



Mechanism of endoplasmic reticulum stress-induced vascular endothelial dysfunction



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ARTICLE INFO

Article history:

Received 13 December 2013

Received in revised form 7 February 2014

Accepted 17 February 2014

Available online 24 February 2014

Keywords:

Endoplasmic reticulum

Endothelial dysfunction

MAPKinase

Oxidative stress

ABSTRACT

Background: We recently reported that ER stress plays a key role in vascular endothelial dysfunction during hypertension. In this study we aimed to elucidate the mechanisms by which ER stress induction and oxidative stress impair vascular endothelial function. **Methodology/principal findings:** We conducted *in vitro* studies with primary endothelial cells from coronary arteries stimulated with tunicamycin, 1 $\mu\text{g}/\text{mL}$, in the presence or absence of two ER stress inhibitors: tauroursodeoxycholic acid (Tudca), 500 $\mu\text{g}/\text{mL}$, and 4-phenylbutyric acid (PBA), 5 mM. ER stress induction was assessed by enhanced phosphorylation of PERK and eIF2 α , and increased expression of CHOP, ATF6 and Grp78/Bip. The ER stress induction increased p38 MAPK phosphorylation, Nox2/4 mRNA levels and NADPH oxidase activity, and decreased eNOS promoter activity, eNOS expression and phosphorylation, and nitrite levels. Interestingly, the inhibition of p38 MAPK pathway reduced CHOP and Bip expressions enhanced by tunicamycin and restored eNOS promoter activation as well as phosphorylation. To study the effects of ER stress induction *in vivo*, we used C57BL/6J mice and p47phox^{-/-} mice injected with tunicamycin or saline. The ER stress induction in mice significantly impaired vascular endothelium-dependent and independent relaxation in C57BL/6J mice compared with p47phox^{-/-} mice indicating NADPH oxidase activity as an intermediate for ER stress in vascular endothelial dysfunction. **Conclusion/significance:** We conclude that chemically induced ER stress leads to a downstream enhancement of p38 MAPK and oxidative stress causing vascular endothelial dysfunction. Our results indicate that inhibition of ER stress could be a novel therapeutic strategy to attenuate vascular dysfunction during cardiovascular diseases.

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1. Introduction

The endoplasmic reticulum (ER) is the cell organelle where protein translation, folding and trafficking occur. Maintenance of calcium homeostasis, and production and storage of glycogen as well as other macromolecules also take place in the ER. Additionally, the ER is the early response site to cellular stresses [1,2]. When there is a biological stress that impairs ER homeostasis or function, the cell responds to the increasing presence of unfolded proteins within the ER by activating a complex signaling network called the unfolded protein response (UPR), which attempts to restore normal ER function [3]. The ER stress is caused by multiple factors including high glucose or energy deprivation, oxidative stress, hypoxia, calcium overload, expression of mutant

proteins incompatible with folding and the exposure to chemicals such as thapsigargin or tunicamycin [4]. Additionally, it has been shown that obesity, diabetes mellitus, neurodegenerative disorders, viral infection, cancer, inflammatory conditions and cardiovascular disorders are associated with ER stress induction [5–8].

Recent studies have shown a relationship between ER stress and oxidative stress in cardiovascular pathology [9–11]. While oxidative stress has been implicated in the apoptotic process triggered by ER stress, the source and its relationship with ER stress are gaps in knowledge that remain to be studied [12,13]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is considered one of the main sources implicated in producing cellular reactive oxygen species (ROS) [14]. The ER stress signaling involving calcium and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) activates NADPH oxidase and increases oxidative stress, which are necessary for ER stress-induced apoptosis [15,16]. However, the issue of whether NADPH oxidase plays a role upstream or downstream to ER stress-induced cardiovascular dysfunction remains controversial.

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Endothelial dysfunction occurs in cardiovascular diseases [17–20] characterized by reduced eNOS activity and/or expression and decreased nitric oxide (NO) bioavailability resulting in part from enhanced oxidative stress [21]. The expression of eNOS is regulated by multiple mechanisms that include transcriptional and post-transcriptional regulation of mRNA, and post-translational regulation of the protein function [22]. It is known that Akt activity enhances phosphorylation of eNOS, which is antagonized by the activation of p38 MAPK [23–26]. The negative effect of ER stress induction on eNOS expression has been recently described in endothelial cells in a model of isolated pulmonary hypertension, and in muscle from CHOP^{-/-} mice [27,28].

Because endothelial nitric oxide synthase (eNOS) plays an important role in maintaining blood pressure and vascular function, our aim is to determine the cellular mechanisms that link ER stress to oxidative stress and how it affects eNOS expression and activity in vascular endothelial cell.

In the present study, we show that ER stress induction by tunicamycin regulates NADPH oxidase and endothelial nitric oxide synthase (eNOS) activity as well as gene expression. We also show that p47phox deficiency prevents ER stress-induced vascular dysfunction.

2. Results

2.1. ER stress induction by Tunicamycin enhances oxidative stress in coronary endothelial cells

The induction of ER stress in coronary endothelial cells exposed to tunicamycin for 6 h was determined by the expression of ER stress markers. Tunicamycin increased phosphorylated protein kinase-like endoplasmic reticulum kinase (PERK), phosphorylated eukaryotic initiation factor 2 α (P-eIF2 α), 78 kDa glucose-regulated protein (Grp78)/Bip, C/EBP homologous protein (CHOP) and activating transcription factor 6 (ATF6) protein expression (Fig. 1A–E). This was accompanied with an increase in CHOP and ATF6 gene expressions (Fig. 1F, G). The enhanced ER stress marker expression was reduced when endothelial cells were pre-treated with ER stress inhibitors (Tudca and PBA). We did not observe changes in the expression of ER stress markers in endothelial cells incubated with vehicle and ER stress inhibitors indicating that the effect of Tudca and PBA on ER stress inhibition is specific (Fig. 1A–G).

The incubation with tunicamycin for 6 h barely impaired DNA studied by the Comet single cell electrophoresis assay. This impairment was prevented after ER stress inhibition by Tudca (Fig. S1).

To determine whether ER stress enhances oxidative stress, we measured Nox2/4 expression, NADPH oxidase activity and superoxide anion production in cells exposed to tunicamycin and pre-treated with or without ER stress inhibitors or apocynin. Thus, Fig. 2 illustrates that ER stress induction triggered an increase in mRNA levels of Nox2 and Nox4 (Fig. 2A, B), and increased NADPH oxidase activity (Fig. 2C) and dihydroethidium (DHE) staining (Fig. 2D), which were prevented when endothelial cells were pre-treated with ER stress inhibitors or apocynin. Interestingly, the down-regulation of Nox2 and Nox4 by specific siRNAs, which led to a 50% decrease in Nox2 and Nox4 basal gene expression, prevented the increase in NADPH oxidase activity and in their mRNA levels in response to tunicamycin (Fig. 2E–G). Transfection of endothelial cells with scrambled RNA (scr) did not change Nox2 or Nox4 mRNA level (Fig. 2E–G).

2.2. ER stress induction by tunicamycin reduces eNOS activity and expression in coronary endothelial cells

To determine how ER stress induction affects endothelial function, eNOS expression and activity were studied in endothelial cells. For this purpose, we measured eNOS promoter activity, as well as its gene and protein expression after incubation with tunicamycin for 6 h.

Transient transfection of endothelial cells with a reporter plasmid containing the eNOS promoter exhibited a high basal luciferase activity that was reduced to 50% after exposure to tunicamycin. This reduction was prevented in cells pre-treated with Tudca (Fig. 3A). To elucidate whether this effect was specifically dependent on ER stress induction, we co-transfected the endothelial cells with the reporter plasmid containing the eNOS promoter and mammalian expression plasmids containing cDNAs encoding for murine CHOP or ATF6. Interestingly, luciferase activity was significantly reduced 1.7-fold and 1.5-fold by the overexpression of CHOP and ATF6, respectively (Fig. 3B).

Surprisingly, the eNOS mRNA levels remained unchanged after incubation with tunicamycin compared with cells incubated with vehicle and in the presence or absence of ER stress inhibitors (Fig. 3C). However, in a time course experiment with tunicamycin, a decrease in eNOS gene expression at longer times than 6 h was observed (Fig. 3D). These results were confirmed by fluorescence *in situ* hybridization (RNA-FISH) analysis of eNOS mRNA. The fluorescence quantification showed a decreased eNOS mRNA expression in cells incubated with tunicamycin for 8–10 h (Fig. 3E).

The discrepancy between the reduction in promoter activity and the sustained eNOS mRNA levels after 6 h of exposure to tunicamycin suggested the implication of post-transcriptional regulatory mechanisms. To test this hypothesis, we performed an assay to measure mRNA stability based on a time course experiment with actinomycin D (transcription inhibitor), in cells incubated with vehicle and cells incubated with tunicamycin in the presence or absence of Tudca. A delay in eNOS mRNA decay was observed in cells incubated with tunicamycin compared to vehicle or cells pre-treated with Tudca (Fig. 3F).

These results in gene expression were supported by a time dependent decrease in total eNOS protein expression (Fig. 3G).

