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Hemodynamics Influences Vascular Peroxynitrite Formation: Implication for LDL Apo B-100 Nitration

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Abstract

Hemodynamics, specifically, fluid shear stress, modulates the focal nature of atherogenesis. Superoxide anion ($O_2^{\cdot-}$) reacts with nitric oxide ($\cdot NO$) at a rapid diffusion-limited rate to form peroxynitrite ($O_2^{\cdot-} + NO \rightarrow ONOO^-$). Immunohistostaining of human coronary arterial bifurcations or curvatures, where oscillatory shear stress (OSS) develops, revealed presence of nitrotyrosine staining, a fingerprint of peroxynitrite; whereas in straight segments, where pulsatile shear stress (PSS) occurs, nitrotyrosine was absent. We examined vascular nitrative stress in models of OSS and PSS. Bovine aortic endothelial cells (BAEC) were exposed to fluid shear stress that simulates arterial blood flow: (1) PSS at a mean shear stress (τ_{ave}) of $23 \text{ dyn}\cdot\text{cm}^{-2}$ and a temporal gradient ($\partial\tau/\partial t$) at $71 \text{ dyn}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$, and (2) OSS at $\tau_{ave} = 0.02 \text{ dyn}\cdot\text{cm}^{-2}$ and $\partial\tau/\partial t = \pm 3.0 \text{ dyn}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ at a frequency of 1 Hz. OSS significantly up-regulated NADPH oxidase (Nox4) expression accompanied with an increase in $O_2^{\cdot-}$ production. In contrast, PSS up-regulated eNOS expression accompanied with NO production (total NO_2^- and NO_3^-). To demonstrate that $O_2^{\cdot-}$ and $\cdot NO$ implicate in $ONOO^-$ formation, we added low density lipoprotein cholesterol (LDL) to the medium in which BAEC were exposed to the above flow conditions. The medium was analyzed for LDL apo B-100 nitrotyrosine by liquid chromatography, electro ionization spray, and tandem mass spectrometry (LC/ESI/MS/MS). OSS induced higher levels of 3-nitrotyrosine, di-tyrosine, and *o*-hydroxy-phenylalanine compared with PSS. In the presence of $ONOO^-$, specific apo B-100 tyrosine residues underwent nitration in the α and β helices: α -1 (Tyr¹⁴⁴), α -2 (Tyr²⁵²⁴), β -2 (Tyr³²⁹⁵), α -3 (Tyr⁴¹¹⁶), and β -2 (Tyr⁴²¹¹). Hence, the characteristics of shear stress in the arterial bifurcations influenced the relative production of $O_2^{\cdot-}$ and $\cdot NO$ with an implication for $ONOO^-$ formation as evidenced by LDL protein nitration.

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Keywords

shear stress; superoxide anion; nitric oxide; nitrotyrosine; LDL

Introduction

The characteristics of shear stress, namely, spatial and temporal variations, influence the focal character of atherosclerosis^{1–3}. Shear stress acting on endothelial cells at arterial bifurcations or branching points regulates both NADPH oxidase^{4,5} and nitric oxide synthase activities^{6,7}. The former is considered a major source of oxygen-centered radicals (i.e., superoxide anion [$O_2^{\cdot-}$]) (oxidative stress), whereas the latter is a source of nitrogen-centered radicals (i.e., nitric oxide [NO]) (nitritative/nitrosative stress). Oxidative and nitritative stress are involved in the modification of low-density lipoprotein (LDL), which occurs at high levels in the athero-prone regions⁸.

At the lateral wall of arterial bifurcations, flow separation and migrating stagnation points create a distinct characteristics of oscillating shear stress (OSS: bidirectional net zero forward flow)⁹. In these OSS-exposed regions, reactive oxygen species (ROS) are reported to be upregulated,^{1,2,10} and OSS may account for production of $O_2^{\cdot-}$ via the membranous NADPH oxidase^{5,11}. Furthermore, an increase in $O_2^{\cdot-}$ production has been implicated in the oxidative modifications of LDL that plays an important role in up-regulation of adhesion molecules and cytokines¹². In contrast, pulsatile shear stress (PSS: unidirectional and positive net forward flow) preferentially develops in the medial wall of arterial bifurcations or the straight regions¹³. PSS down-regulates NADPH oxidase activities and expression of adhesion molecules and cytokines¹⁴, providing an athero-protective mechanism to endothelial cells^{2,11}.

