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## Post-Shock Mesenteric Lymph Induces Endothelial NF- $\kappa$ B Activation

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### Abstract

**Background**—Post-hemorrhagic shock mesenteric lymph (PSML) has been shown to activate pulmonary endothelial cells, and cause lung injury. Although multiple mediators may be involved, most of these effects are mediated by NF- $\kappa$ B activation. Degradation of the inhibitor of kappa B (I $\kappa$ B) is a key regulatory step in the activation of NF- $\kappa$ B. We therefore hypothesized that PSML would cause I $\kappa$ B degradation with subsequent NF- $\kappa$ B phosphorylation and nuclear translocation.

**Methods**—Mesenteric lymph was collected from male rats before shock and each hour after shock for up to 3 hours. (n=5) Buffer (control), buffer + 10% (v/v) lymph, or buffer + TNF (10ng/ml) was incubated with human pulmonary endothelial cells for 30 minutes and then lysed. Immunoblots of lysates were probed for I $\kappa$ B and phospho-p65. Immunohistochemistry was performed on cells grown on glass slides, and then treated as above with the 3<sup>rd</sup> PSML sample. Cells were fixed and then probed for p65. Statistical analysis was performed with student's t-test and analysis of variance with significance set at p< 0.05.

**Results**—Western blots of cell lysates for I $\kappa$ B demonstrated a steady decrease in total I $\kappa$ B with each lymph sample. Phosphorylation of NF- $\kappa$ B, p65 component, steadily increased with each PSML sample, with a maximum reached during the 3<sup>rd</sup> PSML sample, which also significantly increased translocation of NF- $\kappa$ B to the nucleus.

**Conclusion**—Post-shock mesenteric lymph bioactivity is mediated by pathways which involved I $\kappa$ B degradation. These pathways offer novel targets for clinical intervention to prevent the distal organ injury caused by postinjury hemorrhagic shock.

### Keywords

Mesenteric; Lymph; Shock; I $\kappa$ B; NF- $\kappa$ B

### Introduction

Gut ischemia and reperfusion (I/R) is central to the pathogenesis of multiple organ failure after hemorrhagic shock. Although early work focused on the portal circulation as the conduit for

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biological mediators, more recently, mesenteric lymph has been identified as the mechanistic link between gut I/R and neutrophil-mediated acute lung injury.[1-3]

Both *in vivo* and *in vitro* work have focused on the role of neutrophils (polymorph nuclear leukocytes [PMNs]) in mediating lung injury after hemorrhagic shock. We have shown that in a rodent model of hemorrhagic shock, PMN adhesion molecule expression and PMN lung accumulation are diminished with lymph diversion.[4]. Further corroborative work *in vitro* has shown that isolated PMNs exposed to post-shock mesenteric lymph (PSML) increase surface adhesion molecule expression and increase priming for superoxide production.[5]

Although PMNs play a key role in lung injury [6], activation of the pulmonary vascular endothelium is an essential component.[6] Deitch et al. have found that human umbilical vein endothelium (HUVECs) exposed to PSML undergoes cytotoxicity and increased permeability.[2,7] We have shown that pulmonary microvascular endothelial cells (HMVECs) profoundly up regulate surface expression of ICAM after exposure to PSML.[8]

This increase in ICAM-1 has prompted us to examine the role of NF- $\kappa$ B mediated pathways, since NF- $\kappa$ B is a key regulator of the ICAM-1 gene as well as several other pro-inflammatory genes.[9] Components of the active NF- $\kappa$ B are sequestered in the cytosol by specific proteins of the I $\kappa$ B family.[10,11] Upon phosphorylation of I $\kappa$ B, NF- $\kappa$ B components are released and I $\kappa$ B is degraded.[12] Liberation of the NF- $\kappa$ B components allows the molecules to translocate to the nucleus, where they dimerize and initiate transcription of key genes, including ICAM-1. [9,12] Recently, phosphorylation of NF- $\kappa$ B, specifically phosphorylation at the serine 536 site of p65 RelA, by I $\kappa$ K has emerged as a novel mechanism of transcriptional activation.[13]

We therefore hypothesized that endothelial activation by PSML would involve degradation of I $\kappa$ B with subsequent phosphorylation and translocation of NF- $\kappa$ B to the nucleus in HMVECs.

