Assessment and Application of the Biotin Switch Technique for Examining Protein S-Nitrosylation under Conditions of Pharmacologically Induced Oxidative Stress

Michael T. Forrester, Matthew W. Foster, and Jonathan S. Stamler

Protein S-nitrosylation has emerged as a principal mechanism by which nitric oxide exerts biological effects. Among methods for studying protein S-nitrosylation, the biotin switch technique (BST) has rapidly gained popularity because of the ease with which it can detect individual S-nitrosylated (SNO) proteins in biological samples. The identification of SNO sites by the BST relies on the ability of ascorbate to generate a thiol from an S-nitrosothiol, but not from alternatively S-oxidized thiols (e.g. disulfides, sulfenic acids). However, the specificity of this reaction has recently been challenged, prompting several claims that the BST may produce false-positive results and raising concerns about the application of the BST under oxidizing conditions. Here we perform a comparative analysis of the BST using differentially S-oxidized and S-nitrosylated forms of protein tyrosine phosphatase 1B, as well as intact and lysed human embryonic kidney 293 cells treated with S-oxidizing and S-nitrosylating agents, and verify that the assay is highly specific for SNO. Strikingly, exposure of samples to indirect sunlight from a laboratory window resulted in artifactual ascorbate-dependent signals that are likely promoted by the semidehydroascorbate radical; protection from sunlight eliminated the artifact. In contrast, exposure of SNO proteins to a strong ultraviolet light source (SNO photolysis) prior to the BST provided independent verification of assay specificity. By combining BST with photolysis, we have shown that anti-cancer drug-induced oxidative stress facilitates the S-nitrosylation of the major apoptotic effector glyceraldehyde-3-phosphate dehydrogenase. Collectively, these experiments demonstrate that SNO-dependent signaling pathways can be modulated by oxidative conditions and suggest a potential role for S-nitrosylation in antineoplastic drug action.

Nitric oxide (NO) executes a diverse range of cellular functions through the redox-dependent conversion of protein Cys thiols to S-nitrosothiols (SNOs). This post-translational modification, known as S-nitrosylation, has emerged as a highly conserved and spatiotemporally specific signaling mechanism (1). A major contribution to this field was the introduction of the biotin switch technique (BST) by Jaffrey et al. in 2001 (2). Importantly, the BST allows relatively facile identification and quantification of endogenous protein SNOs and thus has greatly contributed to our understanding of protein S-nitrosylation. As evidence, the BST has been employed in over 70 publications, unveiling new roles for S-nitrosylation in events such as apoptosis (3), neurodegeneration (4, 5), insulin signaling (6, 7), and receptor trafficking (8).

The BST consists of three principal steps (supplemental Fig. S1): “blocking” of free Cys thiols by S-methylthiolation with S-methyl methanethiosulfonate (a reactive thiosulfonate), formal reduction of SNOs to thiols with ascorbate (Asc), and in situ “labeling” by S-biotinylation of the nascent thiols with biotin-HPDP, a reactive mixed disulfide. The degree of biotinylation (and hence S-nitrosylation) is determined by either anti-biotin immunoblotting or streptavidin pull down followed by immunoblotting for the protein(s) of interest. Complete blocking of free thiols is requisite for minimizing background biotinylation (i.e. improving signal-to-noise or sensitivity), while the specificity of the BST is predicated on the ability of Asc to reduce SNOs to free thiol, without reducing other Cys-based redox modifications such as S-glutathionylation or S-oxidations (sulfenic, sulfenic, and sulfonic acids). Importantly, transition metal-catalyzed reactions, particularly those that might limit the specificity of Asc, are mitigated through use of metal chelators (e.g. EDTA, DTPA, neocuproine).

