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Pulsatile Versus Oscillatory Shear Stress Regulates NADPH Oxidase Subunit Expression:

Implication for Native LDL Oxidation

Juliana Hwang, Michael H. Ing, Adler Salazar, Bernard Lassègue, Kathy Griendling, Mohamad Navab, Alex Sevanian, and Tzung K. Hsiai

Department of Biomedical Engineering and Division of Cardiovascular Medicine (J.H., M.H.I., A.S., T.K.H.) and Department of Molecular Pharmacology and Toxicology (J.H., A.S.), University of Southern California, Los Angeles, Calif; Division of Cardiology (B.L., K.G.), Emory University, Atlanta, Ga; and Division of Cardiology, Department of Medicine (M.N.), University of California Los Angeles, Calif

Abstract

Shear stress regulates endothelial nitric oxide and superoxide (O_2^-) production, implicating the role of NADPH oxidase activity. It is unknown whether shear stress regulates the sources of reactive species production, consequent low-density lipoprotein (LDL) modification, and initiation of inflammatory events. Bovine aortic endothelial cells (BAECs) in the presence of 50 $\mu\text{g}/\text{mL}$ of native LDL were exposed to (1) pulsatile flow with a mean shear stress (τ_{ave}) of 25 dyne/cm^2 and (2) oscillating flow at τ_{ave} of 0. After 4 hours, aliquots of culture medium were collected for high-performance liquid chromatography analyses of electronegative LDL species, described as LDL^- and LDL^{2-} . In response to oscillatory shear stress, gp91^{phox} mRNA expression was upregulated by 2.9 \pm 0.3-fold, and its homologue, Nox4, by 3.9 \pm 0.9-fold ($P<0.05$, $n=4$), with a corresponding increase in O_2^- production rate. The proportion of LDL^- and LDL^{2-} relative to static conditions increased by 67 \pm 17% and 30 \pm 7%, respectively, with the concomitant upregulation of monocyte chemoattractant protein-1 expression and increase in monocyte/BAEC binding ($P<0.05$, $n=5$). In contrast, pulsatile flow downregulated both gp91^{phox} and Nox4 mRNA expression (by 1.8 \pm 0.2-fold and 3.0 \pm 0.12-fold, respectively), with an accompanying reduction in O_2^- production, reduction in the extent of LDL modification (51 \pm 12% for LDL^- and 30 \pm 7% for LDL^{2-}), and monocyte/BAEC binding. The flow-dependent LDL oxidation is determined in part by the NADPH oxidase activity. The formation of modified LDL via O_2^- production may also affect the regulation of monocyte chemoattractant protein-1 expression and monocyte/BAEC binding.

Keywords

shear stress; NADPH oxidase; LDL oxidation; Nox4; gp91^{phox}

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Correspondence to T.K. Hsiai, MD, PhD, Department of Biomedical Engineering and Division of Cardiovascular Medicine, University of Southern California, Los Angeles, CA. hsiai@usc.edu.

Oscillatory shear stress, cyclic strain, and oxidized LDL increase vascular oxidative stress.^{1,2} Activation of the renin-angiotensin system and shear stress enhance the vascular production of reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), in part through the activation of membrane-bound NADH/NADPH oxidases present in vascular smooth muscle cells, endothelium, fibroblasts, and phagocytic mononuclear cells.¹⁻⁴ Increasing evidence suggests that ROS inactivates nitric oxide (NO), leading to the formation of peroxynitrite ($OONO^-$) and other oxidants.^{5,6} Thus, the enhanced production of ROS impairs endothelium-dependent vasodilation, NO bioavailability, and antiinflammatory responses.

Low-density lipoprotein (LDL) oxidation is one of the fundamental processes in atherogenesis. LDL particles trapped within the subendothelial space undergo oxidative modification, resulting in the formation of minimally modified LDL (MM-LDL) and highly oxidized LDL. Specifically, MM-LDL induces expression of monocyte chemoattractant protein (MCP)-1 on endothelial cells (ECs), leading to monocyte binding and chemotaxis and subsequent trans-endothelial migration.^{7,8} The major fraction, native LDL, represents $\approx 90\%$ to 99% of the total LDL. LDL^- , found in plasma in vivo, is a minimally oxidized subspecies of LDL (resembling MM-LDL) and is characterized by its greater negative charge and oxidized status. LDL^- represents 0.2% to 8% of LDL and is associated with an increased risk of atherosclerosis.^{9,10} However, LDL^{2-} , which harbors a more electronegative charge than LDL^- , constitutes 0.1% to 1% of total LDL and seems to be a more oxidized subfraction of LDL.¹¹ A recent report has elaborated the characteristics of modified LDL species based on electronegative charge and their oxidation characteristics.¹² Three possible sources proposed for LDL^- are the following: (1) oxidation of LDL entrapped in the arterial wall,¹³ (2) ingestion of oxidants or generation from postprandial lipoprotein remnants,¹⁴ and (3) oxidation in plasma.¹⁵

The NADH/NADPH oxidases are multimeric enzymes composed of plasma membrane-associated proteins as well as cytosolic factors. For the phagocytic-type NADPH oxidase, the plasma membrane-associated proteins gp91^{phox} and p22^{phox} comprise the flavocytochrome b558 complex, which forms the catalytic subunit of the oxidase. The cytosolic subunits, including p47^{phox}, p67^{phox}, and the G-protein Rac, provide regulatory function.^{16,17} Several Nox proteins, including gp91^{phox} and Nox4, may contribute to increased intracellular oxidative stress in vascular endothelium; however, the relative contribution of Nox subunits to O_2^- production, as a source of oxidants, remains to be determined.¹⁸

Shear stress, the tangential drag force of blood passing along the surface of the endothelium,¹⁹ imparts profound effects on EC function.²⁰⁻²² Around arterial bends and branches in which the inflammatory responses prevail, the fluid mechanical environment is distinct from the laminar pulsatile environment present in the long, straight sections of the vessel wall. Oscillatory flow with a time-averaged shear stress of zero, characteristic of flow separation points in the arterial branches, upregulates the atherogenic activities of ECs.²³⁻²⁵ Therefore, the characteristics of arterial wall shear stress can influence the recruitment of leukocytes that are fundamental to the initiation of immune responses.^{26,27}

An emerging hypothesis for atherosclerosis is that oxidative stress induces aberrant behavior of the vascular endothelium.^{28,29} Accordingly, the characteristics of shear stress influence oxidative activity that is characterized by increased NADPH oxidase activity and greater production of modified LDL. One consequence of this is the oxidation of LDL to more atherogenic particles that initiate the formation of early atherosclerotic lesions, particularly at the curvatures and lateral walls of vascular branching points.²⁸ Thus, the magnitude, frequency, direction, as well as temporal and spatial components of shear stress may play a distinct role in the production of oxidative stress and oxidatively modified LDL.

