Production of reactive oxygen species caused by dysregulated endothelial nitric-oxide synthase (eNOS) activity is linked to vascular dysfunction. eNOS is a major target protein of the primary calcium-sensing protein calmodulin. Calmodulin is often modified by the main biomarker of nitrosative stress, 3-nitrotyrosine (nitroTyr). Despite nitroTyr being an abundant post-translational modification on calmodulin, the mechanistic role of this modification in altering calmodulin function and eNOS activation has not been investigated. Here, using genetic code expansion to site-specifically nitrate calmodulin at its two tyrosine residues, we assessed the effects of these alterations on calcium binding by calmodulin and on binding and activation of eNOS. We found that nitroTyr−calmodulin retains affinity for eNOS under resting physiological calcium concentrations. Results from in vitro eNOS assays with calmodulin nitrated at Tyr-99 revealed that this nitrination reduces nitric-oxide production and increases eNOS decoupling compared with WT calmodulin. In contrast, calmodulin nitrated at Tyr-138 produced more nitric oxide and did so more efficiently than WT calmodulin. These results indicate that the nitroTyr post-translational modification, like tyrosine phosphorylation, can impact calmodulin sensitivity for calcium and reveal Tyr site-specific gain or loss of functions for calmodulin-induced eNOS activation.

Fundamental biological processes like cell proliferation, gene transcription, cell death, exocytosis, and metabolism are predicated on the tight regulation of cytosolic Ca2+ concentration (1). At the heart of Ca2+ regulation is the Ca2+-sensing protein, calmodulin (CaM),3 which transduces intracellular Ca2+ signals into biological responses. CaM accomplishes this by serving as a Ca2+-binding regulatory subunit of a wide array of enzymes, structural proteins, and membrane transporters. Cellular CaM fluctuates between its Ca2+-bound form (Ca2+-CaM) and Ca2+ and target protein–bound forms (Ca2+–CaM–target), depending on the combination of intracellular Ca2+ levels and target affinity. Cytosolic Ca2+ levels are tightly regulated between ~0.1 μM under resting conditions and ~10 μM after stimulation (2). CaM is an abundant protein with intracellular concentrations up to 10 μM; however, because of the collective concentration of its many target proteins, CaM is the limiting agent in Ca2+ sensing (3, 4).

CaM target proteins in high concentration or that bind CaM with the highest affinity will scavenge the CaM from the cell. At maximal intracellular calcium levels, 25% of the Ca2+-CaM pool in endothelial cells is associated with endothelial nitric-oxide synthase (eNOS) (3). This amount of CaM bound by tight association with eNOS removes sufficient CaM from the intracellular pool to affect other target proteins as seen with the intracellular calcium regulatory channel, plasma membrane Ca2+-ATPase (3). Post-translational modifications (PTMs) on CaM serve as an additional level of control over CaM–target affinity and CaM activity necessary for the intracellular Ca2+ regulatory balance. CaM has two tyrosines, Tyr-99 and Tyr-138, which are phosphorylated at different stoichiometries to regulate target protein association (3, 6). Tyrosine phosphorylation of CaM has been shown to impact Ca2+-dependent CaM interaction with nitric-oxide synthase (NOS). PhosphoCaM-99 binds four times more tightly to neuronal NOS as compared with WT–CaM (4).

CaM is also susceptible to oxidative post-translational modifications (ox-PTMs) by reactive oxygen species and reactive nitrogen and oxygen species, such as H2O2, NO, and peroxynitrite (5–8). Proteomic analysis has shown that reactive oxygen species and reactive nitrogen oxygen species induce a variety of ox-PTMs on CaM in vivo, resulting in an accumulation of significant amounts of tyrosine nitration and methionine sulfoxidation (9–12). Previous studies have indicated that nitration of CaM at tyrosine 99 is a biomarker of oxidative stress and that nitration of CaM at tyrosine 138 is subject to a denitrase activity in activated macrophages (5, 11). Up to 30% of the cellular CaM pool is nitrated on critical regulatory tyrosine residues following macrophage activation (5); however, the effect of tyrosine nitration on CaM function has not been determined. However, given the impact of phosphorylation of these tyrosines on the CaM–NOS interaction, it is perhaps not surprising to think that CaM tyrosine nitration will alter CaM–eNOS affinity.
To evaluate the impact of ox-PTMs on CaM, previous work used standard site-directed mutagenesis with unreactive canonical amino acids to control sites of chemical oxidation. This approach, although the best available at the time, is problematic because altering amino acids to prevent ox-PTM formation can have unknown effects at these regulatory hot spots (11). Genetic code expansion (GCE) provides the means to assess the effects of site-specific protein tyrosine nitration through co-translational installation of nitroTyr into the protein of interest. This methodology has been used recently to show that tyrosine nitration can cause a toxic gain of function for key regulatory sites in heat shock protein 90 (Hsp90) and apolipoprotein A-I, as well as a loss of function in manganese superoxide dismutase (13–15).

The abundance of tyrosine nitration on CaM led us to characterize the effect this ox-PTM has on altering CaM function with site-specifically modified nitroTyr-containing CaM generated with GCE. We focused on CaM target eNOS because it utilizes a significant fraction of the cellular CaM pool and because of the regulatory role of tyrosine phosphorylation in the system. eNOS is a multidomain enzyme that catalyzes the conversion of L-arginine to the biological signaling molecule nitric oxide to regulate vascular tone and angiogenesis (16). In addition, oxidative stress has been shown to alter nitric-oxide levels and compromise the regulation of vascular tone and angiogenesis (12, 18).