Despite the unequivocal ability of the BST to detect SNOs, the specificity of Asc for SNO reduction has not been extensively investigated. Landino et al. (9) suggested that Asc reduces tubulin disulfides, a claim the authors use to challenge the validity of the BST. Furthermore, two other groups have reported that S-glutathionylated proteins yield positive signals in this assay (10, 11). A more recent report by Huang et al. (12) argued that the BST yields “artifactual” Asc-dependent biotinylation of native reduced bovine serum albumin. They extrapolate this finding to argue that the BST may result in false-positive (i.e. SNO-independent) biotinylation, which could cast doubt upon the myriad of studies that have relied heavily on this assay.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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2 The abbreviations used are: NO, nitric oxide; BST, biotin switch technique; SNO, S-nitrosothiol; CysNO, S-nitrosocysteine; GSH, reduced glutathione; GSSG, glutathione disulfide; biotin-HPDP, N-[6-(biotinamido)hexyl]-3’-[(2’-pyridyl-dithio)propionamido]-asc, ascorbate; DHAsc, dehydroascorbate; semi-DHAsc, semidehydroascorbate radical; PTP1B, human protein tyrosine phosphatase 1B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NOS, nitric-oxide synthase; nNOS, neuronal NOS; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DTT, dithiothreitol; HEK, human embryonic kidney.
Shared between these claims of artifactual signals is the idea that Asc must be reducing disulfides (S-glutathionylated or intramolecular disulfides). However, this notion is difficult to reconcile experimentally with the use of S-methyl methanethiosulfonate as a blocking agent (i.e. protein thios are S-methylthiolated to form mixed disulfides). The assay would never work if ascorbate removed the blocking agent, and in fact, the two e⁻ reduction of cysteines of thiols (Cys thiol/disulfide E_{red} = -170 to -320 mV) by Asc is highly thermodynamically unfavorable (Asc/DHAsc E_{red} +70 mV) (13). Thus, one would not expect Asc to directly reduce any biological Cys oxidation products. Instead, the electrochemical measurements favor the reverse reaction (i.e. thiol-dependent reduction of dehydroascorbate (DHAsc⁰ to Asc), a scenario supported by extensive observations (14, 15).

Despite the thermodynamic and empiric evidence against Asc-dependent disulfide reduction (and alternative interpretations for some of the prior results that have formed the basis of challenge to the BST), we felt it was important to evaluate the reinterpretations for some of the prior results that have formed the basis of Protein Modifications and Biotin Switch Technique. —Proteins/lysates were treated with the indicated concentrations of H₂O₂, diamide, glutathione disulfide (GSSG), or CysNO followed by desalting with Bio-Gel P-6DG. The BST was performed on purified PTP1B as described (17) and with several modifications as follows.

Samples were mixed with 2 volumes of HEN buffer followed by addition of freshly prepared S-methyl methanethiosulfonate (10% v/v in N,N-dimethylformamide) and SDS (25% v/v) to final concentrations of 0.1 and 2.5%, respectively. Following frequent vortexing at 50 °C for 20 min, proteins were precipitated with 3 volumes of acetone at −20 °C for 15 min. The proteins were recovered by centrifugation at 5,000 × g for 5 min, followed by gentle resuspending of the pellet with 4 × 1 ml 70% aceton/H₂O. The pellets were then resuspended in 240 µl of HEN buffer containing 1% SDS. For labeling, the blocked samples were mixed with 0.1 volume of biotin-HPDP (2.5 mg/ml in Me₂SO) and 0.1 volume of HEN (control) or freshly prepared sodium Asc in HEN buffer. Labeling reactions were performed in the dark at room temperature for 1 h unless otherwise indicated. Indirect sunlight was introduced by incubating the labeling reactions near a laboratory window without direct exposure to sunlight or change in temperature.

For direct detection of protein biotinylation, 20–40 µg of each labeling reaction was resolved by non-reducing SDS-PAGE, followed by immunoblotting with anti-biotin antibody (1:500). To detect an individual SNO protein from cells or lysates, the labeling reaction was acetone-precipitated as previously described. The washed pellet was resuspended in 250 µl of HEN/10 (HEN diluted 10-fold into H₂O) containing 1% SDS, followed by addition of 750 µl of neutralization buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.5). This material was incubated overnight at 4 °C with 40 µl of a streptavidin-agarose slurry. The beads were washed with 4 × 1 ml of wash buffer (neutralization buffer + 500 mM NaCl), followed by 2 × 1 ml of neutralization buffer. The dried beads were eluted with 50 µl of HEN/10 + 1% β-mercaptoethanol at room temperature for 20 min. The eluted mixture was then analyzed by SDS-PAGE, followed by immunoblotting with anti-PTP1B (1:1000) or anti-GAPDH (1:1000) antibodies.

Thiol Measurements and GSSG Reductase Activity Assays—Thiols were assayed by addition of one volume of 0.1 mg/ml 5,5’-dithiobis(2-nitrobenzoic acid) in HEN. Absorbanes of samples and GSH standards were immediately read at 405 nm. Activity of GSSG reductase was measured as described (18) by following GSSG-dependent consumption of NADPH at 340 nm.

SNO Photolysis—Lysates or purified proteins were incubated in a Pyrex borosilicate NMR tube ~2 cm from a 200-watt mercury vapor lamp (Hanovia) for 3 min, which provides ~4 watts of radiation from ~280–500 nm. During this time, the sample
temperature was unchanged. Immediately following this incubation, the samples were subjected to the BST as described.

RESULTS

Specificity of the Biotin Switch Technique—To evaluate the specificity of the BST, we first sought to perform the assay with an exemplary protein on which we could introduce alternative Cys modifications, including free sulfhydryl (-SH), S-glutathionyl mixed disulfide (-SSG), sulfenic/sulfenic/sulfonic acids (-SOH, -SO2H, -SO3H), and S-nitrosothiol (-SNO). Human protein tyrosine phosphatase 1B (PTP1B), with its well characterized redox-regulated active site Cys residue (19), satisfied these criteria. Treatment of reduced PTP1B with H2O2 resulted in the partially DTT-reversible inhibition of enzyme activity suggestive of a mixture of -SOH/sulfenyl amide (reversible) and -SO2H/-SO3H (irreversible) modifications (Fig. 1A). Incubation of PTP1B with oxidized glutathione (GSSG) or S-nitrosocysteine (CysNO) resulted in S-glutathionylation (Fig. 1B) or S-nitrosylation (Fig. 1C), respectively. Reduced and redox-modified PTP1Bs were then subjected to the BST with various concentrations of Asc. Under these conditions, only the S-nitrosylated PTP1B was biotinylated (Fig. 1D). It should be noted that even with 100 mM Asc, the assay specificity was not compromised. The use of 100 mM Asc did not, however, improve sensitivity (i.e. result in increased biotinylation of SNO-PTP1B) relative to 10 mM Asc, suggesting that under these conditions the reaction with Asc is not a limiting factor.

To investigate the specificity of the assay in situ, and in particular to determine whether any cellular components might limit the specificity of the assay, we subjected HEK-293 lysates to the BST and assayed for the biotinylation of endogenous PTP1B by streptavidin pull down and immunoblotting. CysNO-treated PTP1B was detected by streptavidin pulldown and anti-PTP1B immunoblotting.

FIGURE 1. Specificity of the BST in vitro and in situ: analysis of PTP1B. PTP1B (4.5 mg/ml) was treated with 0.2 mM H2O2, 0.2 mM CysNO, or 10 mM GSSG in HEPES buffer, chemically characterized, and subjected to the BST. A, H2O2-treated PTP1B was incubated in phosphatase assay buffer, and optical absorbance at 405 nm was recorded. B, GSSG-treated PTP1B was analyzed by anti-GSH immunoblot. C, CysNO-treated PTP1B was subjected to the Saville assay, and optical absorbance at 540 nm was recorded. D, each oxidatively modified or S-nitrosylated PTP1B (50 μg) was subjected to the BST with 0, 10, and 100 mM Asc. E, HEK-293 lysates (0.5 mg each) were treated with 0.5 mM H2O2, 10 mM GSSG, or 0.5 mM CysNO and subjected to the BST. Biotinylated PTP1B was detected by streptavidin pulldown and anti-PTP1B immunoblotting.

FIGURE 2. Specificity of the BST in vivo: general analysis. A, lysates from HEK-293 cells (0.5 mg each) were treated with 0.5 mM H2O2, 10 mM GSSG, or 0.5 mM CysNO, subjected to the BST, and biotinylation of samples was assessed by anti-biotin immunoblotting. B, HEK-293 cells were either untreated or incubated with 1 mM H2O2, CysNO, or diamide for 5 min, washed, and lysed in HEK-293 lysis buffer. The extracts (0.5 mg each) were subjected to the BST with or without 100 mM Asc, and biotinylation of each sample was quantified by anti-biotin immunoblotting. C, samples from panel A were probed with anti-GSH antibody.
**Specificity of the Biotin Switch Technique**

**A**

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**D**

![Graph](http://www.jbc.org/)

