Coordinated Endothelial Nitric Oxide Synthase Activation by Translocation and Phosphorylation Determines Flow-Induced Nitric Oxide Production in Resistance Vessels

Xavier F. Figueroa^a Daniel R. González^b Mariela Puebla^a Juan P. Acevedo^a
Daniel Rojas-Libano^a Walter N. Durán^c Mauricio P. Boric^a

^aDepartamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, and ^bDepartamento de Ciencias Básicas Biomédicas, Facultad de Ciencias de la Salud, Universidad de Talca, Talca, Chile; ^cDepartment of Pharmacology and Physiology, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, N.J., USA

Key Words

Endothelial cells · Resistance vessels · Ca^{2+} · Shear stress · Endothelial nitric oxide synthase subcellular location

Abstract

Background/Aims: Endothelial nitric oxide synthase (eNOS) is associated with caveolin-1 (Cav-1) in plasma membrane. We tested the hypothesis that eNOS activation by shear stress in resistance vessels depends on synchronized phosphorylation, dissociation from Cav-1 and translocation of the membrane-bound enzyme to Golgi and cytosol. **Methods:** In isolated, perfused rat arterial mesenteric beds, we evaluated the effect of changes in flow rate (2–10 ml/min) on nitric oxide (NO) production, eNOS phosphorylation at serine 1177, eNOS subcellular distribution and co-immunoprecipitation with Cav-1, in the presence or absence of extracellular Ca^{2+} . **Results:** Increases in flow induced a biphasic rise in NO production: a rapid transient phase (3–5-min) that peaked during the first 15 s, followed by a sustained phase, which lasted until the end of stimulation. Concomitantly, flow caused a rapid translocation of eNOS from the microsomal compartment to the cytosol and Golgi, paralleled by an increase in eNOS phosphorylation and a reduction in eNOS-Cav-1 asso-

ciation. Transient NO production, eNOS translocation and dissociation from Cav-1 depended on extracellular Ca^{2+} , while sustained NO production was abolished by the PI3K-Akt blocker wortmannin. **Conclusions:** In intact resistance vessels, changes in flow induce NO production by transient Ca^{2+} -dependent eNOS translocation from membrane to intracellular compartments and sustained Ca^{2+} -independent PI3K-Akt-mediated phosphorylation. © 2013 S. Karger AG, Basel

Introduction

Nitric oxide (NO) is a ubiquitous signaling molecule that has been recognized as the major endothelium-derived vasodilator [1]. In blood vessels, NO is synthesized in endothelial cells by endothelial NO synthase (eNOS) [2], which is targeted to the plasma membrane by co- and post-translational acylation with myristate and palmitate, respectively. While myristoylation is required for membrane association, palmitoylation determines and stabilizes subcellular targeting of eNOS to caveolae [3–6]. In caveolae, eNOS is associated to caveolin-1 (Cav-1), an integral membrane protein of this microdomain [7, 8]. The

interaction of eNOS with Ca^{2+} -calmodulin during an increase in intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$ releases eNOS from the inhibitory influence of Cav-1 and leads to NO production [9, 10]. Additionally, an increase in Ca^{2+} induces eNOS translocation from the cell membrane to the cytosol [11, 12], or to intracellular perinuclear sites, most likely the Golgi complex [13]. Although in those experiments eNOS translocation was not analyzed in parallel with NO production, translocation was assumed to correspond to an inactivation mechanism of eNOS, because this process was slower than the expected kinetics of NO production [11–13]. However, in the hamster microcirculation in vivo, translocation of eNOS observed in response to acetylcholine (ACh) was concomitant with NO production and vasodilatation [14]. The mechanisms that regulate eNOS translocation and NO function require further studies to elucidate the requirement of Ca^{2+} and the subcellular target dependence on stimulus and type of blood vessel [15–17].

In addition to the subcellular location and protein-protein interactions, several phosphorylation and dephosphorylation sites modulate eNOS activity [18]. In particular, Akt (protein kinase B)-mediated eNOS phosphorylation at Ser-1177 increases the activity of the enzyme and reduces its Ca^{2+} dependency [19, 20].

Fluid shear stress, the dragging force generated by blood flow, is considered the major physiological stimulus for tonic eNOS activation [21–23] and, thereby, for NO-dependent control of peripheral vascular resistance [24–27]. The mechanisms linking shear stress with NO release are incompletely understood. Although it is recognized that eNOS phosphorylation at Ser-1177 by the PI3K-Akt pathway plays a central role in the response to shear stress [19, 28, 29], the contribution of eNOS translocation and the requirement of Ca^{2+} in this physiological regulation are controversial. In this context, results in cultured endothelial cells differ from those reported in isolated arterioles. In cultured endothelial cells, an increase in shear stress induced a biphasic rise in NO release initiated by Ca^{2+} -mediated eNOS activation [30], while, in isolated arterioles, shear stress-induced NO-dependent dilation was not associated with a rise in intracellular Ca^{2+} concentration [31, 32]. Even though shear stress apparently induces translocation of eNOS in cells in culture [33], the role of Ca^{2+} , as well as the interactions between translocation and phosphorylation of eNOS in response to shear stress in arterioles, remains to be determined.

In this study, we tested the hypothesis that increases in shear stress in intact vessels cause eNOS phosphorylation and subcellular translocation from caveolae at the plasma

membrane to intracellular compartments and lead to flow-induced NO release. We evaluated the changes in eNOS subcellular distribution and phosphorylation at Ser-1177 induced by an increase in the perfusion flow rate in the arterial mesenteric bed to analyze in an integrative manner the relevance of eNOS translocation and/or phosphorylation as a function of time. In addition, we analyzed the requirement of extracellular Ca^{2+} in the response to flow changes.

Methods

Animal and Drug Sources

Male Sprague-Dawley rats (290–310 g) were obtained from the Institutional Animal Facilities of the P. Universidad Católica de Chile (PUC). All studies were conducted in compliance with the Committee for Bioethics and Biosecurity of the Facultad de Ciencias Biológicas, PUC, following the Guiding Principles in the Care and Use of Laboratory Animals endorsed by the American Physiological Society.

Unless specified otherwise, all biochemical reagents and inhibitors were purchased from Sigma Chemical Co. (St Louis, Mo., USA) and chemicals of analytical grade were from Merck (Darmstadt, Germany). Monoclonal anti-phospho-Ser1177-eNOS, anti-eNOS and polyclonal anti-caveolin antibodies were purchased from BD-Transduction Labs (Lexington, K.Y., USA). Horseradish peroxidase-linked goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Pierce (Rockford, Ill., USA).

Perfusion of Rat Arterial Mesenteric Bed

Rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.). The mesenteric artery was exposed through a mid-line incision and cannulated. The mesenteric vascular bed was severed from the intestinal wall, perfused with Tyrode solution at 37°C and equilibrated with 95% O_2 and 5% CO_2 to yield a pH ~ 7.4 [34]. We refer to this preparation as the arterial mesenteric bed because only this side of the circulation was perfused and, therefore, the measurements of NO and variations in eNOS location and phosphorylation mainly reflect the response of resistance vessels to changes in flow. Under deep anesthesia, rats were killed by bleeding and pneumothorax. The baseline mesenteric perfusion flow rate was set at 2 ml/min with a peristaltic pump (Gilson Minipuls-3). Perfusion pressure was continuously recorded with a transducer (P23X Statham) connected to a Grass 7D polygraph. In all cases, a 30-min equilibration period was allowed before starting the experimental protocol.