Vascular cells use ROS and reactive nitrogen species (RNS) to modify LDL. ECs produce ROS and RNS from the enzymes and NO synthase and by specific homologues of NADPH oxidase (gp91^{phox}, Nox1, and Nox4).³⁰ We hypothesize that NADPH oxidases are capable of producing high levels of ROS in blood vessels in response to specific stimuli arising from flow conditions. Consequently, more modified LDL species are formed under oscillatory flow conditions that favor ROS production, whereas lower levels of LDL modification occur in response to pulsatile flow conditions that produce fewer ROS.

We demonstrate that the characteristics of shear force experienced by ECs play a critical role in the production of O₂⁻ through the expression of 91^{phox} and its homologue, Nox4, modifying the capacity of ECs to either prevent or promote oxidative modification of LDL. Oscillatory flow (bidirectional net zero forward flow), which occurs at the arterial bifurcations, induces greater NADPH oxidase activities and O₂⁻ production and thus enhances LDL oxidation and upregulation of inflammatory markers. In contrast, pulsatile flow (unidirectional positive net forward flow), which occurs in the straight part of vessel, favors downregulated NADPH oxidase activity that may decrease LDL oxidation. Furthermore, we observed relatively higher levels of Nox4 than gp91^{phox} mRNA expression in response to flow conditions, confirming the former as the major NADPH oxidase homolog in ECs.

Materials and Methods

A Novel Pulsatile Flow System

The pulsatile flow system, which was mounted on the inverted microscope (Olympus FV-IX701), was designed to generate welldefined temporal shear stress gradients (see Figure 1 in the online data supplement, available at <http://www.circresaha.org>).^{31,32} Micro Electro Mechanical Systems were used to monitor real-time shear stress.³³ The theoretical formulation for the pulsatile flow generated by this flow channel can be accessed online at <http://ojps.aip.org/abme>.³⁴ The pH of the circulating DMEM culture medium was monitored and kept at 7.4 (Accumet AP) using 5% CO₂. The flow system allows for real-time recording of shear stress and monitoring of ECs and monocyte binding under both pulsatile and oscillatory flow conditions.

Flow Experiments to Measure Oxidatively Modified LDL

Confluent BAEC monolayers between 3 and 6 passages (see the online data supplement for EC culture) were plated on glass slides and incubated in DMEM (no phenol red), 10% FBS,

1% penicillin-streptomycin, and 0.05% Fungizone. Cells were subjected to pulsatile or oscillatory flow in the presence of 50 $\mu\text{g}/\text{mL}$ of native LDL in the flow channel at 60 cycles/min (1 Hz). Two flow conditions were studied, pulsatile flow at $\tau = 293 \text{ dyne}/\text{cm}^2$ per second with the time-averaged shear stress (τ_{ave}) = 25 dyne/cm^2 and oscillating flow between $\pm 3.0 \text{ dyne}/\text{cm}^2$ with $\tau_{\text{ave}} = 0 \text{ dyne}/\text{cm}^2$. After 4 hours, the medium was aliquoted to determine the extent of LDL modification, as described below. At 4 and 8 hours, BAECs were collected for the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of GP91^{phox}, MCP-1, and endothelial NO synthase (eNOS) mRNA expression.

Measurement of Extracellular Superoxide Formation

The production of $\text{O}_2^{\cdot -}$ was measured by cytochrome *c* reduction rates, as described previously^{35,36} (see the online data supplement).

Intracellular $\text{O}_2^{\cdot -}$ Production Measurements

Dihydroethidium (DHE) was used to localize intracellular $\text{O}_2^{\cdot -}$ production, as previously described³⁷ (see the online data supplement).

Separation of LDL Subspecies by High-Performance Liquid Chromatography

Venous blood was obtained at the Atherosclerosis Research Unit from fasting adult human volunteers under institutional review board approval. Plasma was pooled and immediately separated by centrifugation at 1500g for 10 minutes at 4°C. LDL ($d = 1.019$ to 1.063 g/mL) was isolated from freshly separated plasma by preparative ultracentrifugation using a Beckman L8-55 ultracentrifuge and a SW-41 rotor. The technique used for separating LDL was similar to that described previously³⁸ (see the online data supplement).

Quantitative Real-Time RT-PCR

After BAECs were exposed to the flow conditions, total RNA was isolated using RNeasy kit (Qiagen). Real-time RT-PCR was performed according to the recommendations of PE Biosystems Taq-Man PCR Core Reagent Kit.³⁹ TaqMan probes⁴⁰ were used for added specificity and sensitivity (see the online data supplement).

Cloning of a Bovine Nox4 cDNA Fragment From BAECs

Superscript II RT (Invitrogen) was used to prepare cDNA from 5 μg total BAEC RNA. PCR primers n4+1 and n4-2 were designed to anneal to the coding region of human, rat, and mouse Nox4 sequences (see the online data supplement).

Western Blot Analysis of gp91^{phox}

BAEC protein was size separated in 10% SDS BioRad polyacrylamide electrophoresis gel (BioRad) and electro-transferred to a PVDF membrane (Millipore) (see the online data supplement).

Real-Time Monocyte/Endothelial Cell Interactions in Response to Flow Conditions

Monocytes were isolated using a modification of the Recalde method, as previously described,⁴¹ from healthy volunteers with institutional review board approval. Freshly isolated monocytes (10^5 monocytes/cm³) were introduced into the testing channel under flow conditions, as described above for LDL oxidation (see the online data supplement).

Incubation of BAECs With 2-Deoxyglucose

To demonstrate that NADPH oxidase mediates the extent of LDL oxidation in response to oscillatory flow, we incubated BAECs with 2-deoxyglucose (2-DOG) to block the pentose shunt pathway for NADPH production (see the online data supplement). Once the BAEC monolayers became confluent, growth media was changed to glucose-free media comprised of glucose-free DMEM, 15% heat-inactivated FBS, 100 U/mL penicillin-streptomycin, 0.05% amphotericin B, and 10 mmol/L 2-deoxy-D-glucose (Sigma). Confluent BAEC monolayers were incubated overnight (>12 hours) before the flow exposure and measurement of extracellular O₂⁻ formation.

Statistical Analysis

Data are expressed as mean±SD and compared among separate experiments. For comparisons between two groups, statistical analysis was performed using the two-sample independent-groups *t* test. Comparisons of multiple mean values were made by one-way ANOVA, and statistical significance among multiple groups was determined using the Tukey procedure (for pairwise comparisons of means between static-like and pulsatile flow conditions). *P* values of <0.05 are considered statistically significant.

An expanded Materials and Methods section can be found in the online data supplement, available at <http://www.circresaha.org>.

Results

Relative Expression of NADPH Oxidase Subunit gp91^{phox} and Its Homologue, Nox4, in Response to Pulsatile Versus Oscillatory Flow

Pulsatile versus oscillatory flow had different effects on the relative induction of NADPH subunit gp91^{phox} mRNA expression in BAECs (Figure 1). In response to pulsatile flow (25 dyne/cm², 1 Hz), gp91^{phox} was downregulated relative to static conditions by 44±4% at 4 hours and 33±5% at 8 hours (*P*<0.05, n=5). In contrast, oscillatory flow (±3 dyne/cm², 1 Hz, τ_{ave} ±0) induced sustained upregulation of gp91^{phox} expression by 34±5% at 4 hours and by 32±3% at 8 hours (*P*<0.05, n=5) (Figure 1B). Western blot analysis reflected the relative degree of protein expression of gp91^{phox} in response to pulsatile versus oscillatory flow conditions (Figure 1C).

