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Laminar shear stress enhances endothelial cell survival through a NADPH oxidase 2-dependent mechanism

Hyeona Jeon, Yong Chool Boo *

Department of Molecular Medicine, Cell and Matrix Research Institute, BK21 Medical Education Program for Human Resources, Kyungpook National University School of Medicine, Daegu 700-422, Republic of Korea

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ABSTRACT

The beneficial effects of laminar shear stress (LSS) due to blood flow include inhibition of endothelial cell death, but the associated mechanism is not well understood. This issue was addressed in the present study. In a normal growth medium, the endothelial cell death rate was below 5%, but this value increased beyond 30% when the serum was depleted. However, when cells were exposed to LSS during the serum depletion period, cell viability recovered to the levels of the serum-provided cells. The pro-survival effect of LSS was not affected by L-arginine methyl ester, but it was abrogated by apocynin, indicating that NADPH oxidases (NOX) play key roles in the mechanism. The pro-survival effect of LSS was reduced by NOX2 siRNA, but not by NOX4 siRNA. LSS increased the expressions of $p47^{phox}$ and $p67^{phox}$, the subunits of NOX2 complex. These observations suggest that LSS prevents apoptotic death of endothelial cells through a NOX2-dependent mechanism.

1. Introduction

The endothelium constitutes the inner surface of all blood vessels, forming a physical interface between the blood stream and the vessel wall. It produces a variety of substances that are involved in vasodilatation, blood coagulation, inflammation and immune response. Thus, the structural and functional integrities of the endothelium are the key factors for vascular health. Endothelial injury and dysfunction can lead to the development of cardiovascular diseases such as atherosclerosis, hypertension and heart failure [1,2].

Endothelial cells are normally exposed to the blood stream which carries nutrients and growth factors as well as oxygen that are essential for normal cell function and survival. However, under such conditions as ischemia, angioplasty, and organ transplantation, endothelial cells may experience hypoxia and starvation which can cause endothelial apoptosis. Evidence has supported that oxidative stress and redox-signaling pathways are involved in the hypoxia- or serum starvation-induced endothelial apoptosis [3,4]. Endothelial cell survival is also affected by the shear stress and cyclic strain generated due to the blood flow [5,6]. An irregular disturbed flow can initiate endothelial apoptosis [7] but laminar shear stress (LSS) due to orderly blood flow can inhibit endothelial apoptosis induced by growth factor depletion, tumor necrosis factor-α or oxidative stress [8,9].

Inhibitory effects of LSS against endothelial cell apoptosis have been attributed to enhanced nitric oxide (NO) production [10], protein kinase B (Akt) activation [11], and extracellular-signal-regulated kinase 5 activation [12]. Dr. Dimmeler's group reported that pharmacological inhibition of nitric oxide synthase 3 (NOS3) abrogated the anti-apoptotic effects of LSS, and that exogenous NO donors inhibited apoptosis due to oxidative stress under static conditions [9,10]. Thus, it has been inferred that NO from NOS3 is a critical mediator of the pro-survival effect of LSS, but no further convincing evidence has been provided yet.

The NADPH oxidase (NOX) family has been established as a source of reactive oxygen species (ROS) in cells. After NOX2 ($gp91^{phox}$) was identified as the enzyme responsible for the phagocyte respiratory burst, its membrane subunit ($p22^{phox}$) and cytosolic subunits ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) were discovered [13]. NOX 2 activation requires the assembly of membrane and cytosolic subunits and apocynin is known to inhibit this assembly process [14]. Later, other NOX family members including NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2 were identified [13]. Although the subunit composition of these seven NOX isoforms are different from each other, they share the capacity to transfer electrons from NADPH to oxygen thereby generating ROS.

ROS from the NOX family are known to serve as signaling molecules in the regulation of endothelial cell growth, death, and function [13,15]. Furthermore, LSS has been demonstrated to stimulate the NOX family leading to low levels of ROS production

* Corresponding author. Address: Department of Molecular Medicine, Kyungpook National University School of Medicine, 101 dongin-dong 2-ga, Jung-gu, Daegu 700-422, Republic of Korea. Fax: +82 53 426 4944.
E-mail address: ycboo@knu.ac.kr (Y.C. Boo).

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[16,17]. However, it has not been investigated whether the NOX family is functionally associated with the pro-survival effect of LSS. Therefore, the present study investigated potential role of NOX enzymes in mediating the cell survival-enhancing effects of LSS in Human umbilical vein endothelial cells (HUVECs) exposed to serum-starved conditions.

