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S-Nitrosation of arginase 1 requires direct interaction with inducible nitric oxide synthase

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Abstract

Arginase constrains endothelial nitric oxide synthase activity by competing for the common substrate, L-Arginine. We have recently shown that inducible nitric oxide synthase (NOS2) S-nitrosates and activates arginase 1 (Arg1) leading to age-associated vascular dysfunction. Here, we demonstrate that a direct interaction of Arg1 with NOS2 is necessary for its S-nitrosation. The specific domain of NOS2 that mediates this interaction is identified. Disruption of this interaction in human aortic endothelial cells prevents Arg1 S-nitrosation and activation. Thus, disruption of NOS2–Arg1 interaction may represent a therapeutic strategy to attenuate age related vascular endothelial dysfunction.

Keywords

Arginase; Nitric oxide synthase; Nitrosation; Nitrosylation; Endothelial dysfunction; Aging

Introduction

Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) using L-arginine as substrate is critical in regulating vascular tone and function. Arginase (Arg), an enzyme that also uses L-arginine as a substrate, regulates NOS activity by competing for the common substrate. Increased Arg activity, and consequently, decreased NO production, is described in several pathologies including atherosclerosis [1, 2], hypertension [3], asthma [4], erectile dysfunction [5], ischemia–reperfusion [6], intimal hyperplasia [3], and aging [7, 8]. There are two isoforms of Arg, Arg1 and Arg2, both of which are expressed in both endothelial and smooth muscle cells [7, 9]. Inhibition/ knockdown of Arg improves NO production and vascular endothelial function in a number of diseases [7, 9–12]. Recently, we have demonstrated that Arg1 is S-nitrosated and activated in the aorta of aged rats by stabilization of the active homotrimer of Arg1 [13]. S-nitrosation of Arg1 is dependent on the inducible NOS (NOS2) isoform, which is expressed in the aorta (predominantly but not solely in the endothelium) of aged but not young rats [9, 13–15].

The interaction of NOS2 with a number of its nitrosation targets is emerging. For example, direct interaction with COX2 [16] leads to S-nitrosation and activation of the enzyme. In addition NOS2 forms a multimeric protein complex with cPLA2α [17] leading to S-

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nitrosation and altering its function. In this study, we addressed whether a direct interaction between NOS2 and Arg1 is required for the nitrosation of Arg1. We show that Arg1 binds to the oxygenase domain of NOS2. Disruption of this interaction leads to decreased Arg1 S-nitrosation and enzyme activity in human aortic endothelial cells (HAEC). Given the role of Arg activation in endothelial dysfunction, disruption of the Arg1–NOS2 interaction may represent a potential therapeutic strategy for improving endothelial function in aging.

Materials and methods

HEK293 and RAW264.7 cells were purchased from ATCC. HAEC were purchased from Invitrogen. Plasmids encoding for NOS2 domain mutants as GST fusion proteins were a kind gift from Dr. Sangwon Kim (University of Pennsylvania, School of Medicine) as used to determine iNOS binding to another S-nitrosation target, cyclooxygenase 2 [16]. Purified NOS2 was from Cayman Chemical, and purified Arg1 from XTal Biostructures. Arg1 and GST antibodies were from Santa Cruz Biotech, NOS2 and NOS3 antibodies were from BD Biosciences. RIPA buffer was purchased from Upstate and protease inhibitor cocktail from Roche. GST purification kit was from Pierce (Thermo Scientific). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were from Invitrogen. Endothelial cell media kit comprised of EC media, FBS, P/S, and endothelial cell growth supplement was purchased from ScienCell Research Labs. All other chemicals were from Sigma and of the highest purity commercially available.

Cell cultures

HEK293 and RAW264.7 cells were cultured following ATCC protocol. HAEC were cultured in ECM media following vendor's recommendation and used between passages 7–10.

Animals

The animal protocols used in this manuscript have been approved by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee. NOS2−/− mice and C57BL6 wild type (WT) mice were housed in the Johns Hopkins University Animal Care facility. All animals were fed ad libitum and had free access to drinking water.

