



# Laminar Flow Inhibits ER Stress-Induced Endothelial Apoptosis through PI3K/Akt-Dependent Signaling Pathway

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Atherosclerosis preferentially involves in prone area of low and disturbed blood flow while steady and high levels of laminar blood flow are relatively protected from atherosclerosis. Disturbed flow induces endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). ER stress is caused under stress that disturbs the processing and folding of proteins resulting in the accumulation of misfolded proteins in the ER and activation of the UPR. Prolonged or severe UPR leads to activate apoptotic signaling. Recent studies have indicated that disturbed flow significantly up-regulated p-ATF6 $\alpha$ , p-IRE1 $\alpha$ , and its target spliced XBP-1. However, the role of laminar flow in ER stress-mediated endothelial apoptosis has not been reported yet. The present study thus investigated the role of laminar flow in ER stress-dependent endothelial cell death. The results demonstrated that laminar flow protects ER stress-induced cleavage forms of PARP-1 and caspase-3. Also, laminar flow inhibits ER stress-induced p-eIF2 $\alpha$ , ATF4, CHOP, spliced XBP-1, ATF6 and JNK pathway; these effects are abrogated by pharmacological inhibition of PI3K with wortmannin. Finally, nitric oxide affects thapsigargin-induced cell death in response to laminar flow but not UPR. Taken together, these findings indicate that laminar flow inhibits UPR and ER stress-induced endothelial cell death via PI3K/Akt pathway.

**Keywords:** atherosclerosis, shear stress, endothelial cells, ER stress, unfolded protein response

## INTRODUCTION

Atherosclerosis develops in area of acute curvatures, branch points and bifurcations that are associated with disturbed flow resulting low and reversal vascular wall shear stress (Malek et al., 1999). As a result, disturbed flow induces an inflammatory, thrombotic state and endothelial dysfunction characterized by high expression of cell adhesion molecules and production of inflammatory cytokines. In contrast, steady laminar flow negatively modulates atheroprone responses in a variety of cellular functions including inflammation, apoptosis, angiogenesis, and coagulation (Guo et al., 2007; Nigro et al., 2011). Many previous studies have shown that high, unidirectional, and steady laminar flow maintains endothelial functional integrities against atherosclerosis. Therefore, hemodynamic shear stress is critical for a variety of biologic processes in endothelium (Chen et al., 1999).

Endoplasmic reticulum (ER) stress is caused under stress such as alterations in redox changes, depletion of ER calcium, glucose depletion, hypoxia, and viral infection. ER stress compromises the ability to produce folding proteins and results in the accumulation of misfolded protein in the ER environment and activation of unfolded protein response (UPR) (Brown and Naidoo, 2012; Schroder and Kaufman, 2005). The UPR is mediated by three ER stress-receptors: PKR-like ER kinase (PERK), inositol-requiring protein 1 $\alpha$  (IRE-

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1 $\alpha$ ) and the transcription factor activating transcription factor 6 (ATF6). These UPR signaling pathways coordinate to restore ER homeostasis (Greene and McElvaney, 2010). However, prolonged or severe UPR leads to activate apoptotic signaling in mammalian cells. ER stress-mediated apoptosis is regulated largely by C/EBP homologous protein (CHOP), caspase-12, and JNK pathway (Szegezdi et al., 2006).

It has been suggested that ER stress and the UPR can influence development of atherosclerosis, vascular inflammation, and apoptosis (Tabas, 2010). In the atheroprone regions, endothelium revealed the upregulation of genes linked to ER proteins processing ER stress and the UPR (Lenna et al., 2014). Previous studies have shown that disturbed flow significantly induces ER chaperone-binding protein, p-ATF6, IRE1 $\alpha$ , and its target spliced X-box binding protein-1 (XBP-1) (Davies et al., 2013). Recent study demonstrated that disturbed flow increased activation of XBP-1 expression and splicing, resulting in the induction of apoptosis in endothelial cells (ECs) (Zeng et al., 2009). *In vivo*, the endothelium of atheroprone regions with disturbed flow showed upregulation of genes associated with ER stress. Overall, these studies suggest that disturbed flow mediates partial activation of the UPR and that chronic ER stress is a signature for atherosclerotic endothelial phenotype *in vivo*. However, little is known about the role of laminar flow in UPR and endothelial apoptosis in response to ER stress.

