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Journal of Molecular and Cellular Cardiology 37 (2004) 121–125
Journal of Molecular and Cellular Cardiology
www.elsevier.com/locate/yjmcc

Original Article

Oscillatory shear stress upregulation of endothelial nitric oxide synthase requires intracellular hydrogen peroxide and CaMKII☆

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Received 4 March 2004; received in revised form 12 April 2004; accepted 23 April 2004

Available online 08 June 2004

Abstract

We have previously shown that hydrogen peroxide (H_2O_2) upregulates endothelial nitric oxide synthase (eNOS) expression via a calcium/calmodulin-dependent protein kinase II (CaMKII)-mediated mechanism whereas it also acutely activates eNOS enzyme. We hypothesized that oscillatory shear stress (OSS), which stimulates endogenous H_2O_2, would have effects on eNOS expression and function similar to that of exogenous H_2O_2. Exposure of bovine aortic endothelial cells to OSS (±15 dynes/cm^2) increased eNOS mRNA expression by 3-fold. Pretreatment with either polyethylene glycol-catalase (PEG-CAT, a scavenger of H_2O_2) or KN93, an inhibitor of CaMKII, abolished this response. OSS activated CaMKII in an H_2O_2-dependent fashion whereas unidirectional laminar shear stress (LSS) inhibited CaMKII phosphorylation. Inhibition of c-Src (essential for LSS upregulation of eNOS) had no effect on OSS upregulation of eNOS. Additionally, OSS stimulated $NO^\bullet$ production acutely. Scavenging of H_2O_2 by PEG-CAT attenuated OSS stimulation of $NO^\bullet$ by 50% whereas it had no effect on LSS regulation of $NO^\bullet$ production. These data suggest that intracellular H_2O_2 and CaMKII mediate OSS upregulation of eNOS. The acute activation of eNOS by OSS also partially requires H_2O_2. As OSS has been shown previously to stimulate sustained production of superoxide ($O_2^{\bullet-}$) which would inactivate $NO^\bullet$, these responses may represent attempted compensation to restore $NO^\bullet$ bioavailability in areas exposed to OSS. Simultaneous stimulation of $O_2^{\bullet-}$ and $NO^\bullet$ by this mechanism, however, could facilitate peroxynitrite formation and protein nitration, which may enhance atherosclerotic lesion formation. Both OSS and LSS upregulate eNOS expression but via different signaling mechanisms.
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Keywords: H_2O_2; Oscillatory shear stress; $NO^\bullet$; eNOS; Calcium/calmodulin-dependent protein kinase II; Unidirectional laminar shear stress

1. Introduction

Oscillatory shear stress (OSS) occurs at bifurcations and branch points of the vasculature, such as the carotid bifurcation, the abdominal aorta and the proximal coronary arteries, where atherosclerosis often develops [1]. It was shown previously that OSS stimulates production of superoxide ($O_2^{\bullet-}$) in endothelial cells via both the NAD(P)H oxidase and xanthine oxidase [2,3]. Accumulating evidence has demonstrated an important role of hydrogen peroxide (H_2O_2), the dismutation product of $O_2^{\bullet-}$, in vascular signaling [4,5]. In keeping with this, we and others have recently demonstrated that H_2O_2 can acutely activate endothelial nitric oxide synthase (eNOS) to produce $NO^\bullet$ and over the long-term, H_2O_2 increases eNOS gene expression [6–9]. In addition, we have shown that intracellular H_2O_2, derived from the endothelial cell $p47^{phox}$-containing NAD(P)H oxidase, is responsible for acute $NO^\bullet$ production evoked by angiotensin II [10]. It remains unclear, however, whether endogenously produced H_2O_2 in response to extracellular stimuli, such as OSS, is able to regulate eNOS gene expression. The present study was performed to determine whether OSS modulates eNOS expression and function via redox-sensitive mechanisms, and to gain insight into the signaling mechanisms involved.

