Enhanced External Counterpulsation Attenuates Atherosclerosis Progression Through Modulation of Proinflammatory Signal Pathway

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Objective—Shear stress may be the most crucial local factor affecting atherogenesis. The present study investigated the effect of exposure to increased shear stress promoted by enhanced external counterpulsation (EECP) on the progression of atherosclerosis and the underlying inflammation-related molecular mechanisms in a porcine model of hypercholesterolemia.

Methods and Results—Hypercholesterolemic pigs were subjected to a 7-week EECP intervention while being fed a high-cholesterol diet. EECP resulted in a 34.38% increase of mean wall shear stress and a significantly lower pulsatility index in the brachial artery. The animals receiving EECP showed a marked reduction in atherosclerotic lesion size in the coronary artery and abdominal aorta compared with the hypercholesterolemic control group, associated with a decrease in macrophage accumulation. The expression of a set of genes involved in inflammation (including C-reactive protein [CRP], complement 3a, vascular cell adhesion molecule-1 [VCAM-1], and inducible nitric oxide synthase), mitogen-activated protein kinase (MAPK)-p38 phosphorylation, and nuclear factor-κB (NF-κB) activation, was attenuated.

Conclusion—These findings suggested that long-term EECP exerts a retarding effect on atherosclerosis by downregulating proinflammatory gene expression. The underlying mechanisms are related to chronic exposure to increased pulsatile shear stress promoted by EECP; this exposure suppresses the overactivation of the MAPK-P38/NF-κB/VCAM-1 signaling pathway induced by hypercholesterolemia. (Arterioscler Thromb Vasc Biol. 2010;30:773-780.)

Key Words: atherosclerosis ■ inflammation ■ shear stress ■ MAPK ■ nuclear factor-κB

Atherosclerosis remains the leading cause of morbidity and mortality in many countries. It is now recognized as a chronic inflammatory and immune disease. Oxidation of low-density lipoprotein can elicit a progressive inflammatory response in the arterial wall through activation of inflammation-relevant signaling pathways, resulting in endothelial cell adhesion molecule expression, leukocyte recruitment, macrophage lipid accumulation, and foam cell formation.1,2

Studies have identified that atherosclerotic lesions preferentially develop at distinct sites such as curves, branches, and bifurcations in arteries, areas that experience turbulent blood flow, low fluid shear stress, or flow reversal. Conversely, adjacent regions exposed to undisturbed flow and high mean shear stress are protected from plaque formation. High shear stress with laminar flow is generally beneficial, as it promotes vascular adaptation and dilatation through endothelium-mediated mechanisms and exerts antiinflammatory and prorepair functions on the vasculature.3

Enhanced external counterpulsation (EECP) is a noninvasive modality for the treatment of ischemic cardiovascular disease. EECP therapy is done by sequential inflation of 3 sets of cuffs wrapped around the lower extremities during diastole and deflation of the cuffs during systole. EECP enhances the aortic diastolic blood flow and coronary perfusion,4–6 leading to increased arterial wall shear stress in a pulsatile manner.7–8 Recently, the inhibitory effects of EECP on circulating proinflammatory biomarkers in patients with symptomatic coronary artery disease has been documented.9 However, the exact molecular mechanisms underlying the clinical benefits have not been fully clarified.

This study proposed to examine the effect of EECP on vascular atherosclerosis and inflammation in a porcine model of hypercholesterolemia. Because mitogen-activated protein

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kinases (MAPKs) and NF-κB play central roles in the inflammatory signal transduction, and vascular cell adhesion molecule-1 (VCAM-1) is of pivotal importance in monocyte-endothelial interaction and early atherosclerosis, we investigated these inflammation-relevant molecules and the proinflammatory MAPK-p38/NF-κB/VCAM-1 signaling pathway to provide insight into the molecular mechanisms of the action of EECP.

