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Abstract

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Rational design of a fluorescent NADPH derivative imaging constitutive nitric-oxide synthases upon two-photon excitation

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We report the structure-based design and synthesis of a unique NOS inhibitor, called nanoshutter NS1, with two-photon absorption properties. NS1 targets the NADPH site of NOS by a nucleotide moiety mimicking NADPH linked to a conjugated push-pull chromophore with nonlinear absorption properties. Because NS1 could not provide reducing equivalents to the protein and competed with NADPH binding, it efficiently inhibited NOS catalysis. NS1 became fluorescent once bound to NOS with an excellent signal-to-noise ratio because of two-photon excitation avoiding interference from the flavin-autofluorescence and because free NS1 was not fluorescent in aqueous solutions. NS1 fluorescence enhancement was selective for constitutive NOS in vitro, in particular for endothelial NOS (eNOS). Molecular dynamics simulations suggested that two variable residues among NOS isoforms induced differences in binding of NS1 and in local solvation around NS1 nitro group, consistent with changes of NS1 fluorescence yield. NS1 co-localized with eNOS in living human umbilical vein endothelial cells. Thus, NS1 constitutes a unique class of eNOS probe with two-photon excitation in the 800–950-nm range, with great perspectives for eNOS imaging in living tissues.

In mammals, NO is a gaseous signaling mediator that exerts a wide range of key physiological functions, including blood pressure regulation, neurotransmission, and immune responses (1). NO is formed by nitric-oxide synthases, a family of hemoproteins that catalyzes the oxidation of L-arginine leading to L-citrulline and NO (2, 3). The biological activities of NO are closely linked to its biosynthesis leads to various pathologies (5). Data supported the notion that inhibition of the inducible and neuronal isoforms iNOS and nNOS have therapeutic utility in the treat-ment of a variety of diseases, including septic shock, neurodegeneration, inflammation, and cancer (5, 6). It was shown that the endothelial isoform eNOS participates in tumor initiation and maintenance, thus its inhibition could be beneficial in this context (7, 8). Based on crystallographic studies (9, 10), much effort was dedicated to the development of specific NOS inhibitors by targeting the heme site. However, most of these inhibitors are not specific and cannot avoid NOS uncoupling and formation of deleterious radical oxygen species (ROS) at the reductase domain. Although the structure of nNOS reductase domain has been solved (4), no specific compounds targeting this domain are available, and only diphenyliodonium (DPI) and its analogs nonspecifically and irreversibly react with flavins (11). The NADPH-binding site is highly conserved among redox enzymes. The crystallographic data of NADPH-containing proteins showed that the main recognition elements in their binding site involve conserved arginines that interact electrostatically with the 2′ phosphosphate group of NADPH nucleotide moiety. The nicotinamide part of NADPH can be flexible and undergoes conformational changes depending on the NADP(H) enzymes and their specific catalysis. In recent studies, we have characterized a NADPH analog called nanotrigger, NT1, targeting the NADPH site in NOS reductase domain (12–14) (Fig. L4). NT1 contained a conjugated, photo-activatable chromophore substituted with two donor groups that replaced the nicotinamide moiety of NADPH. Following photo-activation, the terminal amine group of excited NT1 was able to inject electrons to the FAD acceptor, thereby initiating the electron flow. This initiation and synchronization of NOS catalysis upon one- or two-photon excitation (1-PE or 2-PE, respectively) led to light-induced NO formation (12–14).

Here, we designed a unique prototype of NOS inhibitor, called nanoshutter (NS1), with intrinsic fluorescence imaging properties. To this purpose, we designed a compound that meets the following criteria: (i) NS1 should demonstrate a good affinity for NOS, at least comparable to the affinity of the natural hydride donor NADPH; (ii) injection of electrons to FAD should be prohibited by bound NS1 to NOS; (iii) NS1 should have a good two-photon cross-section, compatible with multiphoton imaging; (iv) free and bound NS1 should possess different fluorescence properties. We designed a compound bearing a nucleotide moiety as recognition motif of the NADPH site linked by an alkyl chain to a new chromophore substituted with an acceptor and a donor group, thus engineering a push–pull photoactive element.