2. Methods

2.1. Cell culture

Bovine aortic endothelial cells were cultured as described previously [6,8,10] and studied when confluent. As cultured

☆ This paper is in part presented at the Experimental Biology 2003 scientific conference at San Diego, CA, April, 2003

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doi:10.1016/j.yjmcc.2004.04.012

endothelial cells have low H_4B (tetrahydrobiopterin) content [11], the media was supplemented with sepiapterin overnight (Sigma, St. Louise, MO, 10 μmol/l), a precursor for H_4B, prior to studies of $NO^{\bullet}$ production. A cone-in-plate viscometer was used to create either unidirectional laminar shear stress (LSS) or OSS as previously described [3].

2.2. Measurements of reactive oxygen species and NO

Cell production of H_2O_2 was measured using a peroxidase-based Amplex-red assay (Molecular Probes, Eugene, OR) following manufacturer's instructions. In brief, Amplex red (50 μmol/l) and horseradish peroxidase type II (0.1 U/ml) were added to the media of endothelial cells before and during shear. Fluorescence readings were made in triplicate in a 96-well plate at excitation and emission wavelength of 530 and 580 nm, respectively, using 200-μl samples of media. H_2O_2 concentration was calculated by using a standard curve and was normalized to cellular protein as measured by the Bradford assay [3].

Endothelial cell $NO^{\bullet}$ production was measured using an electron spin resonance (ESR) spectrophotometer and a $NO^{\bullet}$-specific spin trap Fe^{2+}MGD [12]. Briefly, endothelial cells were rinsed by modified Kreb's/HEPES buffer and then exposed to OSS (±15 dynes/cm^2, 60 min) in the presence of freshly prepared Fe^{2+}MGD (0.5:5.0 mmol/l) solution. The post-exposure supernatant was aspirated into 1-ml tuberculin syringes and snap-frozen in liquid N_2. The frozen sample columns were loaded into a finger Dewar and analyzed with a Bruker EMX ESR spectrometer (Bruker Instruments, Billerica, MA) at the following settings: field Sweep: 160 G; microwave frequency: 9.39 GHz; microwave power: 10 mW, modulation amplitude: 3 G, conversion time: 2621 ms, time constant: 328 ms, modulation amplitude: 3 G, receiver gain 1×10^4 and four scans.

2.3. Analysis of eNOS mRNA expression and calcium/calmodulin-dependent protein kinase II phosphorylation

Levels of eNOS mRNA were measured by northern analysis as described previously [8]. Calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylation was determined using a phospho-specific antibody as previously described [8].

2.4. Statistical analysis

Differences in H_2O_2 production between static and sheared endothelial cells were analyzed using a Student's *t*-test. The eNOS mRNA levels and $NO^{\bullet}$ production among static and different shear groups were compared with one-way ANOVA. When differences were indicated, the Dunnet's post-hoc test was employed. Statistical significance was set for $P < 0.05$. All grouped data shown in the figures were presented as mean ± SEM.

3. Results

3.1. Oscillatory shear increases H_2O_2 production

It was previously reported that OSS potently stimulates endothelial cell production of $O_2^{\bullet-}$ [2,3]. To determine whether H_2O_2 was also increased by OSS, endothelial cells were either kept quiescent or exposed to OSS (±15 dynes/cm^2) for 30 and 60 min and H_2O_2 production determined by an Amplex-red assay. As shown in Fig. 1A, OSS significantly increased H_2O_2 production at both time points examined.

3.2. Oscillatory shear upregulation of eNOS requires intracellular H_2O_2 and CaMKII

To examine whether OSS upregulation of eNOS was mediated by endogenous H_2O_2, endothelial cells were pretreated with polyethylene glycol-catalase (PEG-CAT) (a scavenger of intracellular H_2O_2, 50 U/ml for 18 h) and then exposed to OSS (±15 dynes/cm^2, 60 min). Cells were harvested 24 h later. Exposure of endothelial cells to OSS increased eNOS mRNA levels by 3-fold and this was completely prevented by PEG-CAT (Fig. 1B).