Materials and Methods

Animal Model

Thirty-five domestic male pigs were randomized to normal chow (control group; n = 7) or a high-cholesterol diet (n = 28) for 15 weeks. After 8 weeks of a high-cholesterol diet, when hypercholesterolemia was achieved, 17 pigs received EECP intervention while being maintained on the atherogenic diet (CHOL+ EECP group). The remaining 11 pigs served as hypercholesterolemic controls (CHOL group). EECP was performed for 2 hours every other day, for a total of 34 ± 2 hours, with the use of a modified clinical system. The lower extremities and the hips of the pigs were wrapped by 2 sets of modified cuffs. The pressure applied to the cuffs was set at 0.035 to 0.040 MPa/cm². Effective hemodynamic changes of EECP were demonstrated by achieving a diastolic to systolic ratio greater than 1.2 using the plethysmographic technique. All pigs were anesthetized with 5 to 10 mg of midazolam IM and 10 mg/kg per hour 3% pentobarbital IV infusion. Experiments were performed in accordance with institutional guidelines.

Hemodynamic Measurement

Doppler ultrasound flow examinations and arterial wall shear stress calculations were performed in the right brachial artery as previously described. Pulsatility index (PI) as a measure of vascular impedance was calculated according to the formula of Gosling and King: PI= (peak systolic velocity – end diastolic velocity)/mean velocity. Blood pressure and flow volume measurements were performed in the common carotid artery. Maximum pulse flow difference (maximum flow volume–minimum flow volume) was calculated to evaluate the pulsatility of blood flow.

Blood Sample

Peripheral blood samples were taken at baseline (0 weeks), before EECP (8 weeks), in the middle of the EECP treatment course (12 weeks), and at the end of the study (15 weeks) for measurement of blood lipid profiles (Hitachi 7170A, Hitachi, Tokyo, Japan).

Morphological Analysis of Atherosclerotic Lesions

The porcine abdominal aortas were en face stained with Sudan IV solution, cross-sectioned, and then stained with Oil Red O for evaluation of lesions. Left anterior descending coronary artery (LAD) cross-sections were stained with hematoxylin and eosin, elastica Gomori aldehyde fuchsin, and Oil Red O for microscopic quantification of the coronary atherosclerosis. To reveal the cellular and collagen components, smooth muscle α-actin immunohistochemistry and Masson trichrome staining were processed. Segments of the LADs were treated for scanning electron microscopy (Hitachi S520).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA extracted from snap-frozen porcine aortic arches was used to measure VCAM-1 and inducible nitric oxide synthase (iNOS) mRNA expression.

Immunohistochemistry and Immunofluorescence

Serial paraffin-embedded cross-sections of LADs were incubated with primary antibodies specific for phospho-p38 (Cell Signaling Technology, Danvers, Mass.) or CRP (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif.), complement 3a (Santa Cruz Biotechnology), macrophage (Santa Cruz Biotechnology), VCAM-1 (Santa Cruz Biotechnology), iNOS (Santa Cruz Biotechnology), and NF-κBp65 (Cell Signaling Technology). Immunofluorescence images were obtained with a confocal laser scanning fluorescence microscope (Fluoview FV500-IX81, Olympus, Tokyo, Japan).

Western Blot Analysis

The primary antibodies used in the Western blot were polyclonal anti-phospho-p38 and p38 (Cell Signaling Technology), anti-phospho-NF-κB and NF-κB (Cell Signaling Technology), anti-IL6 (Cell Signaling Technology), and anti-CRP (Santa Cruz Biotechnology).

Statistical Analysis

Data are reported as mean±SEM. When homogeneity of variance was assessed, statistical significance regarding multigroup comparison was determined by 1-way ANOVA followed by the Bonferroni post hoc test. When variables violated the assumptions for variance homogeneity, significance was alternatively evaluated by the Kruskal-Wallis ranking test and Mann–Whitney post hoc comparisons adjusted by the Holm method.

A detailed description of the methods is available in the supplemental materials, available online at http://atvb.ahajournals.org.

