Regulation of Endopeptidases EC3.4.24.15 and EC3.4.24.16 in Vascular Endothelial Cells by Cyclic Strain: Role of Gi Protein Signaling

Eoin J. Cotter, Nicholas von Offenberg Sweeney, Paul M. Coen, Yvonne A. Birney, Marc J. Glucksman, Paul A. Cahill, Philip M. Cummins

Objective—Endopeptidase EC3.4.24.15 (EP24.15)- and EC3.4.24.16 (EP24.16)-specific peptide hydrolysis plays an important role in endothelium-mediated vasoregulation. Given the significant influence of hemodynamic forces on vascular homeostasis and pathology, we postulated that these related peptidases may be mechanosensitive. The objective of this study, therefore, was to investigate the putative role of cyclic strain in regulating the expression and enzymatic activity of EP24.15 and EP24.16 in bovine aortic endothelial cells (BAECs).

Methods and Results—BAECs were cultured under conditions of defined cyclic strain (0% to 10% stretch, 60 cycles/min, 0 to 24 hours). Strain significantly increased EP24.15 and EP24.16 soluble activity in a force- and time-dependent manner, with elevations of 2.3±0.4- and 1.9±0.3-fold for EP24.15 and EP24.16, respectively, after 24 hours at 10% strain. Pharmacological agents and dominant-negative G protein mutants used to selectively disrupt Gi- and Gβγ-mediated signaling pathways attenuated strain-dependent (24 hours, 5%) increases for both enzymes. Differences in the inhibitory profile for both enzymes were also noted, with EP24.15 displaying greater sensitivity to Gi(2/3) inhibition and EP24.16 exhibiting greater sensitivity to Gi(1/2) and Gβγ inhibition. Cyclic strain also increased levels of secreted EP24.15 and EP24.16 activity by 2.6±0.02- and 3.6±0.2-fold, respectively, in addition to mRNA levels for both enzymes (EP24.15 ×42%, EP24.16 ×56%).


Key Words: metallopeptidase ■ endothelial function ■ cyclic strain ■ G protein

Mechanical or hemodynamic forces associated with blood flow play an important role in the physiological control of vascular tone, remodeling, and associated pathologies. These include cyclic circumferential strain, which is caused by a transmural force acting perpendicularly to the vessel wall, and fluid shear stress, the frictional force generated as blood drags against cells. Central to the maintenance of vascular homeostasis is the endothelial cell (EC) monolayer, which constitutes a dynamic interface between the vessel wall and bloodstream, where it regulates the physiological effects of hemodynamic forces on vessel wall tone and remodeling events. These forces can modulate EC metabolism by inducing qualitative and quantitative changes in EC gene expression/posttranslational modifications, with downstream effects on vascular cell–fate decisions (eg, migration and proliferation).

Vasoactive peptide hormones and their associated degradative enzymes, primarily extracellularly acting metallopeptidases, also play a crucial role in EC-mediated vasoregulation. Of particular importance are the “thermolysin-like” family of zinc metalloendopeptidases, classified by Barrett et al as belonging to the Clan MA, which hydrolyze peptide bonds in substrates of fewer than 40 amino acids. Several members of this family are known to participate in the metabolism of EC-derived vasoactive peptides, and these include neutral endopeptidase (NEP; EC3.4.24.11), angiotensin-converting enzyme (ACE; EC3.4.15.1), endothelin-converting enzyme (ECE; EC3.4.24.71), endopeptidase EC3.4.24.15 (EP24.15, thimet oligopeptidase), and endopeptidase EC3.4.24.16 (EP24.16, neurolysin). The present study will focus on the related endopeptidases EP24.15 and EP24.16 (for a detailed review, see Shrimpton et al). Both are 75- to 77-kDa metallopeptidases that hydrolyze short peptides and share many of the same cleavage sites on bioactive and synthetic peptides, although they sometimes target different sites on the same peptide. Although they are
primarily cytosolic in nature, both endopeptidases have been widely reported to exhibit secreted and membrane-associated forms in various cell types, including ECs, thereby allowing them to putatively degrade peptides extracellularly. \textsuperscript{7,10}