Nitrotyrosine is considered to be an emergent inflammatory marker for atherosclerosis¹⁵. Superoxide anion ($O_2^{\cdot-}$) reacts with NO at a rapid diffusion-limited rate to form a strong oxidant peroxynitrite ($O_2^{\cdot-} + NO \rightarrow ONOO^-$)^{16–18}. In the presence of $ONOO^-$, the tyrosine residue of proteins undergoes nitration, giving rise to nitrotyrosine, a fingerprint for peroxynitrite¹⁵. In the present study, we hypothesized that the specific characteristics of shear stress influence the formation of peroxynitrite due to the imbalance between $O_2^{\cdot-}$ and NO production in the OSS-exposed regions of vasculatures, and that the presence of $ONOO^-$ induces LDL apo B-100 protein nitration. By immunohistostaining of the explants of human coronary arteries, we demonstrated that OSS-exposed arterial bifurcations were prevalent for nitrotyrosine formation. By a combination of dynamic flow system^{11,19} and liquid chromatography, electro spray ionization and tandem mass spectrometry (LC/ESI/MS/MS)^{20,21}, we showed that OSS influenced the formation of $ONOO^-$ as represented by LDL protein nitration at the tyrosine residues in the α - and β -helices.

Methods

Endothelial Cell Culture

Confluent bovine aortic endothelial cells (BAEC) between passages 4 and 7 were seeded on Cell-Tak cell adhesive (Becton Dickinson Labware, Bedford, MA) and Vitrogen (Cohesion, Palo Alto, RC 0701) coated glass slides (5 cm²) at 3×10^6 cells per slide. BAEC were grown to confluent monolayers in high glucose (4.5 g/L) DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 15% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin-streptomycin (Irvine Scientific), and 0.05% amphotericin B (Gibco) for 48 h in 5% CO₂ at 37°C_[k1].

Immunohistochemistry Analyses of Human Coronary Arteries

Human coronary arteries were obtained from the explanted hearts of cardiac transplant patients with ischemic cardiomyopathy in compliance with the Institutional Review Board. Specific cross-sections of the left and right coronary arteries were analyzed: the lateral wall of arterial bifurcations where oscillatory shear stress develops and the straight segments where pulsatile shear stress occurs^{1,22}. Monoclonal antibodies were used for NOS isoforms (Transduction Labs) and nitrotyrosine (Upstate). Immunostaining was performed with standard techniques in frozen vascular tissue using biotinylated secondary antibodies and peroxidase staining. Nitrotyrosine residues were assessed on paraffin sections after quenching of K treatment for 15 min at room temperature endogenous peroxidase with 3% H₂O₂, proteinase (Dakocytomation), and non-specific adsorption with 3% BSA in PBS for 15 min using a monoclonal anti-nitrotyrosine antibody (1:50 in PBS/1% BSA, overnight incubation at 4°C; Zymed, Clinisciences). Sections were then incubated with a biotin-conjugated goat anti-mouse antibody (1:200 in PBS/1% BSA; Santa Cruz Biotechnology), followed by streptavidin-biotin-peroxidase complex (1 h incubation at room temperature with both agents; Zymed, Clinisciences). DAB were used as a chromogen and the sections were counterstained with hematoxylin for visualization of intima, media, smooth muscle cells, and adventia. Furthermore, endothelial and smooth muscle cells were stained with monoclonal antibodies specific for von Willebrand Factor (1:25 dilution) and β -actin (1:4000 dilution) (Dakocytomation, Carpinteria, CA). Negative controls were performed by omitting the primary antibody. Positive controls included brain and kidney tissues. Immunoreactivity to nitrotyrosine and endothelial NOS (eNOS) were compared between the left main bifurcation and right coronary arteries. A semi-quantitative analysis was performed to determine the percentage of vascular cells staining positive for eNOS and nitrotyrosine.

Flow Experiments to Analyze LDL Protein Nitration

Venous blood was obtained from fasting adult human volunteers under institutional review board approval from the Atherosclerosis Research Unit at the University of Southern California. Plasma was pooled and immediately separated by centrifugation at 1500g for 10 minutes at 4°C. The technique used for separating LDL ($\delta=1.019$ to 1.063 g/mL) was similar to that described previously^{23,24}.

A dynamic flow system was used to deliver temporal variations in shear stress ($\partial\tau/\partial t$); namely, pulsatile (PSS) and oscillatory (OSS) shear stress^{25,26,27}. Confluent BAEC were subjected to the flow conditions in the absence and presence of LDL at 50 $\mu\text{g/mL}$: (1) Control, using cells grown under static conditions ($\tau_{\text{ave}}=0$ dyn $\cdot\text{cm}^{-2}$ at $\partial\tau/\partial t=0$), (2) PSS at a mean shear stress (τ_{ave}) of 23 dyn $\cdot\text{cm}^{-2}$ with a temporal variation ($\partial\tau/\partial t$) at 71 dyn $\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$, and (3) OSS at $\tau_{\text{ave}}=0.02$ with $\partial\tau/\partial t$ at ± 3 dyn $\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$. After 4 hours, BAEC were collected for quantitative RT-PCR and Western blots. In the absence of LDL, the culture medium was collected to identify the differential production of $\text{O}_2^{\cdot-}$ and $\text{NO}_2^-/\text{NO}_3^-$ in response to PSS and OSS, respectively. In the presence of LDL, the culture medium was used to determine apo-B 100 post-translational modifications.

