EDITORIAL

Endothelial nitric oxide synthase activation and nitric oxide function: new light through old windows

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Abstract

The principle mechanisms operating at the level of endothelial nitric oxide synthase (eNOS) itself to control its activity are phosphorylation, the auto-regulatory properties of the protein itself, and Ca^{2+}/calmodulin binding. It is now clear that activation of eNOS is greatest when phosphorylation of certain serine and threonine residues is accompanied by elevation of cytosolic $[Ca2+]_i$. While eNOS also contains an autoinhibitory loop, Rafikov *et al*. (2011) present the evidence for a newly identified 'flexible arm' that operates in response to redox state. Boeldt *et al*. (2011) also review the evidence that changes in the nature of endothelial Ca^{2+} signaling itself in different physiologic states can extend both the amplitude and duration of NO output, and a failure to change these responses in pregnancy is associated with preeclampsia. The change in Ca^{2+} signaling is mediated through altering capacitative entry mechanisms inherent in the cell, and so many agonist responses using this mechanism are altered. The term 'adaptive cell signaling' is also introduced for the first time to describe this phenomenon. Finally NO is classically regarded as a regulator of vascular function, but NO has other actions. One proposed role is regulation of steroid biosynthesis but the physiologic relevance was unclear. Ducsay & Myers (2011) now present new evidence that NO may provide the adrenal with a mechanism to regulate cortisol output according to exposure to hypoxia. One thing all three of these reviews show is that even after several decades of study into NO biosynthesis and function, there are clearly still many things left to discover.

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Editorial

For many years endothelial nitric oxide synthase (eNOS) has been regarded as the source of endothelial NO, which in turn acts as a potent vasodilator with a critical role in cardiovascular physiology. As the control of this Ca^{2+} sensitive isoform has been increasingly dissected at a structure–function molecular level, many different levels of post-translational modification have been identified that can potentially affect activity. In addition to the long recognized binding of the Ca^{2+}/calmodulin complex, eNOS requires dimerization and cofactor binding for activity, and through its activation cycle there are additional myristoylation and phosphorylation events that influence its affinity for the plasma membrane and Cav-1 at rest versus the formation of the eNOS dimer interacting with associated proteins (HSP90 and AKT, etc.) and cofactors in an active complex. An early model of eNOS activation was that eNOS was bound at the membrane to inhibitory Cav-1 and an increase in Ca^{2+}/calmodulin caused its release and dimerization. Thereafter the proposed phosphorylation sites on eNOS that controlled activity were 1177 as an activating site while 495 was thought to be inhibitory. The model was simple, and since NO itself is difficult to measure with any sensitivity, some researchers even attempted to report 1177 phosphorylation in particular as a substitute measure of activity. Nonetheless, others reported that in intact cells, these phosphorylation events could be observed without activation of eNOS, and activation could be observed without changes in phosphorylation. As more phosphorylation sites were identified and activation was studied further in intact cell systems, the concept of a simple off/on role for phosphorylation in regulating eNOS activity seemed increasingly unlikely and phosphorylation was alternatively suggested to be more a way of directing eNOS translocation to specific subcellular compartments within the cell (reviewed in Dudzinski & Michel (2007)) acting much like a 'zip' or 'postal' code (Cale 2005). Further studies have shown, however, that translocation involves many additional

