Assays for S-Nitrosothiols and S-Nitrosylated Proteins and Mechanistic Insights Into Cardioprotection

Douglas T. Hess, PhD; Matthew W. Foster, PhD; Jonathan S. Stamler, MD

Gender differences in the incidence of cardiovascular disease may be ascribed at least in part to the protective effects of estrogen through both long-term and rapid (“nongenomic”) actions. Nitric oxide (NO), generated by endogenous cardiac NO synthases (NOS; NOS1 or neuronal NOS [nNOS], NOS2 or inducible NOS, NOS3 or endothelial NOS), plays a major role in both normal cardiac physiology and cardioprotection (particularly myocardial ischemia/reperfusion injury; see the Figure), and the article by Lin et al in this issue of Circulation contributes to growing evidence that the cardioprotective functions of estrogen are conveyed in significant part by NO. A rapidly expanding body of studies indicates that NO acts in most cellular contexts largely through the covalent modification of protein Cys thiols (to generate an S-nitroso [SNO]-protein, designated S-nitrosylation), and recent analyses using a new generation of analytical approaches (see the Article) both confirm original measurements of SNO-proteins that have been long-standing sources of controversy in the field and point to important roles for S-nitrosylation in NO-derived cardioprotection (see the Figure).

In the myocardium, endothelial NOS is associated primarily with sarcolemmal caveolae and perhaps β-arrestin, and endothelial NOS–derived NO influences β-adrenergic receptor stimulation of myocardial contractility, at least in part through S-nitrosylation and inhibition of the L-type Ca2+ channel and G-protein receptor kinase 2. In contrast, nNOS is localized primarily to the sarcoplasmic reticulum, and nNOS-derived NO S-nitrosylates and activates the sarcoplasmic reticulum–resident ryanodine receptor/Ca2+-channel (RyR2), resulting in cytosolic Ca2+ release and enhanced catecholamine-stimulated contractility. Mice lacking nNOS, but not those lacking endothelial NOS, exhibit hypo-S-nitrosylation of RyR2 and diastolic Ca2+ leakage with arrhythmia characteristic of sudden cardiac death syndrome. Hearts and myocytes from female versus male mice show less isoproterenol-induced sarcoplasmic reticulum Ca2+ loading, which is associated with translocation of nNOS to the sarcolemma and S-nitrosylation of the L-type Ca2+ channel in ischemia. The redistribution of nNOS and upregulation of NOS isoforms appear to contribute significantly to the protection of female hearts from ischemia/reperfusion injury, and upregulation of NOS can be ascribed to 17β-estradiol–dependent gene expression. However, sarcolemmal redistribution of nNOS also is observed in the hearts of humans with idiopathic dilated cardiomyopathy and of rodents with experimental myocardial infarction. Thus, S-nitrosylation of myocardial proteins may exert cardioprotective effects that are coupled to estrogen receptors and, when aberrant, may contribute to the characteristic dysfunction of the failing heart.

This scenario is reminiscent of the well-established cardioprotection conferred by circulating SNO-proteins, particularly S-nitrosoalbumin. However, these early studies were encumbered by controversy over methods of SNO-protein analysis, in which the importance of S-nitrosylation reactions in NO biology was challenged, but that controversy is now coming to resolution. Results obtained with a number of new methodologies to measure SNO-proteins in blood, plasma, and tissues (see the Table), including the approach taken by Lin et al, are providing support for the principal results obtained by a methodology known as Hg-coupled photolysis-chemiluminescence, which was used to detect the first endogenous SNO-proteins and SNO-peptides in extracellular fluids, cells, and cardiac tissues, including SNO-albumin, SNO-glutathione, SNO-hemoglobin, and SNO-RyR2, and which remains a gold standard. In addition, the recent appreciation of the differential reactivity of various SNOs and of the importance of preparative steps that stabilize rapidly degrading SNO-protein pools has revealed the basis of methodological flaws in some widely used assays (particularly triiodide chemiluminescence, which cannot be advocated for measurements of NO-derived species in any complex biological system). Limitations intrinsic to methods of SNO assay suggest that they are often better suited for either blood, plasma, or tissues, except photolysis-chemiluminescence, which has been used in all settings (see the Table).

