Changes of opening angle in hypertensive and hypotensive arteries in 3-day organ culture

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Abstract

To study the effect of pressure changes on the opening angle of arteries in organ culture, tubular segments of porcine common carotid arteries were cultured with pulsatile flow perfusion under hypertensive (150 ± 20 mmHg), normotensive (100 ± 20 mmHg), or hypotensive (30 ± 10 mmHg) pressure while maintaining the artery at a physiological wall shear stress of \(24 \pm 1\) dyn/cm\(^2\) for up to 3 days. Arteries were then cut into short ring segments by sections perpendicular to the axis and then cut open radially to observe the opening angle in aerated phosphate buffered saline solution (37°C). Norepinephrine (NE, 10 \(\mu M\)), carbachol (CCh, 100 \(\mu M\)), and sodium nitroprusside (SNP, 10 \(\mu M\)) were added after the radial cut at 30, 20, and 30 min intervals, the opening angles were measured, respectively. Results show that hypertensive arteries developed a significantly larger opening angle than normotensive and hypotensive arteries, associated with a significant increase in cell proliferation. In addition, with smooth muscle contraction activated by NE, the opening angle decreases significantly in hypertensive arteries but has little change in hypotensive and normotensive arteries, indicating an enhancement of smooth muscle contraction on the lumen side of the hypertensive arterial wall. In comparison, hypotensive pressure has little effect on arterial opening angle and cell proliferation.

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1. Introduction

Arteries are not only passive conduits for blood flow but also remodel significantly in response to changes in blood flow and pressure. An increase in blood pressure would cause myogenic responses in arteries followed by tissue remodeling in the arterial wall (Schubert and Mulvany, 1999; Frize et al., 2003). Arterial wall remodeling would change arterial microstructure, dimensions, mechanical properties, physiologic function, and even lead to cardiovascular diseases. For example, significant changes in wall thickness and mechanical properties have been reported in hypertensive arteries within only a few days after onset of hypertensive pressure (Liu and Fung, 1989; Matsumoto and Hayashi, 1994; Langille, 1996; Zhao et al., 2002). The early stage of vascular response to hypertensive pressure would significantly affect the long-term adaptation in hypertensive arteries.

The opening angle of arteries is a concise parameter directly indicating the residual stress in the vessel (Han and Fung, 1996), which affects the mechanical behavior of the arterial wall. The simple opening angle test can reveal any non-uniform remodeling or anisotropic growth across the arterial wall when the arteries are subjected to hypertensive pressure or other pathological changes such as diabetes and hypoxia (Fung, 1990; Fung and Liu, 1991; Liu and Fung, 1992; Matsumoto et al., 1995). In vivo animal model studies have shown that hypertensive pressure leads to significant increase in the opening angle of arteries (Liu and Fung, 1989;
Matsumoto and Hayashi, 1996). However, though the effects of hypertensive pressure are well established in general, little is known about the effect of hypotensive pressure, partially due to the difficulty in achieving sustained hypotensive pressure in animal models. To fully understand the effect of pressure change on the opening angle of arteries, it is necessary to investigate how hypotensive pressure would affect the opening angle of arteries. In addition, a comparison of the cell proliferation in both hypotensive and hypertensive arteries would help us in understanding the effect of pressure on arterial remodeling.

In normal rabbit arteries, Matsumoto and colleagues demonstrated that smooth muscle contraction affects the opening angle of normal arteries (Matsumoto et al., 1996). However, it is unknown how the smooth muscle contraction would affect the opening angle in hypertensive arteries. Hypertensive arteries demonstrated a stronger overall contractile response to vasoconstriction stimulation (Han and Ku, 2001). But it is unclear whether the contraction is equally enhanced through the wall thickness. It is useful to determine the possible contraction variations since any transmurally uneven changes in contractile function may affect vascular function such as signal transduction and mass transportation through the arterial wall. Examination of opening angle changes provides a window to reveal any transmurally uneven change in smooth muscle contraction since the uneven changes in tissue remodeling and contractile function across the wall would lead to significant changes in the opening angle of the arteries (Fung, 1990).