The activity of eNOS was evaluated by the expression of phosphorylated eNOS (P-eNOS) in Serine 1177 and by nitrite production. The expression of active eNOS was decreased in cells incubated with tunicamycin compared to cells incubated with vehicle and cells pre-treated with Tudca (Fig. 3H). Moreover, nitrite production measured in the supernatants of cell cultures was significantly reduced by tunicamycin in a similar way to cells incubated with L-NAME. The co-incubation with tunicamycin and L-NAME did not have a synergistic effect on the reduction of nitrite content (Fig. 3I).

2.3. ER stress induction modulates extracellular-signal related kinases 1 and 2 (ERK 1/2) and p38 mitogen-activated protein kinase activity (p38 MAPK) in an opposite way

Endothelial cells exhibited a decrease in phosphorylation of extracellular-signal related kinases 1 and 2 (P-ERK1/2) and an increase in phosphorylation of p38 mitogen-activated protein kinase (P-p38 MAPK) after incubation with tunicamycin, which were normalized after ER stress inhibition (Fig. 4A, B).

Due to p38 MAPK activation by ER stress induction, we incubated the endothelial cells with the p38 MAPK inhibitor, SB203580, prior to the addition of tunicamycin to determine whether the effects observed in eNOS activity and expression were mediated by p38 MAPK. In addition, the expression of ER stress markers in the presence of p38 MAPK inhibitor was studied.

The p38 MAPK inhibitor restored to control levels its phosphorylation as expected; however, apocynin did not change p38 MAPK activation by tunicamycin indicating it was independent of O₂⁻ production (Fig. 4C).

Interestingly, the reduction in eNOS phosphorylation was prevented by p38 MAPK inhibitor in cells exposed to tunicamycin (Fig. 4D). Moreover, in cells co-transfected with eNOS promoter plasmid and the mammalian expression plasmid containing cDNA for ATF6, the reduction in

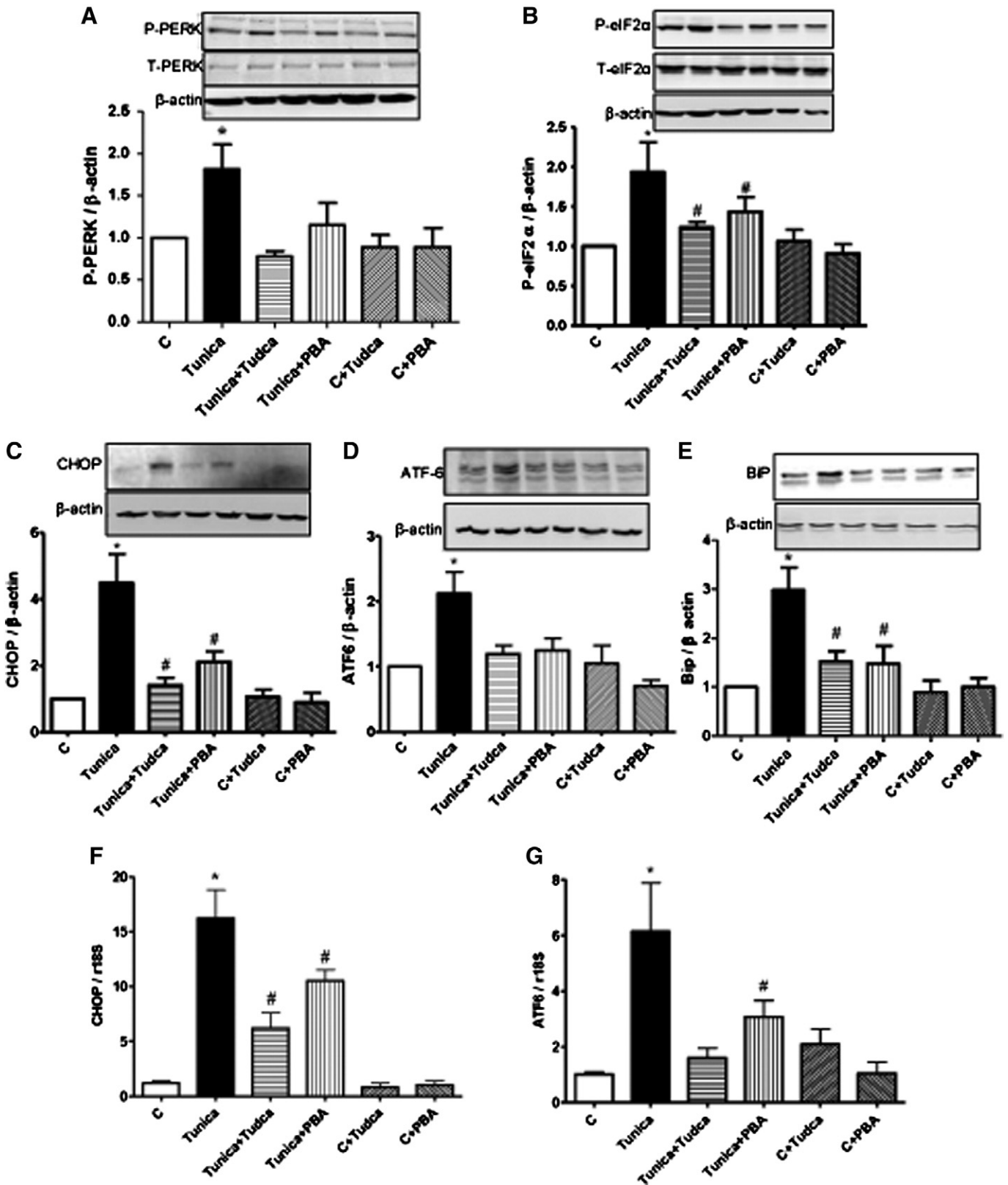


Fig. 1. ER stress markers expression in endothelial cells (EC). A–E. Representative Western blots and quantitative analysis of P-PERK, P-eIF2α, CHOP, ATF6 and grp78 or Bip, n = 5, and; F, G. CHOP and ATF6 mRNA levels, normalized to 18S rRNA, n = 6. The cells were incubated with 1 μg/mL tunicamycin (Tunica) for 6 h and pre-treated or not with ER stress inhibitors (+Tudca/+PBA) 1 h prior to add the tunicamycin. Data are expressed as mean ± SEM. *P ≤ 0.05 for Tunica vs. cells treated with vehicle (C) or cells pre-treated with Tudca or PBA (Tunica + Tudca/PBA); #P ≤ 0.05 for Tunica + Tudca/PBA vs. C or C + Tudca/PBA.

luciferase activity was reversed by p38 MAPK inhibitor. These results indicate that ATF6 is regulating eNOS promoter activity by modulating p38 MAPK action (Fig. 4E, F).

We then studied whether the inhibition of p38 MAPK altered the expression of the ER stress markers P-eIF2α, ATF6, CHOP and BiP. We observed that the pre-incubation with p38 MAPK inhibitor did not

