In vitro protection of vascular function from oxidative stress and inflammation by pulsatility in resistance arteries

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Objective: Resistance arteries remain subject to pulsatility, a potent regulator of large elastic artery tone and structure, but the effect is incompletely understood. Extracorporeal circulation during cardiac surgery is often associated with absence of pulsatility, which may affect vascular tone. To define the role of the vascular wall in the inflammatory process that may occur with or without pulsatility, we studied resistance arteries functions *ex vivo*. We measured vascular reactivity, oxidative stress, and inflammation in the arterial wall.

Methods: Isolated rat mesenteric resistance arteries were mounted in an arteriograph and subjected to pulsatility or not *in vitro*. Arteries were perfused with a physiologic salt solution without circulating cells.

Results: After 180 minutes, flow-mediated dilation was higher and pressure-induced myogenic tone lower in arteries subjected to pulsatility. Without pulsatility, reactive oxygen species and markers of inflammation (monocyte chemoattractant protein 1 and tumor necrosis factor α) were higher than baseline. In perfused mesenteric beds under similar conditions, tumor necrosis factor α was higher in perfusate after 180 minutes of nonpulsatility (5.7 ± 1.6 pg/mL vs 1.1 ± 0.4 pg/mL; $P < .01$). In arteries treated with the antioxidant 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (tempol), flow-mediated dilation and myogenic tone were similar in nonpulsatile and pulsatile arteries; monocyte chemoattractant protein 1 and nuclear factor κ B expression levels were not increased in tempol-treated nonpulsatile arteries.

Conclusions: Absence of pulsatility in resistance arteries increased oxidative stress, which in turn induced inflammation and preferentially altered pressure and flow-dependent tone, which play a key role in control of local blood flow. (J Thorac Cardiovasc Surg 2011;142:1254-62)

Cardiopulmonary bypass (CPB) is used in cardiac surgery to allow interventions to proceed in a bloodless and non-beating operative field. Although this surgical procedure these days is well standardized, the potential benefit and the precise role of pulsatile flow in CPB remain a matter of debate. It is difficult to assess the clinical impact of pulsatile CPB because of the lack of a precise definition and techniques. Observational studies have reported some benefits of pulsatile CPB with respect to hormonal metabolism and postoperative outcome. Prospective randomized trials are still needed, however, for better definition of the effects

of pulsatile flow during CPB and aortic crossclamping.¹ We previously compared the inflammatory responses of patients undergoing coronary artery bypass grafting with either a roller pump or a centrifugal pump delivering nonpulsatile flow.² We found that the terminal complement complex activation and elastase circulating blood level were both increased with use of the centrifugal pump as opposed to the conventional roller pump.³ Such results have been observed by other researchers but to this date remain unexplained.⁴ A recent study has shown that the continuous-flow left ventricular assist device (LVAD) improves end-organ renal and hepatic function in patients with advanced heart failure⁵; however, controversy exists because a higher gastrointestinal bleeding rate has been reported among recipients of nonpulsatile LVADs than among those receiving pulsatile LVADs.⁶

Quantification of pulsatility during CPB is a key issue, especially because the arterial pressure tracing does not necessarily reflect the energy transmitted to the vascular wall.⁷ Although quantification of pulsatility remains controversial, it is now well established that pulsatile flow remains present in even the smallest arterioles.⁸ Indeed, pulsatile perfusion allows efficient exchange between the vascular and extravascular compartments, thus improving cellular metabolism. Although pulsatility is important for proper blood perfusion, excessive pulsatility is associated with an