## Methods

### Animals

Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at Denver Health Medical Center, Denver, Colorado. Adult male Sprague-Dawley rats (Harlan Laboratories, Madison, WI) weighing 320-370 g were housed under barrier-sustained conditions and allowed free access to chow and water before use. All animals were maintained in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*.

### Materials

All materials were purchased from Sigma-Aldrich Corp. (St. Louis, Mo), unless otherwise specified. All tubing was obtained from Fisher Scientific. Pentobarbital sodium was purchased from Abbott Labs (North Chicago, IL) and heparin from American Pharmaceutical Partner Inc. (Schaumburg, IL). Antibodies against I $\kappa$ B $\alpha$  (C-21) and p65 (C-20) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against actin (AC-40) were purchased from Abcam, Inc. Antibodies against phosph-S536-p65 (93H1) were obtained from Cell Signaling Technology, Inc.

### Hemorrhagic shock model

Rats were anesthetized with pentobarbital 50 mg/kg intraperitoneally. The right femoral artery and vein were cannulated with PE-50 tubing. Blood pressure was continuously monitored with a Pro-Paq device (Protocol Systems, Beaverton, Ore). Core body temperature was measured with a rectal thermometer and maintained at 37°C with a heating lamp. A midline laparotomy

was performed to expose the common mesenteric lymphatic duct, which was cannulated with 0.02-in silicone tubing. The tubing was secured with a 7-0 prolene suture and exteriorized via a right flank stab wound. The abdomen and groin were then closed with 4-0 monofilament nylons.

Lymph was collected from rats for 60 minutes before (preshock), during nonlethal hemorrhagic shock (mean arterial pressure 30 mm Hg  $\times$  40 min), and for each hour after shock up to 3 hours (1<sup>st</sup> hour PSML, 2<sup>nd</sup> hour PSML, 3<sup>rd</sup> hour PSML). Animals were resuscitated during the initial 2 hours after shock with half shed blood volume + 4x shed blood volume as normal saline. Lymph was centrifuged at 6,000 g  $\times$  10 minutes at 4°C to remove all cellular components. The supernatant was removed, aliquoted and snap frozen for subsequent analysis.

### Cell Culture

Human microvascular endothelial cells (HMVECs) were obtained from Cambrex Bio Science Inc. (Rockland, ME) and cultured in endothelial growth medium. (Cambrex Bio Science, Inc.) Cells were grown to near confluence at 37°C in 5% CO<sub>2</sub>. After achieving near confluence, cells were incubated with medium, TNF (10ng/ml), or lymph (10% v/v) for 30 minutes at 37°C. To minimize animal-to-animal variability, all post-shock mesenteric lymph samples were compared to the same animal's preshock sample. Supernatants were removed, and cells were lysed in digestion buffer containing SDS. Cell lysates were heated at 100 °C for 10 minutes, aliquoted and frozen for immunoblot analysis.

### Immunoblot Staining

Equal amounts of whole cell lysates were loaded into 8-16% acrylamide gels (Pierce Biotechnology, Inc.), fractionated with SDS-PAGE, and then electrotransferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% nonfat milk solution and immunoprobed. Bound antibodies were detected with enhanced chemoluminescence detection system (Pierce Biotechnology, Inc.). Densitometry was performed using Image J software program (National Institutes of Health, Bethesda, MD). All plots were normalized to actin and results expressed as a percent of control.

### Immunofluorescent microscopy

HMVECs were grown on Lab-Tek Chamber Slides (Nalgene Nunc International, Rochester, NY, USA) and treated with buffer, TNF (ng/ml) or 3<sup>rd</sup> hour PSML (10% v/v) for 45 minutes. The slides were fixed and permeabilized with 70% acetone / 30% methanol solution at -20°C. Non-specific antigen sites were blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS for 60 minutes at room temperature. Slides were then probed with rabbit polyclonal anti-p65 antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in 1% BSA/PBS for 1 hour. Negative control slides were incubated with isotype (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Following three PBS washes, the slides were incubated for 1 hour at room temperature with donkey anti-rabbit Cy3-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and bis-benzimide nuclear stain (Sigma-Aldrich, St. Louis, MO, USA).