These new methods also have provided novel insights into the basis of gender differences in long-QT syndrome, an inherited disease characterized by a prolonged QT interval that can result in fatal arrhythmia. S-nitrosylation can influence QT interval by altering the function of sodium channels that mediate late sodium currents (INa) and of the slowly activating delayed-rectifier K+ channel (IKs). In addition, gender differences in QT duration and in susceptibility to ventricular arrhythmia have been linked to differences in IKs currents. Taken together, these findings suggest that gender differences in cardiac function and pathophysiology can
reflect differences in the localization and activity of NOS, which result in altered S-nitrosylation of critical cardiac proteins, and that these differences are likely to reflect in significant part the effects of estrogen receptor stimulation.

In the study by Lin et al., ovarioctomized mice were infused for 2 weeks with vehicle, 17β-estradiol, or a selective agonist of the β subtype of estrogen receptor, 2,2',bis(4-hydroxphenyl)-proprionitrile (DPN). Isolated hearts were then subjected to ischemia and reperfusion, and cardiac function and infarct severity were assessed. Treatment with either 17β-estradiol or DPN resulted in substantial improvements in functional recovery and decreased infarction. DPN had no cardioprotective effects in knockout mice lacking the estrogen receptor stimulation, Lin et al. surveyed total heart homogenates for S-nitrosylated proteins using a modification of the biotin-switch technique (BST), which is discussed further below. Mass spectrometric analysis identified 11 proteins for which S-nitrosylation was enhanced by treatment with 17β-estradiol or DPN and 3 proteins for which S-nitrosylation was suppressed. All identified proteins were affected by both 17β-estradiol and DPN, although the DPN effects were consistently greater. The enhancement of S-nitrosylation by DPN was eliminated in mice lacking ER-β and by pharmacological NOS inhibition.

The demonstration by Lin et al. that stimulation of estrogen receptors conferred cardioprotection that was abrogated by NOS inhibition indicates a critical role for NO, at least in the case of the ER-β. Although the mechanism of enhanced protein S-nitrosylation was not determined (enhanced NOS expression and/or activation) and the relationship between NOS-dependent cardioprotection and S-nitrosylation is correlative in their study, the proteomic approach represented in this work presages a new era in the study of NO-based cellular mechanisms, which until now has been essentially phenomenological in the cardiovascular system. Indeed, the potentiated S-nitrosylation reported by Lin et al would be at least consistent with a causal role, inasmuch as S-nitrosylation of

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**Table. Methodologies for the Detection and Absolute or Relative Quantification of SNO-Proteins and Other S-Nitrosothiols**