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Design and molecular modelling studies of the nanotrigger NT1 and the nanoshutter NS1. (A) Principles of activation and inhibition of NOS by NT1 and NS1. Because of its terminal nitro-phenyl (electron acceptor) group that cannot inject the reducing equivalents required for the initiation of catalysis, NT1 is expected both to bind to the NADPH site and to inhibit competitively the electron flow to the FAD acceptor (in contrast to the electron donor–containing compound NT1). Additionally, significant modulations of the fluorescence emission by the polarity of the environment are expected. (B) Close-up view of the terminal groups of NT1 diene chromophore and NS1 stilbene chromophore in nNOSred; FAD is omitted for clarity. 

Fig. 1. Design and molecular modelling studies of the nanotrigger NT1 and the nanoshutter NS1. (A) Principles of activation and inhibition of NOS by NT1 and NS1. Because of its terminal nitro-phenyl (electron acceptor) group that cannot inject the reducing equivalents required for the initiation of catalysis, NT1 is expected both to bind to the NADPH site and to inhibit competitively the electron flow to the FAD acceptor (in contrast to the electron donor–containing compound NT1). Additionally, significant modulations of the fluorescence emission by the polarity of the environment are expected. (B) Close-up view of the terminal groups of NT1 diene chromophore and NS1 stilbene chromophore in nNOSred; FAD is omitted for clarity. 

Results

Molecular Modelling. We showed that NS1 fits into the narrow NADPH site of the reductase domain of nNOS (nNOSred) using docking and molecular dynamic simulations based on the structure of this domain (4). We used a stilbene moiety instead of the 1,4-diphenyl-butadiene moiety of NT1 for replacement of the terminal groups of NT1 diene chromophore and NS1 stilbene chromophore in nNOSred; FAD is omitted for clarity (12-14). Such a chromophore is expected to have a high dipole moment caused by the electron delocalization in the excited state that should lead to nonlinear absorption properties (15). The NS1 chromophore carries a conjugated stilbene substituted with the donor–acceptor pair instead of the diene moiety of NT1 (13) (Fig. 1B). Finally, the terminal electron acceptor was chosen to be a nitro group that should block the electron flow, inhibit NOS catalysis, and modulate the fluorescence properties of NS1 with the environment (16). A change in fluorescence emission between free and NOS-bound states of the engineered NS1 compound was then expected. This work paves the way for a unique family of NOS inhibitors with imaging applications.

The synthesis of the target compound NS1 (Fig. 2A) consisted of a seven-step procedure (see Methods and SI Appendix, Fig. S2). The absorption spectra of NS1 and its chromophore was measured using a spectrophotometer (Fig. 2B). The fluorescence emission of NS1 was detected using a fluorimeter (Fig. 2C). The binding isotherm of NS1 was fitted using a one-binding site model, leading to an apparent dissociation constant for NS1 binding of 4.3 ± 0.6 μM (Fig. 2D). No fluorescence emission was detected in Tris buffer upon 2-PE of nNOS alone (Δ) or NS1 alone (30 μM, □); (D) corresponding binding isotherm. The binding isotherm was fitted using a one-binding site model, leading to an apparent dissociation constant for NS1 binding of 4.3 ± 0.6 μM. (E) Inhibition by NS1 of the formation of NO by nNOS; competition experiments between NADPH and NS1. The double-reciprocal plots of nNOS activities measured in the presence of various concentrations of NADPH (up to 100 μM) are shown in the absence (●) or in the presence of S (■, 25 (▲), 50 (●), or 100 (○) μM NS1.