Images were acquired with a Zeiss Axiovert fitted with a Cooke CCD SensiCam using a Chroma Sedat with single excitation and emission filters and a multiple bandpass dichroic and Sutter filter control. The two channel images were then digitally processed using Intelligent Imaging Innovations Slidebook 4.1 software (Intelligent Imaging Innovations, Inc., Denver, CO). Nuclear stains were masked to measure the relative p65 mean fluorescence intensity (MFI) within the nuclei. The MFI of at least 70 cells from each experimental group were analyzed by ANOVA using JMP 5.0.1 software. Statistical significance was accepted as *p* less than 0.001.

## Statistical Analysis

Data were analyzed by repeated measures ANOVA using Proc GLM, followed by the Tukey's multiple comparisons test (SAS Tutorials for Statistical Data Analysis at <http://www.stat.tutorials.com/SAS/>). Contrasts were used to test for linear trends between hours pre/post shock and I $\kappa$ B degradation as well as hours pre/post shock and the phosphorylation state of the p65 component. A *p*-value of less than 0.05 is considered statistically significant.

## Results

### I $\kappa$ B Degradation

In the first set of experiments, PSML was incubated with HMVECs to determine its effects on I $\kappa$ B degradation. (Figure 1.) The I $\kappa$ B degradation level varied significantly with the different PSML samples (Repeated measures ANOVA  $p=0.0092$ ); statistical significant differences were detected between the pre-shock levels and the 2<sup>nd</sup>, 3<sup>rd</sup> and combined post-shock PSML sample (Tukey's multiple comparison test,  $p<0.05$ ). The 3<sup>rd</sup> postshock hour demonstrated the maximal degradation of I $\kappa$ B over control levels with approximately 40% reduction in I $\kappa$ B. Interestingly, combining each hour of lymph and then adding this combined PSML ("Postshock") to HMVECs showed a similar amount of I $\kappa$ B degradation as the 3<sup>rd</sup> postshock hour. A significant linear trend was detected between time (pre, post 1, post 2, post 3 hours) and the I $\kappa$ B degradation level (contrast for linear trend,  $p=0.0051$ ).

### Phosphorylation of NF- $\kappa$ B

Phosphorylation of NF- $\kappa$ B at serine 536 by I $\kappa$ K is a novel pathway. Phosphorylation at Ser536 profoundly increases its translocation and activation.[13] We therefore measured the phosphorylation of NF- $\kappa$ B by assessing the phosphorylation state of the p65 component. (Figure 2.) TNF stimulation demonstrated a 2-fold increase in p65 phosphorylation over control cells. The p65 phosphorylation level varied significantly with the different PSML samples (Repeated measures ANOVA  $p=0.0057$ ). The maximal phosphorylation of p65 occurred after stimulation with the 2<sup>nd</sup> and 3<sup>rd</sup> hours of PSML (Tukey's multiple comparison test,  $p<0.05$ ). In addition, the phosphorylation of p65 observed with the combined PSML sample was significantly higher than the level observed with pre-shock, and the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> post-shock hour samples. A significant linear trend was detected between time (pre, 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> post-shock hour samples) and the phosphorylation of p65 level (contrast for linear trend,  $p=0.0005$ ).

### NF- $\kappa$ B Translocation

Functional activity of NF- $\kappa$ B is dependent on translocation of NF- $\kappa$ B to the nucleus of cells. [11] Using IHC, we were able to detect the amount of NF- $\kappa$ B in different compartments of the cells. (Figure 3a and 3b.) We chose to focus solely on the 3<sup>rd</sup> hour PSML hour.

Control cells demonstrated a nuclear mean fluorescence intensity for p65 of 600. TNF stimulated cell demonstrated a marked increase in p65 translocation with an MFI of approximately 1500. The 3<sup>rd</sup> hour PSML at 10% also induced a significant translocation of p65 to the nucleus, reaching an MFI of approximately 880. This represents a 45% increase in nuclear p65 over control cells.

