eNOS Activation by Physical Forces: From Short-Term Regulation of Contraction to Chronic Remodeling of Cardiovascular Tissues

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transcriptional activity of hypoxia inducible factor in normoxia and hypoxia. The continuum of the influence of eNOS in cardiovascular biology explains its growing implication in mechanosensitive aspects of integrated physiology, such as the control of blood pressure variability or the modulation of cardiac remodeling in situations of hemodynamic overload.

I. INTRODUCTION

As any organism, the cardiovascular system responds to external mechanical stimuli with a vast array of adaptive (and in pathological situations, maladaptive) biological reactions; the complexity of which is commensurate with the variety of the physical forces applied at the organ, cellular, and subcellular levels. Indeed, at the apparently simple level of the vascular wall, both tangential and circumferential forces are conjugated in a temporally defined manner by pulsatile flow to elicit tightly regulated responses within the muscular and endothelial layers to ensure adequate vascular homeostasis. This is well exemplified in the adaptive outward vessel remodeling that contributes to maintain lumen diameter in the face of a stenotic atherosclerotic plaque, as described by Glagov in human coronary arteries (145), and subsequently extended to the general adaptation of vessel diameter through “outward growth” following vessel injury (the failure of which results in restenosis) (300), or changes in flow (217). In all instances, shear forces on the vessel wall were identified as critical for the occurrence of adaptive remodeling (146, 387). Similarly, heterogeneously distributed forces within the cardiac muscle (the pattern of which varies regionally and, importantly, according to changes in the geometry of each cardiac cavity, e.g., during pathological remodeling) exert a profound influence both on short-term and long-term control of cardiac function. The former is illustrated by changes in excitation-contraction coupling, as exemplified by the force-frequency relationship, the Anrep effect, the Frank-Starling law of the heart, or the excitability and susceptibility to arrhythmias; the latter is illustrated by the long-term remodeling of cardiac muscle structure through quantitative and qualitative changes in tissue composition, as occurs in pathophysiological hypertrophy in response to hemodynamic overload. Underlying these functional and structural responses is an extremely complex network of signaling pathways responding to a specific mechanotransduction machinery. Although each cell type within the cardiovascular system is endowed with such ability to respond individually to physical forces, as can be demonstrated with homotypic cell cultures in vitro, the integrated response at an organ level can hardly be predicted by any model that would just be summation of the responses of the individual cell types composing it. This is expected from the difficulty to modelize complex physical forces in any in vitro setting, and, more importantly, from the rich array of cell-to-cell signaling modulating the overall phenotype of the physically challenged organ.

Among the various cell types composing the cardiovascular tissue, the endothelium plays a key role both as a sensor of chemical and physical stimuli and a source of pleiotropic paracrine and endocrine (as well as autocrine) messengers. Although this has been mostly exemplified in vessels, similar paradigms have been verified in cardiac muscle, through the identification of the critical roles of both cardiac endocardial and capillary myocardial cells as integrators of blood-borne and physical stimuli and providers of feedback signaling for coordinate functioning of the cardiovascular system (54). In cardiac muscle, the endothelium was shown to influence inotropism as well as relaxation properties, among others (245, 285). In the vasculature, the endothelium is well-known to maintain a balance between positive and negative signals of hypertrophy, vasoconstriction, and thrombogenicity, justifying the identification of endothelial dysfunction as a first step towards atherosclerosis (102, 295). In both instances, the production of nitric oxide (NO) by endothelial/endocardial cells was singled out as instrumental for these regulatory properties, mostly through activation of the endothelial isoform of NO synthase (NOS), or endothelial NOS (eNOS) (222, 269).

Notably, the production of NO by cardiovascular tissues is exquisitely responsive to mechanostimulation, and its abrogation by genetic deletion of one or several of the NOS isoforms profoundly affects basic physiological responses to physical forces, as will be illustrated, e.g., for flow-mediated dilation of vessels or the Anrep effect of cardiac muscle. This was also verified for the Glagov phenomenon mentioned above, since each NOS isoform, in conjunction with other gene products, was found to exert a distinctive effect on the “outward remodeling” characteristic of this vessel adaptation to changes in shear forces (288, 446). Not surprisingly, the NOS isoform most abundantly expressed in cardiovascular tissues and specifically in endothelium, or eNOS, was shown to be acutely and chronically regulated by tangential shear forces, as will be detailed below. These facts concur to put NO as a central homeostatic regulator in cardiovascular physiology. Although singling out this molecule as the sole mediator (or even modulator) of cardiovascular mechanics would obviously be too reductive, we will illustrate how molecular studies on (e)NOS regulation substantiate the proposition that NO signaling is coordinate regulated to subserve its pleiotropic roles on the
modulation of cardiovascular function in response to mechanical stress.

Among the NOS isoforms identified in cardiovascular tissues, we will therefore focus on eNOS and first review the most recent molecular mechanisms of regulation of its expression and activity; we will proceed with an update on the machinery that transmits mechanosensing at the cellular level, emphasizing the connection to eNOS activation, e.g., in response to shear and stretch in the vasculature and the myocardium; we will expand on the implication of eNOS in chronic remodeling through its specific regulation of progenitor cell biology and finally illustrate the role of eNOS as a mechanosensitive regulator of pathophysiological adaptation to specific injuries at the integrated organ level.

A. Cardiovascular Nitric Oxide Synthases: Which and Where?

Of the three isoforms of NOS that have been cloned (7, 23), namely, the neuronal NOS (nNOS or NOS1, 150-kDa protein, encoded by NOS1 gene), the inducible NOS (iNOS or NOS2, 135-kDa protein, encoded by NOS2 gene), and the eNOS (or NOS3, 135-kDa protein, encoded by NOS3 gene), the latter is most abundantly expressed in the endothelium. Nevertheless, accumulated evidence has now established its expression in other cell types composing cardiovascular tissues, e.g., in cardiac myocytes. Likewise, although nNOS was traditionally considered as a neuronal-specific isoform, recent work demonstrated its expression in vascular smooth muscle cells as well as cardiac myocytes. These two “constitutively” expressed NOS isoforms (nNOS and eNOS) are highly regulated at both transcriptional (expression, abundance) and posttranslational (activity, function) levels, whereas iNOS is mainly regulated transcriptionally, and its expression can be induced in virtually all cell types upon activation with appropriate (mainly proinflammatory) stimuli. Recent work has provided some mechanistic explanation for the cell-specific (predominantly endothelial) expression of eNOS through epigenetic control, as will be detailed below. Such apparent promiscuity of the three NOS isoforms imposes a tight regulation of their activation for coordinate signaling, e.g., through cellular spatial confinement that both ensures accessibility to specific stimuli and restricts the range of NO effects to colocalized downstream targets. Accordingly, we will review the most recent data on the regulation of eNOS expression and activity to provide the necessary context to understand the complex mechanisms of its activation by mechanical forces in the heart and vessels.

B. The eNOS (encoded by the NOS3 Gene): Recap of Its Molecular Structure and Function

Like the other two members of the NOS enzymes family, eNOS (a 1,203 amino acid, 133-kDa protein encoded by NOS3, located on 7q35–7q36 of human chromosome 7) has a bidomain structure and functions as a dimer. Within each monomer, a NH2-terminal oxygenase domain binds a prosthetic heme group, tetrahydrobiopterin (BH₄), oxygen, and the substrate L-arginine and supports the catalytic activity; a peptide sequence containing a consensus calmodulin-binding site links the oxygenase domain to a COOH-terminal reductase domain containing binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and NADPH and critically controls the transfer of electrons in trans from the reductase to the opposite oxygenase domain across the dimer. Catalysis of NO synthesis at the active site requires stepwise transfer of electrons initiated by NADPH binding to its site in the reductase domain. Electrons are transferred from NADPH to FAD and then to FMN, from where an electron is transferred to heme of the oxygenase domain and converts ferric heme to ferrous that binds oxygen actively. The active dimeric form of eNOS is stabilized by heme and L-arginine as well as BH₄, the binding site of which is structurally maintained by a zinc center coordinated by critical cysteine residues (part of two CXXXSC motifs, one contributed by each monomer at the dimer interface) (282, 341, 341). In the reductase domain, the FMN domain is “locked” into an electron-accepting position upon NADPH binding to the calmodulin-free NOS, perhaps through ionic interactions between NADPH and a conserved Arg residue in e- (and n-) NOS that help to orient the COOH-terminal tail in this inhibitory position. Phosphorylation of a COOH-terminal Akt/PKB-dependent phosphorylation site (Ser-1177 on eNOS) would displace the regulatory COOH-terminal tail to relieve repression of NO synthesis. Indeed, mutation of this site to Asp (which mimics the negative charge conferred upon phosphorylation) increases electron transfer rates from the reductase domain in calmodulin-free NOS (1). eNOS (and nNOS), unlike iNOS, also contains a conserved sequence that functions as an autoinhibitory helix by destabilizing calmodulin binding at low calcium levels. Additionally, upon interaction with the FMN- and NADPH-binding domains, the autoinhibitory helix may contribute to the “locked” electron-accepting position of the FMN domain. At high concentrations of Ca²⁺, calmodulin would bind to both the autoinhibitory and calmodulin-binding regions and release the FMN domain for intermodule electron transfer. Accordingly, inhibition of eNOS activity by calmodulin (CaM) inhibitors and calcium removal justifies the traditional definition of eNOS as a calcium-sensitive enzyme. Overall, structural modeling of the holo-NOS enzyme supports a mechanism where both Ca²⁺/CaM bind-
ing and Ser-1177 phosphorylation derepress NO synthesis by displacing the COOH-terminal tail, thus unlocking the FMN-binding domain to promote intermodule electron transfer. Since the two redox partners of the FMN shuttles are located on adjacent polypeptides, dimerization would provide a means for fine-tuning the electron transfer mechanism (142).

C. Regulation of eNOS Activity: Main Transcriptional and Posttranscriptional Mechanisms in Response to Shear

The main mechanisms driving the short- and long-term regulation of NO production in response to shear are illustrated in Figure 1.

1. Transcriptional regulation

Since the early 1990s, many studies have demonstrated that shear stress is an important signal regulating eNOS mRNA and eNOS protein expressions in cultured endothelial cells (209, 342, 412) and in intact arteries (437, 438). Very recently, this demonstration was extended to endothelial progenitor cells (EPC) (401), providing evidence that shear stress may regulate the phenotype of human EPC. Moreover, in vivo, exercise training increases eNOS gene expression, leading to improved NO-mediated endothelial functions as demonstrated in porcine coronary resistance arteries (436) or in rat (181).

c-Src-tyrosine kinase has been identified as a key regulator of this eNOS upregulation in cultured endothelial cells (92) and in mouse vessels (93)(Fig. 2). In the specific context of exercise, Davis et al. (92) documented that contrary to wild-type mice, exercise training did not lead to an increase in eNOS protein levels in c-Src+/− mice. In endothelial cells, these authors showed that while c-Src plays a central role in the modulation of eNOS expression, two different but complementary pathways lead to eNOS upregulation downstream of c-Src, leading to a short-term increase in eNOS transcription and a long-term stabilization of eNOS mRNA. Downstream of c-Src, the increase in mRNA transcription depends classically on the pathway involving Raf, Ras, and ERK1/2 (92). Exposure of endothelial cells to shear results in Ik kinase phosphorylation, and this is blocked by the MEK1/2 inhibitor PD98059 and the c-Src inhibitor PP1, suggesting that these signaling molecules are upstream of NFκB activation (94). Bovine and human eNOS promoters contain a shear stress response element (SSRE), with the core sequence GAGACC allowing the binding of p50/p65, justifying NFκB-dependent eNOS transcription. It was recently proposed that the transactivation of the eNOS promoter is controlled by a negative-feedback loop, ultimately responsible of the short-term increases in eNOS transcription (156). Indeed, laminar shear stress induces a nitrosylation of the NFκB subunit p50 and, consequently, an inhibition of its activity that blunts eNOS enhanced transcription. In a healthy endothelium, shear stress would activate NFκB and promote eNOS expression; the consecutive NO production would ultimately nitrosylate p50 and terminate NFκB-dependent eNOS transcription. In a dysfunctional endothelium (i.e., with diminished NO bioavailability), a deleterious, sustained activation of NFκB may result from the failure of NO to properly inhibit NFκB (156), which will promote the transcription of proinflammatory genes. Therefore, shear stress would
prime endothelial cells for enhanced NFκB-dependent cytoprotective responses while attenuating proinflammatory activation (319). Very recently, a NFκB1 promoter polymorphism ([−94]NFκB1 I/D, where I is insertion and D is deletion) was shown to be associated with the levels of NOS3 gene expression in endothelial cells under physiological levels of laminar shear; homozygous I-I polymorphism was associated with higher NOS3 protein abundance in endothelial cells and higher baseline reactive hyperemic forearm blood flow in volunteers; conversely, in pre- and stage I hypertensive individuals submitted to supervised endurance exercise training, D-D homozygotes were significantly less prevalent in the exercise responder group compared with I-I and I-D genotypes (316). According to this study, increased expression of p50 in I-allele carriers and the resulting abundance of a NFκB signaling pool interact with chronic exercise (that increases hemodynamic shear) to enhance NFκB-mediated NOS3 gene expression. Of note, NOS3 gene transcription may also be developmentally regulated. Indeed, while transcriptional activity from a 1,600-bp eNOS promoter fragment increases in both fetal and adult pulmonary endothelial cells (PAEC) exposed to 8 h of shear stress, activity driven from a 840-bp promoter fragment containing a putative activator protein (AP)-1 binding site was increased only in fetal PAEC. In addition, transcriptionally active phosphorylated c-Jun is elevated only in the nuclei of sheared fetal PAEC. This suggests that different upstream sequences are required for transcriptional activity in the adult and fetal cells and that phosphorylated c-Jun is involved for shear-mediated increases in eNOS transcription (431). Of note, a study has shown that shear stress may also upregulate iNOS in rat lung and in human microvascular endothelial cells as well as in human aortic smooth muscle cells, resulting in increased iNOS protein expression and NOX accumulation. Endothelial iNOS induction by shear stress was dependent on oxidative stress and NFκB in this study (311).

Complementary to these pathways, the laminar shear-dependent transcriptional regulation of eNOS (and other atherosclerosis-related genes) involves the transcription factor LKLF (lung Kuppel-like factor, also identified as KLF2). Dekker and co-workers demonstrated that in HUVECs (105) and in the mouse and human vasculature (100), this endothelium-specific transcription factor, already known to regulate the eNOS promoter (373), is uniquely induced by flow and is required for the transcription of numerous genes that control vascular tone in response to flow. Of note, a decreased expression of KLF2 was measured at human aorta bifurcations to the iliac and carotid arteries, coinciding with neointima formation (100). Recently, the same authors demonstrated that in human umbilical vein endothelial cells (HUVECs), the potent induction of eNOS by prolonged exposure to flow is more likely explained by an increased stability of KLF2 mRNA that results in an increased amount of KLF2 proteins and involves a phosphatidylinositol 3-kinase (PI3K)-dependent mechanism (419). Notably, NFκB and KLF2-mediated transcriptional activation may act coordinately for optimal gene expression thanks to their different kinetics in response to shear induction. Indeed, maximum activation of NFκB was observed as early as 15–30 min after exposure to shear, while maximum induction of KLF2 requires several hours (99). Importantly, KLF2 was demonstrated to inhibit the activity of the p65 subunit of NFκB and to interact with CBP/p300 (373), also critical for NFκB activity, so that KLF2 participates to the late downregulation of NFκB signaling. Another difference may be related to the nature of the shear stimulus itself. Indeed, shear- and NFκB-dependent transcriptional effects were observed after exposure to steady flow; conversely, pulsatile flow applied on HUVECs for 24 h up to 7 days induced sustained KLF2 overexpression as much as 20-fold, whereas steady flow upregulated KLF2 less than 5-fold with a peak around 4 h (99). Altogether, KLF2 appears as a major transcription factor activated by prolonged shear stress to regulate the expression of endothelial genes (including NOS3) that maintain the quiescent and atheroprotective status of the vascular wall under inflammatory conditions. Another transcriptional regula-
tor of eNOS, Foxo-1 was also recently shown to be responsive to shear (80). Foxo-1 acts as a transcriptional repressor of eNOS expression (333), which can be relieved upon nuclear exclusion of Foxo-1 following its phosphorylation by Akt, itself stimulated by shear.

Oscillatory shear stress also increased NOS3 expression in endothelial cells, at least in vitro. Contrary to unidirectional shear, this involves the production, by NADPH oxidase, of reactive oxygen species (ROS), e.g., O2 and H2O2, that in turn activate transcription through a pathway involving calcium/CaM kinase II and Janus kinase 2 (63, 64, 107). Notably, the production of ROS is quantitatively higher upon oscillatory versus unidirectional shear (97). This, combined with the differential regulation of antioxidant molecules, such as intracellular reduced glutathione (289) that critically regulate intracellular redox status and NO bioactivity, determines the prevailing adverse influence of oscillatory shear on the endothelial phenotype and potentially explains the proatherogenic phenotype of endothelia exposed to disturbed flow and shear forces in vivo.

Somewhat unpredictably, NOS3 transcription may also be regulated by microRNAs (miRs). Indeed, Zhang et al. (448) described a 27-nt derived from 27 nt repeats within intron 4 of the NOS3 gene, that represses eNOS expression. Contrary to most miRs that act as inhibitors of translation or degrade mRNA, this intron 4-derived microRNA inhibited transcription, through still uncharacterized mechanisms. Conceptually, this microRNA could provide a negative-feedback mechanism on eNOS expression. It would be expected that the amount of these 27 nt microRNA derived from intronic 27 nt repeats would also vary interindividually according to the number of intronic repeats, perhaps accounting for susceptibility to endothelial dysfunction and cardiovascular risk. Of note, transcriptional inhibition could be reversed by vascular epidermal growth factor (VEGF), itself a NOS3 transcription activator, in parallel with decreased abundance of the 27 nt microRNA. This is contrary to the expectation that increased NOS3 gene transcription would also be accompanied by enhanced expression of the intronic 27 nt microRNA, suggesting that the regulation of this microRNA may be independent of NOS3 transcription and regulated by as yet unidentified mechanisms. Whether expression of this microRNA is sensitive to mechanical forces on the endothelial cells is unknown at present.

Altogether, the transcriptional regulation by NFκB and KLF2, as reviewed above, provides a paradigm for the coordinated NOS3 expression and NO production as an anti-inflammatory and atheroprotective molecule in response to unidirectional and pulsatile shear, which may become inoperative upon excessive ROS production in response to oscillating shear. This paradigm now needs to be refined in light of the experimental evidence on posttranscriptional regulation of NOS3 expression.

2. Posttranscriptional regulation

According to Davis et al. (92), the major component of the steady-state upregulation of NOS3 mRNA by laminar shear stress is the result of prolonged mRNA stabilization. More recently, Weber et al. (429) demonstrated that laminar shear increased expression of NOS3 transcripts with long poly(A) tails that had prolonged half-life (6 h in static conditions versus 18 h in sheared cells), supporting the idea that shear stress modulates NOS3 mRNA stability and translation via increased 3'-polyadenylation.

The group of Harrison (63) had previously shown that H2O2 upregulates NOS3 expression via a calcium/CaM-dependent protein kinase II (CaMKII)-mediated mechanism, whereas it also acutely activates the eNOS enzyme. The same group demonstrated that exposure of bovine aortic endothelial cells (BAEC) to oscillatory shear stress increases NOS3 mRNA expression threefold (64). Moreover, oscillatory shear activates CaMKII in an H2O2-dependent fashion, whereas unidirectional laminar shear stress inhibited CaMKII phosphorylation. Taken together, these data suggest that intracellular H2O2 and CaMKII mediate oscillatory shear stress upregulation of eNOS. According to these authors, as oscillatory shear stress has been shown previously to stimulate sustained production of superoxide (O2•−), which would inactivate NO+ these responses may represent an attempted compensation to restore NO+ bioavailability in areas exposed to oscillatory shear. In agreement with these reports and although surprising at a first glance, another mechanism that may contribute to exercise-induced upregulation of vascular eNOS is therefore the vascular oxidative stress (190). Exercise is known to generate ROS such as superoxide anions through at least two different mechanisms. First, an electron may be transferred in a nonenzymatic fashion during ATP synthesis from coenzyme Q to molecular oxygen (124); this pathway is stimulated during exercise. Second, it is well known that superoxide anions are generated directly in the endothelium in response to shear stress (236), probably through the activation of endothelial NADPH oxidase (97). Although superoxide anion by itself may scavenge NO and reduce its bioavailability, its dismutation by superoxide dismutase (SOD) leads to the production of another reactive oxygen species, namely, hydrogen peroxide, which may, instead, stimulate eNOS expression and activity, as mentioned above. The higher stability and diffusion of hydrogen peroxide (versus superoxide anion) participates in the capacity of this ROS to increase the expression and the activity of eNOS throughout the vascular wall (107, 212). More recently, the use of transgenic mice with endothelial specific overexpression of catalase confirmed the major role of hydrogen peroxide in the exercise-induced upregulation of eNOS (234). In these mice, chronic exercise had
no effect on vascular eNOS expression. Interestingly, SOD isoforms-1 and -3 were shown to be upregulated by exercise, thereby further promoting the conversion of superoxide anions into hydrogen peroxide (134, 357). Similarly, an upregulation of Cu/Zn SOD mRNA and activity was observed in human endothelial progenitor cells subjected to shear probably through enhanced transcription (402). Therefore, in conditions of concurrent ROS and NO production, the expression and activity of the SODs may be critical to derive O$_2^\cdot$ reactivity towards the formation of H$_2$O$_2$, a positive regulator of NOS3 expression, and tilt the balance towards a "protective" phenotype.