Measurement of Extracellular Superoxide Anion ($\text{O}_2^{\cdot-}$) Formation

The production of $\text{O}_2^{\cdot-}$ from BAEC monolayers exposed to the flow conditions as described above was measured as the superoxide dismutase (SOD)-sensitive reduction of cytochrome *c*^{18,20}. In each case, the medium contained 100 μM acetylated-ferricytochrome *c* (Sigma-Aldrich, St. Louis, Mo)²⁸. Control samples were maintained in a cell culture dish with media containing cytochrome *c* (100 μM) and incubated at 37°C. Aliquots of culture medium (300 μL) were collected at 0, 1, 2, 3, and 4 h, and ferrocyanochrome *c* absorbance was measured at 550 nm ($\epsilon_{550}=2.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$)²⁹. For the OSS and PSS conditions, aliquots of medium bathing the BAEC in the flow apparatus were aspirated into a media solution containing

acetylated-ferricytochrome *c* (1mM) and absorbance measured at 550 nm at 0, 1, 2, 3, and 4 h. The specificity of reduction by $O_2^{\cdot-}$ was established by comparing reduction rates in the presence and absence of SOD at 60 $\mu\text{g/ml}$ ³⁰. The corrected rates for SOD-inhibited cytochrome *c* reduction were plotted after computing $O_2^{\cdot-}$ formation.

Analysis of Nitrite (NO_2^-) and Nitrate (NO_3^-)

Quantitative measurements of NO_2^- and NO_3^- were performed as an index of global nitric oxide ($\cdot\text{NO}$) production following methods described previously^{31,32}. $\cdot\text{NO}$ is metabolized or decomposed via various reactions to the metabolites as NO_2^- and NO_3^- , which serve as useful measures of overall $\cdot\text{NO}$ production and metabolism³³. Briefly, the analytical procedure was based on acidic reduction of NO_2^- and NO_3^- to $\cdot\text{NO}$ by vanadium (III) and purging of $\cdot\text{NO}$ with helium into a stream of ozone and detected by an Antek 7020 chemiluminescence- $\cdot\text{NO}$ detector (Antek Instruments, Houston, TX). At room temperature, vanadium (III) only reduced NO_2^- , whereas NO_3^- and other redox forms of $\cdot\text{NO}$ (such as *S*-nitrosothiols) were reduced only if the solution was heated to 90–100°C, so that both NO_2^- and total $\cdot\text{NO}$ could be measured. This allowed for determination of the relative levels of NO_2^- and NO_3^- , which might be indicative of differential oxidative metabolic routes of $\cdot\text{NO}$. Quantification was performed by comparing with standard solutions of NO_2^- and NO_3^- .

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed to study whether one of the NADPH oxidase homologues, Nox4, and eNOS were implicated in the relative production of $O_2^{\cdot-}$ and $\cdot\text{NO}$, respectively. After BAEC were exposed to the flow conditions, total RNA was isolated using RNeasy kit (Qiagen). qRT-PCR was performed according to the recommendations of PE Biosystems TaqMan PCR Core Reagent Kit³⁴. Equal amounts of RNA at 0.5 $\mu\text{g}/\mu\text{L}$ were reverse-transcribed with rTth DNA Polymerase to bring the mixed solution to a final concentration of 1X TaqMan buffer, 5 mM MgCl_2 , 0.2 mM dATP/dCTP/dGTP, 0.4 mM dUTP, 0.1 mM probe, 0.4 μM primers, 0.01 U/ μL AmpErase, and 0.025 U/ μL rTth DNA polymerase. Total cDNA at 0.4 ng/ μL in 5 μL was then transferred to the 96-well plate, followed by RT, beginning with manual ramp rate at 50°C for 2 min, 60°C for 30 min, and then 95°C for 5 min. Next, PCR was performed for 50 cycles at 94°C for 20 s and annealing from 58 to 65°C for 1 min (MJ Research Opticon[®] System). C_T is the threshold cycle number at which the initial amplification becomes detectable by fluorescence. ΔR_n normalizes fluorescence. TaqMan probes were used for added specificity and sensitivity³⁵. Each sample was tested in duplicate. Each experiment was performed 5 times. The difference in C_T values for various flow conditions vs. control were used to mathematically determine the relative difference in the level of NADPH oxidase homologue, Nox4, and eNOS mRNA expression³⁵. For quantification of relative gene expression, the target sequence was normalized to the expressed housekeeping gene GAPDH or β -actin.