## Discussion

The gut has been invoked as the mechanistic link between post-traumatic shock and ensuing MOF, which remains a major cause of morbidity and mortality after major trauma. Work from the Deitch lab [1,7,15-19] as well as work from our group [4,5,8,20,21] has confirmed that PSML is the mechanistic link between gut I/R and MOF.

Our investigation has focused primarily on the effects of PSML on neutrophils and endothelium. *In vitro*, PSML primes neutrophils for an increased physiologic response to a second activator.[5,20] PSML also increases PMN cell surface expression of the adhesion molecule CD11b.[3] Although PMN priming is a critical component in development of lung injury, microvascular endothelial cell activation plays an equally necessary role.[6,22] To that end, PSML also has a diverse array of proinflammatory effects on endothelial cells. The Deitch lab has demonstrated that in umbilical vein endothelial cells, PSML induces cytotoxicity as well as increased permeability.[2,7,19] However, repeated evidence has shown that umbilical vein endothelial physiology is quite different than pulmonary endothelial cells.[23] To that end, endothelial work from our lab has focused on HMVECs. We have previously demonstrated that PSML increases HMVEC ICAM-1 expression, increases *in vitro* PMN-HMVEC adhesion and increases PMN-mediated HMVEC killing.[8]

Despite multiple attempts to identify the relevant mediators, isolation and identification of the actual mediator remains elusive. Positive results to date suggest that the bioactivity of PSML is likely due to either multiple mediators or an interaction of proteins and lipids. [16][20]. Since the specific bioactive substance remains elusive, the next logical mechanistic targeting site for therapy could be obtained by examining signaling pathways.

Prior work from our lab has demonstrated that PSML in HMVECs induces ICAM-1 expression. [8] In endothelial cells, ICAM-1 is an NF- $\kappa$ B driven gene.[9] We therefore conjectured that signaling would converge onto NF- $\kappa$ B through the I $\kappa$ B proteins. Work from Sasaki et al. has demonstrated that phosphorylation of specific sites of the p50 and p65 component lead to changes in transcriptional activity of NF- $\kappa$ B driven proteins.[13] They have also demonstrated that specific I $\kappa$ K complexes not only phosphorylate I $\kappa$ Bs, but also function to specifically phosphorylate NF- $\kappa$ B. The serine 536 residue, for example, is specifically phosphorylated by I $\kappa$ K $\alpha$ , and is independent of I $\kappa$ B activity.

In this study, we have demonstrated that PSML profoundly induces degradation of I $\kappa$ B. This degradation is followed by translocation of free NF- $\kappa$ B to the nucleus with subsequent gene transcriptional activation. We therefore probed further evidence of I $\kappa$ K activation and found that phosphorylation of NF- $\kappa$ B at the serine 536 site is also enhanced after PSML. This suggests that the signaling pathway of PSML is an noncanonical NF- $\kappa$ B pathway.

In this study, the individual PSML lymph time point with the greatest bioactivity was the 3<sup>rd</sup> hour. This is congruent with prior work demonstrating that the 3<sup>rd</sup> postshock hour primed PMNs for an increased response to a second stimulus.[5] This is also congruent with prior work in HUVECs demonstrating the greatest decrease in cell viability after application of 2<sup>nd</sup> and 3<sup>rd</sup> hour of PSML.[17] Of interest, combining samples of PSML from each hour has greater bioactivity than any single time point alone. This suggests that there are different mediators acting as the primary inflammatory signal at different time point. Although the specific mediators have not been fully elucidated, this would suggest that different mediators are acting as the primary pro-inflammatory signal during different time points. Previous work has demonstrated that both proteins and lipids act as bioactive mediators in PSML.[16,20,21] The tremendous increase seen in the phosphorylation of NF- $\kappa$ B with the combined PSML suggests that there may be a synergistic action by combining lymph samples, such as combining protein carriers for stabilization of the lipid component.