2. Materials and methods

2.1. Cell culture and LSS treatments

HUVECs obtained from Clonetics Cambrex (Rockland, ME, USA) were cultured on 0.2% gelatin-coated 100 mm-tissue culture dishes (BD Biosciences, San Jose, CA, USA) at 37 °C and 5% CO_2 [18]. The growth medium was EBM-2 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), endothelial growth supplements (Clonetics Cambrex), and antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B). Cells were seeded at 1×10^6 cells per dish and cultured in the growth medium to cell confluence of ~95%. The medium was replaced with a fresh growth medium with or without serum plus endothelial supplements. The cells were exposed to LSS or kept static for 24 h. LSS was imposed on the cells at 12 dyn cm^{-2} by rotating a Teflon cone (0.5° cone angle) mounted onto a culture dish, as previously described [19,20].

2.2. Small interfering RNAs (siRNAs) and transfection

Human NOX2 siRNA (#1299001, HSS102523), Human NOX4 siRNA (#1299001, HSS121314), and a negative control siRNA with scrambled sequences (#12935200) were purchased from Invitrogen (Grand Island, CA, USA). The nucleotide sequences of NOX2 and NOX4 siRNAs were as follows: NOX2 siRNA, 5'-GGG UUU AUG AUA UUC CAC CUA AGU U-3' (sense) and 5'-AAC UUA GGU GGA AUA UCA UAA ACC C-3' (antisense); NOX4 siRNA, 5'-CCU CAU GAU CAC AGC CUC UAC AUA U-3' (sense) and 5'-AUA UGU AGA GGC UGU GAU CAU GAG G-3' (antisense). For transfection, cells were treated with a mixture of 100 nM siRNA and 1.25 μL mL^{-1} Lipofectamine RNAiMAX (Invitrogen) in 5 mL Opti-MEM (Invitrogen) for 4 h and then the cells were fed with the growth medium. After 2 days, the cells were exposed to serum depletion and/or LSS for 24 h until the cell survival assay or biochemical analysis.

2.3. Cell viability assay

Cell viability was determined by the trypan blue exclusion assay. Briefly, the cells losing attachment were collected by centrifuging (2500 rpm, 5 min) the culture medium, and the attached cells were harvested by trypsinization followed by centrifugation. The cells were collectively suspended in medium. 0.4% Trypan blue solution (Sigma, St Louis, MO, USA) was added to the cell suspension at a one to one ratio. The numbers of stained dead cells and unstained live cells were counted on a hemocytometer under a microscope. Cell death (%) was defined as follows: cell death (%) = $[(A_d)/(A_d + A_v)] \times 100\%$, where A_d is the number of the dead cells and A_v is the number of viable cells.

2.4. DNA ladder assay

DNA ladder assay was done to monitor apoptotic changes of the cells [21]. Harvested cells were lysed in a lysis buffer containing 50 mM Tris–HCl, pH 7.5, 20 mM EDTA, and 1% NP-40. After centrifugation (3000 rpm, 5 min), supernatants were collected and treated with 1% sodium dodecyl sulfate (SDS) and RNAse A (5 mg mL^{-1}) for 2 h at 56 °C followed by incubation with proteinase K (2.5 mg mL^{-1}) for 2 h at 37 °C. DNA was precipitated by addition of 0.5 volume of 10 M ammonium acetate and 2.5 volumes of ice-cold ethanol followed by incubation at −80 °C for 1 h. The DNA was collected by centrifugation (12,000 rpm, 20 min) and its aliquot (4 μg) was subjected to electrophoresis on a 2% agarose gel.