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Abbreviations and Acronyms

CPB	= cardiopulmonary bypass
FMD	= flow-mediated dilation
LVAD	= left ventricular assist device
MCP-1	= monocyte chemotactic protein 1
MRA	= mesenteric resistance artery
NF κ B	= nuclear factor κ B
PSS	= physiologic salt solution
ROS	= reactive oxygen species
TBST	= Tris-buffered saline solution with Tween
TNF α	= tumor necrosis factor α

increased risk of cardiovascular events.⁹ On the other hand, a beneficial effect of pulsatile pressure or flow relative to steady flow has been suggested by in vitro studies, mainly in cultured cells. Shear stress alone stimulates release of vasorelaxing mediators and enhances endothelial survival. Endothelial cell stretch also stimulates nitric oxide release.¹⁰ Although pulsatile flow may exert a protective effect, no experiments have yet been conducted in small (resistance) arteries involved in the local control of blood flow. In resistance arteries, myogenic tone plays a key role in determining sustained vasoconstrictor tone.¹¹ Myogenic tone is a vasoconstriction induced by pressure in arterioles. It relies on stretch-dependent calcium entry followed by *Ras* homolog gene family, member A–dependent sensitization of the contractile apparatus to calcium. In the kidney, the level of contraction obtained in response to a rise in pulsed pressure in preglomerular arterioles is determined by the systolic pressure, not by the mean pressure; this finding reinforces the assumption that pulsatility is essential in the control of microvascular tone.¹¹ The second key issue in the control of local blood perfusion is dilation mediated by flow (shear stress) involving the production of vasoactive agents by the endothelium. Flow-mediated dilation (FMD) in resistance arteries opposes myogenic tone, thus determining a sustained basal tone.¹¹ Unlike myogenic tone, arterial responses to flow are sensitive to mechanical forces.¹² We therefore hypothesized that pressure-induced myogenic tone and FMD are both most probably sensitive to changes in pulsatility. We also aimed to investigate the involvement of inflammatory factors and reactive oxygen species (ROS) in the changes in vascular tone that are associated with reduced pulsatility.

MATERIALS AND METHODS**Arteries and Study Groups**

Four-month-old adult Wistar male rats were anesthetized with isoflurane (5%) and humanely killed with inhaled carbon dioxide. The mesentery was then removed to isolate the mesenteric resistance arteries (MRAs). A

segment of MRA approximately 200 μ m in external diameter was cannulated at both ends and mounted in a video-monitored perfusion system as previously described.¹³ Briefly, cannulated arterial segments were bathed in a 5-mL organ bath containing a physiologic salt solution (PSS) of the following composition: 135.0-mmol/L sodium chloride, 15.0-mmol/L sodium hydrogen carbonate, 4.6-mmol/L potassium chloride, 1.5-mmol/L calcium chloride; 1.2-mmol/L magnesium sulfate, 11.0-mmol/L glucose, and 10.0-mmol/L, N-2-hydroxy-ethylpiperazine-N-2'-ethylsulfonic acid. The pH was maintained at 7.4, the P_{O_2} at 160 mm Hg, and the P_{CO_2} at 37 mm Hg. Arteries were subjected to a pressure of 50 mm Hg. Arterial diameter was measured and recorded continuously with a video monitoring system (Living System Instrumentation Inc, Burlington, Vt). Pressure and flow rate could be changed independently. A rolling pump was connected to the tubing upstream of the artery to generate pulsatility (300 beats/min; Figure 1). In vivo carotid artery pulsatility (Figure 1, A) was compared with the pulsatility measured in vitro in the rat model (Figure 1, B). Arteries were subjected to pulsatile or nonpulsatile conditions (nonpulsatile control) for 30 to 180 minutes. Endothelial and smooth muscle cells functions were then studied, as were biochemical and immunohistochemical factors.

Arterial Tone

Pressure-mediated myogenic tone and FMD were determined as previously described elsewhere.¹⁴ In brief, diameter changes at equilibrium were measured when intraluminal pressures were set at 10, 25, 50, 75, 100, 125, and 150 mm Hg. Arteries were then subjected to 50 mm Hg of pressure and further contracted with phenylephrine (1 μ mol/L). Intraluminal flow was then increased by steps (from 0 to 100 μ L/min), and the diameter was measured to determine FMD.¹⁴ At the end of each experiment, arteries were perfused and superfused with a calcium-free PSS containing ethylene glycol tetraacetic acid (2 mmol/L) and sodium nitroprusside (100 μ mol/L). Pressure steps (from 10 to 150 mm Hg) were then repeated to determine the passive diameter of the vessel in the absence of smooth muscle tone. Diameter measurements made in normal PSS were considered to represent the diameter under active tone, or the active diameter. Pressure and diameter measurements were collected with a Biopac data-acquisition system (Biopac MP 100; BIOPAC Systems, Inc, Goleta, Calif) and analyzed (Acqknowledge software; BIOPAC Systems). Myogenic tone was calculated as the percentage of passive diameter, and FMD was expressed as the percentage dilation of active tone.¹⁴