In summary, this study demonstrates that hemorrhagic shock provokes the release of bioactive agents in PSML, which induce degradation of I $\kappa$ B, phosphorylation of RelA/p65 and NF- $\kappa$ B translocation. This study narrows the specific receptors down to those receptors acting via I $\kappa$ K as mediators of the cytotoxicity of lymph.



### Acknowledgements

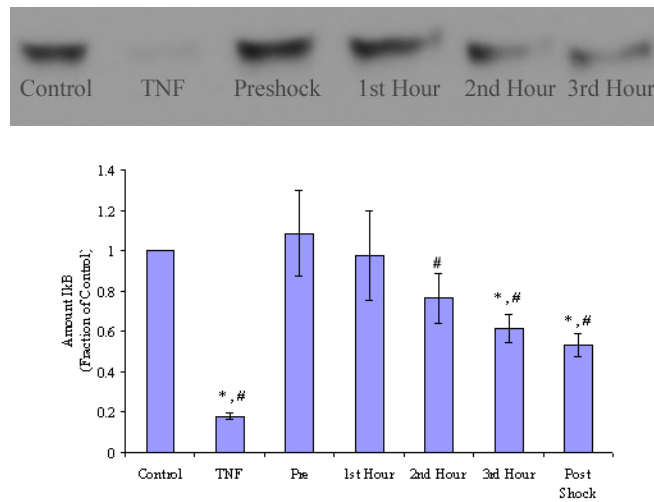
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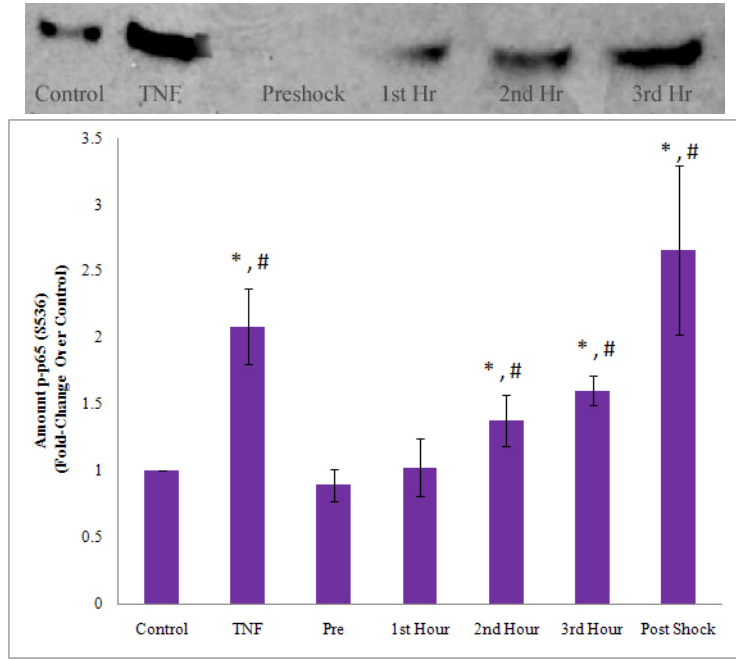
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### Figure 1. PSML induces IκB degradation in HMVECs