Direct roles for EP24.15 and EP24.16 within the vasculature are suggested by several studies. Both endopeptidases have been reported to cleave vasoactive peptides such as angiotensin-I (Ang-I), neurotensin, and bradykinin.\textsuperscript{6} Site-directed inhibitors of both enzymes have been shown to potentiate bradykinin-induced vasodilation\textsuperscript{11} and to decrease myocardial ischemia/reperfusion injury.\textsuperscript{12} More recent studies by Norman et al\textsuperscript{13,14} and Rioli et al\textsuperscript{15} strongly suggest roles for both endopeptidases in metabolism of vasoactivatory peptides in peripheral and cerebral vascular beds. Furthermore, EP24.15 has been shown to hydrolyze Ang-I in vascular smooth muscle cell cultures at the Pro\textsuperscript{2}-Phe\textsuperscript{3} position to generate the vasodilator peptide Ang\textsuperscript{9-11}.

Our central hypothesis is that the expression and function of peptide hormone-degrading metallopeptidases, such as EP24.15 and EP24.16, within vascular ECs are regulated by hemodynamic forces. In support of this hypothesis, previous studies have clearly demonstrated a role for hemodynamic forces in modulating the mRNA expression and enzymatic activity of other members of the thermolysin-like zinc metalloendopeptidase group, namely, ACE and ECE-I, within vascular ECs,\textsuperscript{18,19} whereas various members of this group (eg, EP24.15, ACE, NEP, and ECE-I) exhibit a shear stress–response element (\textsuperscript{−}GAGACC\textsuperscript{−}) within their promoter regions,\textsuperscript{18,20−22} a motif frequently observed in the promoters of mechanistically sensitive genes.\textsuperscript{23}

The present report investigates the influence of cyclic strain on the mRNA expression and enzymatic function of EP24.15 and EP24.16 within vascular ECs. The intracellular transduction of hemodynamic forces in vascular ECs, manifested through changes in gene expression and protein function, remains poorly understood. Several protein candidates have been implicated in this process and include pertussis toxin (PTX)-sensitive (Gi) and -insensitive (Gq) heterotrimeric G proteins, G protein–coupled receptors, ion channels, and integrins.\textsuperscript{2,24−28} Inhibitory guanine nucleotide regulatory G protein (Gi) signaling pathways are of particular interest. Activation of Gi protein subunits and increased GTPase activity occur immediately (within seconds) after mechanical loading, which suggests that these ubiquitous regulatory proteins are strategically located within vascular ECs to facilitate transduction of mechanical stimuli. We subsequently decided to investigate the putative role of Gi\textsubscript{a} and G\textsubscript{b}\textsubscript{y} subunits in mediating the strain-dependent effects observed for EP24.15 and EP24.16 in ECs.

**Methods**
The Methods section is available online at http://atvb.ahajournals.org.

**Results**

**Cyclic Strain–Dependent Increase in EP24.15 and EP24.16 Activity in BAECs**

After exposure of BAECs to varying levels of cyclic strain (0% to 10%) for 24 hours, EP24.15 and EP24.16 activities were both elevated in cells, with increases of up to 2.3±0.4-fold for EP24.15 and 1.9±0.4-fold for EP24.16 at 10% strain (Figure 1a). Force-dependent elevation in endopeptidase activity levels (at 5% strain) proceeded in a time-dependent manner, with increases for both endopeptidases observed within just 3 hours of strain (Figure 1b) and continuing over 24 hours. In parallel with these findings, we observed a 1.9-fold upregulation of cellular endothelial nitric oxide synthase (eNOS) protein expression levels after 5% strain of BAECs for 24 hours (Figure 1c). For all subsequent studies, the cyclic strain regimen consisted of either 0% or 5% strain for 24 hours.

**Attenuation of Cyclic Strain–Dependent Increase in EP24.15 and EP24.16 Activity in BAECs by Pharmacological Inhibitors of Gi Protein Subunits**

BAECs were exposed to cyclic strain (either 0% or 5%) for 24 hours in the absence or presence of PTX and NF023. After PTX treatment, the cyclic strain–dependent increase in endopeptidase activities was completely attenuated (Figure 2a). Furthermore, the suramin analog NF023 attenuated strain-dependent elevation of endopeptidase activities by 84.2±23.1% and 87.2±15.4% for EP24.15 and EP24.16, respectively (Figure 2b).