Fig. 2. Binding of NS1 to nNOS monitored by fluorescence emission upon 2-PE and subsequent inhibition of NOS activity. (A) Structure of NS1; (B) two-photon excitation spectrum of NS1 in DMSO. The fluorescence quantum yield and the two-photon cross-section (δ2) were determined using rhodamine B as a reference and were found to be equal to 0.0045 and 65 GM at 940 nm, respectively (δ2 of approximately 130 GM at 840 nm). (C) Increase in NS1 fluorescence (λex = 940 nm) upon binding to nNOS (5 μM). No fluorescence emission was detected in Tris buffer upon 2-PE of nNOS alone (Δ) or NS1 alone (30 μM, □); (D) corresponding binding isotherm. The binding isotherm was fitted using a one-binding site model, leading to an apparent dissociation constant for NS1 binding of 4.3 ± 0.6 μM. (E) Inhibition by NS1 of the formation of NO by nNOS; competition experiments between NADPH and NS1. The double-reciprocal plots of nNOS activities measured in the presence of various concentrations of NADPH (up to 100 μM) are shown in the absence (●) or in the presence of S (■, 25 (▲), 50 (●), or 100 (○) μM NS1.

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moiety were similar, with a maximum similar to the related 4-N,N-dimethylamino-4′-nitrostilbene (16, 18). NS1 had an absorption maximum at $\lambda_{\text{max}} = 460$ nm with an extinction coefficient $e = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$ in DMSO (SI Appendix, Fig. S3A); upon excitation at 460 nm, NS1 presented a broad emission peak centred at 740 nm (SI Appendix, Fig. S3B). The two-photon cross-section of NS1 was found to be $\sigma_2$ of approximately 130 and 65 GM (Göppert-Mayer; 1 GM $= 10^{-50} \text{ cm}^2/\text{s}/\text{photon}$) in the 800–860 and 920–950 nm ranges, respectively, in DMSO (Fig. 2B). As expected, NS1 fluorescence was strongly sensitive to the presence of water (SI Appendix, Fig. S3 B and C), its fluorescence was undetectable in aqueous solutions under either one-photon excitation condition (at 435 or 460 nm) or two-photon excitation condition ($\lambda_{\text{ex},2\text{-PE}} = 840$ or 940 nm).

**Fluorescence Properties of NS1–NOS Complexes.** Addition of NS1 to purified NOS led to the recovery of bound NS1 fluorescence as compared to free NS1 being non-fluorescent in aqueous buffers. This made possible the biochemical monitoring of NS1 binding to NOS using one- or two-photon fluorescence experiments. Under 1-PE conditions, the intrinsic emission properties of the NS1–nNOS complex were difficult to extract because the fluorescence of nNOS flavins and the fluorescence of NS1 overlapped. NS1 contribution was extracted by monitoring the emission at 700 nm in a spectral region where flavin autofluorescence was not significant. Although NS1 fluorescence was low at 700 nm, NS1 binding to nNOS could be recorded and led to the determination of an apparent $K_d$ of 4.2 µM (SI Appendix, Fig. S4). We reasoned that if the two-photon fluorescence of nNOS is weak (as expected, taking into account the very low $\sigma_2$ value of flavins—see Discussion), the 2-PE approach should be more appropriate for monitoring the fluorescence recovery of NS1 upon binding to nNOS. Indeed, both the intrinsic fluorescence of nNOS and free NS1 fluorescence (up to 30 µM) were undetectable under 2-PE condition (Fig. 2C). Stepwise additions of NS1 to nNOS resulted in an increase of NS1 fluorescence emission (Fig. 2C), which corresponded to pure emission spectra of NS1 in the nNOS–NS1 complex, allowing quantitative analysis of the binding process (Fig. 2D). The apparent $K_d = 4.3 \pm 0.6$ µM was consistent with the 1-PE value. The emission of NS1 was blue-shifted in the protein context (630 nm) compared to DMSO (740 nm), consistent with the influence of solvent polarity on emission maxima of nitrosothiobine derivatives (16). Binding of NS1 to full-length eNOS, iNOS, or eNOS<sub>cyt</sub> yielded comparable results in terms of affinity, but with substantial differences in the quantum yield characterizing each complex (see below).