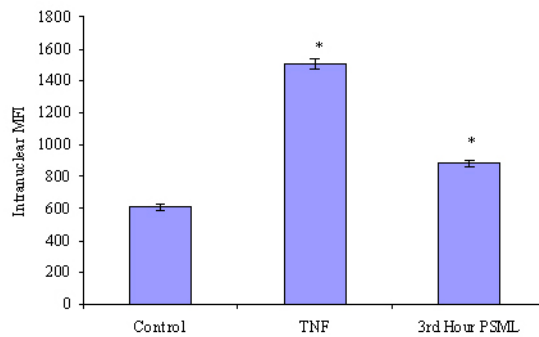
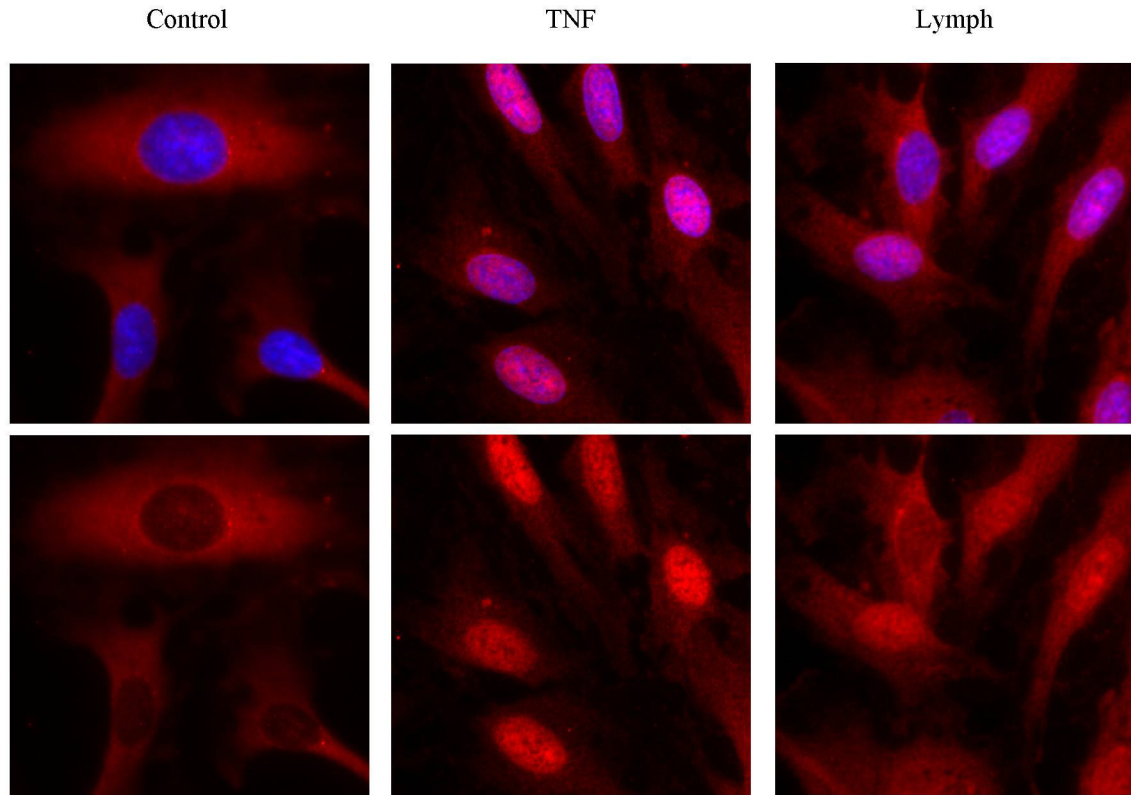
Mesenteric lymph was collected from rats during each hour before and after shock. Lymph was incubated with HMVECs and cells were lysed to determine activation of IκB via IκB degradation. Data are expressed as mean  $\pm$  SEM. \*  $p < 0.05$  vs. control group. #  $p < 0.05$  vs. preshock group. All PSML induces IκB degradation with maximal degradation occurring after stimulation with lymph from the 3<sup>rd</sup> hour and combined PSML. a) Representative blot. b) Densitometry analysis of multiple blots.





**Figure 2. PSML induces NF-κB phosphorylation**

Lymph was incubated with HMVEC for activation. Cells were subsequently lysed and immunoblotted for phospho-p65. \* p < 0.05 vs. control group. # p < 0.05 vs. preshock group. PSML increased phosphorylation of NF-κB with maximal phosphorylation occurring after stimulation with 3<sup>rd</sup> hour PSML and combined PSML. a) Representative blot. b) Densitometry analysis of multiple blots.



**Figure 3. PSML induces nuclear translocation of NF-κB**

The 3<sup>rd</sup> hour of PSML was incubated with HMVECs and probed for p65. (a) Immunofluorescence of NF-κB (RelA/p65, red) nuclear translocation. The nuclear stain is removed in the bottom row. (b) Histogram of p65 nuclear mean fluorescence intensity. \* p < 0.05 vs. control group. PSML increased translocation of p65 to the nucleus in HMVECs by 30%.