Western Blotting Analyses

Western blots were performed to determine if one of the NADPH oxidase homologues, Nox4, was implicated in $O_2^{\cdot-}$ and $\cdot\text{NO}$ production, respectively. BAEC lysate was size-separated in 10% SDS BioRad polyacrylamide electrophoresis gel (BioRad) and electro-transferred to a PVDF membrane (Millipore). Membranes were blocked with 5% nonfat milk in TBS-T and probed with mouse Nox4 antibody (generously provided by Dr. J. David Lambeth Emory University School of Medicine, Pathology and Laboratory Medicine). Membranes were incubated overnight at 4°C and then probed with secondary antibody, goat anti-mouse IgG-HRP conjugated (BioRad). Chemiluminescence detection was used to visualize bands of interest (SuperSignal[™], Pierce) followed by exposure to autoradiography film (Hyperfilm[™] MP, Amersham Pharmacia Biotech). Loading and transfer of equal amounts of protein in each

lane were verified by reprobing the membrane with a monoclonal anti- β -actin antibody from mouse ascites fluid (1:3000 dilution, Sigma-Aldrich). Photographic films were scanned by an imaging densitometer and quantified using the NIH Image software program.

Analyses of LDL Protein Nitration by LC/ESI/MS/MS

After shear stress exposure, LDL suspended in medium was collected for LDL analysis of protein nitration. The extent of total protein-bound nitrotyrosine, *o*-hydroxy-phenylalanine, and di-tyrosine formation in the recovered media was determined by stable isotope dilution liquid chromatography–tandem mass spectrometry³⁶ on a triple quadrupole mass spectrometer (API 4000, Applied Biosystems, Foster City, CA) interfaced with a Cohesive Technologies Aria LX Series HPLC multiplexing system (Franklin, MA). Synthetic [¹³C₆]-labeled nitrotyrosine and [¹³C₁₂]-labeled di-tyrosine internal standard were added to samples for quantification of natural abundance analytes. Simultaneously, a universal labeled precursor amino acid, [¹³C₉, ¹⁵N₁]tyrosine (for nitrotyrosine and di-tyrosine) or [¹³C₉, ¹⁵N₁] phenylalanine (*o*-Phe), was added to both quantify the precursors and assess potential intra-preparative artifactual oxidation during sample handling and analysis. Proteins were hydrolyzed under argon atmosphere in methane sulfonic acid, and then samples passed over mini solid-phase C18 extraction columns (Supelclean LC-C18-SPE minicolumn; Supelco, Inc., Bellefonte, PA) prior to mass spectrometry analysis. Results were normalized to the content of the appropriate precursor amino acid, which was monitored within the same injection. Intra-preparative formation of [¹³C₉, ¹⁵N₁]-labeled nitrotyrosine, *o*-hydroxy-phenylalanine, and di-tyrosine were routinely monitored and were negligible under the conditions employed (i.e. < 5% of the level of the natural abundance product observed).

Analyses of LDL apo B 100 Nitration

Samples of LDL (0.2mg/ml) treated with ONOO⁻ (100uM) were processed for measurements of tyrosine nitration by LC/MS/MS. The tertiary structure of LDL was suspended in 10 μ l 60% formic acid. Chromatographic separation was achieved using a ThermoFinnigan Surveyor MS-Pump with a BioBasic-18 100 mm C 0.18mm reverse phase capillary column. Mass analysis was performed with a ThermoFinnigan LQ Deca XP Plus ion trap mass spectrometer equipped with a nanospray ion source employing a 4.5 cm long metal needle, in the data-dependent acquisition mode. Electrical contact and voltage application to the probe tip took place via the nanoprobe assembly. Spray voltage was set to 2.9 kV and heated capillary temperature at 190° C. The column was equilibrated for 5 min with 95% solution A, 5% solution B (A, 0.1% formic acid in H₂O; B, 0.1 % formic acid in acetonitrile) prior to sample injection. A linear gradient was initiated 5 min after sample injection ramping to 35% A, 65% B after 50 min and 20% A, 80% B after 60 min. Mass spectra were acquired in the *m/z* 400–800 range.

Protein identification was carried out with the MS/MS search software Mascot 1.9 (Matrix Science) with confirmatory or complementary analyses with TurboSequest as implemented in the Bioworks Browsers 3.2, build 41 (ThermoFinnigan). NCBI Sus scrofa protein sequences were used as the primary search database; searches were complemented with the NCBI non-redundant protein database.

Statistical Analysis

Data are expressed as mean $\pm$ 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 analysis of variance (ANOVA), and statistical significance among multiple groups 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.

Result

eNOS and Nitrotyrosine Immunostaining in OSS- versus PSS-Exposed Regions

Explants of human left (LCA) and right (RCA) coronary arteries were compared for eNOS and nitrotyrosine immunostaining. In the OSS-exposed regions (lateral wall of arterial bifurcations or curvatures), eNOS staining was absent in EC, whereas in the PSS-exposed regions, eNOS staining was prevalent throughout the entire EC lining the lumen (n=3) (Fig. 1a–c). Despite the absence of eNOS staining in the OSS-exposed regions, eNOS staining was present in the endothelial lining of vasa vasorum, which may be a potential source of NO needed for ONOO[−] formation. In contrast, nitrotyrosine staining was present in the smooth muscle cells (SMC) in the OSS-exposed regions, including the medial wall of arterial bifurcations and curvatures, but absent in the PSS-exposed regions (n=3) (Fig. 1d–f). Counterstaining with Hematoxylin, von Willebrand factor, and β -actin distinguished EC and SMC, respectively, in the lumen, media, and/or intima.