Recent studies have shown that early stage short-term remodeling of the arterial wall in response to biomechanical or biochemical changes can be investigated in arteries cultured in ex vivo perfusion systems (Bardy et al., 1995; Matsumoto et al., 1999; Han and Ku, 2001). Arteries maintained in organ culture demonstrate normal cellular morphology and functional vasomotor responses (Bardy et al., 1995; Labadie et al., 1996; Matsumoto et al., 1999; Han and Ku, 2001). The organ culture models allow better control of the biochemical and hemodynamic environment than animal models. In animal models such as with an aortic restriction model, it is difficult to achieve a constant hypertensive pressure since the pressure may change dramatically with time due to flow compensation and other in vivo systematic compensation (Liu and Fung, 1989). The effect of hormonal and circulating blood-borne components can hardly be separated from the effect of mechanical factors such as pressure. In organ culture models, hemodynamic parameters can be controlled and changed accurately, consistently, and independently. Individual parameters may be varied independently without affecting other parameters, enabling changes that are hard to achieve for in vivo experiments (Herman et al., 1987). Liu and Fung showed that the opening angle of arteries reaches a peak in about 2 to 4 days after the onset of hypertensive pressure (Liu and Fung, 1989). Therefore, we chose to examine the opening angle of arteries after being cultured for 3 days in organ culture.

The specific aim of this study was to investigate the transmural variation in arterial wall remodeling by examining the opening angle and cell proliferation in arteries being cultured under hypertensive and hypotensive pressures using an organ culture model.

2. Materials and methods

2.1. Artery organ culture

Porcine common carotid arteries were harvested from 6- to 7-month-old American #1 farm pigs with body weight 115–130 kg at a local abattoir. Arterial segments of 4–6 cm in length were prepared and maintained in an organ culture system which has been described in detail previously (Han and Ku, 2001). Briefly, arterial segments were mounted between two thin, stainless-steel cannulae inside a tissue chamber with aseptic technique. The vessel chamber was then filled with preheated bath medium (~100 ml). The cannulae were connected to a medium reservoir from which the medium (~250 ml) was pumped to provide perfusion to the arteries in their in vivo flow direction. The flow loops were then placed in an incubator at 37 °C where both perfusion medium and organ bath medium were aerated with 95% air and 5% CO₂. The distances between the two cannulae in the vessel chambers were adjusted to stretch the arterial segments to an axial stretch ratio of 1.5 with reference to their free lengths to restore their in situ lengths (Han and Ku, 2001) and then held in position through tight fittings. The pump speed was gradually increased to produce a flow rate of 180 ml/min to achieve a physiological wall shear stress with a mean of ~1.5 Pa (15 dyn/cm²) in the arteries. Meanwhile, perfusion pressure was gradually increased by tightening the resistance clamps. The pressure was monitored with a pressure transducer (Harvard Apparatus) connected to the immediate down stream of the vessel chamber. The adjustment was gradually made over a few hours to avoid possible arterial trauma.

Both the bath and perfusion media were composed of Dulbecco’s Modified Eagles Medium (DMEM, Sigma, St. Louis, MO) supplemented with sodium bicarbonate (3.7 g/L, Sigma), l-glutamine (2 mM, Sigma), antibiotics–antimycotic solution (10 ml/L, Gibco, Grand Island, NY) and calf serum (10%, HyClone, Logan, UT). The perfusion medium was also supplemented with Dextran (5% by weight, average molecular weight 282,000, Sigma) to adjust its viscosity to the level of human blood (4 cP). Increasing viscosity allows us to produce a physiologic wall shear stress at a physiologic flow rate.
The medium was adjusted to a pH of 7.4 after all the supplements were added.