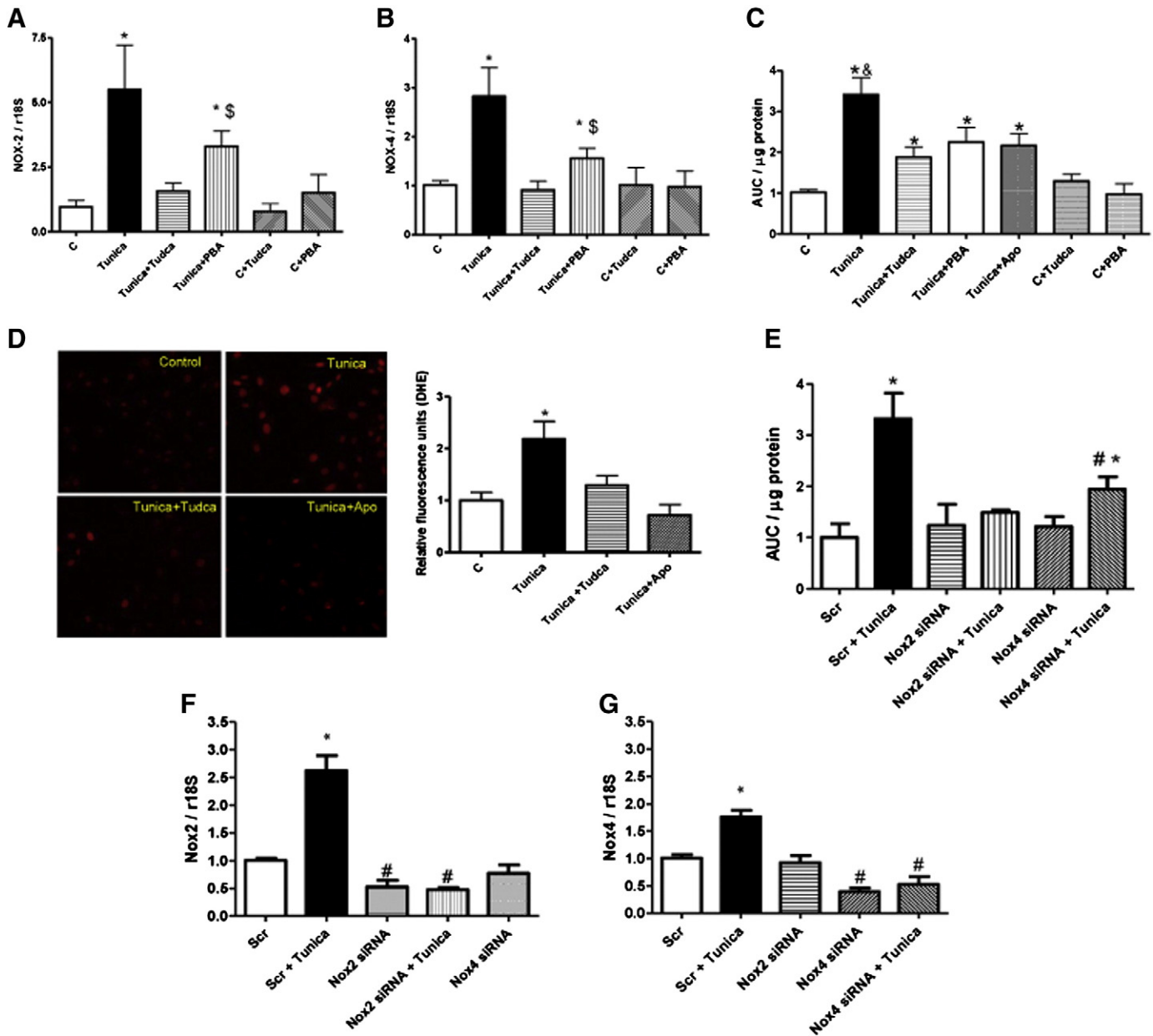


Fig. 2. NADPH oxidase expression/activity induction by ER stress in endothelial cells. A, B. Quantitative real time PCR assessment of Nox2 and Nox4 gene expression, normalized to 18S rRNA, in cells treated with vehicle (C) or tunicamycin (Tunica) in the absence or presence of Tudca or PBA, $n = 6$. C. NADPH oxidase activity measured by lucigenin chemiluminescence in cells treated with vehicle or tunicamycin in the absence or presence of Tudca or apocynin (Apo, 100 $\mu\text{mol/L}$), $n = 6$. D. Representative images of cells stained with dihydroethidium and fluorescence signal quantification, $n = 4$. E. NADPH oxidase activity measured in EC transfected with scrambled RNA (Scr), Nox2 siRNA or Nox4 siRNA, incubated with or without tunicamycin, $n = 5$. F, G. Nox2 and Nox4 gene expression, normalized to 18S rRNA, in EC transfected with either Nox2 siRNA or Nox4 siRNA and incubated with or without tunicamycin, $n = 6$. In all experiments cells were incubated with tunicamycin for 6 h. Data are expressed as mean \pm SEM. * $P \leq 0.05$ for Tunica, Tunica + Tudca/PBA or Tunica + Apo vs. C \pm Tudca/PBA; $\&P \leq 0.05$ for Tunica + PBA vs. Tunica; * $\&P \leq 0.05$ for Tunica vs. Tunica + Tudca/PBA or Tunica + Apo; # $P < 0.05$ for Nox4siRNA/Nox2siRNA \pm Tunica vs. Scr.

change the enhancement of P-eIF2 α or ATF6 expression by tunicamycin but reduced CHOP and Bip expressions (Fig. 4G–I).

2.4. ER stress induction in vivo: effect on vascular reactivity, eNOS and NADPH oxidase activity and expression in vessels from C57BL/6j and p47phox^{-/-} mice

To determine the relationship between the ER stress response, oxidative stress and vascular dysfunction, we examined endothelium-dependent relaxation (EDR) and endothelium-independent relaxation in aorta and in mesenteric resistance arteries (MRA) from C57BL/6j, used as control group, and p47phox^{-/-} mice injected with tunicamycin or saline for two weeks. We selected p47phox^{-/-} because the up-regulation of this subunit of the NADPH oxidase complex has been

showed to be critical in impaired endothelial dependent vasorelaxation and in the uncoupling of eNOS [29,30].

Systolic blood pressure (SBP), measured at the end of the treatment, showed no difference among all groups (Fig. S2). ER stress induction in vivo by tunicamycin was assessed by increased P-eIF2 α /T-eIF2 α , CHOP and ATF6 expressions in aorta and MRA from C57BL/6j (control) and p47phox^{-/-} mice (Fig. 5).

The mRNA levels of CHOP were increased in aorta and MRA from control and p47phox^{-/-} mice, injected with tunicamycin (Fig. 5B, G). These changes in gene expression were accompanied by the corresponding increase in CHOP protein by Western blot in aorta and MRA from control and p47phox^{-/-} mice injected with tunicamycin compared to injected mice with saline (Fig. 5C, H). We also reported an increase in ATF6 gene expression in aortas and MRA from control and

p47phox^{-/-} mice injected with tunicamycin (Fig. 5D, I) that was accompanied by an increase in cleaved ATF6 protein in artery lysates (Fig. 5E, J).

The EDR of aortas from control and p47phox^{-/-} mice in response to acetylcholine (ACh) was reduced by tunicamycin; however, EDR was less impaired in p47phox^{-/-} mice compared with control mice (Fig. 6A). The EDR in MRA was markedly impaired in control group while in p47phox^{-/-} group it was just slightly reduced by tunicamycin (Fig. 7A).

The eNOS phosphorylation was reduced in aorta and MRA from control and p47phox^{-/-} mice injected with tunicamycin. Total eNOS expression was similar in aortas from all groups of mice, while it was significantly reduced in MRA from control and p47phox^{-/-} mice by tunicamycin (Figs. 6C, 7C). These results in eNOS protein were confirmed by eNOS mRNA levels in both types of vessels (Figs. 6D, 7D). The incubation with L-NAME abolished the relaxation in aorta and MRA from control and p47phox^{-/-} mice injected with saline or tunicamycin (data not shown).

The incubation with apocynin had no effect on EDR in aorta from control mice or p47phox^{-/-} mice injected with tunicamycin when compared with their correspondent groups treated with saline (Fig. 6B). In MRA the incubation with apocynin restored the relaxation impaired by tunicamycin in control mice; however, it did not have an effect on EDR of MRA from p47phox^{-/-} (Fig. 7B).

An increase in Nox2 mRNA levels was detected in aortas and MRA from control and p47phox^{-/-} mice groups injected with tunicamycin when compared with their homologous groups injected with saline (Figs. 6E, 7E). Nox4 mRNA levels were similar in all groups (data not

shown). The NADPH oxidase activity was enhanced in arteries from control mice injected with tunicamycin, while it did not change in p47phox^{-/-} mice when compared with their groups injected with saline (Figs. 6F, 7F).

In regard to endothelium independent relaxation, the response to sodium nitroprusside (SNP) in aorta and MRA was shifted to the right in the control mice group injected with tunicamycin when compared with the control mice group treated with saline (Fig. S3A, C). Interestingly, endothelium independent relaxation in aorta was not affected by tunicamycin in p47phox^{-/-} mice, while in MRA it was shifted to the right. The shift in MRA was more pronounced in control mice group than in p47phox^{-/-} mice group (Fig. S3B, D).

3. Discussion

We previously reported that *in vivo* ER stress induction by tunicamycin was associated with impaired vascular endothelial function and an increase in NADPH oxidase activity in aorta and mesenteric resistance arteries [31]. In the present study, we examined the mechanisms by which ER stress induction leads to vascular endothelial dysfunction. Thus, our results show that ER stress induction causes vascular endothelial dysfunction through oxidative stress and p38 MAPK dependent mechanisms.

It is well known that NADPH oxidases (Nox) are the major source for ROS in the cardiovascular system [32,33] and multiple independent studies provide evidence that Nox proteins contribute to oxidative damage in response to a wide variety of stimuli [34,35]. In the present study, we observed an enhancement in NADPH oxidase activity in