Perfused Whole Mesenteric Bed and Measurement of Tumor Necrosis Factor α in Perfusate

Rats were anesthetized with isoflurane (5%). The abdomen was opened, and the superior mesenteric artery was cannulated with polyethylene 90 tubing. Heparinized saline solution was flushed through the mesenteric vascular bed. The vascular bed was then dissected free from the intestine along the intestinal wall and placed in an organ bath maintained at 37°C; it was then perfused and superfused with the PSS described previously at a rate of 2 mL/min. Pulsatility was generated with the rolling pump as described previously in the perfused arteries. The preparation was allowed to equilibrate for 45 minutes before experimentation began. The PSS was then collected after 30, 90, or 180 minutes of perfusion with or without pulsatility. Perfusate tumor necrosis factor α (TNF α) concentration was measured with a commercially available kit (Cayman Chemical Company, Ann Arbor, Mich).

Western Blot Analyses of Monocyte Chemotactic Protein 1 and Nuclear Factor κ B

Western blot analyses of monocyte chemotactic protein 1 (MCP-1, an endothelial activation marker) and nuclear factor κ B (NF κ B) were performed in MRAs as previously described elsewhere.¹² The MRAs (8 rats per group) were homogenized with a lysis buffer (1% sodium dodecyl sulfate, 10-mmol/L tris[hydroxymethyl]aminomethane hydrochloride at pH

FIGURE 1. Typical recording showing blood pressure measurements obtained in vivo in the carotid artery (A) and those obtained in a mesenteric resistance artery perfused in vitro in an arteriograph and subjected to pulsatility (B). When pulsatility was started (arrow, C), pulsatility was detectable both on the diameter recording (C) and on the pressure recording (D). Corresponding control recordings (artery without pulsatility) are shown for diameter (E) and pressure (F). The lower panel (G) shows diameter changes in response to pulsatility (red trace) relative to nonpulsatile arteries. Pulsatility was maintained for 180 minutes. Data are presented as mean $\pm$ SEM (n = 10 rats per group).

7.4, 1-mmol/L sodium orthovanadate, 2.5-mg/L leupeptin, and 5-mg/L aprotinin). Extracts were incubated at 25°C for 30 minutes and then centrifuged (1000 g, 15 minutes, 14°C). Protein concentration was determined with the Micro BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific Inc, Rockford, Ill). After denaturation at 100°C for 5 minutes, equal amounts of proteins (15 μ g) were loaded on a 9% polyacrylamide gel and transferred to nitrocellulose membranes for 12 hours (40 V, 4°C). Membranes were blocked with 10% bovine serum albumin in Tris-buffered saline solution with Tween (TBST, 20-mmol/L tris[hydroxymethyl]aminomethane at pH 8.0, 150-mmol/L sodium chloride, and 0.1% polysorbate 20) for 1 hour and were then incubated with anti-MCP-1 (1/100; BD Pharmingen,) and anti-NF κ B (1/100 BD Pharmingen; Becton, Dickinson and Company, Franklin Lakes, NJ) antibodies for 1 hour at room temperature. After 3 washes with TBST, immunocomplexes were

detected by chemiluminescent reaction (ECL-kit; GE Healthcare Life Sciences, Little Chalfont, UK) with a computer-based imaging system (Fuji LAS 1000 plus; FUJIFILM Medical Systems USA, Inc, Stamford, Conn). Quantification was performed by densitometric analysis.

