Molecular Cell, Volume *31*

Supplemental Data

S-Nitrosylation of β-Arrestin Regulates β-Adrenergic Receptor Trafficking

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Supplemental Methods

Materials: Anti-eNOS and anti-clathrin HC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-adaptin antibody was from Pharmingen (San Diego, CA). Anti-β-arrestin 2 antibody (A2CT) has been described previously (Attramadal et al., 1992). All other reagents were from Sigma unless otherwise noted.

Plasmids: pcDNA3, 1-FLAG-β2AR, pcDNA3-β-arrestin1-FLAG, pcDNA3-β-arrestin2-FLAG and pcDNA3-β-arrestin2-FLAG truncated forms (encoding rat-arrestins) were as described (Nelson et al., 2007). pcDNA3-eNOS was generated by amplification out of a human cDNA library, and cloned into pDNR-CMV (Clontech, CA) at EcoRI and XbaI sites. Clathrin and β-adaptin (AP-2) constructs were obtained from Dr. Marc Caron (Duke University, Durham NC).

Cysteine mutants and truncated forms of β-arrestin 2: To generate single cysteine mutants (C405A, C410N, C410R, C410P, C410M, C410F, C410A and C410S), cysteine residues of the wild-type protein were individually changed to alanine/serine or other (N,R,P,M,F) using the QuickChange XL site-directed kit (Stratagene, La Jolla, CA) and appropriate primers. Truncated forms of β-arrestin 2 were amplified by PCR using appropriate primers, and fragments were inserted into the p3XFLAG-CMV^{TM}-14 expression vector (Sigma-Aldrich, St. Louis, MO).

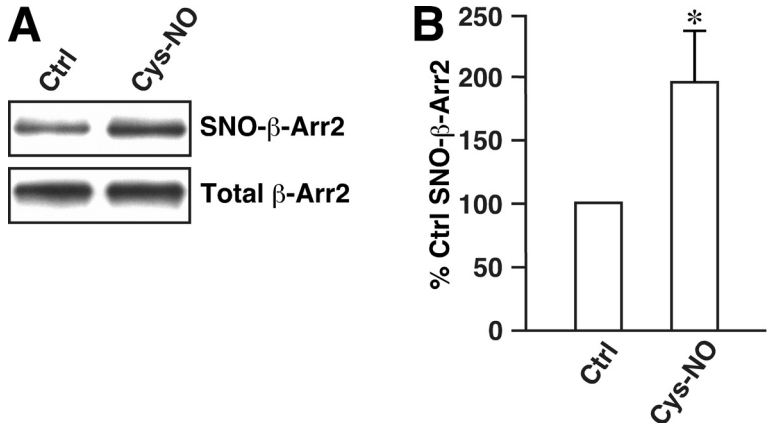
Cell culture and transfection: Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Cells were transfected at ~ 80% confluency using LipofectAMINE2000 (Invitrogen: Carlsbad, CA) according to the manufacturer's instructions. HEK cell lines stably overexpressing the FLAG-β2AR or FLAG-AT_{1A}R were as described (Whalen et al., 2007). The HEK-eNOS line was obtained from William Sessa (Yale University, New Haven CT). HUVECs (passages 2-8) were obtained from the Duke Cell Culture Facility.

siRNA: Pre-designed siRNA for eNOS was purchased from Dharmacon (Dharmacon, Inc., Lafayette, CO) and control siRNA was purchased from Santa Cruz (Santa Cruz, CA). ≈40% confluent cells in 6 cm dishes were transfected with siRNA using the INTERFERin siRNA transfection reagent (Polyplus-transfection Inc., New York, NY) according to manufacturer's instructions. Transfection with β-arrestin 2 (3 μg), clathrin (1. 5 μg) and β-adaptin (1. 5 μg) was at 48 hr after transfection of siRNA, and harvesting and immunoprecipitation analysis was at 96 hr.

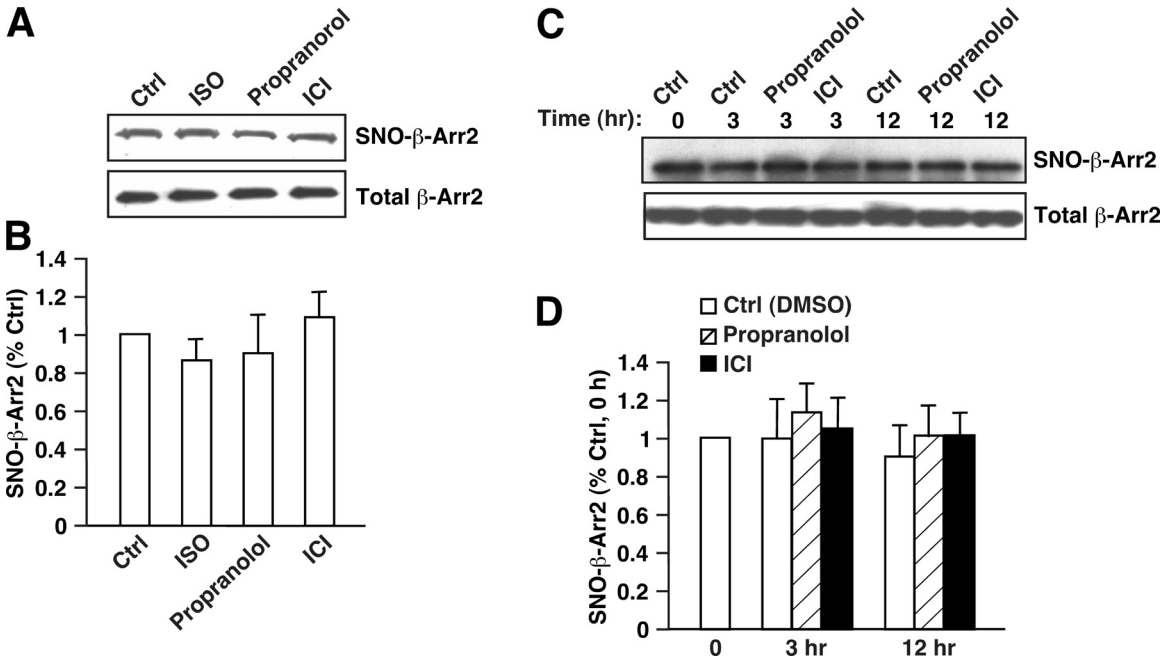
Immunoprecipitation and immunoblotting: Cells were washed with PBS, solubilized in 1 ml of lysis buffer (20 mM Hepes, 150 mM NaCl, 10% glycerol (v/v), 1. 0% Nonidet P-40 (v/v) and 2 mM EDTA) containing a protease inhibitor cocktail (Sigma; St. Louis, MO), and then clarified by centrifugation. For the determination of protein expression, 50 μl of each clarified whole-cell lysate was resolved by SDS-PAGE. Immunoprecipitation was performed using monoclonal M2 anti-FLAG-affinity agarose (Sigma), or anti-FLAG, anti-eNOS, anti-clathrin, anti-β-adaptin or anti-β-arrestin 2 antibodies, with constant agitation for 2 hours at 4°C. Immune complexes were washed three times with lysis buffer and then eluted using 3XFLAG peptide (anti-FLAG agarose or anti-FLAG antibody) or sample buffer. Immunoprecipitated proteins were resolved by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane for immunodetection.

Statistical Analysis: Data were analyzed using paired and unpaired Student's t-tests.

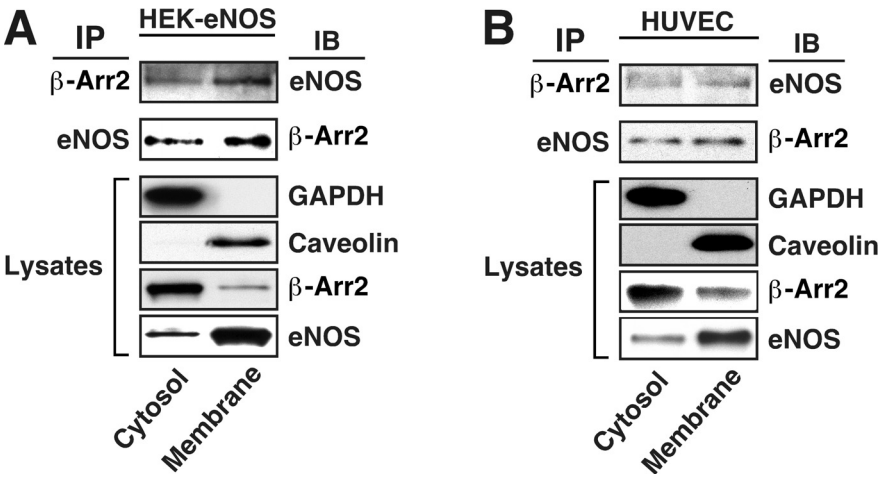
Supplemental Figures



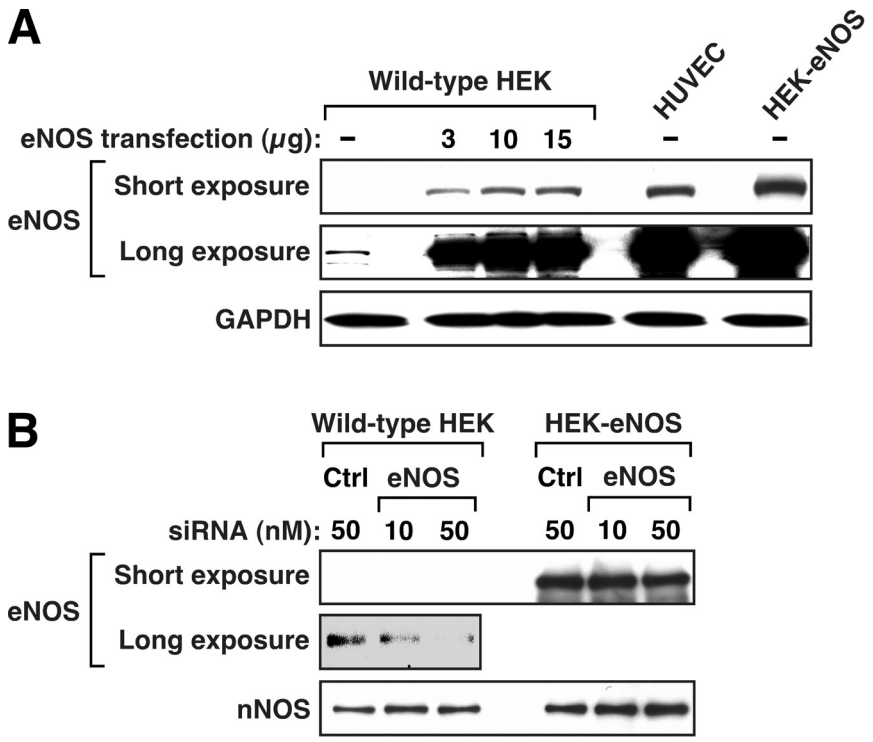
Supplemental Figure 1. *S*-nitrosylation of β-arrestin 2 by Cys-NO in HEK-eNOS cells. (A) HEK-eNOS cells transfected with FLAG-tagged β-arrestin 2 were treated with either saline or Cys-NO (50 μM, 10 min.), and lysates were analyzed by biotin-switch for SNO-β-arrestin 2 (ascorbate-dependent labeling). (B) A histogram presents the results of semiquantitative analysis, in which the amount of SNO-β-arrestin 2 is represented as a percentage of SNO-β-arrestin 2 in control cells and normalized with respect to the level of total β-arrestin 2. Data are means ± SE (n = 3); *P < 0. 05 re. wild-type.



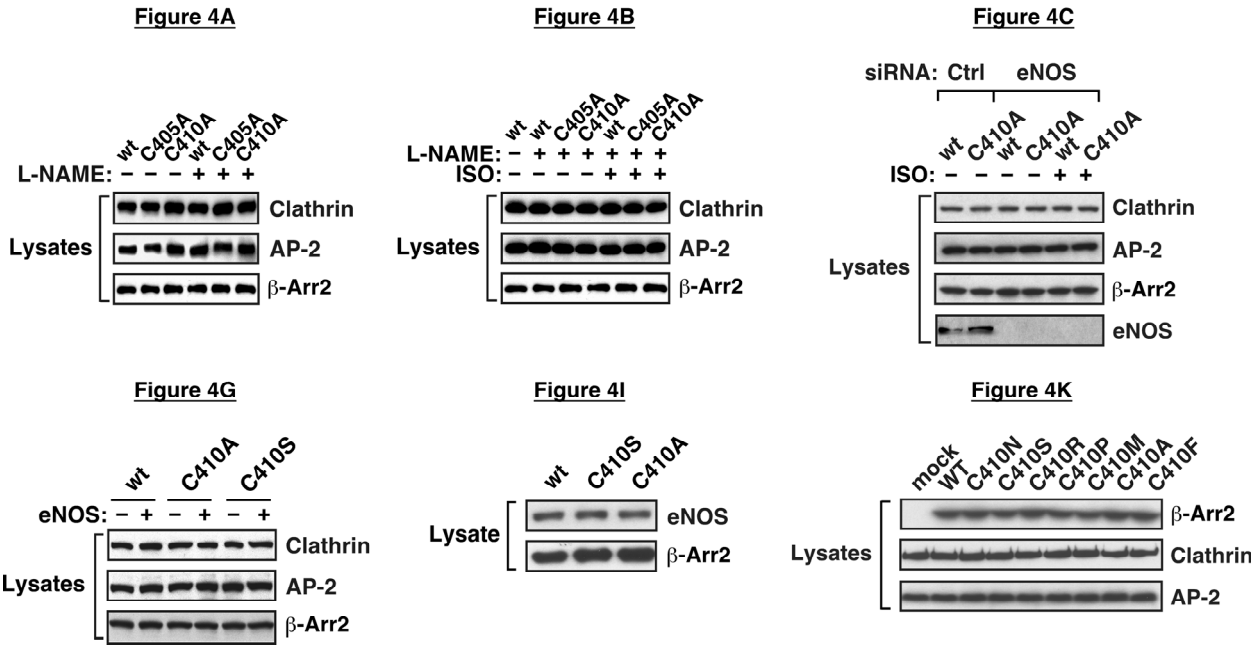
Supplemental Figure 2. Effect of agonist, antagonist and inverse agonist on basal β-arrestin 2 *S*-nitrosylation in HEK-eNOS cells. (A) Western blot showing effects of isoproterenol (ISO), propranolol and ICI 118,551 treatment (10μM, 10 min), on basal SNO-β-arrestin 2 in HEK-eNOS cells. (B) A histogram presents the results of semiquantitative analysis, in which the amount of SNO-β-arrestin 2 is represented as a percentage of SNO-β-arrestin 2 in control cells and normalized with respect to the level of total β-arrestin 2. Data are means ± SE (n = 3); no significant differences. (C) Western blot shows the effects of ISO, propranolol and ICI 118,551 treatment (10μM) for the times indicated on basal SNO-β-arrestin 2 in HEK-eNOS cells. (D) A histogram presents the results of semiquantitative analysis, in which the amount of SNO-β-arrestin 2 is represented as a percentage of SNO-β-arrestin 2 in control cells and normalized with respect to the level of total β-arrestin 2. Data are means ± SE (n = 3); no significant differences.



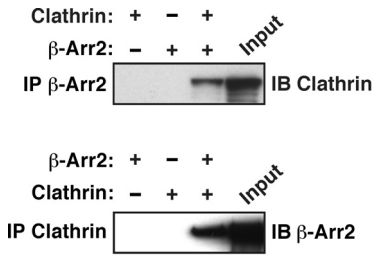
Supplemental Figure 3. **Subcellular localization of the β-arrestin 2/eNOS interaction.** (A) HEK-eNOS cells and (B) human umbilical vein endothelial cells (HUVEC) were transfected with FLAG-tagged β-arrestin 2, and separated into cytosolic and membrane fractions (indicated by the cytosolic marker GAPDH and membrane-localized caveolin). Each fraction (Lysates) was western blotted with anti-GAPDH, anti-caveolin, anti-eNOS or anti-FLAG antibody (β-Arr2), and was immunoprecipitated with anti-eNOS or anti-FLAG antibody followed by immunoblot with anti-FLAG or anti-eNOS antibody respectively.



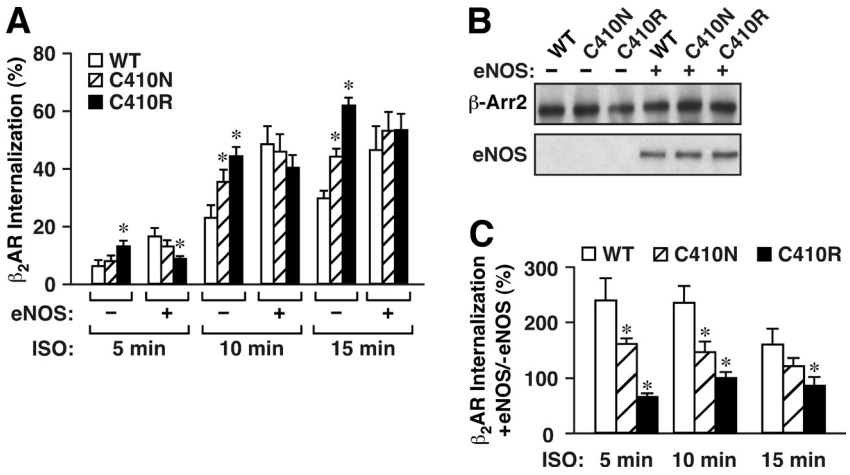
Supplemental Figure 4. Efficiency of eNOS expression following transfection of wild-type HEK cells and of siRNA-mediated silencing of eNOS expression in eNOS-HEK and wild-type HEK cells. (A) Western blots of lysates show expression of eNOS in wild-type HEK cells transfected with vector alone or with increasing amounts of eNOS plasmid, and in human umbilical vein endothelial cells (HUVEC) and HEK-eNOS cells (equal protein loading). (B) Western blots illustrate the efficiency of siRNA mediated silencing of eNOS in both wild-type HEK (complete or near complete silencing) and HEK-eNOS cells (minimal silencing). In (A) and (B), short and long exposure refer to blot exposure times.



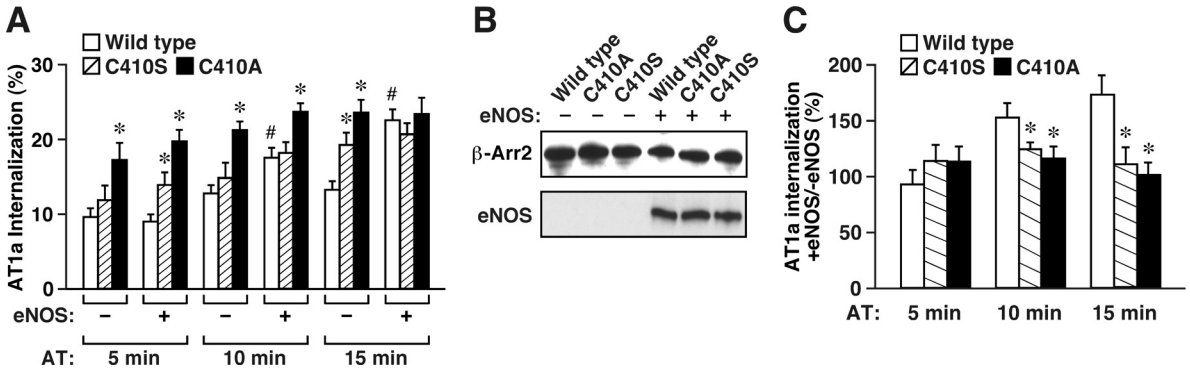
Supplemental Figure 5. Expression controls for Figure 4. Western blots demonstrate equal expression of clathrin, AP-2 and β-arrestin 2 across lystaes employed for immunoprecipitation in Fig. 4A-C, G, I and K.



Supplemental Figure 6. Binding of purified β-arrestin 2 and clathrin *in vitro*. Incubation (4°C, 12 hr) of purified clathrin (300nM; Sigma) with wild-type β-arrestin 2 (300 nM; purified as in Fig. 2) was followed by immunoprecipitation with the anti-β-arrestin 2 antiserum A2CT or with anti-clathrin antibodies. Immunoprecipitates and purified clathrin or β-arrestin 2 (input) were analyzed by western blotting with anti-clathrin or anti-β-arrestin antibodies, respectively.



Supplemental Figure 7. Effects of β-arrestin C410N and C410R mutations on eNOS-potentiated internalization of the β_2AR (A) HEK-β_2AR cells were transfected with wild-type, C410N or C410R β-arrestin 2 and internalization of β_2AR was quantified by flow cytometry following stimulation by isoproterenol (ISO, 10 μM) for the indicated times, with or without transient overexpression of eNOS. Data are means ± SE (n = 6); *P < 0. 05 re. wild-type. (B) Western blot of wild-type, C410N and C410R β-arrestin 2, as well as eNOS, showing comparable expression across groups. (C) A histogram summarizing the results shown in (A). Data are means ± SE (n = 6); *P < 0. 05 re. wild-type.



Supplemental Figure 8. *S*-nitrosylation of β-arrestin 2 facilitates ligand-induced AT1 angiotensin receptor internalization. (A) HEK cells overexpressing the angiotensin 1A receptor (HEK-AT_{1A}R cells) were transfected with wild-type, C410A or C410S β-arrestin 2 and internalization of AT_{1A}R was quantified by flow cytometry following stimulation by angiotensin II (AT, 100 nM) for the indicated times in the presence or absence of transiently overexpressed

eNOS. Data are means ± SE (n = 6); *P < 0. 05 re. wild-type and # indicates P < 0. 01 re. –eNOS vs. +eNOS at each time point. (B) Western blot of wild-type, C410A or C410S β-arrestin 2, and of eNOS, showing comparable expression between groups. (C) A histogram summarizing the results shown in (A). Data are means ± SE (n = 8); *P < 0. 05 re. wild-type.

Supplemental References

Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992). β-arrestin2, a novel member of the arrestin/β-arrestin gene family. J Biol Chem *267*, 17882-17890.

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