These results clearly demonstrated that endogenous H_2O_2 is required for OSS upregulation of eNOS mRNA. In the case of exogenously applied H_2O_2, the serine/threonine protein kinase CaMKII is critical in mediating the increase in eNOS expression [8]. We therefore performed additional experiments to determine whether CaMKII was involved in OSS regulation of eNOS expression. Similar to exogenous H_2O_2 administration, OSS increased CaMKII phosphorylation (Fig. 1D) at 40 min and this was abolished by PEG-CAT, suggesting that OSS activation of CaMKII requires intracellular H_2O_2. To examine a role of CaMKII in OSS regulation of eNOS mRNA expression, endothelial cells were pretreated with KN93 (10 μmol/l) for 1 h prior to OSS stimulation. KN93 in this concentration has been shown to specifically and potently inhibit CaMKII in several cell types including hepatocytes [13], fibroblasts [14], neurons [15], vascular smooth muscle cells [16,17] and endothelial cells [8]. As is evident in Fig. 1B, CaMKII is critical for OSS upregulation of eNOS because KN93 abolished this response.

In striking contrast to OSS, we found that LSS upregulation of eNOS was unaffected by either PEG-CAT or KN93 [18]. This is consistent with our previous observations that LSS upregulates eNOS via a signaling pathway downstream of c-Src that is independent of H_2O_2 or CaMKII [18]. Pharmacological inhibitors of c-Src, PP1 and PP2, have been shown to prevent endothelial activation c-Src in response to a variety of stimuli, such as LSS [18,19], H_2O_2 [6,20], thrombin [21] and fibroblast growth factor 2 [22]. To examine a potential role of c-Src in OSS upregulation of eNOS, endothelial cells were pretreated with PP1 (100 nmol/l) or PP2 (10 μmol/l) for 1 h prior to OSS stimulation. As it is evident in Fig. 1C, PP1 and PP2 had no effect on OSS upregulation of

