *SESN2* suppression and thapsigargin-induced ER stress on protein expression and mRNA profiling of markers of Nrf-2 pathway were thus investigated (Fig. 6). An increase in protein expression of Keap-1 was observed in cells deficient in *SESN2*, while a decrease was noted in cells exposed to thapsigargin both in the presence and absence of *SESN2* siRNA duplexes (Fig. 6A). Treatment of cells with thapsigargin on the other hand caused a marked increase in protein expression of Nrf-2, while the remaining groups showed no obvious effects compared to controls (Fig. 6B). Consistent with protein expression findings, at the mRNA expression level, an increase in the expression of *Keap-1* was also observed in the presence of *SESN2* siRNA compared to controls and thapsigargin alone group, whereas a decrease in *Keap*-1 mRNA expression was observed in cells exposed to thapsigargin alone (Fig. 6C). With regards to Nrf-2 downstream targets, thapsigargin caused an increase in mRNA expression of 6-phosphogluconate dehydrogenase (*pdg*) (Fig. 6D) and quinone oxidoreductase 1 (*NQO1*) (Fig. 6E); however, silencing of *SESN2* only prevented the increase in *pdg* mRNA expression caused by thapsigargin (Fig. 6D).

Altogether, *SENS2* silencing increased reactive oxygen species production (Fig. 4) which was associated with an overexpression of mRNA of antioxidant genes (*SOD-1*, *SOD-2*, *SOD-3*, *HO-1*) (Fig. 5), while the mRNA expression of other antioxidant genes (*pdg*) under the transcriptional action of Nrf-2 were not affected (Fig. 6). This highlights the importance of Nrf-2 pathway in the dysregulation of oxidative balance caused by *SESN2* suppression in endothelial cells.