Detection of ROS by Confocal Microscopy in MRAs

ROS detection was performed on transverse cross sections 7- μ m thick with dihydroethyidine and microfluoroscopy, as previously described elsewhere.¹⁵ Eight different arteries from 8 different rats were used in each group. Fluorescence of the wall after incubation with dihydroethyidine was measured with confocal microscopy to allow quantification with QED image software (Solamere Technology Group Inc, Salt Lake City, Utah). At least 5 measurements were performed per slice and averaged, and at least 5 slices were analyzed per artery.

Immunofluorescence Analysis

Segments of MRAs (n = 6 rats per group) were mounted in embedding medium (Sakura Finetek USA, Inc, Torrance, Calif), frozen in isopentane that had been cooled in liquid nitrogen, and stored at -80°C in 7- μ m thick cross sections. Sections were incubated with anti-TNF α antibodies (1/100 sc1351; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) or anti-MCP-1 antibodies (1/100 554444 BD Pharmingen; Becton Dickinson). Control experiments were performed after incubation without the first antibody, and positive experiments were performed with arterial sections from rats treated with lipopolysaccharide.¹⁵ Fluorescence staining was then visualized with confocal microscopy (Solamere Technology), followed by pixel quantification as previously described elsewhere.¹⁵ At least 5 measurements were performed per slice and averaged, and at least 5 slices were analyzed per artery.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Significance of differences between groups was determined by analysis of variance (1-factor analysis of variance or analysis of variance for consecutive measurements as appropriate).

Animal Care

The procedures followed in the care and humane killing of the study animals were all in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Ministère de l'Agriculture, France, authorization 6422).

RESULTS

In isolated rat MRAs, pulsatile pressure (Figure 1, D) induced significant dilation (Figure 1, C). In the time control experiment, no significant change in diameter was observed throughout the duration of the experiment (Figure 1, E-F). When pulsatility was stopped after 180 minutes, arterial diameter returned to baseline (Figure 1, G). Stepwise increases in flow (shear stress) induced progressive dilation (Figure 2, A), and stepwise increases in pressure induced contraction or myogenic tone (Figure 2, B). FMD was significantly lower ($P < .01$) under nonpulsatile conditions than in arteries subjected to pulsatility for 180 minutes. No difference was observed between pulsatile and nonpulsatile conditions after 30 minutes (Figure 2, A). In contrast, myogenic tone was significantly higher in the absence of pulsatility after 180 minutes but not after 30 minutes ($P < .05$; Figure 2, B). In the absence of pulsatility,

FIGURE 2. Flow-mediated dilation (A) and myogenic tone (B) in mesenteric resistance arteries subjected to pulsatile (P) or nonpulsatile (NP) conditions for 30 (left panel) or 180 (right panel) minutes. Data are presented as mean $\pm$ SEM (n = 10 rats per group). Asterisk indicates $P < .01$ for pulsatile versus nonpulsatile conditions.

a significant ROS level (which was higher after 180 minutes than after 30 minutes) was found in the arterial wall ($P < .05$). No significant amounts of ROS were detected in arteries subjected to pulsatility (Figure 3). With immunohistochemical methods, we found that MCP-1 (Figure 4, A) and TNF α (Figure 4, B) productions were significant in arteries subjected to nonpulsatile conditions after 180 minutes but not after 30 minutes. Under nonpulsatile conditions for 90 and 180 minutes, the quantity of TNF α in the perfusate of whole mesenteric arterial beds was significantly increased ($P < .05$) relative to that seen under pulsatile conditions (Figure 4, C). In arteries treated in advance with the antioxidant 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (tempol), pulsatility induced dilation (Figure 5, A). After incubation in the presence of tempol for either 30 or 180 minutes, FMD and myogenic tone were similar between arteries subjected to pulsatile and nonpulsatile conditions (Figure 5, B and C). In the presence of tempol, the increases in MCP-1 and NF κ B protein levels otherwise associated with the absence of pulsatility were not observed (Figure 5, D and E).