**Attenuation of Cyclic Strain–Dependent Increase in EP24.15 and EP24.16 Activity in BAECs by Molecular Inhibitors of Gi Subunit Function**

After transfection of BAECs with plasmid constructs encoding wild-type and dominant-negative mutant Gi\textsubscript{a1,3} subunits, cells were exposed to cyclic strain (either 0% or 5%) for 24 hours. A consistently high (~40% to 50%) transfection efficiency was achieved throughout these studies, and overexpression of transfected Gi protein subunits (both wild type and mutant) was subsequently confirmed by Western immunoblotting (data not shown). Cyclic strain–dependent elevation of cellular EP24.15 activity in BAECs was found to be attenuated by Gi\textsubscript{a2}-G203T (81.9±36.3%) and Gi\textsubscript{a2}-G202T (102.9±21.8%), whereas Gi\textsubscript{a1}-G202T had no significant effect (P=0.46). Interestingly, the force-dependent increase in cellular EP24.16 activity was strongly attenuated by Gi\textsubscript{a1}-G202T (69.6±18.6%) and Gi\textsubscript{a2}-G203T (101.7±26.7%), whereas Gi\textsubscript{a2}-G202T had no significant effect (P=0.29; Figure 3). In contrast to the dominant-negative mutants, overexpression of the Gi\textsubscript{a1,3} wild-type subunits had no negative effect on cyclic strain–dependent increases in the activity of either endopeptidase in BAECs, and in some instances actually resulted in further, albeit slight, increases in activity (data not shown).

**Attenuation of Cyclic Strain–Dependent Increase in EP24.15 and EP24.16 Activity in BAECs by Molecular Inhibitors of G\textsubscript{b}\textsubscript{y} Dimer Function**

After transfection of BAECs with \beta ARK.c t, cells were exposed to cyclic strain (either 0% or 5%) for 24 hours.
Overexpression of transfected BARK.ct was subsequently confirmed by reduction of melittin-induced[^3H] arachidonic acid release from BAECs, a Gβγ-mediated event[^29] (data not shown). Cyclic strain–dependent elevation of EP24.15 activity in BAECs was only slightly attenuated in cells (12.7±7.3%) by BARK.ct. In contrast, strain-dependent increases in EP24.16 activity were substantially attenuated in BAECs (86.4±2.6%) by BARK.ct (Figure 4).

Cyclic Strain–Dependent Increase in EP24.15 and EP24.16 Secretion From BAECs

After exposure of BAECs to cyclic strain (either 0% or 5%) for up to 24 hours, a time-dependent increase in both endopeptidase activities was observed in BAEC-conditioned media (Figure 5a and 5b). These increases were in excess of constitutive endopeptidase release (0% strain). Significant differences between constitutive and force-dependent endopeptidase release into media were apparent for both enzymes after just 3 hours of strain and had risen to 2.6- and 3.6-fold elevations (for EP24.15 and EP24.16, respectively) after 24 hours. Media levels of lactate dehydrogenase activity, a cytosolic marker used as an index of membrane rupture, remained negligible for up to 24 hours of strain at 5% (data not shown).

Force-Dependent Increase in EP24.15 and EP24.16 mRNA Expression in BAECs

After exposure of BAECs to cyclic strain (either 0% or 5%) for 24 hours, a strain-dependent increase in mRNA expression for both enzymes was observed, with elevations of 42% and 56% for EP24.15 and EP24.16, respectively (Figure 6). Expression of cyclooxygenase-II mRNA was also elevated by 92% in response to strain.

Discussion

Peptidase-mediated metabolism of bioactive peptide hormones plays a vital role in EC-mediated vascular events. Because shear stress and cyclic strain likely represent the principle regulatory stimuli influencing vascular homeostasis and associated pathologies, we postulated that metalloendopeptidase expression and activity in vascular ECs may be regulated by these hemodynamic variables. To further investigate this hypothesis, we examined the relationship between cyclic strain and the enzymatic activity and mRNA expression of both EP24.15 and EP24.16, homologous members of the thermolysin-like zinc metalloendopeptidase family, within vascular ECs. A Flexercell Tension Plus FX-4000T culture system was used, in conjunction with a Bioflex Loading Station, to mechanically strain or deform BAECs cultured on a flexible, matrix-bonded growth surface.[^30] Although this system has been very widely used for studying vascular cells under conditions of defined cyclic strain, a previous review by Brown et al[^31] has highlighted the possibility that fluid motion resulting from cyclical tension of the cell adhesion membrane may possibly result in unintentional shear loading of cells. Although the shear stresses involved are quite likely negligible relative to the applied strain, this
factor should be kept in mind when one interprets results with
the Flexercell strain system, and indeed other systems.