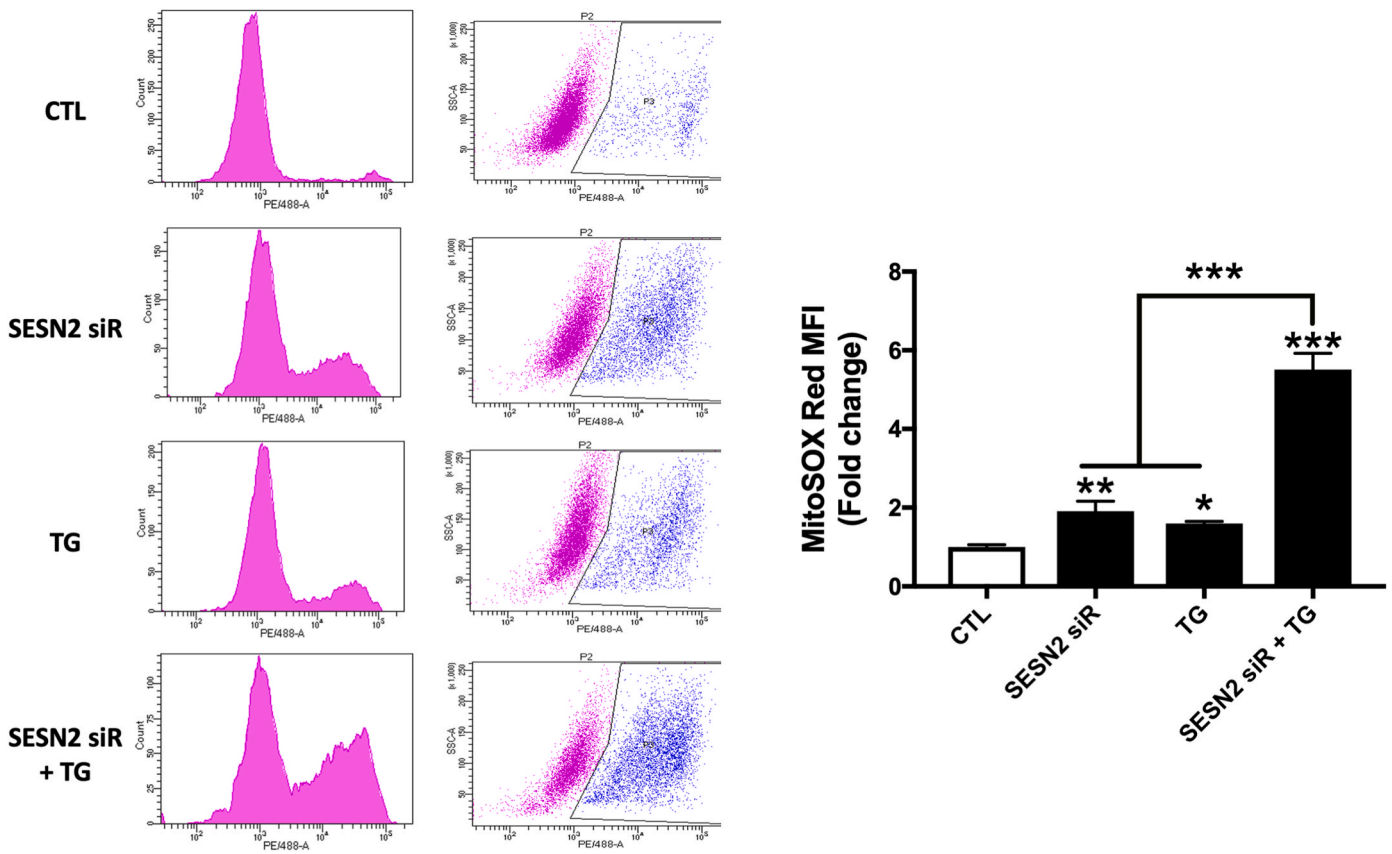

Fig. 4. Effects of *SESN2* suppression and thapsigargin (TG) on reactive oxygen species production. Measurement of reactive oxygen species production in EA.hy926 endothelial cells. Following transfection with *SESN2* siRNA duplexes (48 h), cells were treated overnight with thapsigargin (TG; 100 nM), and reactive oxygen species production was then measured by flow cytometry using mitoSOX red dye. Representative flow cytometry images of fluorescence intensity (PE/488-A) are shown for each experimental group. Bars represent mean fluorescence intensity (MFI) normalized to control (CTL) expressed as fold change (n = 3 per group). Data are presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* CTL or *versus* indicated groups. Treatment groups include CTL, control; *SESN2* siR, *SESN2* silenced; TG, thapsigargin; *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

3.5. SESN2 silencing caused apoptosis in endothelial cells and aggravated pharmacological ER stress-mediated cell death

The assessment of apoptosis by flow cytometry under conditions of thapsigargin-induced ER stress and *SESN2* deficiency in endothelial cells indicated a clear role for SESN2 in endothelial cell survival in general and particularly under ER stress conditions (Fig. 7). As shown in Fig. 7, exposure of cells to thapsigargin (100 nM; 18 h) reduced the percentage of live cells, while increasing the percentage of early and late apoptotic cells; the percentage of total apoptotic cells increased from 5.6 ± 0.35% in controls to 32.3 ± 1.42% in thapsigargin-treated group. *SESN2* silencing alone also reduced the percentage of live cells and increased the proportion of early and late apoptotic cells; the percentage of total apoptotic cells in this group was 27.7 ± 0.65%. Interestingly, the exposure of cells to thapsigargin in the presence of *SESN2* siRNA duplexes caused a dramatic reduction in the percentage of live cells and a staggering increase in the proportion of both early and late apoptotic cells, with total apoptotic cells in this group representing 60.6 ± 1.82% of total cell population, indicating an additive pharmacological effect between *SENS2* silencing and thapsigargin-induced ER stress on cell survival in endothelial cells.

3.6. Effects of SESN2 suppression and thapsigargin on the regulation of AMPK/Akt/mTORC1 axis and autophagy

To better understand the signaling responses implicated in the effects of *SESN2* silencing on oxidative stress and cell survival in the presence or absence of ER stress injury, the regulation of AMPK/Akt/mTORC1 axis that is involved in cell metabolism and survival was assessed. SESN2 works in extreme co-ordination with AMPK and is essential in the regulation of cell growth, survival, and energy homeostasis (Pasha et al., 2017). The effect of *SESN2* silencing on the activation of AMPK was thus studied. *SESN2* silencing markedly decreased the phosphorylation of AMPK at Thr172 (Fig. 8A). Exposure of cells to thapsigargin enhanced the phosphorylation of AMPK at Thr172, while *SESN2* silencing completely prevented this increase (Fig. 8A).

SESNs regulate cellular metabolic networks in close connection with mTORC1 signaling pathway. Excessive increase in the mTORC1 activity increases the risk of ER stress and consequently cellular damage, wherein SESN2 may come as a rescue (Sun et al., 2020). We investigated the activation of mTORC1 signaling pathway under conditions of thapsigargin-induced ER stress in the presence or absence of *SESN2* siRNA duplexes. Both *SESN2* deficiency and exposure to thapsigargin caused an increase in the phosphorylation of mTOR (Ser2448) in endothelial cells (Fig. 8B). The activation of mTORC1 mediated by *SESN2* deficiency and thapsigargin-induced ER stress was associated with a significant increase in the ratio between protein expression levels of LC3-II to LC3-I (indicating conversion of LC3-I into LC3-II), a key marker of macroautophagy (Fig. 8C). However, the expression of more specific macroautophagy markers showed a differential effect on autophagy activation. The phosphorylation of Unc-51-like kinase (ULK)-1 (Fig. 8D) and mRNA expression of Beclin-1 (Fig. 8E) were decreased in the presence of *SESN2* siRNA duplexes. The phosphorylation of ULK-1 was significantly enhanced in the presence of thapsigargin, an effect that was prevented by *SESN2* silencing (Fig. 8D).