Three groups of arterial segments were cultured under hypertensive, hypotensive, and normotensive (control) pressures for 3 days, respectively. All three pressure conditions are pulsatile with a pulse frequency of about 120 pulse per min. Specifically, the hypertensive pressure oscillates from 130 to 170 with a mean of 150 mmHg, the hypotensive pressure oscillates from 20 to 40 mmHg with a mean pressure of 30 mmHg, while the normotensive pressure oscillates from 80 to 120 mmHg with a mean pressure of 100 mmHg. Artery segments from left and right common carotid arteries were cultured as paired specimens with one randomly chosen to be cultured under hypotensive pressure while the other one was cultured under hypertensive pressure. Arteries from a separate group of pigs were cultured under normotensive pressure as control.

2.2. Cell proliferation labeling

Bromodeoxyuridine (BrdU at 5 mg/L, Sigma) was administered to the perfusion medium to label the nuclei of newly proliferated cells.

2.3. Opening angle measurement

Arteries were harvested after being cultured for 3 days. Each arterial specimen was divided into two segments, one for opening angle measurement and the other for histology. Fresh carotid artery segments were also prepared for opening angle measurement and histology.

For the opening angle measurement, arteries were cut into a series of 5–8 short segments (rings, ~2 mm in axial length) by sections perpendicular to the longitudinal axis of the arteries. The rings were arranged in aerated phosphate buffered saline (PBS, 37 °C, Sigma) in a petri dish and then cut open by radial cuts (see Fig. 1). After the radial cut, the rings popped opened into C-shaped sectors and the sectors were allowed to stabilize for 30 min to fully release the residual stress (Han and Fung, 1991). The configurations of the sectors were then either recorded with a JVC video camera or photographed with a Canon camera.

To illustrate the effect of smooth muscle contraction and relaxation on the opening angle of the arteries, norepinephrine (NE, 10 μM), carbachol (CCh, 100 μM), and sodium nitroprusside (SNP, 10 μM) were added in a sequence of 30, 50, and 80 min after the radial cuts. The sectors were photographed 20, 30, and 20 min. after administering the NE, CCh, and SNP, respectively. Norepinephrine is a vasoconstrictor that stimulates smooth muscle contraction, carbachol is an endothelium-dependent vasodilator that relaxes smooth muscle through endothelium NO pathway, and sodium nitroprusside is a strong vasodilator that stimulates smooth muscle relaxation directly.

Later, all images were transferred into a Macintosh computer for image analysis. To characterize the C-shaped sectors, the opening angle of each sector, defined as the angle between the two lines from the midpoint of the inner vascular wall to the tips of the inner wall (Fig. 1, right panel), was measured from these images using NIH Image. The opening angles of the sectors from a vessel were averaged to represent the value for the vessel.

2.4. Tissue processing and immunostaining

Segments from arteries were fixed overnight in 10% formalin and then preserved in 70% alcohol until being processed. Later, the specimens were dehydrated with graded alcohol and embedded in paraffin. Three to four segments from each artery were embedded in each paraffin block. Serial sections (5 μm) were cut and processed for hematoxylin and eosin staining, anti-BrdU staining, and Hoechst counterstaining. Arterial wall structure was observed using sections stained with hematoxylin and eosin under a Nikon light microscope. For the hypertensive and hypotensive specimens, immunostaining was carried out according to the avidin–biotin complex immunoperoxidase procedure (LSAB kit, Dako Co, Carpenteria, CA) to identify proliferating cells with anti-BrdU monoclonal antibody (Chen et al., 1997; Han et al., 2003). For the normotensive control arteries, immunostaining was carried out using an antibody kit (Labeling and Detection Kit I, Roche Diagnostics) with a primary mouse monoclonal antibody to BrdU and a FITC-conjugated secondary antibody (Han et al., 2003; Davis et al., 2005). The BrdU positive nuclei were fluorescently labeled and easier to count. Preliminary study of counting the number of BrdU positive nuclei in a group of sections showed no statistical difference between these two staining protocols. Cell nuclei were counterstained with Hoechst 33258 (1 mg/L, Molecular Probes) in a humid incubator for 30 min at 37 °C for observation under fluorescent microscope.