Using homogeneously nitratated CaM, we found that tyrosine nitration increases the affinity of CaM for its key target protein, eNOS, at reduced calcium levels, and interestingly, site-specific tyrosine nitration also increases its ability to produce the product, NO. This study provides the first evidence that tyrosine nitration of CaM can provide a gain of function to the Ca\textsuperscript{2+}–dependent activation of eNOS signaling.

**Results**

**Production of site-specific nitroTyr–CaM**

We sought to characterize the functional effects of site-specifically nitratating the tyrosine residues on CaM caused by nitroTyr–CaM abundance in cells. We installed nitroTyr co-translationally into CaM at positions 99 and 138 using GCE to generate homogeneous nitroTyr–CaM (Fig. 1). When nitroTyr was withheld from the media, no full-length protein was purified, indicating the fidelity of the engineered aaRS/tRNA pair for nitroTyr (Fig. 1B). The fidelity of site-specific nitroTyr incorporation was verified by MS, with a mass increment of 44 Da corresponding to the addition of a single nitro group (Fig. 1D). The presence of nitroTyr was further confirmed by anti-nitro-
Tyrosine nitration enhances calmodulin sensitivity

To determine whether nitration of CaM changed its Ca\(^{2+}\)-dependent ability to bind target proteins, we supplemented EA.hy926 human endothelial cells with the site-specific nitroTyr-CaM- V5 tag at 20% of the endogenous CaM concentration. Immunoprecipitation of CaM co-precipitated a clearly evident protein with an apparent molecular mass of 130 kDa, consistent with the CaM target eNOS (Fig. 2). Because eNOS was co-immunoprecipitated by both WT- and nitroTyr-CaM-V5 tag, this indicates that nitroTyr-CaM-V5 interacts with eNOS in the presence of other intracellular targets of CaM.

To determine the extent of the impact of nitration of CaM on its Ca\(^{2+}\)-dependent ability to bind eNOS, we employed the use of biosensor interferometry (BLI), outlined in Fig. 3. This method allows for uniform attachment of a target protein–binding region to a BLI optical biosensor tip and measurement of CaM binding in solutions containing 20–225 nM buffered Ca\(^{2+}\). To determine whether CaM nitration alters its affinity for eNOS, we used the synthesized 20-amino acid peptide CaM-binding domain of eNOS with an N-terminal PEG-biotin to aid in solubility and allow for defined surface attachment to BLI streptavidin biosensor tips. The association and dissociation of CaM proteins to immobilized eNOS peptide was fit to a 1:1 interaction model that provides the $K_d$ for different Ca\(^{2+}\) concentrations (Table 1 and Fig. 3) (20, 21).

Consistent with values reported in the literature, WT–CaM binding of eNOS peptide affinity ranges from ~1 nm to undetectable levels as [Ca\(^{2+}\)\textsubscript{free}] is decreased from saturating levels (2 mM) to physiologically resting levels (20–50 nm) (17). Both nitroTyr-CaM species exhibit similar eNOS–peptide binding affinity to WT–CaM at saturating calcium levels. Strikingly, both of the nitroTyr–CaM species retain ~5 nm affinity at physiologically resting [Ca\(^{2+}\)\textsubscript{free}], compared with undetectable affinity for WT–CaM. Because of the retention of high affinity for eNOS at resting [Ca\(^{2+}\)\textsubscript{free}], nitroTyr–CaM has the potential to constitutively activate eNOS in the absence of calcium signal, resulting in a gain-of-function alteration regulating eNOS activity.

## NitroTyr–CaM exhibits a gain-of-function interaction with target protein eNOS

Figure 2. WT–CaM, CaM–nitroTyr-99, and CaM–nitroTyr-138 interact with eNOS protein from HEK293–eNOS and EA.hy926 cell lines. Western blotting analysis of immunoprecipitates of V5-tagged WT–, nitroTyr-99–, and nitroTyr-138–CaM from lysates of HEK293 cells stably expressing eNOS (A) and EA.hy926 human endothelial cells (B) is shown. This confirms that WT– and nitroTyr–CaM interact with eNOS in the context of the cellular milieu containing other CaM target proteins and endogenous CaM. IB, immunoblotting; IP, immunoprecipitation; nY, nitroTyr.

