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Pulsatile Shear Stress Increased Mitochondrial Membrane Potential: Implication of Mn-SOD

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Abstract

Mitochondrial dysfunction is intimately involved in cardiovascular diseases. Mitochondrial membrane potential ($\Delta\Psi_m$) is coupled with oxidative phosphorylation to drive ATP synthesis. In this study, we examined the effect of physiological pulsatile shear stress (PSS) on $\Delta\Psi_m$ and the role of Mn-SOD expression on $\Delta\Psi_m$. Confluent human aortic endothelial cells (HAEC) were exposed to PSS, and $\Delta\Psi_m$ was monitored using tetramethylrhodamine methyl ester (TMRM⁺), a mitochondrial membrane potential probe. PSS significantly increased $\Delta\Psi_m$ and the change in $\Delta\Psi_m$ was a dynamic process. $\Delta\Psi_m$ returned to baseline level after PSS for 2 hours followed by static state for 4 hours. Mitochondrial Mn-SOD expression and activities were also significantly up-regulated in response to PSS. Silencing Mn-SOD attenuated PSS-mediated $\Delta\Psi_m$ increase while adding Mn-SOD mimetic, MnTMPyP, increased $\Delta\Psi_m$ to the similar extent as induced by PSS. Our findings suggest that PSS increased mitochondrial $\Delta\Psi_m$, in part, via Mn-SOD up-regulation.

Keywords

Shear stress; mitochondria; membrane potential; Mn-SOD; endothelial cells

Introduction

Vascular oxidative stress plays an important role in atherosclerosis¹⁻³. Hemodynamics, particularly fluid shear stress, regulates the generation of vascular nitrogen (RNS) and reactive oxygen species (ROS)⁴⁻⁶. While eNOS is the major source of RNS, NADPH oxidase system is considered as a major source of ROS in vascular endothelial cells^{4, 7}. Mitochondria are also an important source of cellular superoxide anion ($O_2^{\cdot-}$) and H_2O_2 ². Mitochondrial function is relevant to metabolic homeostasis⁸. Oxidative phosphorylation in the mitochondria drives the proton translocation across the mitochondrial inner membrane to intermembrane space⁹,

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generating an electrochemical proton gradient that is expressed as mitochondrial membrane potential ($\Delta\Psi_m$)¹⁰.

Mitochondrial $\Delta\Psi_m$ is an important indicator of mitochondrial energetic state and cell viability¹⁰. $\Delta\Psi_m$ is coupled with oxidative phosphorylation to drive ATP synthesis^{11, 12}. During myocardial reperfusion injury, opening of the mitochondrial permeability transition pore (MPTP) collapses $\Delta\Psi_m$ and uncouples oxidative phosphorylation, resulting in ATP depletion and apoptosis^{13, 14}. Fluid shear stress is reported to influence mitochondrial ATP synthesis, which is coupled with $\Delta\Psi_m$ ¹⁵.

The formation of mitochondrial ROS (mtROS) is dependent on $\Delta\Psi_m$ ¹⁶, and mtROS level increases exponentially as $\Delta\Psi_m$ is hyperpolarized above -140 mV¹⁷. In response to oxidative stress, mitochondrial manganese superoxide dismutase (Mn-SOD) is up-regulated¹⁸, leading to dismutation of $O_2^{\cdot-}$ anion to H_2O_2 . In response to laminar shear stress, cytosolic CuZn-SOD expression is also up-regulated¹⁹. However, the potential mechanism whereby shear stress modulates $\Delta\Psi_m$ via Mn-SOD remains unknown.

We hypothesized that pulsatile shear stress (PSS) increased $\Delta\Psi_m$ via an up-regulation of Mn-SOD expression. In this paper, we provided the following new insights: (1) PSS increased $\Delta\Psi_m$ in HAEC, (2) PSS up-regulated Mn-SOD mRNA and protein expression, and (3) adding Mn-SOD mimetic, MnTMPyP, increased $\Delta\Psi_m$ to the similar extent as induced by PSS while silencing Mn-SOD attenuated PSS-induced increase in $\Delta\Psi_m$. Our findings suggest Mn-SOD up-regulation represents a potential pathway whereby shear stress influenced $\Delta\Psi_m$.