Results

Measurement of Hemodynamic Changes

Doppler flow examination of the porcine right brachial artery showed that EECP dramatically enhanced diastolic blood flow velocity but produced no significant change in the systolic flow velocity. This resulted in a 36.30% increase in mean flow velocity (29.08 ± 2.01 versus 21.34 ± 1.69 cm/s, P = 0.007 < 0.05), as well as a 34.38% increase in mean wall shear stress (27.46 ± 8.02 versus 20.43 ± 6.75 dyne/cm², P = 0.019 < 0.05). In contrast, the diastolic peak flow velocity (8.02 versus 20.43 mL/min, P = 0.045 < 0.05), suggesting that EECP decreases the peripheral arterial resistance (details available in the supplemental online materials). We also observed a nonsignificant trend toward a modest decrease in peak systolic flow velocity during EECP in the left common carotid artery compared with before EECP (362.31 ± 38.19 versus 431.79 ± 33.68 mL/min, P > 0.05). In contrast, the diastolic peak flow velocity was significantly elevated by EECP (540.13 ± 57.31 versus 217.14 ± 33.68 mL/min, P < 0.001). During EECP therapy, there were 2 pulses per cardiac cycle, essentially doubling the number of arterial pulsations. EECP also significantly elevated blood pulse flow from 306.82 ± 42.37 to 457.63 ± 48.63 mL/min (P = 0.041 < 0.05), demonstrating an increase in flow pulsatility by both pulsation frequency and magnitude.

Changes in Serum Cholesterol Level

At the end of study, compared with the normal control group, the 15-week high-cholesterol atherogenic diet resulted in a
significant elevation of serum cholesterol level by 4.79-fold in the CHOL group ($P<0.001$) and 3.71-fold in the CHOL+EECP group ($P<0.001$). At the 4 sampling time points of 0, 8, 12, and 15 weeks, there were no significant differences in cholesterol levels between the CHOL group and the CHOL+EECP group ($P>0.05$), as shown in Figure 1A.

Analysis of the Extent and Composition of Arterial Atherosclerotic Lesions
Sudan IV en face staining and Oil Red O lipid staining revealed remarkable atherosclerotic lesions in the abdominal aortas from hypercholesterolemic control animals, especially at branch points, whereas the hypercholesterolemic animals receiving EECP exhibited a significant decrease in their percentage of sudanophilic plaque area (3.33±0.60% versus 12.03±2.15%, $P<0.001$ versus CHOL group), as shown in Figure 1.

The LAD atherosclerotic lesions were characterized by vascular endothelial cell (VEC) transfiguration and desquamation, smooth muscle cell (SMC) hyperplasia and migration, lipid deposition, elastic fibrin and collagen proliferation, and disarrangement in the intima and partial media, as shown in Figure 2. Typical necrotic core and fibrous cap were seldom observed, indicating that the early stage of atherosclerosis was induced by 7-week hypercholesterolemia. Quantitative microscopic inspection revealed that the LAD lesion to media area ratio was as high as

Figure 1. EECP ameliorates atherosclerotic lesion burden in the abdominal aortas from pigs on cholesterol-rich diet. A, Time-related changes of serum total cholesterol levels. B and C, En face Sudan IV–stained porcine abdominal aortas and quantitative analysis of sudanophilic aortic lesion area. D and E, Oil Red O–stained cross-sections of the abdominal aortas and quantification of lesion area. Data are expressed as mean±SEM. *$P<0.01$. CHOL indicates cholesterol.

Figure 2. EECP suppresses the formation of atherosclerotic lesions in the coronary arteries from hypercholesterolemic pigs. A and B, Elastic staining and quantification of lesion to media area. C and D, Oil Red O staining and quantification of lesion area. E and F, Smooth muscle α-actin immunohistochemistry and quantification of positive area. G and H, Masson trichrome staining of collagen and quantification of positive area in LAD sections. Data are expressed as mean±SEM. *$P<0.01$. CHOL indicates cholesterol.
26.57±4.56% in the CHOL group but was only 7.32±1.59% in the animals receiving EECP (P<0.001 versus CHOL group), demonstrating the atheroprotective effect of EECP. EECP animals also presented a 53.78% reduction of SMC fraction (P<0.001 versus CHOL group) and a 30.44% reduction of collagen content (P<0.05 versus CHOL group), suggesting that lesion composition has been altered by EECP.

Coronary Artery Surface Morphology and Macrophage Accumulation
Scanning electron microscopic examination demonstrated many leukocytes (mostly monocytes) adherent to the endothelium of the coronary artery of hypercholesterolemic animals, whereas in the animals receiving EECP, there was less cellular adherence on the arterial endothelium, as shown in Figure 3A.

Macrophage accumulation in the coronary arteries was determined as an index of atherosclerotic burden and also an indicator of inflammatory response. The macrophage-positive staining was much more intense in the LADs of hypercholesterolemic control animals, whereas EECP animals demonstrated a 53.63% reduction in the positive area ratio (5.58±0.49 versus 9.30±0.71, P<0.01; Figure 3B).