Based on the BLI data, we can conclude that nitration of CaM leads to CaM–eNOS association at resting intracellular calcium concentrations; however, this change in affinity only has a regulatory gain of function if these nitration sites on CaM do not abolish eNOS enzyme activity. Electron transfer between eNOS domains depends on the reversible binding of CaM, which is governed through changes in the intracellular Ca\(^{2+}\) concentration. To determine whether the increased binding affinity of nitroTyr–CaM altered eNOS activity, we measured the steady-state NO synthesis activities of WT–eNOS in presence of WT–CaM and nitroTyr–CaMs (Table 2). The coincident rates of NADPH oxidation during the assays were also measured to determine the efficiency of NO production. The cytochrome c reduction assay also provides a means to assess correct electron transfer through the enzyme. Generally, these assays are conducted under saturating calcium levels (2 mM free Ca\(^{2+}\) concentration). At these Ca\(^{2+}\) concentrations, all CaM species bind eNOS with the same affinity (Table 1), so any differences in NO production seen are independent of CaM–eNOS affinity. It is not feasible to perform eNOS assays at very low Ca\(^{2+}\) concentrations because of slow eNOS turnover and detection limits of the oxyhemoglobin, NADPH, and cytochrome c assays. At saturating Ca\(^{2+}\) levels, the eNOS was ~1.3 times more active with nitroTyr–CaM-138 than eNOS with WT–CaM. NitroTyr-CaM-99 had an inhibitory effect and low-er NO synthesis by ~40%. We also compared how these CaMs impact the NADPH oxidation rates of WT–eNOS during NO synthesis from L-Arg. In general, the rate of NADPH oxidation followed the rate of NO synthesis, consistent with the coupling of these processes. Remarkably, the coupling in WT-eNOS with nitroTyr–CaM-138 (3.0 NADPH per NO) was more efficient than in WT–eNOS with WT–CaM (4.4 NADPH per NO). This means that the eNOS works more efficiently with nitroTyr–CaM-138 and enables greater NO synthesis without an increased production of other reactive oxygen species.
The steady-state cytochrome c reductase activity is a useful way to measure the electron flux passing through the NOS FMN subdomain. Because of the closed conformation of the NOS reductase domain, cytochrome c activity is suppressed in the absence of CaM, and cytochrome c activity increases in the presence of CaM. A typical 5-fold increase in cytochrome c activity was induced by the binding of WT–CaM to WT-eNOS in our assays (18). We found that nitroTyr–CaM-138 has a 10% increase in activity as compared with WT–CaM and that nitroTyr–CaM-99 has a 10% decrease in activity as compared with WT–CaM. Cytochrome c reductase activity data also indicate that the eNOS works more efficiently with nitroTyr–CaM-138 than WT–CaM. When supplemented with either of the nitroTyr–CaM species as opposed to WT–CaM, eNOS retained more activity at 225 nM \([Ca^{2+}]_{free}\) (Table 3). This indicates that the gain of function seen with nitroTyr–CaM–eNOS binding is conserved with the full-length protein.

**nitroTyr–CaM interacts with and stimulates the function of eNOS in eNOS-expressing HEK293 cell lysate**

To recapitulate the effects seen in vitro with the pure CaM–eNOS system in a system more closely resembling the intracellular medium, we used lysate from eNOS-producing HEK293 cells, allowing us to still control both the concentration of supplemented CaM and \([Ca^{2+}]_{free}\) and verified that all CaM forms were able to stimulate eNOS activity in HEK293–eNOS lysate. As expected for eNOS we see a calcium concentration-dependent (Fig. 4) and time-dependent (data not shown) increase in activity. This activity is responsive to the NOS inhibitor L-NAME (data not shown), indicating that NO synthesis is a result of eNOS activity. Because of the presence of endogenous CaM, there was a significant eNOS activity without the addition of exogenous CaM (Fig. 4). As predicted from the increased affinity of nitroTyr–CaM-138 for eNOS at low calcium concentrations, the eNOS activity was stimulated to a significantly larger extent in lysate supplemented with nitroTyr–CaM-138 over WT–CaM (Fig. 4), particularly at intermediate calcium levels (225–750 nM \([Ca^{2+}]_{free}\)). Specifically, we see a 60–70% increase in NO production by nitroTyr–CaM-138 over WT–CaM at these intermediate calcium levels.

**Nitration of CaM does not impair calcium binding**

It has been shown that CaM PTMs regulate target affinity and activity by altering interactions with specific targets, but another potential mechanism would be for the PTM to alter CaMs affinity for \(Ca^{2+}\). Dansylated CaM (dansyl-CaM) fluorescence has also been used for monitoring conformational changes in CaM as a result of interactions with \(Ca^{2+}\) (19). To analyze the \(Ca^{2+}\)-induced structural changes of the different dansyl-CaM species, we performed \(Ca^{2+}\) fluorescence titration experiments (Fig. 5). Fitting the data (Equation 2 in "Experimental procedures") gives the EC\(_{50}(Ca^{2+})\), the free calcium

![Figure 3. Outline of biolayer interferometry experiments to determine CaM–eNOS interactions at varying free Ca\(^{2+}\) concentrations. A, the peptide constituting the eNOS CaM-binding domain was synthesized with an N-terminal biotin-PEG that was attached to a streptavidin functionalized BLI tip (light blue lines). The eNOS peptide functionalized tip was then moved to a well containing a particular CaM species (WT–CaM or nitroTyr–CaM, shown in purple) and a buffered Ca\(^{2+}\) solution (Ca\(^{2+}\) shown as green circles). CaM associated (shown as the red curve) with the eNOS peptide on the surface of the tip. White light was transmitted through the BLI tip, and binding events resulted in a shift in the interference of white light, which is measured as a response in nanometers. Following association, the BLI tip is moved to a well containing the same buffered Ca\(^{2+}\) solution but without CaM present. The dissociation of CaM–eNOS peptide (shown as the blue curve) is monitored again as a response in nm over time. Fitting the data for both the association constant and dissociation constant gives the \(K_d\) of the interaction at each Ca\(^{2+}\) concentration. B, representative traces for WT–, nitroTyr-99–, and nitroTyr-138–CaM at 20, 50, and 100 nM free Ca\(^{2+}\) are shown.