2.5. Proliferated cell counting and BrdU index

The slides were examined with light microscopy and fluorescent microscopy. Images (10X objective) of no

Fig. 1. Schematics of cutting to release the residual stress in arteries and the definition of opening angle (θ).
less than 8 fields of the transverse sections (from 2–4 cross-sections) were captured for each specimen and stored on a computer for analysis. Each view field covers the entire wall thickness including the intima, media, and adventitia. For each view field, the anti-BrdU positive nuclei were counted manually while the corresponding total nuclei populations were counted automatically (from the view fields of the corresponding locations in the fluorescent images) using the automatic counting feature of Image-Pro Plus (MediaCybernetics, Silver Spring, MD). The BrdU index was calculated as the percentage of anti-BrdU positive cells over the total number of cells at each field. The mean BrdU index for each specimen was obtained by averaging the values of all fields counted.

To further examine the distribution of proliferating cells across the vascular wall thickness, we divided the wall thickness into five layers, namely, the intima, the inner and outer media layers (each covers half of the medial thickness), the dense adventitial layer, and the loose adventitial layer which includes the loose connective tissues. The numbers of anti-BrdU positive nuclei and the numbers of Hoechst-counterstained total nuclei were counted for all five layers and the corresponding BrdU indices were calculated, respectively.

2.6. Measurement of lumen diameter and wall thickness

The lumen circumferential lengths and wall thickness were measured from the hematoxylin and eosin stained transverse sections of arterial segments using Image-Pro Plus. The lumen diameters were determined by dividing the lumen circumferential lengths with pi. The wall thickness was measured and averaged at four locations along the circumference on each section. The measurements for all the sections were averaged to represent the value for each vessel.

2.7. Statistical analysis

All values are presented as the mean±SD (standard deviation). Statistical significance between means was determined using the Student’s t-test. The significance level was set as a p value less than 0.05.

3. Results

3.1. Opening angle changes due to hypertensive pressure

Hypertensive pressure significantly increased the opening angles of arteries in organ culture (Fig. 2). The opening angles of hypertensive arteries were significantly larger than those of the normal fresh arteries, hypotensive arteries, and normotensive controls (p<0.05). The opening angles of normotensive controls are similar to those of fresh arteries. The opening angles of hypotensive arteries did not differ from that of the normotensive arteries.

3.2. Effect of smooth muscle contraction on opening angles

Smooth muscle contraction and relaxation by NE, CCh and SNP stimulations led to significant changes in opening angles of fresh and 3-day cultured arteries (Fig. 3). In fresh arteries, the opening angles were significantly increased (p<0.05) after NE stimulation and then increased further after SNP stimulation. In hypertensive arteries, however, the opening angles decreased significantly (p<0.05) after NE stimulation and then bounced back slightly after CCh and SNP were administered although the difference was not statistically significant. However, in hypotensive arteries and normotensive controls, the opening angles did not change much with NE and SNP stimulations although they increased slightly in the normotensive arteries and decreased a little in hypotensive arteries. CCh did not change the opening angles overall in all groups though it did affect the opening angle of some individual arteries.

3.3. Distribution of newly proliferated cells

The hypertensive arteries have more BrdU-positive cells than the hypotensive arteries after being cultured for 3 days (Fig. 4), indicating an increase in pressure...
leads to increased cell proliferation in arteries. The corresponding BrdU-index calculated in both the individual layers and the whole arterial wall of hypertensive, normotensive, and hypotensive arteries confirmed this trend (Fig. 5). Across the arterial wall, there are more BrdU-positive cells in the intima-inner media region than in the outer media layer and dense adventitial layer. Among all pressure groups, hypertensive arteries had a significantly higher BrdU index in the intima and media than the normotensive and hypotensive arteries did (\(p<0.05\)). BrdU index was significantly lower in the outer media layer in the hypotensive arteries than in the outer media layer of normotensive arteries (\(p<0.05\)). The cell density was also slightly higher in the hypotensive arteries than in the hypertensive arteries. Interestingly, there were many newly proliferated cells at the very outer boundary of the vascular wall in cultured arteries regardless of the pressure conditions in the arteries (Fig. 6).

