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Buckling Reduces eNOS Production and Stimulates Extracellular Matrix Remodeling in Arteries in Organ Culture

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Abstract

Artery buckling alters the fluid shear stress and wall stress in the artery but its temporal effect on vascular wall remodeling is poorly understood. The purpose of this study was to investigate the early effect of artery buckling on endothelial nitric oxide synthase (eNOS) expression and extracellular matrix remodeling. Bilateral porcine carotid arteries were maintained in an *ex vivo* organ culture system with and without buckling while under the same physiological pressure and flow rate for 3–7 days. Matrix metalloproteinase-2 (MMP-2), MMP-9, fibronectin, elastin, collagen I, III and IV, tissue inhibitor of metalloproteinase-2 (TIMP-2), and eNOS were determined using Western blotting and immunohistochemistry. Our results showed that MMP-2 expression level was significantly higher in buckled arteries than in the controls and higher at the inner curve than at the outer curve of buckled arteries, while collagen IV content showed an opposite trend, suggesting that artery buckling increased MMP-2 expression and collagen IV degradation in a site-specific fashion. However, no differences for MMP-9, fibronectin, elastin, collagen I, III, and TIMP-2 were observed among the outer and inner curve sides of buckled arteries and straight controls. Additionally, eNOS expression was significantly decreased in buckled arteries. These results suggest that artery buckling triggers uneven wall remodeling that could lead to development of tortuous arteries.

Keywords

Arterial buckling; eNOS; MMP-2; Vascular remodeling; Extracellular matrix

INTRODUCTION

Tortuous carotid and coronary arteries are often seen associated with atherosclerosis and aging.^{8,21} However, the underlying mechanism of tortuous artery development is unclear. Recent studies suggested that artery buckling may lead to arterial tortuosity,^{19,22,26} but it is

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CONFLICT OF INTEREST

No conflicts of interest.

unclear how arteries adapt to buckling thus it is important to investigate the vascular remodeling post-buckling.

Artery buckling induces curving of vessel and alters the blood flow. It alters the distributions of fluid shear stress and yields uneven wall stress in arteries.^{21,27,40,43} The changes in wall shear stress in the curved regions are known to be pro-inflammatory and pro-atherogenic.^{6,26,29,38} One of the mechanobiological mechanisms is that lumen shear stress affects endothelial nitric oxide synthase (eNOS) and NO production in endothelial cells that plays a critical role in modulating artery function.^{11,14,16} In the long-term, the changes in lumen shear stress and wall stress due to vessel curving could stimulate arterial wall remodeling as well.^{3,6,12,29,39} This process involves production and degradation of extracellular matrix (ECM) and proliferation of endothelial cells and smooth muscle cells.^{25,30} Adverse wall remodeling can lead to intimal hyperplasia and atherosclerosis.^{2,9,34} Our recent work showed that artery buckling alters wall stress and stimulates cell proliferations in porcine carotid arteries in *ex vivo* 3-day organ culture and *in vivo*.^{40,43} However, the possible temporal effect and the ECM remodeling have not been investigated.

Accordingly, the purpose of this study was to determine the effects of artery buckling on eNOS expression and ECM remodeling in arteries in *ex vivo* organ culture.

MATERIALS AND METHODS

Ex Vivo Arterial Organ Culture System

Bilateral porcine common carotid arteries were obtained from farm pigs (about 100 kg B.W.) post mortem at a local abattoir with the approval from the Texas Department of State Health Service. The arteries were transported to our laboratory in ice-cold phosphate buffer saline (PBS) and cultured in an *ex vivo* perfusion system as described in detail previously.^{23,24,40} Briefly, arteries were cleaned and mounted at their *insitu* length to diameter-matched stainless steel cannulae in vessel chambers which were then filled with bath medium. These arteries were then perfused with perfusion medium driven by a peristaltic roller pump. The perfusion and bath media consisted of cell culture medium DMEM (Sigma), supplemented with sodium bicarbonate (3.7 g/L, Sigma), L-glutamine (2 mM, Sigma), calf serum (10%, Sigma), HEPES (25 mM, Gibco), and antibiotic–antimycotic solution (1%, Sigma). Dextran (50 g/L, Sigma) was added to the perfusion medium to adjust its viscosity close to that of human blood (4cP). The arteries were maintained for upto 3 or 7 days in 5% CO₂ incubators at 37 °C under a flow rate of 160 mL/min, an axial stretch ratio of 1.3, and a pulsatile pressure of 120 ± 20 mmHg at a pulse frequency of ~2.5 Hz.⁴⁰ For 7 day arterial organ culture, the perfusion media was changed once at day 4.

Experimental Groups and Tortuosity Index

Bilateral arteries were randomly assigned as experimental buckling groups and straight control groups and both were cultured for 3 or 7 days to observe the temporospatial change in wall remodeling. To facilitate buckling, long arterial segments were used in the buckling groups, while short arterial segments were used to avoid buckling in control groups.⁴⁰ Our previous theoretical model results demonstrated that the critical buckling pressure of an

artery is approximately inversely related to its length.^{18,20} This approach allows us to achieve buckling without changing the perfusion flow rate and lumen pressure and thus the observed results are buckling specific. The arterial segments were photographed before being set up in organ culture system (load free), during organ culture (under pressure and flow), and later after unloaded from organ culture system to record their dimensions and shapes.

Later, all pictures were analyzed using Image-Pro Plus software to measure their dimensions (diameters and lengths) and shapes. Vessel tortuosity was described by a tortuosity index which was defined as the maximum deflection at the middle point divided by the line distance between the two ends of the vessel segment.⁴⁰ The arterial cross-sectional dimensions were also measured from ring segments harvested after organ culture (unloaded).

Tissue Collection and Processing

After 3 or 7 days of organ culture, three consecutive rings (each ~2 mm in axial length) were cut from the middle of straight and buckled arteries (Fig. 1). One ring was used for immunohistochemistry staining. A small axial slit (less than half way through the wall thickness) was made at the outer curve side of the ring to mark the buckling orientation.⁴⁰ Another ring was cut into 4 pieces of equal circumferential length. The pieces (~5 mm²) from the inner curve and outer curve of the buckled arteries were minced separately for protein analysis. The third ring was used for eNOS measurement by collecting the endothelial cells gently scraped from the ring as described below. Samples for straight controls and fresh controls were collected in the same fashion except that the inner and out curve were randomly chosen since no buckling occurs in these arteries. All the samples including harvested endothelial cells were transferred directly to protein lysis buffer, laemmli sample buffer (4X), then homogenized. Proteinase inhibitor cocktail (Roche) was added to whole cell lysates and the mixture was stored in -70 °C freezer until Western blotting analysis.

Western Blotting for Measuring MMP-2, MMP-9, Fibronectin, TIMP-2, Caspase-3, and eNOS

Western blotting was conducted as previously described.^{16,41} Briefly, the genomic DNA in the whole cell lysates was sheered *via* sonication on ice three times (10 s each). The whole cell lysates were loaded onto SDS polyacrylamide gels for electrophoresis separation. The separated protein bands were blotted onto Immun-Blot PVDF membranes (Bio-Rad) for detection with the following primary antibodies (Abs): mouse anti-MMP-2 (cat#MAB3308, Millipore), rabbit anti-MMP-9 (cat#AB13458, Millipore), rabbit anti-fibronectin (cat#AB1954, Millipore), rabbit anti-TIMP-2 (cat#AB2965, Millipore), rabbit anti-caspase-3 (cat# 9665, Cell Signaling), rabbit anti-eNOS (cat# 610298, BD Bioscience) and mouse anti-GAPDH (cat#CB1001, Calbiochem) as a loading control. The primary antibody bindings were detected with the corresponding secondary Abs conjugated with horse-radish peroxidase (HRP) (Amersham GE Healthcare) and visualized using the standard enhanced chemiluminescence (ECL) detection (Amersham GE Healthcare). Western blotting results were quantified by densitometric measurement using ImageJ (National Institutes of Health, Bethesda, MD).

Immunohistochemistry Staining for Measuring eNOS, Elastin, and Collagen Type I, III, IV

Immunohistochemistry staining was performed following a protocol as previously described.^{40,41,43} Arterial rings were washed with sterile ice-cold PBS and fixed in 2% paraformaldehyde in PBS. The fixed arteries were then transferred in 30% sucrose in PBS until they were processed for embedding in OCT compound. For immunofluorescent staining, 5 μ m frozen cross-sections were permeabilized with 0.2% Triton X-100 in PBS, blocked with 10% fetal bovine serum in PBS, and processed for labeling with rabbit anti-eNOS (cat# 610298, BD Bioscience), anti-collagen I(cat#SAB4500362, Sigma-Aldrich), anti-collagen III (cat#AB757P, EMD Millipore), and anti-collagen IV (cat#ab6586, Abcam), respectively. After washing with PBS, the frozen sections were incubated with Cy3-conjugated (red) goat anti-rabbit antibody (Molecular Probes). DAPI was used to counterstain the nuclei. The slides were then examined under an Olympus CX41 fluorescence microscope and images of 4–8 view fields for each slide were captured with a Qcolor3 camera. Elastin contents were measured with Verhoeff staining.³¹ Fluorescent intensities of elastin, collagen I, III, IV positive area for the whole vessel wall, and the intensity of eNOS for the intima were quantified by using Image-Pro plus 7.0 and ImageJ, respectively.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Staining

Frozen sections were dried and fixed as described above. After being permeabilized with 0.2% Triton X-100, sections were stained with TUNEL kit reagents (Roche Applied Science). Nuclei were counterstained with DAPI. The sections were then examined and photographed using a fluorescent microscope (using 10 $\times$ objective). The TUNEL-positive cells were counted and quantified as percentage of total counterstained cells.

Data Analysis

All values were presented as the mean $\pm$ SD. Statistical differences among fresh control, straight control, outer curve, and inner curve samples were analyzed with Student's *t* test for two-group comparison or ANOVA for multiple-group comparison. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

A total of 28 vessels were used for buckling and straight control groups (7 vessels each) for either 3- or 7-day organ culture. A short segment was cut from each artery of the straight control groups and used as fresh controls. Vessel dimensions measured under no-load condition and tortuosity index measured under perfusion flow are summarized in Table 1.

Vessel Tortuosity

Artery buckling was achieved in the experimental groups with long segments under physiological pressure and flow while no buckling occurred in the straight control groups of short segments (Fig. 1). Buckling under the pressure and flow conditions was visually observed and confirmed by a significant increase in tortuosity index in buckled arteries compared with the straight controls (Fig. 2; Table 1). However, at unloaded condition, there were no differences in the tortuosity index between the arterial segments used for control

and buckled groups at both pre-organ culture (Pre-OC) and post-organ culture (Post-OC). There was no statistical difference between pre-organ culture (Pre-OC) and post-organ culture (Post-OC) for each group, indicating no permanent shape change after 7 days in organ culture.

Artery Buckling Stimulated MMP-2 Expression

Western blotting results demonstrated that MMP-2 expression level was significantly higher in the buckled arteries compared to the straight controls for both 3 and 7 days, and significantly higher at the inner curve than at the outer curve in the buckled arteries of 7 days (Fig. 3). The MMP-2 expression increased in 3–7 days at the inner and outer curve sides of the buckled arteries compared with straight control arteries, but the differences were statistically insignificant. In addition, there was no statistical difference for MMP-9 and TIMP-2 protein levels among straight, outer curve, and inner curve (Fig. 4). These results suggest that artery buckling induces site-specific MMP-2 expression associated with uneven ECM remodeling between the inner and outer curve sides.

Collagen, Elastin, and Fibronectin in the Arterial Wall

In agreement with site specific MMP-2 expression, we observed significant decrease in collagen IV content in buckled arteries. The collagen IV areas measured were significantly reduced compared to straight control arteries, and significantly less collagen IV at the inner curve than the outer curve in 7-day buckled arteries (Fig. 5). But there was no difference between 3-day and 7-day, respectively. In addition, there was no significant difference in Collagen I and III, elastin, and fibronectin among samples from straight, outer, and inner curve sides, respectively, in either 3-day or 7-day groups (Fig. 6). There was no difference in these proteins between 3-day and 7-day.

Effect of Artery Buckling on Cell Apoptosis

TUNEL staining and caspase-3 Western blotting results demonstrated that there was no difference in apoptotic cell death among buckled and straight arteries cultured for 7 days and fresh arteries (Fig. 7), suggesting vessels maintained their cellular viability without significant cell death.

eNOS Expression was Significantly Decreased in the Buckled Arteries

Both immunohistochemistry staining and Western blotting demonstrated that endothelial eNOS expression was significantly decreased in the buckled arteries compared to the straight controls (Fig. 8), suggesting that artery buckling affected endothelial function. There was no statistical difference in eNOS expression between 3 and 7 days.

DISCUSSION

In the present study, we employed an *ex vivo* organ culture model to investigate endothelial eNOS expression and ECM remodeling in buckled arteries. Our results demonstrated that arterial endothelial eNOS expression decreased in response to arterial buckling. MMP-2 expression was significantly higher in buckled arteries than in the controls and higher at the inner curve than at the outer curve of buckled arteries. Collagen IV content was significantly

lower in buckled arteries than in the controls and lower at the inner curve than at the outer curve of buckled arteries. These results suggest artery buckling increased MMP-2 expression and collagen IV degradation in a site-specific fashion. These site-specific differences may contribute to uneven vascular remodeling in buckled arteries.

It is well known that vascular endothelium plays an important role in ECM remodeling.^{29,30} For example, elevated flow induces vessel dilation due to vasomotor response and prolonged elevation in flow leads to enlargement of vessel diameter in the long term due to growth and remodeling. But the vessel dilation response is endothelial cell dependent, and the adaptive remodeling is eliminated if the endothelium is removed.³⁰ In the present study, we found significant decrease in endothelial eNOS level in buckled arteries where shear stress was disturbed. This observation is consistent with a previous report that disturbance in shear stress in curved regions of arteries impairs endothelial function through a downregulation of eNOS gene expression and a decrease of nitric oxide (NO) production in pig carotid arteries.³³ Since eNOS is attributed to NO production in endothelium in arteries,⁴ the change in eNOS indicates possible changes in endothelial cell function. Furthermore, flow and shear stress of different conditions (laminar and perturbed, unidirectional/oscillatory) can differentially regulate eNOS expression.^{37,44} Our previous computational analysis demonstrated that flow in the buckled arteries was laminar and unidirectional,²⁷ similar as in straight control vessels. However, the shear stress magnitude and its spatial distribution are different in buckled and straight vessels. The disturbed shear stress could be the mechanism for the observed changes in eNOS expression.

Previous study showed an association between shear stress, eNOS and NF- κ B expression.⁷ Our previous study showed that cell proliferation increase in buckled arteries was associated with NF- κ B expression.⁴⁰ Buckling-induced shear stress alteration could be the mechanism in the eNOS-mediated regulation of MMP-2 levels possibly through NF- κ B signaling.^{15,17} This could also be a possible mechanism that induces atherosclerosis in tortuous arteries.⁸

MMPs play a key role in wall matrix remodeling associated with atherosclerosis.¹³ High flow and shear stress can mediate endothelial and smooth muscle cells to express MMP-2 and MMP-9 which plays a critical role in flow-induced vascular remodeling.³⁶ A significant increase in MMP2 expression and unchanged MMP-9 expression were observed in the same study.³⁶ Similarly, our current study found that artery buckling significantly increased MMP-2 expression at both the inner curve and outer curve of buckled arteries while there was no change in MMP-9 expression. It is consistent with our previous *in vivo* observation of buckled arteries.⁴³ This observation is also consistent with the report that MMP-2 is the predominant metalloproteinase involved in the early phase of development of vascular remodeling.¹ Associated with increased MMP-2 expression, we observed a significant decrease in collagen IV, the main substrate of MMP-2,³⁵ at the inner curve of the buckled arteries. As inhibition of vascular MMPs is of great interest as a therapeutic target, it could be a potential target for treatment of tortuosity-associated atherosclerosis as well.

Vascular ECM components include elastin, collagen, fibronectin and many other proteins. Besides collagen we also examined fibronectin, which is an important ECM component in the arterial wall that reportedly often change in the early stage of arterial wall remodeling

associated with changes in flow and hypertension.^{5,42} Our result that no significant change in fibronectin in buckled arteries is consistent with the finding that increased MMP-2 expression did not influence fibronectin level in stiff arteries with aging.¹⁰ We also measured elastin and confirmed no change in elastin content in the buckled arteries. Further studies are needed to examine the long-term changes in ECM components.

Our previous fluid–structure interaction (FSI) simulations have demonstrated that arterial buckling increased both lumen shear stress and wall stress and make all the stresses non-axisymmetric in the buckled arteries.²⁷ These stress changes could be the biomechanical mechanism in the observed endothelial dysfunction and ECM remodeling in buckled arteries. The reduction in eNOS could be due to the disturbed lumen shear stress as it acted directly on the endothelium. The decrease in collagen IV in the buckled arteries could be due to both the increases in lumen shear stress and wall stresses,^{5,15,28} though we could not distinguish which stress component led to the difference between the inner and outer curves. Shear stress was found higher at the inner curve of buckled arteries,²⁷ which is most likely the mechanism that led to increased MMP-2 expression, as suggested by Sho *et al.*³⁶ Buckling-induced changes in hemodynamics, mechanical stress, endothelial cell function, and ECM remodeling could be a possible mechanism for the high occurrence of atherosclerosis in tortuous carotid arteries.^{8,29}

In this study, we employed an organ culture model to investigate the artery buckling-induced ECM remodeling. An advantage of using the organ culture model lies in its accurate and independent control of flow, pressure and axial stretch.³² Artery buckling was achieved by using the long arterial segments while subjecting to the same pressure, flow rate, and axial stretch ratio as the control arteries which used short segments and did not buckle. This approach allows us to observe specific effect from artery buckling since the pressure and flow rate remain unchanged. Arteries are normally stable under physiological pressure and axial stretch ratio (1.5), but are prone to buckling at lower axial stretch ratios,³¹ such as in cases of vascular surgery, aged arteries, or genetic deficiencies.²¹ So we used a sub-physiologic axial ratio 1.3 to facilitate buckling. The physiological pulse frequency is 1.5–2 Hz. We used a slightly higher frequency of 2.5 Hz due to the experimental set up, in which the roller pump generated a pulsatile flow of a pulse frequency of 2.5 Hz at the physiological flow rate. While we expect no significant effect from the frequency difference, further study is needed to investigate the effect. Our previous studies have demonstrated that arteries maintain their viability and function for up to 7 days in organ culture.^{23,32} Though there was some difference in eNOS expressions between the fresh and the 3- and 7-day groups, this difference is excluded by comparing the 3- and 7-day buckled arteries with the corresponding straight controls. Another limitation of the current study is the short duration of the observation (7 days) and the tortuosity index was set around a given level. Further studies are needed to examine the effect of different levels of tortuosity index and longer term studies need to be done using an *in vivo* animal model.⁴³

In conclusion, our data demonstrate that artery buckling stimulated uneven MMP-2 expression and decreased collagen IV content and eNOS production. Buckling-induced ECM remodeling and endothelial dysfunction could make arteries locally prone to

atherosclerosis development. Further study is needed to fully understand the atherosclerosis observed in tortuous arteries in humans.

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FIGURE 1.

(a) Schematics of an artery buckled under flow illustrating the inner and outer curve and locations of three artery rings (dot lines). On the right is the cross-sectional view of a tissue ring. A cut was made at the outer curve; shaded areas are the inner and outer curve zones.

(b) Comparison of the shape of a straight (control) artery and a buckled artery. Photographs were taken pre-organ culture (under no-load condition), during organ culture (under flow, for day 1 to day 7, flow direction was left to right), and post organ culture (under no-load condition).

FIGURE 2.

Comparison of tortuosity index (mean $\pm$ SD, $n = 7$) in the straight controls and the buckled groups, ** $p < 0.01$ buckled vs. straight.

FIGURE 3.

Artery buckling induced site-specific MMP-2 expression. (a) Representative immuno blotting images of MMP-2; (b) Comparison of MMP-2 expression levels among straight arteries and the outer curve (Outer) and the inner curve (Inner) of the buckled arteries. # $p < 0.05$, inner vs. outer; & $p < 0.05$ vs. straight.

FIGURE 4.

Effect of artery buckling on MMP-9 and TIMP-2 expression. Top: Representative immunoblotting images of MMP-9 and TIMP-2. Bottom: Comparisons of MMP-9 and TIMP-2 among straight, outer, and inner groups. * $p < 0.05$, 7-day ($n = 6$) vs. 3-day ($n = 7$).

FIGURE 5.

Effect of artery buckling on collagen IV. (a) Immunohistochemistry staining for determining collagen IV (red, 40 $\times$). (b) Comparison of collagen IV positive area among straight, outer, and inner groups. & $p < 0.05$ vs. straight; # $p < 0.05$ inner vs. outer curve. Sample sizes are $n = 6$ for 3-day groups, $n = 4$ for 7-day straight groups, $n = 7$ for 7-day inner and outer groups.

FIGURE 6.

Effect of artery buckling on collagen I, III and elastin contents, and fibronectin expression. There was no difference for fibronectin expression among straight, outer, and inner. Sample sizes: collagen I, $n = 4$ for 3-day and 7-day groups; collagen III, $n = 4$ for 3-day groups, $n = 5$ for 7-day groups; fibronectin expression, $n = 6$ for 3-day groups, $n = 3$ for 7-day groups; elastin: $n = 6$ for 3-day groups, $n = 5$ for 7-day groups.

FIGURE 7.

Effect of artery buckling on cell apoptosis. Representative TUNEL staining and immunoblotting images of caspase-3 and comparison of caspase-3 normalized to GAPDH among fresh, straight, and buckled arteries ($n = 4$).

FIGURE 8.

Effect of artery buckling on endothelial function. Top: Representative immunohistochemistry staining (left) and immunoblotting images (right) for eNOS in endothelial cells of arteries (40×). Bottom: Comparison of eNOS expression levels among fresh, straight, and buckled arteries from immunohistochemistry (left) and Western (right) measurements. Sample sizes are, eNOS expression, $n = 9$ for fresh group, $n = 5$ for both 3-day and 7-day groups; Western, $n = 13$ for fresh, $n = 6$ for both 3-day and 7-day groups. $\#p < 0.05$ buckled vs. straight; $\%p < 0.05$ vs. fresh.

TABLE 1

Summary of porcine carotid artery dimensions and tortuosity index.

	3 day Straight	3 day Buckled	7 day Straight	7 day Buckled
Sample size (<i>n</i>)	7	7	7	7
Unloaded length (mm)	30.3 ± 4.5	61.7 ± 7.4	29.7 ± 2.6	59.6 ± 5.2
Outer diameter (mm)	5.5 ± 0.5	5.6 ± 1.2	5.0 ± 1.1	5.1 ± 1.3
Inner diameter (mm)	2 ± 0.7	2.4 ± 1.4	2.6 ± 1.2	2.4 ± 1.1
Thickness (mm)	1.5 ± 0.2	1.7 ± 0.1	1.2 ± 0.2	1.3 ± 0.2
Tortuosity index (under flow)	0.07 ± 0.01	0.18 ± 0.11 *	0.05 ± 0.01	0.16 ± 0.04 *

All dimensions were measured under no-load condition, but the tortuosity index was measured under flow condition.

* $p < 0.05$ vs. the corresponding straight group.