**Figure 3. Indirect sunlight is responsible for ascorbate-dependent artifactual biotinylation.** A, bovine serum albumin, lactoglobulin, and GAPDH (5 mg/ml each) were pretreated with 10 mM DTT for 30 min at room temperature and then desalted. The reduced samples (1 mg each) were subjected to the BST, and labeling reactions were performed in the presence or absence of 20 mM Asc and/or indirect sunlight from a nearby window for 16 h. B, GAPDH was biotinylated in the presence of Asc/sunlight and treated with 10 mM DTT prior to SDS-PAGE to identify the nature of the protein-bound biotin linkage. C, GAPDH (5 mg/ml) was left untreated or allowed to react with 0.5 mM CysNO prior to performing the BST in the presence or absence of 20 mM Asc under either fluorescent light or indirect sunlight. In each case, protein biotinylation was assessed by anti-biotin immunoblotting. D, biotin-HPDP (0.4 mM) and Asc (20 mM) were incubated in HEN buffer under conditions identical to the BST-labeling reaction. At indicated times, an aliquot was removed and assayed for thiol by 5,5’-dithiobis(2-nitrobenzoic acid) assay.

(lysates) or diamide, a thiol-oxidizing agent (intact cells), and then analyzed by the BST. Under these conditions, protein biotinylation was strictly dependent upon treatment with CysNO and the inclusion of Asc during the labeling step (Fig. 2A and B). To complement these results, we also showed that S-glutathionylated proteins were not reduced in the BST (Fig. 2C).

**Conditions That Promote S-Nitrosothiol-independent Protein Biotinylation**—Although the BST appeared to be highly specific for protein SNOs under our assay conditions, we nonetheless sought to investigate the recent claim of artifactual Asc-dependent biotinylation of reduced bovine serum albumin (12). We reasoned that this SNO-independent, Asc-dependent protein biotinylation must involve reduction of protein disulfides (including S-methylated cysteines) or biotin-HPDP. Ultraviolet radiation is known to facilitate the indirect reduction of protein disulfides (21, 22) as well as the one-e oxidation of Asc to the reactive semi-DHAsc radical, which can reduce non-biological (reactive) aryl disulfides such as 4,4’-dithiopyridine (23). We further reasoned that Asc would rapidly reduce protein thiol radicals (24) generated by UV radiation, providing thiol substrate for biotinylation. Accordingly, we sought to determine whether light could promote artifactual biotinylation during the labeling step of the BST (the assay as originally described was performed in the dark).

Interestingly, pre-reduced bovine serum albumin, lactoglobulin, and GAPDH were biotinylated when the labeling reactions were exposed to indirect sunlight, but not when kept in the dark, even after a 16-h incubation (standard incubations are 1–2 h) (Fig. 3A). Similar results were seen without DTT pre-reduction (data not shown). Further, artifactual biotinylation was fully reversed by excess DTT (Fig. 3C), confirming that the biotin was attached to the protein via a disulfide bond. In addition, the samples exposed to sunlight exhibited a marked yellowing indicative of Asc oxidation to DHAsc, whereas those kept in the dark remained essentially colorless.

To determine the effects of this artifact under typical laboratory conditions, we performed the BST on reduced and S-nitrosylated GAPDH and exposed the labeling reactions to either fluorescent lighting (in a windowless room) or indirect sunlight (a laboratory bench ~ 2 meters from a window) (Fig. 3B).

Under fluorescent lighting, only SNO-GAPDH underwent Asc-dependent biotinylation (Fig. 3B, lanes 1–4); this specific SNO signal was comparable with that obtained in the dark (not shown). In contrast, indirect sunlight led to increases in Asc-dependent biotinylation of both reduced and S-nitrosylated GAPDH (lanes 5–8). Even in the absence of Asc, indirect sunlight potentiated the biotinylation of SNO-GAPDH (lane 3 versus lane 7), consistent with known UV sensitivity of the SNO. Furthermore, the combination of indirect sunlight and Asc dramatically increased the biotinylation of SNO-GAPDH (lane 4 versus lane 8), notwithstanding loss of SNO specificity under these conditions.

To understand the mechanism of this artifact, we examined the ability of Asc to reduce biotin-HPDP to biotin-SH under the conditions of the BST. Interestingly, nearly quantitative reduction of biotin-HPDP occurred in the presence of Asc and indirect sunlight, but not Asc alone (Fig. 3D). The production of high micromolar biotin-SH during the BST would lead to arti-
would seem to be ideal for such analyses. Can differentiate SNOs from other cotemporaneous Cys-based
SNOs. Furthermore, such studies require methodologies that
about the overall role of oxidative stimuli in modulating protein
oxides (33) facilitate
spite reports that GSH depletion (30–32) and exogenous per-
were first subjected to photolysis.