Proinflammatory Molecules in Coronary Artery and Aorta
As shown by immunohistochemical study in Figures 3C and 5C, the brown positive staining of VCAM-1 and iNOS were much stronger in the LADs of hypercholesterolemic control animals and weaker in EECP animals.

Assessed by TaqMan Real Time-PCR, the VCAM-1 mRNA and iNOS mRNA expressions were significantly enhanced in the aortas of the hypercholesterolemic control animals (P=0.02<0.025 for VCAM-1 and P=0.004<0.017 for iNOS versus CHOL group, respectively). In animals receiving EECP, VCAM-1 mRNA levels were significantly decreased (P=0.002<0.017 versus the CHOL group). There was a trend toward lower iNOS mRNA levels in animals receiving EECP (P>0.05 versus CHOL group).

Arterial Complement Deposition and CRP Expression
We found marked deposition of C3a in the LAD atherosclerotic lesions of hypercholesterolemic control animals. The EECP animals demonstrated a 30.06% reduction of C3a positive area ratio compared with hypercholesterolemic control animals (12.98±0.66 versus 18.56±0.90, P=0.001), as shown in Figure 4A, indicating a modifying effect of EECP on complement activation. C3a expression correlated well with the occurrence of atherosclerotic lesions.

As shown in Figure 4, the coronary arteries of hypercholesterolemic control animals presented extensive immunohchemical CRP staining in the intima, media, and adventitia, with the greatest intensity on the edge of plaques. However, less staining was observed in animals receiving EECP. Western blot analysis demonstrated that aortic CRP protein expression was markedly elevated in the CHOL group (P=0.002<0.017 versus control group), whereas it was significantly decreased in the animals receiving EECP (P=0.009<0.025 versus CHOL group).

MAPK-p38 Activation in Coronary Artery and Aorta
Micrographs of porcine LADs with phospho-p38 antibody immunohistochemical staining for the 3 groups are shown in Figure 5. Phosphorylated (activated) p38 protein was localized mainly in the nuclei of endothelial cells and smooth muscle cells of hypercholesterolemic animals, with the greatest intensity on the edge of plaques. In LADs from the EECP
animals, less staining was observed. Consistent with the immunohistochemical study, Western blot analysis demonstrated the p38 activity (expressed as the ratio of phospho-p38 to total p38) was significantly higher in the CHOL group \( (P<0.001 \text{ versus control group}) \) and significantly lower in the CHOL+EECP group compared with the CHOL group \( (P=0.012<0.025) \). These results indicated that the abnormal phosphorylation of p38 induced by hypercholesterolemia could be suppressed by EECP treatment.

**NF-κB Activation and IκB Degradation in Coronary Artery and Aorta**

As shown in Figure 6, the confocal laser scanning fluorescence microscope images of NF-κB p65, visualized in green, were mainly confined to the cytoplasm in the animals fed normal chow, consistent with a quiescent state. In the LAD lesions of the CHOL group, NF-κB p65 protein was abundant not only in the cytoplasm but also in the nuclei (red plus green, visualized as yellow), showing increased nuclear translocation and activation. Decreased nuclear immunofluorescent intensity could be observed in animals receiving EECP, indicating pronounced inhibition of NF-κB p65 activation. Western blotting revealed that the amount of phospho-NF-κB p65 protein was significantly increased in the CHOL group \( (P<0.001 \text{ versus control group}) \). The phospho-NF-κB p65 and total NF-κB p65 protein levels were significantly decreased in animals receiving EECP compared with the CHOL group \( (P=0.014<0.025, \text{ respec-} \)

**Figure 4.** EECP decreases complement activation and CRP expression in hypercholesterolemic porcine coronary arteries. A and C, LAD sections stained for complement 3a and quantification of C3a-positive area. Data are expressed as mean±SEM. B, LAD sections stained for CRP. D and E, CRP Western blot bands of porcine aortas and densitometry quantification. Data show mean±SEM from 3 independent experiments. *\( P<0.025; \) **\( P<0.017. \) CHOL indicates cholesterol.