In the present study, we demonstrate that laminar flow inhibits the UPR and ER stress-dependent endothelial apoptosis. Especially, laminar flow inhibited UPR and ER stress-induced apoptosis via PI3K/Akt signaling pathway.

## MATERIALS AND METHODS

### Cell culture and laminar flow

Human umbilical vein endothelial cells (HUVECs) were cultured in medium M200 (Gibco) containing 5% fetal bovine serum (FBS) and endothelial growth factor supplement (LSGS; Cascade biologics, USA) on 0.2% gelatin coated cell culture dishes. Confluent HUVECs cultured in 100mm dishes were exposed to laminar unidirectional flow (12 dynes/cm<sup>2</sup>) for 24 h using a cone flow system in a 5% CO<sub>2</sub> humidified incubator at 37°C, as described previously (Fleming et al., 1998).

### Reagents and antibodies

Thapsigargin (TG; T9033), tunicamycin (TM; T7765), wortmannin (W1628) and Dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (USA). Compound C (171260), an AMPK inhibitor, was provided by Calbiochem (La Jolla, USA). Antibodies were purchased from the following vendors: KDEL (GRP94, GRP78; ADI-SPA-827) and Cleaved Casp-3 (31A1067) (Enzo Life Sciences, Germany); ATF4 (sc-200) and GADD153 (CHOP; sc-793) (Santa Cruz, CA); PARP-1 (9524), p-eIF2 $\alpha$  (9721), Akt (9272), p-Akt (4056) and p-AMPK (4188) (Cell signaling Technology, USA); XBP-1s (619501) (BioLegend, USA) and  $\alpha$ -tubulin (T5168) (Sigma-Aldrich, USA).

### TUNEL assay

HUVECs were exposed to flow (12 dynes/cm<sup>2</sup>) for 24 h and then treated with 1  $\mu$ M TG or 5  $\mu$ M TM for 24 h. Cells were washed with PBS twice and then fixed with 4% buffered paraformaldehyde for 30 min and permeabilized for 5 min at room temperature. Cells were then blocked with 5% normal goat serum in PBS-0.05% Tween-20 (Sigma-Aldrich), followed by incubation with TUNEL reaction mixture (11684795910) (Roche Diagnostics Australia, Austria) 60 min at 37°C. Nuclear were stained with DAPI, and then TUNEL positive cells were observed by using immunofluorescence microscope.

### Western blotting

HUVECs were lysed with radioimmunoprecipitation assay lysis buffer supplemented with 1 mmol/l phenylmethylsulfonyl fluoride and 0.01 mmol/l protease inhibitor cocktail. Cell lysates were incubated on ice for 15 min and then centrifuged at 15,000 g x g for 10 min at 4°C. Protein concentration in samples was determined from centrifuged supernatant by using a Bradford assay. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were immunoblotted with primary antibodies (1:1,000), followed by immunoblotting with corresponding secondary antibodies (1:5,000). Signals were visualized by using electrochemiluminescence detection reagents (EMD Millipore, USA), according to the manufacturer's protocol.