It is unknown whether gp91^{phox} and its homolog, Nox4, are both responsive to pulsatile shear stress. We performed quantitative real-time RT-PCR and compared the relative mRNA expression between Nox4 and gp91^{phox}. We observed a relatively higher level of Nox4 than that of gp91^{phox} mRNA expression by fluorescent intensity and C_T cycles (Figure 2A). Pulsatile flow downregulated both gp91^{phox} and Nox4 mRNA expression (by

1.8±0.2-fold and 3.0±0.12-fold, respectively, n=4, $P<0.05$), whereas oscillatory flow upregulated the expression of both genes (gp91^{phox} by 2.9±0.3-fold, Nox4 by 3.9±0.9-fold, n=4, $P<0.05$) (Figure 2B). Our findings suggest that shear stress modulates O₂⁻ formation mediated by NADPH oxidase complex. Both gp91^{phox} and Nox4 are expressed in BAECs and responsive to the characteristics of shear stress.

Extracellular O₂⁻ Production in Response to Pulsatile Versus Oscillatory Flow Conditions

Using the cytochrome *c* reduction assay, the level of extracellular O₂⁻ production in BAECs was measured in static cultures (control) and under the two flow conditions (Figure 3). O₂⁻ production in BAECs remained relatively unchanged at 1 hour after exposure to both oscillatory shear stress (±3 dyne/cm², 1 Hz) and pulsatile shear stress (25 dyne/cm², 1 Hz). However, the rate of O₂⁻ production remained steady under static conditions ($P>0.05$, n=5), whereas the rates of production in response to oscillatory versus pulsatile flow diverged steadily starting at 2 hours of exposure ($P<0.05$, n=5). The specificity of reduction by O₂⁻ was established by comparing production rates in the presence and absence of SOD (60 µg/mL). The relative levels of O₂⁻ production were found to correlate with Nox4 mRNA expression (Figure 2B) and gp91^{phox} gene and protein expression in BAECs exposed to the flow conditions (Figure 1).

Intracellular O₂⁻ Production by DHE

To complement the cytochrome *c* reduction assay, measurements of intracellular O₂⁻ production were performed using DHE that specifically reacts with O₂⁻ to form the red fluorescent compound ethidium.⁴² Representative fluorescent photomicrographs of DHE-stained BAECs in response to pulsatile and oscillatory flow and control cultures are shown in Figure 4. Ethidium binds irreversibly to double-stranded DNA, appearing as fluorescent staining in the nuclei (Figure 4B). DHE-stained fluorescent images show that increased intracellular O₂⁻ levels are present at significantly higher intensity in the BAECs exposed to oscillatory flow than to pulsatile flow. This is consistent with previous observations showing significant extracellular O₂⁻ production in response to oscillatory flow.

Correlation of Shear Stress–Induced O₂⁻ Production With the Extent of LDL Oxidation

Pulsatile flow significantly reduced the ratios of oxidatively modified LDL species relative to static conditions by 51±12% for LDL⁻ and 30±7% for LDL⁻², whereas oscillating flow increased these modified LDLs by 67±17% and 30±7%, respectively ($P<0.05$, n=5) (Figure 5). The change in LDL modification coincided with the downregulation of gp91^{phox} and Nox4 mRNA expression and the relative rates of O₂⁻ production in response to pulsatile flow and upregulation in response to oscillatory flow conditions (Figures 2 and 3).

Correlation of Oxidatively Modified LDL With the Relative MCP mRNA Expression and Monocyte/BAEC Binding

The extent of oxidatively modified LDL coincided with MCP-1 expression and the subsequent monocyte/BAEC binding. Introduction of pulsatile flow with τ = 293 dyne/cm² per second and τ_{ave} = 25 dyne/cm² resulted in downregulation of MCP-1 mRNA expression by 2-fold compared with no-flow conditions. In contrast, reversing oscillating

flow induced upregulation of MCP-1 expression by 8.5-fold compared with the no-flow conditions (Figure 6B). The extent of LDL modification also coincided with the numbers of monocyte binding to BAECs (control, 8 ± 2 monocytes per high power field; pulsatile flow, 4 ± 1 ; oscillatory flow alone, 29 ± 3 ; $P < 0.05$, $n = 5$) (Figure 6A). Therefore, the extent of LDL modification coincided with the level of MCP-1 expression and the numbers of monocyte/BAEC binding.

eNOS mRNA Expression in Response to Flow Condition

Although the induction of eNOS expression in response to oscillatory flow remains controversial, it seems to depend on the magnitude of shear stress, the frequency of oscillation, and the duration of flow exposure (Figure 7). This observation suggested flow conditions may influence oxidatively modified LDL formation by regulating the relative levels of eNOS and gp91^{phox} expression and activity.

Effects of 2-Deoxyglucose on Superoxide Production

To demonstrate that NADPH oxidase mediates the extent of LDL oxidation in response to pulsatile versus oscillatory flow, we incubated BAECs with 2-DOG to block pentose shunt pathway for NADPH production. The limitation in NADPH created by 2-DOG resulted in a strong reduction in $O_2^{\cdot -}$ production, as would be anticipated by the substrate requirements of NADPH oxidase activity (Figure 8A). Incubating BAECs with 2-DOG significantly reduced the rates of $O_2^{\cdot -}$ formation between the control and 2-DOG samples, oscillatory flow, and oscillatory flow plus 2-DOG samples. The treatment of BAECs with 2-DOG also reduced the amount of LDL⁻ formation (Figure 8B). Although oscillatory flow significantly increased the extent of LDL oxidation, LDL⁻ formation was attenuated in the 2-DOG-treated BAECs, suggesting the role of NADPH as an important cofactor for $O_2^{\cdot -}$ production and subsequent LDL oxidation.

Discussion

Increasing evidence has shown that NADPH oxidase, a multicomponent enzyme complex, is the major source of ROS in the vessel wall.^{16,37} The present study suggests that induction of both gp91^{phox} and Nox4 expression regulates the activity of NADPH oxidase in vascular endothelium, which in turn modulates the relative amounts of LDL⁻ and LDL⁻² formation. The characteristics of shear stress activate the NADPH oxidase system and enhance the cell-mediated oxidation of LDL.

Shear stress may regulate the balance between the expression of NADPH oxidase subunits and eNOS. Oscillatory flow induces greater oxidative stress by $O_2^{\cdot -}$ production and thus enhances LDL oxidation and upregulation of inflammatory markers. In contrast, pulsatile flow favors upregulation of atheroprotective and antioxidant genes that prevent LDL oxidation.⁴³ We observed that both $O_2^{\cdot -}$ production and the extent of modified LDL (LDL⁻ and LDL⁻²) were associated with the expression of gp91^{phox} subunit of the NADPH oxidase activity, implicating this enzyme system in determining ROS generation and oxidative modification of target molecules under various flow conditions.