**Figure 5.** EECP attenuates the abnormal activation of MAPK-p38 induced by hypercholesterolemia. A, LAD sections stained against phospho-p38. B and C, Western blot bands of phospho-p38 and total p38 of porcine aortas and intensity of bands expressed as the ratio of phospho-p38 to total p38. Data show mean±SEM from 3 independent experiments. D, LAD sections stained for iNOS. E, Quantification of iNOS mRNA of porcine aortas by TaqMan Real Time-PCR. Data are expressed as mean±SEM. *\( P<0.025; \) **\( P<0.017. \) CHOL indicates cholesterol.
tively). IκB protein degradation was significant in the CHOL group (P < 0.002 versus control group), and an improved trend was demonstrated in animals receiving EECP (P < 0.05). These results suggested that the NF-κB overexpression induced by hypercholesterolemia was suppressed by EECP treatment.

Discussion
Atherosclerosis is a chronic lipid-driven inflammatory disease of arteries. Within the atherosclerotic lesions, the accumulation and proliferation of macrophages and T lymphocytes lead to enhanced cytokine secretion and activation of intracellular NF-κB and MAPK signaling pathways, which cooperate to induce more cytokines and other acute-phase reactants to further amplify inflammation. The complement also participates in the development of atherosclerosis. In vessels, the complement system may be activated by antigen-antibody immune complexes, CRP, modified lipoproteins, apoptotic cells, and cholesterol crystals. Because C3 is the central component in complement activation, inhibition of C3 activation leads to the blockade of biologically active end products, including C5b-9, C5a, and C3a, as well as the retardation of atherosclerosis.

It has been widely appreciated that CRP is more than a biomarker of inflammation. Because of its capacity to activate complement, stimulate VCAM-1 to accelerate monocyte-endothelial cell adhesion, and promote uptake of oxidized LDL, CRP plays a direct proatherogenic role. CRP can activate NF-κB and MAPK signaling pathways and evoke an inflammatory endothelial phenotype by decreasing endothelial nitric oxide synthase expression and activity. Studies find that the synthesis and secretion of CRP in the atherosclerotic lesion by paracrine/autocrine loops can result in local concentrations of CRP far in excess of plasma concentrations.

Hemodynamic shear stress may be the most pivotal factor affecting atherogenesis. Accumulating evidence shows that the magnitude of shear stress at the arterial wall is inversely correlated with susceptibility to atherosclerosis. Many endothelial genes contain shear stress–responsive elements in their promoters, which are inducible by shear stress. At sites susceptible to atherosclerosis, low time-averaged or oscillatory shear stress stimulates the endothelial cells to produce adhesion, vasoconstrictor, growth and coagulation molecules, which promote atherosclerosis in synergy with other systemic risk factors. At sites resistant to atherosclerosis, prolonged high shear stress induces the endothelial cells to express antiatherosclerotic genes, which suppress endothelial proinflammatory activation and inhibit cell cycles via multiple mechanisms, including modulating the activities of MAPKs and NF-κB signaling pathways. Experimentally, high shear stress with a clear direction causes only transient activation of proinflammatory and proliferative signaling pathways, which is downregulated, even to below the preshear levels, when such high shear stress is sustained. Our
study supports these findings by demonstrating that EECP increases shear stress to the endothelium, attenuates vascular CRP expression, decreases macrophage accumulation and complement activation, and eventually ameliorates vascular inflammation.

Not only the magnitude of shear stress but also the flow patterns can influence the biology and pathology of endothelium. Studies have revealed that compared with steady laminar flow, pulsatile flow is more effective in suppressing monocyte adhesion to endothelium exposed to oxidized lipids and upregulating endothelial nitric oxide synthase to produce NO. Pulsatile shear stress exerts a greater atheroprotective effect. Furthermore, the duration of exposure to shear stress also influences endothelial gene expression. Acute shear stress induces genes related to endothelial activation, whereas under chronic shear stress, more endothelial genes are suppressed than induced, especially the proinflammatory or atherogenic genes. These findings suggest that any therapeutic approaches directed at optimizing arterial wall shear stress may be beneficial. But few methods have this kind of hemodynamic effect in vivo, and fewer could be used in human beings. Among them, regular physical exercise and control of hypertension can be considered as such modalities. The progression of atherosclerosis can be inhibited and even reversed in some patients who carry out regular physical exercise and modify their cardiovascular risk factors. Longitudinal regular exercise training demonstrates a long-term antiinflammatory effect by improving endothelial function and reducing inflammatory markers. In a manner similar to regular physical exercise, EECP is able to increase cardiac output and blood flow to change the hemodynamic patterns in the lesion-prone area. Our study provides some evidence that long-term EECP treatment increases arterial shear stress and pulsatility in vivo chronically, attenuating the progression of atherosclerosis.