Recently, an interesting study by Kojda et al. (210) documented that although NOS3 expression appears to be normal in NOS3(+/−) mice under basal conditions, these mice are unable to increase NOS3 expression during exercise. These findings show that regulation of NOS3 during exercise (treadmill) requires the presence of both alleles of the gene and might have implications for conditions in which polymorphisms of NOS3 are present in only one allele in humans. These individuals have normal vascular reactivity under basal conditions but may be unable to adapt their vascular reactivity in response to exercise training.

eNOS expression is also regulated posttranscriptionally by the endothelial cell growth status, i.e., NOS3 mRNA was shown to be increased four- to fivefold in proliferating cells compared with confluent cells, and nuclear run-on experiments clearly showed this not to involve increased NOS3 transcription (371). Instead, binding of monomeric actin (G actin) in a ribonucleocore targeted at a 43-nt cis-element in the NOS3 3′-UTR was responsible for downregulation of NOS3 mRNA stability in nonproliferating endothelial cells (370). Notably, the binding of this actin-containing protein complex was linked to changes in the cytoskeletal organization, which may also influence subcellular localization and efficient translation of specific mRNA. Accordingly, in proliferating cells, NOS3 mRNA was preferentially located to cytoskeleton-bound polysomes together with less G-actin binding. Whether the cytoskeletal reorganization in response to shear may similarly affect NOS3 mRNA stability and eNOS expression together with less G-actin binding remains to be formally proven. Notably, treatment of endothelial cells with H$_2$O$_2$ (known to be produced in endothelial cells in response to oscillatory shear, see above) also decreased G-actin binding to NOS3 mRNA 3′-UTR, together with decreased G-actin and increased filamenterous (F-) actin in these cells.

This actin-mediated regulation of eNOS expression seems to be dependent on the endothelial cell type, perhaps related to their embryologic origin or the differential organization of the cytoskeletal actin, as it was shown to be exactly opposite in endothelial cells from pulmonary arteries, where eNOS expression and activity increased during cell growth, together with increased interaction of eNOS with β-actin in Triton-soluble fractions. eNOS activity increased more than the enzyme’s abundance, underlining posttranscriptional regulation of enzyme activity by actin, regardless of actin polymerization state, through still uncharacterized mechanisms (215, 390).

Recent work has emphasized the role of miRs as posttranscriptional regulators of gene expression in virtually all, including endothelial cells as mentioned above. Silencing of Dicer, a protein critical for microRNA maturation, was shown to upregulate eNOS in endothelial cells, and subsequent microRNA screening has led to the identification of few miRs abundantly represented in endothelial cells, including miR221 and miR222, that may be involved in the control of eNOS expression (391). Earlier work had identified a longer, 662-nt antisense NOS3 mRNA that was expressed in nonendothelial cells but could mediate posttranscriptional downregulation of eNOS upon overexpression in endothelial cells (352). However, very little is known on the transcriptional regulation of miRs themselves, and no evidence to date has implicated these as part of the posttranscriptional regulation of eNOS in response to mechanical forces, although this is an attractive hypothesis.

Increases in NOS3 abundance only partly predict the overall enzyme’s output that is amenable to a very rich modulation of the protein activity, as can be anticipated from the earlier description of the structural basis of its catalytic activity (see above). Accordingly, we will now concentrate on the posttranslational regulation of eNOS through mechanisms that were shown to be responsive to physical forces.

### 3. Posttranslational regulation

At the posttranslational level, eNOS activity is highly regulated by lipidation, direct protein-protein interactions, phosphorylation, O-linked glycosylation, and S-nitrosylation; in addition, substrate and cofactor availability, as well as endogenous inhibitors, all concur to fine-tune the NO output; finally, oxidative and nitrosative metabolism influence the fate of the NO produced and specificity/efficiency of downstream signaling (for reviews, see Refs. 127, 136).

#### a) Regulation of NOS3 activity by lipidation and subsequent localization. The NH$_2$-terminal domain of eNOS contains a glycine residue necessary for cotranslational and irreversible myristoylation that ensures membrane attachment and proper coupling to activators (121). In addition, reversible, double palmitoylation of cysteine-15 and -26 residues directs eNOS specifically to caveolae (31, 122). These invaginations of the plasmaembran membrane, enriched in cholesterol and sphingolipids, are determined by the membrane association and homo- and hetero-oligomerization of caveolins, which also serve as
scaffolds for the assembly of multiproteins signaling complexes, including eNOS, at the plasma membrane. As the thiopalmitoyl bonds can be disrupted by acyl-protein thioesterase 1 (APT-1) (445), the targeting of eNOS to caveolae is dynamically regulated, and upon specific stimuli, the enzyme can shuttle to intracellular compartments. Indeed, intracellular calcium increases translocation of eNOS from the detergent-insoluble fraction (caveolae) to the detergent-soluble fraction (Golgi complex and cytoskeleton) (98). Dissociation from caveolin interaction is also a critical early step in eNOS activation, as detailed below. Shear stress promotes such dissociation and calmodulin binding to eNOS for its full activation (350). Of interest, a single nucleotide polymorphism (SNP) in the NOS3 gene coding for a substitution of Glu by Asp at position 298 (Glu298Asp variant) was shown to be associated with decreased activation of NO production in endothelial cells in response to shear, together with decreased basal association of the variant eNOS to caveolae and less dissociation from caveolin-1 upon application of shear (194). Although a full mechanistic explanation is still lacking (e.g., because the amino acid substitution is remote from the known caveolin binding or phosphorylation sites on eNOS, and phosphorylation of the variant eNOS is unperturbed), this observation may be one explanation for the association of this eNOS polymorphism with adverse cardiovascular prognosis (428).

The effect of shear was also examined on the plasmalemmal versus peri-Golgi localization in intact vessels by en face microscopy (79). Although shear stress did increase the total expression of eNOS, its relative distribution between these two compartments seemed unaffected.

**B) Regulation of NOS1 Activity by Phosphorylation.** eNOS is regulated by phosphorylation on serine, and, in specific circumstances, also on tyrosine and threonine residues. Phosphorylation at Ser-1177 (for human)/1179 (for bovine eNOS) (104, 135), Ser-635 and Ser-617 (279) increases activity, while phosphorylation at Ser-116 and Thr-495 decreases it. Notably, Ser-1177 phosphorylation requires efficient membrane targeting of eNOS, since myristoyl- or palmitoyl-deficient eNOS failed to be phosphorylated by agonists (149). Shear stress induces Ser-1177 phosphorylation and activation of eNOS through PKB-dependent activation of Akt (protein kinase B) (129), eNOS where serine-1177 is mutated to aspartate (S1177D, which, again, mimics the negative charge conferred upon phosphorylation) retains enzymatic activity despite low levels of calcium or CaM both in vitro and in situ. The proposed mechanism is that Ser-1177 displaces the COOH-terminal tail, thus freeing the FMN-binding domain and promoting electron transfer from the reductase to oxygenase domain (see above). Other kinases, e.g., protein kinase A (278), protein kinase G (61), AMP-activated kinase (76), and calcium/CaM-dependent protein kinase II also phosphorylate eNOS on serine-1177. PKA also induces phosphorylation on Ser-635, e.g., upon shear (40).

Conversely, phosphorylation on threonine-495 by protein kinase C (PKC) and AMPK decreases the binding of calmodulin to eNOS and decreases activity (128); dephosphorylation at this residue (probably via PP2A or PP1, in response to agonists such as histamine or bradykinin), together with the activating phosphorylation on serine-1177, may coordinate these enzyme activities. It was also suggested that the phosphorylation state on Thr-495 may balance the output of NO and superoxide by eNOS (252).

Src kinase was also shown to phosphorylate eNOS on Tyr-83, with a resultant activation of the enzyme (135). Whether this is a direct effect of phosphorylation on catalytic activity or results from modulation of signaling through the recruitment of proteins with Src homology domains (SH3) is uncertain, but may have special relevance in the context of shear-mediated activation of Src.

Recently, oxidant radical production by mitochondria has been implicated as an element of signaling (338), e.g., in response to shear; the concurrent production of NO in response to shear, resulting in the formation of peroxynitrite, was implicated in the activation of PKC-ζ and downstream activation of AMPK through its upstream kinase, LKB1 (440); in this setting however, eNOS may be implicated upstream, but not downstream of AMPK.

**C) Regulation of NOS1 Activity by Glycosylation.** Similar to phosphorylation, growing evidence suggests that O-linked glycosylation may regulate the function of cytosolic and nuclear proteins and perhaps involves reciprocal modification of the same serine/threonine sites in some cases (434). Accordingly, hyperglycemia or glucosamine inhibits eNOS activity through postranslational glycosylation near the PKB/Akt phosphorylation site (108).

**D) Regulation of NOS1 Activity by Nitrosylation.** NOS can be self-inhibited by continuous high concentrations of NO (155); nitrotyrosylation of the functional NOS heterodimer causes monomerization and subsequently inactivation of the enzyme (343). In intact cells, however, eNOS targeted at the cell membrane is constitutively S-nitrosylated at the zinc-tetrathiolate cysteines, and agonist stimulation promotes denitrosylation together with translocation of the enzyme and its activation, paralleled with increased Ser-1177 phosphorylation (116).

Altogether, this emphasizes the importance of the subcellular localization of eNOS for its proper activation (e.g., through Akt-dependent phosphorylation) and sustained catalytic activity. In particular, its caveolar association and "chaperoning" through interaction with specific protein partners are key for these late regulatory steps, which are also mechanosensitive, and will be reviewed in the next section.
E) PROTEIN PARTNERS. A number of protein partners have been shown to interact with eNOS in diverse cellular compartments, where they modulate the enzyme’s activation through either direct steric inhibition of CaM binding [for caveolin (119, 248)] or interfering with upstream or downstream signaling events in the eNOS pathway. Accordingly, eNOS is part of a multiprotein complex (or “signalosome”) in which each partner associates with or dissociates from the complex in a temporally controlled manner for coordinate signaling. A common sequence shared by many stimuli activating eNOS (including shear) is the successive association of CaM (263, 296), 90-kDa heat shock protein (Hsp90) (141, 420), and Akt leading to the sustained activity of the enzyme in its Ser-1177 phosphorylated state (Fig. 3). Signal extinction will, in part, involve the subsequent association of phosphatases to dephosphorylate the enzyme. Upstream elements of signaling include many G protein-coupled receptors (109, 195), as well as integrins implicated in mechanotransduction of shear, such as PECAM-1 (see below). Downstream elements include protein partners involved in cellular trafficking of eNOS [such as NOSIP (98), NOSTRIN (452), endoglin (407), a cdc37 homolog (167); transmembrane ion fluxes [porin (394)], or arginine transport [CAT-1 (292)]; additional partners include caveolin (407), a caveolin-1 (167) and, more recently, SIRT-1 (271). Among these eNOS regulators, caveolin-1 and HSP90 will be discussed further given their implication in mechanotransduction, which will be detailed in later sections.

1) Caveolin. A direct interaction was shown between eNOS and recombinant caveolin-1, or GST-caveolin fusion proteins in vitro; eNOS is also communoprecipitated with anti-caveolin-1 antibodies from endothelial cell extracts (and anti-caveolin-3 antibodies in extracts of cardiomyocytes), and colocalized with caveolin in plasmalemmal caveolae (although deletion of caveolin-1 does not prevent membrane association of the prenylated eNOS) and maintains eNOS in an inactive state. Proper caveolar targeting is critical for eNOS activation by other signaling elements [e.g., G protein-coupled receptors (GPCRs) concentrated in the same locale], although the proportion of total cellular eNOS targeted to caveolae may vary among cell types and a substantial proportion of eNOS resides in the peri-Golgi area, where it was also found to be active. Consistent with the steric inhibition of eNOS by caveolin in vitro, mice genetically deficient in caveolin-1 exhibit a hyporesponsiveness to constrictor agonists and enhanced vasorelaxation ex vivo (106, 344), as well as decreased blood pressure variability in vivo (103), attributable to increased NO release. Likewise, our group showed that statins potentiate eNOS activity by decreasing caveolin-1 abundance in vitro and in vivo, at least in macrovascular endothelial cells where the caveolin pool is lower and the proportion of caveolin-bound eNOS is higher (51).

2) Hsp90. This heat shock protein of 90 kDa is a chaperone mostly expressed in the cytosol; it is very abundant (up to 1–2% of total cellular protein content) even in unstressed conditions and is involved in the proper folding of specific protein substrates, as well as in the conformational regulation of signal transducing molecules. These include members of the Src-kinase family of nonreceptor tyrosine kinases, Raf and other serine/threonine kinases, transcription factors and eNOS, among others. The essential role of Hsp90 is illustrated by the lethality of homozygous disruption of its only homolog in Drosophila and disruption of many signaling pathways.

FIG. 3. Calcium-dependent and phosphorylation-dependent activation of eNOS. In basilar conditions, caveolin (cav-1 and cav-3) maintains eNOS in its inactivated state, thereby limiting the production of NO. Increase in intracellular calcium in response to agonist stimulation (e.g., VEGF) leads to the disruption of the caveolin/eNOS inter-action by calcium-bound calmodulin. Hsp90 consecutively binds eNOS and favors the recruitment of Akt, which in turn phosphorylates eNOS on serine-1177. Activation of Akt results from the activation of signaling pathways including the stimulation of phosphatidylinositol 3-kinase (PI3K) in response to a variety of agonists and also statins. The phosphatase calcineurin is similarly recruited in the eNOS vicinity via hsp90 binding; the subsequent dephosphorylation of eNOS on threonine-405 also contributes to maintain NO release, independently of any further changes in intracellular calcium.
after heterozygous disruption. Hsp90 is associated with eNOS in the resting state, and stimulation of endothelial cells with VEGF, estrogen, histamine, statins, and shear stress increases the association between the two proteins, concomitant with enhanced NO production. The mechanism for this activation could involve an allosteric modulation of eNOS resulting in enhanced affinity of calcium/CaM for the enzyme, as was demonstrated for nNOS (33). Notably, Hsp90 also recruits agonist-activated PKB/Akt in the eNOS multiprotein complex and maintains Akt activity both by preventing proteosomal degradation of upstream PKB (433) and impeding protein phosphatase (PP)2A-mediated dephosphorylation of PKB (362), thereby maintaining late phosphorylation-dependent activation of eNOS and preventing calmodulin dissociation (50). Indeed, Hsp90 binds by nonoverlapping sequences of its M domain to both the COOH-terminal half of the oxygenase domain of NOS3 and PKB (amino acids 442–600 and 327–340 of Hsp90, respectively), supporting the proposition that Hsp90 serves as adapter for the kinase and its substrate (132). In contrast, binding sequences to the COOH-terminal half of the oxygenase domain of eNOS for caveolin (amino acids 350–358) and Hsp90 (amino acids 300–400) clearly overlap, arguing for reciprocal competition. Agonist stimulation or shear stress recruits more Hsp90 on eNOS, together with less eNOS binding to caveolin, compatible with competitive binding to a common region. Accordingly, CSD peptides completely abrogated the recruitment of Hsp90, Akt, and the phosphorylation of eNOS in endothelial cells. This emphasizes the importance of the temporal sequence and the interrelationship between these events, especially in the context of the additional reciprocal binding of caveolin and calmodulin to its nearby binding site (21). Using co precipitation methods with a carefully controlled temporal resolution, our group has shown that agonist stimulation (with VEGF) produces an early (0.5 min) disruption of the eNOS-caveolin complex, followed (at 2 min) by recruitment of Hsp90 on eNOS. These early steps are calcium dependent and necessary for the subsequent recruitment of activated Akt to phosphorylate eNOS. Once phosphorylated, eNOS activity becomes sustained and less calcium sensitive, compatible with the conformational change in its COOH-terminal tail, as detailed above (Fig. 4). Accordingly, our group showed increased recruitment of phosphorylated Akt, phosphorylated eNOS, and long-term NO-dependent capillary tube formation in endothelial cells transduced with Hsp90 constructs (50). Hsp90 also interacts with and activates PP2B/calcineurin, which additionally promotes eNOS catalytic activity through dephosphorylation of its Thr-495 residue in vitro and in vivo (225).

Finally, Hsp90 association with eNOS controls the balance between the enzyme’s production of NO versus superoxide anions (336). Recently, this mechanism has been implicated in the decreased oxidant radical production and preserved NO bioavailability in a high shear model of pulmonary hypertension in sheep (392).

4. Regulation of NOS3 activity by modulation of substrate and cofactors availability

Substrate (l-arginine) availability and NO scavenging by oxidant radicals determine the efficiency of NO production by all NOS isoforms. Under physiological conditions, the intracellular l-arginine concentration is in excess of the $K_m$ for eNOS (395), owing to l-arginine uptake via cationic amino acid transporters, including CAT-1, kinetically characterized as $y^+$ transport system. Under pathological conditions, NO-dependent endothelial dysfunction has been linked with reduced L-arginine transport, i.e., eNOS might become substrate limited (197). This may be due to competition with NOS for L-arginine as substrate by other enzymes coexpressed in the same cell type, including arginase, an enzyme converting L-arginine to urea and L-ornithine, and arginase decarboxylase, a mitochondrial enzyme which produces carbon dioxide and agmatine (169). Recently, the activity of arginase 1 was shown to be activated by S-nitrosylation on two regulatory cysteine residues (C168 and C303); notably, nitrosylation of arginase 1 was increased in vessels of old rats, together with upregulation of the inducible NOS, or iNOS, as the source of nitrosylating species, providing an attractive mechanism for the occurrence of endothelial dysfunction in aging vessels (361).

Upon deficiency of l-arginine and/or reduced tetrahydrobiopterin (BH$_4$), NOS no longer produces NO but instead transfers electrons to oxygen, thereby producing free oxygen radicals (127, 136, 199, 206, 421). Free oxygen radicals, in turn, can lead to further oxidation of BH$_4$ (as
well as other cofactors) and perhaps loss of heme following disruption of the zinc-thiolate bond, thereby aggravating the whole process (12). The precise role of BH$_4$ in the formation of NO still remains unclear, but it is likely to have an effect as an allosteric and/or redox cofactor (230). In atherosclerosis, during ischemia-reperfusion, and/or during inflammation, oxidation of BH$_4$ is paralleled with NOS-dependent generation of superoxide (i.e., NOS “uncoupling”) and subsequent nitrosative stress secondary to peroxynitrite formation. The critical role of BH$_4$ availability was highlighted from the protection against endothelial dysfunction upon endothelial-specific overexpression of DDAH-1 (95, 388, 416), and transgenic overexpression of DDAH-1 disease states, such as the degree of atherosclerosis (38, 95, 388, 416), and transgenic overexpression of DDAH-1 was shown to increase NO production and reduce blood pressure in vivo (95). Conversely, genetic deletion of DDAH was associated with endothelial dysfunction and high blood pressure, confirming the pathophysiological importance of this enzymatic system for vascular disease (241). Under certain conditions when NO formation increases, S-nitrosylation diminishes DDAH activity leading to accumulation of ADMA and subsequently NOS inhibition, as a type of regulatory feedback (240).

5. Endogenous NOS inhibitors

NOS activity can be decreased by endogenous substances such as asymmetric N$^\omega$-methylated derivatives of L-arginine, e.g., asymmetric dimethylarginine (ADMA). The intracellular ADMA concentration is controlled by dimethylarginine dimethylaminohydrolase (DDAH) (293). Levels of ADMA are known to correlate with certain disease states, such as the degree of atherosclerosis (38, 95, 388, 416), and transgenic overexpression of DDAH-1 was shown to increase NO production and reduce blood pressure in vivo (95). Conversely, genetic deletion of DDAH was associated with endothelial dysfunction and high blood pressure, confirming the pathophysiological importance of this enzymatic system for vascular disease (241). Under certain conditions when NO formation increases, S-nitrosylation diminishes DDAH activity leading to accumulation of ADMA and subsequently NOS inhibition, as a type of regulatory feedback (240).