Continuous endothelial cell lining was observed in the cultured arteries. Examination of the cross sections demonstrated a normal morphology in the cultured arteries. The overall dimensions of the arteries did not change much by the pressure difference in 3 days. Hypertensive arteries had slightly larger diameter than the hypotensive and normotensive arteries while the hypotensive arteries had slightly thicker media than the other groups (Fig. 7). However, neither of these differences was statistically significant.

4. Discussion

Using an ex vivo artery organ culture model, we demonstrated that an increase in pressure leads to an increase in the opening angle of arteries which is associated with an increased cell proliferation on the lumen side of the arterial wall. In addition, contraction of smooth muscle stimulated by norepinephrine leads to a decrease in the opening angle of hypertensive arteries. Hypotensive pressure has little effect on the opening angle and cell proliferation in arteries in comparison to hypertensive pressure.

4.1. Organ culture model

A major advantage of the arterial organ culture system lies in its excellent control over experimental
conditions. In our organ culture system, the flow rate and pulse frequency were controlled by the pump speed while the mean and oscillation amplitudes of pressure were controlled by the clamps and T-end length. Therefore, the means and amplitudes of oscillation of pressure as well as wall shear stress and flow rate could be adjusted independently over a wide range. Hemodynamic parameters can be monitored continuously over the culture period. Therefore, many cellular and vascular changes that happen in hours to a few days in response to biomechanical and biochemical alterations can be studied using the artery culture model (Bardy et al., 1995; Davies, 1995; Langille, 1996; Chesler et al., 1999; Han and Ku, 2001). In addition, the effect of mechanical factors is separated from any possible effects of circulating blood components since there are no circulating blood components in the organ culture system.

4.2. Viability

Arteries are viable and functional after 3 days in organ culture. We have previously reported that arteries are viable and functional in organ culture for up to 7 days (Han and Ku, 2001). Arteries in this study were cultured using the same techniques as the previous study for 7-day organ culture. Though we did not conduct the contractile relaxation assay in terms of diameter response (Han and Ku, 2001), the change of opening angle in response to norepinephrine and sodium nitroprusside also verified the viability of the vessel. In addition, histology sections demonstrated a normal microstructure of the arterial wall with endothelial cells lining on the lumen surface. The viability of the cultured arteries was also supported by the low cell death rate as examined by ethidium staining. Arteries in cultured groups demonstrated similar numbers of ethidium-positive cells as did the fresh arteries reported earlier (Han et al., 2003).

4.3. Hypotension models

Though hypertensive pressure can be achieved in vivo by various methods such as aortic constriction (Liu and Fung, 1989; Friderz et al., 2003), hypotensive pressure is hard to achieve in animal models. The constriction to arteries is limited by the need of blood supply to keep the distal organ alive. A temporal hypotensive pressure may be generated at the distal side of an aortic restriction but the pressure is non-stable, uncontrollable, and can only last a very short period (Liu and Fung, 1989; Li et al., 2002). In addition, alteration of blood pressure in animal models in vivo often leads to changes in blood flow (Li et al., 2002). Changes in blood flow...
itself may lead to changes in the opening angle of arteries (Lu et al., 2001). In comparison, both hypertensive and hypotensive pressures can be easily achieved in the organ culture model without changing perfusion flow rate. Prolonged hypertensive or hypotensive pressure conditions are difficult to achieve in animals in vivo but can be easily achieved with consistency in organ culture.

4.4. Implication of opening angle changes

This study demonstrated a significant increase in opening angle in arteries cultured under hypertensive pressure for 3 days. This change is similar to the opening angle increase observed in hypertensive arteries in hypertensive animal models (Liu and Fung, 1989, 1992; Fung and Liu, 1991; Matsumoto and Hayashi, 1994, 1996; Matsumoto et al., 1999). This similarity demonstrated that arteries respond to hemodynamic changes similarly in the organ culture model and the in vivo models.