LDL from different batches contained different levels of LDL⁻, which, along with other properties, would contribute to the extent of the LDL oxidation. However, in this study, LDL particles were derived from pooled plasma of healthy subjects. Our goal was to compare the levels of LDL oxidation in response to two distinct flow patterns. Each set of flow experiments (pulsatile versus oscillatory flow) was performed using the same LDL sample.

As observed in our laboratory and reported by Rueckschloss et al,⁴⁴ the NADPH oxidase subunits p22^{phox} and p47^{phox} are expressed at similar levels in both ECs and leukocytes, whereas gp91^{phox} is expressed at a much lower level in ECs than in leukocytes, as determined by real-time RT-PCR (Figure 2A). We have observed a relatively higher level of Nox4 than that of gp91^{phox} mRNA expression by the quantitative real-time RT-PCR method (Figure 2B). Our findings suggest that shear stress modulates O₂⁻ formation mediated by the NADPH oxidase complex. Both gp91^{phox} and Nox4 are expressed in BAECs and responsive to the characteristics of shear stress; however, Nox4 seems to be the dominant source of NADPH oxidase-derived O₂⁻. Based on real-time RT-PCR data, Nox4 expression is ≈4 times greater than gp91^{phox}. Assuming that both gp91^{phox} and Nox4 are equal in their capacity to generate O₂⁻, we estimate that Nox4 contributes ≈4 times more to O₂⁻ production. Several Nox proteins, including gp91^{phox} and Nox4, may contribute to increased intracellular oxidative stress. Monocytes express almost exclusively gp91^{phox}, whereas the most abundant gp91^{phox} homologue in ECs, smooth muscle cells, and fibroblasts is Nox4.⁴⁵ In atherosclerotic arteries, gp91^{phox} is localized to the central region of plaque shoulder. Nox4 expression is evident in the media and in the intima surrounding the central core of the plaque. In response to oxidized LDL, gp91^{phox} is expressed at a much lower level in ECs than in leukocytes.⁴⁴

DHE staining showed a significant level of intracellular O₂⁻ production in response to oscillatory flow. This finding, taken together with results from cytochrome *c* reduction assay for extracellular O₂⁻ production, suggests that vascular endothelium and NADPH oxidase subunits, gp91^{phox} and Nox4 expression, generate the bulk of O₂⁻. It is possible that the increased NADPH oxidase-derived O₂⁻ levels in BAECs consume flow-induced endothelium-derived NO, decreasing its signaling function with increased formation of other oxidants, such as ONOO⁻. There is also evidence for mitochondria as a source of cytosolic O₂⁻ that may transit via the outer membrane voltage-dependent anion channels.⁴⁶

A subclass of LDL described on the basis of its greater electronegativity and oxidative status has been previously characterized.^{9,38,47} These particles, which were referred to as LDL⁻, are enriched in lipid peroxides and other peroxidation products compared with the bulk of the unmodified, native LDL recovered from human plasma. We have demonstrated that LDL⁻ is a major carrier of lipid hydroperoxides and cholesterol oxides associated with plasma LDL and that LDL⁻ may arise from oxidative events in the vasculature.⁴⁸ Decreased rates of O₂⁻ production in glucose-free medium containing 2-DOG additionally implicate NADPH as an important cofactor for NADPH oxidase activity and the necessity of this activity to produce O₂⁻ and modify LDL. The variation in LDL⁻ levels formed by BAECs under pulsatile versus oscillatory flow conditions points to a role for NADPH oxidase activation in cell-mediated LDL modification.

The electronegative and oxidative status of LDL impacts the regulation of MCP-1 expression and monocyte/EC binding, key factors in the pathogenesis of inflammatory processes associated with atherogenesis. LDL oxidation and inflammation are based on seminal observations that LDL must be oxidatively modified for it to be taken up by macrophages.^{49,50} At the arterial bifurcations or branching point, ECs become hyperpermeable in the presence of hyperlipoproteinemia, which may favor intimal uptake and retention of LDL, resulting in local oxidative degradation of trapped LDL.⁵¹⁻⁵³ LDL particles trapped within the subendothelial space undergo oxidative modification, resulting in the formation of MM-LDL and highly oxidized LDL. MM-LDL and LDL⁻ induce expression of MCP-1 on ECs,^{9,54} leading to monocyte binding, chemotaxis, and subsequent trans-endothelial migration.^{7,54}

Conclusion

Pulsatile (positive net forward flow) versus oscillatory (zero net forward flow) flow regulates the relative induction of the NADPH subunits, gp91^{phox} and its homologue Nox4 mRNA, which affects the conversion of native LDL to more electronegative LDL particles, namely, LDL⁻ and LDL⁻². The modification of LDL seems to be NADPH oxidase dependent, the activity of which is influenced by the nature of shear forces. Pulsatile versus oscillatory shear stress regulates the production of ROS and RNS and the extent of LDL modification. The electronegative status of LDL may subsequently affect the regulation of MCP-1 expression and monocyte/EC binding as important inflammatory and proatherogenic events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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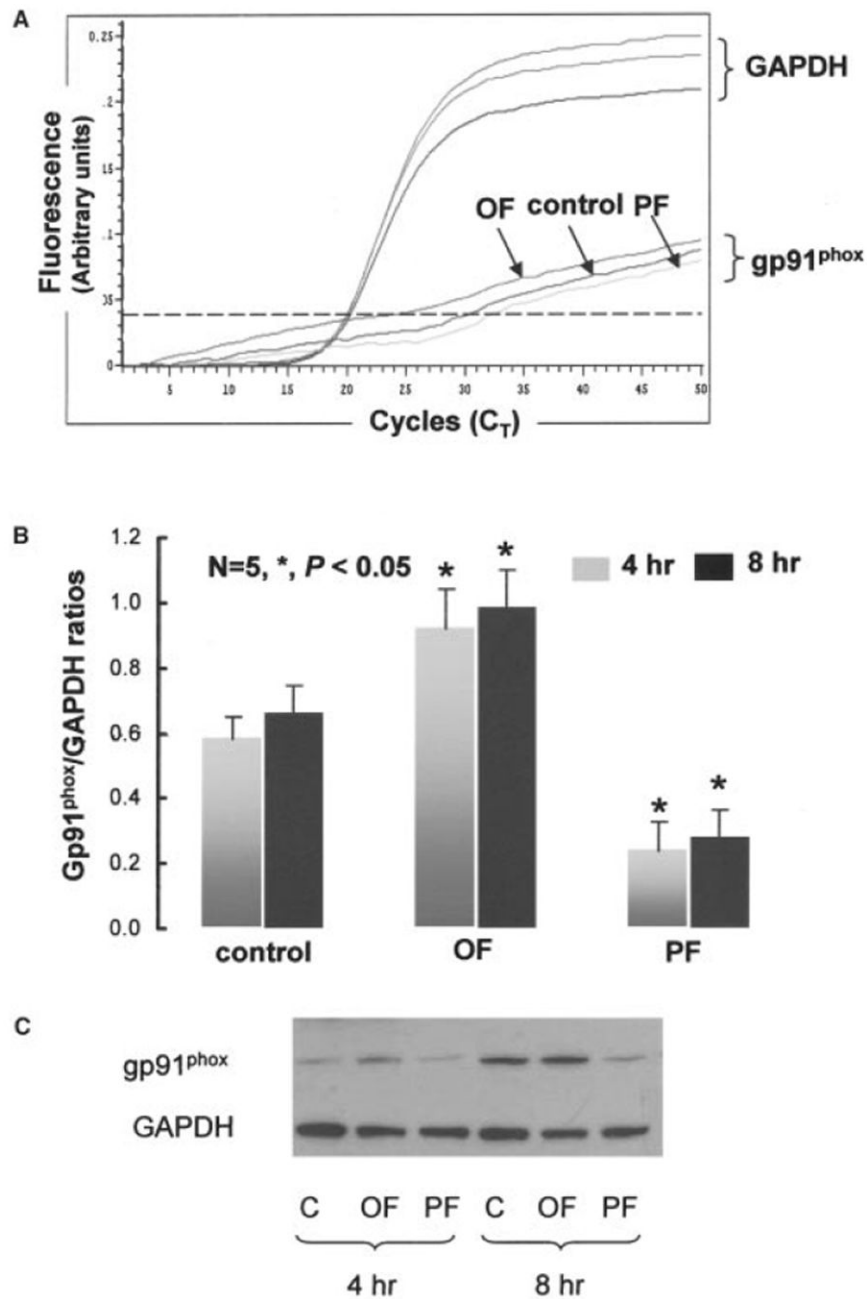