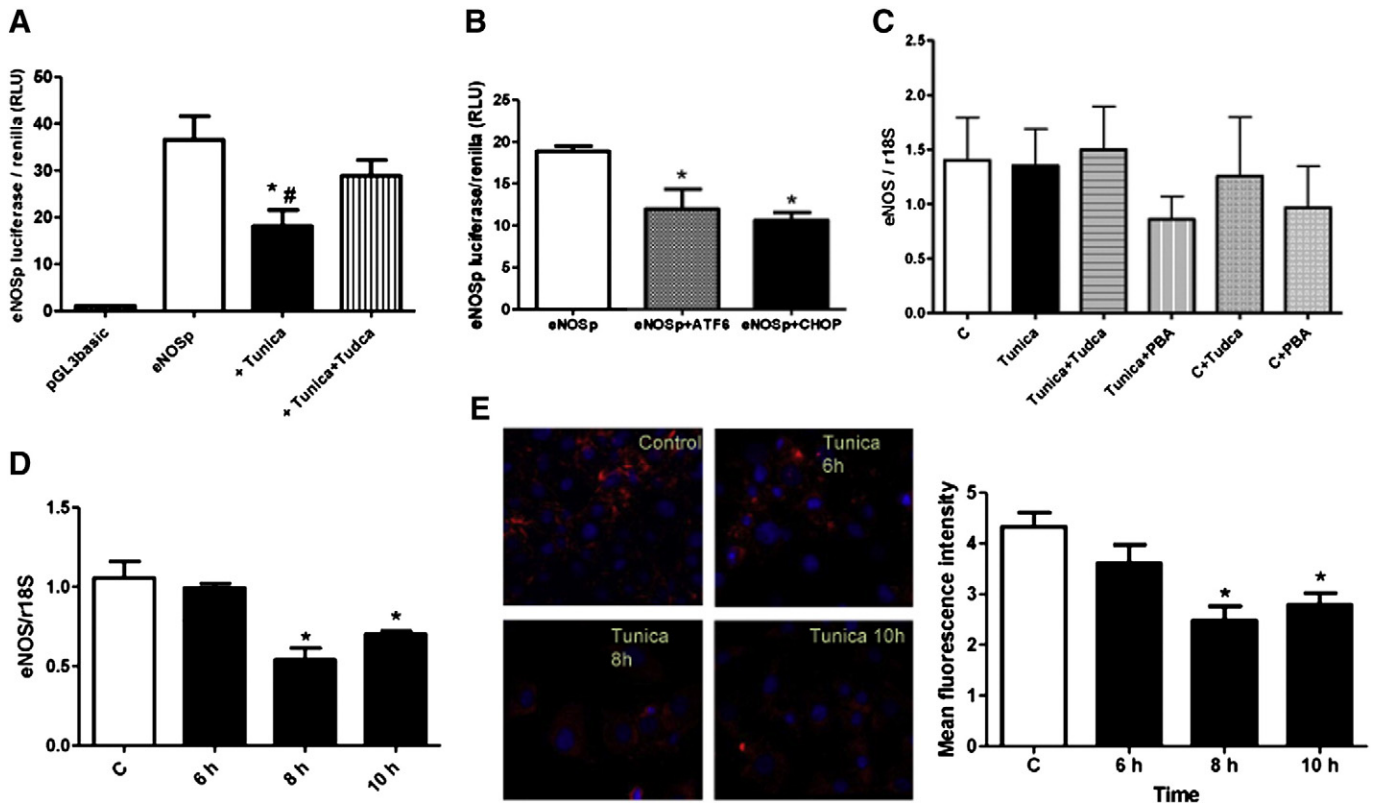


Fig. 3. Effect of ER stress on eNOS activity/expression in endothelial cells. A, B. eNOS promoter activity in transfected EC with plasmid containing eNOS promoter in cells added with vehicle (C) or incubated with tunicamycin (Tunica) \pm Tudca/PBA and in cells co-transfected with plasmids overexpressing CHOP and ATF6, respectively, $n = 5$. C. Quantitative evaluation of eNOS mRNA levels, normalized to 18S rRNA, in cells with vehicle or incubated with tunicamycin \pm Tudca/PBA, $n = 6$. D. Quantitative evaluation of eNOS mRNA levels, normalized to 18S rRNA, in a time course assay with tunicamycin, $n = 5$; E. Representative pictures of eNOS mRNA detected by RNA-FISH analysis with quantitative fluorescence evaluation, $n = 3$; F. eNOS mRNA levels in a time course experiment with actinomycin D after incubation with tunicamycin with or without Tudca, $n = 5$; G. Representative Western blot for eNOS and quantitative analysis in a time course assay with tunicamycin, $n = 4$; H. Representative Western blot for phosphorylated eNOS (P-eNOS), total eNOS and quantitative analysis in cells with vehicle (C) or incubated with tunicamycin (Tunica) \pm Tudca/PBA, $n = 4$; and I. Nitrite production measured in supernatants of cultured EC with vehicle (C) or incubated with tunicamycin (Tunica) in the absence or presence of Tudca, PBA, Apocynin or L-NAME (100 μ mol/L), $n = 6$. In these experiments cells were incubated with tunicamycin for 6 h. * $P \leq 0.05$ for Tunica vs. C or C + Tudca; # $P \leq 0.05$ for Tunica vs. Tunica + Tudca; &#P ≤ 0.05 for Tunica + L-NAME vs. control + L-NAME.

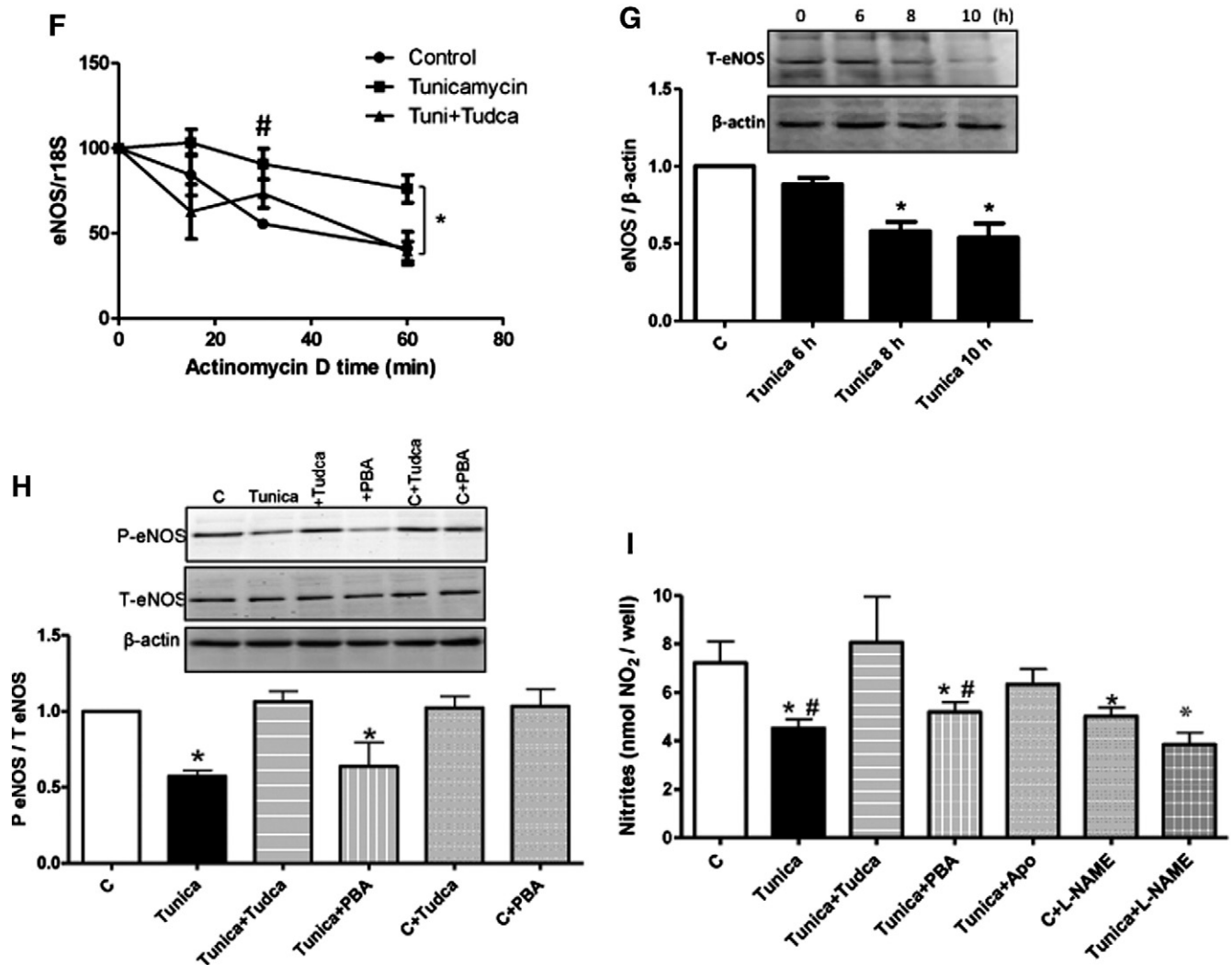


Fig. 3 (continued).

tunicamycin-challenged endothelial cells that was associated with an increase in Nox2 and Nox4 gene expression. In addition, knockdown of either Nox2 or Nox4 drastically reduced NADPH oxidase activity and abolished the increments in Nox2 and Nox4 gene expression after tunicamycin exposure, respectively, indicating that both are involved in oxidative stress generation after ER stress induction. These results are supported by previous studies where enhanced superoxide generation and up-regulation of Nox2, Nox4 and NADPH oxidase p47phox subunit were associated with impaired endothelial vasorelaxation [15,29]. In terms of vascular reactivity, the inhibition of reactive oxygen species generation by apocynin did not affect the endothelium dependent relaxation (EDR) in vessels from p47phox^{-/-} mice, but it significantly improved EDR in control mice injected with tunicamycin. The endothelium-independent relaxation was impaired in aorta and MRA from control mice administration with tunicamycin, while in the p47phox^{-/-} mice injected with tunicamycin, only the MRA relaxation was slightly affected indicating the importance of oxidative stress derived from ER stress induction in vascular function. Furthermore, in the present study, the absence of functional p47phox *in vivo* prevented the increase in NADPH oxidase activity induced by tunicamycin in aorta and MRA. Moreover, we found a substantial increase in Nox2 mRNA levels in aorta and MRA from both C57BL/6J and p47phox^{-/-} mice treated with tunicamycin, which is consistent with our results in endothelial cells. Our findings are supported by previous studies where Nox2 and CHOP-mediated ER stress was responsible for apoptosis in high

glucose treated endothelial cells. Furthermore, a study using Nox2-deficient mice showed a reduction in CHOP expression and apoptosis in response to ER stress induction suggesting a complex interplay between oxidative stress and ER stress [36,14]. The eNOS protein and gene expression were reduced in MRA from tunicamycin treated mice, while they did not change in aorta. These data indicate a differential effect in terms of mechanistic that causes vascular endothelial dysfunction between resistance arteries vs. conductance arteries.