Materials and Methods

Methods

Endothelial Cell Culture—Human aortic endothelial cells (HAEC) were purchased from Cell Applications (San Diego, CA) and cultured in endothelial growth medium (Cell Applications). Endothelial cells (ECs) between passages 4 and 7 were seeded on Cell-Tak cell adhesive (Becton Dickson Labware, Bedford, MA) and Collagen Type I (BD bioscience, San Jose, CA) coated glass slides at 1.5×10^5 cells per slide (5 cm^2). ECs were grown to confluent monolayers in endothelial growth medium for 48 hours in 5% CO_2 at 37°C before being set up into the dynamic flow system.

Dynamic Flow System—Two-dimensional dynamic flow channels were used to implement shear stress simulating physiologic flow profiles in human carotid arterial bifurcation. Please see supplemental materials and methods for details.

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi_m$)—After exposing the cells to flow conditions, mitochondrial membrane potential was measured with a cationic fluorescent dye, tetramethylrhodamine methyl ester (TMRM⁺) (Molecular probes, Carlsbad, CA). For details, please see supplemental materials and methods.

Conversion of TMRM⁺ Fluorescent Intensity to Voltage—Calculation of $\Delta\Psi_m$ from fluorescence intensity was performed essentially as described by Nicholls²⁰. To account for the plasma membrane potential ($\Delta\Psi_p$) in the intact cells, we monitored $\Delta\Psi_p$ by “Membrane Potential Assay kit” (Molecular Devices, Sunnyvale, CA). The fluorescence intensity was captured at an excitation wavelength of 488 nm and emission of 527 nm (Olympus IX70). Calibration curve for $\Delta\Psi_p$ was established by probing the fluorescent intensity at the potassium concentration, ranging from 3.9 mM to 80 mM, followed by application of the Nernst equation²⁰.

Measurement of Mitochondrial Superoxide Production: Mitochondrial superoxide was measured with flow cytometry (FACS) after staining with mitochondrial superoxide specific dye MitoSOX Red (Invitrogen) as described²¹.

Quantitative RT-PCR—Mn-SOD mRNA expression is measured with quantitative RT-PCR. Please see supplemental materials and methods for details.

Mn-SOD Activity Assay: Cells exposed to static condition or shear stress were scrapped into PBS and spun down. The cells were lysed in Mn-SOD assay sample buffer (10mM HEPES, pH7.9, 420mM NaCl, 1.5mM MgCl₂, 0.5mM EDTA, 0.1% Triton X-100) for 20 minutes in ice. After spin at 12000g for 5 minutes, the supernatant was collected for Mn-SOD activity assay. Mn-SOD activities were measured using the SOD assay kit from Cayman Chemicals (#706002 Ann Arbor, MI) in the presence of 2mM potassium cyanide, which inhibited the activities of Cu-Zn-SOD and EC-SOD but not Mn-SOD. Relative SOD activities were expressed as absorbance at 450 nm in the absence SOD less absorbance with sample or SOD standard. Reading in the presence of 1U/ml of SOD standard was used as blank. Relative Mn-SOD activities were normalized to protein concentration of samples.

Transfection of siRNA in Human Aortic Endothelial Cells—Silencer siRNAs for Mn-SOD were custom-designed by Ambion (Austin, TX). The sequences were as follows: sense sequence: GGC CUG AUU AUC UAA AAG Ctt, anti-sense sequence: GCU UUU AGA UAA UCA GGC Ctg. Confluent human aortic endothelial cells (HAEC) were trypsinized and re-suspended to 100,000 cells/mL in standard growth medium. siPORT NeoFX transfection reagent (Ambion, Austin, TX) was diluted in OPTI-MEM I (Invitrogen, CA) medium and incubated at room temperature for 10 minutes. Mn-SOD siRNA at a final concentration of 30 nM was diluted in OPTI-MEM I and mixed with diluted siPORT NeoFX reagent at room temperature for additional 10 minutes. The transfection solution was dispensed into the 6-well plates, followed by adding HAEC in suspension. The medium was changed to standard growth medium after 24 hours. The medium was replaced every other day until confluent HAEC monolayer developed. The cells were cultured for another 24hours and then used for experiments. Quantitative RT-PCR and Western blot were performed to assess the levels of silencing. Scrambled siRNA were used as the control.

Western Blot—Cells were harvested, washed with phosphate-buffered saline and lysed with RIPA buffer. The lysate was centrifuged at 12,000g for 10 minutes, and the resulting supernatants were used as the whole cell lysate. 15µg protein samples were size-separated in 10% SDS BioRad polyacrylamide electrophoresis gel (BioRad, CA). Mn-SOD and β-tubulin (loading control) were detected with rabbit Mn-SOD antibody (Upstate), mouse anti-β-tubulin antibody (Millipore). Chemiluminescence signal was developed with Supersignal Western Pico (Pierce) and recorded with FluorChem FC2 (Alpha Inotech Inc). Densitometry scan of western blot was done with the software come with FlorChem FC2 machine.