D. The Fate of NO in the Vasculature: Regulation of Its Bioactivity

The bioavailability of NO depends not only on its production but also its transport, storage, and interaction with molecules on its path, e.g., hemoglobin in circulating erythrocytes of the vascular lumen that critically limit its diffusibility (for review, see Ref. 368). Chemical reactions driving its inactivation mostly belong to oxidative and nitrosative metabolism of NO (204).

1. Oxidative metabolism of NO

Upon reaction with oxygen in the presence of low superoxide anions (O$_2^-$) levels, NO more readily forms nitrogen dioxide (NO$_2$) which, upon reaction with another NO molecule, forms the nitrosating species N$_2$O$_3$, subsequently hydrolyzed to nitrite. In the presence of high O$_2$ levels, NO equimolarly reacts with O$_2^-$ to form peroxynitrite (ONOO$^-$), which can decompose to nitrate, or lead to the formation of NO$_2$ and highly damaging hydroxyl radicals.

However, peroxynitrite may not simply be detrimental because low concentrations of peroxynitrite were suggested to play subtle roles in signal transduction processes (83, 281). Furthermore, low levels of peroxynitrite could be detoxified by enzymatic and nonenzymatic systems (57).

2. Nitrosative metabolism of NO

Many signaling functions of NO are mediated by stimulation of soluble guanylyl cyclase (sGC) with formation of the second messenger cGMP. In addition, thiol nitration, or “S-nitrosylation,” a posttranslational protein modification independent of the sGC/cGMP pathway, has been implicated in several control mechanisms in health and disease (133), such as oxygen delivery to tissues, modulation of the function of transcription factors, enzymes, membrane receptors and ion channels, and participates in transnitrosation reactions. S-nitrosylation can occur by means of oxidative nitrosylation (rather than NO oxidation) (56), reaction with peroxynitrite, NO-thiol interactions in the presence of electron acceptors, and transnitrosation reactions.

Notably, not all protein cysteine residues with a free-thiol become nitrosylated, but the residue environment critically determines its reactivity. Since the rate of S-nitros(yl)ation is not enzymatically determined, the concentration of NO and the related species as well as its localization (i.e., subcellular compartmentation) are critical. Accordingly, some S-nitrosylatable proteins are topographically associated to nNOS or eNOS, the subcellular targeting of which, understandably, becomes key in signaling specificity and is tightly regulated (see above). Likewise, denitrosylation is tightly controlled in cells through the activity of a specific denitrosating enzyme, S-nitrosogluthathione reductase (337).

Altogether, this complex, multisite regulation of eNOS activity emphasizes the need to carefully consider parameters such as cellular compartment, cell status (i.e., proliferating versus quiescent), type, and intensity of stimulus (e.g., oscillating versus unidirectional shear), availability and/or activity of substrate, cofactors (e.g., BH$_4$), allosteric modulators (calmodulin, caveolin), chaperones (Hsp90), as well as other regulators (SODs, ADMA) of NO bioavailability or eNOS itself, to predict the overall NO output. Nevertheless, many studies of eNOS activation in vitro or in vivo, as reviewed above, demonstrate an amazing coordinate regulation of many of these parameters for
optimal enzyme activity. The next section will review how such sophisticated regulation can be orchestrated by the molecular machinery that translates mechanical stimuli into intracellular signaling.

II. ENDOTHELIAL NITRIC OXIDE SYNTHASE AND MECHANOTRANSDUCTION IN THE ENDOTHELIUM

The vascular endothelium is permanently subjected to mechanical forces in the form of cyclic stretch and shear stress, respectively, due to the pulsatile nature of blood pressure and flow. Much of the chronic cyclical mechanical strain is experienced by the smooth muscle cells of the media, whereas shear stress, the tangential component of the hemodynamic forces, acts primarily on endothelial cells. Endothelial shear stress is proportional to the product of blood viscosity and the velocity gradient between adjacent layers of flowing fluid. The cardiac cycle, the velocity of the flow, the blood vessel geometry, and the presence or absence of lumen stenosis are accountable for the arterial hemodynamics and the different patterns of shear stress.

Endothelial cells are ideally located at the border between bloodstream and the vessel wall to constitute a permanent signaling interface that regulates major homeostatic properties, among them hemostasis, thrombosis, inflammatory responses, vasoactivity, vasopermeability, and vascular remodeling. Specifically, mechanotaxis (246) refers to fluid shear stress-induced migration of endothelial cells in the direction of flow, a critical step of angiogenesis and postangioplasty wound healing. Many of the above effects are related to increased NO release by endothelial cells exposed to shear stress. In addition, fluid filtration across the endothelium (transendothelial flow), another important regulator of endothelial functions, was also proposed to trigger NO production, presumably via shear stress within intercellular clefts (60, 70).

Exercise increases heart rate, which in turn increases blood flow and vascular shear stress. In the vascular tissues, several studies have reported an important role of endogenous production of NO in the beneficial effect of exercise. In vivo studies in animals have shown more than a decade ago that exercise results in an increased vascular NO production. As early as 1993, Hintze and colleagues (426) reported that chronic exercise training in dogs (2 h each day at a speed of 10.9 km/h for 7 days) enhanced acetylcholine-induced vasodilation in a nitro-L-arginine-dependent way (426). The enhancement of NO-dependent dilation by exercise training was simultaneously identified in rats (101). In humans, evidence for a direct relationship between exercise, NO, and functional improvement of the endothelial function was provided by several independent studies. The relevance of improved endothelial function for coronary blood flow was first confirmed in patients with stable coronary artery disease (CAD) (164) and in others with chronic heart failure (CHF) (162). The exercise protocols consisted for the CAD patients of 4 wk of six bicycle ergometer exercise units of 10 min/day and for the CHF patients, in 6 mo of home-based ergometer exercise training for 20 min/day. The CAD patients responded to intracoronary acetylcholine with a significantly attenuated paradoxical vasoconstriction, and the CHF patients presented a significant reduction in peripheral resistance. In another study, the same group showed that endothelial dysfunction in patients with CHF could be corrected by both dietary supplementation with L-arginine (the substrate for NOS) and regular physical exercise (4 wk of daily hand-weight training). Both interventions together produced additive effects with respect to endothelium-dependent vasodilation of the radial artery (163). Altogether, these studies emphasize the critical role of NO in the vascular benefit of exercise secondary to increased shear forces.

A. Molecular Machinery of Shear Stress Sensing in the Endothelium

Numerous studies have reported a biphasic production of NO in response to flow: application of shear induces a burst of NO, while the steady shear that follows evokes a production of NO maintained as long as the stimulus is applied (70, 220). This has later been linked to tyrosine kinase (291) activity and more specifically to phosphorylation of eNOS serine-1177 and serine-633 by the kinases Akt (104), PKA (40, 41, 105), PKC (430), or AMPK (129). The so-called “calcium-independent” activation of eNOS in response to shear, which results in maintained production of NO, was attributed to the activation of P3K and Akt and the subsequent phosphorylation of eNOS on Ser-1177 (104, 126). Besides, an enhanced eNOS expression has been documented in vessels of exercised animals including rats (101, 400) and pigs (193, 436). The events that link physical stretch to NO production and, more generally, sensing mechanisms by which cells perceive mechanical stimuli to convert them into a physiologically relevant response, more probably arises from a concerted action of multiple mechanotransducer molecules. The major molecular elements that orchestrate this initiation process in endothelial cells include the extracellular matrix (ECM), cell-ECM adhesion, cell-cell adhesion complexes, and membrane components such as ion channels, caveolae, and surface receptors as well as cytoskeletal filaments (see Fig. 5). Indeed, although fluid shear stress is strictly applied on the luminal surface of endothelial cells, the shear forces are transmitted throughout the cell (nucleus and organelles) to cell junction and to cell-extracellular matrix adhesions. These
structures are all interconnected to trigger a number of events, including NO production. This involves activation of ion channels, heterotrimeric G proteins, Src family, and vascular endothelial growth factor receptor 2 (VEGFR2) tyrosine kinases in or at the membrane. Downstream effectors include extracellular signal-regulated kinases (ERKs), c-Jun NH\(_2\)-terminal kinases (JNKs), p38 mitogen-activated protein kinase, AKT serine/threonine kinases, and PKC. Some of these signaling events are translated to activation of transcriptional regulators such as c-fos, c-jun, and NF\(_\text{B}\). Downstream increases of gene expression for intracellular adhesion molecule (ICAM), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-\(\beta\), and NOS, in turn, mediate slower responses to shear.

1. Glycocalyx

One of the most striking properties of this surface glycocalyx is its ability to sense shear forces and to translate them into intracellular responses (e.g., mediating shear stress-induced NO release). The glycocalyx may transduce extracellular signals via proteoglycans and associated core proteins, which interact with the cytoskeleton. Only a few experimental studies have attempted to characterize the precise role of the glycocalyx in mechanotransduction; the first evidence was provided by experiments where specific components were degraded, followed by a reassessment of function. Indeed, flow-dependent dilation of perfused rabbit mesenteric arterioles is inhibited after preincubation with neuraminidase, which removes part of the membrane glycocalyx (332), and hyaluronic acid glycosaminoglycans seem to detect and amplify the shear forces that trigger the endothelium-derived NO production in canine femoral arteries (283). In a very recent study, the use of albumin in an isolated perfused organ model (constant pressure perfusion mode) led to a substantial increase in coronary flow. This phenomenon seems to result largely from NO production induced by shear stress and transmitted via the glycocalyx on the endothelial surface as it was possible to inhibit the flow-dependent dilatation by stripping away the main part of the glycocalyx almost as efficiently as by blocking NOS (186). In addition, it was demonstrated that heparan sulfates participate in pressure-mediated mechanotransduction in lung microvascular endothelial cells. Heparanase pretreatment significantly reduced the pressure-induced increase in ROS production, suggesting that cell-surface heparan sulfates directly participate in mechanotransduction that results in NO/ROS production and increased permeability (110). A study recently demonstrated that specific depletion of heparan sulfate (HS), hyaluronic and sialic acid but not chondroitin sulfate (CS), blocked the shear-induced NO production (312). This confirms Florian’s findings that enzymatic removal of HS abolishes the NO production evoked by steady or oscillatory shear stress as measured in BAECs (131). Pahakis et al. (312) interpreted these data in the context of Davies’ centralized and decentralized models of mech-

![Diagram of endothelial cells showing elements of shear mechanosensing](image-url)
brane heterodimers working in pairs composed of an integrin refers to a family of more than 24 transmembrane proteins activation or expression. The chemical composition of ECM proteins bind to different integrins and could activate different signaling pathways. Whereas EC plated on fibronectin or fibrinogen activate NFκB in response to flow, cells on collagen or laminin do not (308). Shear stress causes recruitment of Shc by αβ1 in EC plated on laminin but not fibronectin or vitronectin while αβ3-mediated mechanotransduction is triggered on cells plated on fibronectin and vitronectin but not collagen or laminin (187). Moreover, a shear-induced increase in eNOS mRNA and protein expression was demonstrated in endothelial cells seeded on laminin (147). This was abolished by the peptide YIGSR, which blocks the cellular binding to laminin I via a 67-kDa laminin-binding protein that colocalizes with stress fibers of the cytoskeleton and with part of the focal adhesion kinase complex, α-actinin and vinculin.

B) PLATELET ENDOTHELIAL CELL ADHESION MOLECULES. In addition to widely expressed junctional adhesion molecules, endothelial cells also contain the immunoglobulin family receptor platelet endothelial cell adhesion molecule (PECAM-1). Cell-cell adhesion sites constitute with focal adhesion sites, rigid regions of the plasma membrane particularly enriched with signal transduction molecules that could participate in mechanotransduction despite the lack of direct exposure to fluid flow. Shear stress is therefore transmitted from the apical surface through the cytoskeleton to points of attachment that allow the endothelial cells to resist to physical forces; consequently, points of attachment undergo changes in mechanical tension and could serve as mechanotransducers.

PECAM-1 (also called CD31) is the cell-cell adhesion molecule most abundantly expressed in endothelial cells. Once a cell-cell contact is constituted, PECAM-1 accumulates on contact sites, and via homophilic extracellular domains, establishes interactions with PECAM-1 expressed on neighboring cells. In addition to key roles in platelets/leukocytes-endothelial cells interaction, and in forming and maintaining the contact-inhibited state in endothelial cells, PECAM-1 is a logical candidate component of shear signaling pathway. Unequivocal evidence came from studies demonstrating that flow-mediated dilation is impaired in gracilis muscle (20) and coronary arteries (20, 253) from PECAM-1 deficient mice, as is the shear-induced increase in intracellular cGMP (129) (see Fig. 6). Earlier observations had already argued in favor of such a role for PECAM; not only is PECAM concentrated at cell junctions, in regions highly receptive to shear forces, but it is connected to remote cellular sites through the actin cytoskeleton. PECAM was also reported to co-localize with eNOS in the cell membrane (151), and finally, PECAM is tyrosine phosphorylated in its short intracellular domain in response to shear. PECAM contains two distinct immunoreceptor tyrosine-based inhibitory motifs (ITIMs) centered around the targeted tyrosine residues and the SH2-containing protein tyrosine phospho-
tase-2 (SHP-2) is recruited to ITIM-phosphorylated PECAM-1 in endothelial cells (270). The adaptor molecule Gab1, that translocates from the cytosol to the endothelial cell junctions in response to flow, has also been shown to colocalize with PECAM-1 and SHP-2 at endothelial borders after exposure to fluid shear stress (309). This favors the possibility that Gab1/PECAM-1 and SHP-2 form ternary signaling complexes in response to shear and is in agreement with a role for PECAM-1 as a scaffold. Of note, Fleming's group demonstrated recently that shear stress elicited the formation of a signalosome that includes Gab1, eNOS, SHP2 and the catalytic subunit of PKA, participating to the PKA-dependent phosphorylation of eNOS (105).

NO plays an important role in the dynamic regulation of the intercellular junctions of the endothelium. An enrichment of eNOS was observed at these junctions in bEnd.3 endothelial cells where PECAM and eNOS colocalize (151). In PECAM-1 genetically deficient mouse aorta, the eNOS junctional localization is absent (112). Interestingly, junctional eNOS staining is also lost in wild-type aorta near intercostal branches, regions reportedly exposed to changing levels of fluid shear stress. The physical interaction between PECAM-1 and eNOS, assessed by reciprocal coimmunoprecipitation, decreases eNOS activity in vitro. Single pulse and pulsatile shear stimulation induce a transient dissociation of PECAM-1/eNOS and a concomitant augmentation of eNOS activity as measured by cGMP production (112). However, the dynamics of the PECAM-1/eNOS colocalization and their signaling consequences still raise major controversies. Indeed, Fleming et al. (129) reported that fluid shear stress induces tyrosine phosphorylation of PECAM-1 that enhances its association with eNOS. Downregulation of PECAM-1 using a siRNA approach attenuates the shear stress-induced phosphorylation of Akt and eNOS and accumulation of cGMP, as does the absence of PECAM-1 in endothelial cells from PECAM-1-deficient mice (see Fig. 6).

Recently, it was shown that high shear stress applied in vivo upregulates PECAM-1 without modification of the relative distribution of eNOS to the PECAM-1-positive cell membrane region, indicating that eNOS and PECAM-1 may be increased to similar amounts in response to shear (79). Another recent study added to the complexity of PECAM implication in flow-mediated dilation. Gutterman and collaborators (253) provided evidence that although flow-mediated dilation is reduced in coronary arteries from PECAM-1 knockout (KO) mice, the NOS-dependent component is not; in fact, the levels of NO and O$_2$$^-$$^2$ are elevated, and this accounts for the formation of ONOO$^-$$^2$, which would contribute to the impaired EDHF and cyclooxygenase (COX) components of flow-mediated dilation observed in PECAM-1-deficient vessels (253).

PECAM may also be responsible for integrin activation in response to fluid shear stress. Tzima et al. (411) recently demonstrated that PECAM-1 (which directly transmits mechanical force), VE-cadherins (which function as adaptor molecules), and VEGFR2 (which activates PI3K and is closely linked to integrin activation, as mentioned above) form a mechanosensory complex within seconds after the onset of shear stress. Therefore, PECAM seems a central piece in a signalosome induced by shear forces that contain eNOS and mediate vasorelaxation, although the latter may involve more than NO as a mediator.

3. The endothelial cytoskeleton

Functional and structural adaptations to shear forces including activation of ion channels, G proteins, and transmembrane adhesion occur within seconds after the
onset of shear stress. As all these events occur almost simultaneously at the apical, junctional, and basal surfaces of the cell, it is very probable that the cell structure itself plays a key role in transmitting mechanical forces throughout the cell to specific locations, to promote assembly and activation of signaling complexes. Besides, a hallmark of all EC responses to fluid shear stress is the rearrangement of microfilaments and microtubules and their elongation along the direction of shear. A specific role has been attributed to the intermediate filament protein vimentin in vascular endothelium. The evidence was provided by the phenotype of vimentin-deficient mice (V−/−) that exhibit a blunted, acute, flow-induced arterial vasodilatation (173, 366); this may be the consequence of altered focal contact dynamics, as in vimentin siRNA-treated cells exposed to flow, the size, structure, and motility of focal contacts were altered (409). In the late 1990s already, initial evidence showed that intermediate filaments effectively mediated force transfer to the nucleus (261); force redistribution to the karyoskeleton under flow generates changes in gene expression through interactions among nuclear intermediate filaments proteins, nuclear lamin DNA, and histones. Dystrophin deficiency also induces a selective defect in flow-dependent mechanotransduction, therefore attenuating flow-mediated dilation and eNOS expression in resistance arteries (256, 257).

Focal adhesion sites (Fas), where integrins colocalize with extracellular matrix, cytoskeleton proteins and signaling molecules constitute key components of the EC response to flow, especially in the context of the “tensility” model proposed by Ingber (261), which suggests that integrins constitute the mechanoreceptor and that the underlying signal is transduced through the cytoskeleton. Accordingly, shear stress induces a polarized recruitment of FAK at new Fas and remodeling of existing Fas, which may be crucial for shear-evoked directional migration (246). Finally, Tai et al. (396) demonstrated that shear stress stimulates PYK2 tyrosine phosphorylation through a ROS and intracellular calcium increase in endothelial cells (396). This shear stress-induced PYK2 pathway is important in endothelial cell functions related to focal adhesions as PYK2 is localized at focal adhesions and participates in ERK1/2 activation.

4. Signaling through kinases and G proteins

Many of the signal transducing molecules needed to integrate mechanical signals and to convert them into biochemical events concentrate in the focal adhesion complex, as mentioned above. Several recent studies demonstrate that tyrosine kinases (FAK and c-Src) constitute part of the mechanotransduction machinery in endothelial cells submitted to flow. Inhibition of FAK signaling by interfering with autophosphorylation at Tyr-397 impairs flow-induced phosphorylation of Akt (Ser-473) and eNOS (Ser-1179) and flow-induced dilation of coronary arterioles (218). This suggests that FAK plays a pivotal role in flow-induced dilation by regulating Akt and eNOS activation and may constitute the link between shear stress-evoked integrin signaling and eNOS activation. Numerous studies have documented the flow-stimulated phosphorylation of eNOS at serine-1179, via the P3K-Akt-eNOS signaling pathway (104, 126), as detailed above, and these studies have been completed by the demonstration that the pathway requires the Src kinase-dependent transactivation of VEGFR2 (flk-1) (191). Inhibition of VEGFR2 kinase with selective inhibitors blocks flow-induced activation of Akt and eNOS and production, while decreasing VEGFR2 expression with antisense VEGFR2 oligonucleotides significantly attenuates activation of Akt and eNOS, confirming the key role of Flk-1 in eNOS flow-induced activation. This emphasizes the key role of VEGFR2 in mechanotransduction of shear to eNOS activation, as supported by its identification in a shear-induced signalosome, as discussed above. Downstream of VEGFR2, the docking protein Gab1, when tyrosine-phosphorylated in response to shear associates with the P3K subunit p85 and mediates Akt and eNOS phosphorylation (192). Recent work from Berk’s group suggests that flow-induced Akt and eNOS activation require Glutaredoxin (Grx) (425). Indeed, overexpression of Grx increases Akt and eNOS phosphorylation, while pretreatment of endothelial cells exposed to a steady laminar flow with a Grx inhibitor or a Grx siRNA reduce both. Additional effects on other kinases that can be glutathionylated and could impact on NOS activity (namely PKA) cannot be ruled out.