PSS and OSS Regulated the Relative Production of O₂[−] and NO (NO₂[−] and NO₃[−])

To demonstrate that PSS and OSS induced the relative production of O₂[−] and NO, we used our dynamic flow system³⁷. We observed that OSS was a stronger inducer than PSS in up-regulating Nox4 expression (Fig. 2a). However, PSS was a stronger inducer than OSS in up-regulating eNOS mRNA at 4 h and 8 h (Fig. 2b). Consequently, OSS induced a higher rate of O₂[−] production than did PSS (Table 1). OSS also induced a higher rate of NO formation accompanied with a significantly higher ratio of O₂[−]/NO than did PSS (Table 1), suggesting that the imbalance in O₂[−] and NO production was likely to contribute the presence of nitrotyrosine in the arterial bifurcations as demonstrated by the Immunostaining of peroxynitrite (Fig. 1). Furthermore, the elevated level of total NO formation (NO₂[−] and NO₃[−])^{31,32} was likely due to the presence of peroxynitrite (Table 1).

PSS and OSS Differentially Influenced the Formation of Peroxynitrite

ONOO[−] reacted rather specifically with tyrosine residues in proteins to yield 3-nitrotyrosine. To measure the production of ONOO[−] as protein-bound nitrotyrosine, confluent BAEC were exposed to PSS and OSS in the presence of LDL at 50 μ g/mL. After shear stress exposure, LDL particles suspended in medium was collected for apoB-100 protein modifications. The levels of nitration in nitrotyrosine residues of apoB-100 were higher in response to OSS than to PSS (Table 2). LC/ESI/MS/MS analyses demonstrated differential levels of protein modifications in response to PSS versus OSS: [3-nitrotyrosine] $\ll$ [di-tyrosine] $<$ [*o*-Phe]. Together with the Immunohistochemistry analyses (Fig. 1) and the imbalanced production of O₂[−] and NO (Table 1), the higher levels of apoB-100 protein nitration in response to OSS suggest that vascular nitrative stress is likely to develop within the lateral walls of arterial bifurcations and curvatures. The pathophysiologic implication of d-tyrosine and *o*-hydroxy-phenylalanine remains to be determined.

Peroxynitrite Modified Specific Protein Nitration

To further investigate the molecular mechanisms by which protein underwent nitration in the presence of ONOO[−], we performed LC/MS/MS analyses of LDL (0.2mg/ml) in the presence of 100 μ M of ONOO[−] (Table 3). The tyrosine residues of LDL apoB-100 were susceptible to nitration in the α -1, α -2, α -3, and β -2 helices; specifically, α -1 (Tyr¹⁴⁴), α -2 (Tyr²⁵²⁴), α -3 (Tyr⁴¹¹⁶), β -2 (Tyr³²⁹⁵), and β -2 (Tyr⁴²¹¹). Relative Mascot and Sequest scores were obtained by software that demonstrated LDL modifications. Mascot is the score obtained using matrix science software to analyze the actual observed masses, followed by searching the data base of proteins to determine the sequence of the digested protein, whereas Sequest scores are obtained using Thermo Finnigan (Excaliber) software to determine the most likely peptide

from actual observed peptide masses by searching a protein database³⁸. The representative MS/MS spectrum illustrates a tryptic peptide, NLQNNAEWVYQGAIK, which was modified with tyrosine residues 14 (Fig. 3). The Y and B ion series reflected the direction in which the mass of the ion was observed. The Y6 ion in the MS/MS spectrum showed the presence of tyrosine nitration as evidenced by an additional mass of 45 Daltons ($\text{NO}_2 = 46$ daltons; but the replacement a hydrogen atom resulted in a mass of 45 daltons). Further evidence for an additional 45 Daltons was identified in ions Y7, Y9, Y10, Y11, Y13, B13 and B14. The mass of the modified and non-modified peptides bear a mass of one half of the theoretical for doubly charged ions and one third of the theoretical for triply charged ions. A mass difference of 22.5 daltons was observed for the nitrotyrosine containing peptide as compared to the unmodified peptide. Ion mass to charge ratio (m/z) of entire peptide demonstrated that the observed mass of the modified peptide NLQNNAEWVYQGAIK for the doubly charged ion was 911.23 Daltons, whereas the unmodified peptide mass was 888.57 Daltons, consistent with the presence of nitrotyrosine.