Tyrosine nitration enhances calmodulin sensitivity
Tyrosine nitration enhances calmodulin sensitivity

Discussion

Previous work has shown that eNOS is almost exclusively CaM-free under resting conditions but is half-bound at roughly a quarter of maximum intracellular [Ca^{2+}]_{free} (50% saturation of the eNOS peptide at a [Ca^{2+}]_{free} of 228 nM) (27). Although unmodified CaM is inactive under resting [Ca^{2+}]_{free}, tyrosine phosphorylation of CaM increases binding affinity for NOS compared with unphosphorylated CaM (4). The sites of phosphotyrosine regulation on CaM are also shared by tyrosine nitration, which makes it important to understand how this ox-PTM alters CaM signaling. CaM is maintained at low basal levels of tyrosine phosphorylation and nitration.

In response to insulin signaling or infection by Rous sarcoma virus, the amount of phosphotyrosine increases up to 50%, and nitrotyrosine levels increase up to 30% because of oxidative stress and immune responses (5, 20). The balance of CaM phosphorylation and nitration further broadens the pool of regulatory components that control eNOS function (5, 21–23).

Our results show nitroTyr–CaM–138 remains bound even at 20 nM [Ca^{2+}]_{free} indicating that eNOS in the presence of nitroTyr–CaM–138 is active under resting conditions, analogous to what was seen for phosphoCaM–99. Both of the nitroTyr–CaM species bind the same as the WT–CaM with identical subnanomolar affinities under saturating [Ca^{2+}]_{free}; however, they exhibit a slight decrease in affinity for the eNOS peptide at the resting [Ca^{2+}]_{free} of 50 nM. Previously, phosphorylation of eNOS at Ser-1179 by Akt or PKA has shown a gain of function by allowing CaM to bind at lower [Ca^{2+}]_{free} levels, effectively lowering the calcium signal required for eNOS activation (24).

In the same vein, nitration of CaM at Tyr-138 provides a similar but newly uncovered mechanism for enhancing CaM interactions and thereby activating eNOS independent of an increase in the [Ca^{2+}]_{free} level (Fig. 6). Tyr-138 serves as a structural coupler between the N-terminal domain and the central linker of CaM through hydrogen bonding with Glu-82 (Fig. 6) (25), potentially explaining the alteration in eNOS association seen following nitration at this site. Although phosphorylation of CaM has been shown to alter the affinity of CaM for eNOS, it is also clear that phosphorylation of CaM or eNOS also influences the activity of eNOS in a manner independent from CaM–eNOS affinity. Based on this, we expect that tyrosine nitration of CaM will impact eNOS function in addition to the changes to affinity.

To study the influence of CaM nitration on the catalytic activity of full-length WT-eNOS, we monitored NO production, NADPH oxidation, and cytochrome c reduction. Previous work with the phosphomimetic CaM mutation Y99E showed a 40% decrease in NO production, whereas the control mutation Y99Q showed only a 20% decrease in NO production (23). At 2 mM [Ca^{2+}]_{free} similar to the Y99E mutation, nitroTyr–CaM–99 resulted in a 40% decrease in NO production and was less efficient with a 10% decrease in NADPH to NO ratio as compared with WT–CaM. Strikingly nitroTyr–CaM–138 was able to support more NO production and more efficiently than WT–CaM, with a 33% increase in NO output and a 62% efficiency increase. This increase in output and efficiency is comparable with what concentration at which the dansyl-CaM is half-saturated with Ca^{2+}, and n, the Hill cooperativity constant (Table 4). The EC_{50}(Ca^{2+}) values for all CaM forms were ~0.3 μM [Ca^{2+}]_{free}. This unaltered Ca^{2+} affinity for the WT– and nitroTyr–CaMs is in good agreement with previously published dansyl-CaM Ca^{2+} affinity data (27). The Ca^{2+} fluorescence titration did show a difference for the Hill cooperativity coefficient for the WT–CaM as compared with the nitroTyr–CaMs (Table 4). This indicates that there is slightly lower cooperativity for the nitroTyr–CaMs as compared with the WT–CaM. The fluorescence titration data confirm that tyrosine nitration on CaM does not independently change its affinity for Ca^{2+}, which indicates the increased nitroTyr–CaM–eNOS affinity at low Ca^{2+} concentrations is due to cooperative binding of both target eNOS and Ca^{2+} binding to nitrated CaM.
Tyrosine nitration enhances calmodulin sensitivity

Figure 6. Potential effects of CaM nitration on eNOS function. The two sites of physiological tyrosine nitration in calmodulin are shown, with Tyr-99 in yellow, Tyr-138 in orange, and Ca$^{2+}$ shown as green spheres corresponding to the van der Waals radius. Hydrogen bonding between Glu-82 in purple and Tyr-138 is key to structural coupling between the N- and C-terminal lobes of CaM. Nitration of Tyr-138 lowers the pKa of the residue resulting in a weakened hydrogen bond, likely lowering interlobe coupling. The carbonyl oxygen of Tyr-99 (yellow) involved in chelating Ca$^{2+}$ in EF-hand III is also shown. A gain of function may occur if holoCaM (Protein Data Bank code 3CLN) binds to a target protein, like eNOS, with greater affinity (eNOS–CaM complex, Protein Data Bank code 1NIW). Further, a gain of function could occur if nitroTyr–CaM binding induces increased target protein activity. Nitration of these sites leads to different effects on eNOS, and the gain-of-function change caused by nitroTyr–CaM-99 leads to decreased activity of eNOS, whereas the gain of function caused by nitroTyr–CaM-138 leads to increased activity of eNOS.

was seen for eNOS regulation by the phosphomimetic mutations S1179D or S617D (18, 26).

Because we do not expect the affinity of nitroTyr–CaM for eNOS to be altered at saturating [Ca$^{2+}$]$_{free}$ as compared with WT–CaM, we may be able to ascribe the change in eNOS function to a shift in eNOS domain dynamics (16). Under intermediate calcium conditions (225 nM [Ca$^{2+}$]$_{free}$), both nitroTyr–CaMs retained 10% more eNOS activity than the WT–CaM, indicating that nitration will regulate CaM to increase NO at such intermediate calcium levels in the cell.

Because CaM regulates many proteins, the nitroTyr–CaM pool may impact target proteins other than eNOS. Regulation of eNOS is also multifaceted and is also dependent on interactions with proteins other than CaM and subcellular localization. To approach this problem, we first assessed eNOS–nitroTyr–CaM association in HEK293–eNOS and EA.hy926 human endothelial cell lysate. We saw that both nitroTyr–CaM species when supplemented to cell lysate at 20% of the total CaM pool interacted with eNOS via immunoprecipitation of eNOS via the V5 tag on CaM. Assessing eNOS function, we saw that supplementation with nitroTyr–CaM, particularly at site 138, led to significantly more eNOS activity at intermediate calcium levels representing a gain-of-function even in the presence of native CaM, other CaM target proteins, and other eNOS regulatory interactions. Under this condition, the nitroTyr–CaM-138 does stimulate 60–70% more eNOS activity in cell lysate than does WT–CaM, consistent with a gain of function.

Conclusion

Here we sought to explore whether the nitroTyr PTM can alter the function of regulatory protein (27), in our case CaM. The central role CaM plays in regulating calcium signaling, abundant nitration of its tyrosine residues, and the potential presence of a cellular denitrase for nitrated CaM (5) make it an ideal candidate for assessing the impact of tyrosine nitration. Because nitroTyr can be incorporated site-specifically using GCE, it provides excellent opportunity to evaluate whether it can modulate protein function. Our results show that tyrosine nitration of CaM at sites 99 and 138 increase binding and activity of eNOS at lower Ca$^{2+}$ concentrations. Most strikingly, CaM nitrated at site 138 binds eNOS even under resting physiological conditions, indicating that although only a subset of cellular CaM will be nitrated on tyrosine 138 at any given time, because of its gain of function, this subpopulation will activate eNOS at reduced [Ca$^{2+}$].

Proper regulation of eNOS is important for healthy vascular function, and tyrosine nitration enhances CaM calcium sensitivity and activation of eNOS. CaM binding to NOS activates NOS by relieving the suppression of the electron transfer process. Under conditions of limited arginine substrate or improper coupling of N-terminal oxygenase domain and C-terminal reductase domain NOS has also been observed to produce superoxide through electron transfer to molecular oxygen (28). Because eNOS produces both superoxide and nitric oxide, which can react to form peroxynitrite (8, 29), the nitrating agent of CaM, tyrosine nitration may serve as a positive feedback mechanism to stimulate eNOS function. Based on the efficiency of nitric oxide production, we would predict from these results that nitration of CaM at Tyr-99 will lead to increased eNOS decoupling and therefore tyrosine nitration with lower NO bioavailability, whereas nitration at Tyr-138 will decrease uncoupling and the level of tyrosine nitration but increase the overall amount of bioavailable NO.

Experimental procedures

Antibodies used in this study

Nitrotyrosine antibody was obtained from Cayman Chemical (nitrotyrosine polyclonal antibody, item no. 10189540), V5 tag mAb was obtained from Invitrogen (V5 tag monoclonal antibody, mouse, catalog no. R960-25), NOS antibody was obtained from Cell Signaling Technology (NOS (pan) antibody no. 2977), and anti-rabbit and anti-mouse secondary antibodies were obtained from Li-Cor Biosciences (IRDye 800CW donkey anti-rabbit IgG secondary antibody P/N 926-32213; and IRDye 800CW donkey anti-mouse IgG secondary antibody P/N 926-32212).

Immunoblotting

Western blotting samples were separated on 15% SDS-PAGE gels, transferred to PVDF membrane, blocked with 5% nonfat milk in TBST, and probed with antinitrotyrosine (1:500) primary antibody, rocking for 16 h at room temperature. After rinsing three times with TBST, the membranes were than...
incubated with Li-Cor IRDye 800CW goat anti-rabbit IgG (1:10,000) secondary antibody, rocking for 1 h at room temperature, and washed three times for 5 min in TBST. The membrane was then scanned using a Li-Cor Odyssey 9120 imaging system.

Recombinant expression of homogenous site-specifically modified nitroCaM

The human CaM sequence was codon optimized for expression in Escherichia coli (GenScript) and cloned into a pBad vector to include a C-terminal His6 affinity tag (Invitrogen). The protein was expressed via DH10B E. coli cells in autoinduction medium in the presence of 100 μg/ml ampicillin for 24 h at 37 °C with shaking at 250 rpm. The cells were pelleted at 5500 × g, resuspended in −10 ml binding/wash buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 7.4) at 4 °C, and lysed once with a Microfluidics M-110P microfluidizer set at 18,000 p.s.i. Cell debris was pelleted in Oakridge tubes at 20,900 × g for 25 min at 4 °C. Approximately 75 ml of supernatant was passed through an Acrodisc 32-mm syringe filter fitted with a 0.45-μm Supor membrane. The supernatant was loaded onto a 5-ml HisTrap nickel–nitrilotriacetic acid column at 1 ml/min, washed Supor membrane. The supernatant was loaded onto a 5-ml His-Trap nickel–nitrilotriacetic acid column at 1 ml/min, washed

Purified peak fractions determined via SDS-PAGE analysis were

100 mM NaCl, pH 7.5) twice, and fresh storage buffer was added

for overnight dialysis. The purified CaM was concentrated
corresponding to 150–200 mM imidazole. The pure protein
fractions then had CaCl2 added to a concentration of 5 mM and
loaded on a 5-ml HiTrap phenyl-Sepharose column, washed
with 20 ml of wash buffer (50 mM Tris-HCl, 1 mM CaCl2, 500 mM NaCl, pH 7.5), and eluted with 0–100%, 30-ml linear gradient of elution buffer (20 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 7.4) using an Amersham Biosciences AKTA explorer. Peak elutions were between 30 and 40% elution buffer, corresponding to 150–200 mM imidazole. The pure protein

Expression and purification of CaM containing nitroTyr

E. coli DH10B was transformed with pBad–CaM–(99 or 138
TAG) and pDule–nitroTyr–5B (Addgene plasmid no. 85498) (30). The orthogonal aminocayl-tRNA synthetase and cognate amber suppressing tRNA for incorporation of nitroTyr are
expressed from the plasmid pDule containing the aforementioned synthetase/tRNA pair, a p15 origin, and a tetracycline resistance marker. The pBad–CaM plasmids contain CaM with
an amber stop codon at the site of interest (99 or 138) to direct incorporation of nitroTyr. Similar to WT–CaM, expression of nitroTyr-containing CaM was in autoinduction medium (31) containing 100 μg/ml ampicillin, 25 μg/ml tetracycline, and 1 mM nitroTyr. Purification of nitroTyr-containing CaM was identical to purification of WT–CaM. Yields of ~300 mg (liters of culture)−1 were obtained for nitroTyr–CaM-99 and nitro-

Tyrosine nitration enhances calmodulin sensitivity

Tyr–CaM-138, compared with that of ~375 mg (liters of culture)−1 for WT–CaM expression (31).

Mass spectrometry

Purified CaM samples were diluted to a concentration of 10 μM, desalted on Millipore C4 zip tips, and analyzed using an FT LTQ mass spectrometer at the Oregon State University Mass Spectrometry Facility. The samples included WT–CaM and nitroTyr-containing CaM. Spectra were collected using Tune-Plus (Thermo, version 2.2) page of Xcalibur (Thermo, version 2.0.5) using parameters described in Ref. 32. The spectra were averaged over the 3-min CaM eluted from the ZipTip using the Qual Browser (Thermo, version 2.0). The data were exported as text files containing two columns of m/z versus intensity to be analyzed by custom programs written in MatLab (32). The raw and deconvoluted MS data were deposited online.

Calmodulin dansylation and fluorescence measurements

Dansyl-CaM was prepared as previously described (23). CaM (1 mg/ml) was buffer-exchanged into 10 mM NaHCO3 and 1 mM EDTA (pH 10.0) at 4 °C. 30 μl of 6 mM dansyl chloride (1.5 mol/mol of CaM) in acetone was added to 2 ml of CaM while it was being stirred. After incubation for 12 h at 4 °C, the mixture was buffer-exchanged into fluorescence buffer. Labeling yields were determined from absorbance spectra using an ε325 of 3400 M−1 cm−1 and were compared with actual protein concentrations determined using the Bradford method with WT CaM used as the protein standard.

Fluorescence emission spectra were recorded using a photon technology international (London, Canada) QuantaMaster spectrofluorimeter. Fluorescence measurements were taken on 50-μl samples consisting of dansyl-CaM (2 μM) in 30 mM MOPS, 100 mM KCl, and 10 mM EGTA (pH 7.2) with an increasing concentration of free Ca2+. The free Ca2+ concentration was controlled using the suggested protocol from the calcium calibration buffer kit from Invitrogen. The excitation wavelength for all of the dansyl-CaMs was set to 340 nm, and emission was monitored between 400 and 600 nm. Silt widths were set at 2 nm for excitation and 1 nm for emission. The relative fluorescence was calculated with the following equation,

\[
\frac{(F - F_0)}{(F_{\text{max}} - F_0)}
\]

where \(F\) is the measured intensity, \(F_{\text{max}}\) is the maximal intensity, and \(F_0\) is the intensity without added Ca2+. The relative fluorescence was calculated using the fluorescence emission intensity at 475 nm. The Ca2+ sensitivities of the different dansyl-CaMs we determined as the EC50(Ca2+) values, which were derived from fits of the relative fluorescence intensity increase upon addition of Ca2+ using the following equation,

\[
\frac{[\text{Ca}^{2+}]_{\text{free}}}{[\text{Ca}^{2+}]_{\text{free}} + [\text{EC}_{50}(\text{Ca}^{2+})]^n}
\]

where relative fluorescence is obtained from Equation 1, and \(n\) is the Hill coefficient.
Tyrosine nitration enhances calmodulin sensitivity

Octet Red96 biolayer interferometry measurements

All BLI measurements were made on a forteBIO (Menlo Park, CA) Octet Red96 system using streptavidin sensors. The assays were performed in 96-well microplates at 37 °C. All sample volumes were 200 µL. The eNOS peptide was purchased from Genscript (Piscataway, NJ) homogeneously biontinnated at the N terminus. Tips were loaded with three different concentrations of biotin–eNOS peptide (33). After loading biotinylated eNOS peptide onto streptavidin baseline, a baseline was established in buffer composed of 30 mM MOPS, 100 mM KCl (pH 7.2), and varying free calcium concentration. Free calcium was controlled by mixing two buffers containing 10 mM EGTA and 10 mM Ca2+–EGTA in varying ratios. Free calcium concentration was calculated either from the Thermo Fisher scientific calcium calibration kit 1 instructions or the Maxchelator proteination was calculated either from the Thermo Fisher scientific

Steady-state eNOS assays

NO synthesis and NADPH oxidation rates were determined using the oxyhemoglobin assay. The NO synthesis activity was determined by the conversion of oxyhemoglobin to methemoglobin using an extinction coefficient of 38 mM−1 cm−1 at 401 nm. The NADPH oxidation rates were determined following the absorbance at 340 nm, using an extinction coefficient of 6.2 mM−1 cm−1. Reaction mixtures (total volume 400 µL) contained 0.1–0.2 µM bovine eNOS, 0.3 mM dithiothreitol, 4 µM FAD, 4 µM FMN, 10 µM H2B, 2 mM L-Arg, 0.1 mg/ml BSA, 2 mM CaCl2, 0.2 mM EDTA, 2–5 µM CaM (WT or nitroTyr depending on the experiment), 100 units/ml catalase, 60 units/ml superoxide dismutase, 5 µM oxyhemoglobin, and 150 mM NaCl in 40 mM EPPS buffer, pH 7.6. The reaction was initiated by adding the NOS co-factors listed above and calcium concentration in the lysate along with 5 mM CaCl2. The lysate with recombinant CaM was incubated at 4 °C overnight with 2 µl of mouse anti-V5 antibody (Invitrogen). The mixture was further incubated with 20 µl of protein A/G magnetic beads for 6 h at 4 °C. The magnetic beads were washed four times with lysis buffer containing 5 µM CaCl2, resuspended in 40 µl of Laemmli buffer, and incubated at 55 °C for 10 min. Western blotting samples were separated on 4–22% gradient SDS-PAGE gels, transferred to PVDF membrane, blocked with 5% nonfat milk in TBST, and probed with anti-NOS (Pan) (1:1000) or anti-V5 (1:5000) primary antibodies rocking for 16 h at room temperature. After rinsing three times with TBST, the membranes were then incubated Li-Cor IRDye 800CW goat anti-rabbit or anti-mouse IgG (1:10,000) secondary antibody, rocked for 1 h at room temperature, and washed three times for 5 min in TBST. The membrane was then scanned using a Li-Cor Odyssey 9120 imaging system.

eNOS-lysat reaction conditions

Reactions to determine eNOS activity in eNOS expressing HEK293 cell lines were carried out. Lysate extracted as above was supplemented with 2 mM L-arginine, 1 mM NADPH, 20 µM BH4 in solution containing 3 mM DTT, and 500 µM WT– or nitroTyr–CaM. For those reactions containing Ca2+– or EGTA, they were supplemented with 10 mM of either, whereas those reactions were supplemented with L-NAME to a concentration of 1 mM. The reaction was initiated by the addition of the NOS co-factors listed above and incubated for 30 min at 37 °C. The eNOS reactions were carried out at a 50-µl scale in triplicate and stopped via freezing in dry ice.

eNOS-activity assays

The amount of NO produced in lysate was measured amperometrically by an AmiNO700 NO selective electrode (Innovative Instruments, Inc.) as described previously (35).