Figure 1.

A, Fluorescent signals versus PCR cycles of gp91^{phox} versus GAPDH. B, Relative NADPH subunit gp91^{phox} mRNA expression normalized to GAPDH in response to pulsatile flow (PF) and oscillatory flow (OF) at 4 and 8 hours. C, Western blots of gp91^{phox} at 4 and 8 hours.

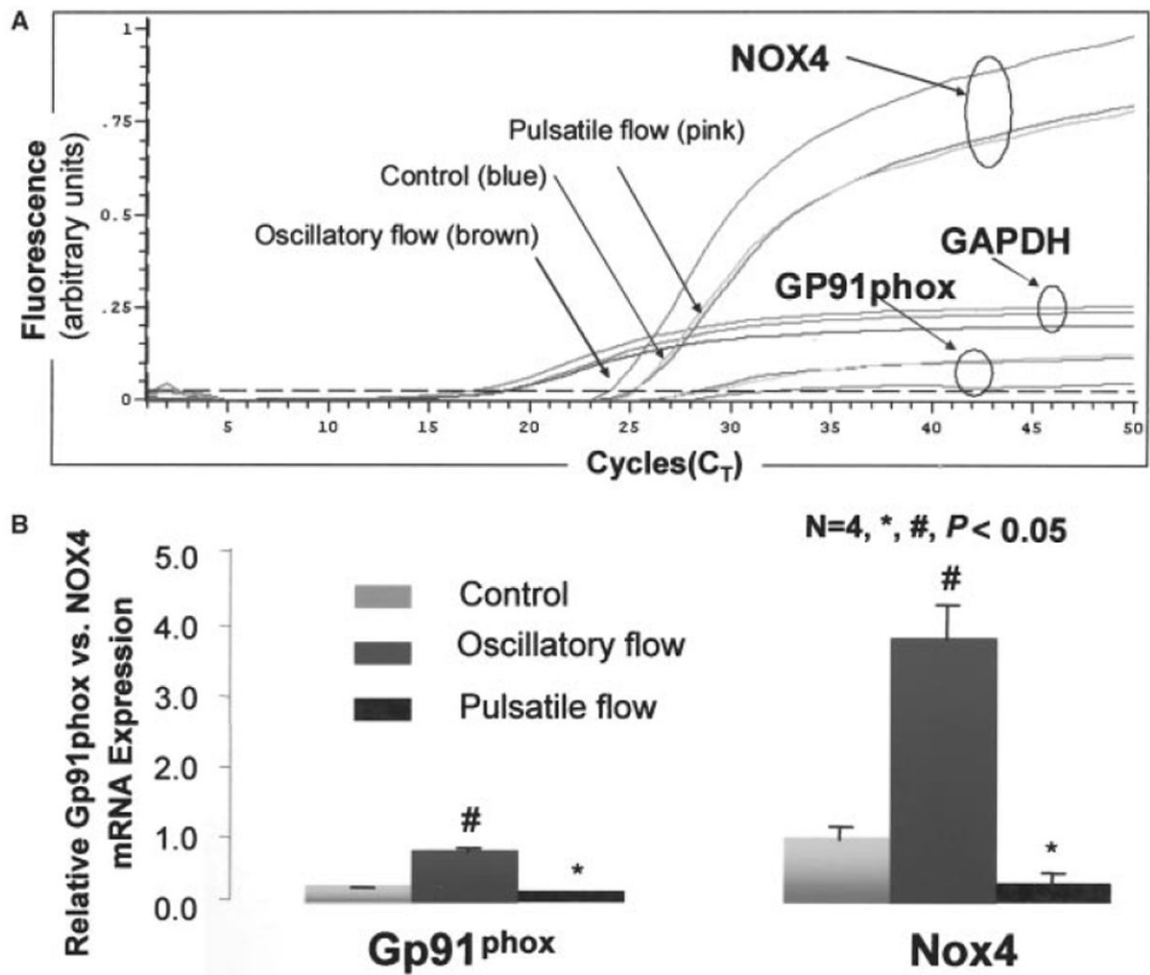


Figure 2.

A, Fluorescence signal versus cycle number for Nox4 and gp91^{phox} and GAPDH. B, Bar graphs show relative mRNA expression for gp91^{phox} and Nox4 in response to pulsatile versus oscillatory flow conditions. Values are expressed as mean±SEM. # $P < 0.05$ gp91^{phox} vs control; * $P < 0.05$ Nox4 vs control.