<table>
<thead>
<tr>
<th>SNO Manipulation</th>
<th>Detection Method</th>
<th>Applicability</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV photolysis (with and without Hg&lt;sup&gt;2+&lt;/sup&gt;)&lt;sup&gt;14&lt;/sup&gt;</td>
<td>Ozone-based chemiluminescence</td>
<td>Plasma, blood, cells, tissue homogenates</td>
</tr>
<tr>
<td>UV photolysis&lt;sup&gt;32&lt;/sup&gt;</td>
<td>Radical trapping by nitrene (method in development)</td>
<td>Plasma, blood, cells, tissue homogenates?</td>
</tr>
<tr>
<td>Cu/Cys (2C, 3C) or Cu/ascorbate reduction&lt;sup&gt;24,25&lt;/sup&gt;</td>
<td>Ozone-based chemiluminescence; NO electrode</td>
<td>Plasma (online)</td>
</tr>
<tr>
<td>Decomposition by organoselenium&lt;sup&gt;26&lt;/sup&gt;</td>
<td>SNO sensor</td>
<td>Plasma</td>
</tr>
<tr>
<td>Decomposition by gold nanoparticle&lt;sup&gt;27&lt;/sup&gt;</td>
<td>HPLC/MS</td>
<td>Plasma</td>
</tr>
<tr>
<td>Decomposition by organomercury&lt;sup&gt;16&lt;/sup&gt;</td>
<td>Colorimetric (Saville); fluorescent (DAN/DAF-2)</td>
<td>Cells</td>
</tr>
<tr>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt; displacement&lt;sup&gt;28&lt;/sup&gt;</td>
<td>SNO-specific antibody</td>
<td>Cells, tissue homogenates</td>
</tr>
<tr>
<td>None&lt;sup&gt;30&lt;/sup&gt;</td>
<td>Affinity tagging or fluorescent labeling of nascent thiol&lt;sup&gt;21&lt;/sup&gt; (eg, BST); differential gel electrophoresis with multiple fluorescent tags&lt;sup&gt;2&lt;/sup&gt;; labeling with isotope-coded affinity tags&lt;sup&gt;35&lt;/sup&gt; (eg, SNO-CAP); isotopic labeling of SNO-peptides after resin-assisted capture (SNO-RAC)&lt;sup&gt;31&lt;/sup&gt;</td>
<td>Cells, tissue homogenates</td>
</tr>
</tbody>
</table>

UV indicates ultraviolet; HPLC, high-performance liquid chromatography; and MS, mass spectrometry.
multiple proteins, including cyclooxygenase, hypoxia-inducible factor α, complex I, and caspase, has been shown to be cardioprotective. It is of note that the majority of substrates for which enhanced S-nitrosylation was reported by Lin et al consist of metabolic or mitochondrial enzymes that are present at high cellular abundance, which may suggest that energy conservation contributes in some way to cardioprotection.

Notably, the accumulation of evidence demonstrating the ubiquitous action of S-nitrosylation has been exponential because, although absolute quantification of SNO levels in cells, tissues, and purified proteins has long been possible (the Table), methodology only recently has emerged that may be applied facilely to identify individual S-nitrosylated substrates and the sites and degree of S-nitrosylation within those proteins. This history is hardly unique in form, given the importance of isotopic labeling for the analysis of post-translational protein modification by phosphorylation. The principal advance in methodology was provided by the introduction of the BST. In this approach, free Cys thiols were used to chemically code biotin tags or, alternatively, isobaric labeling methods that allow quantification of S-nitrosylation, as Lin et al encountered. For all current BST-based methods, sensitivity of SNO-protein detection remains an issue, although improvements have recently been made (see SNO-RAC). Photolysis-chemiluminescence is not limited by sensitivity and can readily provide SNO stoichiometry, but it is not adaptable to proteomic analysis. Several new techniques, including amperometric SNO sensors based on organoselenium and nanogold technology, have been adapted for online measurements in plasma (see the Table). These techniques have the added advantage of assaying in real time and indicate that SNO-proteins are highly abundant in the bloodstream (micromolar). It seems likely that in the near future additional methodological advances, conceivably involving the direct labeling of S-nitrosothiols in situ, will again provide a quantum improvement in our ability to characterize dynamic protein S-nitrosylation in the context of cellular signal transduction and disease.

**Source of Funding**

Dr Stamlers work is supported by National Institutes of Health grant 5P01-HL075443.

**Disclosures**

Dr Stamlers owns equity in LifeHealth, a company developing assays for the detection of NO-based molecules. The remaining authors report no conflicts.

**References**


**Key Words:** Editorials / cardiomyopathy / endothelium-derived factors / hypoxia / myocardial infarction / nitric oxide
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Circulation. 2009;120:190-193; originally published online July 6, 2009;
doi: 10.1161/CIRCULATIONAHA.109.876607

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/120/3/190

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