Isolation of endothelial cells from mouse aorta

Endothelial cells were isolated from mouse aorta as described previously [1, 18]. In brief, mice were anesthetized and perfused with PBS containing heparin (1000 U/ ml). Aorta was then dissected and immersed in DMEM containing 10% FBS and 1000 U/ml heparin, cannulated and ligated with a silk thread. The lumen was washed with serum free DMEM and filled with collagenase type II (2 mg/ml in serum free DMEM). After a 45 min incubation, EC were recovered by flushing with 5 ml of DMEM containing 10% FBS. Cells were recovered by centrifugation and plated on 35 mm dishes coated with collagen. After 2 h, plates were rinsed with PBS and 2 ml of complete endothelial cell medium (ScienCell Labs) were added. Cells were cultured for two passages and used.

S-nitrosation assays

Arginase 1 S-nitrosation was determined using the biotin switch [19] and S-FLOS [20] assays as described in the literature. In brief, for the biotin switch assay, 100 μg proteins were blocked using methylmethane thiosulfonate (MMTS; 50 mM; 50°C, 1 h with vigorous shaking). Proteins were separated from unreacted MMTS by ice-cold acetone precipitation, resuspended and reduced with 5 mM ascorbate, and biotinylated with HPDP-biotin (4 mM, 1 h, room temperature with vigorous shaking). Biotinylated proteins were enriched using

streptavidin coated agarose, resolved by SDS PAGE, and Arg1 determined by western blotting. For the S-FLOS assay, proteins were blocked, precipitated, and resuspended as above. Proteins were then reduced with ascorbate and labeled with maleimide conjugated Cy3 or Cy5 (Saturation dyes from GE Healthcare). Samples were immunoprecipitated with Arg1 antibody and resolved by SDS PAGE. Gels were scanned on a Typhoon scanner. For both experiments, a negative control was performed as follows: samples with highest expected nitrosation signal were blocked and labeled in the absence of ascorbate (non-reduced) to confirm that signals in the assay were nitrosation specific.

Arginase activity assay

Arginase activity was measured colorimetrically as described previously [13].

Co-immunoprecipitation experiments

RAW264.7 and HAEC cultured in 100 mm dishes to 90% confluence and stimulated with interferon γ (IFN γ; 100 units/ml) and bacterial lipopolysaccharide (LPS; 5 μg/ ml) for 6 h in the presence and absence of NOS2 inhibitor 1400 W [13]. Media was then aspirated, cell monolayers rinsed three times with 1×PBS, and lysed by scraping in 200 μl of lysis buffer (1×RIPA buffer supplemented with protease inhibitors). After centrifugation, protein concentration was determined in the supernatant (BioRad Protein Assay Reagent). Equal amounts of protein (500 μg) were removed and immunoprecipitated using NOS2 antibody followed by western blotting with Arg1 antibody. An isotype IgG was used as a negative control.

Co-transfection experiments

HEK293 cells were grown to 80% confluence and used. Myc-His-tagged Arg1 and NOS2 domain mutants (GST-tagged) were co-transfected using Lipofectamine 2000 (Invitrogen) following manufacturer's protocol. Cells were grown for an additional 36 h, rinsed, lysed in 500 μl lysis buffer, and proteins recovered by centrifugation. 500 μg proteins were immunoprecipitated using Arg1 or Arg2 antibody. Co-immunoprecipitation of NOS2 domain mutant was determined by western blotting using GST antibody.

Binding assay

Purified NOS2 (5 μg) and Arg1 (5 μg) were incubated in 500 μl PBS for 30 min and immunoprecipitated with NOS2 antibody or control IgG. The NOS2 domain chimera 115–499 (NOS-DM) was transfected and overexpressed in HEK293 cells, and purified using spin GST purification kit following manufacturer's protocol. NOS-DM (~2.5 μg) and Arg1 (5 μg) were incubated (30 min in PBS) and immunoprecipitated with GST antibody. Last, 5 μg each of NOS2, Arg1, and NOS-DM were incubated and immunoprecipitated with NOS2 antibody. Co-precipitation of Arg1 was determined by western blotting.