After removing excess CysNO, one sample of SNO-GAPDH was incubated for
2 min near a mercury vapor lamp. The samples (1 mg each) were then sub-
tected to the BST in the presence or absence of 50 mM Asc, and biotinylation
was measured by anti-biotin immunoblotting. HEK-293 cells were either
untreated or incubated with 0.5 mM CysNO for 5 min, followed by lysis with or
without later exposure for 3 min to a UV-visible mercury vapor lamp. The BST
was performed in the presence or absence of 50 mM Asc, and biotinylation
was analyzed by anti-biotin immunoblotting.

factual protein biotinylation via thiol/disulfide exchange with blocked (i.e. S-methylthiolated) proteins.

UV Photolysis as a Control for the Detection of Protein S-Nitrosothiols—Under some experimental conditions, Asc
dependence for biotinylation may be the primary control for confirming an endogenous protein SNO by the BST. For ex-
pample, numerous experiments have demonstrated endogenous constitutively S-nitrosylated proteins that are largely insensitive
to NOS inhibitors (25–28). To complement the Asc control in instances such as these, we sought to introduce a separate
method to independently confirm the existence of an SNO moiety. Because UV light will efficiently cleave (photolyze) an
SNO (29), we reasoned that it should eliminate SNOs. We thus
exposed SNO-GAPDH to UV radiation prior to the BST. This
treatment resulted in a significant decrease in Asc-dependent
biotinylation of SNO-GAPDH (Fig. 4A). The generality and
biological utility of this approach were confirmed using CysNO-treated HEK-293 cells (Fig. 4B), which also demonstr-
ated a strong attenuation of biotinylation when the lysates
were first subjected to photolysis.

Effects of Oxidative Stresses on Protein S-Nitrosylation—De-
spite reports that GSH depletion (30–32) and exogenous per-
oxides (33) facilitate S-nitrosylation, relatively little is known about the overall role of oxidative stimuli in modulating protein
SNOs. Furthermore, such studies require methodologies that
can differentiate SNOs from other cotemporaneous Cys-based
redox modifications. The BST coupled with SNO photolysis
would seem to be ideal for such analyses.

To this end, we examined the effects of BCNU (carmustine), a clinically employed alkylating/carbamoylating agent often
used to treat astrocytomas, on general Cys redox status and
S-nitrosylation. Consistent with previous reports (34, 35),
BCNU efficiently inhibited GSSG reductase in HEK-293 cells
(Fig. 5A), which has been shown to increase the cellular GSSG/
GSH ratio and thereby induce oxidative stress (36). Furthemore,
these conditions resulted in a loss of cellular protein thi-
ols (Fig. 5B), demonstrating the effects of BCNU on Cys redox
status of both low molecular weight thiols and proteins. Next,
HEK-293 cells stably expressing nNOS were treated with
BCNU and subjected to the BST. A dose-dependent increase in
SNO-GAPDH was observed (Fig. 5C) that correlated with both
GSSG reductase inhibition (Fig. 5A) and protein thiol oxidation
(Fig. 5B). To confirm that modification of GAPDH by S-ni-
trrosylation was consequent upon BCNU-induced oxidative
stress (and hence to differentiate SNO from other Cys redox
modifications), we employed SNO photolysis prior to the BST.
As expected, UV pretreatment of samples resulted in a marked
decrease in biotinylated GAPDH (Fig. 5D).

DISCUSSION

Jaffrey and Snyder (2, 3, 37–39) have provided unequivocal
demonstrations of the utility of the BST for identifying SNOs.
Given the widespread use of the assay, it remains important to
show that assay specificity, which has to date been examined for
a limited number of proteins (2, 39), in fact captures the general
behavior of SNO proteins in complex biological systems. Here,
we confirm that the BST is in fact highly selective for S-nitroso-
lated versus S-oxidized (sulfenic acid and disulfide) groups in
proteins and that assay specificity, which is a unique feature of
the chemistry between Asc and SNO, can be generalized to
many classes of proteins.