Data Availability

The mass spectrometry data are publicly accessible online at Figshare (https://doi.org/10.6084/m9.figshare.11346536.v1).4

4 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
human atherosclerotic plaque and dysfunctional. J. Biol. Chem. 289, 10276–10292 CrossRef Medline
15. Neumann, H., Hazen, J. L., Weinstein, J., Mehli, R. A., and Chin, J. W. (2008) Genetically encoding protein oxidative damage. J. Am. Chem. Soc. 130, 4028–4033 CrossRef Medline
16. Campbell, M. G., Smith, B. C., Potter, C. S., Carragher, B., and Marletta, M. A. (2014) Molecular architecture of mammalian nitric oxide synthases. Proc. Natl. Acad. Sci. U.S.A. 111, E3614–E3623 CrossRef Medline
17. Spratt, D. E., Taiakina, V., Palmer, M., and Guillemette, J. G. (2008) FRET conformational analysis of calmodulin binding to nitric oxide synthase peptides and enzymes. Biochemistry 47, 12006–12017 CrossRef Medline
18. Haque, M. M., Ray, S. S., and Stuehr, D. J. (2016) Phosphorylation controls endothelial nitrite-nitric oxide synthase by regulating its conformational dynamics. J. Biol. Chem. 291, 23047–23057 CrossRef Medline
19. Piazza, M., Taiakina, V., Guillemette, S. R., Guillemette, J. G., and Dieckmann, T. (2014) Solution structure of calmodulin bound to the target peptide of endothelial nitric oxide synthase phosphorylated at Thr495. Biochemistry 53, 1241–1249 CrossRef Medline
20. Benaim, G., and Villalobo, A. (2002) Phosphorylation of calmodulin: functional implications. Eur. J. Biochem. 269, 3619–3631 CrossRef Medline
21. Joly, J. L., and Sacks, D. B. (1994) Insulin-dependent phosphorylation of calmodulin in rat hepatocytes. J. Biol. Chem. 269, 30039–30048 Medline
22. Villalobo, A. (2018) The multifunctional role of phospho-calmodulin in pathophysiological processes. Biochem. J. 475, 4011–4025 CrossRef Medline
23. Piazza, M., Futrega, K., Spratt, D. E., Dieckmann, T., and Guillemette, J. G. (2012) Structure and dynamics of calmodulin (CaM) bound to nitric oxide synthase peptides: effects of a phosphomimetic CaM mutation. Biochemistry 51, 3651–3661 CrossRef Medline
24. Sessa, W. C. (2004) eNOS at a glance. J. Cell Sci. 117, 2427–2429 CrossRef Medline
25. Mukherjea, P., Maune, J. F., and Beckingham, K. (1996) Interlobe communication in multiple calcium-binding site mutants of Drosophila calmodulin. Protein Sci. 5, 468–477 Medline
26. Tran, Q. K., Leonard, J., Black, D. J., Nadeau, O. W., Boulantikov, I. G., and Persechini, A. (2009) Effects of combined phosphorylation at Ser-617 and Ser-1179 in endothelial nitric-oxide synthase on EC50(Ca2+)
27. radi. Proc. Natl. Acad. Sci. U.S.A. 106, 23047–23057 CrossRef Medline
28. Yang, Y.-M., Huang, A., Kaley, G., and Sun, D. (2009) eNOS uncoupling and endothelial dysfunction in aged vessels. Annu. J. Physiol. Heart Circ. Physiol. 297, H1829–H1836 CrossRef Medline
29. Cooley, R. B., Feldman, J. L., Driggers, C. M., Bundy, T. A., Stokes, A. L., Karplus, P. A., and Mehli, R. A. (2014) Structural basis of improved second-generation 3-nitro-tyrosine TRNA synthetases. Biochemistry 53, 1196–1199 CrossRef Medline
30. Rady, R. (2013) Protein tyrosine nitration: biochemical mechanisms and structural basis of functional effects. Acc. Chem. Res. 46, 550–559 CrossRef Medline
31. Stuehr, D. J., Tejero, J., and Haque, M. M. (2009) Structural and mechanistic aspects of flavoproteins: electron transfer through the nitric oxide synthase flavoprotein domain. FEBS J. 276, 3959–3974 CrossRef Medline
32. Yang, Y.-M., Huang, A., Kaley, G., and Sun, D. (2009) eNOS uncoupling and endothelial dysfunction in aged vessels. Annu. J. Physiol. Heart Circ. Physiol. 297, H1829–H1836 CrossRef Medline
33. Cooley, R. B., Feldman, J. L., Driggers, C. M., Bundy, T. A., Stokes, A. L., Karplus, P. A., and Mehli, R. A. (2014) Structural basis of improved second-generation 3-nitro-tyrosine TRNA synthetases. Biochemistry 53, 1196–1192 CrossRef Medline
34. Hamill, J. T., Miyake-Stoner, S., Hazen, J. L., Jackson, J. C., and Mehli, R. A. (2007) Preparation of site-specifically labeled fluorescent proteins for 3P-NMR structural characterization. Nat. Protoc. 2, 2601–2607 CrossRef Medline
35. Rhoads, T. W., Williams, J. R., Lopez, N. I., Morré, J. T., Bradford, C. S., and Beckman, J. S. (2013) Using theoretical protein isotopic distributions to parse small-mass-difference post-translationally modified via mass spectrometry. J. Am. Soc. Mass. Spectrom. 24, 115–124 CrossRef Medline
36. Abdičević, Y., Malashock, D., Pinkerton, A., and Pons, J. (2008) Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet. Anal. Biochem. 377, 209–217 CrossRef Medline
37. Bers, D. M., Patton, C. W., and Nuccitelli, R. (2010) A practical guide to the preparation of Ca2
28+ buffers. Methods Cell Biol. 99, 1–28 CrossRef Medline
38. Boo, Y. C., Tresell, S. L., and Jo, H. (2007) An improved method to measure nitrate/nitrite with an NO-selective electrochemical sensor. Nitric Oxide 16, 306–312 CrossRef Medline