**Inhibition of NOS Catalytic Activity by NS1.** The ability of NS1 to inhibit the NADPH-mediated hydride transfer to nNOS was studied by stopped-flow measurements. Fast mixing of nNOS with NADPH resulted in a fast decrease of the NADPH absorption, corresponding to a two-phase model (SI Appendix, Fig. S5). A significant slowdown of the fast phase of NADPH consumption that contributed approximately 65% of the decay at 340 nm was observed in the presence of NS1. NS1 competed with NADPH on NOS reduction in a dose-dependent manner, presenting a typical saturation curve, and led to an apparent inhibition constant $K_i = 15 \pm 4$ µM. This value compares well with those obtained previously with NT1, $K_i = 7 \pm 3$ µM (13, 17), showing that NT1 and NT1 display similar affinities for the NADPH site of constitutive NOS, as suggested by our molecular modelling studies.

Because NS1 abolished the electron-transfer flux from NADPH to the flavins in the reductase domain of NOS, NO formation should also be inhibited. The ability of NS1 to inhibit NO formation was assessed using the oxyhemoglobin assay. NS1 inhibited the formation of NO catalyzed by nNOS in a dose-dependent manner with a half-inhibitory concentration (IC$_{50}$) at 65 ± 8 µM in the presence of 100 µM NADPH and L-arginine (SI Appendix, Fig. S6A). We then investigated the mechanism of inhibition by varying NADPH and NS1 concentrations. The double-reciprocal plot of NO formation activity as a function of [NADPH] led to a $K_m$ value of 5.5 ± 0.5 µM in the absence of NS1 (intercept with the x axis; Fig. 2E). NS1 inhibition was competitive with NADPH as demonstrated by changes of the apparent $K_m$ values ($K_m^{\text{app}}$) with significant effect on the $V_{\text{max}}$ values with varying NS1 concentration. The plot of $K_m^{\text{app}}$ values as a function of [NS1] led to a $K_i$ value ranging between 13 and 18 µM (SI Appendix, Fig. S6B). The value of $K_i = 3.4$ µM deduced from IC$_{50} = 65$ µM using the Cheng-Prusoff relationship is consistent with the values of $K_d = 4.3$ µM and $K_i = 15$ µM obtained in 2-PE and stopped-flow experiments, respectively, and compares well with NADPH affinity ($K_m = 5.5 \pm 0.5$ µM). Further experiments using the dilution method showed that the inhibition of NO formation by NS1 was reversible. NS1 appears as a unique prototype inhibitor that competes with NADPH for binding in the reductase domain.

**In Vitro Selectivity in the Fluorescence Enhancement of Protein-Bound NS1.** The relative two-photon fluorescence intensity of NS1 bound to various proteins was next assessed. NS1 did not display significant fluorescence enhancement in the presence of proteins lacking a NADPH-binding site (Fig. 3A). For instance, NS1 non-specifically binding to the fatty acid–binding protein (FABP) accounted for only 5% of the fluorescence signal observed with eNOS-bound NS1. Thus, NS1 differs from non-specific probes such as the well-known ANS compound (anilinonaphthalene sulfonic acid), whose fluorescence is largely enhanced by multiple interactions in inner protein cavities, in particular upon binding to FABP (19). Among the NADPH-binding proteins, including the three NOS isoforms, NS1 binding to eNOS and nNOS led to the largest fluorescence enhancement (eNOS > nNOS > iNOS). The fluorescence quantum yield of NS1 was found to be strongly dependent on the protein context, indicating that NS1 within the NADPH-binding site probed distinct local solvent environment in each NOS isoform. In contrast, NS1 was only weakly fluorescent when bound to ferredoxin reductase or glucose-6-phosphate dehydrogenase (G6PDH). Interestingly, the two-photon fluorescence emission of eNOS-bound NS1 was found to be even higher than the value found in DMSO (approximately 45-fold higher) (Fig. 3A, Inset). Thus, NS1 is highly selective between constitutive and inducible NOS. These results were promising for further cellular investigations.

**Structural Insight of NS1 Binding to NOS Isoforms Based on Molecular Dynamics Simulations.** We attempted to explain the modulation in fluorescence yields of NS1 bound to the three NOS isoforms by additional structural insight; homology modelling was used to generate eNOS and iNOS reductase domains (17). NS1 bound to nNOS or eNOS by three arginine residues interacting with its phosphate group (SI Appendix, Fig. S7). Among these arginines, R1400 in nNOS was replaced in iNOS by S1130 unable for forming an H-bond with NS1 phosphate. Note that S1130 is located on the same linker as the catalytic D1123, allowing a unique interaction in NOS isoforms. In contrast, NS1 was only weakly fluorescent when bound to ferredoxin reductase or glucose-6-phosphate dehydrogenase (G6PDH). Interestingly, the two-photon fluorescence emission of eNOS-bound NS1 was found to be even higher than the value found in DMSO (approximately 45-fold higher) (Fig. 3A, Inset). Thus, NS1 is highly selective between constitutive and inducible NOS. These results were promising for further cellular investigations.
the proximity of NO₂ to a well-packed hydrophobic patch limited access to water-solvating D1127 in eNOS; thus, NS1 terminal probed a more hydrophobic environment. This hypothesis fits well with NS1’s probing the most hydrophobic environment in eNOS, thus the largest fluorescent yield. Indeed, specific solvent effects depending on the water content modulate NS1 fluorescence yield without λₑₓₘ,ᵣₓ shift in solution and in NOS isoforms (SI Appendix, Fig. S3 and Fig. 3A).

**NS1 Imaging in Endothelial Cells.** The fluorescence selectivity of NS1 for constitutive NOS prompted us to test if NS1 would specifically highlight eNOS in living cells. A rapid uptake of NS1 (maximum 10 min) by living human umbilical vein endothelial cells (HUVECs) was easily monitored upon excitation in the near infrared, showing the suitability of NS1 for 2-PE fluorescence studies of living cells (Fig. 4). (A comparison between 1- and 2-PE images for identical living cells is shown in SI Appendix, Fig. S9). NS1 displayed an intense fluorescence signal in the cytoplasm, especially in the perinuclear region and, to a smaller extent, at the cell membrane (Figs. 4A and 5A and D). The dots at the cell membranes were prominent at high cell confluence, in agreement with the role of eNOS in the maintenance of tight cell-to-cell junctions (21). The subcellular localization of NS1 within organelles was then further investigated by colocalization experiments. NS1 mainly colocalized with the Golgi apparatus (Fig. 4 E–H). NS1 did not colocalize with the endoplasmic reticulum, cell nucleus, or mitochondria, whereas only partial colocalization was observed with early endosome (EEA1) (SI Appendix, Fig. S10). The two main locations of NS1 (Golgi and plasma membrane) are compatible with the subcellular localization of eNOS (22). Using specific immunostaining of eNOS combined to one- (Fig. 5A–C) or two-photon (Fig. 5D–F) imaging of NS1, we observed an excellent colocalization of NS1 and eNOS in the perinuclear region and at the plasma membrane level, showing that NS1 actually targets eNOS in HUVEC cells. Because the 2-PE process avoided cellular autofluorescence, the signal-to-noise ratio was remarkably higher using two-photon compared to one-photon imaging, as judged by the more restricted colocalization area obtained using λₑₓ = 840 nm instead of 488 nm.

**Discussion**

NS1 fulfilled a double functionality: (i) a new prototype of a reversible NOS inhibitor, targeting the reductase domain in a specific manner and competing with NADPH; (ii) a unique fluorescent compound with 2-PE properties when bound to constitutive eNOS or nNOS. These two functions result from a rational design of a push–pull nitroaminostilbene chromophore moiety that cannot inject reducing equivalents required for triggering NOS catalysis, and that is highly sensitive to solvent polarity and to the presence of water in terms of fluorescence quantum yield (16).

Available NOS inhibitors include analogs of substrate L-arginine, calmodulin antagonists, and irreversible flavin inhibitor DPI and its analogs (11) as nonspecific inhibitors of electron transfer. NS1 represents a unique prototype of NOS inhibitor that competes with the initial hydride donor NADPH for binding to the reductase domain. Consequently, NS1 appears as a site-specific, reversible inhibitor that prevents both NO formation and NOS uncoupling by blocking the electron flow (flavins and heme). As such, avoiding the formation of toxic ROS and NO concentrations offers great perspectives, in particular for therapeutic strategies for, on the one hand, targeting nNOS and iNOS in the treatment of neurodegeneration, inflammation, and cancer (5), and, on the other hand, controlling eNOS activity and inhibiting deleterious effects of NO produced by eNOS in metastasis (7). Because eNOS constitutes the major source of NO production in endothelial cells and is an important regulator of cardiovascular homeostasis, further work is in progress for improving the isoform selectivity of NS1 in terms of molecular recognition, as well as for developing a photoactivation strategy for spatial and temporal control of eNOS inhibition by a two-photon–induced process.

In terms of NS1 imaging, a specific fluorescence enhancement upon binding to constitutive NOS was observed, with a preference for eNOS over nNOS. Compared to available NOS ligands,
NS1 has the additional benefit of real-time imaging eNOS in living endothelial cells with excellent signal-to-noise ratio. This is of special interest because confinement effects on the subcellular location of eNOS have a profound effect on eNOS activity and are altered in pathological states (23). NS1 was highly photostable, without fading or blinking compared to fluorescent proteins (FP) or quantum dots (QD), respectively, and probed endogenous expression profiles of eNOS in contrast to exogenous fusion or QD-labeled proteins. Moreover, NS1 internalization did not require permeation mandated for immunostaining when using antibodies against eNOS. The excellent signal-to-noise ratio primarily relies on NS1 fluorescence enhancement by binding to eNOS with negligible fluorescence either free or with nonspecific proteins. Additionally, NS1 is well-suited for two-photon studies because it has a much higher two-photon cross-section (σ2 = 130 and 65 GM at 840 and 940 nm, respectively) than that of flavins and NAD(P)H (24, 25) [σ2 (flavins) = 0.8 and 0.1 GM at 700 and 900 nm, respectively, or σ2 (NADPH) = 5 × 10−22 and 5 × 10−24 GM at 700 and 900 nm, respectively]. Thus, the intrinsic advantage of NS1 relies on its two-photon absorption properties and ability for monitoring native eNOS using 2-PE without background or free NS1 contribution.

In conclusion, NS1 is a promising tool for noninvasive imaging of living tissues and isolated organs with an enhanced spatial resolution and a better light penetration in living tissues, leading to potential applications for monitoring eNOS within tumor cells as melanoma. Improvement of the selectivity of the NS1 prototype toward nNOS should afford an interesting pharmacological and imaging tool for neurodegenerative diseases.

Methods

Molecular Modelling. Molecular dynamics simulations of nNOS–NS1 and nNOS–NT1 complexes were carried out using the program NAMD with the CHARMM22 force field (26). eNOS and iNOS reductase domains were generated by homology modelling based on the structure of the nNOS isoform (1TLL) (4). The solvent was treated explicitly. Electrostatic interactions were calculated with no truncation, using the particle mesh Ewald summation algorithm. The van der Waals interactions were smoothly shifted to zero between 10.0 Å and 12.0 Å. The list of the nonbonded interactions was calculated with no truncation, using the particle mesh Ewald summation algorithm. The van der Waals interactions were smoothly shifted to zero between 10.0 Å and 12.0 Å. The list of the nonbonded interactions was calculated with no truncation, using the particle mesh Ewald summation algorithm. The van der Waals interactions were smoothly shifted to zero between 10.0 Å and 12.0 Å. The list of the nonbonded interactions was calculated with no truncation, using the particle mesh Ewald summation algorithm.