Disruption of Arg1–NOS2 interaction

The domain mutant corresponding to amino acids 115–499 was identified as the major binding partner of Arg1. This plasmid was introduced into HAEC by electroporation (Amaxa Corp) using Primary Cells Electroporation kit (Lonza) following vendor's instructions. Cells were cultured for an additional 48 h, stimulated with IFN γ/LPS as described above. Arginase activity was measured as described earlier [13]. Expression of Arg1, NOS2, and domain mutant were determined by western blotting. Arginase S-nitrosylation was determined by the S-FLOS assay [20].

Statistics

Data in bar graphs are presented as mean ± SEM. Comparisons between treatment groups were performed by one-way ANOVA with Tukey post-test (*P <0.05, **P <0.01).

Results

Arg1 nitrosation is NOS2 dependent

Endothelial cells (ECs) isolated from freshly dissected aorta of WT and NOS2−/− mice were cultured for two passages and used. Cells were stimulated with IFNγ/LPS for 6 h. S-nitrosation of Arg1 increased in stimulated ECs from WT but not NOS2−/− mice as determined by the S-FLOS and biotin switch assays [19, 20] (Fig. 1a). However, treatment of cells from NOS2−/− mice with S-nitrosoglutathione (GSNO; 100 μM, 1 h) led to increased Arg1 S-nitrosation. NOS2 was expressed in ECs from WT mice in response to stimulation. Arg1 expression was constant (Fig. 1b).

Arg1 and NOS2 co-immunoprecipitate in cells

As described previously, stimulation of HAEC and RAW264.7 cells with IFNγ/LPS leads to increased Arg1 S-nitrosation and activity [13]. In this study, cells were stimulated with IFNγ (100 units/ml) and LPS (5 μg/ml) for 6 h in the presence and absence of the NOS2 inhibitor 1400 W (20 μM) and lysed. 500 μg proteins were immunoprecipitated with NOS2 or NOS3 antibody. Co-precipitation of Arg1 and Arg2 were determined by western blotting. Arg1 co-precipitated with NOS2 in both HAEC and RAW264.7 cells and the interaction of the two proteins was unaffected by 1400 W (Fig. 2a, c). Arg2 did not co-precipitate with NOS2. Neither Arg isoform co-precipitated with NOS3. Arg activity increased in both cell types with stimulation and was restored to baseline by 1400 W (Fig. 2b, d). NOS2 was expressed in stimulated cells but not in unstimulated cells. Arg1 expression remained constant in all treatment groups (Fig. 2a, c).

Oxygenase domain of NOS2 interacts with Arg1

GST-tagged NOS2 domain constructs (Fig. 3a) were co-transfected with full length Arg1 (myc-tagged) into HEK293 cells. Proteins were recovered after 36 h and immunoprecipitated with Arg1 or Arg2 antibody and blotted with anti-GST antibody. A NOS2 fragment corresponding to amino acid sequence 115–499 (NOS-DM), CAM binding domain, and oxygenase domain co-precipitated with Arg1 (Fig. 3). NOS-DM did not precipitate with control IgG (rightmost band). No interactions were observed with Arg2. Next, the interaction was confirmed using purified proteins (Fig. 3c). Arg1 bound to both full length NOS2 and NOS-DM in vitro. The interaction with full length NOS was inhibited by NOS-DM.

NOS-DM interferes with Arg1 S-nitrosation in HAEC

NOS-DM or vector control was introduced into HAEC by nucleofection (Amaxa). Cells were then stimulated with IFNγ/LPS for 6 h. Controls were transfected with vector but left unstimulated. NOS-DM, Arg1, and NOS2 expression levels were determined by western blotting (Fig. 4a). Arg1 S-nitrosation increased in stimulated cells and returned toward unstimulated controls in NOS-DM transfected samples (Fig. 4a). Similarly, Arg activity increased in vector-transfected stimulated cells compared to unstimulated controls. NOS-DM transfection restored Arg activity toward unstimulated controls (Fig. 4b).