As mentioned above, shear stress evokes the tyrosine phosphorylation and kinase activity of FAK, followed by association of FAK with Grb2 (247), which are critical for shear activation of ERK and JNK and may impact on shear modulation of more profound aspects of vascular biology. Indeed, shear activated ERK1/2 is attenuated in endothelial cells expressing a dominant negative FAK mutant (247). Other studies provide evidence that the shear-dependent activation of integrins leads to the Flk-1-Cbl association, a recruitment that is essential for ERK activation (427). Blockade of Shc results in attenuation of the shear-induced activation of ERK and JNK and the consequent gene transcription. Shay-Salit et al. (377). showed that shear stress induces a rapid induction as well as nuclear translocation of the VEGFR 2 and promotes the binding of the VEGFR2 and the adherens junction molecules, VE-cadherin and β-catenin, to the endothelial cytoskeleton, as mentioned above. These changes are accompanied by the formation of a complex containing the VEGFR2-VE-cadherin-β-catenin. In endothelial cells lacking VE-cadherin, shear stress did not augment nuclear translocation of the VEGFR2 and phosphorylation of Akt1.
and P38 MAPK as well as transcriptional induction of a reporter gene regulated by a shear stress-responsive promoter. This suggests that VEGFR2 and the adherens junction act as shear-stress cotransducers, mediating the transduction of shear signals into gene expression in vascular endothelial cells.

The small GTPases (namely, Rac, Rho, and cdc42), acting downstream of integrins, are critical for both cytoskeletal reorganization and changes in gene expression in response to shear stress (recently reviewed by Tzima et al., Ref. 410). Specifically, activation of the small GTPase Ras by shear stress peaks at 1 min and returns to baseline after 10 min (250). Moreover, shear stress increases the amount of S-nitrosylated GTPase p21Ras in endothelial cells (178), thereby participating in its long-term activation.

Numerous studies have demonstrated the stimulation of PKC isoforms in endothelial cells exposed to shear which affects both their expression and intracellular distribution (179). PKCs are important in all stages of NO release (early and late production) as demonstrated by Wedgwood et al. (430) in ovine fetal pulmonary arterial endothelial cells exposed to shear stress in the presence of the PKC inhibitor calphostin. The initial release of NO would be related to the activation of preformed enzyme and is more probably associated with eNOS phosphorylation at serine-1177 as proposed by Partovian et al. (318). In contrast, the second NO release coincided with an increase in eNOS mRNA and protein and was stimulated by factors that regulate eNOS gene transcription.

5. Ion channels

Some of the many ion channels present on the plasma membrane of endothelial cells are activated by flow. Flow-sensitive channels have been considered for a long time as major flow sensors as their activation is one of the most rapid response to shear stress (for review, see Refs. 25, 72). Early studies of Olesen et al. (307) have identified a K+ selective, shear stress-activated ionic current in single arterial endothelial cells exposed to controlled levels of laminar shear stress, that led to cell membrane hyperpolarization. This K+ current varies in magnitude and duration as a function of shear stress, desensitizes slowly, and recovers rapidly and fully upon cessation of flow (307). Treatment of endothelial cells with blocking agents to the K+ channel such as barium chloride or tetraethylammonium inhibited the induction of NO by shear stress and the induced expression of eNOS and TGF-β (305, 306, 412), supporting the importance of this channel in mediating shear stress responsiveness.

In addition, flow activates an outward-rectifying Cl- channel generating a membrane depolarization that rapidly follows the transient K+ current evoked-hyperpolarization (143). The notion that K+ channels respond more quickly to shear stress than the Cl- current may explain the differential activation of these channels depending on the temporal pattern of flow exposure. How these events regulate downstream signaling remains unclear. A predictable target may be changes in intracellular Ca2+ concentration resulting from alterations of cell membrane potential generated by flow-sensitive ion channel activation. Moreover, Chatterjee and colleagues (72, 73) observed that cessation of flow results in endothelial cell membrane depolarization and subsequent generation of ROS, Ca2+ release, and activation of NOS. Shear stress also directly activates cation channels. A recent study of Kohler et al. (209) demonstrated that shear stress-induced dilation of the rat carotid artery involves a calcium current through TRPV4 channels. Although [Ca2+]i increases could directly impact on NO production, it may also act on nonspecific cation channels, Ca2+-activated K+ channels, and Cl- channels (298) or other intracellular signaling cascade(s). Of note, the NO production evoked by laminar shear stress exceeds that produced by maximal increases in intracellular calcium, arguing for the implication of additional mechanisms.

Nevertheless, in cultured endothelial cells, shear stress is a well-established trigger for increases in intracellular calcium concentration (72, 73, 144, 171, 172, 378). Application of a step increase in shear stress from 0.08 to 8 dyn/cm2 to confluent BAEC monolayers resulted in a transient increase in [Ca2+]i, the magnitude of which increased in direct proportion to the applied shear stress (378). However, purely oscillatory flow fails to increase [Ca2+]i in BAEC monolayers, while steady flow, as well as nonreversing and reversing pulsatile flows, increased [Ca2+]i. The dynamics of the response are dependent on the flow pattern. Both internal Ca2+ release and extracellular Ca2+ entry appear to be involved in these [Ca2+]i increases. Ca2+ responses to laminar flow appear less clear when measured on pressurized vessels. In cremaster arterioles, dilation to intraluminal flow is associated with an increased endothelial [Ca2+]i (117). In contrast, only minor changes in endothelial [Ca2+]i were observed concomitantly with a NO-mediated dilation in coronary or gracilis skeletal muscle arterioles exposed to flow (292, 414). Of note, shear stress-activated Ca2+ influx is completely abolished after removal of sialic acid in the glycoscalyx of endothelial cells, as discussed above (298). Therefore, changes in calcium dynamics may be another mechanism for the differential response of endothelial cells to various flow patterns, e.g., oscillatory versus pulsatile flow, although its applicability to pressurized vessels in vivo remains doubtful.

A critical question that remains is how flow activates ion channels. As recently reviewed by Barakat et al. (25), candidate mechanisms of flow-sensitive ion channel activation may include I) a physical interaction between flow and the ion channel with potential effect on channel confor-
mation; 2) membrane bilayer distortion that alters channel conformation influencing opening and closing rates; and 3) effect of flow on cellular cytoskeleton to which flow-sensitive ion channels are directly coupled to open the channel pore.

6. Membrane bilayer/caveolae

A role for caveolin-1 in mechanosensing was convincingly demonstrated from the description of the phenotype of mice genetically deficient for caveolin-1 (cav-1⁻/⁻). Vessels from these mice had impaired shear stress regulation of their diameter both in vitro and in vivo, and these defects were rescued when endothelial caveolin-1 was reexpressed (447). In addition to the critical role of direct interaction with caveolin-1 or caveolar compartmentation of eNOS (that will be discussed later), several data point to caveolae as players in mechanotransduction in vascular cells where their density is particularly high. Accordingly, caveolae may represent mechanosensing microdomains converging shear stress to intracellular signaling events.

Chronic exposure to blood flow dynamically regulates caveolar density (315); exposure of BAEC to shear stress for 1 h increased invaginated caveolae by threefold (315), while longer exposure (1–3 days) increased the total number of caveolae by 45–48% above static conditions (45). Whereas caveolin-1 shows a balanced location between plasma membrane and Golgi under static conditions, chronic shear stimulates caveolae formation by translocating caveolin-1 from the Golgi to the plasma membrane (45). Enhanced caveolin expression at the luminal endothelial cell surface is concomitant with a marked increase in caveolae surface density in endothelial cells subjected to sustained physiological shear stress (351) (see Fig. 7). A preferential location at the luminal side of the endothelial cells reinforces the pivotal role of caveolae as mechanosensors. Similarly, a crucial role of caveolae has also been demonstrated in vascular smooth muscle cells subjected to cycle stretch. Specifically, mechanosensitive activation of the PKC/Akt pathway requires intact caveolar structure and caveolin-1 (372). Interaction of caveolin-2 with caveolin-1 could also modulate mechano-signal transduction in response to shear. Under static conditions, a pool of caveolin-2 colocalizes with the cis-Golgi, but unlike caveolin-1, this Golgi-associated pool is maintained after a day of shear exposure (44).

Early work documented that cholesterol or cholesterol-rich membrane compartments play a key role in shear stress sensing, as shear-dependent activation of ERK, but not JNK, in BAEC was inhibited by cyclodextrin, a membrane-impermeable cholesterol binding drug (315). This inhibition is not related to membrane permeabilization and is independent of the number of invaginated caveolae (314). Similarly, a neutralizing caveolin-1 antibody was found to inhibit shear-dependent activation of ERK (but not JNK). Shear-sensitive signaling molecules that regulate the ERK pathway therefore seem to be assembled in caveolae-like domains by binding to the scaffolding domain of caveolin-1 in their inactive state. Changes in shear stress level may then specifically trigger rapid, organized, and compartmentalized signaling cascades to activate ERK, but not other pathways (315).

Although the basal level of caveolin phosphorylation is not affected by mechanical forces (380), shear stress appears to actively modulate caveolin-1 phosphorylation. Indeed, when measuring the phosphorylation of caveolin in whole cell lysates, Shin et al. (380) showed that caveolin-1 is acutely and transiently dephosphorylated on tyrosine-14 after exposure to shear stress (probably following tyrosine phosphatase μ transient activation). In contrast, caveolin-1 specifically located at the luminal membrane of BAECs is phosphorylated on tyrosine-14 when exposed to 10 dyn/cm² of laminar shear in a β1-integrin-dependent way (340), as this is inhibited by the β1-integrin blocking antibody J1B1A. The consequence of caveolin tyrosine phosphorylation is the recruitment of Src-like kinase to phospho-caveolin-containing integrin complexes. Ultimately, these events culminate in a downstream signal that initiates cytoskeletal rearrangement (340). While β1-integrin was scarcely detected in caveolae derived from BAECs kept in static culture, shear stress induces β1-integrin transposition to the caveolae. Moreover, cholesterol depletion attenuated integrin-dependent caveolin-1 phosphorylation, Src activation, and Csk association with β1-integrin. This demonstrates that β1-integrin-mediated mechanotransduction is mediated by the assembly of a protein complex in caveolar microdomains (339). The next section will examine the specific implication of caveolin-1 and eNOS in this complex.

B. Caveolar Compartmentation of eNOS: Relationship to Its Short-Term Activation by Shear

A proper intracellular localization of eNOS is mandatory for its regulated activity, raising the question of its caveolar versus intracellular distribution in response to shear stress. Subcellular fractionation to purify luminal endothelial cell plasma membranes and their caveolae directly from rat lungs has revealed that eNOS is concentrated and enzymatically active in caveolae. Interestingly, increasing vascular flow and pressure in situ rapidly activates caveolar eNOS with apparent eNOS dissociation from caveolin and association with CaM leading to increased activation (350) (see Fig. 7). Rizzo et al. (351) reported that 90% of detected eNOS colocalizes to caveolar vesicles in shear preconditioned endothelial cells.
Acute shear stress applied to static endothelial cell cultures induces the phosphorylation of both caveolin-1 (phospho-tyrosine-14) and eNOS (phospho-serine-1177) present at the luminal cell surface. Additional challenge to flow-acclimated cultures enhances and accelerates phosphorylation of both caveolin-1 and eNOS, the latter persisting even after 6 h of flow preconditioning (351). Caveolin-1-deficient mice have impaired flow-dependent arterial remodeling and vasodilation (447), as previously mentioned. Interestingly, the abnormal remodeling of vessels from \textit{cav-1}^{-/-} mice in response to flow is reminiscent of similar alterations of vessels from eNOS-deficient mice. Moreover, Yu et al. (447) showed that the phosphorylation (phospho-serine-1177) and subsequent activation of eNOS was reduced in \textit{cav-1}^{-/-} carotids without alteration of eNOS localization, suggesting that flow activation of upstream kinases is impaired. Importantly, these effects were rescued in carotids from \textit{cav-1}^{-/-} mice reconstituted with a transgene expressing caveolin-1 specifically in endothelial cells, supporting the crucial role of caveolin-1 in response to shear stress in vivo (447). eNOS is probably not the only protein affected by caveolin-1 deficiency. Specifically, we have previously shown that VEGFR2, which was recently shown to be crucial in...
mechanosensing (377), as reviewed above, was mislocalized together with an impaired ability to activate eNOS and ERK in aortic endothelial cells from caveolin-1-deficient mice (382). Therefore, caveolin-1, in addition to its direct allosteric modulation of eNOS activity, also acts as a primary scaffold protein for the assembly of a shear-sensitive signalosome connecting integrins to other mechanosensitive signaling proteins, such as VEGFR2 that, in turn, activate eNOS phosphorylation and promote its sustained catalytic activity. This probably involves the recruitment to the complex of kinases, such as Akt to phosphorylate eNOS and tyrosine kinases to phosphorylate caveolin-1 and recruit other Src-like kinases mediating cytoskeletal rearrangements. Again, the central role of eNOS is supported from the similarly abnormal vascular remodelling in NOS3−/− (356) and cav-1−/− mice (447).

C. Differential Effects of Laminar/Oscillatory Shear on Endothelial NO Production and Bioavailability

Distinct roles are attributed to laminar and oscillatory flow patterns in the regulation of the cell cycle of vascular endothelial cells. While steady laminar flow, as in the straight parts of the arterial tree, is known to be atheroprotective, the disturbed, oscillatory flow that occurs in branching points and the aortic root may promote the development of atherosclerosis. In coronary arteries where shear-mediated dilation is NO dependent, oscillatory flow without a net forward component does not cause flow-dependent dilation except after incubation with SOD. Indeed, as reported by Kelly and Snow (203), in the presence of SOD, endocardial vessels dilate even to a greater degree in response to oscillatory flow than to steady flow. Therefore, they proposed that steady flow induces the production of NO that overwhelms any possible concomitant O$_2$

$^*$ production, resulting in NO-dependent dilation, whereas during oscillatory flow, O$_2$

$^*$ production prevails and impairs the bioavailability of the simultaneously produced NO.

Moreover, asynchronous hemodynamics as observed in the coronary circulation was demonstrated to induce a differential pattern of gene expression compared with regions subjected to more synchronous flow. Indeed, coronary arteries have reduced eNOS mRNA levels compared with aortic regions, whereas endothelin mRNA shows an opposite trend (88). Fully reversing shear stress is known to inhibit eNOS activation and NO release while enhancing oxidative stress (125). Therefore, oscillatory stress is proposed to induce an endothelial dysfunction marked by an important deficiency in NO bioavailability, whereas unidirectional shear would preserve endothelial function. Ex vivo exposure of porcine carotid segments to oscillatory versus unidirectional shear reduces eNOS gene and protein expression and NO-mediated vasodilation. In addition, an altered eNOS phosphorylation profile combining increased threonine-495 and decreased serine-1177 phosphorylation was observed in response to bradykinin (138). As mentioned above, these data may seem at odds with the results of Cai et al. (64) who demonstrated an upregulation of eNOS expression after oscillatory shear. Contrary to laminar shear stress that upregulates eNOS via a signaling pathway downstream of c-Src but independent of H$_2$O$_2$ and CaMKII, oscillatory shear stress-evoked eNOS upregulation is dependent on the simultaneous production of O$_2$

$^*$ and H$_2$O$_2$, as it is abolished upon treatment with PEG-catalase. Despite or due to this up-regulation of eNOS and NO production, oscillatory shear may enhance atherosclerotic lesion formation as the simultaneous production of NO and O$_2$

$^*$ would facilitate the production of peroxynitrite and ensuing nitrosative stress.

Guo et al. (158) recently tried to elucidate the molecular basis underlying the regulation of the endothelial cell cycle by these distinct flow patterns. On the basis of flow-channel experiments, they demonstrated that laminar flow causes a transient activation of both AMPK and Akt, while oscillatory flow activated only Akt, with AMPK being maintained at its basal level. Measurements of phosphorylation of mTOR Ser-2448 and S6K Thr-389 showed that AMPK, by counteracting Akt under laminar flow, induced only a transient activation of S6K. Under oscillatory flow, however, because of the lack of concurrent AMPK activation, S6K is activated in a sustained manner. These results support the proposition that AMPK activation would attenuate cell cycle progression in response to both laminar and oscillatory flows, and that, in contrast, AMPK inhibition would promote endothelial cell cycle progression.

Altogether, these mechanistic studies provide a potential explanation for the adverse endothelial phenotype associated with oscillatory flow observed at athero-prone segments of the vasculature, in conjunction with altered NO bioavailability.

D. Stretch Versus Shear: Differential Impact on eNOS Activation

Perfusion in vivo is by nature pulsatile, combining both phasic shear and stretch. Therefore, in addition to the tangential forces exerted by blood flow, endothelial cells are also submitted to the circumferential forces that are generated by the transmural pressure gradient. Tedgui and collaborators (239) have demonstrated striking differences in the transduction of steady versus pulsatile stretch on whole rabbit aorta. Although both stimuli activate ERK1/2 pathway, steady stretch strongly activates FAK in an integrin-extracellular matrix interaction and
c-SRC-dependent manner, while pulsatility appears to have little impact on FAK phosphorylation. Specifically, the mechanical stretch caused by increased blood pressure has been proposed to play a major role in NO production. As a corollary, wall stiffness would suppress protective NO production, and endothelial dysfunction would not only contribute to arterial stiffening but also derive from an altered distensibility of the vessel wall. As early as the 1990s, several in vitro studies provided evidence that physiological levels of cyclic strain are able to increase eNOS gene expression and protein (18) and eNOS activity (19) in cultured endothelial cells. The up-regulation of eNOS in response to cyclic stretch is mediated via \([Ca^{2+}]_i\) increase through stretch-activated channels in an early phase, followed by activation of the PI3K/Akt pathway in a later phase (398). In BAECs cultured within distensible tubes, reduced wall distensibility altered pulse pressure-induced eNOS/Akt mechanosignaling and subsequent cytoprotection against oxidant stress (326). In isolated coronary arterioles, changes of intraluminal pressure concomitantly with shear stress activate mechanosensing mechanisms that modify vascular tone via \(H_2O_2\) and NO production (214). In the pulmonary microcirculation in vivo, Kuebler et al. (221) showed that the circumferential forces evoke PI3K- and Akt-dependent eNOS phosphorylation and NO production that appear independent of the concomitant increase in shear stress. In the very specific context of the pulmonary microcirculation, however, NO production has no apparent effect on vessel tone, as this vascular tree is already fully diluted in a healthy pulmonary circulation. Conversely, the antiadhesive and antiaggregating role of NO may subserve protective effects in response to stretch, resulting in attenuation of the proinflammatory effects of increased pressure or overventilation. Accordingly, treatment of endothelial cells with S-nitrosoc-N-acetylpenicillamine (SNAP) or eNOS overexpression reduced cyclic strain-induced monocyte chemotactic protein (MCP)-1 expression and superoxide levels, supporting a role for NO to downregulate redox-sensitive gene expression in endothelial cells constantly under hemodynamic stress (439). Indeed, endothelial cells respond to mechanical strain by upregulating the expression of adhesion molecules, e.g., VCAM-1 through redox-sensitive activation of NFκB (9) by mitochondrial-derived reactive oxygen species. These mitochondria-derived ROS could additionally trigger FAK phosphorylation through a signaling pathway that involves PKC, as demonstrated in HUVEC (8) and in rat femoral arteries (413). More recently, Csiszar et al. (84) proposed that in endothelial cells, high pressure-related cell stretch promotes NAD(P)H oxidase-derived \(H_2O_2\) generation, which leads to IkB degradation and nuclear translocation of NFκB. The ensuing oxidant stress accounts for the increased expression of BMP-2, a transforming growth factor-\(\beta\) superfamily member cytokine that appears to play important roles both in vascular development and pathophysiological processes including the development of atherosclerosis. This confirms the importance of stretch-induced NO production to tamper these signaling pathways that concur to promote proatherogenic endothelial activation and monocyte adhesion.

E. Role of NO in the Phenotypic Adaptation of Endothelial Cells to Shear In Vitro and In Vivo

Shear-mediated NO production regulates many aspects of endothelial cell functions, including antithrombogenic and antiadhesive effects, inhibition of vascular smooth muscle cell proliferation, and regulation of arterial contraction and tone, as discussed above. In this section we will discuss how this translates into vessel remodeling in particular physiological situations highlighting the role of NO.