Discussion

This study examined whether or not spatial specific characteristics of shear stress in the arterial bifurcations or straight segments influenced vascular ONOO^- formation with an implication for protein nitration at the tyrosine residues. Immunostaining of the human coronary arteries from ischemic cardiomyopathy patients revealed nitrotyrosine staining present in the OSS-exposed arterial regions (bifurcations or curvatures), but absent in the PSS-exposed regions (straight segments). In contrast, eNOS staining was absent in the OSS-exposed regions, but prevalent in the PSS-exposed regions. Next, we showed that OSS promoted an imbalance between $\text{O}_2^{\cdot-}$ and NO formation that influenced formation of ONOO^- as measured by the presence of 3-nitrotyrosine, d-tyrosine, and *o*-hydroxy-phenylalanine in the LDL apoB-100 protein. Finally, our LC/ESI/MS/MS analyses revealed specific tyrosine residue nitration in the α - and β -helices of the apoB-100 protein at α -1 (Tyr¹⁴⁴), α -2 (Tyr²⁵²⁴), β -2 (Tyr³²⁹⁵), α -3 (Tyr⁴¹¹⁶), and β -2 (Tyr⁴²¹¹).

Within the lateral walls of arterial bifurcations or curvatures, disturbed flow (including OSS) is considered to be an inducer of oxidative stress that favors the production of ROS through the endothelial NADPH oxidase system^{5,39,40}. In contrast, in the medial wall of bifurcations or straight regions where pulsatile flow develops, vascular endothelial cells are protected from atherosclerosis.¹⁴ Temporal variations in shear stress (OSS and PSS) in these regions are likely to account for the relative expression of NADPH oxidase homologues, Nox4, and eNOS. The increased vascular activities of NAD(P)H oxidases enhance the production of reactive oxygen species (ROS), including $\text{O}_2^{\cdot-}$ and hydrogen peroxide (H_2O_2). $\text{O}_2^{\cdot-}$ can inactivate NO, leading to the formation of OONO^- , thereby lowering NO bioavailability^{41,42}. While others have shown the presence of eNOS staining in the mouse vasculatures^{43,44}, we revealed that eNOS staining was absent in the OSS-exposed regions of coronary arteries in patients with ischemic cardiomyopathy.

While eNOS expression is absent in the luminal endothelial cells of athero-prone regions of coronary arteries, eNOS expression is present in the luminal endothelial cells lining the vasa vasorum (Fig. 1b). Our observation is consistent with the previous report that reduced NO release in atherosclerotic segments was accompanied by marked reduction of immunoreactive eNOS in luminal endothelial cells. However, endothelial cells of vasa vasorum and the endothelium of normal arteries remained positive for eNOS⁴⁵. In the absence of disease, the vasa vasorum nurture the outer component of the vessel wall, and the intima is fed by oxygen diffusion from the lumen. As disease progresses, the intima thickens, and oxygen diffusion is impaired. As a result, vasa become the major source for nutrients to the vessel wall^{46,47}. Furthermore, apolipoprotein E (apoE) (–/–)/low-density lipoprotein (LDL) (–/–) double

knockout mice developed vasa vasorum in association with advanced lesion formation⁴⁸. Vasa vasorum plays a significant role in maintaining vessel integrity and the vasa vasorum may contribute to the initiation and progression of different types of vascular disease in the systemic circulation. In this context, NO production from vasa vasorum likely attributes to the formation of peroxynitrite despite the absence of eNOS expression in the luminal endothelium of the coronary arteries.

Smooth muscle cells (SMC) were stained with β -actin in the left main bifurcation and right coronary artery (RCA) (Fig. 1d). We observed that nitrotyrosine staining is prevalent in the oscillatory shear stress-exposed regions, but it was relatively sparse or absent in figure 1f. This observation is likely due to the relative straight segments of RCA from which immunostaining for nitrotyrosine was performed. The luminal endothelial cells in the straight segment were known to be exposed to pulsatile flow⁴⁹, which down-regulated NADPH oxidase (NOX4) expression, but up-regulated eNOS expression (Fig. 2). Despite the presence of eNOS staining in SMC, the relative rate of superoxide anion and NO production is small in response to pulsatile shear stress (Table 1). In this context, pulsatile shear stress-exposed regions seemed to be protected from oxidative stress, whereas oscillatory shear stress-exposed regions (the bifurcations or curvatures) seemed to be prone to oxidative stress as shown by the peroxynitrite formation (Fig. 1e).

Less eNOS is present in oscillatory shear stress (OSS) (Fig. 2a), but NO production is comparable to pulsatile shear stress (PSS) in table 1. The Griess reaction is the most frequently used analytical approach to quantify the major metabolites of NO, i.e. nitrite (NO_2^- decay product) and nitrate (NO_3^- decay product). NO_2^- is the measurement of total usable nitric oxide: $\text{NO} + \text{HO} \cdot \rightarrow \text{HONO} \rightarrow \text{NO}_2^- + \text{H}^+$, whereas total NO_3^- is the degradation product of peroxynitrite ($\text{ONOOH} \rightarrow \text{NO}_3^-$) or the degradation product of nitrating intermediates: $\text{ONOOH} \rightarrow \text{NO}_2 \cdot + \text{HO} \cdot \rightarrow \text{HONO}_2 \rightarrow \text{NO}_3^-$.