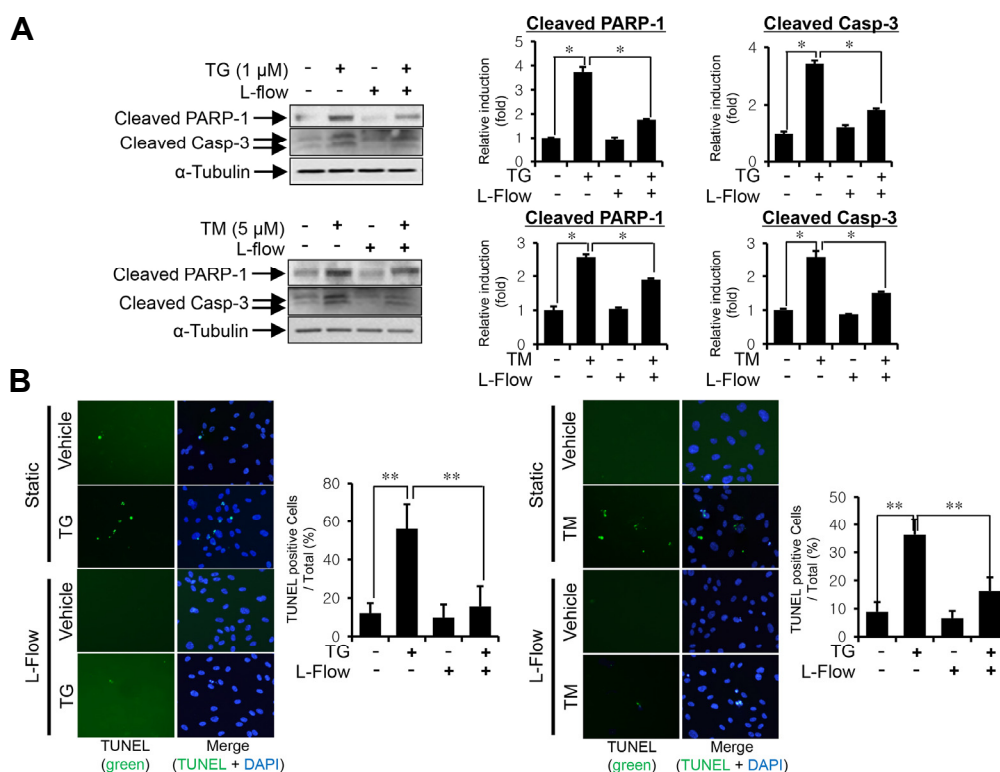
### Statistical analysis

Data in the bar graphs are presented as mean  $\pm$  S.D. for three independent experiments. Statistical significance of difference was performed with Student's *t*-test, and *p* < 0.05 was considered statistically significant. *p* values less than 0.05 are indicated by \*, and *p* values less than 0.01 are indicated by \*\*.

## RESULTS

### Laminar flow inhibits ER stress-induced endothelial cell death

It is well known that prolonged ER stress leads to inflammatory signaling, and unmitigated and excessive stress leads to apoptotic cell death (Schroder and Kaufman, 2005). In contrast, many studies have reported that laminar flow has anti-inflammatory and anti-apoptotic effect (Kim et al., 2012; Kuchan et al., 1994; Li et al., 2005). However, the role of laminar flow on ER stress-dependent endothelial cell death has not been studied. Therefore, we first investigated if laminar flow affects ER stress-induced endothelial cell death, which were exposed to 12 dynes/cm<sup>2</sup> flow for 24 h and then treated with thapsigargin (TG) or tunicamycin (TM), well-known ER stress inducers. As shown in Fig. 1A, TG- and TM-induced cleaved forms of PARP-1 and caspase-3 were significantly inhibited by laminar flow. In addition, we also confirmed the effect of laminar flow on ER stress-induced endothelial apoptosis with TUNEL assay in HUVECs. Consistent with the immunoblotting data, laminar flow markedly inhibited TG- and TM-induced TUNEL-positive cells (Fig. 1B).



**Fig. 1. Laminar flow inhibits ER stress-induced endothelial apoptosis.** HUVECs were treated with 1 μM thapsigargin (TG) or 5 μM tunicamycin (TM) for overnight after exposure to laminar flow (L-flow, 12 dynes/cm<sup>2</sup>) for 24 h. (A) Protein level was analyzed by immunoblotting with specific antibodies against PARP-1, Cleaved Casp-3 and α-Tubulin. Bar graphs present the densitometric quantification of Western blot bands. Results are expressed as means SDs and are representative of three independent experiments. \**p* < 0.05; \*\**p* < 0.01 compared with control (n = 3). (B) Representative photomicrographs showing TUNEL (apoptotic, green), DAPI (nuclei, blue) signals and their merged images (original magnification ×400). Bar graphs present number of TUNEL positive cells from total endothelial cell counted. Results are expressed as mean ± SDs and are representative of three independent experiments. \**p* < 0.05; \*\**p* < 0.01 compared with control (n = 3).

These data suggest that laminar flow inhibits ER stress-induced endothelial apoptosis.