A new finding of this study is that the opening angle of the hypertensive arteries decreases when the smooth muscle is stimulated by NE, indicating that the smooth muscle function is more enhanced on the lumen side of hypertensive arteries. The opening angle of hypertensive arteries was significantly decreased when NE was administered while the opening angles of hypotensive arteries remained rather constant and the opening angle of the fresh normotensive arteries increased. The NE-induced opening angle decrease in hypertensive arteries is totally different from the reported opening angle increase in normotensive rat arteries in response to NE stimulation (Matsumoto et al., 1996). Opening angle changes reveal uneven changes or anisotropic tissue remodeling across the arterial wall. The opening angle of the open arterial sectors decreases when the lumen side of the wall shrinks or the other side expands. Here, we found that contraction of smooth muscle stimulated by norepinephrine leads to a decrease in the opening angle of hypertensive arteries. Previously, we showed that the overall contractile response was stronger in hypertensive arteries (Han and Ku, 2001). Therefore, we conclude that the decrease in opening angle in the hypertensive artery after administration of norepinephrine is a result of stronger smooth muscle contraction at the lumen side of the arterial wall, indicating an enhancement of smooth muscle contraction at the lumen side of the hypertensive arterial wall. In contrast, the opening angle of arteries cultured under hypotensive pressure slightly increases when NE is administered but the change is statistically insignificant.

Fig. 7. Comparisons of the lumen diameters and medial thickness in arteries after being cultured for 3 days under normotensive, hypertensive, and hypotensive pressures. \( n = 6 \) for each group.

4.5. Cell proliferation and wall remodeling

We quantified the cell proliferation at individual layers across the arterial wall. The distribution of the newly proliferated cells as measured by BrdU index was highest at the intima and it was higher at the inner layers...
of the media than at the outer layers of the media. Hypertensive pressure increases BrdU positive cells in the intima and media regions with the most dramatic increase occurring at the intima and the inner-half layer of the media. The large number of BrdU labeled cells on the lumen side of hypertensive arteries suggests that the increase in cell density may contribute to the increase in contractile response. An increased number of BrdU positive cells may indicate increased cell proliferation or DNA repair of damaged cells. Though we did not measure the number of injured/dead cells, both cell proliferation and DNA repair mean improvement in overall cell viability and function. Nevertheless, an increase in the number of BrdU positive cells is related to an increase in local contractility enhancement.

It was shown that the wall remodeling in arteries under hypertensive pressure might be due to the increasing circumferential stretch, which stimulates vascular remodeling through activation of ERK1/2 pathway (Birukov et al., 1997). The current study demonstrated that significant increases in opening angle and cell proliferation occur in arteries cultured under hypertensive pressure for 3 days. These results revealed that the arterial wall remodels quickly under hypertensive pressure, similar to arteries in vivo that remodel within a few days after onset of hypertensive pressure (Liu and Fung, 1989). This conclusion is also supported by the evidence that DNA synthesis, matrix metalloproteinases (MMPs) activity, and ECM production including fibronectin production increases quickly in arteries within a few days after onset of hypertensive pressure (Bardy et al., 1995, 1996; Chesler et al., 1999).

We also showed that the opening angle and cell proliferation in hypotensive arteries did not change much from the control arteries. These data may suggest that the remodeling in hypotensive arteries was at the same level/pattern as the normotensive arteries indicating little remodeling was induced from the hypotensive pressure. Studies have shown that a much less significant change in vasomotor responses occurs in hypotensive arteries than in hypertensive arteries (Zulliger et al., 2002). Slower and less significant dimensional changes were observed in hypotensive animal models (Li et al., 2002). These results may suggest that arteries under hypotensive pressure remodel differently than arteries under hypertensive pressure and may be through different mechanisms.

Studies have shown that hypertensive pressure would affect mass transport through the arterial wall (Lever and Jay, 1994). Our results suggest that the contractility may differ across the arterial wall. The relatively strong contraction on the lumen side of the arterial wall may be one of the factors that affect the mass transport and signal transduction in hypertensive arteries.

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