DISCUSSION

This is the first time that a study has identified a mechanism for the alteration in vascular reactivity induced by the absence of pulsatility in isolated resistance arteries. We found that the oxidative stress that was induced initially was followed by inflammation and changes in vascular

tone, with increased myogenic tone and reduced FMD, thus favoring alteration in blood flow. The experimental setup we used allowed the arteries to be subjected to pulsatile or nonpulsatile conditions while controlling pressure and flow and also eliminating any influences of circulating cells and surrounding tissues. Under these conditions, changes occurring in the absence of pulsatility could be attributable only to a response in the vascular wall. This provides new insights into the systemic inflammatory response seen after cardiac surgery, which has generally been believed to be a process generated by blood cell activation.¹⁶

Controversial findings have been described in clinical trials.¹⁷ Some authors have reported that pulsatile CPB improves postoperative outcome, whereas others consider that linear flow might be well tolerated in clinical practice. In particular, clinical experiences with patients in whom pulseless LVADs (which provide totally linear flow) have been implanted support this belief.⁵ There are marked differences, however, between the clinical and surgical settings of patients with LVADs and those of patients undergoing routine CPB for cardiac surgery.¹⁸ It is important to isolate the effects of the absence of pulsatility in the surgical contexts in which various protocols and devices (pumps, oxygenators, filter, etc) may be used.¹⁹ This persistent controversy led us to study the effect of pulsatility alone in isolated arteries in vitro.

First, we found that FMD was selectively reduced and that myogenic tone was higher in arteries exposed to

FIGURE 3. Reactive oxygen species (ROS) detection with dihydroethyldine (DHE). Reactive oxygen species level was determined in mesenteric resistance arteries subjected to pulsatile (P) or nonpulsatile (NP) conditions for 0, 30, or 180 minutes. Data are presented as mean $\pm$ SEM (n = 10 rats per group). Asterisk indicates $P < .01$ for pulsatile versus nonpulsatile conditions.

nonpulsatile conditions for 3 hours. These changes in vascular reactivity argue in favor of a vasospastic phenotype. Because myogenic tone and FMD are sensitive to ROS

production, we measured ROS concentrations in the arteries subjected to pulsatile or nonpulsatile conditions. Indeed, a rise in ROS production in both endothelial and

FIGURE 4. Monocyte chemotactic protein 1 (*MCP-1*, A) and tumor necrosis factor α (*TNF α* , B) levels were determined with immunohistochemical methods (*IHC*) in mesenteric resistance arteries subjected to pulsatile or nonpulsatile conditions for 30 or 180 minutes. Typical images are shown on the left side. Positive (*Pos.*) and negative (*Neg.*) controls are shown for each protein. Tumor necrosis factor α level was then measured in the perfusate of whole mesenteric arterial beds perfused under pulsatile or nonpulsatile conditions (C). Perfusate was collected after 30, 90, and 180 minutes. Data are presented as mean $\pm$ SEM (n = 10 rats per group). Asterisk indicates $P < .01$ for pulsatile versus nonpulsatile conditions.

smooth muscle cells might be the cause of the vasospasticity observed in nonpulsatile arteries. Myogenic tone and FMD both play key roles in the control of local blood flow, especially in organs with tightly controlled blood flow autoregulation. In the cerebral circulation, for

example, myogenic tone and FMD closely interact to control blood flow.²⁰ We have shown that myogenic tone and FMD were affected (increased and reduced, respectively) at a very early stage in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and

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FIGURE 5. Effect of treatment with the antioxidant tempol on pulsatility-induced dilation (A), on flow-mediated dilation (B and D), and on myogenic tone (C and E). Nuclear factor κ B (*NFκB*, F) and monocyte chemoattractant protein 1 (*MCP-1*, G) protein levels were measured in arterial walls subjected to pulsatile (P) or nonpulsatile (NP) conditions in the presence or absence of tempol. Flow-mediated dilation and myogenic tone were determined after 30 (B and C) or 180 (D and E) minutes. Protein level was determined with Western blot analysis. Data are presented as mean $\pm$ SEM ($n = 10$ rats per group). Asterisk indicates $P < .01$ for pulsatile versus nonpulsatile conditions. Hash mark indicates $P < .01$ for the effect of tempol.