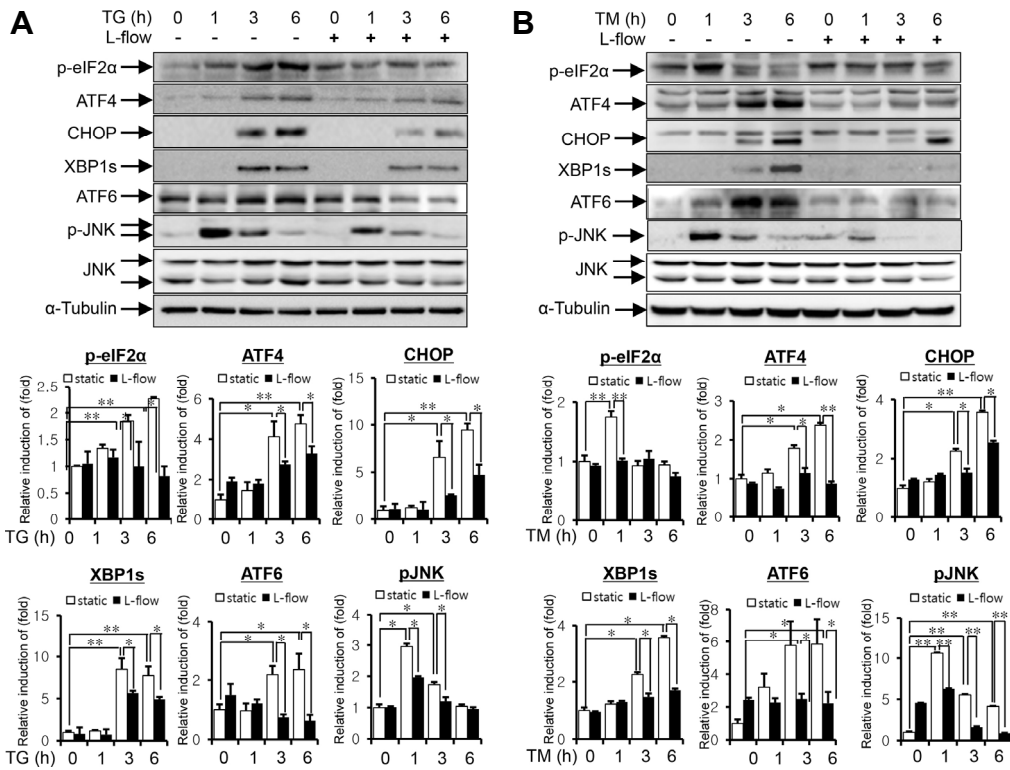
### Effect of laminar flow on ER stress inducers-induced unfolded protein response

Accumulation of unfolded proteins in the ER initiates IRE1α, ATF6, and PERK cascades, leading to a transcriptional/translational response known as unfolded protein response (UPR) (Tabas, 2010). We thus examined whether laminar flow affects TG- or TM-induced UPR in HUVECs. To determine the specificity of TG- or TM-induced UPR, HUVECs exposed to laminar flow for 24 h were treated with TG or TM for 1, 3, and 6 h. As shown in Fig. 2, TG (and TM) activated eIF2α-ATF4-CHOP pathway, spliced XBP-1, ATF6 and JNK pathway and these inductions were inhibited by laminar flow. These results indicate that laminar flow inhibits UPR signaling pathway.

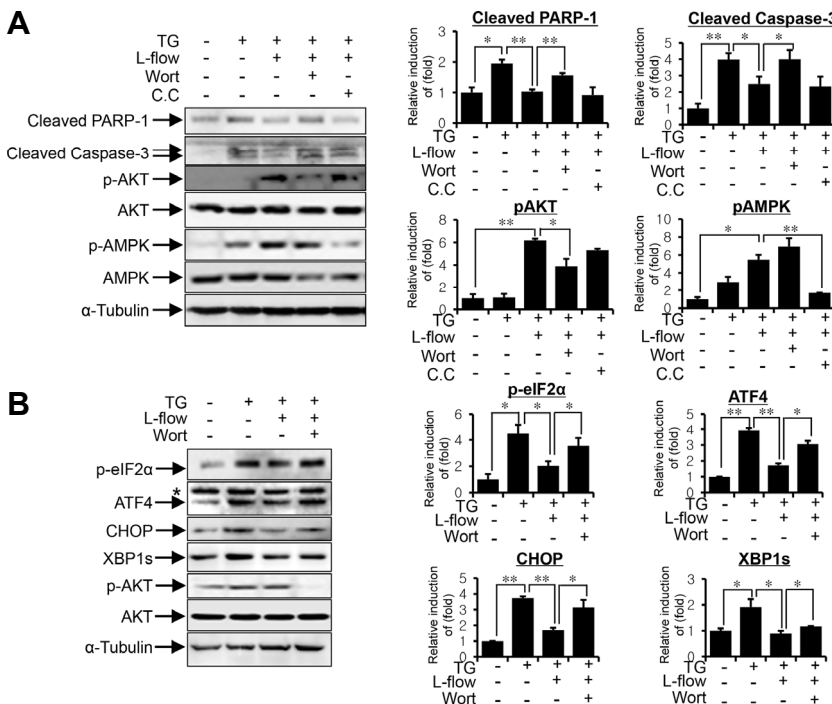
### Laminar flow inhibits ER stress-induced UPR and endothelial apoptosis through PI3K/Akt signaling pathway