To further harness the impact of *SESN2* silencing and thapsigargin-

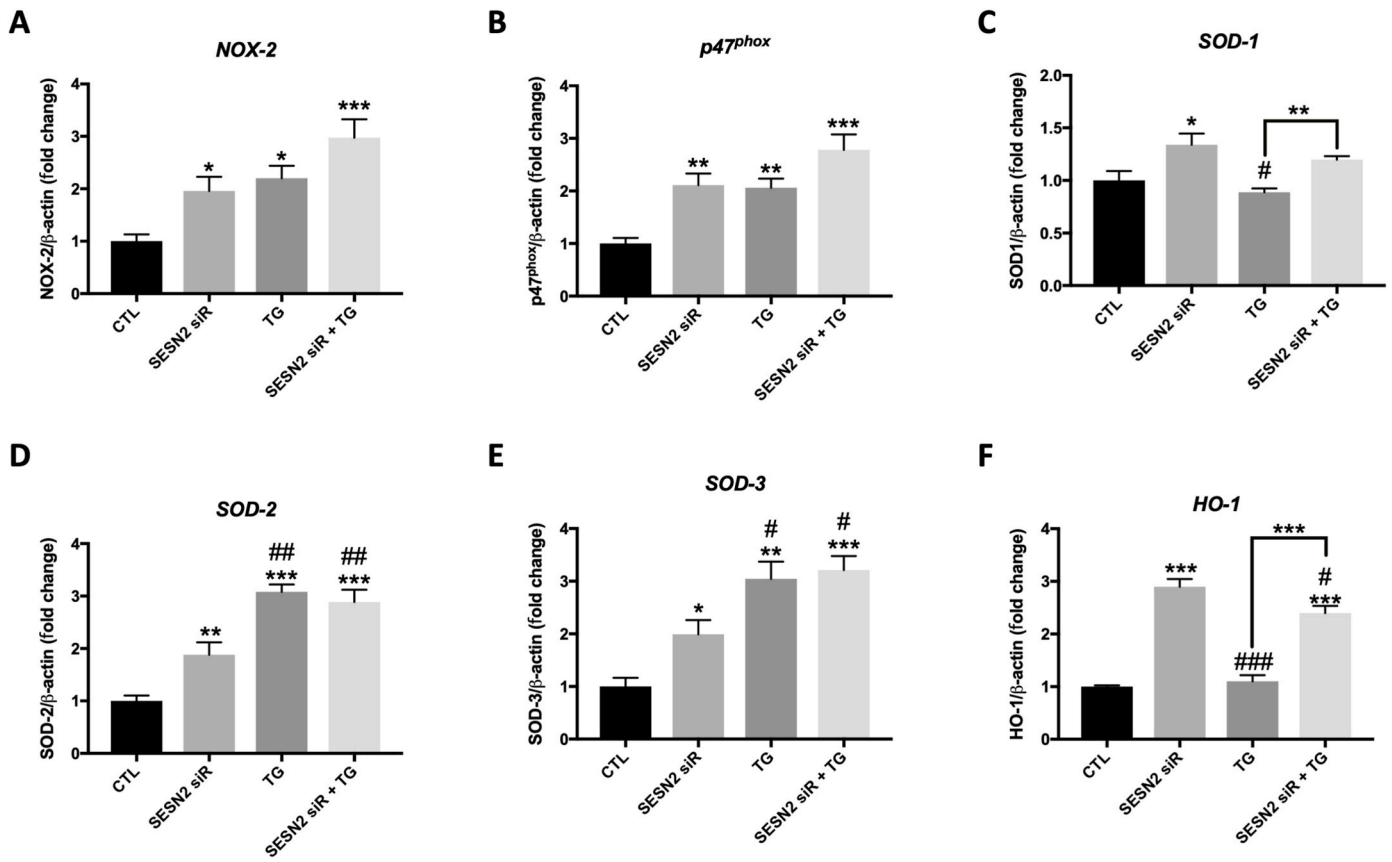

Fig. 5. Effects of *SESN2* suppression and thapsigargin (TG) on mRNA expression of prooxidant and antioxidant genes. Relative mRNA expression of *NOX-2* (A), *p47*^{phox} (B), *SOD-1* (C), *SOD-2* (D), *SOD-3* (E), and *HO-1* (F), normalized against β-actin housekeeping gene (n = 5) in cells treated with TG (100 nM) for 4 h in the presence or absence of *SESN2* siRNA. Results are expressed as mean ± S.E.M. as fold change relative to control (CTL). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* CTL or *versus* indicated groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus SESN2* siR + TG group. Treatment groups include CTL, control; *SESN2* siR, *SESN2* silenced; TG, thapsigargin; *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

induced ER stress on the regulation of mTORC1 and endothelial cell survival, the activation of Akt was also assessed. As shown in Fig. 8F, the silencing of *SESN2* caused a dramatic and significant decrease in protein expression of Akt which also drove a strong decrease in the phosphorylation of Akt at Ser473, indicating a reduced activation. Exposure of cells to thapsigargin did not affect neither the expression nor the phosphorylation of Akt compared to control (Fig. 8F). This decrease in the expression of Akt caused by *SESN2* suppression may contribute to the reduced survival of endothelial cells silenced for *SESN2*. Additionally, we assessed the impact of *SESN2* and thapsigargin on the proliferative signal p42/p44 MAPK. As shown in Fig. 8G, both *SESN2* silencing and thapsigargin enhanced the phosphorylation of p42/p44 MAPK.