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

Results

Characterization of Mitochondrial Membrane Potential ($\Delta\Psi_m$) in Human Aortic Endothelial Cells (HAEC)

We employed TMRM⁺ dye to measure mitochondrial membrane potential ($\Delta\Psi_m$). Mitochondrial respiratory chain inhibitors and uncouplers were used to validate the method in intact HAEC. Addition of FCCP, a protonophore to uncouple oxidative phosphorylation, depolarized $\Delta\Psi_m$ in the intact HAEC and decreased the TMRM⁺ intensity by 30% compared to the control ($P < 0.05$, $n=5$). Treatment with rotenone, a NADH dehydrogenase inhibitor, depolarized $\Delta\Psi_m$ and decreased the TMRM⁺ intensity by 56% ($P < 0.05$, $n=5$). Treatment with oligomycin, an ATP synthase inhibitor, hyperpolarized $\Delta\Psi_m$ and increased TMRM⁺ intensity by 2.5-fold ($P < 0.05$, $n=5$). Furthermore, cyclosporine-A, an inhibitor of mitochondrial permeability transition pores (MPTP), increased the TMRM⁺ intensity by 2.6-fold ($p < 0.05$, $n=5$, supplemental Fig. 1A). The change in TMRM⁺ intensity were converted to $\Delta\Psi_m$ (mV) as described in materials and methods with background level of $\Delta\Psi_m$ set at -140mV. In control condition, $\Delta\Psi_m$ was $-140\text{mV} \pm 6.9$. In the presence of FCCP, Rotenone, Oligomycin and cyclosporine A, $\Delta\Psi_m$ was $-118.6\text{mV} \pm 5$, $-107.8\text{mV} \pm 7.3$, $-165\text{mV} \pm 4.2$ and $-165.3\text{mV} \pm 9.8$, respectively (supplemental Fig. 1B). These data were in agreement with published results^{22, 23}. Hence, the dynamic range of TMRM⁺ provided a basis to characterize shear stress-induced $\Delta\Psi_m$.

Pulsatile Shear Stress Increased Mitochondrial Membrane Potential ($\Delta\Psi_m$)

In response to pulsatile shear stress (PSS) simulating arterial flow at a time-averaged shear stress (τ_{ave}) of 23 dyn cm^{-2} for 2 and 4 hours, endothelial cells became energized as evidenced by an increase in $\Delta\Psi_m$ (Fig. 1A. static: $-140 \text{ mV} \pm 4.9$; PSS 2 hours: $-170.5 \pm 1.5 \text{ mV}$, $P < 0.05$, $n=4$; PSS 4 hours: $-187.5 \pm 5.6 \text{ mV}$, $P < 0.05$, $n=4$). The dynamic changes in $\Delta\Psi_m$ were consistent with those of physiological range in mammalian cells between -90 and -180 mV¹⁷. Fluorescence microscopy indicated that PSS increased TMRM⁺ uptake by HAEC (Fig. 1B), consistent with an increase in mitochondrial membrane potential. In parallel, PSS increased mitochondrial superoxide production (Fig. 1C). PSS is considered to be cardioprotective²⁴ and our observations suggest that one of the possible mechanisms whereby PSS confers cardioprotection may be due to an increase in $\Delta\Psi_m$ that is important for oxidative phosphorylation and ATP synthesis¹⁰.

Pulsatile Shear Stress Up-regulated Mn-SOD Expression in HAEC

In response to PSS for 4 hours, Mn-SOD mRNA level was up-regulated by 3.6-fold as compared to that of static condition ($P < 0.01$, $n=3$) (Fig. 2A). Mn-SOD protein expression was also increased by 1.9-fold (Fig. 2B). In parallel, Mn-SOD activities were increased by 40% (Fig. 2C). Next, HAEC were subjected to PSS for 2 hours, followed by static condition for 4 hours. Mitochondrial $\Delta\Psi_m$ was increased to $-160 \pm 5.3 \text{ mV}$ in response to PSS for 2 hours (Fig. 3A), a similar trend shown in Fig. 1A. $\Delta\Psi_m$ returned to near baseline value at $-145 \pm 6.4 \text{ mV}$ (baseline was -140 mV) after 4 hours in static condition. This finding suggests that changes in $\Delta\Psi_m$ are a dynamic process in response to shear stress. Despite the static condition, the Mn-SOD protein and activity levels remain elevated (Fig. 3B & 3C), consistent with Mn-SOD mRNA up-regulation at 4 hours in response to PSS as previously reported in bovine aortic endothelial cells²⁵.