Both endopeptidase activities were routinely detected in
the soluble fraction of BAECs cultured under static (un-
strained) conditions. Basal levels were determined to be
4.4 ± 0.6 and 6.6 ± 0.6 nmol of QFS hydrolyzed per hour per

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upregulation of soluble EP24.15 and EP24.16 activity in
BAECs, with inductions of 1.9- and 2.3-fold, respectively, at
10% strain. In concurrence with previous reports, eNOS
protein expression was also monitored and found to increase
significantly (1.9-fold) in response to chronic cyclic strain. Initial experiments clearly demonstrated a strain-dependent
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ties was also found to be time dependent, with induction of activity observed after 3 hours of strain and continuing over 24 hours. These observations lead us to conclude that cyclic strain can potentially regulate the levels of EP24.15 and EP24.16 activity within BAECs.

The putative involvement of G protein signaling pathways in mediating these events was also considered in this study. Previous investigations have clearly implicated a role for Gi proteins in the transduction of mechanical stimuli in vascular ECs.\textsuperscript{2,24,28,29} We therefore decided to investigate the putative role of Gi\textsubscript{a} and Gβγ subunits in mediating the strain-dependent increase in EP24.15 and EP24.16 activities observed in BAECs.

Pharmacological inhibitors of Gi protein signaling were initially used to investigate this role. PTX, which catalyzes the ADP ribosylation of the α-subunit in the intact heterotrimeric G protein signaling complex, was used to inhibit Gi\textsubscript{a} activity. PTX treatment was followed by transient transfection of BAECs with βARK.ct, which is a dominant negative mutant of Gi\textsubscript{a}. Cells were allowed to recover overnight before application of cyclic strain (0% or 5%) for 24 hours. Results are expressed as fold change in specific activity relative to unstrained control (mock-transfected cells at 0%). Statistical differences (Student’s t test) were established at ***P≤0.001 vs unstrained control and at δP≤0.05 and δδP≤0.01 vs strained control (mock-transfected cells at 5%). Data are the result of 3 sets of experiments.

**Figure 4.** Attenuation of the cyclic strain–dependent increase in EP24.15 and EP24.16 activity by molecular inhibition of Gβγ-protein function. After transient transfection of BAECs with βARK.ct, cells were allowed to recover overnight before application of cyclic strain (0% or 5%) for 24 hours. Results are expressed as fold change in specific activity relative to unstrained control (mock-transfected cells at 0%). Statistical differences (Student’s t test) were established at ***P≤0.001 vs unstrained control and at δP≤0.05 and δδP≤0.01 vs strained control (mock-transfected cells at 5%). Data are the result of 3 sets of experiments.

**Figure 5.** Cyclic strain–dependent increase in EP24.15 and EP24.16 secretion from BAECs. Elevation of (a) EP24.15 and (b) EP24.16 enzymatic activities in BAEC-conditioned media after exposure to cyclic strain (0% or 5%) for varying times (0 to 24 hours). Results are expressed as picomoles of substrate hydrolyzed per minute per milliliter per microgram of protein. Statistical differences (ANOVA) were established at *P≤0.05 and **P≤0.01 vs unstrained controls. Data are the result of 3 sets of experiments.
mer, thereby uncoupling the G protein from its receptor, and NF023, a suramin analogue that blocks GDP dissociation from the α-subunit, have been used extensively by others for this purpose.\(^{33-36}\) Both agents were demonstrated to strongly attenuate strain-dependent upregulation of activity for both endopeptidases, which suggests a role for Gi protein signaling in these events. The use of pharmacological tools to delineate G protein–mediated signaling events must be interpreted with caution, however, because potential effects on Gi protein–independent pathways cannot be ruled out.