4. Discussion

In the present study, we investigated, the impact of *SESN2* suppression on oxidative stress and cell survival in endothelial cells exposed to pharmacologically (thapsigargin)-induced ER stress and studied the underlying cellular pathways. We report here that *SESN2* deletion, though did not specifically induce a strong ER stress response, it aggravated thapsigargin-induced ER stress effects on reactive oxygen species production that was more than additive in the presence of thapsigargin and *SESN2* silencing using specific siRNA duplexes. This effect was associated with a dysregulation of the antioxidant Nrf-2 pathway and an upregulation of mRNA expression of NADPH oxidase, *NOX-2* and *p47*^{phox}, subunits. Furthermore, *SESN2* deficiency aggravated, in an additive manner, apoptosis caused by thapsigargin-induced ER stress, suggesting the activation of additional anti-survival cellular signals. Enhanced apoptosis caused by *SESN2* silencing was associated with AMPK inactivation, activation of mTORC1 and enhanced activity of p42/44 MAPK. Importantly, we observed that *SESN2* deficiency, unlike thapsigargin, caused a dramatic decrease in protein expression and phosphorylation of Akt, a key pro-survival signaling pathway, which may contribute to the additional effects of *SESN2* silencing on reduced cell viability in the presence of ER stress inducer, thapsigargin.

We observed here that the induction of ER stress caused the overexpression of SESN2. This is in agreement with several studies that have reported that ER stress upregulated SESN2 in various cell types (Bruning et al., 2013; Ding et al., 2016; Park et al., 2014; Saveljeva et al., 2016); however, our study is the first to report the upregulation of SENS2 in endothelial cells to be directly mediated by pharmacological (thapsigargin) ER stress induction. As observed in our current study, ER stress activation is associated with selective expression of ATF-4, which has been shown to directly control the mRNA expression of *SESN2* (De Sousa-Coelho et al., 2012; Gomez-Samano et al., 2017).

Our data indicate that *SESN2* deficiency in endothelial cells did not dramatically affect mRNA and protein expression of key ER stress markers; however, prolonged exposure of cells to *SESN2* siRNA duplexes (72 h–48 h of transfection and 18 h in the presence of thapsigargin) led to a decrease in protein expression of BiP and CHOP compared to controls. Our findings are consistent with a previous study by Hwang et al. (2017) who assessed protein expression of CHOP, and phosphorylation of PERK and IRE-1α, as markers of ER stress. Authors reported that *SESN2* deficiency in HUVECs did not affect the expression of these ER stress markers (Hwang et al., 2017). However, in our study, we observed that *SESN2* silencing caused a reduction in mRNA (*ATF-4*, *BiP*, *GRP94*,