Recently, a relationship between ER stress induction and eNOS expression has been established [28]. Indeed, we observed a decrease in eNOS gene expression in cells subjected to tunicamycin for 6 h preceded by the reduction in eNOS promoter activity. However, the eNOS protein and mRNA levels remained the same at this time suggesting that exposure to tunicamycin for 6 h was not sufficient to produce changes in eNOS protein. Additionally, the delay in eNOS mRNA decay in the actinomycin D time course assay in cells exposed to tunicamycin indicated an increase in eNOS mRNA stability as a compensating mechanism. These data are supported by numerous studies that indicate post-transcriptional mechanisms governing the eNOS expression by modulating eNOS mRNA stability through the intervention of stabilizing/destabilizing proteins binding to the 3'UTR region of the messenger [37,38].

Recently, it has been reported that ER stress induction activates MAPKs [39–41]. Tunicamycin reduced ERK1/2 phosphorylation in endothelial cells that were restored after ER stress inhibition. These

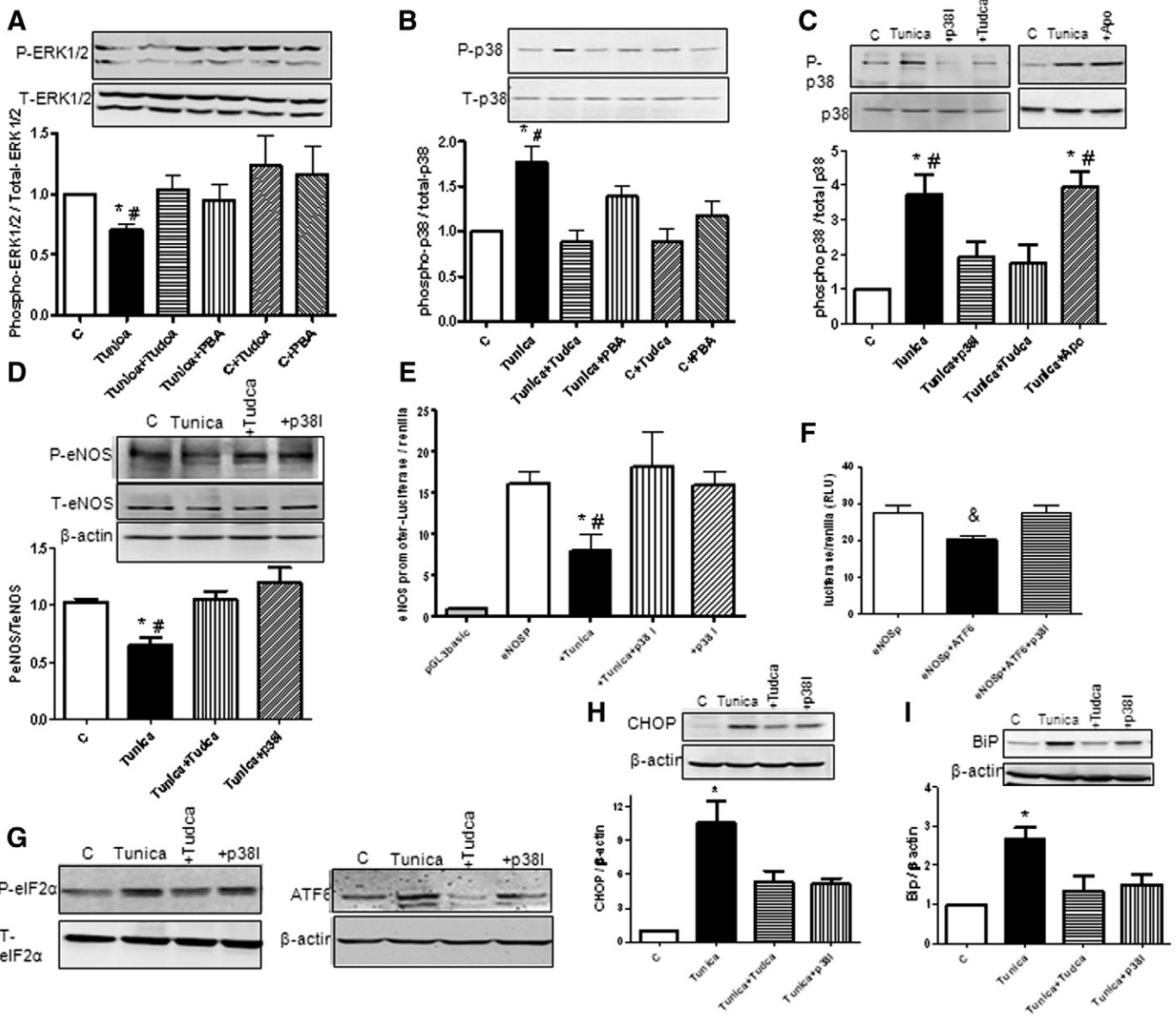


Fig. 4. Effect of p38 MAPK activation by ER stress on eNOS. A, B. Representative Western blots and quantitative analysis for P-ERK 1/2 and P-p38 MAPK in EC with vehicle (C) or incubated with tunicamycin (Tunicam) in the absence or presence of Tudca or PBA, n = 5; C. Representative Western blots and quantitative analysis for P-p38 in EC treated with tunicamycin in the absence or presence of p38 inhibitor (\pm p38i), SB203580 (10 μ mol/L), or apocynin (Apo), n = 4; D. P-eIF2 α and ATF6 and E, F. CHOP and BiP representative blots and quantitative analysis, n = 4; G. Representative blot and quantitative data of phosphorylated eNOS, n = 4, and H. eNOS promoter activity in transiently transfected EC incubated with or without tunicamycin \pm p38i, n = 5; I. eNOS promoter activity in cells co-transfected with the plasmid expressing ATF6 \pm p38i, n = 5. *P \leq 0.05 for Tunicam or Tunicam + Apo vs. C; #P \leq 0.05 for Tunicam or Tunicam + Apo vs. Tunicam + Tudca or Tunicam + p38i; &P \leq 0.05 for eNOSp + ATF6 vs. eNOSp + ATF6 + p38i or eNOSp.

results are supported by previous studies showing that ER stress induction reduces ERK1/2 activation and that TUDCA and PBA prevent cell death by interrupting classic pathways of apoptosis and restoring ERK activity [42–44]. In contrast, we detected an increase in p38 MAPK phosphorylation by tunicamycin. The activation of this MAPK signaling pathway in response to ER stress has been previously described in endothelial cells stimulated with thapsigargin [45]. Interestingly, the inhibition of p38 MAPK in the presence of tunicamycin, restored eNOS activation and normalized promoter activity suggesting that the activation of p38 MAPK by ER stress contributes to down-regulation of eNOS expression. Our results are supported by previous studies showing the negative effect of p38 MAPK activation on eNOS promoter regulation [46,47]. The inhibition of p38 MAPK reduced CHOP and BiP expressions but did not affect ATF6 expression or eIF2 α phosphorylation suggesting that p38 MAPK can modulate CHOP activity and expression and its targeted genes like BiP/GRP78 [48,49]. Moreover, the inhibition of p38

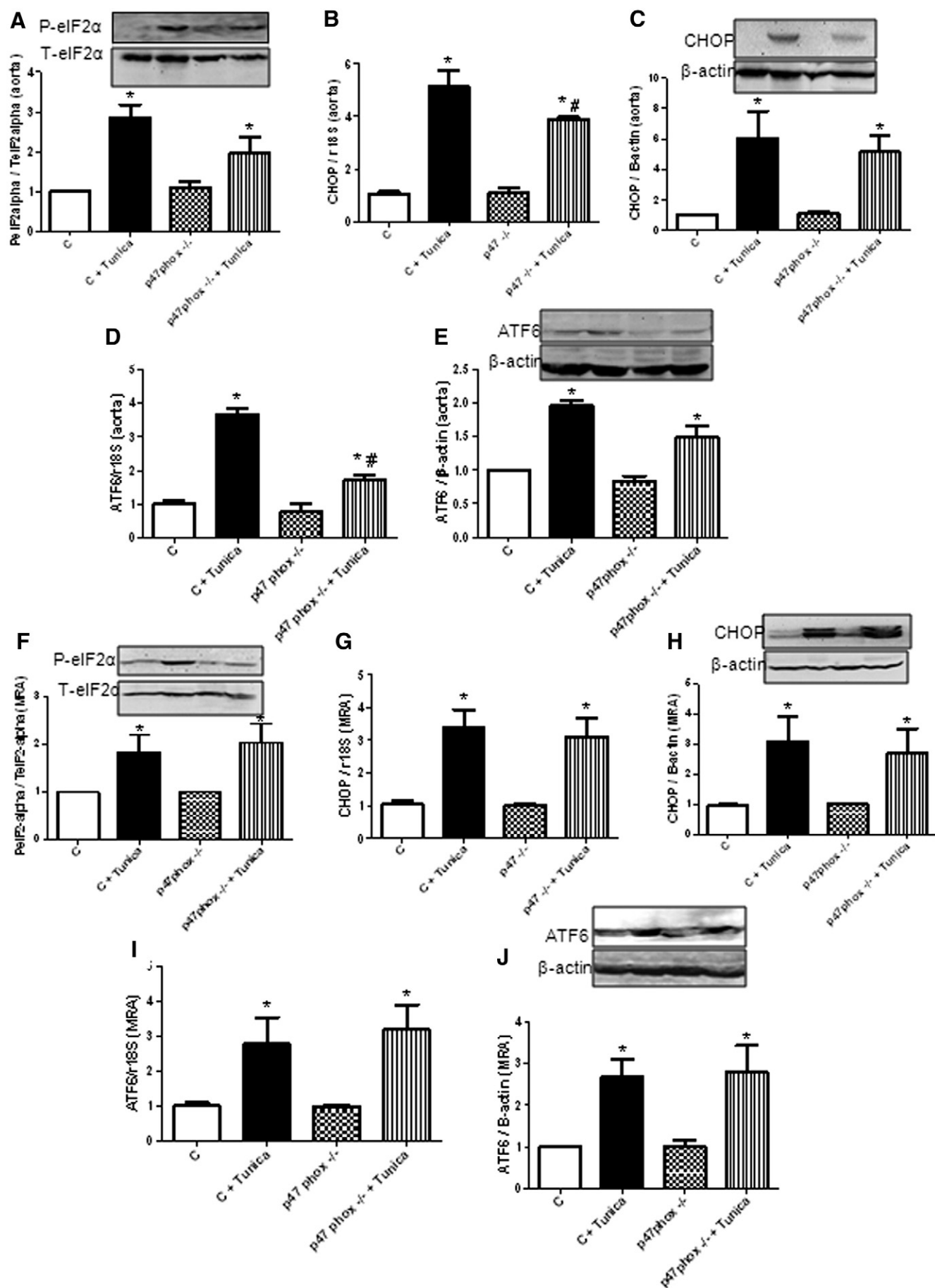
MAPK in transiently transfected endothelial cells overexpressing ATF6 restored the eNOS promoter activity indicating that p38 MAPK activity is downstream to ATF6 action.