Subsequent studies therefore focused on selective ablation of individual Gi protein subunits at the molecular level. Plasmids encoding dominant-negative mutants of Gi\(_{\alpha1}\) subunits (ie, Gi\(_{\alpha1}\)-G202T, Gi\(_{\alpha1}\)-G203T, and Gi\(_{\alpha1}\)-G202T) were subsequently used to selectively ablate the function of their endogenous wild-type counterparts in BAECs.\(^{37}\) βARK.ct was also used to selectively inhibit Gβγ signaling pathways.\(^{38}\) Strain-dependent upregulation of both endopeptidase activities was found to be strongly attenuated (80% to 100%) after overexpression of Gi\(_{\alpha1}\)-G203T. By contrast, only strain-dependent elevation of EP24.15 activity was inhibited by Gi\(_{\alpha1}\)-G202T (100%), whereas EP24.16 activity was significantly inhibited by Gi\(_{\alpha1}\)-G202T (69.6%). A putative role for Gβγ signaling is also implicated in these mechanotransduction events.\(^{39}\) βARK.ct, while only marginally blocking the strain-dependent increase in EP24.15 activity in BAECs (12.7%), substantially blocked the increase in EP24.16 activity (86.4%). These findings confirm our previous observations with PTX and NF023 and reinforce the involvement of multiple Gi protein subunits in transduction of these strain-induced responses. Furthermore, the differential involvement of Gi protein subunits in the strain-dependent changes for either endopeptidase suggests distinct signal transduction pathways. The reasons for this divergence are presently unclear; however, significant differences exist between EP24.15 and EP24.16, despite their structural and functional similarities, which may underlie this divergence. In addition to differences in their substrate specificities, both enzymes are separate gene products (~60% homology) under the control of distinct promoters, with additional evidence to indicate that EP24.16 expression is subject to alternative promoter usage, thereby enabling targeting of some EP24.16 to the mitochondrial compartment.\(^{40}\) Mechanoregulation in vascular ECs by divergent signaling pathways has also been reported by other workers. In this regard, the ability of hemodynamic forces to differentially regulate signaling molecules such as mitogen-activated protein kinases in ECs by PTX-sensitive and -insensitive pathways,\(^{41}\) in addition to modulating the expression and function of G proteins themselves,\(^{42}\) is also highly relevant. Also significant is the ability of hemodynamic forces to regulate expression of vasoactive enzymes such as eNOS by divergent signaling pathways.\(^{43}\) Further investigations will subsequently focus on the role of Gi protein subunits in the regulation of EP24.15 and EP24.16 expression at the transcriptional level by cyclic strain in vascular ECs.

Although predominantly cytosolic, secreted forms of EP24.15 and EP24.16 have also been widely reported for various cell types, including ECs, which subsequently enables them to function extracellularly.\(^{7-10,13}\) Several studies have detailed the effects of cyclic strain on secretory processes in vascular cells. Given the regulatory influence of cyclic strain on soluble levels of EP24.15 and EP24.16 activity described here, it was decided to examine its potential influence on endopeptidase release from BAECs. Exposure of BAECs to 5% cyclic strain led to a time-dependent increase in EP24.15 and EP24.16 mRNA expression in BAECs. Cyclic strain (0% or 5%) was applied to BAECs for 24 hours. After mRNA extraction and reverse transcription–polymerase chain reaction (as described in Methods), all amplified products were analyzed by agarose gel electrophoresis on 2.5% gels and visualized by ethidium bromide staining. Representative gels (inverted) shown are as follows: lanes 1 and 2, 0% strain; lanes 3 and 4, 5% strain; lanes 5 and 6, 0% strain; and lanes 7 and 8, 5% strain. Lanes 1 to 4 for each of the 3 gels are GAPDH. Lanes 5 to 8 are EP24.15 (upper gel), EP24.16 (middle gel), and cyclooxygenase-II (lower gel). Densitometric analysis of individual gels is summarized in the graph beneath the gels. GAPDH was used for normalization of gel loading. Statistical differences (Student’s t test) were established at *\(P<0.05\) vs unstrained control. Data are the result of 3 sets of experiments.