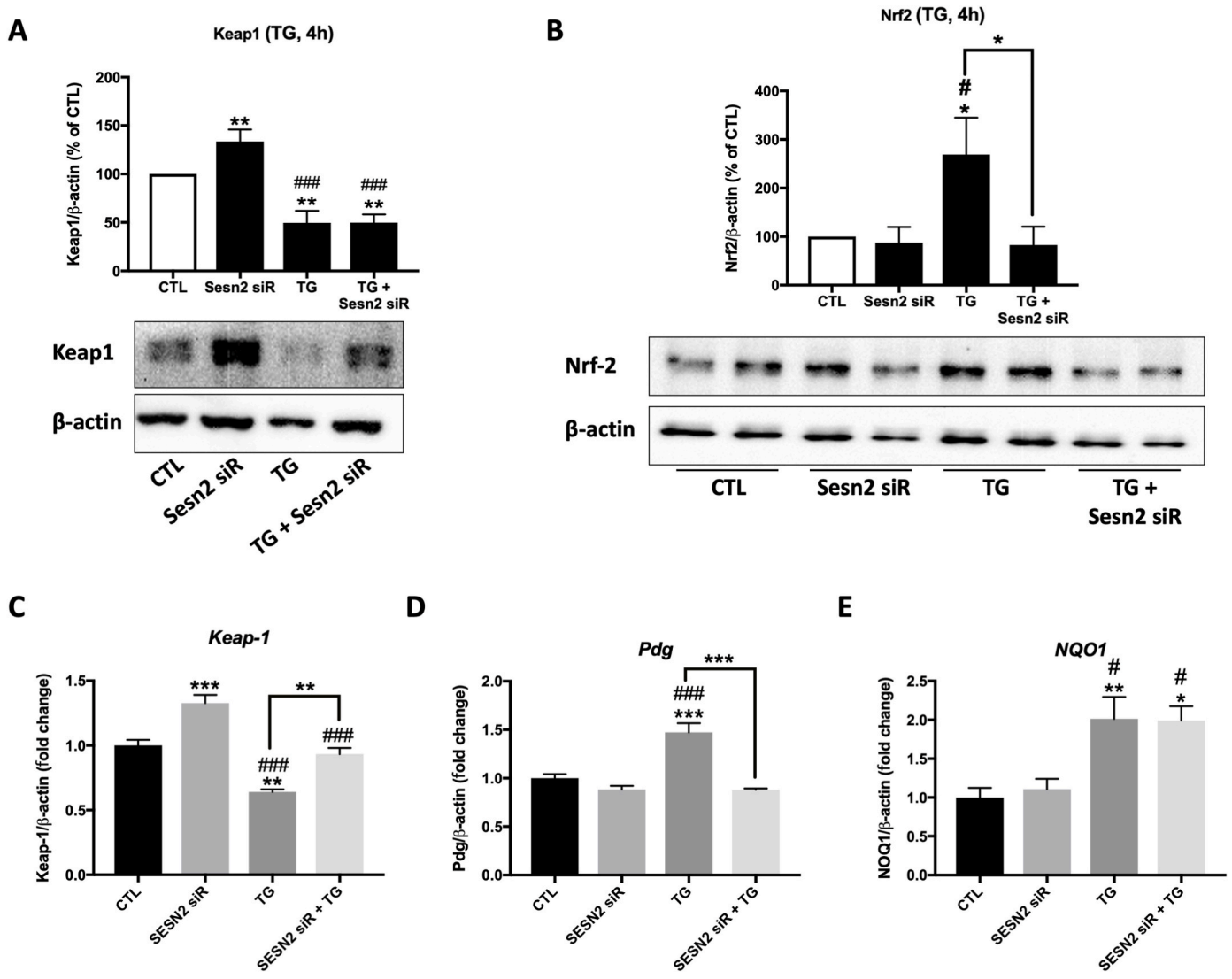

Fig. 6. Effects of *SESN2* suppression and thapsigargin (TG) on the activation of Nrf-2 pathway. (A, B), western blot analysis showing protein expression levels of Keap-1 (A) and Nrf-2 (B) normalized against β-actin loading control. Bars represent pooled densitometry data normalized to total amount of β-actin expressed as percentage of control (% of CTL) (n = 3 in each group). Data are presented as mean ± S.E.M. (C–E), Relative mRNA expression, expressed as fold change relative to control (CTL), of *Keap-1* (C), *Pdg* (D), and *NQO1* (E) normalized against β-actin housekeeping gene (n = 5 per group). Data are presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* CTL or indicated groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *versus SESN2* siR group. Treatment groups include CTL, control; *SESN2* siR, *SESN2* silenced; TG, thapsigargin; *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

CHOP, *TRIB3*) and protein expression (BiP and CHOP) of all ER stress markers studied in cells treated with thapsigargin compared to thapsigargin alone although levels were still significantly higher compared to control (untreated condition). This is not consistent with the observations of Hwang et al. (2018) who found that *SESN2* silencing enhanced ER stress response caused by LPS (Hwang et al., 2018). Discrepancy may be explained by the mechanism of action for ER stress induction, depletion of Ca^{+2} stores for thapsigargin in our study and via interaction with Toll-like receptors (TLR) for LPS in the other report.

We observed here that exposure of cells to thapsigargin stimulated reactive oxygen species release. Furthermore, *SESN2* silencing alone elevated reactive oxygen species release in endothelial cells; however, when cells were challenged with thapsigargin in the presence of *SESN2* siRNA duplexes, reactive oxygen species production was significantly higher in an additive fashion. Our findings are in line with previous reports indicating that *SESN2* deficiency leads to oxidative stress in endothelial cells (Hwang et al., 2018) and renal mesangial cells (Eid et al., 2013). However, our study further demonstrates that *SESN2* deficiency aggravates, in an additive fashion, the effects of pharmacologically induced ER stress on oxidative stress in endothelial cells, indicating overlapping molecular mechanisms between *SESN2* deficiency and thapsigargin in inducing oxidative stress. ER stress is intimately associated to both oxidative stress and endothelial cell dysfunction (Maamoun et al., 2019b). Higher demand for protein synthesis within the ER lumen causes excess formation of non-native disulfide bonds, which eventually exhausts intracellular stores of glutathione and subsequent oxidative stress (Maamoun et al., 2019a). ER stress disrupts Ca^{2+} homeostasis enhancing thus reactive oxygen species release (Carreras-Sureda et al., 2018; Gorlach et al., 2006). Conversely, during ER stress response, the activity of ERO-1α is also increased leading to excess H_2O_2 (Wu et al., 2019). The antioxidant actions of SESN2, though not fully understood, can be mediated by inhibiting reactive oxygen species both directly through their enzymatic activity (redox-regulating actions) and by regulating specific cellular metabolic networks (Pasha et al., 2017). SESN regulate and contribute to the homeostasis of metabolic functions through upstream regulation of mTORC1 and AMPK pathways, that are crucial for nutrient sensing and energy in cells (Budanov and Karin, 2008; Sun et al., 2020). SESN2