Mn-SOD Expression Modulated PSS-Mediated Mitochondrial Membrane Potential

To test the role of Mn-SOD on $\Delta\Psi_m$, we first treated HAEC with Mn-SOD mimetic, MnTMPyP, for 4 hours. MnTMPyP treatment resulted in an increase in $\Delta\Psi_m$ to a similar extent as induced by PSS (Fig. 4A, $p < 0.05$, $n=3$). To examine the effect of Mn-SOD level on PSS-

mediated $\Delta\Psi_m$ change, we transfected HAEC with Mn-SOD siRNA (siMn-SOD). siMn-SOD decreased Mn-SOD mRNA expression by 80% compared to scrambled siRNA in HAEC (Fig. 4B). siMn-SOD significantly attenuated PSS-mediated $\Delta\Psi_m$ (Fig. 4C, $p < 0.05$, $n = 3$). Taken together, our findings support the notion that PSS-induced Mn-SOD expression plays a role on changes in $\Delta\Psi_m$.

Discussion

In this study, we employed a dynamic fluorescent technique to monitor mitochondrial membrane potential ($\Delta\Psi_m$). We demonstrated that pulsatile shear stress (PSS) increased $\Delta\Psi_m$ in the intact aortic endothelial cells (HAEC). PSS also up-regulated mitochondrial Mn-SOD expression. Treatment of HAEC with Mn-SOD siRNA attenuated PSS-mediated increase in $\Delta\Psi_m$ while Mn-SOD mimetic, MnTMPyP, increased $\Delta\Psi_m$ to similar extent as PSS. Our findings suggest that PSS hyperpolarized $\Delta\Psi_m$ via *Mn-SOD up-regulation*.

Maintenance of $\Delta\Psi_m$ at physiologic range ($\sim -90\text{mV}$ to -150mV) is critical for cellular function¹⁷. Important factors that influence $\Delta\Psi_m$ include (1) proton leakage across the inner membrane, (2) ATP synthesis or hydrolysis, (3) substrate availability, (4) electron flux through the respiratory chain, and (5) ion transport²⁶. The methyl esters of tetramethylrhodamine (TMRM⁺) is useful for monitoring mitochondrial depolarization relevant to cytosolic Ca^{++} transient and for imaging time-dependent mitochondrial membrane potential²⁷. The precise concentration of TMRM⁺ was critical to detect step changes in $\Delta\Psi_m$. At a low concentration (10 nM), TMRM⁺ operates at the distribution/redistribution mode. When the mitochondrial membrane is depolarized, TMRM⁺ in mitochondria undergoes dequenching, rendering a decrease in fluorescent intensity. Distribution mode is applicable to our experiments that involved slow step changes in $\Delta\Psi_m$ at a low TMRM⁺ concentration²⁷.

Several lines of evidence support that shear stress increases exogenous ATP release. ATP production is a process dependent on $\Delta\Psi_m$ ⁸. Kudo *et al.* demonstrated an increase in $\Delta\Psi_m$ and ATP production in endothelial cells exposed to laminar shear stress for 48 hours in a parallel plate flow system¹⁵. Our data showed a hyperpolarized $\Delta\Psi_m$ in response to pulsatile shear stress within 2 hours. An increase in $\Delta\Psi_m$ is accompanied with an increase of ATP release and the generation of mitochondrial reactive oxygen species (mtROS) when $\Delta\Psi_m$ is above -140mV ^{11, 12}. In response to PSS, mitochondrial superoxide generation in HAEC was increased compared to static condition (Fig. 1C). Thus PSS can modulate $\Delta\Psi_m$ and mitochondrial redox state with an implication for endothelial function.

Over-expression of Mn-SOD or SOD mimetic has been shown to protect against beta-amyloid-induced neuronal death and improved mitochondrial respiratory function²⁸. Mn-SOD was also reported to suppress selenite-induced decrease in mitochondrial membrane potential²⁹. Furthermore, over-expression of Mn-SOD reduced polarization of colorectal cancer cells³⁰. In this study, we demonstrated that PSS increased $\Delta\Psi_m$ in endothelial cells, which was at least partially mediated via up-regulation of Mn-SOD expression. Hence, Mn-SOD is important in maintaining homeostasis of endothelial cell function as an antioxidant^{31, 32}. Although the precise mechanisms remain to be identified, Mn-SOD-mediated membrane potential increase is likely related to its antioxidant activity since the activity of proton pumps depends on the reductive power at the respiratory chain of mitochondria.