Experimental Protocols

Flow Change

Perfusate output was collected every minute in test tubes for determination of NO. A 20-min experiment was performed in the following sequence: 5 min at 2 ml/min (baseline), 10 min at 1, 5 or 10 ml/min (stimulus), and 5 min at 2 ml/min (recovery). One group was perfused at 2 ml/min for the duration of the experiment as a time control. The flow rate was changed by rapid acceleration of the pump (~ 0.8 ml/min/s); perfusate was not collected during flow-change intervals.

To confirm NOS as the source of NO, some mesenteric beds were stimulated with flow changes in the presence of 30 μM N^{ω} -nitro-L-arginine (L-NNA). Application of L-NNA was initiated 45 min before starting the experimental protocol, and maintained throughout the experiment by continuous perfusion.

Effect of Ca^{2+} Removal

To test the effect of extracellular Ca^{2+} , mesenteries were equilibrated for 30 min with normal Tyrode and basal NO release was measured for 3 min. Thereafter, the solution was changed to a modified Tyrode solution, without Ca^{2+} and containing 1 mM EGTA (Ca^{2+} -free buffer). The mesentery was submitted to the 20-min flow-change protocol (5 ml/min stimulus). The role of intracellular Ca^{2+} was assessed in separate mesenteries perfused throughout the equilibration (30 min) and experimental periods with Ca^{2+} -free buffer supplemented with 10 mM of caffeine to deplete intracellular Ca^{2+} stores. In addition, to study the early component of flow-induced NO release in separate experiments, mesenteries were equilibrated and perfused with normal or Ca^{2+} -free buffer, and the perfusate output was collected every 15 s during the first minute after raising the flow from 2 to 10 ml/min. To test the role of the PI3K-Akt pathway in eNOS phosphorylation, additional mesenteries were perfused with 1 μM wortmannin for 15 min, and then stimulated with the 5 ml/min flow-change protocol.

eNOS Translocation and Phosphorylation

To ascertain changes in the subcellular location and phosphorylation of eNOS, we homogenized mesenteries at different times of the stimulation protocol. The sampling times were based on the measurements of NO release in response to flow changes to evaluate the basal (min-5), transient (min-6), sustained (min-12) and recovery (min-17) responses. These studies were performed with the flow stimulus of 10 ml/min. The association of eNOS with Cav-1 was determined in basal conditions (min-5) and after 1 min of stimulation (min-6). All biochemical analyses were performed in whole homogenized mesentery, which includes venular vessels.

eNOS Subcellular Location

To directly assess the eNOS distribution in endothelial cells of intact resistance vessels, some mesenteries were processed for immunofluorescence analysis by confocal microscopy. Mesenteries were prepared, as described above, and then the vasculature was fixed by perfusing 2% paraformaldehyde for 5 min in control conditions (2 ml/min) or 1 min after increasing the flow rate from 2 to 10 ml/min. Mesenteries were kept in 2% paraformaldehyde for 48 h and a resistance artery of 120–180 μm was isolated to perform the immunofluorescence procedure.

Measurements

Determination of NO

The perfusate content of NO plus nitrite was quantified by chemiluminescence using an NO analyzer (Sievers 280, Boulder, Colo., USA) [35–37]. The equipment sensitivity detection threshold was ~ 10 pmol/ml (0.5 pmol in 50- μl sample volume). The net NO/nitrite mesenteric output was expressed in pmol/min.

Subcellular Fractionation

The external, nonglandular portions of the mesentery were rapidly excised (~ 15 s) and homogenized with an Ultraturrax in

1 ml of cold antiprotease-lysis buffer. The crude extract was centrifuged for 5 min at 150 g to obtain a clear total homogenate, which was then centrifuged for 30 min at 10,000 g and for 90 min at 100,000 g to obtain Golgi, microsomal and cytosolic fractions, as described previously [14].

Immunoprecipitation

The degree of association of eNOS with Cav-1 was assessed by immunoprecipitation using slight modifications of previously reported methods [37]. Total homogenate (1 mg protein) was pre-cleared with protein-A bearing *Staphylococcus aureus* (Pansorbin, 450 μl 10%-wt/vol) and incubated for 1 h with 10 μg of anti-caveolin polyclonal antibody. Bound protein was precipitated with 450 μl of Pansorbin, resuspended in Laemmli's buffer and submitted to SDS-PAGE and Western blot for eNOS and Cav-1.

Western Blotting

Protein samples of each fraction (30–100 μg , Bradford method) were separated by 7.5% SDS-PAGE and blotted onto a PVDF or nitrocellulose membrane (Bio-Rad, Hercules, Calif., USA). The membrane was incubated with a primary antibody overnight at 4°C and then with an appropriate secondary antibody (2 h at room temperature). The primary antibodies were used at the following dilutions: anti-eNOS 1:2000; anti-Cav-1 1:2500; anti-P-eNOS 1:1000. The same membrane was analyzed first for phosphorylated eNOS and then for total eNOS protein, after stripping. The protein bands were visualized with standard methods, using Western LightingTM Chemiluminescence reagent (Perkin Elmer, Boston, Mass., USA), SuperSignal[®] West Femto (Pierce, Rockford, Ill., USA) or with 0.01% 3,3'-diaminobenzidine, 0.5% H_2O_2 in the dark according to the strength of the signal. Western blots were scanned and evaluated by densitometric analysis using NIH-image software. A pooled sample of 8 naïve mesenteries was used as internal standard to standardize the signal strength among different blots when comparing the ratio of P-eNOS/total eNOS.

Immunocytochemistry

The expression of eNOS was analyzed by immunohistochemistry in cross sections and by immunofluorescence in whole mount intact vessels. For immunohistochemistry, mesenteries were perfused with Bouin's fixative for 5 min at 2 ml/min and postfixed overnight. Then, tissues were dehydrated, embedded in paraffin, sectioned at 10 μm , placed in silanized slides and deparaffinized using standard procedures. Tissue sections were blocked with 3% BSA in TBS (pH 7.4) for 1 h at room temperature and prepared as indicated by the Mouse/Rabbit ImmunoDetector System (Bio SB, Santa Barbara, Calif., USA) protocol. After blocking the endogenous peroxidase activity, sections were incubated overnight at 4°C with an anti-eNOS monoclonal primary antibody and the signal was developed using the biotin link secondary antibody (10 min), HRP label and DAB chromogen of the Mouse/Rabbit ImmunoDetector System. The sections were observed with an Olympus BX 41 microscope and a Jenoptik ProRes C5 camera. For immunofluorescence in intact vessels, isolated resistance arteries fixed with 2% paraformaldehyde were cut longitudinally and permeabilized with 0.01% Triton X-100 and blocked with 3% BSA. Triton X-100 and BSA were diluted in TBS (pH 7.4) and applied for 24 h at 4°C. The permeabilized and blocked arteries were incubated for 24 h at 4°C with anti-eNOS monoclonal primary antibody, and then with Alexa-568-labeled goat anti-mouse secondary antibody (Molecular Probes, Eugene,

Fig. 1. Flow-induced NO production. Isolated rat mesenteries were perfused at different flow rates and the content of NO in the perfusate was determined by chemiluminescence at 1-min intervals. **a** Time course of NO release in mesenteries submitted to a step change in perfusion flow. Initial flow was 2 ml/min for all groups and, after 5 min baseline collection, in 3 groups flow was suddenly changed to 1, 5 or 10 ml/min for 10 min and returned to 2 ml/min (horizontal bars). In a fourth group, flow was kept at 2 ml/min as a time control. Highly significant differences in NO

release as a function of time were observed in the 3 groups submitted to flow change ($p < 0.0001$, one-way ANOVA). Data are mean $\pm$ SEM. **b** The values at min-6 (peak transient response), or the average value obtained between min 12 and 15 (established sustained response) are plotted as a function of flow. Open symbols are control responses corresponding to the same tissues depicted in **a**; black symbols denote NO release in mesenteries perfused with 30 μ M L-NNA, an NOS inhibitor. Asterisks indicate significant differences versus control ($p < 0.05$, unpaired t test).

Oreg., USA) for 4 h at 4°C. The fluorescent signal was examined with an Olympus LSM Fluoview 1000 confocal microscope.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Paired or unpaired Student's t tests and one-way ANOVA plus Newman-Keuls post hoc test were used to assess the significance of variations over time within or between the groups, as appropriate. A value of $p < 0.05$ was considered statistically significant. Nonparametric Mann-Whitney or Kruskal-Wallis tests were used to compare the ratios of phospho-Ser1177-eNOS/eNOS densitometric intensities.

Results

Flow-Induced NO Production

At basal flow rate (2 ml/min), the perfusion pressure was 8.7 ± 0.7 mm Hg ($n = 16$). During stimulation, this perfusion pressure changed linearly with flow, averaging 5.8 ± 0.6 , 8.6 ± 1.3 , 15.1 ± 3.1 and 27.6 ± 2.0 mm Hg at 1, 2, 5 and 10 ml/min, respectively ($n = 4$ in each experimental group). In all instances, perfusion pressure returned to baseline immediately after the end of stimulation.

Baseline NO production was 282 ± 14 pmol/min (range 210–440 pmol/min, $n = 24$). In time-control experiments, NO release observed during perfusion at 2 ml/min showed a slow decay along the 20-min protocol (fig. 1a). Raising flow to 5 or 10 ml/min induced a proportional increment in NO production that exhibited two clearly distinct phases (fig. 1a). The flow rise produced an initial NO peak in the first minute, followed by a gradual decrease in the next 2–3 min to a stable level that remained above baseline until the end of the stimulus. We named these phases as transient and sustained, respectively. The latter phase was not a washout of the initial peak, because NO release remained above and parallel to the time-control group for several minutes and returned abruptly to control level at the end of the stimulation period (fig. 1a). Contrariwise, changing the flow to 1 ml/min caused a rapid reduction in NO production, which was sustained at about one-half of baseline and returned to control at the end of the experimental period (fig. 1a).

Figure 1b depicts the average data of transient peak and sustained NO production as a function of flow. In this and following figures, we used the values determined at

Fig. 2. Effect of Ca^{2+} removal on flow-induced NO production. Mesenteries perfused with different media were stimulated by a rapid flow change from 2 to 5 ml/min during 10 min, as in figure 1. **a** After 3 min of control sample collection, perfusate medium was changed for a Ca^{2+} -free medium (indicated by open bar), thereafter the mesentery was submitted to the flow-change protocol (as indicated). **b** Mesenteries were perfused for 30 min in Ca^{2+} -free buffer containing 10 mM of caffeine, and then submitted to the flow-change protocol. In both plots (**a**, **b**), the control response of mesenteries perfused with normal medium (data from fig. 1) is shown in dotted lines for comparison. **c** Net increment in NO re-

lease above baseline in response to flow increase from 2 to 5 ml/min at the peak (min-6) and sustained (average min 12–15) phases in mesenteries perfused in control medium (data from fig. 1), Ca^{2+} -free buffer (**a**) and Ca^{2+} -free plus caffeine buffer (**b**). * $p < 0.05$ versus control (one-way ANOVA, Newman-Keuls test); † $p < 0.05$ versus peak (paired t test). **d** Early transient phase of flow-induced NO production. Mesenteries perfused in control medium or in Ca^{2+} -free plus EGTA medium were submitted to a flow increase from 2 to 10 ml/min. NO production was measured every 15 s. Flow change took ~10 s.

min-6 to analyze the transient peak, and the average of values observed between min-12 and min-15 to evaluate the sustained phase. Using a logarithmic scale for the range of flow rates, figure 1b shows that NO production is a direct function of flow in the mesenteric vessels.

In mesenteries treated with 30 μM L-NNA, basal NO release was reduced (fig. 1b) and the increase in NO production induced by stimulation with 5 and 10 ml/min was dramatically blocked, confirming the measurement of NOS-derived NO. In addition, consistent with the ab-

Fig. 3. Flow-induced eNOS translocation and dissociation from Cav-1. Mesenteries were homogenized and processed for subcellular fractionation or immune-precipitation just before (min-5) or 1 min after (min-6) increasing perfusate flow from 2 to 10 ml/min, as in figure 1. **a** Western blots showing subcellular distribution of eNOS in the microsomal, cytosolic and Golgi-enriched fractions. Similar content of Cav-1 in the microsomal fraction, and eNOS in total homogenate, attest for equal protein load from endothelial origin. One representative of 3 similar experiments is shown. Protein load was 30 μ g per lane (microsomal and Golgi) or 70 μ g per lane (cytosolic and total homogenate). **b** Mesenteries were perfused either with control medium or with Ca²⁺-free medium and homogenized just before or 1 min after increasing perfusate flow

from 2 to 10 ml/min. One milligram of total mesenteric protein was immunoprecipitated with anti-Cav-1 antibody and analyzed by Western blot to identify eNOS and Cav-1 (immunoprecipitate). Total homogenate eNOS content was similar (100 μ g protein per lane). A representative Western blot is shown above, and the resultant average band intensity ratios for eNOS/Cav-1 in the immunoprecipitate, expressed in arbitrary units, are shown in the graphs below. * $p < 0.001$ versus basal, the number of experiments appears inside the columns. NO release (pmol/min) measured in the same mesenteries at the moment of homogenization was: in control, 126 ± 12 at 2 ml/min and 608 ± 42 at 10 ml/min; and in Ca²⁺-free conditions, 120 ± 21 at 2 ml/min and 477 ± 52 at 10 ml/min.

sence of myogenic tone in this preparation, inhibition of NOS did not affect flow-dependent changes in perfusion pressure (data not shown).

Requirement of Ca²⁺

Perfusion with a Ca²⁺-free medium supplemented with EGTA did not affect baseline NO production (fig. 2a). Increasing flow to 5 ml/min produced a rapid increment in NO that showed only the sustained phase while the transient component of the response was absent (fig. 2a). Depletion of intracellular Ca²⁺ stores by prolonged (30 min) perfusion with Ca²⁺-free buffer plus caffeine also produced loss of the initial transient NO peak (fig. 2b).

The magnitude of the NO release response induced by stimulation at 5 ml/min in mesenteries perfused with

control medium and two different Ca²⁺-free media was analyzed by comparing the peak and sustained phases (fig. 2c). This analysis confirmed that absence of Ca²⁺ abolished the transient component of NO release but did not impact the sustained phase.

In addition, in separate experiments, we analyzed the early part of the response by collecting samples every 15 s during the first minute of stimulation with 10 ml/min (fig. 2d). This enhanced time resolution revealed that, in control conditions, flow-induced NO release was rapid, peaking in the first 15 s and starting to decrease gradually thereafter. In the absence of extracellular Ca²⁺, the rise in NO was equally rapid, but NO levels immediately attained a steady level (fig. 2d).

Fig. 4. Immunocytochemical analysis of eNOS distribution in mesenteric vessels. Immunohistochemistry of mesentery's cross sections, showing eNOS expression in an arteriolar (a)-venular (v) pair (**a**) and in a resistance artery (**b**). Note that the eNOS immunolabeling signal was detected only at the endothelium. **c, d** Confocal immunofluorescence images of eNOS in the endothelium of intact resistance vessels. 150- to 180- μ m arterioles were fixed just before or 1 min after increasing perfusion flow from 2 to 10 ml/min and processed for confocal immunofluorescence. Endothelial cells found at the bottom of the vessel are shown. Under basal flow (2 ml/min), eNOS signal was uniformly distributed in endothelial cells, but also a Golgi-like perinuclear distribution (arrowheads) can be distinguished (**c**). Flow increase to 10 ml/min (**d**) caused a change in eNOS distribution to a patchy cytosolic pattern (arrows), with a clear increase in the perinuclear Golgi-like signal (arrowheads). Scale bars = 20 μ m.

eNOS Dissociation from Cav-1 and Translocation

Subcellular analysis of the mesenteric vessels revealed that under basal flow perfusion at 2 ml/min, eNOS was mainly located in the microsomal fraction (fig. 3a) of endothelial cells (fig. 4a–c), but was also found in the Golgi-enriched fraction and cytosol. Flow increase to 10 ml/min for 1 min caused eNOS translocation to the cytosolic and Golgi fractions (fig. 3a), which was confirmed by immunofluorescence analysis of intact mesenteric resistance vessels (fig. 4d).

Concomitantly with eNOS translocation, flow increase caused a rapid dissociation of eNOS from Cav-1 (fig. 3b). At basal flow, a clear eNOS band was detected in the Cav-1 immunoprecipitate, confirming the association of both proteins in intact vessels. After a 1-min stimulation with 10 ml/min, the amount of eNOS co-precipitated with Cav-1 was drastically reduced, in agreement with the decrease in microsomal eNOS content (fig. 3a). In contrast, co-immunoprecipitation analysis carried out in mesenteries perfused in the absence of Ca^{2+} indicated that flow-induced NO production in these conditions occurred without detectable dissociation of eNOS from

Cav-1 (fig. 3b). The reduced eNOS content in the Cav-1 immunoprecipitate observed at 10 ml/min in control conditions can only be ascribed to dissociation between the two proteins, because total eNOS content as well as the amount of immunoprecipitated Cav-1 was similar in mesenteries perfused at 2 or 10 ml/min, in control and Ca^{2+} -free conditions (fig. 3b).

Based on these results, we performed a time-course analysis of the effect of flow on the microsomal eNOS content, both in the presence and absence of extracellular Ca^{2+} . In normal Ca^{2+} containing medium, flow increase produced a rapid and reversible reduction of microsomal eNOS content, which mirrored NO production measured in the same mesenteries (fig. 5a–c). Microsomal eNOS was reduced by $48 \pm 8\%$ relative to the control, during the peak of the initial phase (min-6), which was associated with a 4-fold increase in NO production (fig. 5a, c). During the sustained phase (min-12) microsomal eNOS was not different from basal, while NO release remained at twice the baseline (fig. 5a, c). Two minutes after ending the stimulus (min-17), microsomal eNOS content and NO release returned to the level observed in basal condi-

Fig. 5. Transient NO production is associated with Ca^{2+} -dependent reduction on microsomal eNOS content. Mesenteries were perfused with control medium (left) or with Ca^{2+} -free medium containing 1 mM of EGTA (right), and homogenized and processed for subcellular fractionation at different times before, during and after stimulation with 10 ml/min, following the protocol depicted in figure 1. **a** NO production determined in the mesenteries just before homogenization; the number of experiments appears inside the columns. One-way ANOVA indicated significant differences in control ($F_{(3,12)} = 51.7$, $p < 0.00001$), and Ca^{2+} -free ($F_{(3,8)} = 9.4$, $p < 0.006$) conditions; $^{\dagger} p < 0.001$ versus all other groups; $* p < 0.05$ versus min-5 and min-17, Newman-Keuls post hoc test. **b** Representative Western blots showing eNOS content of the microsomal fraction in each condition (70 μg protein). **c** Densitometric analysis of all experiments; the number of tissues processed in each condition appears inside the columns. In control conditions there were significant differences in microsomal eNOS content between time points ($F_{(3,22)} = 4.70$, $p < 0.02$, one-way ANOVA; $* p < 0.05$ vs. min-5 and min-17, Newman-Keuls post hoc test). No differences were detected in Ca^{2+} -free conditions ($F_{(3,8)} = 0.54$, $p < 0.67$, n.s.). **d** A significant inverse correlation between NO production and microsomal eNOS content was found in mesenteries perfused with normal medium; whereas no significant correlation between both variables was detected in Ca^{2+} -free conditions (these analyses include all experiments in which both measurements were performed in parallel, data from panels **a** and **c**).

tions. Statistical analysis confirmed the inverse relationship between NO production and microsomal eNOS content (fig. 5d). Consistent with the results observed in Cav-1 co-immunoprecipitation, microsomal eNOS content did not change throughout the Ca^{2+} -free stimulation protocol (fig. 5b, c), though NO production increased to approximately twice the baseline value during the period of perfusion at 10 ml/min, and remained at the same level in measurements performed at min-6 and min-12, confirming the lack of a transient phase in this condition (fig. 5a).

Accordingly, statistical analysis showed no correlation between NO production and microsomal eNOS content in the absence of extracellular Ca^{2+} (fig. 5d).

eNOS Phosphorylation at Ser1177

We then studied the role of the PI3K-Akt pathway in flow-induced eNOS activation. In control mesenteries perfused at basal flow, either with a normal or a Ca^{2+} -free medium, there was a detectable level of phospho-Ser1177-eNOS (fig. 6a). After a 1-min stimulation with 10 ml/min,

Fig. 6. Flow induces eNOS phosphorylation at Ser1177. **a** Mesenteries were perfused either in control medium or Ca²⁺-free medium and homogenized at basal flow (2 ml/min, min-5) or after 1 min of stimulation with high flow (10 ml/min, min-6) and processed to detect phospho eNOS (Ser1177, P-eNOS) and total eNOS content. Representative Western blots and corresponding densitometric analysis; the number of tissues processed in each condition appears inside the columns. P-eNOS/eNOS signal ratio is expressed in arbitrary units relative to an internal standard included in each blot. Flow caused rapid eNOS phosphorylation regardless of Ca²⁺ availability. * $p < 0.05$ min-6 versus min-5, Mann-Whitney test. **b** Representative Western blots of P-eNOS and total eNOS

content, and densitometric analysis of P-eNOS to total eNOS content ratio in six similar experimental series. Persistent eNOS phosphorylation was determined in mesenteries perfused in control medium and homogenized at different times before, during and after stimulation at 10 ml/min (as indicated). * $p < 0.05$ versus basal, Kruskal-Wallis test. **c** Increased P-eNOS (ser1177) was detected in the cytosolic fraction. Mesenteries perfused in control medium were homogenized at basal flow (2 ml/min) or after 1 min of stimulation with 10 ml/min, submitted to subcellular fractionation and processed to detect p-eNOS and total eNOS content in subcellular fractions. * $p < 0.05$ versus basal, Mann-Whitney test.

a similar increment in eNOS phosphorylation was evident in both conditions. As expected, this increase in phospho-Ser1177-eNOS was associated with an increase in the level of phosphorylated Akt at Ser473 (data not shown).

To analyze the phosphorylation of eNOS (P-eNOS/total eNOS) in relation to the changes in eNOS subcellular distribution observed as a function of time, we used the same time points chosen to assess eNOS translocation. This analysis (fig. 6b) corroborated that eNOS was phosphorylated during the transient phase (min-6), remained phosphorylated during the sustained phase (min-12) and returned to basal phosphorylation levels after the end of stimulation (min-17).

In order to assess the spatial relation between eNOS phosphorylation and translocation, we also analyzed the ratio of P-eNOS/total eNOS in subcellular fractions before and after the flow change. This analysis showed an increment in the phosphorylation only in the enzyme located at the cytosol (fig. 6c).

The relevance of the PI3K-Akt pathway in the eNOS phosphorylation and NO production in response to

changes in flow was assessed using the PI3K blocker wortmannin. Although application of 1 μ M wortmannin did not affect the baseline of NO release, this treatment abolished the sustained phase of NO production induced by stimulation with 5 ml/min (fig. 7) and reduced in a similar magnitude the transient peak (fig. 7).

Discussion

The major finding of the present study is that, in intact resistance vessels, flow-associated shear stress leads to NO production by the coordinated activation of two signaling pathways: the Ca²⁺-dependent translocation of eNOS from the membrane to intracellular compartments and the Ca²⁺-independent phosphorylation of eNOS at serine 1177 through the PI3K-Akt pathway. Although both signaling pathways are activated rapidly in parallel, eNOS translocation is transient, and then only contributes to the initial phase of flow-induced NO release. As a result, the tonic NO production observed in response to flow is maintained by the phosphorylation of the enzyme.

Fig. 7. Inhibition of PI3-kinase suppressed the sustained phase of flow-induced NO release. Mesenteries perfused at 2 ml/min in control conditions or in the presence of the PI3K blocker wortmannin (1 μ M) were stimulated by increasing flow to 5 ml/min for 10 min, as in figure 1; the periods of flow change are indicated. **a** Time course of NO production. * $p < 0.05$ versus control (un-

paired t test). **b** Net increment in NO release above baseline at the peak (min-6) and sustained phase (average min 12–15) in each group of mesenteries (details as in fig. 2). * $p < 0.05$ versus control (unpaired t test); † $p < 0.005$ versus respective peak value (paired t test).

Translocation and phosphorylation of eNOS are not two independent mechanisms, since our results suggest that flow induces the translocation of phospho-Ser1177-eNOS, which may contribute to enhance the initial, transient NO production.

Experimental Model and Flow Stimulus

We chose to study the mechanisms of flow-induced eNOS activation in the isolated perfused arterial mesenteric bed because this preparation offers several advantages to the study of the transduction signals connecting shear stress with eNOS activation: first, intact resistance vessels can be stimulated in controlled conditions of flow; second, changes in NO production can be recorded as a function of time, and third, the subcellular distribution and phosphorylation degree of eNOS can be evaluated in a single mesentery [35]. We specifically avoided the use of vasoconstrictor agents because it has been shown that $[Ca^{2+}]_i$ changes in smooth muscle cells can spread to adjacent endothelial cells leading to increases in NO release [38–40]. In our model, perfusion pressure changed in direct proportion to flow, confirming that the arterial mesenteric bed preparation does not develop myogenic constriction, which facilitates our goal to assess the mechanisms of endothelial cell signaling in response to changes in shear stress, in intact vessels, without interference of $[Ca^{2+}]_i$ variations in smooth muscle cells.

We used a Newtonian fluid; therefore, we are confident that flow changes were directly translated into equivalent changes in shear stress. Based on the diameter of the superior mesenteric artery (cannula's outer diameter 0.965 mm) and assuming a viscosity of 0.007 poise for bicarbonate buffer at 37°C, we calculated that shear stress at the major perfused arterial branch was 1.3, 2.6, 6.6 and 13 dynes cm^{-2} at 1, 2, 5 and 10 ml/min, respectively. These values are within the physiological range for medium-size arteries [41], and it is reasonable to think that shear stress levels in downstream vessels were also within their respective physiological values because perfusate flow was allowed to distribute freely through the normal branching pattern of the rat mesenteric arterial network. Moreover, as arterial smooth muscle tone was not affected by the flow-change stimulus, the observed changes in NO production can be attributed only to a direct activation of endothelial cells. Although the lack of vasomotor tone precluded the assessment of the impact of NO release on vessel diameter, it has been reported that blood flow increases similar to those used here (4–6 times above baseline) caused maximal dilatation of arcading arterioles in vivo in this vascular bed [42], as well as in skeletal muscle [43]. In addition, we have shown that similar amounts of NO release to those recorded in this study in response to flow increase caused significant relaxation in phenylephrine-constricted perfused rat mesenteric arteries in re-

sponse to endothelium-dependent agonists [36] or perivascular nerve stimulation [35]. Therefore, we are confident that the measured changes in NO production translate into significant vasodilatation of resistance vessels in *in vivo* conditions.

Flow-Induced NO Release

The stimulation protocol produced temporary and sustained changes in NO production in the arterial mesenteric bed, while removal of extracellular Ca^{2+} only abolished the initial transient peak without affecting the sustained phase. These findings are consistent with those previously reported in other preparations [44]. Likewise, transient and sustained phases in cumulative NO release have been observed in cultured endothelial cells exposed to laminar flow [30, 45]. In those works, both phases of the response were proportional to the intensity of the flow rate applied; however, only the first phase was abolished by calmidazolium, a Ca^{2+} -calmodulin inhibitor.

The initial NO release, characterized by a conspicuous rapid peak and a gradual decrease during the following 3–5 min to the sustained level, may be interpreted as the result of eNOS activation triggered by the brief period of flow acceleration (approximately 10 s to change from 2 to 10 ml/min). Consistent with this interpretation, we found that NO production reached its maximum within 15 s after starting stimulation (fig. 2). On the other hand, sustained NO production attests that the prevailing flow constitutes an efficacious stimulus for persistent eNOS activation in arteriolar endothelium. It is likely that flow-induced stable NO production largely contributes to the NO-dependent vasodilator tone exposed by NOS inhibition *in vivo* [24–27]. In contrast, NO production elicited by receptor-dependent stimulation is essentially transient, despite continuous application of agents like noradrenaline, clonidine [36], bradykinin or ATP [46, 47].

Subcellular eNOS Distribution

We took advantage of the fact that eNOS is only expressed in endothelial cells of mesenteric vessels (fig. 4a, b); thus, changes in eNOS signal in fractions of the whole homogenized mesentery necessarily represent changes in endothelium. Only the arterial side of the circulation was perfused and, consequently, only these vessels were stimulated by flow changes. Therefore, perfusate output corresponds to the arterial mesenteric bed; however, biochemical analysis of changes may have been underestimated by proteins belonging to the nonstimulated venular and lymphatic endothelium. By combining analysis of eNOS band intensities ($n = 5$) and the total protein

content of each fraction ($4,020 \pm 190 \mu\text{g}$ cytosolic, $530 \pm 17 \mu\text{g}$ microsomal, $450 \pm 70 \mu\text{g}$ Golgi; $n = 17$), we estimated that in mesenteries perfused at 2 ml/min, approximately 50% of total mesentery eNOS was membrane bound, and distributed 3:2 between the microsomal and Golgi fractions. This proportion of eNOS associated to membrane is similar to that found in rat lungs by immunolocalization [8] and slightly smaller than that reported in the hamster cheek pouch *in vivo* [25]. In cultured endothelial cells, it has been extensively reported that the eNOS content associated to membrane reaches 90–100% of total eNOS [3, 6, 11–13, 48]. These observations suggest that constant stimulation by blood flow maintains a certain amount of eNOS trafficking in the cytosolic and Golgi compartments.

Consistent with this hypothesis, our results indicate that a substantial fraction of eNOS was translocated from the microsomal compartment to the cytosolic and Golgi compartments during the first minute of post-flow stimulation (fig. 3), concurrent with the maximal enzyme activation in the transient phase. The subcellular localization of eNOS is ascribed according to operational definitions as microsomal fraction, Golgi compartment and cytosol, based on ultracentrifugation in tandem with Western blotting, as well as assessment by microscopy as we have shown previously [14, 17]. In this context, we have documented the relevance of eNOS translocation to Golgi in response to the endothelium-dependent vasodilator ACh in resistance arteries *in vivo* [14] and in cultured endothelial cells [17]. The eNOS translocation toward the Golgi compartment was further confirmed in this work by the confocal analysis of eNOS distribution in endothelial cells of intact mesenteric vessels (fig. 4c). Then, in subsequent experiments, we only evaluated the effects of flow on the enzyme content in the microsomal compartment because the membrane-bound eNOS correspond to the activatable enzyme pool [8–10, 49]. Our results show that, in control conditions, an increase in flow reduces microsomal eNOS content in an inverse relationship with NO production. Indeed, we found a significant correlation between both variables in tissue samples collected during the basal, transient, sustained and recovery stages of the flow-change protocol (fig. 5). During the period of maximal enzyme activation, a sizeable fraction of eNOS translocated away from membrane; however, during the sustained phase, there was no significant reduction in microsomal eNOS. Interestingly, in the absence of extracellular Ca^{2+} , flow stimulation did not cause a reduction in the microsomal eNOS content (fig. 5). These results support the idea that eNOS redistrib-

bution is associated with Ca^{2+} -dependent enzyme activation as we proposed previously for the ACh-induced NO production observed in the hamster microcirculation in vivo [14].

Translocation of eNOS from membrane to the cytosol and/or Golgi has been detected by immunofluorescence in resting cultured endothelial cells in response to ACh, bradykinin or estradiol [12, 13, 17]. While the reported ACh-induced eNOS translocation matches the time course of our experiments [17], maximal eNOS translocation induced by bradykinin and estradiol was observed 5 min after drug application [12, 13]. Because bradykinin induces transient NO release within seconds in endothelial cells [50], translocation was interpreted as an inactivation mechanism of eNOS [12]. The apparent discordance with our results could be explained by the different nature of the stimulus and/or the source and physiological state of endothelial cells, as well as by inherent differences between studies performed ex vivo versus in vitro.

eNOS is mainly found in plasma membrane caveolae in an inhibitory association with Cav-1 [7, 9, 51]. Other signaling molecules, such as receptors, G proteins, Ca^{2+} channels/transporters, calmodulin, protein kinases and phosphatases are also present in caveolae [52, 53]. Caveolar location provides eNOS with special proximity to intracellular modulators that may facilitate its release from Cav-1 and subsequent activation. Consistent with this notion, disruption of caveolae in cultured endothelial cells and in rabbit aorta perfused ex vivo renders eNOS unable to be activated by flow [54]. In the present study, we used immunoprecipitation to explore whether eNOS activation by translocation implies Ca^{2+} -dependent dissociation of the eNOS-Cav-1 complex. To avoid time-dependent spurious eNOS dissociation, we incubated fresh homogenates with anti-Cav-1 antibody for a relatively short period (1 h). Our results clearly confirm the association of eNOS with Cav-1 in mesenteries perfused at 2 ml/min either in the presence or absence of Ca^{2+} (fig. 3). In control conditions, flow stimulation rapidly (1 min) caused a reduction in this association, suggesting that initial eNOS activation involves dissociation of the enzyme from Cav-1, which probably triggers the translocation of the enzyme. As expected, eNOS dissociation from Cav-1 was prevented by the absence of extracellular Ca^{2+} , which is also consistent with our finding that Ca^{2+} is required for the eNOS translocation induced by flow. Although these results are in agreement with the flow-dependent eNOS dissociation from Cav-1 and association to Ca^{2+} -calmodulin observed in the luminal surface of perfused rat lungs [8], in those experiments, caveolar eNOS con-

tent was not affected by flow, which may be explained by the time elapsed between stimulation and tissue sampling that was relatively long (>10 min), perhaps allowing for eNOS relocation to caveolae. Taken together, the present evidence strongly supports the notion that eNOS dissociation from Cav-1 and further translocation is a process involved in the initial enzyme activation induced by an increase in shear stress and suggests that translocation accounts for the transient, Ca^{2+} -dependent component of NO release. In this context, the activity of acyl-protein thio-esterase-1, which preferentially hydrolyses the palmitoylation of Ca^{2+} -calmodulin-bound eNOS to plasma membrane [55], may constitute the link between Ca^{2+} availability and eNOS translocation.

eNOS Phosphorylation

Consistent with previous reports, we found that eNOS phosphorylation at Ser-1177 via the PI3K-Akt pathway contributes to flow-induced NO production. This conclusion is supported by the action of wortmannin, which inhibited the sustained phase of NO release without affecting the transient phase (fig. 7). This is in agreement with the wortmannin-sensitive vasodilation observed in the hamster cheek pouch [56]. eNOS phosphorylation at Ser-1177 increased in the first minute after raising flow and remained phosphorylated throughout the stimulation period, which supports the notion that phosphorylation at Ser-1177 accounts for the sustained phase of flow-induced eNOS activation, but also contributes to the magnitude of the transient, Ca^{2+} -dependent NO production activated by flow at the initiation of the stimulation. In addition, the rapid increase in phosphorylation of Ser-1177 was also detected after removing Ca^{2+} from the extracellular solution, which suggests that the persistent association of eNOS with Cav-1 observed in these conditions does not preclude the activation of eNOS by phosphorylation. Therefore, these results indicate that an increment in flow triggers two parallel and additive mechanisms of eNOS activation: an increase in Ca^{2+} influx and the Ca^{2+} -independent phosphorylation of the enzyme.

Although it has been proposed that eNOS localized at the plasma membrane as well as the Golgi complex may be regulated by phosphorylation [57], the localization of eNOS at caveolae appears to be necessary for its shear stress-induced phosphorylation at Ser-1177 (Ser-1179 in bovine) in endothelial cells [54]. Interestingly, in the present study, the rapid increase in phosphorylation of Ser-1177 detected after 1 min of stimulation with flow was observed in the cytosolic compartment (fig. 6c), which suggests that most likely eNOS was phosphorylated in ca-

veolae at the plasma membrane and rapidly translocated to the cytosol. Flow also induced eNOS translocation to Golgi after 1 min of stimulation (fig. 3a) and a concomitant increase in phosphorylation was not observed in this subcellular compartment (fig. 6c), suggesting that phosphorylation of Ser-1177 may delay the traffic of eNOS from membrane to Golgi.

In summary, our results fully support the working hypothesis that in intact resistance vessels, flow-induced NO release involves two parallel mechanisms of eNOS activation: (a) enzyme dissociation from Cav-1 and translocation away from the plasma membrane, which is short lasting and entirely dependent on extracellular Ca^{2+} , and (b) there is a rapid, but more persistent, eNOS phosphorylation at serine 1177 via the PI3K-Akt pathway. Our results highlight Ca^{2+} as a mediator of shear stress-induced eNOS activation, mostly relevant for the first minute of the stimulus. This rapid component may be important in

the context of flow-induced dilatation for rapid adaptations to the change in network flow conditions. Conversely, sustained NO production attests that the prevailing flow constitutes an efficacious stimulus for persistent eNOS activation in arteriolar endothelium. Taking all together, we propose that recurrent flow fluctuations constitute a persistent stimulus reducing microsomal eNOS content, likely accounting for the smaller amount of enzyme found in this compartment in vivo, as compared with cultured endothelial cells grown in stationary conditions.

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References

- Moncada S, Palmer RM, Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109–142.
- Michel T, Feron O: Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 1997;100:2146–2152.
- Busconi L, Michel T: Endothelial nitric oxide synthase: N-terminal myristoylation determines subcellular localization. *J Biol Chem* 1993;268:8410–8413.
- Robinson LJ, Michel T: Mutagenesis of palmitoylation sites in endothelial nitric oxide synthase identifies a novel motif for dual acylation and subcellular targeting. *Proc Natl Acad Sci USA* 1995;92:11776–11780.
- Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG, Michel T: Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem* 1996;271:6518–6522.
- Sowa G, Liu J, Papapetropoulos A, Rex-Haffner M, Hughes TE, Sessa WC: Trafficking of endothelial nitric-oxide synthase in living cells: quantitative evidence supporting the role of palmitoylation as a kinetic trapping mechanism limiting membrane diffusion. *J Biol Chem* 1999;274:22524–22531.
- Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, Michel T: Endothelial nitric oxide synthase targeting to caveolae: specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *J Biol Chem* 1996;271:22810–22814.
- Rizzo V, McIntosh DP, Oh P, Schnitzer JE: In situ flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association. *J Biol Chem* 1998;273:34724–34729.
- Michel JB, Feron O, Sacks D, Michel T: Reciprocal regulation of endothelial nitric-oxide synthase by Ca^{2+} -calmodulin and caveolin. *J Biol Chem* 1997;272:15583–15586.
- Michel JB, Feron O, Sase K, Prabhakar P, Michel T: Caveolin versus calmodulin: counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J Biol Chem* 1997;272:25907–25912.
- Michel T, Li GK, Busconi L: Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1993;90:6252–6256.
- Prabhakar P, Thatte HS, Goetz RM, Cho MR, Golan DE, Michel T: Receptor-regulated translocation of endothelial nitric-oxide synthase. *J Biol Chem* 1998;273:27383–27388.
- Goetz RM, Thatte HS, Prabhakar P, Cho MR, Michel T, Golan DE: Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1999;96:2788–2793.
- Figueroa XF, Gonzalez DR, Martinez AD, Duran WN, Boric MP: ACh-induced endothelial NO synthase translocation, NO release and vasodilatation in the hamster microcirculation in vivo. *J Physiol* 2002;544:883–896.
- Duran WN, Breslin JW, Sanchez FA: The NO cascade, eNOS location, and microvascular permeability. *Cardiovasc Res* 2010;87:254–261.
- Omura M, Kobayashi S, Mizukami Y, Mogami K, Todoroki-Ikeda N, Miyake T, Matsuzaki M: Eicosapentaenoic acid (EPA) induces Ca^{2+} -independent activation and translocation of endothelial nitric oxide synthase and endothelium-dependent vasorelaxation. *FEBS Lett* 2001;487:361–366.
- Sanchez FA, Savalia NB, Duran RG, Lal BK, Boric MP, Duran WN: Functional significance of differential eNOS translocation. *Am J Physiol Heart Circ Physiol* 2006;291:H1058–H1064.
- Fleming I, Busse R: Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol* 2003;284:R1–R12.
- Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM: Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601–605.
- Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC: Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999;399:597–601.
- Pohl U, Herlan K, Huang A, Bassenge E: EDRF-mediated shear-induced dilation opposes myogenic vasoconstriction in small rabbit arteries. *Am J Physiol* 1991;261:H2016–H2023.

- 22 Cooke JP, Rossitch E Jr, Andon NA, Loscalzo J, Dzau VJ: Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. *J Clin Invest* 1991;88:1663–1671.
- 23 Kuo L, Chilian WM, Davis MJ: Interaction of pressure- and flow-induced responses in porcine coronary resistance vessels. *Am J Physiol* 1991;261:H1706–H1715.
- 24 Chu A, Chambers DE, Lin CC, Kuehl WD, Cobb FR: Nitric oxide modulates epicardial coronary basal vasomotor tone in awake dogs. *Am J Physiol* 1990;258:H1250–H1254.
- 25 Figueroa XF, Martinez AD, Gonzalez DR, Jara PI, Ayala S, Boric MP: In vivo assessment of microvascular nitric oxide production and its relation with blood flow. *Am J Physiol Heart Circ Physiol* 2001;280:H1222–H1231.
- 26 Vallance P, Collier J, Moncada S: Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* 1989;2:997–1000.
- 27 Rees DD, Palmer RM, Moncada S: Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 1989;86:3375–3378.
- 28 Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J, Jo H: Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. *J Biol Chem* 2002;277:3388–3396.
- 29 Li Y, Zheng J, Bird IM, Magness RR: Mechanisms of shear stress-induced endothelial nitric-oxide synthase phosphorylation and expression in ovine fetoplacental artery endothelial cells. *Biol Reprod* 2004;70:785–796.
- 30 Kuchan MJ, Frangos JA: Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *Am J Physiol* 1994;266:C628–C636.
- 31 Muller JM, Davis MJ, Kuo L, Chilian WM: Changes in coronary endothelial cell Ca^{2+} concentration during shear stress- and agonist-induced vasodilation. *Am J Physiol* 1999;276:H1706–H1714.
- 32 Ungvari Z, Sun D, Huang A, Kaley G, Koller A: Role of endothelial $[\text{Ca}^{2+}]_i$ in activation of eNOS in pressurized arterioles by agonists and wall shear stress. *Am J Physiol Heart Circ Physiol* 2001;281:H606–H612.
- 33 Kolluru GK, Sinha S, Majumder S, Muley A, Siamwala JH, Gupta R, Chatterjee S: Shear stress promotes nitric oxide production in endothelial cells by sub-cellular delocalization of eNOS: a basis for shear stress mediated angiogenesis. *Nitric Oxide* 2010;22:304–315.
- 34 McGregor DD: The effect of sympathetic nerve stimulation on vasoconstriction responses in perfused mesenteric blood vessels of the rat. *J Physiol (Lond)* 1965;177:21–43.
- 35 Boric MP, Figueroa XF, Donoso MV, Paredes A, Poblete I, Huidobro-Toro JP: Rise in endothelium-derived NO after stimulation of rat perivascular sympathetic mesenteric nerves. *Am J Physiol* 1999;277:H1027–H1035.
- 36 Figueroa XF, Poblete MI, Boric MP, Mendizabal VE, Adler-Graschinsky E, Huidobro-Toro JP: Clonidine-induced nitric oxide-dependent vasorelaxation mediated by endothelial α_2 -adrenoceptor activation. *Br J Pharmacol* 2001;134:957–968.
- 37 Duran WN, Seyama A, Yoshimura K, Gonzalez DR, Jara PI, Figueroa XF, Boric MP: Stimulation of NO production and of eNOS phosphorylation in the microcirculation in vivo. *Microvasc Res* 2000;60:104–111.
- 38 Dora KA, Doyle MP, Duling BR: Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. *Proc Natl Acad Sci USA* 1997;94:6529–6534.
- 39 Figueroa XF, Duling BR: Gap junctions in the control of vascular function. *Antioxid Redox Signal* 2009;11:251–266.
- 40 Figueroa XF, Isakson BE, Duling BR: Connexins: gaps in our knowledge of vascular function. *Physiology (Bethesda)* 2004;19:277–284.
- 41 Lipowsky HH, Kovalcheck S, Zweifach BW: The distribution of blood rheological parameters in the microvasculature of cat mesentery. *Circ Res* 1978;43:738–749.
- 42 Smiesko V, Lang DJ, Johnson PC: Dilator response of rat mesenteric arcading arterioles to increased blood flow velocity. *Am J Physiol* 1989;257:H1958–H1965.
- 43 Koller A, Kaley G: Endothelium regulates skeletal muscle microcirculation by a blood flow velocity-sensing mechanism. *Am J Physiol* 1990;258:H916–H920.
- 44 Ayajiki K, Kindermann M, Hecker M, Fleming I, Busse R: Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. *Circ Res* 1996;78:750–758.
- 45 Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG: Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res* 1996;79:984–991.
- 46 Harris MB, Ju H, Venema VJ, Liang H, Zou R, Michell BJ, Chen ZP, Kemp BE, Venema RC: Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation. *J Biol Chem* 2001;276:16587–16591.
- 47 Wagner S, Groschner K, Mayer B, Schmidt K: Desensitization of endothelial nitric oxide synthase by receptor agonists. *Biochem J* 2002;364:863–868.
- 48 Liu J, Garcia-Cardena G, Sessa WC: Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. *Biochemistry* 1995;34:12333–12340.
- 49 Sessa WC, Garcia-Cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman IM, Desai KM: The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J Biol Chem* 1995;270:17641–17644.
- 50 Malinski T, Taha Z: Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. *Nature* 1992;358:676–678.
- 51 Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J, Li S, Lisanti MP, Sessa WC: Dissecting the interaction between nitric oxide synthase (NOS) and caveolin: functional significance of the NOS caveolin binding domain in vivo. *J Biol Chem* 1997;272:25437–25440.
- 52 Anderson RG: The caveolae membrane system. *Annu Rev Biochem* 1998;67:199–225.
- 53 Saliez J, Bouzin C, Rath G, Ghisla P, Desjardins F, Rezzani R, Rodella LF, Vriens J, Nilius B, Feron O, Balligand JL, Dessy C: Role of caveolar compartmentation in endothelium-derived hyperpolarizing factor-mediated relaxation: Ca^{2+} signals and gap junction function are regulated by caveolin in endothelial cells. *Circulation* 2008;117:1065–1074.
- 54 Lungu AO, Jin ZG, Yamawaki H, Tanimoto T, Wong C, Berk BC: Cyclosporin A inhibits flow-mediated activation of endothelial nitric-oxide synthase by altering cholesterol content in caveolae. *J Biol Chem* 2004;279:48794–48800.
- 55 Yeh DC, Duncan JA, Yamashita S, Michel T: Depalmitoylation of endothelial nitric-oxide synthase by acyl-protein thioesterase 1 is potentiated by Ca^{2+} -calmodulin. *J Biol Chem* 1999;274:33148–33154.
- 56 Aramoto H, Breslin JW, Pappas PJ, Hobson RW 2nd, Duran WN: Vascular endothelial growth factor stimulates differential signaling pathways in in vivo microcirculation. *Am J Physiol Heart Circ Physiol* 2004;287:H1590–H1598.
- 57 Fulton D, Babbitt R, Zoellner S, Fontana J, Acevedo L, McCabe TJ, Iwakiri Y, Sessa WC: Targeting of endothelial nitric-oxide synthase to the cytoplasmic face of the Golgi complex or plasma membrane regulates Akt-versus calcium-dependent mechanisms for nitric oxide release. *J Biol Chem* 2004;279:30349–30357.