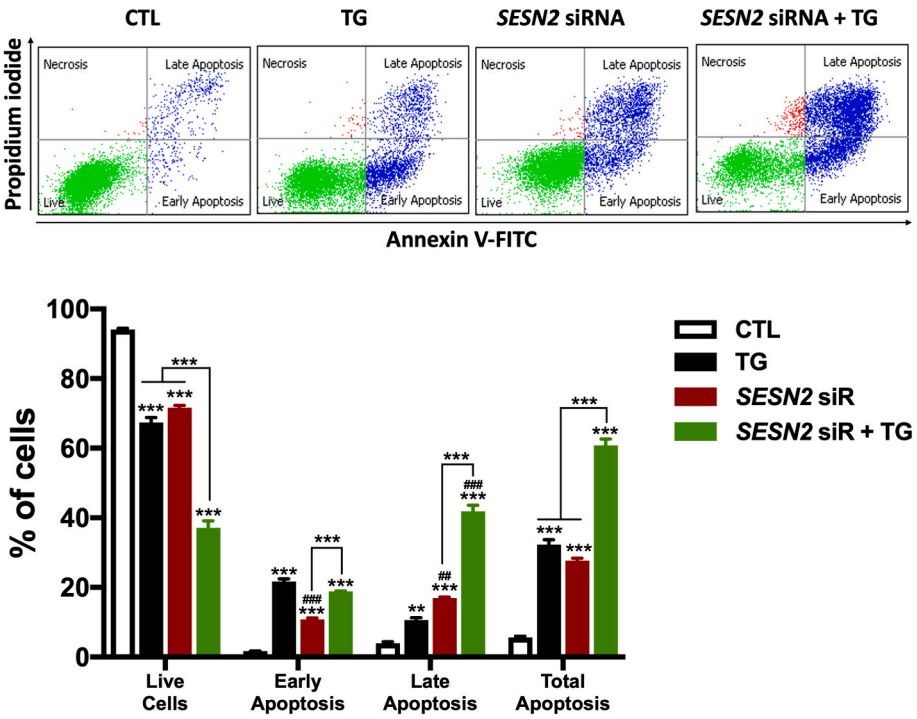

Fig. 7. Effects of *SESN2* suppression and thapsigargin (TG) on endothelial cell death. Assessment of apoptosis by flow cytometry using Annexin V and PI staining (n = 3 per group) in EA.hy926 cells left untreated (CTL) or incubated with TG (100 nM; overnight) in the presence or absence of *SESN2* siRNA duplexes (after 48 h of transfection). Bars represent pooled data expressed as percentage of live cells, early apoptotic cells, late apoptotic cells, and total apoptotic cells in each group. Representative flow cytometry images of fluorescence intensity are shown for each experimental group. Data are presented as mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* CTL or indicated groups; $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *versus* TG group. The groups include untreated cells or control (CTL); *SESN2* siR, *SESN2* silenced cells; TG, thapsigargin-treated cells (100 nM; overnight); *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

upregulation enhances the degradation of Keap-1 via autophagy under stress states, which directs Nrf-2 for degradation (Shin et al., 2012). We report here that increased reactive oxygen species production caused by both *SESN2* deficiency or thapsigargin in endothelial cells was associated with an increase in mRNA expression of *NOX-2* and its regulatory subunit $p47^{phox}$ in addition to *SOD-2* and *SOD-3*; however, *SOD-1* and *HO-1* were only increased in cells deficient for *SESN2*. This differential expression in antioxidant enzymes between cells treated with thapsigargin in the presence or absence of *SESN2* siRNA, indicate overlapping pro-oxidant cellular mechanisms, further explaining the additive effects on reactive oxygen species production observed with concomitant *SESN2* silencing and thapsigargin-induced ER stress. Our data corroborate our previous observations that thapsigargin-induced ER stress is associated with elevated reactive oxygen species production in endothelial cells and enhanced activity of NADPH oxidase (phosphorylated $p47^{phox}$) (Maamoun et al., 2017). In the current study, we also uncover a differential action between thapsigargin-induced ER stress and *SESN2* deficiency in regulating Nrf-2 pathway. While thapsigargin reduced the expression Keap-1 and enhanced Nrf-2 expression and its downstream targets (*NQO-1* and *Pdg*), the silencing of *SESN*2 in endothelial cells caused a decrease in the expression of Keap-1, indicating a lower activation of Nrf-2 in *SESN2*-silenced cells. Despite its documented role in the antioxidant actions of SESN2, our study is the first to show that Nrf-2 activation is reduced in endothelial cells deficient for *SESN2*. In accordance with previous studies, including in endothelial cells (Hwang et al., 2017, 2018), we showed here that *SESN2* deletion caused a reduction in the phosphorylation of AMPK; however, we also extend our observation to an increase in the activation of mTORC1 pathway and subsequent inhibition of autophagy, both of which have been linked to the antioxidant actions of SESN2 (Pasha et al., 2017). Interestingly, thapsigargin-induced ER stress was associated with enhanced phosphorylation of AMPK, prevented by *SESN2* deletion, and activation of mTORC1 and autophagy, further highlighting the discrepancy in mechanisms mediated by *SESN2* deficiency and thapsigargin, contributing to the additive pharmacological effects on oxidative stress and apoptosis.