Synthesis of NS1. The substituted aldehyde 3 was prepared from N-ethyliamine and phthalimido-acetaldehyde 1 in the presence of NaBH₄ in anhydrous DMF assembled the chromophore S and the 2-3′-iso-propylidenes adenosine 5-carboxylic acid 6 (28). After HCl depredation of the iso-propylidene moiety of compound 7, diol 8 was phosphorylated at position 2′ and 3′ by treatment with chlorodiethylphosphate in anhydrous CH₂Cl₂. Further acidic hydrolysis of the diethylphosphate afforded the
expected NS1 as a mixture of isomeric compounds 9 and 10 bearing the \( \text{P(O)}_2\text{O}_3\text{H}_2 \) group at positions 2’ and 3’ in a 40-60 ratio, respectively (see SI Appendix, Fig. S2).

Expression and Purification of Proteins. Recombinant rat nNOS, murine iNOS, and bovine eNOS were expressed in Escherichia coli BL21 cells whereas eNOS reductase domain was expressed in yeast and purified as described (29, 30).

Spectroscopic Characterization of Free or NOS-Bound NS1. UV-visible absorption spectrum of NS1 was carried out with an Uvikon spectrophotometer. Fluorescence excitation and emission spectra under one-photon excitation condition were recorded on an Eclipse (Varian) fluorimeter. Two-photon excitation and emission spectra were recorded using a home-built setup. Briefly, an 80-MHz mode-locked Mai-Tai Ti:Sapphire tunable laser (690-1,040 nm, 100-fs laser pulse; Spectra Physics) was focused onto the sample placed in a quartz micro cell. The fluorescence was collected at 90°, filtered by a Semrock FF01-842/SP filter, and focused into an optical fiber connected to a SpectraPro-275 digital spectrograph coupled to CCD detector (Princeton Instruments).

Stopped-Flow Experiments. Kinetics of the reaction between nNOS (5 μM) and NADPH were measured at 24°C in an anaerobic chamber (Coy Laboratory Products Inc.) using a Bio-SEQUENTIAL DX-18MV stopped-flow instrument (Applied Photophysics). The solutions of NADPH (25 μM) and NS1 (0–100 μM) were prepared in oxygen-free buffer. Reaction rates were calculated by fitting data to single- or double-exponential functions using Applied Photophysics software.

Effect of NS1 on the Formation of NO Catalyzed by nNOS. The initial rates of NO formation were determined at 37°C in 1-cm path-length cuvettes (150 μM) on a Uvikon 941 spectrophotometer using the oxyhemoglobin assay. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored by repetitive scanning between 380 and 480 nm every 0.2 min. All values are means ± SD from 3–4 experiments.

Imaging of Living HUVEC Cells in the Presence of NS1 and Colocalization Experiments. NS1 was incubated with HUVECs (Sigma, at fourth passage) for 30 min using fresh medium before observation. One- and 2-photon images of living NS1-treated HUVECs were obtained using a SP2 confocal microscope (Leica Microsystems) and a laser at 488 nm or an 80-MHz mode-locked Mai-Tai Ti:Sapphire laser (720–920 nm, 100-fs laser pulse; Spectra Physics) tuned to 840 nm, respectively (the emission range was set between 520 and 680 nm). Colocalization experiments of NS1 with endoplasmic reticulum (ER), mitochondria, or Golgi complex were performed on living HUVEC cells using ER-Tracker Red dye, Mitotracker Deep Red FM, or BODIPY TR ceramide complexed to BSA (Invitrogen), respectively. Colocalization of NS1 with early endosome (EEA1) or eNOS was performed by immunostaining on fixed cells using rabbit polyclonal to EEA1 marker (Abcam) or purified mouse anti-eNOS/NOx Type III (BD-Biosciences), respectively, as primary antibodies.

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