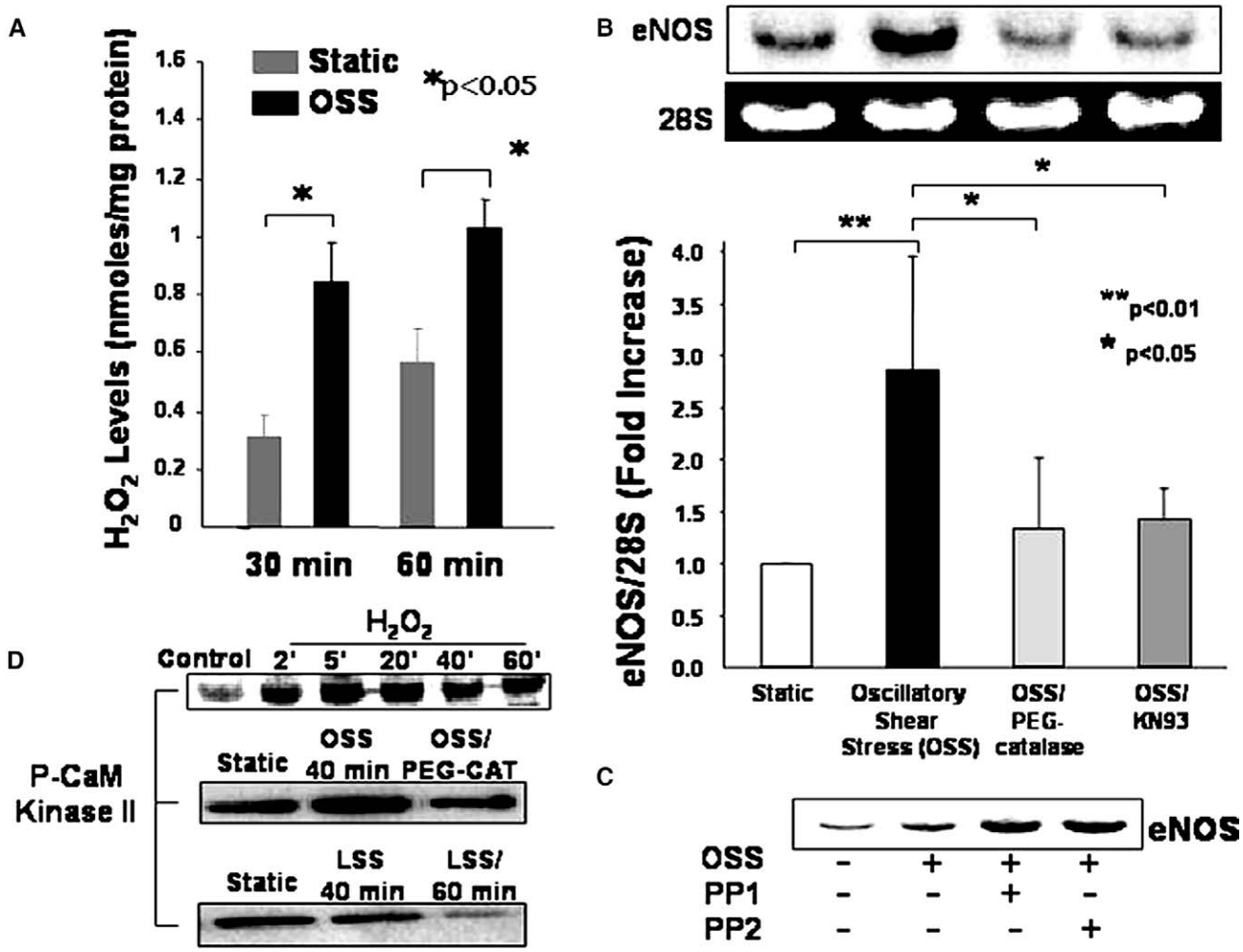

Fig. 1. Role of intracellular H_2O_2 and CaMKII in eNOS gene regulation by OSS. (A) Effects of OSS on H_2O_2 production. Endothelial cells were exposed to OSS (±15 dynes/cm^2) for 30 and 60 min and H_2O_2 production determined by an Amplex-red assay. (B) Effects of H_2O_2 scavenging and CaMKII inhibition on OSS regulation of eNOS mRNA expression. Endothelial cells were pretreated with PEG-CAT (50 U/ml) for 18 h or KN93 (10 μmol/l) for 1 h prior to 1-h OSS. Cells were harvested at 24 h and subjected to northern analysis of eNOS mRNA. The upper panel shows the representative eNOS northern blot and the 28S bands as a loading control. Grouped densitometric data from four separate experiments are presented in the lower panel. (C) Effects of Src inhibition on eNOS upregulation by OSS. Endothelial cells were preincubated with Src inhibitors PP1 (10 μmol/l) or PP2 (100 nmol/l) for one hour prior to OSS stimulation and measurement of eNOS mRNA by northern analysis. (D) Effects of OSS and unidirectional LSS on CaMKII phosphorylation. Endothelial cells were exposed to OSS for 40 min in the presence or absence of PEG-CAT (preincubated overnight) and harvested for western analysis of CaMKII threonine 286 phosphorylation using a phospho-specific antibody; 40 and 60 min LSS-ed (15 dynes/cm^2) endothelial cells were also examined for CaMKII phosphorylation following identical procedure.

eNOS mRNA expression assessed by northern blotting. Thus, these findings suggest that OSS and LSS both increase eNOS expression, but using completely different signaling pathways.

3.3. Role of H_2O_2 in acute activation of eNOS by oscillatory shear

We and others have recently shown that both exogenous H_2O_2 and angiotensin II-stimulated endogenous H_2O_2 can acutely activate eNOS [6,7,10]. We performed additional experiments to determine whether OSS also stimulates endothelial $NO^\bullet$ production in an H_2O_2-dependent fashion. Endothelial cells were exposed to OSS for 60 min and $NO^\bullet$ production detected using ESR and a $NO^\bullet$-specific spin trap Fe^{2+}MGD. OSS caused a 3-fold increase in endothelial $NO^\bullet$ production and this was reduced by half in cells treated with PEG-CAT, suggesting that intracellular H_2O_2 is partially involved in this response (Fig. 2A). In contrast, LSS increased $NO^\bullet$ production to a greater extent (5-fold), and this was unaffected by PEG-CAT (Fig. 2B).

4. Discussion

The present study characterizes a novel role of intracellular H_2O_2 in eNOS upregulation by the mechanical force OSS. In an earlier study we found that CaMKII is responsible for transcriptional regulation of eNOS by H_2O_2 [8]. Our current study indicates that CaMKII is involved in transcriptional regulation of eNOS by OSS. OSS is associated with sustained production of $O_2^{\bullet-}$ in endothelial cells, which in turn

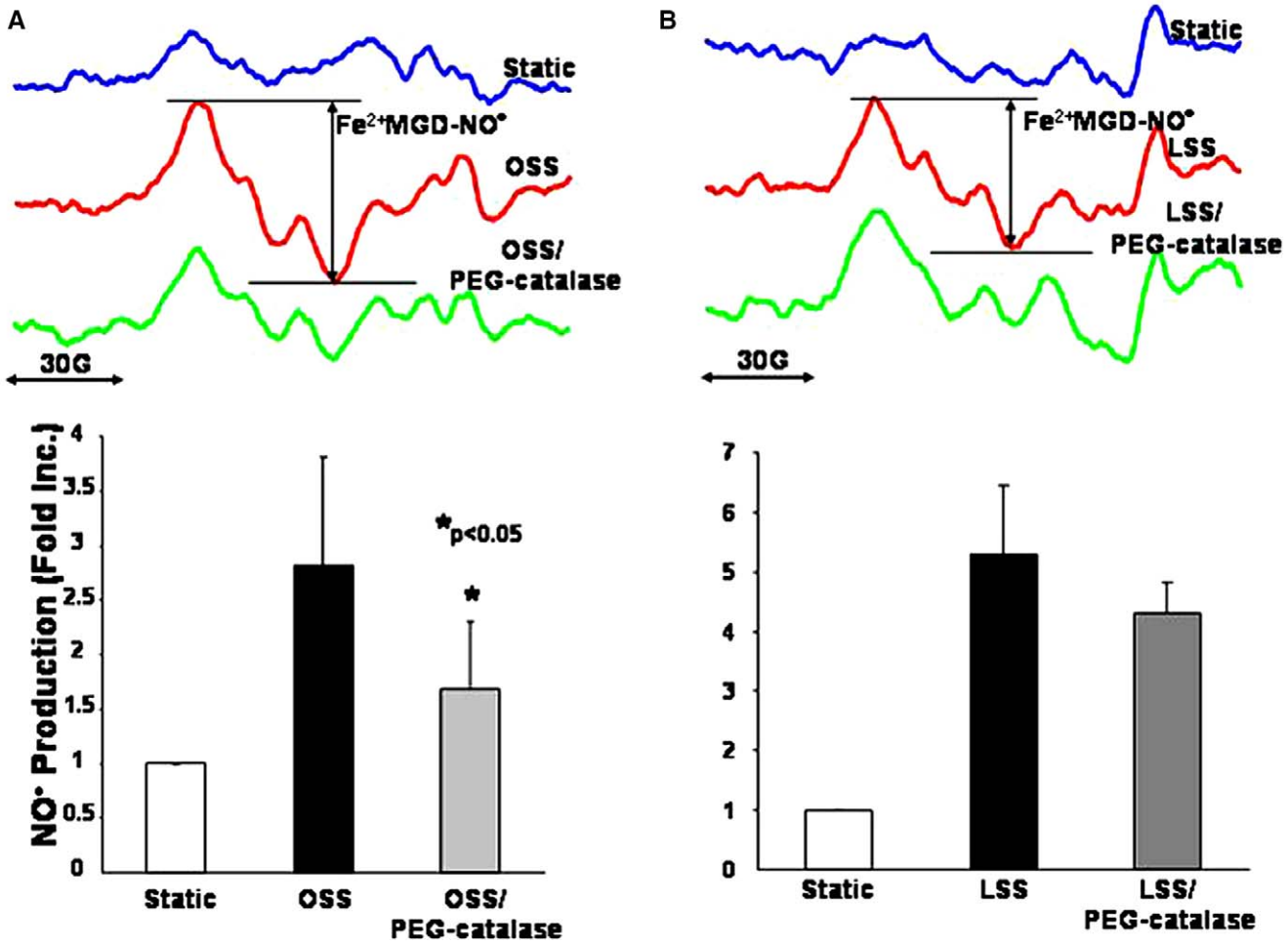

Fig. 2. Role of intracellular H_2O_2 in acute activation of eNOS by OSS. (A) Effects of H_2O_2 scavenging on OSS stimulation of $NO^•$. Endothelial cells were pretreated with sepiapterin (10 μmol/l) overnight and then exposed to OSS (±15 dynes/cm^2) for 60 min in the presence or absence of PEG-CAT. $NO^•$ production was examined using ESR and a $NO^•$-specific spin trap Fe^{2+}MGD. (B) Effects of H_2O_2 scavenging on LSS stimulation of $NO^•$. Endothelial cells exposed to LSS (15 dynes/cm^2) were examined for $NO^•$ production in the presence or absence of PEG-CAT pretreatment.

inactivates $NO^•$. Upregulation of eNOS expression and activity by OSS may thus represent an attempted compensatory mechanism to maintain sufficient bioavailable $NO^•$. On the other hand, the simultaneous production of $O_2^{•-}$ and $NO^•$ could facilitate peroxynitrite ($ONOO^-$) formation and tyrosine nitration. The latter has been found in human atherosclerotic lesions [23,24] and may mediate predisposition to atherosclerosis of OSS-exposed vascular areas [24–26]. More recently, it has been shown that myeloperoxidase, released by inflammatory cells, is taken up by endothelial cells to catalyze the formation of nitrotyrosines in the presence of the co-substrates H_2O_2 and $NO^•$. The nitration of specific proteins may have pathophysiological significance [27,28]. For example, nitrated fibrinogen was recently found to accelerate clot formation in vitro and its levels were increased in patients with coronary artery disease [28]. Likewise, prostacyclin synthase has been shown to be nitrated on tyrosine, leading to altered vasomotion [29]. Thus, protein nitration in response to the simultaneous production of $O_2^{•-}$, H_2O_2 and $NO^•$ may augment atherosclerotic lesion formation.

In contrast to OSS, which occurs at the bifurcations of branch points of the vasculature, unidirectional LSS occurs in unbranched vascular regions and seems to be vasoprotective. While OSS stimulates a sustained production of $O_2^•$ in endothelial cells [2,3], LSS stimulates only a transient increase in $O_2^{•-}$. As shown in the present study, both OSS and LSS upregulate eNOS expression and activity but require very different intracellular signaling pathways. OSS upregulation of eNOS requires intracellular H_2O_2 and CaMKII, while LSS utilizes a c-Src-dependent signaling pathway to upregulate eNOS expression, which is H_2O_2 and CaMKII independent.

We have also found that intracellular H_2O_2 is partially involved in the acute activation of eNOS by OSS. Scavenging of H_2O_2 with PEG-CAT reduced acute stimulation of $NO^•$ by OSS by 50% while it had no effect on LSS stimulation of $NO^•$. We and others have shown that the acute activation of eNOS in response to H_2O_2 is only partially calcium dependent, and that H_2O_2 stimulates phosphorylation of serine 1177 in a PI3-kinase-dependent fashion [6,7]. It is likely that OSS activation of eNOS is also mediated via a similar signaling pathway. We have performed experiments examining a role of CaMKII in the acute activation of eNOS by OSS. CaMKII appears not involved because KN93 had no effect

on OSS stimulation of $NO^{\bullet}$. On the other hand, inhibition of PI3-kinase (with Wortmannin) or ERK1/2 (with PD98059) reduced OSS stimulation of $NO^{\bullet}$ production by approximately 50%. This is consistent with our previous findings that the calcium-independent activation of eNOS by $NO^{\bullet}$ is mediated by synergistic effects between PI3-kinase/Akt pathway and activation of ERK1/2 [6].

In summary, our present study identifies a novel role of intracellular H_2O_2 in modulating eNOS expression and activity in response to OSS. Redox-sensitive activation of CaMKII is necessary for OSS upregulation of eNOS mRNA. The simultaneous production of $NO^{\bullet}$ and $O_2^{\bullet-}$ in response to OSS, however, may facilitate peroxynitrite formation and protein nitration, which may in turn predispose to atherosclerosis. Both OSS and unidirectional LSS upregulate eNOS expression and activity but via distinct signaling mechanisms.

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