In agreement with a previous report (Hwang et al., 2018), we found here that *SESN2* deletion increased the percentage of apoptotic endothelial cells. Furthermore, and in line with our previous report (Maamoun et al., 2017), we also found that thapsigargin-induced ER stress caused an increase in apoptosis. Of particular interest, we show here that *SESN2* silencing caused an additive apoptotic effect in endothelial cells exposed to thapsigargin, suggesting the activation of non-overlapping apoptotic mechanisms in the presence of both conditions, which may further contribute to the additive effect on oxidative stress observed in these cells. Several mechanisms may contribute to this situation. First, the inactivation of AMPK was shown to contribute to cell death mediated by *SESN2* deficiency (Pasha et al., 2017). Furthermore, the activation of mTORC1 and subsequent inhibition of autophagy in cells deficient for *SESN2* may also contribute to enhanced apoptotic signals. The inhibition of mTORC1 has been shown to prevent low shear stress-induced apoptosis and oxidative stress in endothelial cells by preventing downregulation of SESN1 (Zhang et al., 2014). One focal observation in our current study is the downregulation of Akt protein expression, and subsequent reduction in its phosphorylation and hence activation, in endothelial cells deficient for *SESN2* compared to controls and cells treated only with thapsigargin. This is a novel finding which may explain the additive apoptotic effect of *SESN2* deletion in thapsigargin-treated cells because of the well-documented endothelial pro-survival role of Akt (Gerber et al., 1998; Shiojima and Walsh, 2002). Akt is a major signaling hub for the regulation of metabolism and cell survival and its activation is intimately associated with the regulation of AMPK and mTORC1 pathways. In addition, Akt is a critical regulator of endothelial cell function and mediates the activation of eNOS and increases NO production in endothelial cells (Dimmeler et al., 1999). Moreover, the overexpression of *TRIB3* associated with *SESN2* deficiency and thapsigargin treatment may further contribute to the inactivation of Akt (Du et al., 2003). An increase in the activation of p42/44 MAPK pathway was observed in cells exposed to both thapsigargin and *SESN2* siRNA. As a proliferative pathway, this may be a compensatory mechanism to overcome the loss of activity of Akt and strong activation of apoptotic signals.

Taken together, these data suggest that although *SESN2* silencing *per se* did not cause a strong increase in ER stress response and did not potentiate the expression of key ER stress markers caused by thapsigargin, it did aggravate the consequences of ER stress response induction