2.5. Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

Total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). To prepare cDNA, 1 μg of cellular mRNA was reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster city, CA, USA). The kit utilizes random hexamers primers and MultiScribe™ Reverse Transcriptase. PCR was conducted using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) in a reaction mixture (20 μL) containing SYBR® Green PCR Master Mix (Applied Biosystems), 60 ng cDNA, and 2 picomole of gene-specific primer sets (Macrogen, Seoul, Korea). The reactions were performed with the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a dissociation protocol. Single peaks in the melting curve analysis supported the homogeneity of the amplicons. The mRNA expression level relative to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was calculated by the comparative threshold cycle method [32]. The sequences of the PCR primers were as follows: NOX2 (CYBB, $gp91^{phox}$) (GeneBank accession number, NM_000397.3) 5'-TGT TAG TGG GAG CAG GGA TTG-3' (forward) and 5'-TCA GAT TGG TGG CGT TAT TGC-3' (reverse); NOX4 (NM_016931.3, NM_001143836.1, NM_001143837.1) 5'-GCA TGT GGC TGC CCA TCT-3' (forward) and 5'-GCC AGG AAC AGT TGT GAA GAG A-3' (reverse); RAC1 (Rac1) (NM_006908.4) 5'-TAG ATG GAA AAC CGG TGA ATC TG-3' (forward) and 5'-CCG CAC CTC AGG ATA CCA CTT-3' (reverse); CYBA ($p22^{phox}$) (NM_000101.2) 5'-ACT TTG GTG CCT ACT CCA TTG TG-3' (forward) and 5'-GGC ACC GAG AGC AGG AGA T-3' (antisense); NCF4 ($p40^{phox}$) (NM_000631.4, NM_013416.3) 5'-GCA GCA CTC CCC TAT TGA AAG A-3' (forward) and 5'-CCT CAG CGT CCC GGT AAT T-3' (reverse); NCF1 ($p47^{phox}$) (NM_000265.4) 5'-CAG CCA GCA CTA TGT GTA CA-3' (forward) and 5'-GAA CTC GTA GAT CTC GGT GAA-3' (reverse); NCF2 ($p67^{phox}$) (NM_000433.3, NM_001127651.2, NM_001190794.1, NM_001190789.1) 5'-CCT CCA CCC AGA CCG AAA A-3' (forward) and 5'-CCG TGG CCC AGT TAT CAT TG-3' (reverse); GAPDH (NM_002046.3) 5'-ATG GGG AAG GTG AAG GTC G-3' (forward) and 5'-GGG GTC ATT GAT GGC AAC AA-3' (reverse).

2.6. Statistical analysis

Data are presented as the means ± SEM. The statistical analyses were conducted using the Sigma Stat 3.1 software program. Significant differences among the groups were determined with one-way ANOVA. Duncan's multiple-range test was conducted if differences were identified between the groups at a significance level of $p < 0.05$.

3. Result

To examine the effect of LSS on cell survival, HUVECs were exposed to an arterial level of LSS (12 dyn cm^{-2}) for 24 h in the presence and absence of serum (10% fetal bovine serum plus endothelial growth supplements). As shown in Fig. 1A and B, cell death was below 5% under a normal *in vitro* culture condition (serum, static), but the value increased beyond 30% when serum was not included in the growth medium (no serum, static). However,