However, the Griess reaction is specific for nitrite. Analysis of nitrate by this reaction requires chemical or enzymatic reduction of nitrate to nitrite prior to the diazotization reaction. Because there are numerous interferences in the analysis of nitrite and nitrate in biological fluids and because there is a desire to analyze these anions simultaneously, the Griess reaction has been repeatedly modified and automated. In recent years, the Griess reaction has been coupled to HPLC for post-column derivatization of chromatographically separated nitrite and nitrate. However, there are particular analytical and pre-analytical factors and problems that may affect the quantitative analysis of nitrite and nitrate in these matrices by assays.

We performed the analytical procedure based on acidic reduction of NO_2^- and NO_3^- to NO by vanadium (III) and purging of NO with helium into a stream of ozone and detected by an Atek 7020 chemiluminescence NO detector. At room temperature, vanadium (III) only reduced NO_2^- , whereas NO_3^- and other redox forms of NO (such as S-nitrosothiols) were reduced only if the solution was heated to 90–100°C, so that both NO_2^- and total NO_x could be measured. Peroxynitrite has a half-life of about 7 day⁵⁰, and it will decay to nitrite. However, our experimental samples were frozen, and then processed for NO_2^- and heating to 90–100 °C for NO_3^- . Thus, the presence of peroxynitrite might partly contribute to the elevated total NO production in response to OSS.

Previously, our lab and others have reported that the production of O_2^- influences the capacity of endothelial monolayers to modulate oxidative modification of LDL apo B-100^{11,51}, and a significant correlation may be observed between generation of O_2^- and the expression of NADPH oxidase subunit, p22^{phox}, or oxidized LDL from directional coronary atherectomy specimens⁵². In this study, we revealed that peroxynitrite reacted specifically with tyrosine residues in proteins leading to the formation of nitrotyrosine. Peroxynitrite is a potent oxidant,

exerting its toxic effects by undergoing redox cycling, by interfering with signal transduction, or by becoming incorporated into the microtubules to distort the cytoskeleton⁵³. Emerging evidence has shown that the exposure of LDL to ONOO⁻ resulted in the (a) nitration of apoB-100 tyrosine residues⁵⁴, (b) peroxidation of lipid^{55,56}, (c) depletion of lipid-soluble antioxidants^{57,58}, and (d) conversion of the lipoprotein into a high uptake form for macrophages^{5,59}. Thus, nitrotyrosine is considered an emerging inflammatory marker¹⁵.

The pathways leading to the nitration of tyrosine residues in proteins have been extensively studied *in vitro* and *in vivo*^{36,60–71}. Tyrosine nitration, a post-translational modification of proteins *meta* position of tyrosine residues⁶⁰, has been through the addition of a nitro (NO₂) group in the detected under physiological settings and in a number of pathological states including inflammatory and septic conditions^{64,68–72}. At the protein levels, these modifications implicate a number of potential consequences such as alterations in secondary structure, function, and susceptibility to proteolysis^{64,68–72} as well as the behavior of LDL particles⁷³. At the physiologic level, C57 mice undergoing exercise protocol, that was associated with an augmentation in shear stress, decreased the level of protein nitration (3-nitrotyrosine level)⁷⁴. In this context, protein-bound nitrotyrosine is considered to be an emergent predictor for cardiovascular disease risk assessments^{15,59,75–78}.

The α -2 and α -3 helices of apoB-100 have the highest content of tyrosine residues (4.8% and 6.8% tyrosine, respectively). An aromatic amino acid tyrosine assumes a flat and stackable structure to intercalate into the hydrophobic space between adjacent negatively charged phospholipids of the α helices that are stabilized by lysine and arginine positive charges⁷⁹. The most observed nitration of apoB-100 occurs in the α helical domains which likely account for the unfolding of LDL. This LDL unfolding has been demonstrated in various LDL modifications, including PLA2 treated LDL⁸⁰ (LDL- subfraction) and *in vivo* LDL⁸¹. ApoB-100 was also sensitive to nitration/oxidation in the β -2 domain which is upstream from the LDL-Receptor site (3359–3369)⁷⁹. Furthermore, nitration of tyrosine residues is likely to change the hydrophilic properties of the apoB-100 particle; rendering hydrophilic and likely disrupting hydrophobic interactions with the outer hydrophobic lipid core. Further investigation of posttranslational protein modification by nitration, including signaling molecules, enzymes, and receptors would provide insights into the biologic effects of nitrative stress in the initiation of atherosclerosis.

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Fig. 1.

Immunostaining of representative sections of coronary arteries (n=3). (a) Endothelial cells (EC) were stained with von Willebrand factor in both the lateral wall of left main bifurcation (OSS-exposed region) and straight regions (PSS-exposed regions) of right coronary artery (RCA). The inserts (20X) detailed EC lining the inner lumens. En face staining of the human left main bifurcation was beyond the field of view at the lower magnification (10X). (b) A section of left main bifurcation revealed that eNOS staining was absent in the luminal EC, but was present in both smooth muscle cells and the vasa vasorum. (c) A representative PSS-exposed section of RCA revealed that eNOS staining was prevalent throughout the entire luminal EC. (d) Smooth muscle cells (SMC) in the media of left main bifurcation were counterstained with β -actin. β -actin was observed in the intima and media of the OSS-exposed section, suggesting SMC migration (data not shown). (e) OSS-exposed section of the left main bifurcation revealed nitrotyrosine staining in the media. The insert (20X) further showed nitrotyrosine staining in the SMC. (f) A representative PSS-exposed section in RCA revealed a lack of nitrotyrosine staining.