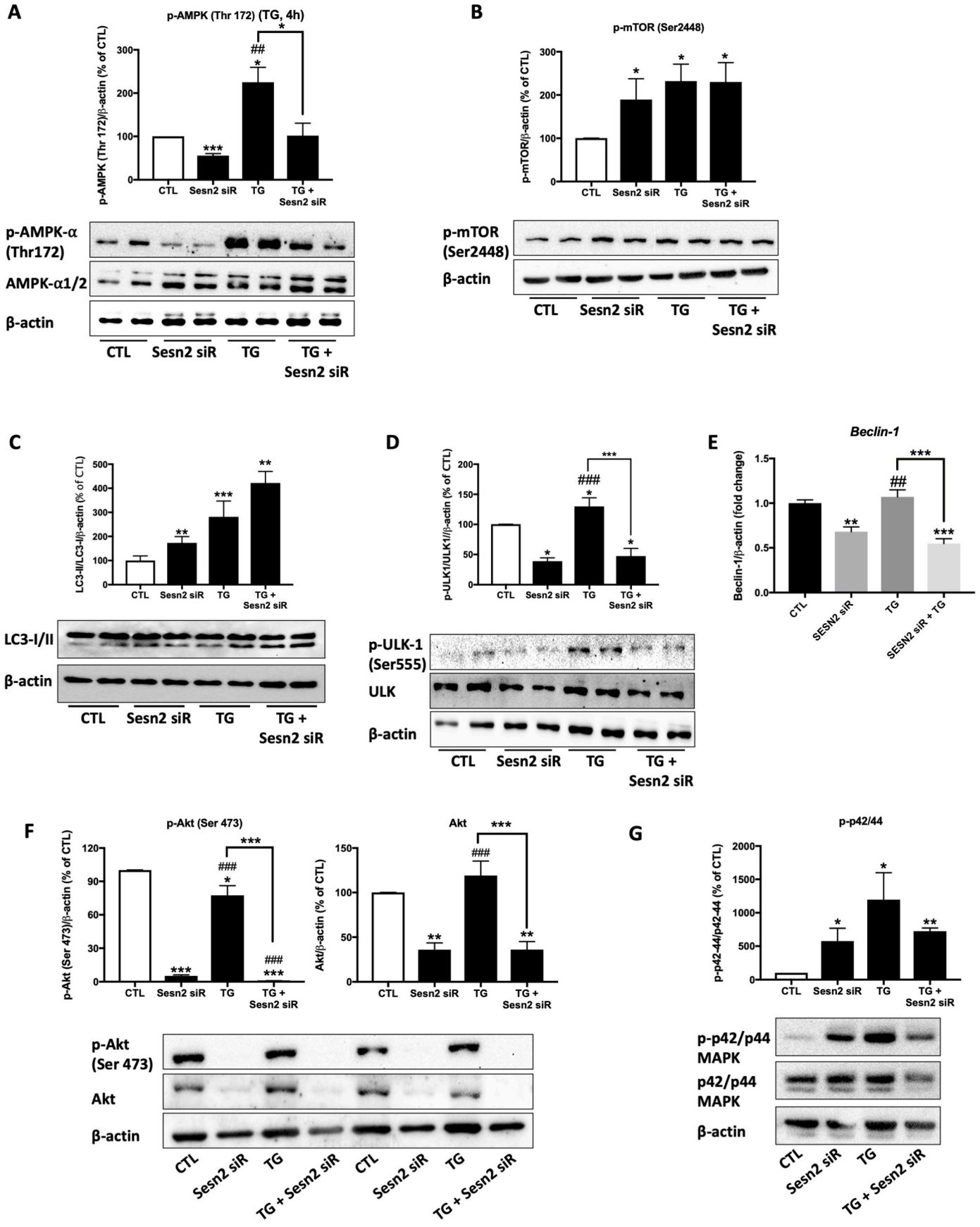

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Fig. 8. Effects of *SESN2* suppression and thapsigargin (TG) on the regulation of AMPK/Akt/mTORC1 axis and autophagy. (A–D), Western blot analysis showing protein expression levels of p-AMPK (Thr172) (A), p-mTOR (Ser2448) (B), LC3-I/II (C), and p-ULK-1 (Ser555) (D), normalized against respective total proteins and/or β-actin. Bars represent pooled densitometry data normalized to total amount of respective proteins and/or β-actin loading control expressed as % of control (CTL) (n = 3 per group). (E), Relative mRNA expression of *Beclin-1* normalized against β-actin housekeeping gene (n = 5 per group). (F,G), Western blot analysis of p-Akt (Ser 473) & Akt (F), and p-p42/44 MAPK & p42/44 MAPK (G) in cells left untreated or incubated with TG (100 nM, 4 h) in the presence or absence of *SESN2* siRNA duplexes (after 48 h of transfection). Bars represent pooled densitometry data normalized to β-actin loading control or respective total protein (Akt or p42/44) expressed as % of control (CTL) (n = 3 per group). Cells were treated with *SESN2* siRNA for 48 h, and then exposed to thapsigargin (100 nM; 4 h), where specified. Data are presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* CTL or indicated groups; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *versus SESN2* siR. The groups include untreated cells or control (CTL); *SESN2* siR, *SESN2* silenced cells; TG, thapsigargin-treated cells (100 nM; 4 h); *SESN2* siR + TG, *SESN2* silenced cells treated with TG.

in endothelial cells. This inflammatory pathway is intimately associated with several metabolic and cardiovascular pathologies and is involved in the pathogenesis of endothelial dysfunction (Maamoun et al., 2019a).

5. Conclusion

These data suggest that patients with disease conditions characterized by the activation of ER stress response are at higher risk for cardiovascular complications if their endogenous capacity to increase and/or maintain expression levels SESN2 is not optimal. In line with this, it has recently been reported that mRNA *SESN2* levels are significantly reduced in patients suffering from dyslipidemia, and diabetes with and without dyslipidemia (Sundararajan et al., 2020). Therefore, identifying novel or repurposing existing pharmacological strategies (e.g., metformin) to enhance or maintain the expression levels of SESN2 would be beneficial in pathological states associated with sustained ER stress activation such as diabetes, obesity, or atherosclerosis.

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CRediT authorship contribution statement

Munazza T. Fatima: performed the experiments and collected data, performed formal analysis, wrote the manuscript and prepared the figures with input from all authorsall authors. **Maram Hasan:** performed the experiments and collected data. **Shahenda S. Abdelsalam:** performed the experiments and collected data. **Siveen K. Sivaraman:** performed the experiments and collected data. **Heba El-Gamal:** performed the experiments and collected data. **Muhammad A. Zahid:** performed the experiments and collected data. **Mohamed A. Elrayess:** performed formal analysis, revised the manuscript critically for important intellectual content. **Hesham M. Korashy:** performed formal analysis, revised the manuscript critically for important intellectual content. **Asad Zeidan:** performed formal analysis, revised the manuscript critically for important intellectual content. **Aijaz S. Parray:** performed formal analysis, revised the manuscript critically for important intellectual content. **Abdelali Agouni:** conceptualized the research study idea, designed the research plan, acquired resources and funding, supervised the experiments, and curated the data, performed formal analysis, wrote the manuscript and prepared the figures with input from all authors, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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