Discussion

Arginase negatively regulates NOS3 dependent NO production in endothelial cells. Thus, increased Arg activity is shown to contribute to reduced NO bioavailability in several pathologies including vascular dysfunction [7, 9, 13], atherosclerosis [1, 2, 21], asthma [4, 22], erectile dysfunction [23, 24], and aging [7, 25–27]. Arginase activity increases in the vasculature of aging rats [7, 10, 25] and constrains NO production by NOS3. Pharmacologic inhibition of Arg or knockdown of Arg1 using antisense to Arg1 mRNA improves NO production and vascular function. Existing literature also demonstrates an age-associated increase in NOS2 expression in endothelial cells [13–15]. Using the NOS2 inhibitor 1400 W, we have previously shown a NOS2 dependent S-nitrosation of Arg1 which leads to increased Arg1 activity in cultured endothelial cells and ex vivo in rat aorta [13]. However, 1400 W can inhibit other NOS isoforms, albeit with lower efficiency. In this study, endothelial cells isolated from NOS2 knockout mice aorta were used to confirm that NOS2 is indeed the isoform responsible for Arg1 S-nitrosation. Direct interaction of the two proteins was confirmed using purified Arg1 and NOS2 and by co-immunoprecipitation in cellular models. The interaction of Arg1 with NOS2 was specific as Arg2 did not co-precipitate with NOS2 and Arg1 did not co-precipitate with NOS3. We determined that the oxygenase domain of NOS2 is involved in this interaction using different deletion mutants of NOS2 [16]. The interaction was strongest with amino acids 115–499 (NOS-DM) contained in the oxygenase domain. NOS-DM disrupted the Arg1–NOS2 interaction in stimulated endothelial cells and reversed the effect of NOS2 suggesting that this interaction is necessary for Arg1 S-nitrosation. Disruption of the Arg1–NOS2 interaction could represent a mechanism to diminish Arg1 S-nitrosation, thus restoring its activity toward baseline, and could be of therapeutic value in treating age-associated vascular dysfunction. The generation of NO by NOS2 is important in mediating immune response and cytotoxicity to kill invading pathogens. Conversely, increased Arg activity, producing ornithine and urea can support the growth of parasitic pathogens and bacteria. For example, increased Arg activity and associated decrease in NOS2 activity in macrophages has been shown to restrict host defense to *Helicobacter pylori* [28]. Furthermore, mice deficient in macrophage Arg1 have higher NO in tissues associated with *Mycobacterium tuberculosis* infection and improved host control of the organism as measured by granuloma size [29]. Thus, Arg inhibition/constraint of activity can represent a mechanism to treat infections in which Arg isoforms are shown to modulate the pathology of infection, particularly in intracellular organisms [30–32]. Thus, the interaction of Arg1 and NOS2 has important implications for normal host immunity as well as for vascular pathobiology such as aging in which endothelial NOS 2 is upregulated.

Conclusions

Arg1 S-nitrosation by NOS2 requires a direct interaction between the two proteins. The oxygenase domain of NOS2 participates in the interaction with Arg1. Disruption of this interaction restores Arg1 S-nitrosation and activity toward baseline in endothelial cells. This could represent a potential therapeutic route toward preventing increased S-nitrosation of Arg1 in aging vessels, and thus treating age-associated increases in vascular stiffness.

Acknowledgments

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Abbreviations

Arg1	Arginase 1
NOS2	Inducible NOS
NOS2-DM	GST-fusion NOS2 domain corresponding to amino acids 114–599