Previous studies have shown that in patients with refractory angina, EECP treatment reduces arterial stiffness and improves arterial wall properties and wave reflection characteristics. In the present study, hypercholesterolemic animals subjected to 34 hours of EECP over a 7-week period displayed a significant shear stress augmentation in their brachial arteries, associated with a decreased PI. The PI reflects downstream vascular resistance of arteries and can be used as an estimate of vascular impedance and severity of vascular damage. PI values ≥ 1.2 are considered to indicate increased distal resistance to blood flow in human beings. Low mean velocity and high PI have been documented in multiple vessels by Doppler in patients with ischemic stroke diffuse intracranial disease, diabetes, transient monocular blindness, and cigarette smoking. In our study, although a normal range of PI has not been defined in the porcine model, significantly higher mean flow velocities and lower PI in the brachial arteries of hypercholesterolemic pigs were observed during EECP compared with baseline. This is indicative of vasodilation and reduced peripheral vascular resistance, contributing to the beneficial hemodynamic effects of EECP.

Many survival signaling pathways sensitive to atheroprone stimuli converge on MAPKs, including the p38 cascade, which regulates inflammation through activation of proinflammatory transcription factors such as NF-κB. Phosphorylation and activation of MAPKs was observed in endothelial cells exposed to low shear stress and mediated low shear stress–induced proinflammatory gene expression. Shear stress has also been found to modulate the expression of NF-κB. Analysis of vascular regions prone to atherogenesis revealed that low or disturbed shear stress primes the endothelial cells to respond to proatherogenic stimuli through upregulation of NF-κB.

Our current data provide evidence that limiting MAPK-p38 and NF-κB activation in plaques could represent a molecular mechanism by which EECP modulates inflammatory responses to hypercholesterolemia, contributing to the observed clinical benefits of EECP.

EECP is a noninvasive device capable of improving blood flow, shear stress, and pulsatility systematically, thereby modulating the inflammatory response to hypercholesterolemia. Long-term EECP intervention may be an effective alternative therapeutic strategy to protect the cardiovascular system from inflammation and atherosclerosis.

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**Disclosures**

John C. K. Hui served as chief technology officer and senior vice president of Vasomedical and is the principal inventor of several patents related to enhance external counterpulsation (EECP).

**References**


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Supplemental Material

Hemodynamic Measurement

At the end of the study, just before the animals were sacrificed, right brachial artery flow was examined in animals receiving EECP by a color Doppler ultrasound system (ALT- HDL-5000, Phillip Com.) equipped with a 5-10 MHz multifrequency linear probe immediately before and during EECP. The internal diameter (ID) and blood flow velocity (V) in the right brachial artery were measured just before and during the last hour of EECP treatment. Flow velocities were detected using the smallest size of the sample volume placed in the center of the vessel and recorded as the mean of 3 cardiac cycles. The peak systolic maximum velocity, end-diastolic minimum blood velocity, and mean blood velocity were determined by integration of the outer envelope in the flow profile. Blood viscosity (\( \eta \)) at the shear rate of 200/s was measured in vitro just before the Doppler examination with the use of a cone/plate viscometer (Rheocalc V 2.1, Brookfield Engineering Laboratory, Inc.). The intensity of arterial wall shear stress (\( \tau \)) was calculated according to the following formula \( \tau \) (dyne/cm\(^2\)) = 4\( \eta \)V/ID as previously described.\(^1,2\) Pulsatility index (PI) as a measure of vascular impedance was calculated according to the formula of Gosling and King: pulsatility index (PI) = (peak systolic velocity - end diastolic velocity)/ mean velocity.\(^3\)

At the same time, measurement of blood pressure and flow volume in the common carotid artery was performed before and during EECP. A Dopper flowmeter (TS420
Transit Time Perivascular Flowmeter, Transonic System Inc.) was placed in the left common carotid artery to record blood flow volume. A pressure transducer (Micro-tip Catheter Transducer, Millar Instrument) was placed in the right common carotid artery to record blood pressure. The maximum pulse flow difference value (Maximum flow volume – minimum flow volume) was calculated to evaluate the pulsatility of blood flow.