As recently illustrated in vivo by Kelly and Snow in large-conduit arteries (203), responses to shear stress constitute a double protection system, i.e., through the shear-induced NO production, endothelial cells minimize the increases in shear stress associated with increasing blood flow and inhibit the process of cell adhesion to the arterial wall (known to occur in response to high shear, Ref. 71). In addition to the already discussed beneficial effects of exercise and the associated shear, another illustration of the physiological importance of shear forces in vasoregulation is the dramatic decrease in placental and uterine vascular resistances and their associated increase in blood flow that occur to meet the increasing nutrient and metabolic needs of the growing fetus. Increases in physiological shear stress and the subsequent increases in eNOS expression and NO production act therefore synergistically with other growth factors to modulate fetoplacental perfusion (249).

Adaptive morphological changes to high flow and high shear stress are characterized by vessel enlargement, elongation, and tortuosity. Conversely, in response to low wall shear, remodeling includes arterial shortening and constriction with intimal proliferation and thickening (89). The key roles of the endothelium (233) and eNOS-derived NO (356) in chronic vessel remodeling in response to hemodynamic forces have been known since the late 1980s, as supported by a large body of in vitro and clinical data (for a review, see Ref. 356). This applies to the pathological (e.g., atherosclerotic) remodeling described by Glagov in human arteries (146, 217), as discussed in section 1, where eNOS may act in conjunction with other NOS isoforms in the vascular wall (446). Indeed, inhibiting NO production diminished vessel enlargement even when high flow persists, whereas eNOS overexpression enhances vessel enlargement. Remodeling of the extracellular matrix is critical for vessel remodeling.
including during the development and progression of atherosclerosis which is highly dependent on shear stress, as atherosclerotic plaques preferentially localize to areas of the vasculature with complex laminar or oscillatory blood flow. In cultured endothelial cells, oscillatory but not unidirectional shear increases matrix metalloproteinase (MMP-9) mRNA expression as well as secretion of the MMP-9 protein. The expression of MMP-9, therefore, is not only activated by shear but differentially sensitive to the flow pattern (260). NO exerts a key role in the control of the MMP-9 activity involved in flow-induced remodeling. Indeed, in a high-flow remodeling model in mesenteric resistance arteries, inhibition of eNOS by L-NAME prevented MMP-9 activity, suggesting that a sequential activation of eNOS, and, subsequently, MMP-9 occurred in high-flow induced remodeling (111). Moreover, a bidirectional influence seems to exist between MMP-9 and eNOS, as step increases of flow evoke a dilation of mouse mesenteric arteries that is strongly potentiated in mice genetically deficient in MMP-9; this effect has been linked to an increased eNOS expression in MMP-9 KO mice, as inhibition of eNOS is associated with a reduction in the potentiated flow-induced dilation of MMP-9 KO mice (389). Therefore, the reciprocal influence of eNOS and MMP-9 may contribute to vessel remodeling in high-flow areas and perhaps, in athero-prone segments where disturbed flow would promote MMP-9 expression.

Age-related arterial stiffening is also implicated in the progression of endothelial dysfunction as demonstrated in young versus old rat aorta (384). Indeed, impaired shear stress-induced NO production in aorta from older rats was correlated with reduced eNOS and Akt phosphorylation. This led to the proposal that age-dependent vascular stiffening would attenuate vessel stretch, thus reducing a component of Akt activation leading to NO-dependent endothelial dysfunction. At the whole organism level, this may contribute to increased variability of systolic blood pressure that is correlated with adverse clinical outcomes, as will be discussed in later sections.

Many studies describe the activation and/or upregulation of eNOS in response to shear, with its ensuing antioxidant and atheroprotective effects, as described above; much less is known about the consequences of differences in shear magnitude and spatial gradient. Irrespective of the shear gradient, low shear stress is atherogenic with a reduced expression of eNOS. It is generally accepted that plaques do not develop under relatively high shear conditions, and recently, it was demonstrated that variations in shear stress patterns affect the initiation of atherosclerosis and induce the development of atherosclerotic plaques with a vulnerable phenotype (78); while relatively low shear stress (as observed in the inner curvature of the aortic arch) induces the development of large regions with a vulnerable plaque phenotype, oscillatory shear (expected to occur at the beginning of side branches) promotes the formation of more stable lesions. Of note, the different shear patterns not only lead to variable plaque vulnerability but also to different responses in chemokine expression and the composition of shear-induced atherosclerotic lesions (77). Another argument in favor of a major role of hemodynamic stresses in atherogenesis comes from the observations that distribution of atherosclerotic lesions changes with age as does the pattern of near-wall flow (6). Moreover, there are significant differences between coronary arteries and the proximal aorta in the expression of genes that could predispose to atherosclerosis. Indeed, the asynchronous in vivo hemodynamics observed in the coronary arteries result in a differential gene pattern, specifically for eNOS and ET-1 in endothelial cells, relative to more synchronous hemodynamics typical of the aorta (89). This correlates with the observation that upregulation of eNOS expression is part of the antiatherogenic properties of increased shear stress. Indeed, in apoE KO mice fed with an atherogenic western diet, eNOS is elevated in vessel segments with cast-induced increased shear stress (79), together with protection from atherosclerotic lesions in the same regions. Laminar shear stress was also shown to increase the S-nitrosylation of proteins such as caspase-3 and thioredoxin specifically in young endothelial cells (178), contributing to the antiapoptotic and antioxidative role of steady shear.

Finally, inflammatory mechanisms are critical for the development of cardiovascular diseases, and many studies correlate tumor necrosis factor (TNF)-α signaling with the extent of vascular injury. Shear stress differentially regulates TNF-α expression in remodeling large arteries. Indeed, while low shear acutely upregulates large artery TNF-α, high shear evokes a delayed and sustained expression associated with an outward remodeling (310). TNF-α has been shown to reduce NO bioavailability by decreasing eNOS expression or by increasing NO inactivation through stimulation of NADPH oxidase. Moreover, upregulation of TNF-α levels with aging is associated with a decrease in flow-mediated vasodilation leading to higher levels of shear stress (14).

In summary, the previous sections both illustrate the role of NO as a mechanosensitive mediator of short-term vasodilation and chronic vessel remodeling, and its congruence with the experimental demonstration of the activation of eNOS expression and activity, as well as NO bioavailability by physical forces; notably, many elements of the cellular mechanosensing machinery interact (or overlap with) regulatory elements of the eNOS pathway, consistent with a strategic position of eNOS in signal transduction in response to physical forces. This is supported by the subcellular confinement of (at least part of) cellular eNOS in caveolar structures now recognized as key signaling platforms for the assembly of mechanosensitive signalosomes. Although most of the evidence re-
viewed above pertains to endothelial cells, more recent work (including from our group) has identified a similar subcellular localization and some common mechanisms of activation of eNOS in cardiac myocytes. This will now be detailed in the next section.

III. STRETCH AND ENDOTHELIAL NITRIC OXIDE SYNTHASE IN THE HEART: NITRIC OXIDE-MEDIATED REGULATION OF CARDIAC PERFORMANCE

In addition to endothelial cells, eNOS is expressed in many other cell types of the cardiac muscle, including cardiac myocytes, together with nNOS and, in inflammatory states, also iNOS. However, the evidence so far identifies eNOS as the sole isoform participating in mechanosensitive regulation of cardiac function. In the following section, we will systematically review the cellular distribution of eNOS, compared with the other isoforms in the heart, its mechanisms of activation by physical forces in cardiac cells, and its impact on cardiac performance at the single cardiomyocyte as well as integrated organ level.

A. Expression and Compartmentation of the NOS in the Heart and Cardiac Myocyte

The three NOS isoforms are abundantly represented in the diverse cell types composing the myocardial tissue, albeit with differential gradients across the myocardial layers and with a specific subcellular localization. The concept of compartmentalization of cardiac NOS signaling has gained support over the last few years (27, 165, 453), as accumulated evidence demonstrated that the net effect of NO on cardiac physiology depends on a specific stimulus acting on a specific isoform at a particular subcellular location.

nNOS is expressed in orthosympathetic (369) and parasympathetic (286) nerve terminals as well as in intra-cardiac neurons (383) but not in intrinsic cardiac adrenergic cells. In the normal ferret heart, nNOS is detectable at low levels in both right and left ventricles, predominating in left ventricular endocardium and septum (47). In rat hearts, nNOS predominates in atria (280).

During embryogenesis, cardiac iNOS expression is prominent between embryonic days 9–14, but declines abruptly thereafter, and disappears at birth (37). iNOS is not expressed in normal and hypertrophic human heart. While absent in cardiomyocytes from intact hearts, iNOS expression is increased in cardiomyocytes from pacing-induced heart failure in rabbits (5), as well as in endocardial and coronary arteriolar endothelium in lipopolysaccharide-induced rabbit cardiomyopathy (274).

In normal ferret hearts, eNOS is abundantly expressed and clearly predominates over nNOS. eNOS expression is most prominent in the left ventricular apical and midventricular epicardium, is intermediate in the right ventricular free wall, and is markedly reduced or absent in the left ventricular endocardium and left ventricular side of the septum, following a gradient opposite to nNOS (see above). eNOS is also highly expressed in right atrial and sinoauricular node (47). Notably, at all these sites, eNOS colocalizes with extracellular, membrane-bound SOD. A similar distribution was found for eNOS (with a predominant epicardial expression) in human left ventricular tissue (47), as well as abundant expression in normal human atrial myocardium (432).

Quantification in the canine cardiac vasculature indicated that coronary microvessels contain 15-fold more eNOS mRNA than larger arteries. Among larger, epicardial arteries, the circumflex coronary artery had the highest eNOS mRNA content, followed by the right coronary artery, left anterior descending coronary artery, and aorta, respectively (137). It has been estimated that ~20% of cardiac eNOS is associated with cardiomyocytes (148). This, however, does not preclude from physiologically important signaling in the cardiomyocytes themselves, because of the spatial confinement, as emphasized below.

1. Myocyte subcellular localization of the NOS

In cardiomyocytes, nNOS was found to be expressed in/or coimmunoprecipitate with proteins of the sarcoplasmic reticulum (27, 442) and the sarcolemma (85, 86, 443), but apparently not with caveolin-3 (27). When induced, iNOS mainly localizes to the cytosolic compartment (277), but may be enriched in the perinuclear space, Golgi complex, mitochondria, and plasma membrane (443), along contractile fibers and even in the nuclear envelop of rat cardiomyocytes (59), but not in the sarcoplasmic reticulum (442). Conversely, eNOS is predominantly located in caveolae (119) both from the external envelope and t-tubular sarcolemma (268) in adult (242, 317), but not neonatal cardiomyocytes, which lack t-tubules (304). Although eNOS was not found at the sarcoplasmic reticulum itself by electron microscopy (442), a more recent report suggested the association of the reductase domain of eNOS with the ryanodine receptor in cardiac myocytes (264).

In endothelial cells, eNOS may also be docked to the mitochondrial outer membrane through a pentabasic amino acid sequence in the autoinhibitory domain of eNOS (residues 628–632 of bovine NOS3) (140), or translocated to the cytoskeleton (370), allowing signaling in response to shear stress, as reviewed above; such cytoskeletal localization, however, has not yet been demonstrated in cardiomyocytes.
B. Modulation of Cardiac Function by NO in Response to Physical Forces

At the whole organ level, the heart is submitted to a complex array of heterogeneously distributed physical forces that dictate adaptive responses through changes in contractile force, relaxation, and excitability on a beat-to-beat basis, as well as long-term trophic changes. Although NO has been implicated in virtually all these responses, in this section we concentrate on short-term reactions, where eNOS is the predominant player, while we will address chronic remodeling in later sections.

1. Paracrine/autocrine production of NO in the contracting heart

A first indication supporting the involvement of NO in mechanoregulation of cardiac function comes from early observations of its production in the coronary effluent of actively contracting hearts (205, 229). Understandably, these measurements could not distinguish the respective cellular sources of the NO produced, which probably integrated endothelial cells from the vasculature and cardiac myocytes, although the majority of the NO produced in the coronary vasculature can be attributed to the endothelial NOS more abundantly expressed in macro- and microvascular coronary endothelial cells. Accordingly, these cells represent a significant source of paracrine NO for the regulation of cardiac muscle function at the level of capillary microvascular endothelial cells as well as endocardial cells closely apposed to cardiomyocytes (13). Within the contracting muscle, these endothelial and endocardial cells probably respond to compression and shear forces through the same molecular machinery as described above in vessels, although this has been less formally studied in the heart. At the whole organ level, NO production was measured ex vivo in the nonbeating rabbit heart after mechanical compression; as the NO signal was lost after endothelial destruction with Triton X, this production was attributed mainly to endothelial cells (330).

As commented on before, the fact that any measurable NO signal from the heart is lost after endothelial destruction does not mean that this cell type is the only source of functionally important NO. Their number and abundant production of NO should not detract from the quantitatively lower (and therefore hardly detectable), but nevertheless physiologically important autocrine NO production within cardiomyocytes because of the spatially confined signaling by this short-lived radical close to its targets colocalized in subcellular compartments (30).

2. Paracrine effect of NO on contraction

Following early demonstrations of the role of endocardium (55) and endothelial cells on the mechanical performance of cardiac muscle, several studies have naturally focused on the regulation of cardiac contraction by NO. Studies using isometrically contracting papillary muscle preparations have identified a small positive inotropic effect at lower concentrations of NO or cGMP analogs, followed by a negative inotropic effect at higher concentrations (269, 284, 321). Notably, the direction and magnitude of this inotropic effect were dependent on the level of adrenergic stimulation, with the negative influence of NO being more prominent after β1-adrenergic activation (345). Importantly, similar paradigms were confirmed in atrial trabeculae and left ventricular papillary muscle of human origin (130). This effect of NO to predominantly attenuate β-adrenergically stimulated inotropism recapitulates the initial paradigm of the role of endogenous NO first proposed in single cardiac myocytes (24). A distinctive feature of the regulation of papillary muscle isometric contraction by NO was the observation of an abbreviation of the developed contraction due to the earlier onset of relaxation (a phenomenon also amplified in the presence of adrenergic prestimulation). This was confirmed in vivo by the observation of a small decrease in left ventricular (LV) developed pressure (in absence of changes in LV dP/dt) and early relaxation in human hearts after infusion of sodium nitroprusside (322) or substance P to activate the paracrine production of NO by coronary endothelial cells (323). This emphasized the ability of paracrine NO to promote LV relaxation, with beneficial effects through an increase of diastolic reserve and preservation of overall pump function, despite the attenuation of peak LV pressure. Of note, such regulation of diastolic function was conserved in patients with hypertrophic cardiomyopathy (272).

Therefore, from a physiological point of view, the activation of NO production by mechanical activity of the cardiac muscle, as mentioned above, perhaps also rhythmically coincident with the development of maximal force at the end of systole, as suggested from measurements with a porphyrinic sensor (330), would suitably contribute to cardiac muscle relaxation during the ensuing diastole; at the same time, NO would contribute to decrease the duration of contraction, thereby lengthening the diastolic interval and promoting muscle perfusion especially in subendocardial layers. This would be particularly important at higher cardiac frequencies, since the effect of NO on twitch duration and contraction-relaxation time intervals was shown to be operative over a higher range of contraction frequencies (334); accordingly, the calcium-dependent NOS activity of cardiomyocytes and its effect on contraction were similarly proportional to stimulation frequency (200), suggesting that paracrine and autocrine NO may combine their influence in the intact heart. Finally, NO also regulates the chronotropic state of the heart, e.g., through potentiation of the vagal tone at the presynaptic level (by nNOS in the in
parasympathetic terminals), as well as postsynaptic level (by eNOS in cardiac cells) (320). The latter was demonstrated in vivo by the potentiation of the bradycardic effect of muscarinic cholinergic agonists in mice with cardiomycocyte-specific expression of eNOS (at unchanged blood pressure) (268). Altogether, these effects of eNOS (and nNOS) would cooperatively modulate contraction frequency and diastolic interval for optimal cardiac perfusion and filling.

Mechanistically, the relaxation-promoting effect of NO (and, possibly, part of its negative inotropic effect) has been attributed to a cGMP- and PKG I-mediated desensitization of cardiac myofilaments (374) coincident with increased phosphorylation of cardiac troponin I (cTnI) (201, 237). Other intracellular mechanisms for the inotropic influence of NO have been extensively reviewed elsewhere (266) and are beyond the scope of this review. The ensuing increased distensibility of the cardiac muscle may conceptually also participate in the length-dependent recruitment of contractile reserve according to the Frank-Starling relation. This has been suggested on the basis of a study in isolated guinea pig hearts where the increase in cardiac output following increased preload was attenuated by NOS inhibition (335). NO would then promote diastolic lengthening through myofilament desensitization and, simultaneously, increase force development. Although the action of catecholamines would provide another example of such concurrent, dual inotropic and lusitropic effects on cardiac contraction, possibly both attributable, in part, to changes in cTnI phosphorylation on its NH2 terminus, the true mechanism(s) for such a dual effect of NO probably involves other targets that still need further characterization. Before that, it remains to be verified that the length-promoting effect of NO (hastening of relaxation) is maintained after stretch, as data suggest this effect to be lost in stretched papillary muscles (321).

3. Autocrine production of NO by mechanical forces: activation of cardiomycocyte eNOS by stretch and effect on excitation-contraction coupling

Using single rat and mouse ventricular myocytes, Petroff et al. (327) showed that NO was an obligatory intermediate for the slow increase in calcium and contractile force following stretch (resulting in 8–10% increase in sarcomere length). Confocal microscopic analysis of fluo 4-loaded cardiomycocytes revealed that stretch resulted in an increase in calcium spark rate (despite no change in spark characteristics, such as distributions of spontaneous spark amplitudes); preincubation of single cardiomycocytes with the NOS inhibitor L-NAME fully abrogated the effect of stretch on Ca2+-spark rate, as well as on the increase in Ca2+-transients after electrical stimulation (Fig. 8). This effect was assigned specifically to eNOS activation by stretch within the cardiomycocytes, as it was totally absent in cells from eNOS-deficient mice. The production of NO from single, isolated cardiomycocytes in response to stretch was also demonstrated using the NO-sensitive fluorescent dye DAF2. Notably, another independent study using specific NOS-deficient cells confirmed the involvement of eNOS (but not nNOS) as the mechanosensitive isoform responsible for NO production by single cardiomycocytes in response to stretch (113).

The mechanism for the increased calcium sparks rate (reflecting SR calcium release) did not implicate an increased SR calcium load (unchanged by stretch), nor an indirect effect due to stretch-activated nonspecific cationic currents, as it was unaffected by treatment with the peptide from Grammostola Spatulata (or gaddolinium). As the L-type calcium current (the trigger for SR calcium-induced calcium release) is known to be unchanged by stretch, these experiments suggested that intracellular NO production acted as a gain amplifier for excitation-contraction coupling. Notably, the NO-mediated effect on Ca2+ sparks was independent from cGMP, as it was unaffected by treatment with the guanylcylic cyclase inhibitor ODQ. On the other hand, independent evidence has demonstrated that the cardiac-specific ryanodine receptor (RyR2) can be regulated by N-nitrosoylation of critical cysteine residues, and Petroff et al. (327) showed that the effect of stretch on Ca2+ spark rate can be reproduced with S-nitrosoacetyl-penicillamine or S-nitrosothiocyante, that both release the S-nitrosylating species NO+, while the reducing agent dithiothreitol prevented it (327). This suggested a cGMP-independent effect of autocrine NO directly on RyR2, that would be supported by the close colocalization of eNOS in t-tubular caveolae with the sarcoplasmic reticulum RyR2 in the cardiac dyad (268).

Of interest, stretch activation of eNOS also implicated PI3K, as both the increase in Ca2+ spark rate and phosphorylation of Akt/protein kinase B and eNOS on Ser-1177 (reflective of its activation) were abrogated by pretreatment with LY294002. This dependence on PI3K was also observed in another independent study (113). Whether PI3K is activated by oxidant radicals produced by NADPH oxidase (following AT1R stimulation by autocrine/paracrine production of angiotensin II), as proposed for other stretch-activated channels (52), remains an open question. Another candidate coupling molecule could be the muscle-specific protein melusin, a cytoplasmic, CHORD protein interacting with surface integrins in costameres near the Z disk. In cardiac muscle, genetic deletion of melusin results in complete abrogation of Akt/protein kinase B phosphorylation in response to acute pressure overload (48). Conversely, cardiac-specific transgenic overexpression of melusin resulted in increased basal as well as stretch-induced phosphorylation of Akt (96). Although melusin may not represent the only mediator of
stretch-induced downstream signaling to specific kinases, it may be critical for the recruitment of multiprotein complexes (including Akt) in the vicinity of Z-disk proteins implicated in both acute and chronic regulation of cardiac mechanics in response to stretch. Whether eNOS is part of such “interactome” is the object of ongoing work.