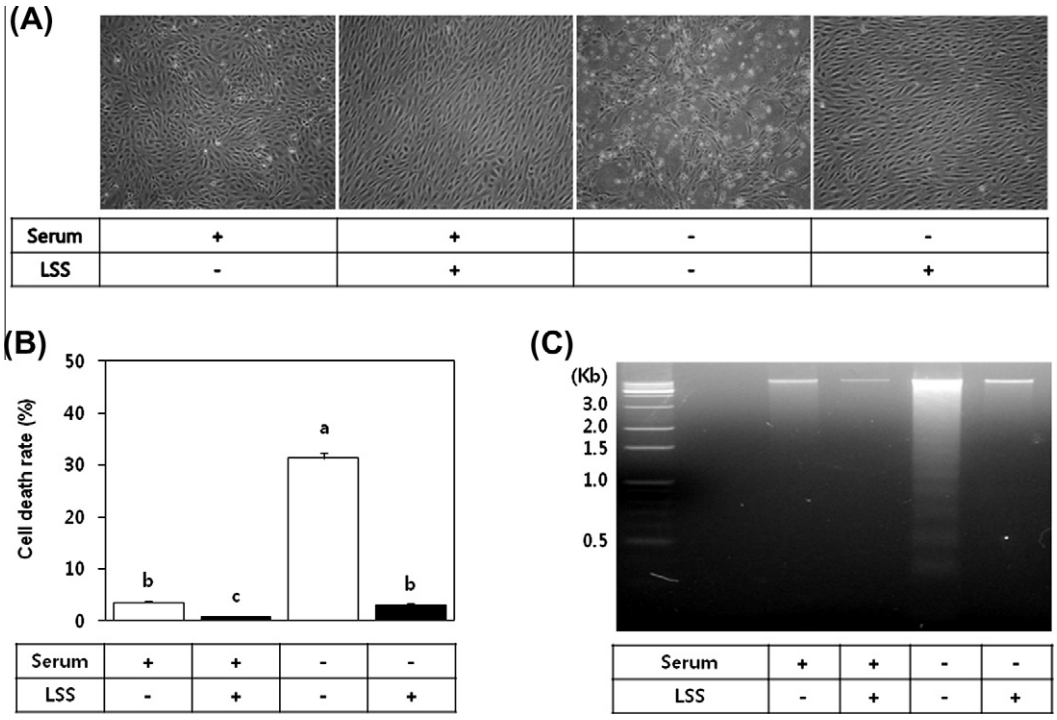

Fig. 1. Laminar shear stress (LSS) prevents endothelial cell death due to serum depletion. Cells were exposed to an arterial level of LSS (12 dyn cm^{-2}) or kept static for 24 h in the presence and absence of serum (10% fetal bovine serum plus endothelial growth supplements). After treatments, cells were observed under a microscope (A) and the cell death rate (% of total cells) was determined with the trypan blue assay (B). Apoptotic changes (DNA fragmentation) of the cells were monitored with the DNA ladder assay (C). Bar graphs represent the means ± SE ($n = 3$). Data not sharing the same letter are significantly different from each other ($p < 0.05$).

when cells were exposed to LSS in a serum-depleted medium, cell death was reduced back to the value of the serum-provided cells. The DNA ladder assay indicated that the endothelial cells committed apoptosis due to serum depletion and the apoptosis was inhibited by LSS treatment (Fig. 1C). These data verified that LSS stimulated survival signals in endothelial cells under serum-depleted conditions.

Cellular NO and ROS are known to serve as signaling molecules in cell growth, death, and function [13,15]. Therefore, we compared the relative contribution of NO from the NOS family and ROS from the NOX family to the cell survival enhancing effects by LSS. As shown in Fig. 2A and 2B, serum depletion-induced cell death was suppressed by LSS even in the presence of L-arginine methyl ester (L-NAME), a NOS inhibitor. In contrast, the cell survival enhancing effect was completely diminished by apocynin, a NOX inhibitor. The DNA ladder assay also showed that the anti-apoptotic effects of LSS was abrogated by apocynin but not affected by L-NAME (Fig. 2C). These results implicate that the NOX family producing ROS rather the NOS family producing NO may be a key player mediating cell survival signals triggered by LSS.

To further verify the functional role of the NOX family in cell survival by LSS, cells were treated with siRNAs to selectively deplete NOX2 and NOX4 known to be expressed in endothelial cells [22,23]. Surprisingly, the effects of the siRNAs for NOX2 and NOX4 were quite different from each other. As shown in Fig. 3A and B, cell death and apoptotic DNA laddering due to serum depletion were suppressed by LSS in cells treated with the control siRNA or NOX4 siRNA but the suppression was much less in cells treated with NOX2 siRNA. The specific gene silencing effects of NOX2 and NOX4 siRNAs were verified by qRP-PCR (Fig. 3C). Thus, it is indicated that LSS enhances cell survival under serum-depleted conditions through a NOX2-dependent mechanism.

Then how does LSS stimulate NOS2? As shown in Fig. 4A, the NOX2 complex consists of the membrane proteins NOX2 (CYBB, $gp91^{phox}$) and $p22^{phox}$ (CYBA), and cytosolic proteins such as $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac1 GTPase, whereas the NOX4 complex that only has the membrane proteins NOX4 and $p22^{phox}$ [13]. The effect of LSS on the expression of these subunits was determined by quantitative PCR. The results showed that LSS did not have a significant effect on the expressions of NOX2, $p22^{phox}$, and Rac1 (Fig. 4B). However, LSS increased the $p47^{phox}$ and $p67^{phox}$ expressions and decreased the NOX4 and $p40^{phox}$ expressions. The changes in $p67^{phox}$ were the most prominent. These observations suggest that LSS might stimulate NOX2 activation through the expressional upregulation of $p67^{phox}$ and/or $p47^{phox}$.

4. Discussion

Both NO and ROS are known to be key signaling molecules and determinants of endothelial cell fates under various physiological and pathophysiological conditions [24,25]. Such reactive species can induce either cell death or cell survival depending on where and how much they are produced. Therefore, it is important to define their functional roles under specified physiological conditions. In the present study, we examined the potential role of NO *versus* ROS in cell death and survival due to serum depletion and/or LSS treatments. This *in vitro* model is physiologically relevant because endothelial cells are normally exposed to LSS due to blood flow and they could experience ischemia under certain pathological or accidental conditions.

The results of the current study confirmed that serum depletion can induce apoptotic cell death and this phenomenon can be prevented by LSS (Fig. 1). Based on previous reports [9,10], we initially inferred that LSS might enhance cell survival under serum-depleted conditions by a NO-dependent mechanism. However, our experimental data did not support this assumption because the cell sur-

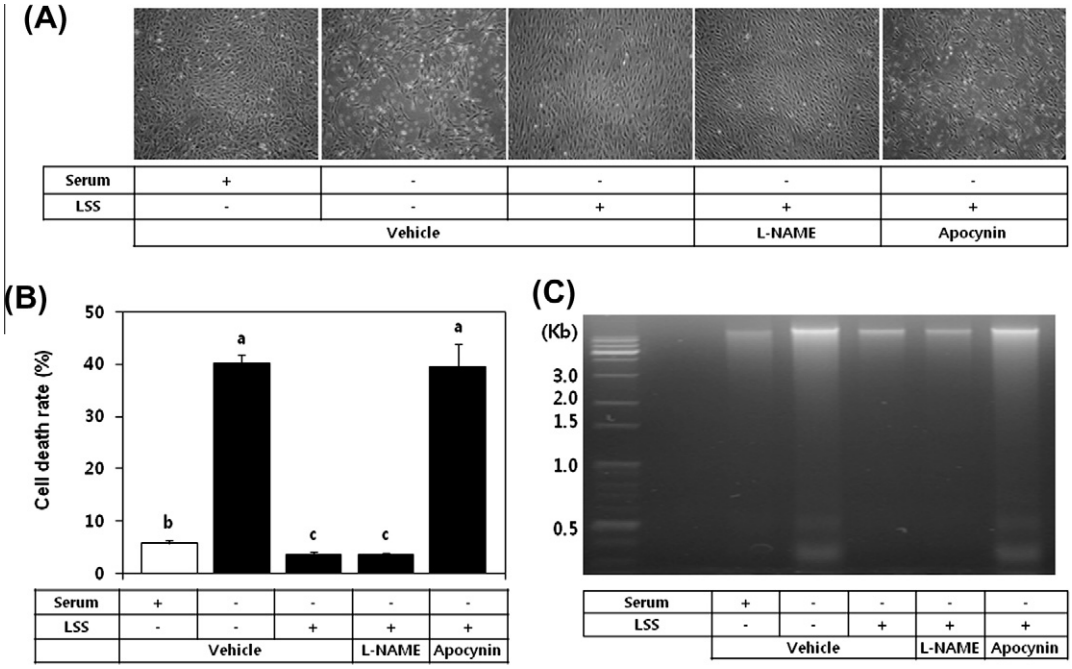

Fig. 2. Apocynin, but not L-arginine methyl ester (L-NAME), abrogates the pro-survival effect of LSS under serum-depleted conditions. Cells were treated with 1 mM L-NAME (a NOS inhibitor) or 1 mM apocynin (a NOX inhibitor) and exposed to LSS at 12 dyn cm^{-2} for 24 h in the presence and absence of serum (10% fetal bovine serum plus endothelial growth supplements). The cells were observed under a microscope (A) and the cell death rate (% of total cells) was determined by with the trypan blue assay (B). Apoptotic changes (DNA fragments) of the cells were monitored with DNA ladder assay (C). Bar graphs represent the means ± SE ($n = 3$). Data not sharing the same letter are significantly different from each other ($p < 0.05$).

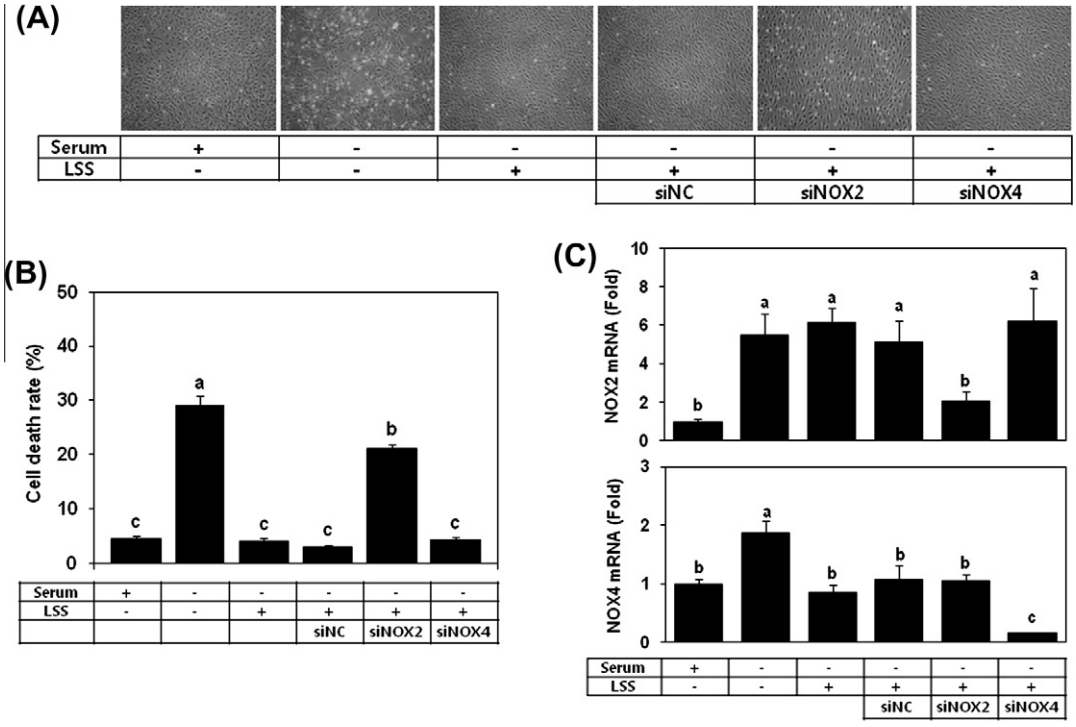

Fig. 3. The siRNA-mediated depletion of NOX2, but not NOX4, abrogates the cell survival enhancing effects of LSS under serum-depleted conditions. Cells were transfected with siRNAs prior to exposure to LSS in the absence of serum. The cells were observed under a microscope (A) and the cell death rate (% of total cells) was determined with the trypan blue assay (B). The mRNA levels of NOX2 and NOX4 relative to the internal control, GAPDH, were determined by qRT-PCR (C). Bar graphs represent the means ± SE ($n = 3$). Data not sharing the same letter are significantly different from each other ($p < 0.05$).

vival enhancement of LSS was not reduced by the pharmacological inhibition of its activity with L-NAME (Fig 2). In contrast, pharmacological inhibition of the NOX family with apocynin abrogated the cell survival enhancing effects of LSS under serum depleted conditions (Fig. 2), implicating that NOX-derived ROS might be the key mediator involved in the cell survival signal transduction triggered by LSS in endothelial cells. The current study further demonstrated that LSS may promote endothelial cell survival through a