Rates of Superoxide Production in Response to Flow Conditions

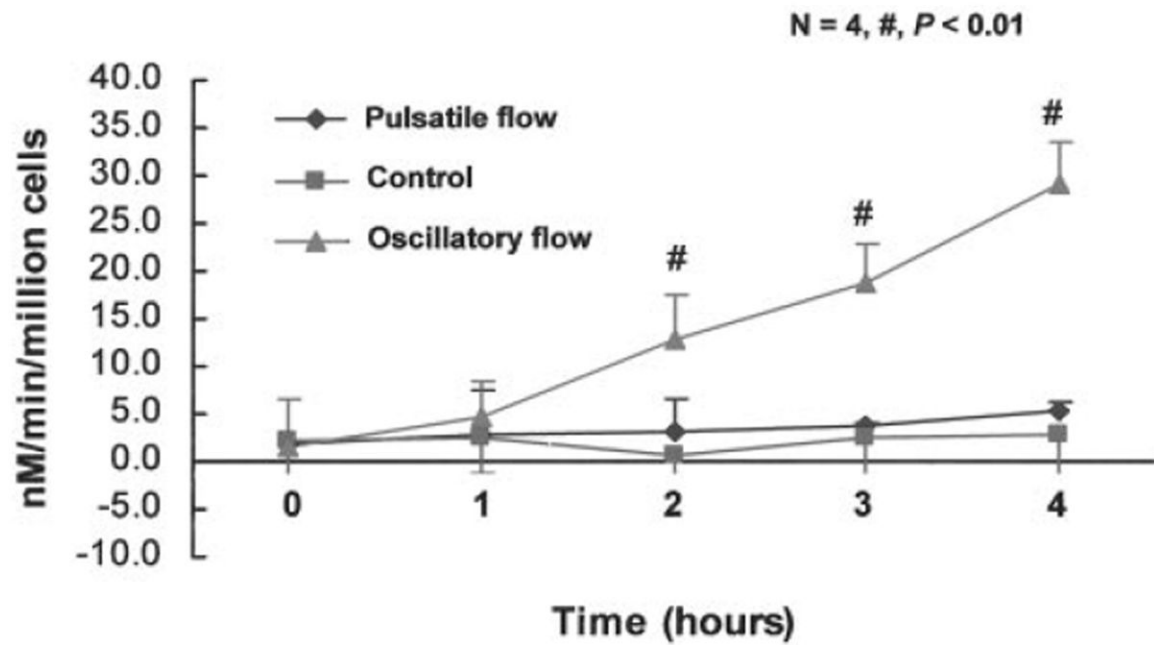


Figure 3. Extracellular superoxide-measured production in BAECs by cytochrome *c* reduction assay. Rate of superoxide production remained unchanged under the static state (control). However, the rates in response to oscillatory versus pulsatile shear stress diverged starting at 2 hours of exposure.

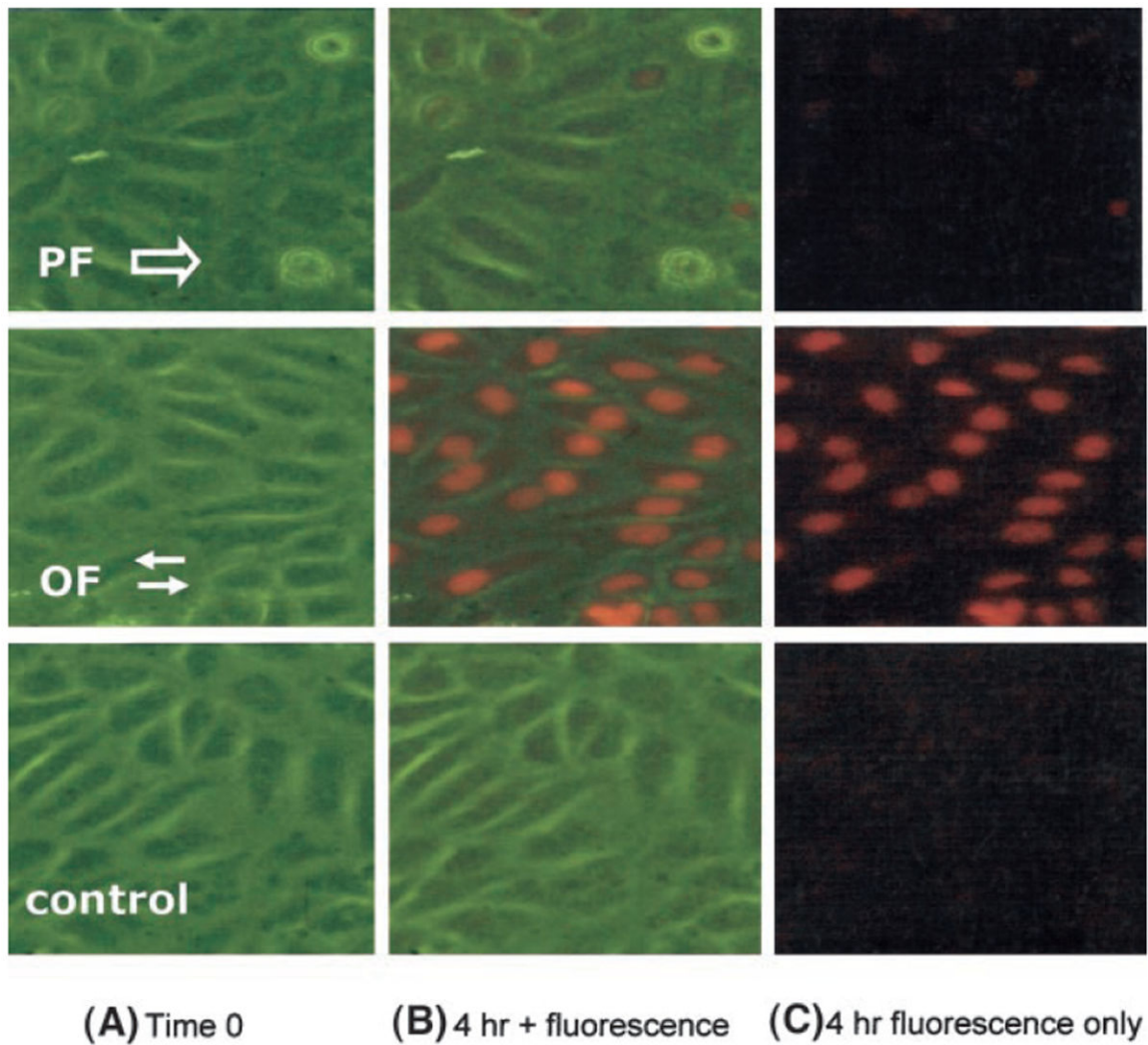


Figure 4.

Real-time intracellular superoxide production in response to flow measured by DHE. A, At time zero, column A illustrates BAECs under 3 conditions: pulsatile flow (PF), oscillatory flow (OF), and static state (control). B, Real-time merged images of phase and fluorescence at 4 hours demonstrate the localization of red fluorescence in the nuclei. In the presence of superoxide, DHE was converted to ethidium, which intercalated into the double-stranded DNA in the nuclei. C, Real-time fluorescent microscopy underscores the superoxide production as red fluorescence.

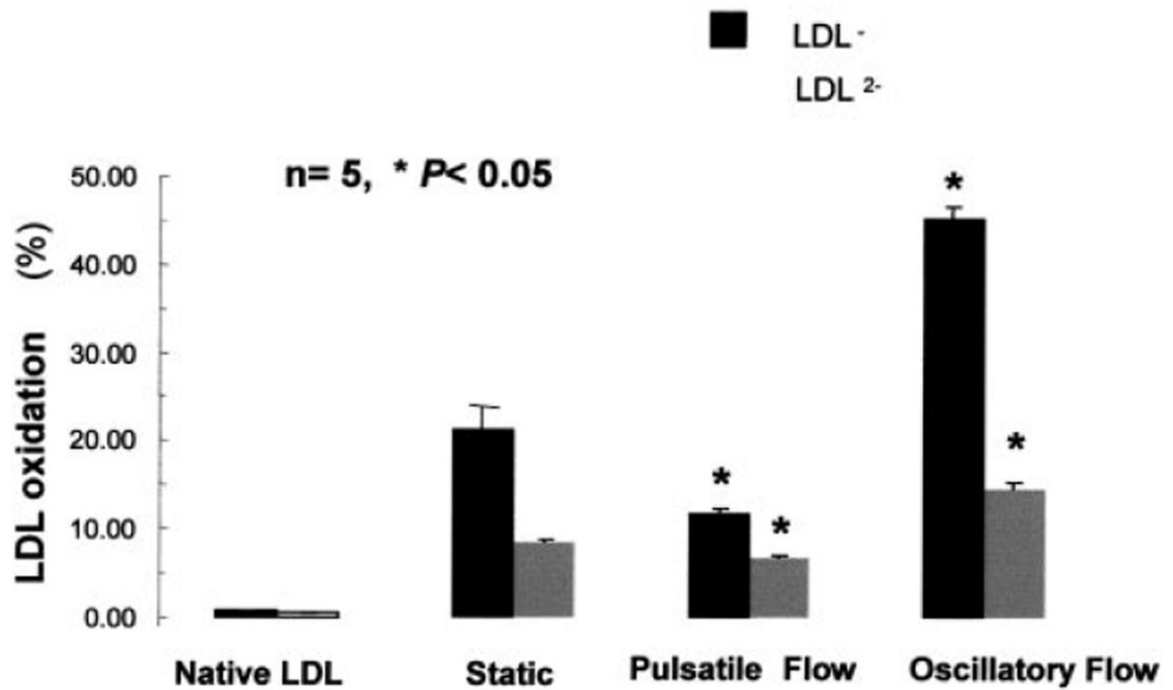


Figure 5.

Flow regulation of native LDL oxidation. Pulsatile flow significantly reduced the ratios of oxidatively modified forms of LDL relative to static conditions by $51 \pm 12\%$ for LDL⁻ and $30 \pm 7\%$ for LDL²⁻, whereas oscillating flow increased LDL oxidation by $67 \pm 17\%$ and $30 \pm 7\%$, respectively (* $P < 0.05$, $n=5$).

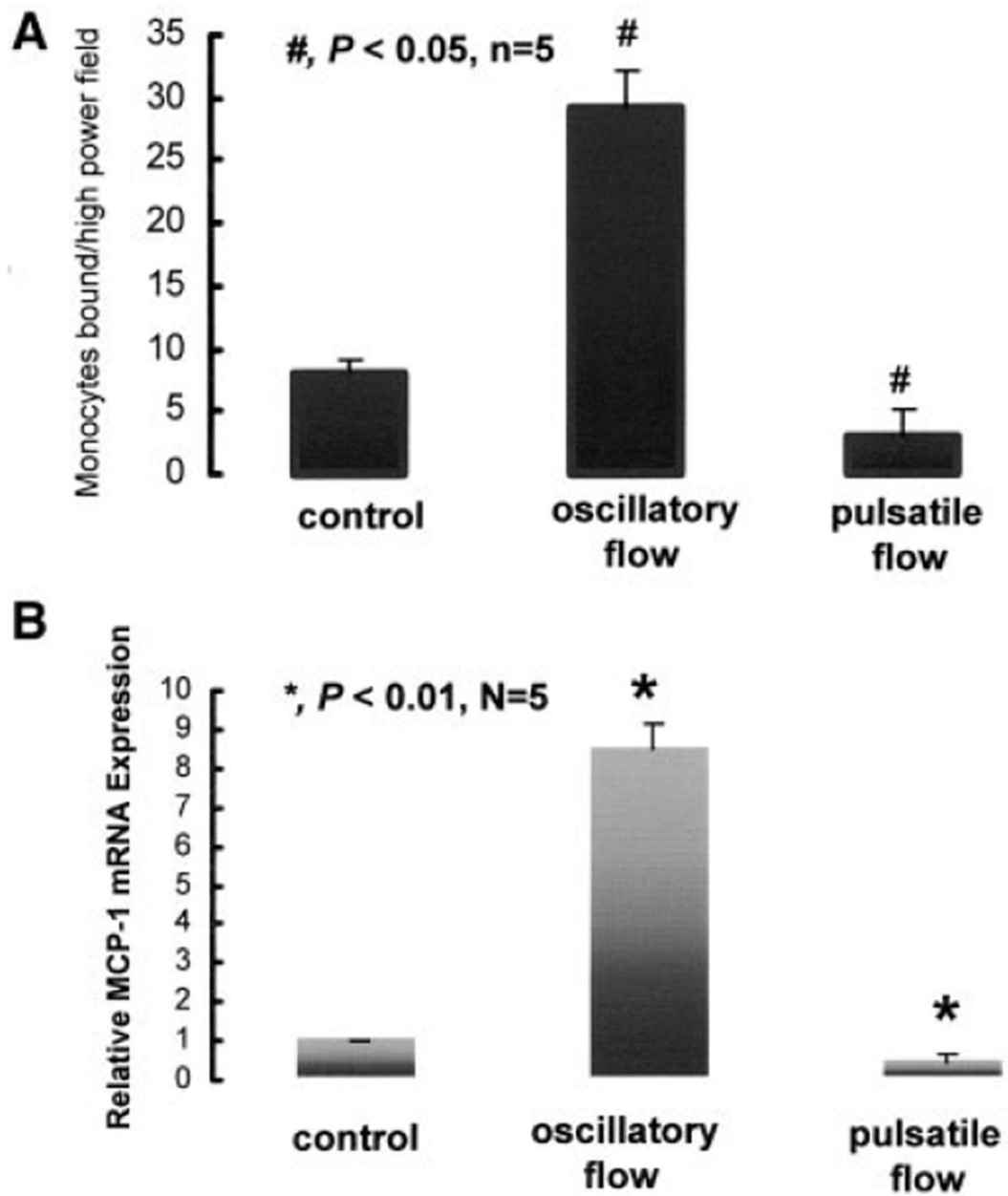


Figure 6.

Monocytes establishing solid adhesion to ECs were captured in response to pulsatile flow and oscillatory flow. A, Numbers of monocytes bound per high-power field in response to control (static condition), oscillatory, and pulsatile flow at 4 hours. B, Relative changes in MCP-1 mRNA expression.