Aside from (or in addition to) melusin, other proteins participating in the anchoring of integrins to actin may be responsive to NO and modify the adaptive response of the myocardium to increased mechanical forces. Among these is ENA/VASP (enabled vasodilator-stimulated phosphoprotein), a well-known target for cGMP-activated PKG. ENA/VASP also binds to zyxin, which critically controls its localization at cell adhesion points for the modulation of actin assembly and organization. Although deletion experiments have shown both zyxin and VASP to be dispensable for proper cardiac development and basal function (168), expression of a dominant negative VASP results in dilated cardiomyopathy with early mortality (114), pointing to a role in the dynamic regulation of sarcomere-plasmalemma anchoring. How this function is related to the attenuating role of NO on cardiac hypertrophy (see below) is unknown. These observation still point to VASP/eNOS as potential “modifier genes” affecting the biological response of the myocardium to mechanical stress.

C. eNOS-dependent regulation of cardiac contraction in response to stretch: an integrated view

The eNOS-mediated increase in Ca\textsuperscript{2+} spark rate results in the slow (i.e., >10 min) increase in whole cell Ca\textsuperscript{2+} transient and cell shortening in electrically stimu-
lated cardiac myocytes (327), that was proposed to participate to the intrinsic cardiac reserve capacity accounting for (at least part of) the Anrep effect (i.e., the slowly evolving increase in cardiac contractility beyond that immediately achieved as the result of ordinary length-dependent mechanisms). At the whole organ level, NO produced by eNOS in the cardiomyocyte and in surrounding capillary endothelial cells may cooperatively orchestrate the length-dependent changes in contractility in response to increased preload, as coronary endothelial NO, produced in response to shear, will favor myocyte relaxation through cGMP-dependent desensitization of cardiac myofilaments (see above; Refs. 201, 269), and the ensuing myocyte elongation will favor the slow stretch-dependent adaptive increase in contractility through autocrine NO production by eNOS in cardiomyocytes (Fig. 9).

At the same time, paracrine and autocrine NO will attenuate the positive inotropic (and chronotropic; Ref. 268) effect of maximal β-adrenergic stimulation, while reinforcing its lusitropic effect for optimal adaptation of contraction-relaxation time intervals (favoring coronary perfusion) and recruitment of diastolic reserve, while sparing oxygen consumption (including through direct effects on mitochondrial respiration; reviewed in Ref. 269). Notably, some of these autocrine effects may be shared by nNOS, also expressed in cardiomyocytes, and similarly implicated in direct lusitropic effects, as well as attenuation of β-adrenergic signaling (22), although the implication of this isoform in mechanotransduction has not been identified so far. Overall, combination of these effects of eNOS (and, to some extent, nNOS) results in an equivalent of a “smart β-adrenergic blockade” through built-in endogenous signaling.

Altogether, this section highlighted the cooperativity between endothelial/endocardial and cardiomyocyte eNOS for the short-term regulation of cardiac function, thereby extending the physiological role of mechanically stimulated NO far beyond shear-mediated vasodilatation. Furthermore, the next section examines the chronic influence of eNOS in cardiovascular remodeling, with emphasis on endothelial and cardiac progenitor cells.

**Fig. 9.** Integrated influence of paracrine and autocrine NO on cardiac function. eNOS is expressed in endothelial cells and in cardiac myocytes, where the enzyme is probably associated with different caveolar pools, i.e., at the peripheral plasmalemma and in t-tubules, in close apposition to the sarcoplasmic reticulum. nNOS is expressed in parasympathetic nerve terminals (and also in cardiomyocytes, not illustrated). Shear forces and compression activate NO production from endothelial cells; NO then diffuses to neighboring cardiomyocytes and increases myocyte distensibility through desensitization of myofilaments. eNOS in peripheral caveolae is activated by muscarinic and β-adrenergic receptor stimulation by acetylcholine and norepinephrine, respectively, where NO attenuates the β-adrenergic effect and potentiates the cholinergic effect, resulting in negative chronotropic effects. This is reinforced by nNOS activity in parasympathetic nerve terminals, which increases acetylcholine release in the synaptic cleft. The overall effect is increased lusitropy with abbreviated contraction and increased diastolic interval, both of which promote ventricular perfusion and filling. The increased distensibility also promotes the recruitment of contractile reserve by stretch, which results in eNOS activation and NO release in the dyad, increased SR calcium release, and the slow increase in calcium transient and contraction force (Anrep effect, see Fig. 8, A–D). Together these combined effects on excitation-contraction coupling and diastolic reserve contribute to eNOS-dependent optimization of cardiac function, while preventing from the adverse effects of excessive β-adrenergic stimulation (“built-in β-blockade”). ec, Endothelial cell; cm, cardiac myocyte; ps, parasympathetic; os, orthosympathic; sr, sarcoplasmic reticulum; Ach, acetylcholine; Nad, noradrenaline.
IV. ENDOTHELIAL NITRIC OXIDE SYNTHASE, NITRIC OXIDE, AND CARDIOVASCULAR REMODELING: ROLE OF ENDOTHELIAL AND CARDIAC PROGENITOR CELLS

Regular physical activity is an important factor in the prevention of cardiovascular diseases and the reduction of mortality in patients with ischemic diseases. Exercise increases maximal myocardial oxygen uptake and leads to several physiological adaptations in cardiac and skeletal muscles including the development of new capillaries (angiogenesis). A fall in \( P_{O_2} \) occurring within muscle during exercise is a critical trigger of acute and chronic adaptation processes (262, 424), with mitochondria acting as oxygen-sensing organelles (259). In ischemic diseases, the decrease in oxygen availability leads to a similar response of the compromised organ to promote the survival of the hypoxic tissues and to remodel the vascular network to restore a normal perfusion. Although ischemia involves an accumulation of toxic metabolites and a reduction in energy substrates like glucose, hypoxia represents the major signal to trigger the corrective response. This will generally imply the acute regulation of vascular contractility and on the longer term, the development of a neovascularure to decrease the oxygen diffusion distances. Several studies have reported an important role of NO in mediating the protective effects of exercise and the recovery from an ischemic insult (154, 211, 234, 262). The mechanisms governing the regulation of the vessel tone, in particular the impact of shear stress on NO bioactivity, has been detailed in previous sections of this review. We now focus on the capacity of NO to induce a gene/protein expression program supporting angiogenesis and metabolic adaptation by interfering with the major hypoxia-driven transcription factor HIF-1. We then review recent data documenting how NO (mostly produced by eNOS in endothelial cells) may support the formation of a new vascular network by promoting postnatal vasculogenesis. Finally, we show how NO may orchestrate these different processes (i.e., those driving vascular homeostasis and remodeling) in the specific context of the estrogen regulation of the cardiovascular system.

A. Influence of Hypoxia: the Interplay Between NO and HIF

1. NO drives the stabilization of HIF-1\(\alpha\) under normoxia

Cells exposed to low oxygen conditions respond by initiating adaptation and survival mechanisms. The major regulatory pathway switched on by hypoxia involves the family of transcription factors HIF or hypoxia-inducible factors, which bind the promoter of different families of genes such as those involved in angiogenesis and glycolysis (see Table 1). The HIF-1 protein is the most extensively studied HIF isoform; HIF-1 is actually a heterodimeric transcription complex consisting of an inducible and a constitutively expressed subunit, HIF-1\(\alpha\) and HIF-1\(\beta\), respectively. The real \( O_2 \)-sensing protein however is not HIF-1 itself but a family of prolyl hydroxylases (PHDs) that hydroxylate HIF-1\(\alpha\) in conditions of normoxia and thereby favor its degradation. Hydroxylation of 402/564 proline residues in HIF-1\(\alpha\) leads to its interaction with the von Hippel-Lindau protein (pVHL), which in turn promotes the ubiquitination and the consecutive proteasomal degradation of HIF-1\(\alpha\) (Fig. 10). In the absence of \( O_2 \), HIF-1\(\alpha\) rapidly accumulates, interacts with HIF-1\(\beta\), and facilitates the recruitment of cofactors (e.g., p300/ CBP). The translocation of the HIF-1 heterocomplex and the binding to promoter specific regions called HRE (hypoxia-response elements) consecutively drives the activation of specific families of genes (Fig. 10).

<table>
<thead>
<tr>
<th>HIF-1 Target Genes</th>
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<tbody>
<tr>
<td>Angiogenesis</td>
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<tr>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>Angiopoietin-2</td>
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<tr>
<td>Adrenomedullin</td>
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<tr>
<td>Transforming growth factor-(\beta3)</td>
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<tr>
<td>Anaerobic metabolism</td>
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<tr>
<td>Aldolase-A and -C</td>
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<tr>
<td>Enolase-1</td>
</tr>
<tr>
<td>Glucose transporter-1 and -3</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>Carbonic anhydrase-9 and -12</td>
</tr>
<tr>
<td>Hexokinase-1 and -2</td>
</tr>
<tr>
<td>Lactate dehydrogenase-A</td>
</tr>
<tr>
<td>Pyruvate kinase-M</td>
</tr>
<tr>
<td>Phosphofructokinase-L</td>
</tr>
<tr>
<td>Phosphoglycerate kinase-1</td>
</tr>
<tr>
<td>6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase-3</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
</tr>
<tr>
<td>Vascular tone</td>
</tr>
<tr>
<td>Endothelin-1</td>
</tr>
<tr>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>iNOS</td>
</tr>
<tr>
<td>(\alpha,\beta)-Adrenergic receptor</td>
</tr>
<tr>
<td>Erythropoiesis/iron metabolism</td>
</tr>
<tr>
<td>Erythropoietin</td>
</tr>
<tr>
<td>Transferrin (receptor)</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>Matrix metabolism</td>
</tr>
<tr>
<td>MMP-2</td>
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<tr>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>Collagen prolyl-4-hydroxylase-(\alpha)</td>
</tr>
<tr>
<td>Fibronectin-1</td>
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<tr>
<td>Cell proliferation/survival</td>
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<tr>
<td>Insulin growth factor-2</td>
</tr>
<tr>
<td>Insulin growth factor-binding protein-1, -2, and -3</td>
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<tr>
<td>Transforming growth factor-(\alpha)</td>
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<tr>
<td>Cyclin G2</td>
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<td>p21</td>
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HIF, hypoxia inducible factor; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase.
Signals other than hypoxia are known to participate in HIF-1 signaling, including growth factors and NO. Interestingly, the NO-driven stabilization of HIF-1α was reported to occur under normoxic conditions (276, 360). Different mechanisms support the stimulatory influence of NO on HIF-1α (Fig. 11, top). Metzen et al. (276) reported that the exogenous NO donor GSNO dose-dependently inhibits the PHD enzyme activity but not the interaction of a synthetic peptide mimicking the hydroxylated oxygen-dependent degradation domain of HIF-1α. They concluded that the PHD activity was supporting the NO-driven HIF-1α accumulation during normoxia. Simultaneously, Sumbayev et al. (393) documented that in vitro GSNO could provoke massive S-nitrosation of purified HIF-1α, a process attenuated by the addition of GSH or ascorbate. In cells, the same drugs produced S-nitrosation of HIF-1α on maximum four thiol residues (versus 15 residues in purified HIF-1α) and the addition of N-acetylcysteine was shown to destabilize HIF-1α in close correlation to the disappearance of S-nitrosated HIF-1α (393). Yasinska and Sumbayev (444) then identified the Cys-800 residue within the HIF-1α sequence as a key target for S-nitrosation. This residue is, indeed, critical for the recruitment of the p300 coactivator protein that is necessary for the transcriptional activity of the HIF-1 complex. GST pull-down assays suggested that S-nitrosation of Cys-800 could stimulate the recruitment of p300 and its interaction with HIF-1α (444) (see Fig. 11). The definitive demonstration of the relevance of the direct nitrosation of HIF came from studies examining the effects of endogenous formation of NO (via the induction of iNOS) (244, 393, 450). In particular, the work by Li et al. (244) revealed that HIF-1α is S-nitrosated at Cys-533 in the oxygen-dependent degradation domain (see Fig. 11). This modification prevents HIF-1α degradation, independently of alterations in PHD activity. Interestingly, these authors further showed that the selective disruption of this S-nitrosation...
significantly attenuated macrophage-induced activation of HIF-1α. These data unravel a direct link between inflammation and HIF-1α, with NO playing a pivotal role in ensuring the transcription of HIF-target genes under normoxia (see Table 1 for major HIF-regulated genes).

2. NO favors the degradation of HIF-1 under hypoxia

Paradoxically, while NO may promote HIF-1α stabilization under normoxic conditions, NO may favor the degradation of HIF-1α under hypoxia. In this context, the target of NO is not directly HIF-1 but the cytochrome-c oxidase. This enzyme which forms the complex IV of the mitochondrial respiratory chain is responsible for 90% of the cell oxygen consumption in mammals (Fig. 11, bottom). Recent work has shown that NO (in the same range of concentrations as that reported to activate soluble guanyl cyclase) can compete with oxygen and lead to the inhibition of cytochrome-c oxidase (53). This situation called “metabolic hypoxia” is characterized by the incapacity for the cell to use available oxygen (287); this differs from true hypoxia in which O2 is not or poorly available.

The paradox that arises from these conditions is that in the context of a moderate hypoxia (between 1 and 3% O2), low concentrations of NO may prevent the stabilization of HIF-1α through the reduction of the (residual) cell O2 consumption. The explanation is the redistribution of oxygen towards nonrespiratory oxygen-dependent targets such as the prolyl hydroxylases PHDs (3, 160) (see Fig. 11). The unusually elevated K_m of the PHDs renders such regulation possible, allowing small changes in the O2 availability to substantially affect the enzyme activity. Moreover, besides the increased activity of PHD because of the relative increase in O2 availability, other parameters have been identified that concur to support the NO-mediated degradation of HIF-1. Accordingly, the combination of exposure to a NO donor and hypoxia (but not hypoxia alone) was shown to lead to the upregulation of PHD2, a major member of the PHD family (160) as well as to an increase in free iron and 2-oxoglutarate (two cofactors required for PHD catalytic activity) (65, 219).

The above paradigm was pushed one step further by Quintero et al. (338) who suggested that in endothelial cells where eNOS is constitutively expressed, the primary role of mitochondria may not be to generate ATP but to act under the control of NO as signaling organelles. Indeed, they showed that in endothelial cells exposed to low O2 concentration (1–3%), endogenously produced NO prevents the accumulation of HIF-1α (as normally observed in other cell types such as smooth muscle and epithelial cells). In their hands, endothelial cells need to be exposed to deep hypoxia (<1%) to lead to HIF-1α stabilization. Such a mechanism would be critical to avoid unwanted stimulation of angiogenesis and thereby to maintain the quiescence of endothelial cells facing hypoxia. The work by Martinive et al. (265) explored the impact of intermittent hypoxia on the endothelial cell phenotype expanding this paradigm. We found that in the specific conditions of cyclic hypoxia (i.e., fluctuations between moderate and deep hypoxia), HIF-1α could accumulate in endothelial cells to a larger extent than in response to prolonged hypoxia. Endothelial cells were shown to consecutively become more resistant to pro-apoptotic stresses but also to increase their “angiogenic” potential (i.e., the capacity to migrate and organize in tubes). These data were further confirmed in vivo in a model of tumors where fluctuating changes in PO2 are known to occur in response to the chaotic perfusion within the disorganized tumor vascular network. Altogether, these studies indicate that NO-producing vascular endothelial cells register hypoxia under very low O2 concentrations. This apparent lower sensitivity to hypoxia is not deleterious for endothelial cells, since glycolysis (and not oxidative phosphorylation) is the main source of ATP generation in these cells (338). The diversion of O2 away from endothelial cell mitochondria by NO could even facilitate the oxygenation of vascular smooth muscle cells. In response to intermittent hypoxia, however, HIF-1α may accumulate, probably because of the increased ROS production that scavenge the NO production. The cell response is an increased resistance to apoptosis and angiogenesis that may favor not only tumor progression (265) but also neovascularization of chronically (and intermittently) hypoxic vascular tissues in general.

3. NO-driven HIF-1-dependent angiogenic program

Since almost 100 genes are known to be regulated at the transcriptional level by HIF-1, NO-mediated inhibition of respiration under moderate hypoxia or stimulation of HIF-1 activity under normoxia may profoundly modify intracellular signaling. Table 1 presents a nonexhaustive classification of the major families of HIF-regulated genes. The reading of this list in the light of the previous paragraphs allows one to understand how NO may activate or block the expression of a broad range of genes/proteins including those supporting glucose uptake, red blood cell production, and the formation of new blood vessels via angiogenesis. The specific roles of these gene/protein families have been extensively reviewed elsewhere (46, 202, 423). Among these genes, the various VEGF isoforms play critical roles to stimulate the neangiogenic process within the ischemic tissues, in particular after acute myocardial infarction (238). VEGF is released and diffuses to nearby preexisting blood vessels to induce capillary formation. Notably, VEGF expression decreases with the age of the patient (349), together with the impaired induction of HIF-1 and activity of eNOS. Another
key family of HIF-dependent upregulated genes are those encoding glucose transporters and enzymes of the glycolytic pathway, which allow hypoxic cells to compensate for the diminished ATP production due to the reduced oxidative phosphorylation in mitochondria. However, as emphasized above, these adaptations barely concern endothelial cells that are already largely glycolytic under basal conditions.

4. Limitations to the bimodal regulation of HIF-1 by NO

A first note of caution on the differential regulation of HIF-1α by NO is the nature of the NO compound(s) which interact(s) with HIF itself, PHD, or the cytochrome-c oxidase. For instance, in the study by Sumbayev et al. (393), the S-nitrosation of purified HIF-1α was observed in response to GSNO and SNAP, which are NO⁺ donors but not following exposure to true NO radical donating compounds.

Second, the influence of ROS may considerably tune the expected impact of NO on the regulation of HIF-1α biology. ROS, in particular superoxide anion, are produced by the mitochondria precisely in the context of the moderate hypoxic conditions that drive the inhibition of cytochrome-c oxidase by NO and the consecutive degrada-
tion of HIF-1α. The blockade of complex IV is known to favor ROS production by complex I, which should exacerbate the oxidative stress. The mutual influence of NO and the superoxide anion as highly reactive radicals, to influence their respective bioavailability is therefore likely to influence their regulation of HIF-1α biology (208). Also, ROS, produced in response to the effects of NO on mitochondria, may stimulate the AMP-activated protein kinase, another key defense mechanism for endothelial cells (180) and cardiac myocytes (359). Moreover, the interpretation of the protective effects of AMPK and HIF through the NO-driven modulation of the mitochondrial respiratory chain are further complicated by the recently described AMPK-dependent activation of eNOS (243, 367, 449).

B. eNOS, Caveolin, and Endothelial Progenitor Cells in Vasculogenesis

HIF-responsive cytokines, such as VEGF, exert pleiotropic proangiogenic effects, many of which depend on downstream activation of eNOS. In the following section, we will review the role of eNOS as regulator of the biology of progenitor cells involved in vasculogenesis. The term angioblasts is used to name the population of endothelial cell precursors or EPC present in the adult blood (348). Studies using bone marrow transplantation have shown that angioblasts reside in the bone marrow. Other reports have then proposed that this angioblast population could correspond to the hemangioblasts, a term so far reserved to describe in the embryo, the common endothelial/hemato-
poietic precursor of endothelial cells (ECs) (365). Al-
though some authors may still dispute the classification, it is now clear that EC-generating activity in bone marrow-derived cells reside in the hematopoietic stem cell compartment and may be described as the adult hemangioblast population. This does not exclude the existence of nonhematopoietic stem cells that may also contribute to the formation of endothelial cells.

1. Mobilization from the hematopoietic niches

The distribution of hematopoietic progenitor cells is organized in the bone marrow between the endosteum of the bone and the regions directly in contact with blood vessels (Fig. 10). This localization responds to a dynamic process where stem cells can move from one niche to another, i.e., from a microenvironment promoting their quiescence (“endosteal or stromal niches”) to sinusoidal vessels (“vascular niches”) where they undergo differentiation and ultimately mobilization to the blood (216). The bone marrow microvasculature functions as a barrier between the bone marrow parenchyma and the peripheral circulation. This obstacle needs to be overcome in either direction, to allow mobilization of progenitor cells towards ischemic tissues but also to recolonize the bone marrow in the context of transplantation. Opposite gradients of cytokine/growth factors govern the progenitor cell homing to the bone marrow and the mobilization of hematopoietic stem cells. Proteolytic enzymes allow the cleavage of specific mobilization actors such as the Kit ligand and SDF-1. In particular, activation of the collagenase MMP-9 accounts for the cleavage of the membrane-bound Kit ligand from bone marrow stromal cells and the consecutive release of the soluble Kit ligand (sKitL, also named stem cell factor) (170) (Fig. 12, left). As a result, quiescent hematopoietic stem cells expressing c-Kit (the receptor of sKitL) are translocated to a permissive zone where they may extravasate in the circulation after proliferation and differentiation.