We next sought to determine the molecular mechanisms by which laminar flow regulates TG-induced endothelial cell

death via laminar flow-dependent signaling pathways. It is well known that laminar flow activates various kinases including Akt and AMPK (Guo, Chien et al., 2007; Yan et al., 1999). We thus determined if laminar flow regulates ER stress-induced apoptotic cell death by Akt and AMPK. Initially, HUVECs were exposed to laminar flow 24 h and then were treated with wortmannin (a specific inhibitor of PI3K) or Compound C (a specific inhibitor of AMPK) for 1 h before incubation with 1 μM TG for 24 h. As shown in Fig. 3A, laminar flow strongly inhibited TG-induced cleaved forms of PARP-1 and Caspase-3, but these were increased by the addition of wortmannin. Compound C does not affect laminar flow-mediated inhibition of endothelial apoptosis induced by TG. Thus it was examined whether TG-induced UPR could be regulated via PI3K/Akt pathway in HUVECs. As shown in Fig. 3B, laminar flow inhibits TG-induced protein expression of eIF2α-ATF4-CHOP pathway and spliced XBP-1, but these inductions were not blocked by the addition of wortmannin. Therefore, laminar flow inhibits TG-induced UPR via PI3K-Akt signaling. These data suggest that laminar flow inhibits ER stress-induced endothelial apoptosis via PI3K/Akt pathway.

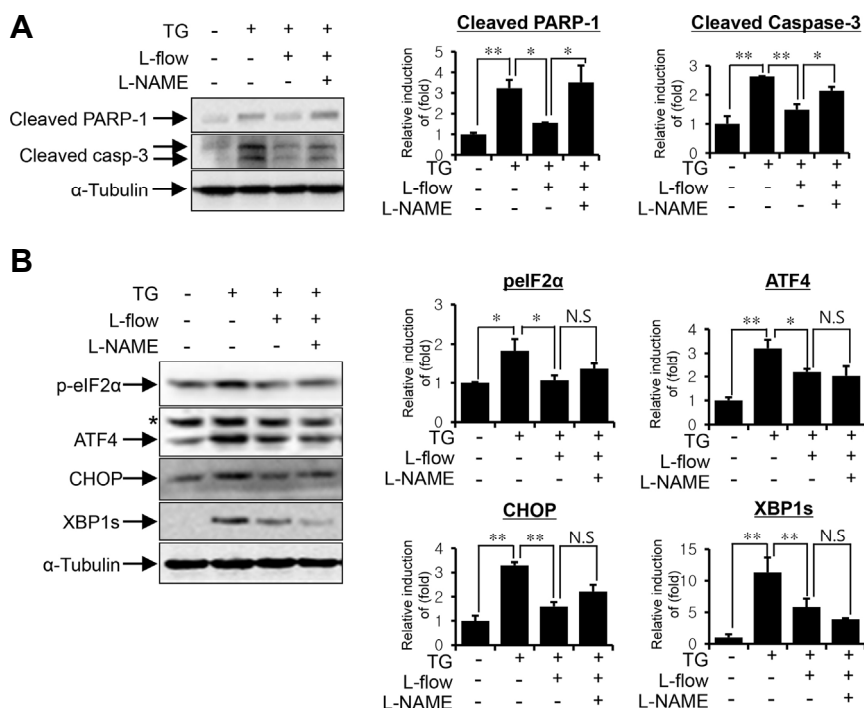


**Fig. 2. Laminar flow inhibits TG- or TM-induced unfolded protein responses.** HUVECs were treated with 1 μM TG or TM for 1, 3, or 6 h after exposure to L-flow (12 dynes/cm<sup>2</sup>) for 24 h. Protein level was analyzed by immunoblotting with specific antibodies against spliced-XBP1, ATF4, CHOP, p-eIF2α, ATF6, pJNK, JNK and α-Tubulin. Bar graphs present the densitometric quantification of western blot bands. Results are expressed as means SDs and are representative of three independent experiments. \*p < 0.05; \*\*p < 0.01 (n = 3).