**Figure 6.** Cyclic strain–dependent increase in EP24.15 and EP24.16 mRNA expression in BAECs. Cyclic strain (0% or 5%) was applied to BAECs for 24 hours. After mRNA extraction and reverse transcription–polymerase chain reaction (as described in Methods), all amplified products were analyzed by agarose gel electrophoresis on 2.5% gels and visualized by ethidium bromide staining. Representative gels (inverted) shown are as follows: lanes 1 and 2, 0% strain; lanes 3 and 4, 5% strain; lanes 5 and 6, 0% strain; and lanes 7 and 8, 5% strain. Lanes 1 to 4 for each of the 3 gels are GAPDH. Lanes 5 to 8 are EP24.15 (upper gel), EP24.16 (middle gel), and cyclooxygenase-II (lower gel). Densitometric analysis of individual gels is summarized in the graph beneath the gels. GAPDH was used for normalization of gel loading. Statistical differences (Student's t test) were established at *\(P<0.05\) vs unstrained control. Data are the result of 3 sets of experiments.
in EP24.15 and EP24.16 activity in BAEC-conditioned media, increases that were significantly in excess of constitutive endopeptidase release. Differences between constitutive and force-dependent endopeptidase release were apparent for both enzymes after just 3 hours of strain, with fold differences of 2.6 and 3.6 observed for EP24.15 and EP24.16, respectively, after 24 hours. On the basis of these observations, we can conclude that cyclic strain potentially modulates release of EP24.15 and EP24.16 from BAECs and thus may have a significant influence on their extracellular function(s). Future work with this model will extend these investigations to include a more detailed examination of the effects of mechanical strain on the EP24.15/16-specific hydrolysis of extracellular peptide substrates (e.g., bradykinin and Ang-I) with a view to elucidating the functional consequences of the hemodynamic regulation of these important metallopeptidases in vascular ECs.

To determine whether the strain-dependent effects observed thus far were posttranscriptional in nature, we decided to investigate the effect of cyclic strain on gene expression for both endopeptidases. Chronic strain led to an upregulation of steady-state mRNA expression for both EP24.15 and EP24.16 in BAECs, in parallel with a strain-dependent upregulation in mRNA expression for cyclooxygenase-II, a well-characterized strain-inducible component of ECs. This suggests that transcriptional events may contribute to the strain-dependent elevation in EP24.15 and EP24.16 activity in BAECs, although posttranscriptional processes cannot be ruled out.

In conclusion, therefore, we describe for the first time the effects of cyclic strain on the activity and expression of EP24.15 and EP24.16, 2 closely related members of the thermolysin-like zinc metalloendopeptidase family, in vascular ECs. Our findings suggest that both peptidases are subject to mechanoregulatory control in BAECs, possibly via distinct signal transduction pathways. These results complement previous studies that report on the hemodynamic regulation of other members of this enzyme family. In this regard, shear stress has been shown to reduce both mRNA expression and the enzymatic activity of ACE in bovine pulmonary artery ECs, as well as mRNA expression of both ECE-1 and ET-1 in BAECs and human umbilical vein ECs, events that were both force and time dependent. These shear-dependent decreases likely lead to a significant reduction in levels of vasoconstrictive peptide products such as Ang-II and ET-1 (coupled with an increase in levels of the vasodilator peptide, bradykinin) and subsequently an attenuation in vascular tone and inhibition of smooth muscle cell proliferation, in concordance with the central effects of laminar shear stress on the vessel wall. A more recent study by Masatsugu and coworkers has also demonstrated that the shear-dependent reduction in ECE-1 and ET-1 expression is putatively mediated by oxidative stress. These findings are in contrast to the increases in activity and expression seen here for EP24.15 and EP24.16, albeit in response to a different hemodynamic stimulus, cyclic strain. It is tempting to speculate that the physiological consequence of all of these observations is a collective regulation of the expression and function of the entire thermolysin-like zinc metalloendopeptidase family of enzymes by hemodynamic loading (ie, both shear stress and circumferential cyclic strain), with consequences for the net balance of pressor versus depressor peptides within the blood vessel. This may subsequently lead to downstream effects on EC proliferation and endothelium-mediated remodeling events (possibly via alterations in EC-derived nitric oxide/prostacyclin production and release, among other mechanisms). One can expect a clearer picture to emerge as more data become available.

To the best of our knowledge, this is the first instance in which a member of this enzyme family has been shown to be regulated by cyclic strain, and indeed, the first attempt to delineate the signaling components associated with their mechanoregulation. We believe these studies will have an important impact on our overall understanding of how metallopeptidases participate in the hemodynamic control of vessel wall remodeling and the initiation and progression of vascular diseases.

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