In summary, we conclude that oxidative stress generation and p38 MAPK activation are intermediates in the impairment of vascular endothelial function by chemically induced ER stress in our model. Our data provides further insights on the complex relationship among ER stress homeostasis, oxidative stress and endothelial cell biology.

4. Methods

4.1. Endothelial cell culture, transient transfection and Luciferase assay

Mouse coronary artery endothelial cells were purchased in CelProgen (San Pedro, CA, USA) and were cultured according to the manufacturer's instructions with CelProgen Complete Growth media. Cultures were



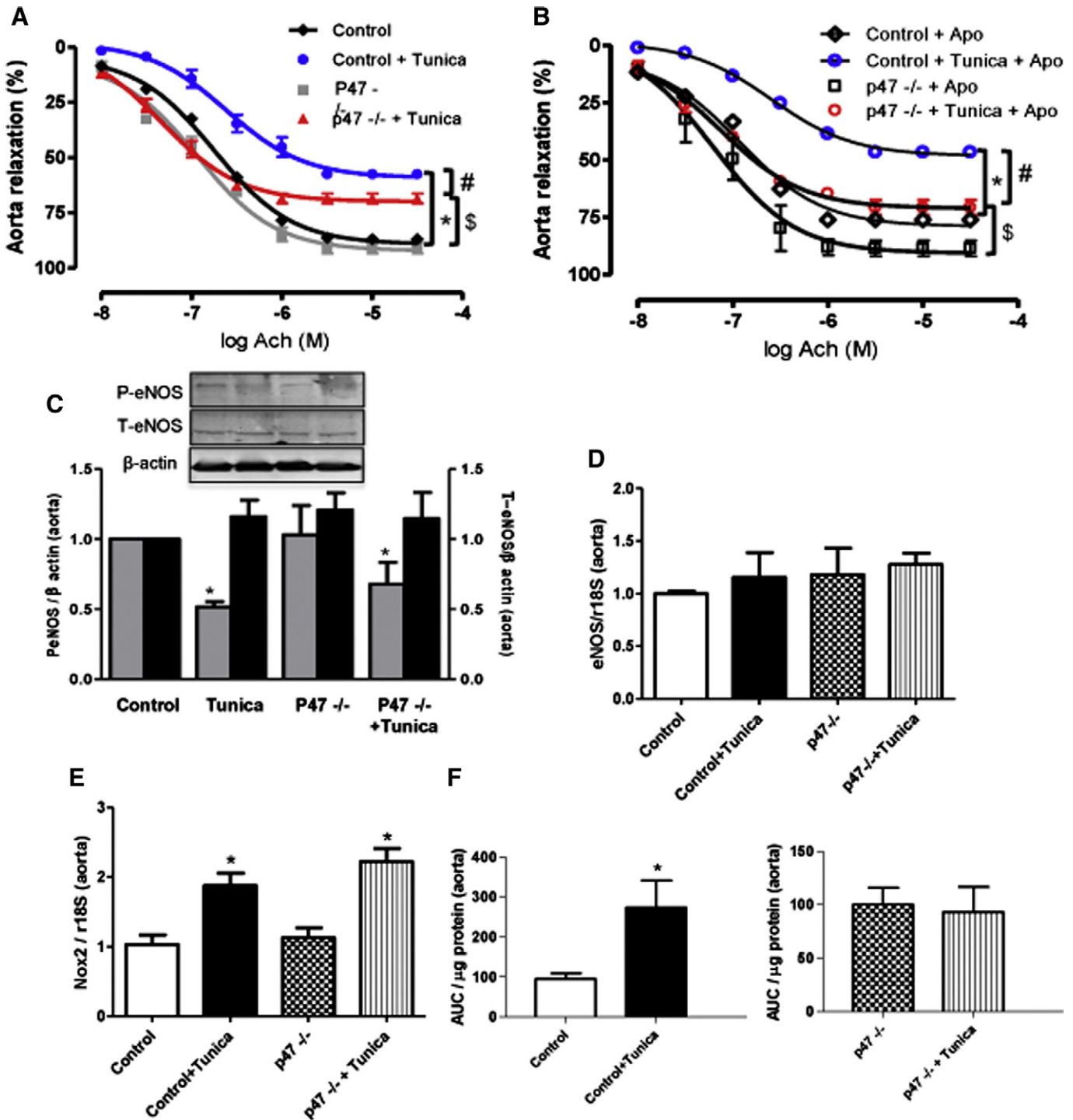


Fig. 6. Endothelium dependent relaxation, eNOS and NADPH oxidase in aortas from control and p47phox^{-/-} (p47^{-/-}) mice. A. Endothelium dependent relaxation (EDR) in response to ACh, n = 6; B. EDR in response to ACh in the presence of apocynin (Apo), n = 6; C, D. Western blot analysis for P-eNOS and T-eNOS, normalized to β-actin, n = 4, and eNOS mRNA levels, normalized to 18S rRNA, n = 6; D, E. Nox2 mRNA levels, normalized to 18S rRNA, n = 6; and F. NADPH oxidase activity in tissue lysates of vessels from control and p47phox^{-/-} mice injected with saline or tunicamycin, n = 5. *P < 0.05 for control + Tunica or p47^{-/-} + Tunica vs. control and p47^{-/-}; #P < 0.05 for control + Tunica vs. p47phox^{-/-} + Tunica; \$P < 0.05 for p47phox^{-/-} vs. p47phox^{-/-} + Tunica.

incubated at 37 °C in a humidified 5% CO₂ incubator and the medium was changed every 2 days. Confluent cell monolayers were harvested by a brief incubation with 0.25% trypsin–EDTA solution (GIBCO, Invitrogen,

Gran Island, NY, USA) and subcultured in order to perform further experiments between passages 3 and 6. The endothelial cells were incubated with vehicle or tunicamycin (1 μg/mL) [50] for 6 h and were pre-

Fig. 5. ER stress markers expression in aorta and MRA. A, F. Western blot analysis and quantitative data for P-eIF2-α normalized to eIF2-α in aorta and MRA, n = 4; B–C, G–H. CHOP mRNA levels, normalized to 18S rRNA, n = 6, and representative blot for CHOP protein and quantitative data respectively in aorta and MRA, n = 4; D–E, I–J. ATF6 mRNA levels, normalized to 18S rRNA, n = 6, and representative blots for cleaved ATF6 and quantitative data respectively in aorta and MRA from control (C) and p47phox^{-/-} mice untreated or treated with tunicamycin (Tunica), n = 4. *P < 0.05 for C + Tunica or p47phox^{-/-} + Tunica vs. C and p47phox^{-/-}; #P < 0.05 for p47phox^{-/-} + Tunica vs. C + Tunica.