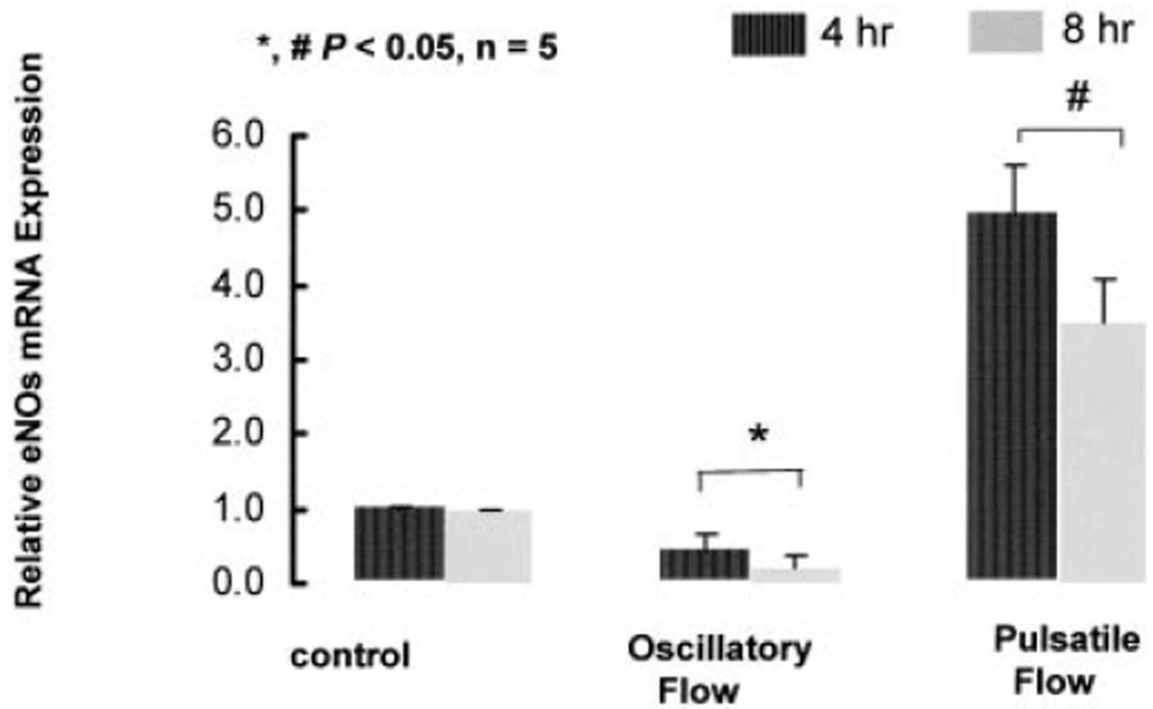


Figure 7. Flow regulation of eNOS mRNA expression normalized with GAPDH in response to control, pulsatile flow, and oscillatory flow at 4 and 8 hours.

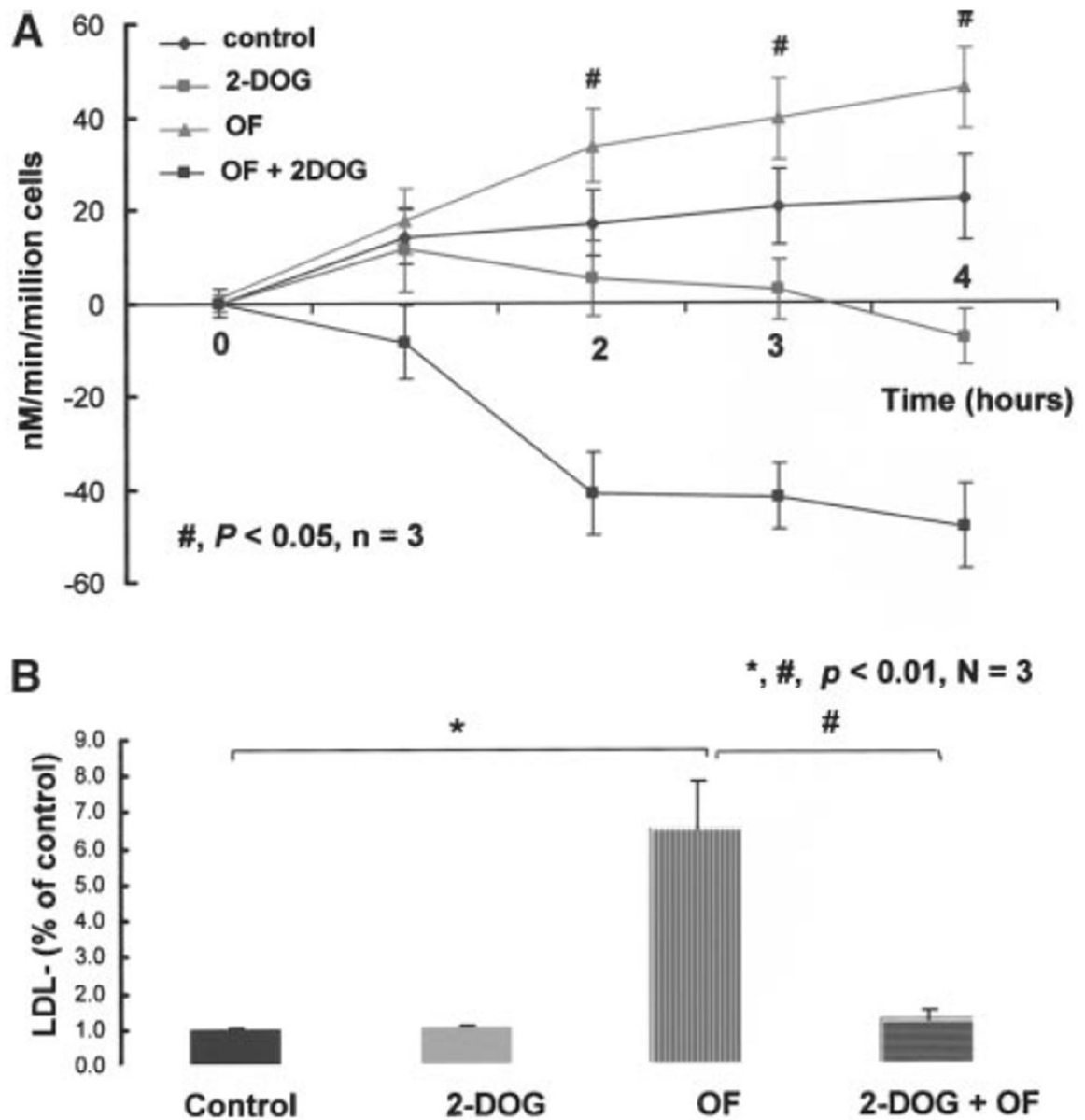


Figure 8.

Rates of superoxide production in the presence of 2-DOG between static and oscillatory flow (OF) conditions. A, BAECs were incubated with 10 mmol/L of 2-DOG for 12 hours before SOD inhibited ferric cytochrome *c* reduction assay. The presence of 2-DOG significantly reduced the rates of superoxide formation between the control and 2-DOG samples, OF, and OF+2-DOG samples. The negative rates of superoxide production were attributable to the use of SOD. B, LDL oxidation in response to OF. The presence of 2-DOG significantly attenuated the extent of LDL oxidation, suggesting the role of NADPH as an important cofactor for superoxide production and the subsequent LDL oxidation.