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processes and protein–protein interactions beyond simple phosphorylation (Dudzinski & Michel 2007). The activation model has thus been further refined, with the proposal that some phosphorylation sites are indeed associated with activation, but also that they are not sufficient alone to bring about activation, and instead they serve to increase the efficiency of electron transfer between the complimentary functional domains of two eNOS proteins when forming a functional dimeric complex (for review see Dudzinski & Michel (2007) and also Rafikov *et al.* (2011) in this issue). In this study, the key refinement is the recognition finally that under these classic serine/threonine phosphorylation models eNOS does still need a physiologic elevation in Ca^{2+} above a basal of ~40–60 nM to achieve proper activation. The lower $[Ca2+]_i$ is in the basal state, the harder it is for phosphorylation at these serine and threonine based sites to achieve any given degree of activation. A recent publication now shows it is the combined phosphorylation at positions 1777 and 615 that most sensitize eNOS to activation by Ca^{2+}, and in that bi-phosphorylated state, a level of 100 nM [Ca2+] is sufficient for 50% activation and 300 nM gives full activation (Tran *et al.* 2009). This brings the eNOS story full circle and emphasizes that a true understanding of eNOS activation by a wide variety of agonists requires knowledge of Ca^{2+} signaling for the agonist under study. Furthermore, in intact cells it is not the initial agonist induced rise in $[Ca2+]_i$, but more the secondary sustained elevation of $[Ca2+]_i$ that most determines NO output (Lin *et al.* 2000). Recent studies of Ca^{2+} signaling in the context of eNOS activation also show that changes in this same endothelial sustained phase Ca^{2+} signaling are not constant and are in fact altered in different physiologic states (see review by Boeldt *et al.* (2011) in this issue). Thus physiologic changes in eNOS activation may also be critically regulated at the level of sustained phase Ca^{2+} signaling adaptation. Such adaptation occurs through alterations at the level of capacitative entry itself, and it is this important variable that critically determines gain and indeed duration of NO production over and above that due to any change in eNOS protein expression or transient phosphorylation. This adaptive change in capacitative entry mechanisms is particularly observed throughout the vasculature during pregnancy, and profound physiologic relevance is indicated in preeclampsia where a failure of this signaling adaptation response is associated with potentially life threatening hypertension (see also review by Boeldt *et al.* (2011)).

Beyond eNOS phosphorylation at the classic serine/threonine phosphorylation sites, or the relative role of Ca^{2+} in activation, eNOS is now increasingly found to be subject to phosphorylation on several Tyr sites through alternate kinase pathways such as Src. In addition, the 'autoinhibitory loop' that has been known for some years to be important in changes in activation in the monomeric versus dimeric state, is now joined by a further regulatory sequence element, the flexible arm. In the accompanying review in this volume, Rafikov *et al.* (2011) describe studies that newly identify this flexible arm and further suggest it is in fact a redox sensor, so raising the exciting possibility that eNOS can cycle into an active versus inactive state according to redox state.

Another recent development is in the area of the action of NO *in vivo*. While commonly acknowledged as a regulator of vascular function, a number of studies has shown eNOS to be present within steroidogenic cells. Early studies in rats suggested adrenal steroid production could be altered by exogenous NO donors, but eNOS was only expressed in the zona glomerulosa (ZG), and not the zona fasciculate (ZF; see the review by Ducsay & Myers (2011) in this issue). So why would eNOS be used in one zone but not another? Studies in sheep also showed eNOS was not only absent in the ZG but was expressed throughout the ZF (sheep have no clearly defined zona reticularis (ZR)), while in the rhesus adrenal it was contained within the steroid producing cells of the ZG and ZR but not the ZF (Peterson *et al.* 2001). It is important to understand that all the P450 family members that catalyze reaction steps in the adrenal are proteins with iron coordinated heme reaction centers responsible for binding and then presenting molecular oxygen in the reaction cycle. Furthermore, NO can also coordinate with heme in competition with oxygen, so inhibiting the reaction cycle. The question is does it happen *in vivo*? Peterson *et al.* (2001) proposed that the rhesus distribution of eNOS matched the P450 enzymes most essential to zone-specific steroid end products that are also required to undergo three molecular oxygen reaction cycles as opposed to one. In the human/primate these are P450aldo in the ZG to make aldosterone, and P450c17 in the ZR to make dehydroepiandrosterone (DHEA) . Of note, while P450c17 is also expressed in the ZF, it only undertakes one reaction cycle in the ZF pathway to cortisol. In both the ZG and ZR however, P450aldo and P450c17, respectively, undergo three rounds of reaction cycle, and through competition with molecular oxygen for the heme, would thus be most susceptible to NO inhibition. But zonation of eNOS is also different by species, so is that always true? Recent studies by Ducsay *et al.* suggest in the sheep, where eNOS zonal expression is predominant in the ZF, that NO can indeed regulate cortisol output if the sheep are exposed to the state of hypoxia, and that such regulation is likely mediated at many different levels including control of SF-1 transcription factor activity (see Ducsay & Myers (2011)). This is an interesting twist and the fact NO has now been shown to act as a regulator of cortisol production under physiologic conditions (and particularly pregnancy) is an important advance in this field. Clearly we not only continue to make advances in our understanding of the control of the production of NO but also the diverse actions of NO.

Declaration of interest

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