leukoencephalopathy) syndromes, which are characterized by reduced blood flow to the brain and eventually by irreversible dementia.²¹ Similarly, FMD is the hallmark of endothelial disorders with a reduced dilatory response, occurring at an early stage in most cardiovascular and metabolic diseases that are associated with vascular dysfunction.²² The hypothesis that an excessive production of ROS might increase myogenic tone and reduce FMD is strongly supported by our data, which show that treating arteries with the antioxidant tempol could prevent the

changes in vascular reactivity induced by different pulsatile conditions. We also found inflammatory factors (*MCP-1*, *TNFα*, and *NFκB*) in arteries subjected to nonpulsatile conditions. To confirm this inflammation, we measured the production of *TNFα* in the perfusate of a whole mesenteric bed and found it to be significantly elevated in the absence of pulsatility. These findings are in agreement with those of a previous study conducted in patients undergoing coronary artery bypass grafting, which showed that endotoxemia (caused by bacterial

translocation from splanchnic hypoperfusion and injury) was increased in patients undergoing nonpulsatile CPB.²³ It is interesting that this elevation was more pronounced during aortic crossclamping than throughout the duration of CPB itself, because the native pulsatility from the beating heart was completely abolished by cardioplegic arrest. The residual pulsatile flow in this condition was provided only by the arterial pump used for CPB. This is a condition that is not encountered during long-term circulatory support,¹⁸ because the heart does not need to be arrested as it is during heart surgery. This may explain the fairly good results with respect to end-organ function, such as those recently published by Russell and colleagues,⁵ when native pulsatile oscillatory activity generated by the beating (although not working) heart is still present in recipients of nonpulsatile LVAD. Onorati and colleagues²⁴ have reported on a series of clinical studies maintaining the use of an intra-aortic balloon pump during aortic crossclamping in CPB in critically ill patients. They found that some intra-aortic balloon pump–induced pulsatile perfusion during this critical period was associated with a reduction in inflammatory response (particularly MCP-1) relative to that observed after nonphysiologic linear perfusion.²⁴ The oscillatory energy provided by pulsatile flow may protect the endothelial cells, which are normally exposed and presumably adapted to a normal level of shear stress in the range of 5 to 20 dyn/cm².²⁵ Following this reasoning, the inflammatory response would be generated at the endothelial level, which is consistent with our results reported here. Until now, the surgical literature has considered the inflammatory response to be a blood cell and plasma activation process, with subsequent microcirculatory injury. We found that pulsatility in itself was protective. In the absence of flow, the residual pulsatility imposed by the roller pump was sufficient to prevent the changes in myogenic tone and FMD that were seen in the absence of pulsatility. Similarly, in no-flow conditions but with pulsatility, ROS, MCP-1, TNF α , and NF κ B were not found in arteries even after 3 hours. Steady shear stress activates phosphoinositide-3 kinase, leading to phosphorylation and activation of Akt in isolated cells.²⁶ Activated Akt in turn phosphorylates nitric oxide synthase, contributing to sustained nitric oxide release and stimulating kinases and transcription factors to increase cytoprotection.²⁶ Cyclic stretch also enhances Akt activity,²⁷ and when both stimuli are combined, as with pulsatile perfusion in a compliant conduit, Akt and nitric oxide synthase phosphorylation are further increased.²⁸

Finally, we found that inflammation could be detected after 3 hours in the arterial wall or after 90 minutes in the perfusate, whereas ROS levels were significantly elevated after 30 minutes in the absence of pulsatility. This finding suggests that ROS production might precede and possibly even induce inflammation. This speculation is confirmed

by our data showing that the treatment of the arteries with an antioxidant prevented the rise in ROS level and inflammation within the mesenteric arteries. Thus ROS production induced by the absence of pulsatility in turn induced both inflammation and a change in vascular reactivity in favor of vasospasm. Nevertheless, our study does not allow involvement of the inflammatory response in the change in vascular reactivity. Indeed, inflammation is rather known to induce vasodilatation.

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