**Fig. 3. Laminar flow inhibits TG-induced endothelial apoptosis and UPR via PI3K/Akt pathway.** (A) HUVECs were exposed to L-flow (12 dynes/cm<sup>2</sup>) for 24 h. After 24 h, HUVECs were incubated with 10 nM wortmannin (Wort) or 2 μM Compound C (C.C.) for 1 h before treatment with 1 μM TG for 24 h. Protein level was analyzed by immunoblotting with specific antibodies against cleaved PARP-1, Cleaved Caspase-3, p-AKT, AKT, p-AMPK, AMPK and α-Tubulin. Bar graphs present the densitometric quantification of western blot bands. ANOVA: \*p < 0.05; \*\*p < 0.01. (B) HUVECs were exposed to laminar flow (12 dynes/cm<sup>2</sup>) for overnight, then incubated with wortmannin for 1 h before treatment with 1 μM thapsigargin for 3 h. Protein level was analyzed by immunoblotting with specific antibodies against spliced-XBP1, ATF4, CHOP, p-eIF2α and α-Tubulin. The asterisk (\*) indicates a non-specific band detected by the anti-ATF4 antibody. Bar graphs present the densitometric quantification of western blot bands. Results are expressed as means SDs and are representative of three independent experiments. \*p < 0.05; \*\*p < 0.01 (n = 3).





**Fig. 4. Nitric oxide is involved in laminar flow-mediated inhibition of TG-induced endothelial apoptosis, but not UPR.** (A) HUVECs were exposed to L-flow (12 dynes/cm<sup>2</sup>) for 24 h, then incubated with 300 μM L-NAME for 1 h before treatment with 1 μM TG for 3 h. Protein level was analyzed by immunoblotting with specific antibodies against PARP-1 Cleaved Caspase-3 and α-Tubulin. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: \**p* < 0.05; \*\**p* < 0.01. (B) Protein level was analyzed by immunoblotting with specific antibodies against spliced-XBP1, ATF4, CHOP, p-eIF2α and α-Tubulin. The asterisk (\*) refers to a non-specific band detected by the anti-ATF4 antibody. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: \**p* < 0.05; \*\**p* < 0.01; N.S (Not significant) (n = 3).

### Nitric oxide regulates laminar flow-mediated inhibition of TG-induced endothelial apoptosis, but not UPR

Akt, a multifunctional serine/threonine-specific protein kinase, is a major downstream effector of PI3K. It is also an upstream regulator of endothelial nitric oxide (NO) synthase (eNOS) activity. Studies *in vitro* have shown that Akt can directly phosphorylate eNOS and activate the enzyme, leading to NO production (Shiojima and Walsh, 2002). In cultured endothelial cells, eNOS is rapidly phosphorylated after the application of fluid shear stress (Davis et al., 2004; Xiao et al., 1997). We next examined whether eNOS-derived NO production was involved in UPR. HUVECs were exposed to laminar flow 24 h and then were treated with or without the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) for 1 h before incubation with 1 μM TG for 24 h. As shown in Fig. 4A, TG-induced cleaved forms of PARP-1 and caspase-3 were prevented by laminar flow, but these inhibitions were restored by the addition of L-NAME. In addition, it was evaluated if TG-induced UPR could be regulated via eNOS-NO pathway in HUVECs. TG-induced protein expression of eIF2α-ATF4-CHOP pathway and spliced XBP-1 was reduced under laminar flow at 3 h, but these inhibitions were not affected by L-NAME (Fig. 4B). These results suggest that NO regulates laminar flow-mediated inhibition of TG-induced endothelial apoptosis, but not UPR.

### DISCUSSION

It has been established that ER stress plays a critical role in atherosclerosis. Especially, prolonged activation of the UPR and chronic ER stress are linked to atherosclerotic regions, the altered expression of pro-inflammatory NF-κB and ox-

idant pathways, and apoptosis. In the present study, we found that laminar flow prevents ER stress-induced endothelial apoptosis by PI3K/Akt signaling pathway. Also, laminar flow reduced the protein levels of the eIF2α, ATF4, CHOP, spliced XBP-1, ATF6 and JNK pathway. Interestingly, nitric oxide (NO) is involved in laminar flow-mediated inhibition of TG-induced cell death, but not UPR. Collectively, our results indicated that laminar flow inhibits UPR and ER stress-dependent endothelial apoptosis via PI3K/Akt signaling pathway.