During EECP, effective hemodynamic changes were monitored by achieving a diastolic- to- systolic peak pressure ratio > 1.2 using finger plethysmography.

**Morphological Analysis of Atherosclerotic lesions**

The porcine abdominal aortas were collected. Proximal parts were cross- sectioned, OCT- embedded and stained with Oil Red O. Distal parts were en face stained with Sudan IV solution for evaluation of lesion coverage. For the microscopic quantification of the coronary atherosclerosis, 1-cm segments of the left anterior descending coronary artery (LAD) were collected and cut into 3 segments, embedded in paraffin, then cut into cross sections to stain with hematoxylin and eosin (H & E) and elastica Gomori aldehyde fuchsin. To reveal the cellular and collagen components, smooth muscle α-actin immunohistochemistry and Masson trichrome staining were processed. 0.5- cm segments were OCT- embedded and sections were Oil Red O stained. LAD lesion area and medial area were measured at ×40 magnification with the use of a Zeiss-KONTRON IBAS 2.5 Automatic Image Analysis System (Zeiss, Munich, Germany). The percentage of the intimal area occupied by the smooth
muscle α-actin- positive area and collagen- positive area were assessed. For each LAD, at least 3 separate sections from different segments were calculated. Morphological evaluation was performed by technicians who were blinded to the group assignment.

Other segments of LADs were treated for scanning electron microscopy (Hitachi S520).

**Quantitative Real-Time PCR**

Total RNA extracted from snap frozen porcine aortic arches was used to measure VCAM-1 and iNOS mRNA expression. Porcine-specific primers and probe target sites were designed through Primer Express 2.0 software. For VCAM-1, the forward primer was 5'-CTC CCA GGG ATA CGA CCA TCT-3'; TaqMan probe was 5'-FAM-TCC ACC CTG GAG GAG GGC AGT-TAMRA-3'. For iNOS, the forward primer was 5'-AAC ATC AGG TCG GCC ATC AC-3'; TaqMan probe was 5'-FAM-TGT TCC CCC AGC GGA GCG-TAMRA-3'. Standard curves for expression of each gene were generated by 10-fold dilution of aortas total RNA. Relative quantities of PCR product in each sample were measured against the standard curve and normalized with GAPDH. Samples were run on an ABI (Applied Biosystems, Foster City, CA) 7500 real time PCR system.

**Immunohistochemistry and Immunofluorescence**

Serial paraffin-embedded cross sections of LADs were incubated with primary antibodies specific for phospho-p38 (at 1:100 dilution; Cell Signaling Technology, Danvers, Mass), or CRP (at 1:100 dilution, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), Complement 3a (at 1:100 dilution, Santa Cruz), Macrophage (at 1:100 dilution, Santa Cruz), VCAM-1 (at 1:50 dilution, Santa Cruz), iNOS (at 1:100 dilution,
Santa Cruz). Negative controls in the absence of primary antibodies were also performed. For each LAD, at least 3 separate sections from different segments were stained. The macrophage accumulation and Complement 3a protein expression were quantified as the mean ratio of positive stained area to the total area on five randomly chosen high magnification fields (×400) of 3 different sections of each LAD. Indirect immunofluorescence analysis was used to identify the expression of NF-κB. Paraffin-embedded cross sections of LADs were incubated with rabbit polyclonal NF-κBp65 antibody (at 1:50 dilution, Cell signaling Technology, Danvers, Mass) overnight, then incubated with goat anti- rabbit secondary antibody tagged with fluorescein isothiocyanate (FITC). The nuclei were stained with DAPI (Molecular Probes Inc, Eugene, OR). Immunofluorescence images were obtained with a confocal laser scanning fluorescence microscope (CLSM, Fluoview FV500- IX81, Olympus, Tokyo, Japan).