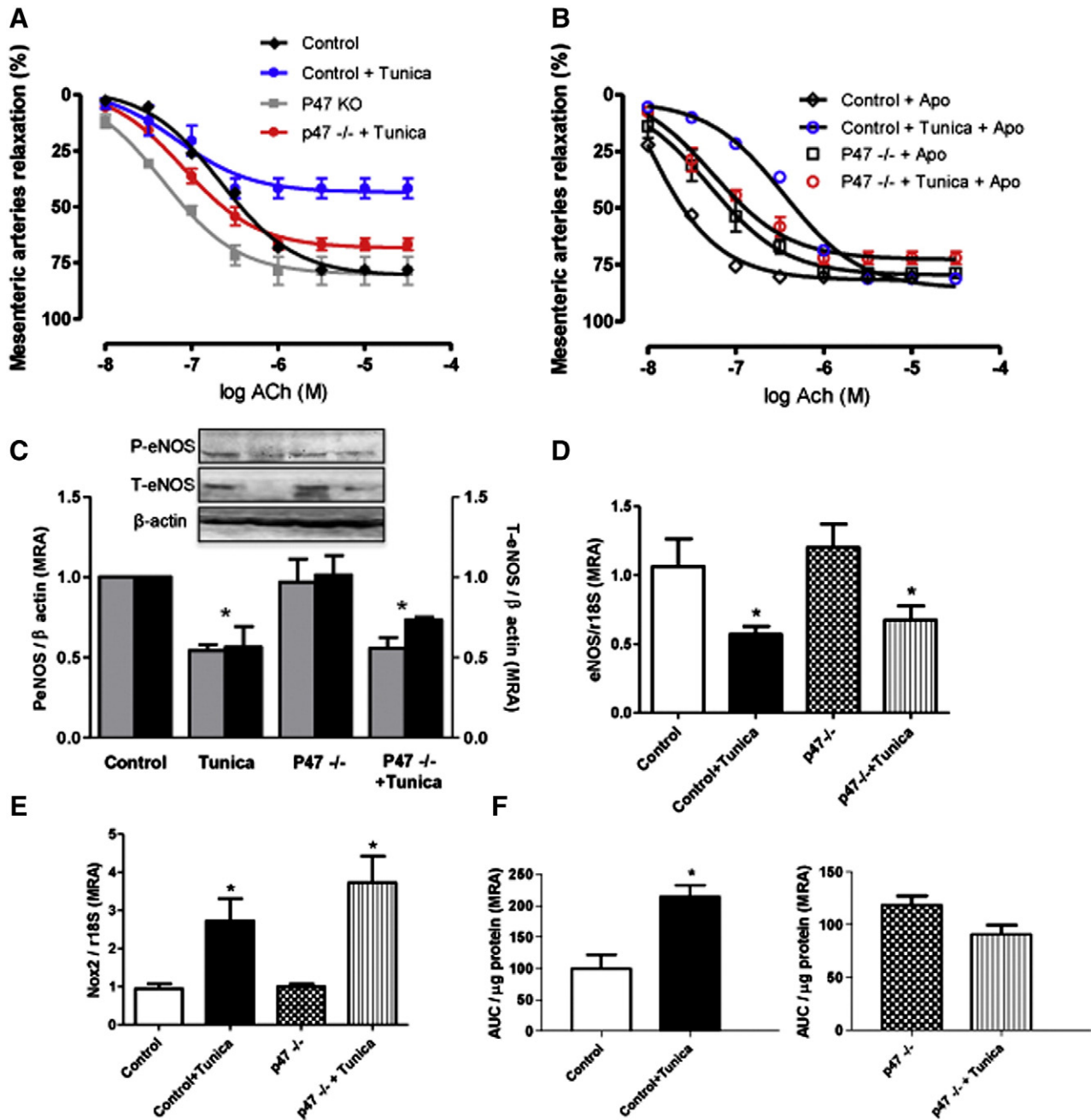


Fig. 7. Endothelium dependent relaxation, eNOS and NADPH oxidase in MRA from control and p47phox^{-/-} (p47^{-/-}) mice. **A.** Endothelium dependent relaxation (EDR) in response to ACh, n = 6; **B.** EDR in response to ACh in the presence of apocynin (Apo), n = 6; **C, D.** Western blot analysis for p-eNOS and T-eNOS, normalized to β-actin, n = 4, and eNOS mRNA levels, normalized to 18S rRNA, n = 6; **E.** Nox2 mRNA levels, normalized to 18S rRNA, n = 6; **F.** NADPH oxidase activity measured in tissue lysates of vessels from control and p47phox^{-/-} mice injected with saline or tunicamycin, n = 5. *P < 0.05 for control + Tunica or p47phox^{-/-} + Tunica vs. control and p47phox^{-/-}; #P < 0.05 for control + Tunica vs. p47phox^{-/-} + Tunica; SP < 0.05 for p47phox^{-/-} vs. p47phox^{-/-} + Tunica.

treated with or without ER stress inhibitors, tauroursodeoxycholic acid (Tudca, 500 μg/mL) [42,43] or phenylbutyric acid (PBA, 0.1 mM) [44] or in the presence of the p38 MAPK inhibitor, SB203580 (10 μM). We tested the induction of ER stress by studying ER stress markers expression in a time course experiment of 6, 8, 10 and 18 h with tunicamycin (1 μg/mL) and observed that incubation for 6 h was enough to trigger ER stress induction.

To perform transient transfection experiments, ECs were grown at 70% confluency in 12-well culture dishes and co-transfected with 1 μg of the reporter plasmid pGL2-eNOS Promoter-Luciferase (which contains a 1621 base pair fragment of human eNOS proximal promoter driving the luciferase gene), 0.25 μg of the reporter plasmid pRL-TK

(Promega) in the presence or absence of 0.5 μg of the mammalian expression vectors pcDNA1-CHOP or pcDNA3.1-ATF6 using lipofectamine LTX and Plus transfection reagents (Invitrogen) and transfection was performed following the manufacturer's instructions. The plasmids pGL2-eNOS Promoter and pcDNA1-CHOP were purchased from Addgene (ref. 19297 and ref. 21902, respectively). Transfection was followed by an incubation of 6 h with vehicle or with tunicamycin in the presence or absence of ER stress inhibitors. At the end of the treatment, cells were lysed in lysis buffer (Promega), and luciferase and renilla activities were measured with the dual Luciferase Assay System (Promega) with a luminometer (Fluoroskan Ascent FL, Labsystems). The renilla activity was used to normalize the transfection efficiency.

4.2. The siRNA-mediated gene silencing

Endothelial cells were transfected with scrambled siRNA (sc-37007), siRNAs against murine gp91-phox (sc-35504) or Nox4 (sc-41587) (Santa Cruz Biotechnology, Inc) following the manufacturer's instructions and using lipofectamine LTX and Plus transfection reagents. After 18 h of transfection, the media was replaced, and, 24 h later cells were incubated with or without tunicamycin for 6 h and then assayed for Nox2 or Nox4 mRNA levels, respectively, by real time PCR.

4.3. Impaired DNA analysis by Comet assay

The presence of DNA damage was examined by single-cell electrophoresis (Comet assay) by the manufacturer's protocol (OxiSelect Comet Assay Kit; Cell Biolabs, Inc., San Diego, CA). Briefly, endothelial cells were treated with tunicamycin for 6 h with or without ER stress inhibitors. After the culture period, the cells were harvested and washed with PBS, cell suspension was mixed with agarose at 37 °C, and 75 µL of this mixture was instantly added to comet slides. Slides were immersed in lysis buffer at 4 °C for 1 h and then replaced with pre-chilled alkaline buffer and placed at 4 °C for 30 min followed by electrophoresis (35 V for 15 min) in a horizontal electrophoresis chamber filled with Tris borate EDTA (TBE) buffer. Then, slides were transferred to a container filled with cold water for 2 to 4 min and then placed in 70% ethanol for 5 min and air dried overnight at room temperature. After staining with Vista Green DNA dye for 15 min at room temperature, comets were observed by fluorescent microscopy (Nikon, Tokyo, Japan) with $\times 20$ magnification. The images were analyzed using the software NIS-Elements BR 3.0 (Nikon, Tokyo, Japan).

4.4. General protocol in mice

All experiments were performed according to the American Guidelines for the Ethical Care of Animals and were approved by Tulane University Animal Care and Use Committee. The mice, C57BL/6J and B6(Cg)-Ncf1m1J/J (p47phox^{-/-}), 8 weeks-old males, were purchased from Jackson Laboratories (Bar Harbor, ME), housed in groups of five and maintained at a temperature of 23 °C with 12 h light/dark cycle. Mice were fed on a solid standard diet (Na⁺ content 0.4%) and water.

The mice were divided into four groups: 1) control group (control received saline, n = 12); 2) control group that received intra-peritoneal injection of tunicamycin (Tunica, 0.75 mg/kg, 2 injections/week for two weeks, control + Tunica, n = 12); 3) p47phox^{-/-} group that received saline, n = 12 and 4) p47phox^{-/-} group that received intra-peritoneal injection of tunicamycin (Tunica, 0.75 mg/kg, 2 injections/week for two weeks, p47phox^{-/-} + Tunica, n = 12).

At the end of the treatment period, mice were euthanized using an overdose of isoflurane (100%) followed by thoracotomy to ensure death of the animals. This method is chosen because it is the least traumatic. This method is consistent with the recommendation of the Panel on Euthanasia of the American Veterinary Medical Association with the AVMA guidelines on euthanasia.

Organs like aorta, heart and mesenteric resistance arteries (MRA) were harvested immediately, placed in PSS solution (composition in mM: NaCl 118; KCl 4.7; CaCl₂ 2.5; KH₂PO₄ 1.2; MgSO₄ \times 7H₂O 1.2; NaHCO₃ 25 and glucose 11), pH = 7.4 or processed appropriately for further studies.