Interestingly, the one e− reduction potential of GSH/GSNO
is −180 mV (40), indicating that Asc cannot directly reduce
the S-NO bond (the Asc/semi-DHAsc radical $E_{\text{red}}$ is +280 mV)
The oxidation of Asc to DHAsc by sunlight, probably plays a critical role in the process. The semi-DHAsc radical, an intermediate in the thiolysis (9–12), which we had not previously observed even at high Asc concentrations (8, 17), revealed that indirect sunlight is the likely cause. The semi-DHAsc radical, an intermediate in the oxidation of Asc to DHAsc by sunlight, probably plays a role. Though semi-DHAsc cannot drive the single e\(^{-}\) reduction of biological Cys disulfides (Cys disulfide radical/disulfide \(E_{\text{red}}\) is \(-1500\) mV) (42), it has been shown to reduce reactive synthetic disulfides such as 4,4\(^{`}\)-dithiopyrindine (23), that are similar to biotin-HPDP. Indeed, we show that Asc in the presence of indirect sunlight reduces biotin-HDPDP to biotin-SH, which would inevitably incorporate into protein disulfides to generate artifactual (i.e. SNO-independent) S-biotinylation, consistent with our findings and those of Huang et al. (12).

Alternatively, the data might be explained by UV light-induced tryptophan photoexcitation, enabling the transfer of one e\(^{-}\) to an adjacent disulfide to generate a disulfide radical (21, 22), which transiently dissoclates into free thiol plus thyl radical. Thermodynamically, Asc should drive this reaction by further reducing Cys thyl radical to thiol (Cys thiol/thyl radical \(E_{\text{red}}\) +920 mV) (43), which would then undergo biotinylation. Of relevance, Walmsley et al. (44) applied this rationale to explain their discovery that ambient daylight reduced 5,5\(^{`}\)-dithiobis(2-nitrobenzoic acid) in the presence of Asc. Whatever the exact sequence of electron transfer that creates the artifact, we can recapitulate the result with indirect sunlight and Asc.

Interestingly, our experiments demonstrate that, even in the absence of Asc, indirect sunlight promotes the biotinylation of an SNO during the labeling step of the BST. Indirect sunlight likely contains sufficient UV light for SNO homolysis (\(\lambda_{\text{max}}\) 334 nm). Although it is generally thought that thyl radicals proportionate to disulfides or are further oxidized, our experiments and those of others (21, 22) suggest that protein Cys thyl radicals are, at least in small part, reduced to thiols that can subsequently react with biotin-HDPDP. That Asc potentiates the effects of indirect sunlight on SNO biotinylation would be consistent with Asc-mediated reduction of thyl radical (as described previously). Alternatively, the semi-DHAsc radical (semi-DHAsc/DHAsc \(E_{\text{red}}\) -174 mV) (41) may directly reduce an SNO to thiol (they have similar reduction potentials; see above). Increased sensitivity of the BST as a result of sunlight, however, comes at the expense of specificity; the labeling step should therefore be performed under fluorescent light or in the dark.

A number of anti-cancer agents employed in clinical practice create an oxidative stress. Assessment of protein oxidation versus S-nitrosylation under these conditions has been a challenge. The introduction of the BST coupled to SNO photolysis has allowed us to rigorously demonstrate that pharmacologically induced oxidative stress in fact promotes GAPDH S-nitrosylation. Mechanisms for this increase in SNO-GAPDH may include a loss of reduced cellular GSH or oxidation of proteins necessary for the break down of S-nitrosothiols. Given the well established role of SNO-GAPDH in mediating cellular death (3, 37, 45), our data raise the idea that clinically employed cancer chemotherapies (e.g. BCNU) may execute their effects through S-nitrosylation rather than exclusively by Cys oxidation.

Notwithstanding the reproducibility and specificity of the BST for the detection of SNO proteins, we show here that SNO photolysis prior to the assay may be useful as a separate control. Whereas Asc generates an SNO-dependent signal, photolysis eliminates it. Photolysis may thus find wide application in instances where the BST does not respond to NOS agonists or inhibitors (which increase or eliminate endogenous SNO signals) or under conditions of diverse redox stresses (e.g. BCNU). The BST (when performed protected from sunlight) combined with SNO photolysis should provide a highly specific method for future investigations of S-nitrosylation, particularly under conditions that require differentiation of SNOs from other Cys-based modifications.

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REFERENCES

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