**Western-blot analysis**

Equal amounts of protein (30 µg) from the arch of the aorta were run on SDS-PAGE using 10% gels, electrophoretically transferred to Hybond-PVDF membranes (Amersham Phamacia Bio) and incubated overnight with primary antibody including phospho- p38 antibody (at 1:1000 dilution, Cell Signaling), phospho- NF-κB antibody (at 1:1000 dilution, Cell Signaling), IκB antibody (at 1:1 000 dilution, Cell Signaling), or CRP (at 1:500 dilution, Santa Cruz). A HRP-conjugated secondary antibody (at 1:2000, Cell Signaling) and an enhanced chemiluminescence (ECL) kit (Cell
Signaling) was used.

The membranes were then stripped and incubated with a total p38 antibody (at 1:1000 dilution, cell signaling) or total NF-κB antibody (at 1:1000 dilution, cell signaling). After exposure, the membranes were stripped again, then blocked, incubated with total actin antibody and detected as internal controls.

**Statistical analysis**

Data are expressed as mean±SEM. When homogeneity of variance was assessed by Levene variance homogeneity test, statistical significance regarding multigroup comparison was determined by one-way ANOVA followed by Bonferroni’s post-hoc test with \( p < 0.05 \) representing significant differences. When variables violated the assumptions for variance homogeneity, significance was alternatively evaluated by Kruskal-Wallis ranking test and Mann-Whitney post hoc comparisons adjusted by Holm’ method. Order the \( p \) values for the \( n \) ( \( n=3 \) groups in our study), hypotheses being tested from smallest to largest. It was used, for example, in the results presented in Figure 2 of CRP Western-blot protein expression, \( p < \frac{\pi}{n} \) ( \( 0.05/3 = 0.017 \)) was considered statistically significant for the first comparison, \( p < \frac{\pi}{(n-1)} \) ( \( 0.05/2 = 0.025 \)) for the second comparison and \( p < 0.05 \) for the third comparison. For data of multiple time-point measurements, repeated-measures analysis was performed with General Linear Methods-general factorial ANOVA, followed by the aforementioned methods. The SPSS 13.0 software was used for all statistical calculations.

Reference

1. Irace C, Cortese C, Fiaschi E, Carallo C, Farinaro E, Gnasso A. Wall shear stress is


Supplemental Figure 1. **EECP enhanced blood flow velocity in a pulsatile manner.**

Ultrasound Doppler blood flow velocity waveforms of porcine right brachial artery immediately before (Upper panel) and during EECP (lower panel). Amplified flow velocity waveforms of cardiac cycles (right). EECP caused a significant diastolic augmentation of flow velocity and doubled the number of arterial pulsations per cardiac cycle. R for R wave of ECG, S for systolic flow velocity, D for diastolic flow velocity.
Supplemental Figure 2. Measurement of blood pressure and flow volume in the common carotid artery before and during EECP. The ECG (upper), flow volume (middle) recorded in the left common carotid artery and blood pressure (lower) recorded in the right common carotids.
Supplemental Figure 3. The ECG (upper) and finger plethysmography (lower) recorded during EECP. ① The signal of cuff inflation, ② The signal of cuff deflation, and ③ Diastolic augmentation. Effective hemodynamic changes of EECP were monitored by achieving a diastolic- to- systolic peak pressure ratio > 1.2 using finger plethysmography.
Supplement table 1. Hemodynamic parameters of hypercholesterolemic pigs in right brachial artery after 15- weeks of high-cholesterol diet.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before EECP (n=13)</th>
<th>During EECP (n=13)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic peak velocity, cm/s</td>
<td>50.77±3.77</td>
<td>55.08±3.09</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Diastolic peak velocity, cm/s</td>
<td>24.62±1.31</td>
<td>56.93±4.02</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>mean flow velocity, cm/s</td>
<td>21.34±1.69</td>
<td>29.08±2.01</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Systolic peak wall shear stress, dyne/cm²</td>
<td>53.19±4.77</td>
<td>56.35±3.68</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Diastolic peak wall shear stress, dyne/cm²</td>
<td>23.92±2.02</td>
<td>53.18±3.81</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>mean wall shear stress, dyne/cm²</td>
<td>20.43±1.87</td>
<td>27.46±2.22</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Pulsatility index (PI)</td>
<td>1.83±0.13</td>
<td>1.52±0.06</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM