The Biotin Switch Method for the Detection of *S*-Nitrosylated Proteins

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Abstract

Many of the effects of nitric oxide are mediated by the direct modification of cysteine residues resulting in an adduct called a nitrosothiol. Here, we describe a novel method for detecting proteins that contain nitrosothiols. In this three-step procedure, nitrosylated cysteines are converted to biotinylated cysteines. Biotinylated proteins can then be detected by immunoblotting or can be purified by avidin-affinity chromatography. We include examples of the detection of *S*-nitrosylated proteins in brain lysates after in vitro *S*-nitrosylation, as well as the detection of endogenous *S*-nitrosothiols in selected neuronal proteins.

INTRODUCTION

Nitric oxide (NO) donors and endogenously produced NO exert a variety of effects in biological systems, including smooth muscle relaxation, cellular proliferation, apoptosis, neurotransmitter release, neurotoxicity, and differentiation. In mediating vasorelaxation, NO stimulates 3′-5′ guanosine monophosphate (cGMP) formation by binding to heme at the active site of soluble guanylyl cyclase, which leads to a conformational alteration that augments enzyme activity (*1*). The effect of NO is not completely blocked by drugs that inhibit guanylyl cyclase, however. A component of the NO-dependent relaxation of vascular smooth muscle appears to utilize a poorly characterized cGMP-independent process (*2*). In fact, other than vasorelaxation, many of the known effects of NO appear to be wholly independent of cGMP.

An important mechanism by which these effects of NO are mediated appears to be through a chemical modification of cysteine residues, termed *S*-nitrosation or *S*-nitrosylation (*3-5*). The adduct of cysteine is termed a nitrosothiol and accounts for the NO-dependent alteration of the activity of proteins including H-ras (*6*), the olfactory cyclic nucleotide-gated channel (*7*), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*8*). Nitrosothiols are exceptionally labile because of their reactivity with intracellular reducing agents, such as ascorbic acid and glutathione (GSH) (*9*), and with reduced metal ions, especially Cu(I) (*10*). This lability results in tissue half-lives of seconds to a few minutes (*9*). The reversible regulation of protein function by *S*-nitrosylation has led to suggestions that nitrosothiols function as posttranslational modifications analogous to phosphorylation or acetylation (*3*, *11*).

The principal methods used to study phosphorylation rely on radiolabeled precursors, such as [^{32}P]ATP or [^{32}P]PO_4. The presence of a phosphate can be detected by determining if the radiolabel copurifies with a protein of interest. Depending on the specific activity of the radiolabeled precursor, autoradiography and scintillation counting are sufficiently sensitive to detect attomole quantities of a stoichiometrically phosphorylated protein.

In the case of signaling by *S*-nitrosylation, however, radioactive isotopes of nitrogen or oxygen (the atoms that compose the adduct) are not available, necessitating the development of novel sensitive methods by which to detect the nitrosothiol moiety. Indeed, using a photolytic-chemiluminescence technique (*12*), Stamler and associates have provided evidence that certain proteins, including hemoglobin (*13*), the ryanodine receptor (*14*), and albumin (*12*), possess nitrosothiol moieties in their endogenous states. This methodology has two limitations: first, the equipment and methodology used in this method is complex, precluding widespread application. Second, this methodology is useful only to test if a particular purified protein possesses a nitrosothiol. The photolytic-chemiluminescence technique cannot identify *S*-nitrosylated proteins in a complex mixture, such as cytosol.

We have developed a method that results in the labeling of *S*-nitrosylated proteins with a biotin moiety specifically on *S*-nitrosylated cysteines (*15*) (Fig. 1A). In the first step, free thiols are blocked by incubation with the thiol-specific methylthiolating agent methyl methanethiosulfonate (MMTS) (*16*). Sodium dodecyl sulfate (SDS) is used to ensure access of MMTS to buried cysteines. Under the conditions used, MMTS does not react with nitrosothiols or preexisting disulfide bonds (*16*). After the blocking of free thiols, nitrosothiol bonds are selectively decomposed with ascorbate, which results in the reduction of nitrosothiols to thiols. In the last step, the newly formed thiols are reacted with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP), a sulfhydryl-specific biotinylating reagent. Because MMTS can compete with biotin-HPDP for thiol groups, it is necessary to remove MMTS completely by a spin column or acetone precipitation before treatment with biotin-HPDP. Labeled proteins can easily be detected by immunoblotting with antibodies against biotin (anti-biotin), following SDS-polyacrylamide gel electrophoresis (SDS-PAGE), or purified using immobilized streptavidin, a biotin-binding protein.

This method allows the detection of proteins that are *S*-nitrosylated by NO donors or that are endogenously *S*-nitrosylated by NO produced endogenously by isoforms of nitric oxide synthase (NOS). In the former case, detection is performed by anti-biotin immunoblotting. In the latter case, signals are typically not strong enough to detect proteins readily by anti-biotin immunoblotting. To overcome this limitation, larger quantities of tissue are obtained and subjected to the *S*-nitrosylation assay to biotinylate nitrosocysteines. The biotinylated proteins are then enriched by streptavidin-affinity chromatography, and retained proteins are eluted and blotted with antibodies specific to particular candidate proteins.

The *S*-nitrosylation assay was used successfully to detect proteins in a brain lysate that are *S*-nitrosylated after incubation with an NO donor, *S*-nitroso-glutathione (GSNO) (Fig. 1B). Nitrosylated proteins were detected by their immunoreactivity with an anti-

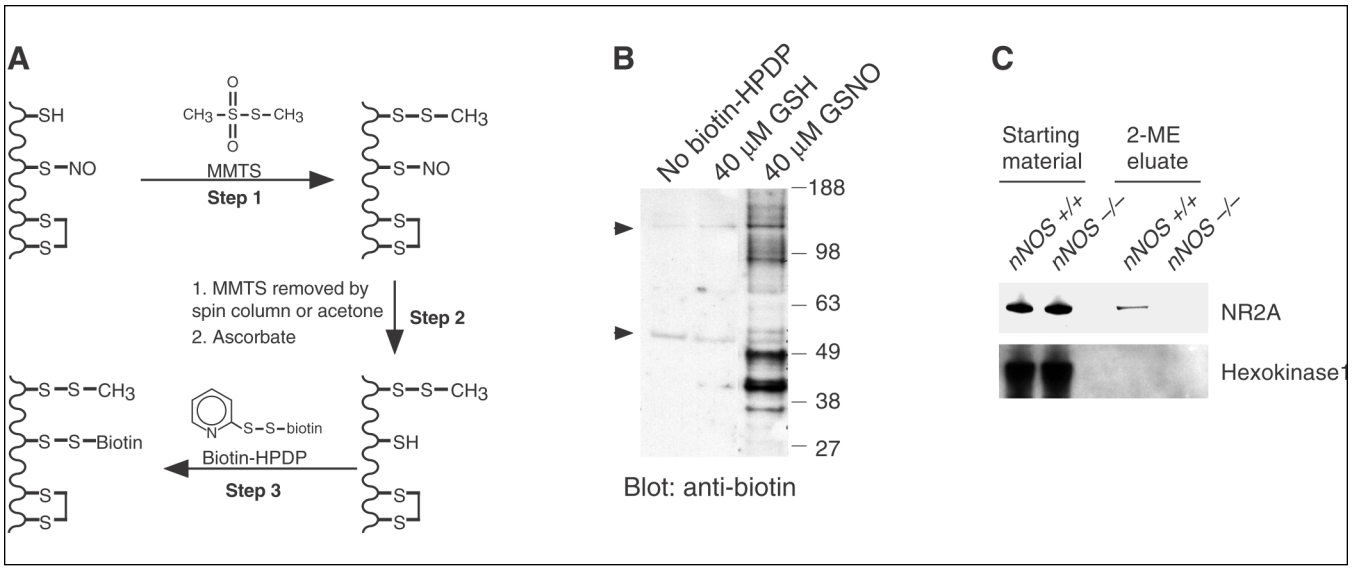

Fig. 1. Examples of experiments in which *S*-nitrosylation is detected. **(A)** Schematic diagram of the *S*-nitrosylation assay. A theoretical protein is indicated with cysteines in the free thiol, disulfide, or nitrosothiol conformation. In a preliminary step of the procedure, free thiols are made unreactive by methylthiolation with MMTS. This modification can be reversed by reduction with 2-ME. Unreacted MMTS is removed in a subsequent step either by passing the protein mixture through a spin column or by acetone precipitation. In the final step, nitrosothiols are selectively reduced with ascorbate to re-form the thiol, which is then reacted with the thiol-modifying reagent biotin-HPDP. **(B)** In vitro *S*-nitrosylation of brain extracts. Brain extracts were incubated with 40 μM GSNO, vehicle (HEN buffer), or the control compound GSH, and then subjected to the *S*-nitrosylation assay. Treatment of lysates with GSNO resulted in the *S*-nitrosylation of a discrete subset of proteins. In samples that were not treated with biotin-HPDP, the endogenously biotinylated proteins are detectable (arrowheads). **(C)** Endogenous *S*-nitrosylation of *N*-methyl-D-aspartate receptor, 2A subunit (NR2A) by nNOS. Brain lysates from wild-type and *nNOS*$^{-/-}$ mice were subjected to the *S*-nitrosylation assay, and biotinylated proteins were purified on streptavidin-agarose followed by 2-ME elution. NR2A was detected by immunoblotting with the indicated antibodies. The presence of proteins in 2-ME eluates derived from *nNOS*$^{+/+}$ mice, but not from *nNOS*$^{-/-}$ mice, indicates a requirement for nNOS in the *S*-nitrosylation of NR2A. A different protein, hexokinase, is not *S*-nitrosylated in either wild-type or *nNOS*$^{-/-}$ mice.

biotin antibody in an immunoblot. This method has also been used to determine if the *N*-methyl-D-aspartate receptor, 2A subunit (NR2A), is endogenously *S*-nitrosylated (Fig. 1C). In this experiment, *S*-nitrosylated proteins from wild-type and *nNOS*$^{-/-}$ mice were subjected to the *S*-nitrosylation assay, and the biotinylated proteins were recovered on streptavidin-agarose. These proteins were selectively eluted with 2-merceptoethanol (2-ME), and the presence of the potentially nitrosylated target protein was assayed by immunoblotting with specific antibodies.

MATERIALS

Acetone

Anti-biotin mouse monoclonal antibody (Sigma-Aldrich)

Bromophenol blue

3-([3-Cholamidopropyl]dimethylammino)-1-propanesulfonate (CHAPS)

Dimethylformamide (DMF)

Dimethylsulfoxide (DMSO)

Ethylenediaminetetra acetic acid (EDTA)

Glycerol

Hepes

2-Mercaptoethanol (2-ME)

Methyl methanethiosulfonate (MMTS) (Sigma-Aldrich)

Micro Bio-Spin P6 prepacked columns (Bio-Rad)

N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) (Pierce)

Neocuproine (Sigma-Aldrich)

NO donor GSNO (Sigma-Aldrich)

Glutathione (GSH) (Sigma-Aldrich)

Sodium dodecyl sulfate (SDS)

Sodium ascorbate

Streptavidin agarose (Sigma-Aldrich) or Neutravidin (Pierce)

Triton X-100

RECIPES

Recipe 1: HEN Buffer

Hepes-NaOH pH 7.7	250 mM
EDTA	1 mM
Neocuproine	0.1 mM

Recipe 2: 10% CHAPS Stock

Prepare a 10% solution in dH_20.

Recipe 3: MMTS Stock

Prepare a 2-M solution in DMF.

Recipe 4: Blocking Buffer

HEN Buffer (Recipe 1)	9 volumes
SDS (25% w/v in H_2O)	1 volume

Adjust to 20 mM MMTS with MMTS Stock (Recipe 3).

Recipe 5: HENS Buffer

Adjust HEN Buffer (Recipe 1) to 1% SDS by addition of a 1:25 volume of 25% (w/v) SDS solution.

Recipe 6: Ascorbate Solution

Prepare a 50-mM solution of sodium ascorbate in deionized water.

Recipe 7: Biotin-HPDP Stock

Prepare biotin-HPDP as a 50-mM suspension in DMSO. Freeze at −20°C until needed.

Recipe 8: Labeling Solution

Just before use, thaw the stock suspension of biotin-HPDP (Recipe 7) and vortex to resuspend the biotin-HPDP. Dilute with DMF to a final concentration of 4 mM. Vortex to ensure that the biotin-HPDP is in solution.

Recipe 9: SDS-PAGE Sample Buffer (Nonreducing)

50 mM Tris-HCl, pH 6.8	7 ml
Glycerol	3 ml
SDS	1.028 g
Bromophenol blue	1.2 mg

Note: Other sample buffers may be appropriate, depending on the type of gel system used; however, the sample buffer must be made without 2-ME or dithiothreitol (DTT).

Recipe 10: Neutralization Buffer

Hepes-NaOH, pH 7.7	20 mM
NaCl	100 mM
EDTA	1 mM
Triton X-100	0.5%

Recipe 11: Neutralization Buffer + NaCl

Prepare Neutralization Buffer (Recipe 10) with 600 mM NaCl.

Recipe 12: Elution Buffer

Hepes-NaOH, pH 7.7	20 mM
NaCl	100 mM
EDTA	1 mM
2-ME	100 mM

INSTRUCTIONS

Preparation of Protein Samples

The following steps are performed at 4°C. Protein samples can be obtained from homogenized tissue and can include or exclude the membrane fragments. Alternatively, purified proteins may be used, providing that any low molecular weight thiol contaminants have been removed.

Preparation of cytosolic proteins

1. Homogenize the tissue in about 20 volumes of HEN Buffer (Recipe 1).
2. Centrifuge at 100,000*g* for 1 hour at 4°C.
3. Recover the supernatant.
4. Assay for protein concentration using the Biuret or other method.

Note: Protein concentration must not exceed 0.8 μg per μl. Add HEN Buffer (Recipe 1) to adjust concentration as needed.

Preparation of membrane fragments and cytosolic proteins

1. Homogenize the tissue in approximately 20 volumes of HEN Buffer (Recipe 1).
2. Centrifuge at 2000*g* for 10 min at 4°C.

3. Recover the supernatant.
4. Assay for protein concentration using the Biuret or other method.

   Note: Protein concentration must not exceed 0.8 μg per μl. Add HEN Buffer (Recipe 1) to adjust the concentration as needed.

5. Adjust the sample to 0.4% CHAPS with 10% CHAPS Stock (Recipe 2). Vortex the sample to dissolve any membrane constituents. Avoid introducing bubbles while vortexing.

   Note: This step ensures that the gel filtration columns used for removing NO donors will work properly.

Preparation of purified proteins

To use a purified protein, ensure that low molecular weight thiols, such as 2-ME, DTT, or GSH, have been removed.

1. Dialyze the protein against HEN Buffer (Recipe 1) or exchange the protein solution with a HEN Buffer-equilibrated Micro Bio-Spin P6 column following the instructions of the manufacturer.
2. Recover the sample.
3. Assay for protein concentration using the Biuret or other method.

   Note: Protein concentration must not exceed 0.8 μg per μl. Add HEN Buffer (Recipe 1) to adjust the concentration as needed.

In Vitro *S*-Nitrosylation

The following procedure describes the use of GSNO as the NO donor. Other NO donors are also acceptable, such as *S*-nitroso-*N*-acetylpenicillamine (Sigma) or 1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA NONOate) (Alexis Chemicals), and are used at concentrations similar to those used for GSNO. A range of NO donor concentrations are recommended; however, *S*-nitrosylation signals obtained with 1 μM or less of NO donor are more likely to represent physiologic targets. A negative control reaction should always be included. For the NO donor GSNO, GSH is the appropriate negative control.

NO donors and the following reactions are labile. The NO donors should be stored protected from light and frozen at −80°C when not in use. To prevent light-induced degradation of the NO donors or protein nitrosothiols, the reactions should be protected from light by covering the tubes and containers with foil or by performing the reactions in a dark room.

1. Add NO donor (GSNO) or inactive donor control (GSH) to 100-μl protein sample to achieve a range of final concentrations from 0.1 μM to 100 μM.

   Note: All samples should be protected from light by covering tubes and containers in foil, or by performing the experiments in a dark room.

2. Incubate in the dark for 20 min at room temperature.

Biotinylation of *S*-Nitrosylated Proteins

It is advisable to remove the NO donor at this time, because it may nitrosylate cysteines that are physiologically inaccessible after the proteins are denatured in the subsequent steps. After the NO donor has been removed, the remaining thiols are blocked with MMTS, and then unreacted MMTS is removed. Two possible methods are described for removing the MMTS: acetone precipitation or spin columns. For samples greater than 100 μl, acetone precipitation is recommended.. For sample volumes of less than 100 μl, we use spin columns, such as the Micro Bio-Spin P6 column. One column is prepared for every condition used above. The column preparation and use are performed as recommended by the manufacturer.

Removal of the NO donor

1. Invert the spin column resin to form a suspension.
2. Allow the resin to drain and then resuspend the resin in 500 μl of HEN Buffer (Recipe 1).
3. Allow the resin to drain and then resuspend the resin in another 500 μl of HEN Buffer (Recipe 1). Allow the resin to drain.
4. Centrifuge the column at 1000*g* for 2 min to remove remaining buffer.
5. Apply the sample (in a volume of 75 μl) to the center of the resin.
6. Centrifuge the column at 1000*g* for 4 min.
7. Recover the sample.

Blocking of protein samples (>100 μl) and removal of MMTS by acetone precipitation

1. Add four volumes of Blocking Buffer (Recipe 4) to the sample from Step 7 above.
2. Incubate at 50°C for 20 min, vortexing frequently.
3. Add 10 volumes of –20°C acetone (pre-chilled) to each sample.
4. Incubate for 20 min at –20°C.
5. Centrifuge at a minimum of 2000*g* for 10 min at 4°C.
6. Resuspend the pellet in 0.1 ml HENS Buffer (Recipe 5) per mg of protein in the starting sample.

Blocking of protein samples (≤100 μl) and removal of MMTS with spin columns

1. Add four volumes of Blocking Buffer (Recipe 4) to the sample from Step 7 in "Removal of the NO donor," above.
2. Incubate at 50°C for 20 min, vortexing frequently.
3. Exchange the blocking solution with HEN Buffer (Recipe 1) three times with three Micro Bio-Spin P6 columns pre-equilibrated in HEN buffer. Final volume of the third eluate should be approximately 50 to 70 μl.

   Note: Each sample requires these exchanges because each exchange only removes ~95% of the MMTS. Therefore, three buffer exchanges are required to reduce the MMTS level to 1:10,000 of the original amount. We recommend preparing three separate columns for each sample because it takes too long to wash the columns between each spin, and time is critical because of the lability of the nitrosothiols.

Biotinylation of nitrosothiols

1. Prepare Labeling Solution (Recipe 8) just before use. Vortex before use to ensure even suspension of the biotin-HPDP.
2. Add 1:3 volume of Labeling Solution (Recipe 8) and 1:50 volume of Ascorbate Solution (Recipe 6) to the blocked protein samples.
3. Incubate for 1 hour at 25°C.

   Note: At this point, samples no longer need to be protected from light.
4. As a control, treat some samples with DMF vehicle solution without the biotin-HPDP added.

Detection of Biotinylated Proteins

Detection of biotinylated proteins by immunoblotting

1. Add SDS-PAGE Sample Buffer (Nonreducing) (Recipe 9).

   Note: Do not boil samples or use reducing agents in the SDS-PAGE sample buffer. We recommend that sample buffer be added directly to samples and immediately loaded onto the gel.
2. Perform SDS-PAGE.
3. Transfer the electrophoresed samples to nitrocellulose for immunoblotting.
4. Detect the biotinylated proteins with anti-biotin mouse monoclonal antibody.

Purification of biotinylated proteins

1. To the samples from "Biotinylation of nitrosothiols," add two volumes of –20°C acetone and incubate for 20 min at –20°C to remove the biotin-HPDP.
2. Centrifuge the samples at a minimum of 2000*g* for 10 min at 4°C.
3. Discard the supernatant, which contains the biotin-HPDP.
4. Gently rinse the walls of the tubes and the surface of the pellets with –20°C acetone to remove traces of biotin-HPDP.
5. Resuspend the pellets in 0.1 ml of HENS Buffer (Recipe 5) per mg of protein in the initial protein sample.
6. Add two volumes of Neutralization Buffer (Recipe 10).
7. Add 15 μl of packed streptavidin-agarose per mg of protein used in the initial protein sample, to purify biotinylated proteins.

   Note: Neutravidin-agarose (Pierce) has been used with identical results.
8. Incubate the biotinylated proteins with the resin for 1 hour at room temperature.

9. Wash the beads five times with 10 volumes of Neutralization Buffer + NaCl (Recipe 11). Centrifuge at 200*g* for 5 s at room temperature between each wash.
10. Incubate the beads with Elution Buffer (Recipe 12) to recover the bound proteins.
11. Add SDS-PAGE Sample Buffer with reducing agent.