A host of factors modulates mitochondria membrane potential. While Mn-SOD may contribute to PSS-mediated increase in $\Delta\Psi_m$, other factors such as metabolic status and ATP level may dominate. Our data showed that $\Delta\Psi_m$ returned to baseline level (-145mV) after 2 hours of PSS followed by 4 hours of static condition. At the same time, both Mn-SOD protein levels and activities remained elevated. These data are consistent with shear stress-induced Mn-SOD

mRNA at 4 hours and the ensuing protein expression observed in bovine aortic endothelial cells²⁵. While membrane potential returned to baseline after shear stress exposure, the persistent elevated Mn-SOD may play an important role in dismutating mitochondrial superoxide production to maintain mitochondrial homeostasis⁸.

In summary, PSS favors mitochondrial energetic state with an implication for mitochondrial function in endothelial cells¹⁵. In this paper, we linked pulsatile shear stress with mitochondrial $\Delta\Psi_m$ in the intact vascular endothelial cells via Mn-SOD regulation. Regulation on $\Delta\Psi_m$ may represent one of the mechanisms whereby pulsatile shear stress confers a cardioprotective effect. The precise mechanisms will be of important interest for future investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Pulsatile shear stress (PSS) increased mitochondrial membrane potential ($\Delta\Psi_m$)

A: HAEC were exposed to static condition or PSS for 2 and 4 hours. After PSS exposure, cells were incubated with 10 nM TMRM⁺ for 30 minutes. TMRM⁺ intensity was measured by FACS and converted to voltage (mV) as described in “Material and Methods” (**p* < 0.05 versus static condition, *n* = 4). **B:** HAEC were exposed to static condition or PSS for 2 hours. Cells were incubated with 10 nM TMRM⁺ for 30 minutes. Images were taken with fluorescence microscope. The images were representative of 4 independent experiments. **C:** HAECs were exposed to PSS for 30 minutes or 2 hours. Mitochondrial superoxide production was measured by flow cytometry using mitochondrial superoxide probe MitoSOX Red. Relative superoxide production was calculated based on fluorescence intensity.

Fig. 2. Pulsatile shear stress up-regulated Mn-SOD expression and activities

A: HAEC were exposed to static condition or pulsatile shear stress (PSS) for 4 hours. RNAs were isolated and the expression of Mn-SOD mRNA were measured by quantitative RT-PCR and normalized to 18S rRNA (* $p < 0.01$, $n = 3$). **B:** HAEC were exposed to static condition or PSS for 4 hours. Mn-SOD protein expression was assessed by western blot. The relative expression of Mn-SOD protein was normalized to β -tubulin from density scan data. **C:** HAEC were exposed to static condition or PSS. The cells were lysed and Mn-SOD activities were measured as described. Relative Mn-SOD activities were normalized to protein concentration (* $p < 0.01$, $n = 3$).

Fig. 3. Changes of mitochondrial membrane potential in response to PSS were a dynamic process HAEC were subjected to three conditions: (1) static state for 2 hours, (2) PSS exposure for 2 hours, and (3) PSS exposure for 2 hours followed by static state for 4 hours. Mitochondrial membrane potential (**A**), Mn-SOD protein levels (**B**) and Mn-SOD activities (**C**) were measured. (* $p < 0.02$ vs. static state, $n = 3$; ** $p < 0.05$ vs. PSS, $n = 3$).

Fig. 4. Mn-SOD mediated PSS-increased mitochondrial membrane potential

A: Treatment of HAEC with 50 μ M of Mn-SOD mimetic, MnTMPyP, for 4 hours resulted in an increase in $\Delta\Psi_m$ to a similar extent as induced by PSS for 4 hours. **B:** HAEC were transfected with scrambled control siRNA (Scr) or siRNA for Mn-SOD (siMn-SOD). Mn-SOD mRNA expression was measured by quantitative RT-PCR. Mn-SOD siRNA treatment induced an 80% reduction in Mn-SOD mRNA expression compared to scrambled siRNA. **C:** HAEC were transfected with control siRNA (Scr) or siMn-SOD (si) followed by exposure to shear stress or static condition. Membrane potential was measured. siMn-SOD attenuated PSS induced mitochondrial membrane potential as compared to control siRNA (* $p < 0.05$, $n = 3$).