4.5. Systolic blood pressure (SBP) by tail-cuff method

The systolic blood pressure (SBP) was measured at the end of treatment using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, USA) [51]. Arterial blood pressure measurements were performed at the same time of the day (between 9 a.m. and 11 a.m.) in order to avoid the influence of the circadian cycle, and the value for SBP was obtained by calculating the average of 10 measurements.

4.6. Vascular reactivity

Thoracic aorta and MRA from all groups of mice were carefully cleaned of fat and connective tissue and cut into rings (2 mm in length). Aorta and MRA were mounted in a small vessel dual chamber myograph for the measurement of isometric tension. Two steel wires (25 µmol/L) were introduced through the lumen of the mesenteric resistance artery (MRA) and mounted according to the method described by Mulvany and Halpern [52]. After a 30 min equilibration period in PSS solution bubbled with carbogen at 37 °C and pH = 7.4, arteries were stretched to their optimal lumen diameter for active tension development. After a second 30 min equilibration period, arteries were stimulated with phenylephrine (PE, 10⁻⁵ mol/L) followed by acetylcholine (ACh, 10⁻⁶ mol/L) to assess endothelial function. After pre-constriction with PE (10⁻⁴ mol/L) and steady maximal contraction, cumulative concentration response curves were obtained for ACh and SNP (1 \times 10⁻⁸ to 3 \times 10⁻⁵ mol/L). The arterial lumen diameter and the contraction in response to PE were similar in all groups of mice.

To determine the role of eNOS and NADPH oxidase in impaired endothelium-dependent relaxation in mouse vessels, aorta and MRA were incubated with L-NAME (100 µmol/L) or apocynin (100 µmol/L) for 30 min and then endothelium-dependent relaxation was assessed after pre-contraction with PE.

4.7. Western blot analysis

Mice were sacrificed and heart, aorta and MRA were immediately harvested and frozen in liquid nitrogen and then stored at -80 °C. Endothelial cells and tissue lysates were prepared as previously described [53]. Western blot analysis for P-eNOS, T-eNOS, P-PERK, T-PERK, BiP(GRP78), P-eIF2- α , T-eIF2- α , CHOP, P-p38 and T-p38MAPK, P-ERK1/2 and T-ERK1/2 (1:1000 dilution, Cell Signaling Technology, Inc, USA), and ATF6, HuR or β -actin (1:500 dilution, Santa Cruz Biotechnology, Inc) was performed using specific antibodies as previously described [53].

4.8. RT and real-time PCR analysis

CHOP, ATF6, Nox and eNOS mRNA levels were determined in aorta, MRA and heart samples from all groups as previously described [51]. The RNA extraction from endothelial cells was obtained by using TRIzol (Invitrogen). A total of 1 µg of DNase I-treated RNA was reverse-transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) in a 20 µL reaction. Assays-on-Demand (Applied Biosystems) of TaqMan fluorescent real time PCR primers and probes were used for Chop (Mm00492097_m1), Atf6 (Mm01295317_m1), Nox-2 (Mm01287743_m1), Nox-4 (Mm00479246_m1) and Nos3 (Mm00435217_m1), and 18S rRNA (Hs99999901_s1), which was used as endogenous control in tissues or cells respectively, to normalize the results. Quantitative RT-PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following conditions: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative mRNA levels were determined using the 2^{- $\Delta\Delta$ Ct} method. Results are expressed as the relative expression of mRNA in tunicamycin treated mice compared with untreated mice.

4.9. NADPH oxidase activity assay

Superoxide anion levels generated by NADPH oxidase activity were measured in mouse endothelial cells and in lysates of tissues prepared in sucrose buffer containing KH₂PO₄ 50 mmol/L, EGTA 1 mmol/L, sucrose 150 mmol/L; pH = 7.0 and protease inhibitor cocktail (complete mini, Roche Diagnostics, IN, USA). As a negative control of NADPH oxidase activity, we used cells pre-treated with apocynin (100 µmol/L).

In the case of tissue, lysates were prepared in sucrose buffer containing 1% SDS, 1% NP-40, 1 mmol/L Na_3VO_4 , 1 mmol/L DTT and $1 \times$ protease inhibitor cocktail complete C (Roche) using a Tissue Dounce homogenizer on ice, and aliquots of the homogenates were used immediately or stored at -80°C . To start the assay, a volume of 100 μL of each lysate was used in a total volume of 1 mL PBS buffer preheated at 37°C and O_2^- production was measured in the presence of 5 $\mu\text{mol/L}$ of lucigenin and 100 $\mu\text{mol/L}$ of NADPH. Chemiluminescence was determined every 30 s for 10–15 min in a luminometer (Turner Biosystems 20/20, single tube luminometer) until enzymatic activity reached a plateau. A blank was subtracted from each sample prior to adding lucigenin. The area under the curve was used to quantify chemiluminescence. Data are expressed as relative light units (RLU) normalized to protein content (μg protein).

4.10. Detection of ROS by fluorescence microscopy

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate O_2^- production *in situ* in endothelial cells. Hydroethidine freely permeates cells and is oxidized in the presence of O_2^- to ethidium bromide, which is trapped by intercalation with DNA. Briefly, coronary endothelial cells were plated on special cell chambers for microscopy analysis and incubated with tunicamycin with or without ER stress inhibitors or apocynin for 6 h. Afterwards, cells were loaded with DHE (10 μM) in cell culture medium for 30 min at 37°C and 5% of CO_2 . Images were then acquired with a fluorescence microscope (Nikon Eclipse T300, objective $\times 20$), captured using a digital spot camera (Diagnostic) and processed using Metamorph image analysis software. Non-stimulated endothelial cells were daily imaged in parallel using the same image settings during the course of the study. DHE fluorescence was quantified in individual cell nuclei (number of nuclei 10–20/image/experimental condition) and averaged. Then, we assigned the value 1 to the control situation and expressed the effects of the different drugs as fold increase over control.

4.11. Nitrite assay

Endothelial cells were plated into six-well plates, cultured until confluence and then PBA, Tudca, L-NAME (100 $\mu\text{mol/L}$) or apocynin (100 $\mu\text{mol/L}$) were added 1 h before incubation with tunicamycin for 6 h. Finally, treated cells were incubated for 30 min with ACH (10^{-6} mol/L) and supernatants were harvested. NO production was determined by measuring nitrite content in 225 μL from each well with 75 μL of Griess reagent. Absorbance was measured and nitrite concentration was determined by interpolation of a calibration curve for standard sodium nitrite concentration *versus* absorbance.

4.12. Fluorescence *in situ* hybridization (FISH) RNA

The FISH analysis target was a 476-bp DNA fragment containing bases from 2821 to 3299 of mouse eNOS included in the NCBI Reference Sequence NM_008713.4, which was generated by amplification using as a template the reverse transcribed cDNA from total RNA extracted from heart tissue and the oligonucleotides: eNOSF: 5'-GCCCGGTACTACTCTGTCA-3' and eNOSR: 5'-CTGTCTCAGGAGGTCTTGC-3'. The 476 bp amplicon was purified from agarose gel and cloned into pGEM-T vector (Promega, Madison, WI, USA) and verified subsequently by sequencing using the T7 primer. Two clones of p-GEM-T-eNOS (476 bp) construct containing the insert in opposite directions were picked in order to synthesize the antisense probe for eNOS mRNA detection and the sense probe as a negative control for hybridization assay.

The probes were synthesized and labeled with Alexa Fluor 594 dye using the FISH Tag RNA kit (Molecular probes, Invitrogen). The RNA probe synthesis and labeling with fluorescent dye, hybridization and detection of fluorescent signals were performed as described by the kit protocol and following the manufacturer's instructions. The

fluorescence intensity was quantified in captured images, taken with a fluorescent microscope (Nikon, Tokyo, Japan) with $\times 40$ magnification, using the software NIS-Elements BR 3.0 (Nikon, Tokyo, Japan).

4.13. Drugs

Phenylbutyric acid (PBA), phenylephrine hydrochloride, acetylcholine chloride, sodium nitroprusside, U46619, apocynin, NADPH and L-NAME were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tauroursodeoxycholic acid (Tudca), tunicamycin and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) were purchased from Calbiochem (USA and Canada). Stock solutions of drugs were prepared in ultrapure water or in DMSO following the manufacturer's instructions, stored at -20°C and appropriate dilutions were made on the day of the experiment.

4.14. Statistical analysis

Data are expressed as mean \pm SEM. Concentration–response curves were analyzed using the GraphPad Prism 4.0 software (GraphPad, USA). One-way or 2-way ANOVA was used to compare each parameter when appropriate. Values of $P \leq 0.05$ were considered significant. Differences between specified groups were analyzed using the Student's *t* test (two-tailed) comparing the two groups with $P \leq 0.05$ considered as statistically significant.

Sources of funding

This work was supported by the National Institutes of Health (1R01HL095566; PI: Dr. Matrougui) and (5R01HL097111; PI: Dr. Trebak). Modar Kassin is supported by the post-doctoral fellowship from the AHA (16850060).

Disclosures

None.

Acknowledgements

We thank Dr. Ryosuke Satou (Hypertension and Renal Center of Excellence, Tulane University, New Orleans) for providing the protocol to run the FISH analysis assay.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2014.02.009>.

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