    Note: At this point, it is not important to keep the samples unreduced, so any recipe for reducing SDS-PAGE sample buffer is suitable.
12. Perform SDS-PAGE.
13. Transfer the electrophoresed samples to nitrocellulose for immunoblotting.
14. Stain the nitrocellulose with Ponceau S and excise the proteins or test for the protein of interest with specific antibodies.

RELATED TECHNIQUES

Detection of Endogenous *S*-Nitrosothiols

To detect endogenously *S*-nitrosylated proteins, protein samples can be prepared as described above from a minimum of 750 mg of tissue derived from *nNOS*$^{-/-}$ or *nNOS*$^{+/+}$ wild-type mice, without the addition of NO donors. Because no NO donor is added, following the preparation of the protein sample, the procedure begins with the blocking step ("Blocking of protein samples") and then biotinylation ("Biotinylation of nitrosothiols"). The biotinylated proteins are purified following the steps outlined in the "Purification of biotinylated proteins," and can be immunoblotted with antibodies to candidate proteins whose nitrosylation status is being queried. Additionally, samples are protected from light immediately after homogenization, up until the biotin-HPDP Labeling Solution (Recipe 8) is added.

TROUBLESHOOTING

False Positive Signals

This *S*-nitrosylation assay is based on the assumption that only an *S*-nitrosylated cysteine residue will become biotinylated following the various treatments described above. However, if all cysteines have not been successfully blocked with the methylthiolating reagent MMTS, then the non-nitrosylated cysteines may produce a signal in this assay. SDS is included to promote denaturation, which should ensure accessibility of MMTS to each thiol. A cysteine that is inaccessible to MMTS may become accessible to biotin-HPDP during the labeling step if time-dependent or DMSO-dependent denaturation occurs and results in the unhindered exposure of a thiol. To ensure the maximal accessibility of cysteines to MMTS, a minimum ratio of SDS to protein is essential to ensure maximal protein denaturation. Thus, protein samples that exceed a concentration of 0.8 µg per µl are more prone to be incompletely blocked by MMTS.

Negative Controls for in Vitro Nitrosylation

The best way to ensure that a signal is due to *S*-nitrosylation is to include an inactive NO-donor control. Thus, in experiments designed to detect proteins susceptible to *S*-nitrosylation in brain extracts (Fig. 1B), the NO donor was GSNO and the inactive compound was the corresponding denitrosylated molecule GSH. A vehicle control is also useful, because the inactive control compound (GSH) is a thiol-containing molecule, which may have effects on cellular thiols and nitrosothiols. Bands that are present in the NO donor lane, but not in lanes in which control compounds were used, represent proteins that are *S*-nitrosylated.

Negative Controls for Detection of in Vivo Nitrosylation

Because the nitrosylation assay includes biotin immunoblotting, proteins that are endogenously biotinylated can be a source of significant background. These enzymes, which perform carboxytransferase reactions, are found at different levels in different animals. For example, the brains of C57/BL6 mice contain these endogenously biotinylated proteins at levels 20 times higher than that found in Norwegian white rats. The presence of endogenously biotinylated proteins can actually be beneficial, because they can serve as loading controls. That is, their uniform intensity in different lanes can ensure that equivalent amounts of protein were used in different experiments. A key control in the identification of these proteins is the use of a DMF vehicle control, instead of biotin-HPDP, in the biotinylation labeling reaction. A blocked sample that has been subjected to DMF treatment rather than biotin-HPDP treatment shows two bands, which represent endogenously biotinylated proteins (Fig. 1B).

This protocol outlines the methods for the detection of both in vitro *S*-nitrosylated proteins, by biotin immunoblotting, and endogenously biotinylated proteins, by purification of *S*-nitrosylated proteins through streptavidin-affinity chromatography followed by candidate-specific immunoblotting. In this latter method, some of the same troubleshooting issues arise, but the required controls are different. To ensure that a signal is due to NOS activity and not to incomplete blocking, tissue samples that are devoid of NOS

are ideal. Thus, protein samples prepared from *NOS*$^{-/-}$ mice provide ideal controls. Alternatively, if NOS-transfected tissue culture cells are used, mock-transfected cells would be useful. If these types of controls are unavailable, then samples prepared with NOS inhibitors, such as nitroarginine, can also be used as negative controls.

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