2. NO and EPC mobilization

NO is a major regulator of the biological processes driving progenitor cell maturation and egression from the bone marrow. A key set of experiments documented that while the transplantation of bone marrow cells from eNOS knock-out mice (eNOS−/−) to wild-type mice gives rise to mobilization of progenitor cells, the opposite was not true. Indeed, in eNOS−/− mice, transplantation of wild-type bone marrow cells failed to elicit mobilization (4). Intravenous injection of hematopoietic stem cells collected from eNOS−/− mice however improved the neo-
vascularization in ischemic hindlimb. A defect in MMP-9 expression and activity was reported in eNOS−/− mice.
Together with a reduced capacity to release sKitL in response to mobilizing drug treatment (Fig. 13). These authors further showed that EPC mobilization could be rescued by the infusion of sKitL. The interplay between MMP-9 and eNOS is also supported by data identifying MMP9 as a direct target for S-nitrosylation (157).

Besides the sKitL/cKit axis, other actors including VEGF and SDF-1 play key roles in the dynamic process of hematopoietic stem cell mobilization and involve NO at some stages of their signaling. An increase in circulating EPC following VEGF administration leading to corneal neovascularization was initially reported by Isner and colleagues (16). SDF-1 was then identified in subsequent studies as another major factor released by ischemic/hypoxic tissues and stimulating the mobilization of bone marrow-derived EPC (Fig. 12). Activation of eNOS is recognized as one of the major pathways switched on in response to VEGF (313, 418, 451), and SDF-1 (176) stimulation.

3. eNOS-activating drugs and EPC

With the identification of eNOS as one of the key effectors of VEGF-driven proangiogenic effects, NO became the subject of many studies addressing its role as mediator of postnatal vasculogenesis downstream of other stimuli. Stimulation of NO production is today considered as a necessary event accounting for progenitor cell mobilization in response to major cardiovascular drugs, including statins (HMG-CoA reductase inhibitors) (42, 404) and angiotensin converting enzyme (ACE) inhibitors (404). The provasculogenic effects of these substances were revealed in animal models of myocardial infarction. In the early postinfarction phase, increased ROS production and impaired MMP-9 activity are indeed commonly observed in the bone marrow (404). Both statins and ACE inhibitors through transcriptional and post-translational modifications of eNOS were shown to correct this defect. Similar observations were reported in response to estrogens that can increase EPC mobilization after an infarct through a facilitating effect on eNOS activity and subsequent activation of MMP9 in the bone marrow (184). These data may account for part of the sex difference in the occurrence of cardiac events (i.e., lower incidence of ischemic cardiac events in premenopausal women) and suggest a potential benefit of hormone replacement therapy for the management of ischemic diseases in postmenopausal women (see below). Finally, it is noteworthy that moderate exercise training may similarly promote NO-mediated EPC mobilization (235) (although the final target of NO remains unknown so far) and that a direct correlation between increased circulating EPC and elevated plasma VEGF levels was reported in response to exercise in ischemic patients (2).
provided for information.
3 days after a single 5-FU injection; densitometric quantification is
ligand (skitL)
A: gelatin zymography of bone marrow plasma before and
measured on day 6 after 5-FU treatment (53).
Aicher et al. (4).
deficiency abrogates MMP9 activation and release of sKitL. [From
(157). The potential role of caveolin/caveolae in pro-
produce NO (364) and thus to regulate MMP-9 activity
abundance modulates the capacity of the enzyme to
regulation of caveolin, and any change in caveolin
above). eNOS activation is, indeed, under the close
for caveolin, the structural protein of caveolae (see
lature formation, an interest progressively developed
mechanisms governing bone marrow-derived neovascu-
aging) (379, 405). These results together with the direct
functions in either condition (i.e., increased ADMA or
(379). Statins were documented to restore normal EPC
was also established in a genetic mouse model of aging
between aging and a defect in NO-mediated vasculogenesis
was also established in a genetic mouse model of aging
(379). Statins were documented to restore normal EPC
functions in either condition (i.e., increased ADMA or
aging) (379, 405). These results together with the direct
effect of NO on MMP-9 identify NO as a key actor of
postnatal vasculogenesis.

4. eNOS inhibitors and EPC

The plasma concentration of asymmetric dimethyl-
arginine (ADMA) known to act as endogenous inhibitor of
eNOS (see above) is known to be related to the severity of
coronary artery disease and correlates inversely with the
number of circulating progenitor cells (405). A link be-
tween aging and a defect in NO-mediated vasculogenesis
was also established in a genetic mouse model of aging
(379). Statins were documented to restore normal EPC
functions in either condition (i.e., increased ADMA or
aging) (379, 405). These results together with the direct
effect of NO on MMP-9 identify NO as a key actor of
postnatal vasculogenesis.

5. Caveolin signaling and EPC

After the initial discovery of the NO-dependent
mechanisms governing bone marrow-derived neovascu-
lature formation, an interest progressively developed
for caveolin, the structural protein of caveolae (see
above). eNOS activation is, indeed, under the close
regulation of caveolin, and any change in caveolin
abundance modulates the capacity of the enzyme to
produce NO (364) and thus to regulate MMP-9 activity
(157). The potential role of caveolin/caveolae in pro-
genitor cell biology and mobilization led us to compare
the posts ischemic vasculogenic process in wild-type and
caveolin-deficient (cav-1−/−) mice (363).

Mouse phenotypes after the resection of short fem-
oral artery/vein segments were dramatically different.
Wild-type mice completely recovered normal hindlimb
perfusion, whereas cav-1−/− mice presented a perma-
nent drop in blood flow in the operated hindlimb.
Furthermore, while transplantation of wild-type mice with
either eNOS−/− or wild-type bone marrow cells indifferently led to a complete recovery of limb perfusion
(4), the transplantation of cav-1−/− bone marrow CD45+ cells
led to distinct patterns of response. When cav-1−/−
CD45+ cells were used, the restoration of blood flow in
the proximal operated limb was delayed with the partial
or complete loss of the animal’s foot. In contrast, in
wild-type CD45+ cell-reconstituted wild-type mice, no dis-
tal limb atrophy was detectable. Furthermore, cav-1−/−
mice recovered from the ischemic insult when CD45+
cells from wild-type mice were used to reconstitute the
bone marrow or Sca-1+ Lin− bone marrow cells were
directly injected in the blood.

In an attempt to understand the reasons of the
deficit in hematopoietic stem cells mobilization from
cav-1−/− mice, we focused on VEGF and SDF-1 signal-
pathways and found that both were altered due to the
lack of caveolin (Fig. 14). A deficit in the coupling of
VEGFR2 receptor and downstream eNOS activation
was identified as a consequence of a deficit in caveolar
platforms in caveolin-deficient mice (382). In endothe-

cells isolated from cav-1−/− mice, VEGF failed to
induce Ser-1177 eNOS phosphorylation and Thr-495 de-
phosphorylation, two hallmarks of eNOS activation.
Interestingly, caveolin transfection in cav-1−/− endo-

celial cells redirected the VEGF-induced eNOS activa-
tion that differentiation of EPC to EC and further NO-

colony assays (255). A deficit in VEGF/eNOS coupling
was further identified in late EPC (not shown), suggest-
ing that differentiation of EPC to EC and further NO-
mediated angiogenesis may be modulated according to
the caveolin density. This may account for part of the
deficit in vasculogenesis associated with major cardio-
vascular diseases (364). As for SDF-1-dependent signal-
ing, the alteration stemmed from the incapacity of this
cytokine to induce the internalization of its cognate
receptor CXCR4 within caveolae (absent because of the
lack of caveolin-1). As a result, EPC remain attached to
the bone marrow stromal cells, thereby impairing the

FIG. 13. Influence of NO on MMP-9-driven release of soluble Kit
ligand (skitL). A: gelatin zymography of bone marrow plasma before and
3 days after a single 5-FU injection; densitometric quantification is
provided for information. B: soluble skitL in bone marrow plasma was
measured on day 6 after 5-FU treatment (n = 6–8 per group). eNOS
deficiency abrogates MMP9 activation and release of sKitL. [From
Aicher et al. (4).]
mobilization process. Considering that this step of caveolae fission (and consequently, of CXCR4 internalization) is favored by the activation of the VEGF/NO pathway in endothelial cells (74, 118) and in EPC (unpublished data), these data further emphasize the role of caveolin/caveolae in bridging a deficit in eNOS signaling and a deficiency in postnatal vasculogenesis.

As estrogens exert part of their nongenomic signaling through similar activation of the caveolin/eNOS axis, the next section will expand on the implication of eNOS in their vasculoprotective properties.

C. eNOS, Estrogens, and Vascular Remodeling

1. Estrogens, NO, and cardiovascular protection

The impact of estrogen on cardiovascular health arose from two sets of observations from population-based studies: 1) premenopausal women have much less coronary artery disease than men (28), and 2) the incidence of the disease rises after menopause (329). A third set of evidence arises from observational research on postmenopausal hormone replacement therapy (HRT) that suggests a reduction of the risk of coronary heart disease to premenopausal levels (385). This latter point was however questioned by randomized clinical trials which identified increased cardiovascular risks after HRT. Factors such as late initiation of HRT and the association of progestin with estrogen could account for these discrepancies (81). Still, animal studies and clinical studies are concordant in suggesting that when administered in the early time after menopause, HRT may be cardioprotective (353).

The mechanisms underlying the protective effects of estrogens involve the regulation of the lipid balance (29) but also a direct action on the blood vessel wall (275). The latter effect is particularly obvious at the onset of menopause when the concentrations of 17β-estradiol (E2) fall to levels that are equivalent to those in males or during the menstrual cycle (wherein circulating E2 levels fluctuate) (67). In postmenopausal women (or after ovariectomy in young women), endothelium-dependent vasodilation and blood flow response to NO antagonism are lower than in premenopausal women. Also, studies evaluating changes in brachial artery diameter during the menstrual cycle revealed that the endothelial vasodilatory response to reactive hyperemia or bradykinin paralleled serum E2 levels, with the lowest levels seen during menarches and reaching a peak at midcycle (69, 115). Importantly, in these different studies, when tested, the vascular response to a NO donor is generally unaltered. The serum levels of nitrites/nitrates (i.e., the major circulating NO metabolites) are subject to parallel changes with higher serum levels at mid-cycle (or before menopause if compared with postmenopausal women) (354, 355).

The reversible characteristic of the loss of the endothelial-dependent NO-mediated vasorelaxation was provided by HRT studies which documented that estrogen replacement could significantly restore a normal NO activity (174, 251). Interestingly, progesterone when included in postmenopausal HRT blunted these effects on NO production.

Insights on the source of NO underlying the estrogen effects may be found in the numerous experimental studies performed in rodents. In animals, estrogen was indeed shown to protect the endothelial function or restore it.
after vascular injury in a NO-dependent manner. The eNOS isoform was rapidly suspected to primarily mediate the estrogen effects on the vascular wall (406). Ex vivo evaluation of the reactivity of rings of vessels collected from animal preexposed to estrogen revealed an increase in the NO-sensitive endothelium-dependent relaxation (for review, see Ref. 67). Increase in steady-state eNOS mRNA levels and eNOS protein expression in different vessels were also documented in response to E₂ treatment. Of note, the capacity to reverse endothelial dysfunction by increasing eNOS expression was also recently extended to the SERM raloxifene (324, 454) and to phytoestrogens (11).

These studies, however, pointed out another key aspect of the estrogen action on the NO system, namely, the acute effects on eNOS activation. Indeed, several authors have reported that E₂, when acutely applied to ex vivo isolated arterioles, causes a rapid, NO-mediated relaxation (82). This however was not observed in all the vascular beds, some of which showed an endothelium-independent E₂-mediated relaxation (376). There is also evidence of short-term effects of estrogen on eNOS function in humans. Ethinyl E₂ was, for instance, found to cause a direct decrease in coronary vasomotor tone when administered intravenously and a greater vasodilatory response to ACh when intracoronarily coadministered (346). NOS antagonism could prevent at least in part the augmentation in the ACh response.

Altogether, the above studies in animals and in humans indicate that estrogen may cause both long-term upregulation of eNOS expression and rapid enhancement of eNOS enzyme activation. Note that the implication of the inducible NOS isoform was formally excluded by studies using genetically deficient mice for this isoform and documenting that the protective effects of estrogen were conserved.

2. Estrogens and bone marrow

Another source of sex difference in the frequency and gravity of cardiac events may also be found in the capacity of estrogen to modulate EPC mobilization and differentiation in postischemic recovery (see also previous section). For example, Iwakura et al. (184) used a model of carotid injury in ovariectomized wild-type mice receiving either 17β-estradiol or placebo. They showed that estradiol treatment significantly increased the number of circulating EPC and their incorporation into injured arterial segments, thereby leading to an acceleration of the reendothelialization process. Importantly, they further documented that estradiol did not accelerate EPC-driven reendothelialization in eNOS-deficient mice. In another study, the same authors confirmed that estradiol could augment the mobilization of bone marrow-derived EPC after myocardial infarction (MI) and increase their incorporation into sites of ischemia-induced neovascularization (161). Again, in eNOS-null mice, the effects of estradiol on both the myocardial function recovery and the mobilization of EPCs were lost. Of note, although the genetic deficit in eNOS may account for the incapacity of cells to produce NO in response to estrogens, the direct interaction between eNOS and the estrogen receptor ERα within caveolae (68, 90) could also account for a loss of functional ER when the scaffold eNOS protein is absent. This was indeed suggested in a recent study where the reendothelialization promoting properties of E₂ were unaffected by NOS inhibition, but abrogated in eNOS-deficient mouse vessels or endothelial cells (35) (Fig. 15).

Interestingly, a study by Grassi et al. (153) focused on the role that NO plays in ovariectomy-induced modifications of bone turnover. NO produced by bone cells in response to estrogen is indeed known to regulate bone maturation and remodeling. In their experiments, however, the deficit in estrogen associated with ovariectomy led to a stable increase in eNOS expression. By comparing bone mineral density in eNOS-deficient and wild-type mice, they further documented that NO was actually limiting the bone-remodeling process in ovariectomized wild-type mice.

3. Genomic regulation of eNOS expression by estrogens

The increase in eNOS abundance in response to long-term E₂ exposure has been reported in endothelial cells (177) and in cardiac myocytes (301, 302). Note that nonvascular cells such as osteoblast-like cells (15) and carcinoma cells (152) were also shown to increase eNOS expression in response to E₂ stimulation. These effects are blocked by specific estrogen antagonists like tamoxifen, confirming the role of ER in the changes in eNOS transcript expression. Furthermore, eNOS upregulation by E₂ was fully prevented in neonatal rat cardiac myocytes by the ERβ-specific antagonist RR-tetrahydrochrysene (THC), indicating a primary role for that ER subtype (302). Nuclear run-on assays showed that the increase in eNOS mRNA abundance is related to greater ENS gene transcription, and not to increased stability of eNOS mRNA (207). Although no classical estrogen response elements (EREs) can be found in the eNOS promoter sequence, EMSA studies have shown a greater DNA-protein complex formation between a putative Sp1 binding element and estrogen. Posttranslational regulation of eNOS has also been documented, in particular through changes in the extent of its interaction with caveolin (375). As amply detailed above, the close interaction between eNOS and caveolin maintains the enzyme in its inactivated state in the absence of agonist stimulation (120, 122). An upregulation of caveolin, as observed in
response to ovariectomy (i.e., chronic estrogen depletion), was therefore not surprisingly reported to account for the loss of endothelium-dependent NO-mediated relaxation (together with the decrease in eNOS expression) (189, 325, 441); estrogen replacement normalized the vasorelaxation and the expression of both eNOS and caveolin. Of note, the interaction between eNOS and Hsp90 was shown to be stimulated by E2, thereby promoting the active conformation of eNOS (358, 367).

4. Nongenomic regulation of eNOS by estrogens

The acute effects of estrogen on eNOS activation in endothelial cells are not blocked by inhibition of gene transcription but are attenuated by ER antagonists and stimulated by recombinant ERα expression (75). The dissection of the downstream signaling cascade led to the identification of MEK/MAPK and PI3K/Akt as key enzymes activated by E2 stimulation. Although the final target of MAPK remains unknown, the E2-dependent Akt activation was shown to lead to phosphorylation of eNOS on the serine-1177 (374). Moreover, this E2-induced PI3K/Akt signaling seems to entail direct interaction between ERα (and not ERβ) and the p85 subunit of PI3-kinase (381). The caveolin-binding protein striatin was consecutively identified as the molecular scaffold that localizes ERα to the membrane and organizes the functional, multiprotein complex with eNOS, ERK, and Akt (258). More recently, a direct interaction between Gαi and ER was claimed to play a direct role in the estradiol-induced eNOS activation (223). Of note, in these experiments, the vascular protection was conferred by the attenuation of monocyte adhesion.

D. eNOS and Cardiac Stem Cells

Aside from the recruitment of endothelial progenitor or hematopoietic stem cells to the injured heart for tissue healing/repair, a number of recent studies have highlighted the regenerative potential of resident cardiac stem cells in specific “niches” within the adult heart, from which these cells may divide and, upon proper signaling differentiate into mature cell types composing the myocardium, including cardiac myocytes (26, 49). The role of NO as a paracrine modulator of such differentiation, however, has remained elusive so far.

Early studies had demonstrated that eNOS favors the maturation and cardiomyogenesis of murine embryoid bodies in vitro; indeed, chronic (9 days) NOS inhibition in these embryonic cells or treatment with the guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo-[4,3-a]-quinoxalin-1-one (ODQ), resulted in defective or incomplete myofibrillogenesis and differentiation arrest; all these phenomena were reversed upon incubation with the NO donor spermine-NOate (37). Likewise, NO donors and human iNOS-gene adenoviral transfection in mouse embryonic stem cells cultured in embryoid bodies facilitated their differentiation into beating cardiomyocytes (198).

The regenerative potential of cardiac stem cells would be expected to be enhanced by factors promoting their proliferation or resistance to cell death upon cardiac injury. It has been proposed that estrogens may promote cardiac repair by enhancing the recruitment or mobilization of bone-marrow-derived stem cells (254). Estrogen replacement was reported to increase the number of bone-marrow-derived stem cells in the damaged myocardium (326). Moreover, estrogen administration was shown to enhance the expression of the stem cell marker, c-kit (CD117), in the bone-marrow-derived stem cells injected into the injured heart (143). This increase in c-kit expression was associated with enhanced retention of these cells in the infarcted myocardium (143).

Although the role of estrogen in cardiac repair remains to be fully elucidated, these studies suggest that estrogen replacement may enhance cardiac repair by promoting the mobilization or recruitment of bone-marrow-derived stem cells and facilitating their differentiation into mature cell types that can contribute to myocardial repair.
stress, such as ischemia. Cardiac stem cells were indeed shown to be preserved from apoptosis in mice with transgenic overexpression of insulin-like growth factor I (IGF-I) (408). IGF-I overexpression also promoted new myocyte formation in adult mice (347). As IGF-I exerts its effects at least partly through the PI3K-eNOS pathway in cells that express eNOS, including endothelial cells (182), this raises the possibility of NO being a critical paracrine signaling molecule for both the preservation and, perhaps, the differentiation of cardiac stem cells. Recent experiments testing the effects of exogenous NO or inhibitors of endogenous NOS in P19 cells expressing GFP under a cardiac-specific promoter to monitor their cardiomyocyte differentiation lend further support to such a proposition (87).

A number of in vivo studies are also suggestive of a beneficial role of endogenous NO on cardiac repair, although the involvement of cardiac stem cells remains to be formally proven. Infarcted mice bearing a cardiomyocyte-restricted NOS3 transgene exhibit less LV dysfunction and remodeling compared with wild-type mice, despite similar collagen content (188). Remote, noninfarcted myocytes were also less hypertrophied in transgenic mice, but the potential beneficial impact of cardiac stem cells was not investigated.

Also, as previously mentioned, Landmesser et al. (231) demonstrated that the favorable role of atorvastatin on endothelial progenitor cell mobilization (231), myocardial neovascularization in the infarct border, protection from LV dysfunction, interstitial fibrosis, and survival after myocardial infarction in wild-type mice was lost in eNOS−/− mice. Therefore, an increase of endogenous eNOS activity is responsible for the beneficial effect of statins on cardiac regeneration, although again, the specific role of resident cardiac stem cells was not directly addressed. The therapeutic potential of these and other drugs known to enhance eNOS expression/activity through the potentiation of cardiac stem cell differentiation remains to be formally tested.