Fig. 2.

Fig. 2a eNOS mRNA expression in response to shear stress at 4 and 8 hours. Pulsatile shear stress (OSS) induced a sustained increase in eNOS expression by 5 ± 0.85 -fold and 4.48 ± 0.47 -fold at 4 and 8 hours, respectively, whereas oscillatory shear stress (OSS) down-regulated eNOS expression by 2.5 ± 0.7 -fold ($n=5$, $P<0.05$).

Fig. 2b Nox4 protein expression in response to shear stress. OSS up-regulated Nox4 protein by 2 ± 0.8 -fold, whereas PSS up-regulated Nox4 protein by 1.60 ± 0.7 -fold at 4 hours ($n=4$, $P<0.05$). Nox4 protein was normalized to β -actin. Controls were performed under static conditions.

Fig. 3.

A representative MS/MS spectrum of nitro-modified peptide. The tryptic peptide, NLQNNAEWVYQGAI R, revealed that Y6 ion is the largest blue peak. The molecular weight of Y6 is greater in the modified peptide (752.4 Daltons) than the non-modified (707.3 Daltons) by 45 Daltons. Only representative peaks were labeled in the spectrum.

Table 1

Relative rates of $O_2^{\cdot-}$ and $\cdot NO$ production in response to PSS and OSS. The rates of $O_2^{\cdot-}$ and $\cdot NO$ production remained steady under the static state. OSS induced a higher rate of $O_2^{\cdot-}$ production while PSS promoted a higher rate of $\cdot NO$ (total NO_2^- and NO_3^-) formation. OSS also induced a higher level of $\cdot NO$ formation and a higher ratio of $O_2^{\cdot-}$ to $\cdot NO$ production than did PSS.

	control	OSS	PSS
$dO_2^{\cdot-}/dt$ [nmoles/min/10 ⁶ cells]	1.2±0.8 [*]	25.7±5.1 [*]	5.4 ±4.2 [*]
$d\cdot NO/dt$ [nmoles/min/10 ⁶ cells]	1.9±0.3 [#]	37.5±4.1 [#]	48.6±5.1 [#]
$(dO_2^{\cdot-}/dt)/(d\cdot NO/dt)$	0.63	0.68	0.11

^{*,#} $P < 0.05$, n=4)

Table 2

PSS and OSS influenced LDL protein nitration. Liquid chromatography/electron spray ionization/mass spectrometry/mass spectrometry (LC/ESI/MS/MS) analyses of LDL apoB-100 revealed that OSS induced higher levels of LDL nitrotyrosine, di-tyrosine, and *o*-hydroxy-phenylalanine (*o*-Phe) than did PSS. di-tyrosine and *o*-Phe appeared to be the predominant forms of protein nitration. The differences between control and PSS were statistically insignificant.

	Control	OSS	PSS
3-nitrotyrosine [mmol/mol]	0.15±0.08	0.17±0.09 [*]	0.09±0.04 [*]
di-tyrosine [mmol/mol]	16.0±4.4	21.1±3.4 ^{##}	13.0±2.7 ^{##}
<i>o</i>-hydroxy-phenylalanine [mmol/mol]	54.5±4.1	82.0±4.4 ⁺	52.0±3.1 ⁺

^{*},^{##},⁺ P < 0.05, n=3)

Table 3

Tyrosine nitration of LDL apo B-100. Six tryptic peptides were identified to have undergone tyrosine nitration. The nitrotyrosines are denoted by asterisks. “AA” represents modified tyrosine residue, “Peptide” the numerical sequence of amino acid in the tryptic fragment, “Charge” the ion precursor charge of the tryptic fragment, “Sequence” the amino acid sequences of the tryptic fragment, “M” the Mascot score, Xcor the Sequest score, and “ Δ cn” the difference between two closely matched peptides.

AA	Peptide	Charge	Sequence	M	Xcor	Δ cn
Y144	140 – 157	3	QVFLY*PEKDEPTYILNIK	42	3.2	.328
Y2524	2523 – 2534	2	M*Y*QM*DIQQELQR	71	4.4	.264
Y2524	2523 – 2534	2	M*Y*QM*DIQQELQR	79	4.5	.286
Y3295	3292 – 3311	3	VPSY*TLILPSLELPVLHVPR	78	5.9	.558
Y4116	4107 – 4121	2	NLQNNAEWVY*QGAIR	97	4.7	.478
Y4211	4202 – 4213	3	FQFPKPGIY*TR	34	2.5	.271