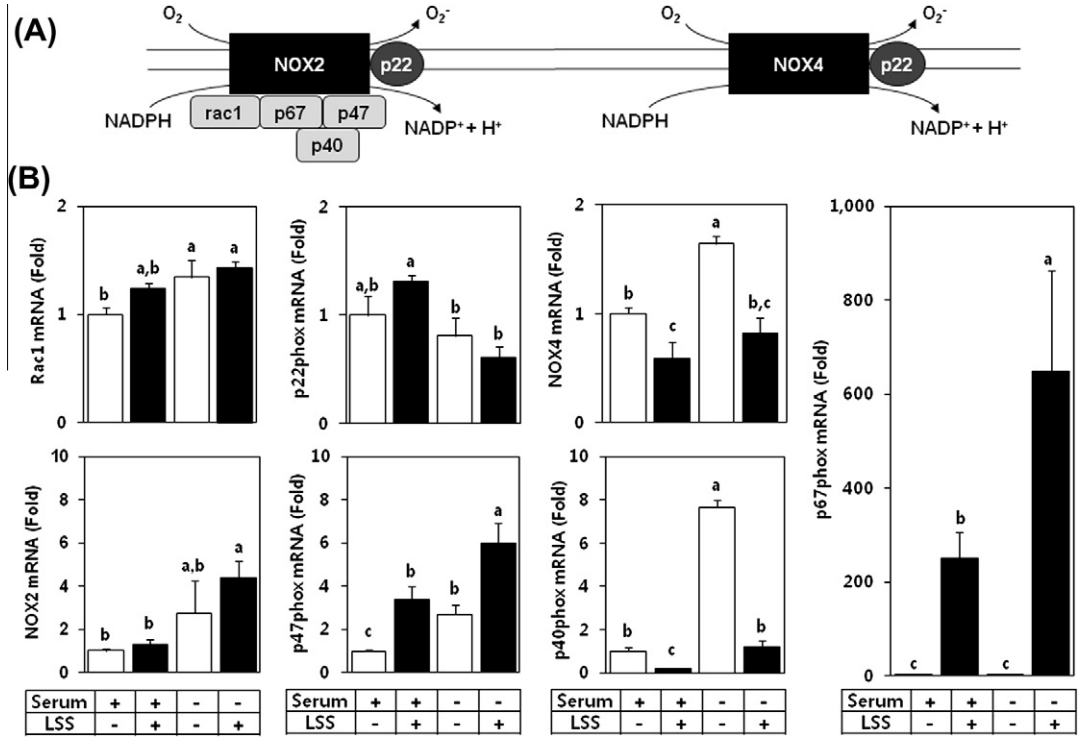

Fig. 4. Effects of LSS and serum depletion on the expressions of NOX2 and NOX4 subunits. NOX2 and NOX4 complexes consist of multiple subunits (A). Cells were exposed to an arterial level of LSS (12 dyn cm^{-2}) or kept static for 24 h in the presence and absence of serum (10% fetal bovine serum plus endothelial growth supplements). The mRNA levels of NOX2, NOX4, Rac1, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ relative to GAPDH were monitored by qRT-PCR analysis (B). Bar graphs represent the means ± SE ($n = 3$). Data not sharing the same letter are significantly different from each other ($p < 0.05$).

NOX2-dependent mechanism, with a gene-silencing approach using siRNAs (Fig. 3).

It is established that NOX-derived ROS can trigger apoptosis by stimulating apoptotic signaling pathways directly and indirectly [4,26,27]. In some circumstance, however, NOX-derived ROS exhibit pro-survival effects [23,28,29]. It has been shown that NOX2 and Rac1 could prevent apoptosis to support cell survival in endothelial cells [23,28]. Thus, it is not surprising to observe the critical role of NOX2 in mediating the pro-survival effect of LSS in the present study.

It is an intriguing issue how different isotypes of NOX influence the cell fate differently. Although NOX family members share common biochemical property producing ROS, they are different in terms of subcellular localization, subunit composition, enzyme activation mechanism, catalytic mechanism producing specific ROS and so on. Of interest, NOX4 is known to generate hydrogen peroxide in preference of superoxide anion radical, unlike other NOX isoforms [30,31]. Superoxide mediates one-electron signaling via iron ions while hydrogen peroxide mediates two-electron signaling via thiols [32], and their relative concentrations are a key factor in determining the fates of cells, survival *versus* death [33]. In this regard, the activation of different NOX isoforms producing either superoxide or hydrogen peroxide preferentially would have different effects on cell fates. Based on the mRNA levels of various subunits of NOX2 and NOX4 complexes (Fig. 4), the preferential activation of NOX2 relative to NOX4 by LSS is believed to increase the local concentration of superoxide relative to hydrogen peroxide, providing cell survival signals.

Although NOX2 was observed in the current study to mediate endothelial cell survival enhancement by LSS, its physiological functions may not be simply designated as "good" or "bad" because not only survival and but also death signals at the cellular levels may be necessary for the healthy life of the whole organism. Furthermore, expression levels of NOX isoforms are different from cell to cell and their function can be affected by their physiological contexts. In this regard, it is not surprising to observe divergent outcomes from *in vivo* studies using genetically modified mouse models [34]. Previously, global deletion of NOX2 or $p47^{phox}$ genes was shown to protect against atherosclerosis [34]. However, this result may not be simply attributed to the deletion of endothelial NOX2, since NOX2 is more abundantly expressed in the phagocytic cells of wild type animals. Further studies are needed to define the specific function of endothelial NOX2 in more sophisticated animal models with an endothelial cell-specific conditional knockout or expression of NOX2 subunits.

In conclusion, the current study suggests that LSS prevents apoptotic death of endothelial cells under serum-depleted conditions through a NOX2-dependent mechanism.

Acknowledgments

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