In summary, the evidence reviewed in this section extends the role of eNOS beyond the acute modulation of vessel tone or cardiac function in response to physical forces, to chronic tissue healing through modulation of progenitor-driven vasculogenesis, and, perhaps, cardiac regeneration. This involves eNOS expressed both in stromal cells of specific “niches” and in the progenitors themselves, following its activation by hormones (e.g., estrogens) and cytokines (e.g., VEGF, under the transcriptional control of HIP, itself regulated by NO). Strikingly, most mechanistic studies again highlighted the association of eNOS to multiprotein complexes assembled in microdomains enriched in caveolin-1, further generalizing the functional importance of such protein-protein interaction for the pleiotropic effects of eNOS in cardiovascular biology.

With the hindsight of these mechanistic studies, the next section will examine specific pathophysiological situations highlighting the involvement of cardiac or vascular eNOS in cardiovascular function and repair under the influence of mechanical forces at the whole organ level.

V. ENDOTHELIAL NITRIC OXIDE SYNTHASE AND MECHANOTRANSDUCTION IN INTEGRATED PHYSIOLOGY AND PATHOPHYSIOLOGY

A. Vascular eNOS Influence on Pulse Wave Velocity and Blood Pressure Variability

eNOS-mediated mechanotransduction plays a significant role in the homeostatic mechanisms that control blood pressure and organ perfusion. Any deviation of blood pressure from its set-point elicits a number of physiological correction mechanisms acting both systemically (such as baroreceptor reflexes) and locally (i.e., in the vascular wall). The latter include regulation of the myogenic tone, local action of catecholamines, and the renin-angiotensin system; all of these, in turn, are controlled by paracrine production of NO from the endothelium. The speed at which these feedback mechanisms exert their action on blood pressure, however, may differ greatly. Neuronal mechanisms mediating the baroreceptor reflexes act quickly, whereas humoral influences through activation of circulating autacoids take longer time to develop their action and will result in slower regulation of blood pressure variability. This has led to the proposition that each component of these regulatory mechanisms could be identified through decomposition of the complex variability of systolic blood pressure by frequency analysis. Accordingly, we will review the evidence supporting the identification of specific components of variability that respond to changes in NO production, among other regulators.

Spectral analysis of systolic blood pressure (SBP) in animals (and to a lesser extent in humans) has provided some identification of frequency bandwidths reflecting each of the above-mentioned regulatory components. In most animal studies, sympathetic modulation of vascular tone is reflected by variability at low frequencies (0.2–0.6 Hz in rats; and lower, from 0.075 to 0.15 Hz, in humans), the so-called Mayer waves (196), that may differ between vascular beds; a theory postulates that the arterial baroreflex exhibits a resonance frequency at the frequency of Mayer waves (34). Myogenic vasoconstriction typically develops with a slower time course than catecholamine-induced constriction, which is reflected by an influence on even lower frequencies (below 0.2 Hz) in the blood pressure (BP) variability spectra, as observed experimentally after infusion of calcium channel blockers (that typ-
Later studies in mice with genetic deletion of NOS3 (294) showed increased variability in the frequency range of 0.05–0.4 Hz, but again, the interpretation of this observation is confounded from the chronic elevation of blood pressure in these mice (403). Based on the modifications of the power spectra in wild-type (C57Bl6, WT) mice treated with a NOS inhibitor, we found frequencies between 0.05 and 0.4 Hz (VLF) to be responsive to acute changes in NO production. We verified that these spectral bandwidths were affected by NO independently from changes in absolute levels of BP by analyzing the effect of exogenous NO (low doses of sodium nitroprusside) at unchanged levels of BP. SNP dose-dependently and quantitatively decreased BP variability independently of BP levels, both in the presence or absence of NOS inhibition. This was associated with increased phosphorylation of VASP (reflecting NO bioactivity) in aortic extracts from the same mice that negatively correlated with VLF variability. Changes in BP variability did not result from NO-dependent regulation of autonomic nervous system activity or systemic neurohormones, which may affect SBP power spectra in partly overlapping frequencies (see above), since the effect of NO was insensitive to blockade of the renin-angiotensin system with enalapril or adrenergic system with α- or β-adrenoceptor blockers (or both) at unchanged absolute BP levels. Conversely, the influence of the adrenergic system (e.g., Mayer waves) on VLF is also modulated by NO through postsynaptic effects, as demonstrated from the NOS-dependent attenuation of the vasopressor effect of phenylephrine in vivo (103). Therefore, in the C57Bl6 mouse strain at least, NO affects SBP variability in the frequency range of 0.05–0.40 Hz, and we showed this to be independent from BP changes. Within these frequency bandwidths, NO may additionally attenuate the effects of catecholamines and, perhaps modulate myogenic tone, if its target frequencies below 0.2 Hz, as identified in the rat, also applies to mice.

To validate the influence of NO in the VLF, we applied spectral analysis of BP variability in mice genetically deficient in caveolin-1 (cav-1−/−). As mentioned in earlier sections, caveolin-1 is the structural protein of caveolae where it colocalizes and interacts with eNOS in endothelial cells. This interaction tonically inhibits eNOS enzyme activity and is relieved upon agonist- or stimulus-induced increases in intracellular calcium and the subsequent displacement of caveolin-1 by the activator calcium-calmodulin complex. Genetic deletion of caveolin-1 was shown to result in enhanced endothelium-dependent relaxation of isolated vessels in vitro, that was abrogated by NOS inhibition, pointing to a potentiation of NO release from a deinhibited eNOS. However, this was never formally associated with changes in blood pressure obtained by conventional measurements, which is at odds with previous evidence of increased circulating nitrate/nitrite levels in cav-1−/− mice, raising doubts about the relevance of the eNOS/caveolin-1 interaction or, more broadly, of NO regulation of vascular tone for blood pressure homeostasis in vivo. On the other hand, as these mice have unchanged blood pressure, they represented a suitable model for the characterization of the independent influence of eNOS on variability. We first verified that cav-1 deletion results in endothelium-derived increased vascular NO production through electron paramagnetic resonance (EPR) measurements of NO production in intact aortic segments of cav-1−/− mice ex vivo, and in circulating Hb-NO by EPR spectroscopy. In these animals, SBP variability in the VLF (0.05–0.40 Hz) was also decreased (reversibly under NOS inhibition), further confirming the “buffering” effect of NO on SBP variability in our selected bandwidths in vivo. From a complete analysis of BP profiles in the time domain, we can exclude independent effects of BP levels or central effects, since SBP was strictly unchanged in cav-1−/− compared with wild type, and the circadian regulation was preserved. To exclude other chronic secondary adaptations to cav-1 genetic deletion, we examined the effect of the acute modulation of eNOS activity through delivery of a fusopeptide containing a CSD domain of caveolin-1, which is sufficient to inhibit eNOS activation, in vitro and in vivo, at least in the microvasculature (58).
After validation of its ability to displace endogenous caveolin-1 and bind to eNOS in intact endothelial cells, we confirmed that, after intraperitoneal administration in vivo, the peptide inhibited both NO production in aortic segments and circulating Hb-NO levels in wild-type and cav-1−/− mice. Although it did not increase SBP, CSD peptide infusion increased VLF variability in wild-type and cav-1−/− mice, the mirror effect of cav-1 deletion. That this involved a reduced buffering effect of endogenous NO was confirmed from the absence of supplemental effect of NOS inhibition in vivo. Similarly, the CSD peptide abrogated the BP-lowering effect of carbachol infusion and attenuated carbachol-induced VASP phosphorylation in vivo in both mouse strains, confirming its ability to interrupt eNOS-mediated vascular signaling leading to vasorelaxation (103).

In addition to validating the frequency bands responsive to NO, our use of this CSD peptide also provided key evidence that vascular NO deficiency is a cause of altered BP variability rather than a consequence of high BP producing increased variability per se, thereby demonstrating the pathophysiological importance of NO-dependent endothelial function for BP homeostasis. This role of NO may extend beyond the peripheral control of vascular contractility, to the central regulation of BP in the time domain, i.e., its circadian variation. Indeed, a recent study demonstrated that NO upregulates the promoter activity of the clock gene Period and that NO administration could restore the circadian variation of BP in elderly animals with deficient endogenous NO synthesis (224).

B. eNOS and Vascular Remodeling in Hypertension

A major adaptative response to chronic elevations of the arterial pressure is the vascular remodeling that contributes to normalize wall tension. The implication of NO in such vascular remodeling was demonstrated in the early 1990s both in vitro and in vivo. Chronic oral treatment with l-NAME for 2 wk is associated with marked medial thickening and perivascular fibrosis in coronary microvessels (183). The long-term blockade of NO synthesis causes coronary microvascular remodeling that implicates increased wall-to-lumen ratio and perivascular fibrosis in rats in vivo by a mechanism independent from arterial hypertension (303). In essential hypertension, while large conduit arteries present hypertrophic remodeling, resistance artery remodeling remains eutrophic and is characterized by a reduced lumen (43). Specifically, in resistance arteries, chronic increases in blood flow, in vivo, induce structural and functional remodeling that requires NO production and MMP activation, while low-flow remodeling remains independent of NO and MMPs (111). Similarly, inward remodeling was shown to occur in vessels cultured under no-flow conditions and is inhibited by flow-dependent NO synthesis (331). As reviewed above, endothelial expression of caveolin-1 and caveolae are critical for such chronic NO-dependent adaptive remodeling (447).

C. Cardiac and Vascular eNOS and Myocardial Hypertrophy

The role of eNOS on the remodeling of the cardiac muscle in response to chronic changes in mechanical constraints (i.e., volume or pressure overload) is equally important, as it will inevitably impinge on contractile performance. Evidence for its modulatory role was provided from the description of the phenotype of genetically modified mice with either deletion or overexpression of eNOS, as reviewed recently (267). Most of the evidence points towards an inhibition of prohypertrophic stimuli (including pressure overload secondary to trans-aortic constriction) (62) by the vascular and myocyte eNOS, at least at moderate stimulus intensity, resulting in preserved LV function (and survival) on the long term. Aside from the direct effects through changes in pre- and afterload by the vascular eNOS, the protection could be explained by optimization of excitation-contraction coupling and preserved contractility (see above), enhanced diastolic reserve, attenuation of deleterious effects of catecholamines (e.g., by antagonizing β1-adrenergic effects, Ref. 24), cGMP and PKG-mediated inhibition of prohypertrophic signaling (e.g., through calcineurin/NFAT, Ref. 123) promotion of angiogenesis and progenitor cells differentiation, or a combination of all the above.

It is important to recognize that, as with all the NOS, eNOS is susceptible to “uncoupling” of its catalytic activity, resulting in transfer of electrons to molecular oxygen to produce O2− in lieu of/NADH addition to NO, thereby enhancing oxidant stress, as reviewed above. This has been observed in vivo in conditions of relative deficiency in BH4 relative to the amount of expressed eNOS, as in endothelial-targeted eNOS overexpressing mice (32). At “endogenous” levels of eNOS, a similar situation is obtained upon BH4 depletion by exposure to oxidant stress, as produced during ischemia/reperfusion or upon excessive chronic mechanical overload. The latter was shown to paradoxically produce more damage (dilated cardiomyopathy and increased mortality) in eNOS expressing versus eNOS-deficient mice, because of increased eNOS-dependent oxidant radicals production (399). This raises the interest for unveiling new molecular mechanisms of protection of eNOS from uncoupling, which would preserve the enzyme’s beneficial effects in the face of prevailing oxidant stress (see below).
D. eNOS and Vascular Remodeling in Postischemic Healing

A clinical priority in myocardial ischemia caused by an occluded coronary artery is rapid reestablishment of perfusion. However, reperfusion also leads to organ dysfunction, including arrhythmias, myocardial stunning, and microcirculatory disturbance (422). Overexpression of eNOS in endothelial cells or cardiac myocytes may protect the heart against these ischemia/reperfusion insults through a variety of effects including vasorelaxation, inhibition of platelet aggregation and leukocyte recruitment (185), prevention of myocyte necrosis/apoptosis (39, 139), and induction of hibernation (175). Interestingly, the cardioprotective effects of insulin (139), corticosteroids (159), and VEGF (226) were found to arise from eNOS phosphorylation at the serine residue 1177 (Ser-1177; human sequence) following receptor-specific induction of the PI3K/Akt-signaling pathway. This observation led us and others to propose that an increase in vascular NO production does not necessarily require an increase in eNOS abundance but may also stem from specific post-translational modifications of endogenously expressed eNOS. As mentioned earlier, we have reported that the Akt-driven phosphorylation of eNOS on serine-1177 implies assembly of a multiprotein complex in which Hsp90 acts as a scaffold, recruiting activated Akt in the eNOS vicinity (21, 50). In consecutive studies, we therefore examined whether overexpression of Hsp90 could promote NO-mediated protection of the myocardium from the deleterious effects of ischemia-reperfusion. In a pig model of infarct, we found that recombinant Hsp90 expression in the ischemic region of the heart led to a one-third reduction in infarct size and prevented the increase in posts ischemic LV end-diastolic pressure observed in mock-transfected animals (225) (Fig. 16). These effects were completely abrogated by administration of the NOS inhibitor L-NAME. We further documented that the cardioprotective effects of Hsp90 were not only due to its capacity to act as an adaptor for Akt, thereby promoting eNOS serine-1177 phosphorylation, but also to recruit the phosphatase calcineurin, which in turn may promote eNOS threonine-495 dephosphorylation (Fig. 16). This reinforces the impact of Hsp90 regulation on eNOS activation. As reviewed above, Ser-1177 phosphorylation was indeed proposed to improve the electron flux through the enzyme and to increase its affinity for CaM (273, 397), whereas dephosphorylation of Thr-495 is thought to suppress the steric inhibition for CaM association to its binding site (76, 128). Pfosser et al. (328) also explored the potential of regional Hsp90 overexpression to induce therapeutic neovascularization in vivo in a chronic rabbit hindlimb ischemia model. Compared with mock-treated controls, Hsp90 transfection increased capillary/muscle fiber ratio and collateral formation in a L-NAME-sensitive manner. These data further identified the capillary level as an initial site of Hsp90-induced neovascularization, followed by growth of larger conductance vessels, resulting in an improved limb perfusion. More recently, Kupatt et al. (227) investigated whether retroinfusion of a lipocomplex containing a cDNA encoding for the phosphomimetic eNOS construct (eNOS S1177D) could affect neovascularization and function of the ischemic myocardium. A pig model of hibernation was used wherein a reduction stent

![Fig. 16. Effects of Hsp90 transfection on posttranslational regulation of eNOS in posts ischemic myocardium. A: effects of Hsp90 transfection on postischemia/reperfusion ventricular dysfunction. Left ventricular end-diastolic pressure (LVEDP; mmHg) was determined before (pre) and 24 h after (post) ischemic insult (n = 6 per group; #P < 0.05 vs. mock group). B: fragments of cardiac tissues from control (CTL) area (nonischemic), area at risk (AAR), and infarct zones were collected from mock- and Hsp90-transfected pigs. Corresponding lysates were immunoblotted with antibodies directed against Hsp90, eNOS, and the indicated phosphorylated residues. Hsp90 overexpression prevents posts ischemic LV function degradation in a NOS-dependent manner. This is paralleled with increased Ser-1177 phosphorylation and Thr-495 dephosphorylation. [Adapted from Kupatt et al. (225).]
A graft was percutaneously implanted into the left anterior descending artery, inducing total occlusion within 4 wk (Fig. 17). The eNOS S1177D overexpression increased capillary and collateral growth and regional myocardial perfusion in a 1-NAME-sensitive manner. The expression of the constitutively active form of eNOS resulted in a gain of perfusion, enabling an improved functional reserve of the hibernating myocardium. These results confirmed a previous study by the same authors (226) where the same phosphomimetic eNOS construct was shown to decrease the infarct size in pigs together with a decrease in NFkB activation and PMN infiltration in the infarct region. The results with the S1177D construct were recently confirmed by Atochin et al. (17) in transgenic mice with exclusive expression of the phosphomimetic form of eNOS. These mice showed greater vascular reactivity, develop less severe strokes, and have improved cerebral blood flow in a middle cerebral artery occlusion model than mice expressing an unphosphorylatable (S1179A) form. Altogether, these data demonstrate the in vivo relevance of posttranslational modification of eNOS on the preservation of ischemic cardiovascular tissue and also explain how multiple cardiovascular risks, such as diabetes and obesity, may be centrally integrated on changes in eNOS phosphorylation to influence progressive cardiovascular diseases in vivo.

VI. CONCLUSION

This review examined the involvement of NO, produced by eNOS, in the complex molecular machinery translating the effect of physical forces into intracellular signaling events driving the adaptation of individual cells, tissues, or organs to acute or chronic mechanical stimulation. It is remarkable that, at virtually all levels, genetic evidence (through isoform-specific deletion) or pharmacological approaches (with NOS inhibitors or eNOS activators) support the physiological importance of eNOS activation as a homeostatic mechanism of mechanically challenged organisms, be it for the acute and chronic regulation of vessel diameter in response to altered shear forces, fine-tuning of cardiac inotropic, lusitropic, and chronotropic states in response to stretch, or cardiovascular tissue repair through eNOS’s influence of progenitor cell biology. In a way, the very ancient phylogenetic origin of the NO synthetic pathway (identified in primitive organisms such as the horseshoe crab) would make NO a believable candidate as mediator of such fundamental processes as the reaction of any organism to physical forces in its environment. It certainly does not make it the only one, as eNOS-deficient animals do develop and survive (albeit with profoundly altered cardiovascular function), but a critical modulator at the very least. Genetic epidemiological data also point to eNOS as a modifier gene in pathological processes that clearly involve chronic adaptation to hemodynamic forces, such as ath erosclerosis and hypertension, or exercise-induced adaptation of peripheral vascular resistance, supporting its relevance to human disease. To further substantiate causality, supplying with exogenous NO should then correct the disease associated with its insufficient production. This has been verified in some aspects (such as acute vasodilatation with NO donors), but not all. Although disappointing at first glance, this fact is also a clear reminder of two things: 1) eNOS is one of several (probably redundant) modulators of these processes, as stated before; and 2) exogenous NO supply in no way reconstitutes the minute regulation of eNOS activity in response to specific stimuli in discrete subcellular compartments (and in distinct cell types). The very complex molecular regu-

[Fig. 17. Forced eNOS S1177D expression improves perfusion and function of ischemic myocardium. A: regional myocardial function was assessed by subendocardial segment shortening (SES) 3 wk after lipofection in the distal left anterior descending artery (LAD) region at rest or after atrial pacing (120/min and 140/min); n = 5/group. B: number of collaterals detected 3 wk after transfection (postmortem angiogram); n = 6 per group. [Adapted from Kupatt et al. (227).]]
loration of eNOS appears indispensable for coordinate signaling with such a versatile radical, the effect of which is also critically dose and environment dependent. An example is the bimodal effect on cardiac inotropism, or the switch from beneficial to detrimental effect of NO on tissue remodeling in the context of increased oxidant stress. Nevertheless, it is striking that the mechanosensing machinery coordinately activates pathways that result in acute and sustained eNOS activation, increased eNOS expression, and preservation of NO bioactivity in physiological situations (such as unidirectional laminar shear in vessels), whereas deficiency in activation of any or several of these steps results in maladaptive phenotypes together with altered eNOS function. This again emphasizes the need for preservation of an optimally regulated eNOS for adaptive responses to mechano- (and other) stimuli. Rather than trying to grossly substitute the effect of physiologically produced NO with exogenous sources, in many instances, the real added value would be a strategy that restores endogenous NO production from eNOS in situ. This has proven efficacious with some clinically used drugs, such as statins and angiotensin-converting enzymes which, among other effects, increase eNOS abundance and activity, with beneficial effects demonstrated in diseases such as atherosclerosis or postinfarction remodeling. Other attractive strategies could aim to restore proper activation of the enzyme, e.g., through modulation of caveolin-1 (or -3 in muscle cells) that couples it to mechanical (and other) stimuli, or catalytic activity, e.g., by restoring substrate/cofactors supply, or proper conformation, phosphorylation state, and coupling with Hsp90. Alternative strategies could target upstream mechanosensitive elements of signaling (e.g., membrane receptors, such as VEGFR2; integrins, or associated proteins, such as melusin in muscle cells; other downstream effectors, such as specific PI3K isoforms), knowing that these may not exclusively signal to eNOS. Although some of these interventions have been tested in genetically modified mice, their therapeutic applicability needs further confirmation.

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