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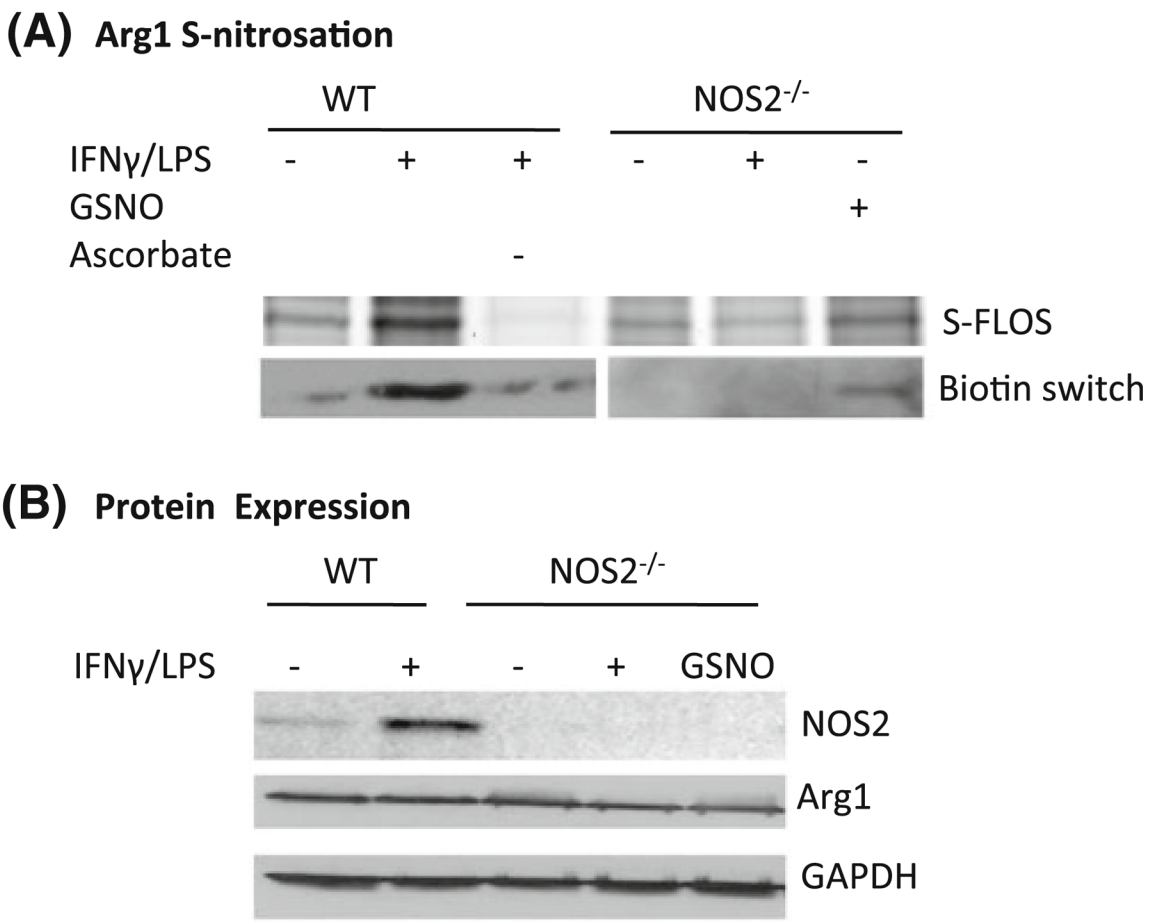

Fig. 1.

Arg1 S-nitrosation is mediated by NOS2 in endothelial cells. **a** Stimulation with IFN γ (100 units/ml) and LPS (5 μg/ml) for 6 h led to increased Arg1 S-nitrosation (measured by the S-FLOS (*top*) and Biotin switch (*bottom*) assays) compared to unstimulated in ECs isolated from the aorta of WT but not NOS2−/− mice; specificity of S-nitrosation signal was confirmed by blocking stimulated samples followed by labeling in the absence of ascorbate (third lane from *left*); GSNO (100 μM, 1 h) treatment led to increased S-nitrosation in ECs from NOS2−/− mice. **b** NOS2 was expressed in WT stimulated cells but not in NOS2−/− cells as determined by western blotting; Arg1 expression was unchanged. GAPDH was used as loading control

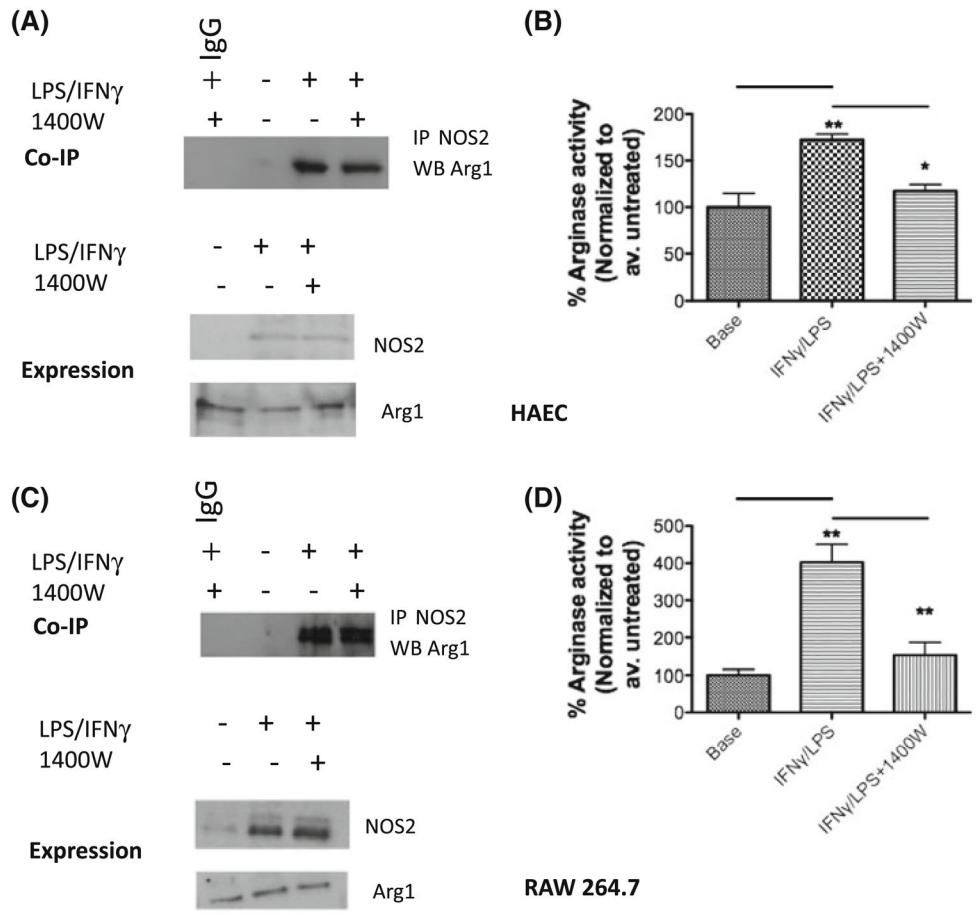

Fig. 2.

Arg1 and NOS2 co-precipitate in cells. Cells were stimulated with IFN γ (100 units/ml) and LPS (5 μg/ ml) in the presence and absence of NOS2 inhibitor 1400 W (20 μM) for 6 h; 500 μg of proteins were used to determine co-immunoprecipitation of Arg1 with NOS2. Isotype IgG was used to confirm specificity of the co-precipitation. Arg1 co-precipitated with NOS2 (*top* panels) in stimulated **a** HAEC and **c** RAW264.7 cells independent of NOS2 inhibition by 1400 W; Arg1 abundance was unchanged and NOS2 was expressed in stimulated cells (*bottom* panels; GAPDH was used as loading control); data are representative of four independent experiments; Arg activity increased in stimulated **b** HAEC and **d** RAW264.7 cells and reversed by 1400 W ($n = 4$; $^{*}P < 0.05$ by one-way ANOVA with Tukey post-test); $^{**}P < 0.01$ by one-way ANOVA with Tukey post-test)

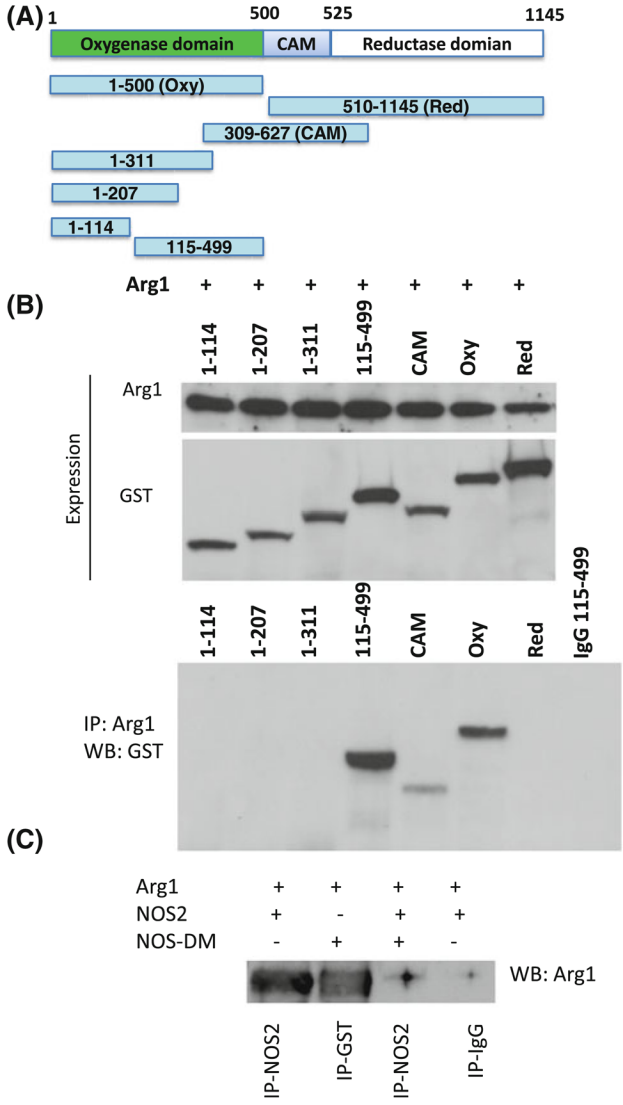

Fig. 3.

The Oxygenase domain of NOS2 interacts with Arg1. **a** Domain mutants of NOS2 used in this study; all are GST-tagged; **b** Arg1 and NOS2 domain mutants were co-transfected in HEK293 cells and cultured for an additional 36 h. 500 μg proteins were immunoprecipitated with Arg1-specific antibody and blotted using GST antibody. The oxygenase and CAM binding (309–627) domains interact with Arg1 with the strongest interaction perceived in the domain chimera comprised of amino acids 115–499 (NOS2-DM) within the oxygenase domain; **c** Purified Arg1 was incubated with full length NOS2, NOS-DM, or both and immunoprecipitated as indicated; Arg1 binds with full length NOS2 and NOS-DM in vitro; NOS-DM disrupts NOS2–Arg1 interaction

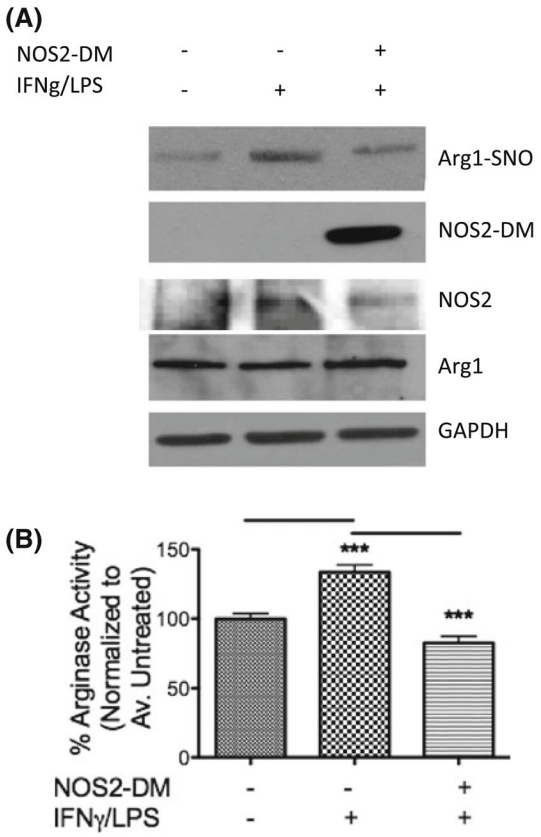

Fig. 4.

Overexpression of NOS2-DM in HAECs by nucleofection prevents Arg1 S-nitrosation by Arg1. HAECs were nucleofected with NOS2-DM (+) or empty vector (−) and treated with IFNγ/LPS as indicated. **a** Arg1 S-nitrosation increased in stimulated cells (*middle* lane) compared to unstimulated (*right* lane). Nucleofection with NOS2-DM restored Arg1 S-nitrosation toward unstimulated (*left* lane); NOS2-DM, NOS2, and Arg1 abundance was determined by western blotting. GAPDH was used as control; blots are representative of three independent experiments; and **b** Arg activity increased in stimulated cells and was restored toward unstimulated controls in cells expressing NOS2-DM; ($n = 6$; ***$P < 0.001$ by one-way ANOVA with Tukey post-test)