Vascular ECs are directly subjected to changes in vascular wall shear stress, which affects the function of EC, such as, vascular remodeling, coagulation, proliferation, apoptosis, migration, and permeability (Li et al., 2005). ECs transduced responses to laminar flow through mechano-sensors (Chen et al., 1999; Hahn and Schwartz, 2009). Laminar flow mediates Krüppel-like factor 2 that induces endothelial nitric oxide synthase (eNOS) expression and mediates atheroprotective signaling pathway (van Thienen et al., 2006). Also, laminar flow activates NF-E2 related factor 2, which contributes to atheroprotective effects, in an ERK5-dependent manner (Kim et al., 2012). In contrast, low and disturbed blood flow induces endothelial inflammation and apoptosis leading to endothelial dysfunction and subsequent development of atherosclerosis (Nigro et al., 2011; Takabe et al., 2011). There are numerous pieces of evidence that the endothelial cells in response to disturbed flow reveal the upregulation of genes associated with ER proteins processing ER stress and the UPR (Lenna et al., 2014). Recently, disturbed flow was found to directly cause sustained GRP78, p-ATF6α, p-IRE1α, spliced XBP1 and CHOP in the ER stress response (Chung et al., 2015; Davies et al., 2013). Many studies have shown

that inhibition of ER stress induced by disturbed flow has a strategic importance in the prevention and treatment of atherosclerosis. However, there is little evidence regarding any direct correlations between laminar flow and ER stress in ECs. Herein, our data showed that laminar flow inhibited UPR pathways (Fig. 2). In our results, laminar flow inhibited protein levels of eIF2 $\alpha$ , ATF4, CHOP, spliced XBP-1, and ATF6.

Our data suggested that laminar flow inhibits ER stress-induced endothelial apoptosis and UPR pathways. However, it is not known whether laminar flow is able to block the specific target for ER stress signaling pathway. Here, we used the effectiveness of two different strong inducers of ER stress; tunicamycin (TM) and thapsigargin (TG). TM, a homologous nucleoside antibiotics, inhibits N-linked glycosylation (N-glycans) that disturbs glycoprotein synthesis thereby inducing protein unfolding in eukaryotic cells (Ettlinger et al., 1986; Vai et al., 1987). TG is a non-competitive cell permeable inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA). TG increases cytosolic intracellular calcium concentration by blocking the ability of the cell to calcium pump into the sarcoplasmic and endoplasmic reticulum (Osowski and Urano, 2011). Although TM and TG induce UPR via different mechanisms, signaling pathways of UPR induced by both chemicals are quite similar. The precise mechanism underlying the regulation of ER stress by laminar flow needs to be further investigated in the future studies.

Prolonged or severe UPR leads to inflammatory signaling and apoptosis in mammalian cells. Apoptosis in response to ER stress is mainly regulated through induction of CHOP, caspase-12 and JNK pathway (Szegezdi et al., 2006). Furthermore, it has been reported that disturbed flow induces endothelial apoptosis and atherosclerosis via XBP1 splicing and sustained activation (Zeng et al., 2009). In particular, it was known that CHOP is a key regulator for ER stress-dependent apoptosis pathway (Zinszner et al., 1998). The depletion of CHOP with siRNA against CHOP inhibited ER stress-induced apoptosis in ECs and elevated cell viability (Fujii et al., 2008). Interestingly, it was recently found that methylglyoxal induces ER stress and CHOP is responsible for methylglyoxal-induced endothelial apoptosis. In the present study, laminar flow prevented ER stress-induced endothelial apoptosis. These effects are abrogated by pharmacological inhibition of PI3K/Akt with wortmannin and L-NAME, an inhibitor of NOS (Fig. 1, 3, 4). These results suggest that laminar flow inhibits ER stress-induced endothelial apoptosis via PI3K/Akt/eNOS signaling pathway *in vitro*.

In conclusion, we found that laminar flow inhibits UPR and ER stress-induced endothelial apoptosis via PI3K/Akt signaling pathway. This study suggests that laminar flow provides an atheroprotective effect in response to ER stress-mediated atherosclerosis.

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