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Minireview

The determination of *S*-nitrosothiols in biological samples—Procedures, problems and precautions

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ABSTRACT

Despite the considerable number of published studies in the field of *S*-nitrosothiols (RSNO), the determination of these compounds in biological samples still represents an analytical challenge, due to several technical obstacles and often long sample preparation procedures. Other problems derive from the intrinsic lability of RSNO and the absence of certified reference material, analytically validated methods or suitable internal standards. Also, thiols and nitrites are usually present at high concentrations in biological matrices, and all precautions must be adopted in order to prevent artifactual formation of RSNO. Preanalytical steps (sampling, preservation and pre-treatment of samples) are particularly critical for the obtainment of reliable measurements. Three main mechanisms have been identified capable of compromising the assays: metal-catalyzed RSNO decomposition, reduction of the S-NO bond by thiols (transnitrosylation reactions) and enzymatic degradation of *S*-nitroso-glutathione (GSNO) by endogenous γ-glutamyltransferase (GGT) activity possibly present in the sample. If not adequately controlled, these factors likely contribute to the wide dispersion of values reported in the literature for RSNO and GSNO concentration in biological fluids, blood in the first place. The use of metal chelators, thiol reagents and GGT inhibitors appears therefore mandatory.

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Introduction

Modulation of cell signalling and function by nitric oxide is involved in a number of pathophysiologic processes and conditions, of which inflammatory processes represent an important portion. The formation of *S*-nitrosothiols (RSNO) on specific cysteine residues in peptides and proteins represents a major mechanism of post-translational modification, with a significance comparable to protein phosphorylation (Hess et al., 2005). Recent literature has thus known a remarkable increase in the number of studies exploring the possible implications of RSNO in clinics and therapy of cardiovascular, respiratory and neurodegenerative diseases (Foster et al., 2009).

Regardless of the model under investigation (cell cultures, subcellular fractions, biological material of clinical origin) adequate analytical approaches is mandatory, in order to warrant the obtainment of unequivocal information. However, the determination and quantification of RSNO in biological samples constitute a complex

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and somewhat confuse matter. Despite the abundance and variety of published methods, the field still represents a challenge for analytical biochemistry and has often been the subject of discussion and controversy (Gow et al., 2007; Giustarini et al., 2007; MacArthur et al., 2007). A major obstacle to the reliable measurement and speciation of RSNO is represented by the lack of validated sample collection/preservation procedures capable of assuring RSNO stability during the pre-analytic steps, as well as by the lack of validated methods and certified reference material. As a result of this situation, the definition of reference RSNO concentration values in complex biological matrices remains elusive, and the range of basal RSNO levels reported in the literature still extends over 3 orders of magnitude, from low-nanomolar up to micromolar values (MacArthur et al., 2007). Several factors can affect the accuracy of determinations, and while strategies to cope with some of these interferences are widely established, others have only recently emerged.

Critical factors in preanalytical steps

As in other determinations, sample obtainment and preparation represent a delicate issue, due to possible artifactual generation of RSNO or degradation of specific metabolites. Samples should be collected in the presence of 10–12 mM *N*-ethylmaleimide (NEM), in order to alkylate free –SH and make them unavailable for possible transnitrosylation reactions, such as e.g. between albumin and glutathione. Moreover, free thiols are capable of reducing cupric, Cu(II), to cuprous ions, Cu(I), which can trigger RSNO decomposition (Dicks and Williams, 1996). The employment of NEM is therefore mandatory, in order to avoid artifactual loss of the analyte. Metal chelators ethylen-diamino-tetraacetic (EDTA) or diethylen-triamino-pentaacetic acid (DTPA) should also be added (0.5–2.5 mM), to chelate adventitious metals which may catalyse RSNO decomposition. Recent observations have documented the light sensitivity of RSNO, suggesting that all preparation procedures should be carried out avoiding exposure of samples to light (Wu et al., 2008). In summary, treatment of samples with alkylating agents (NEM), metal chelators (EDTA or DTPA) and avoiding direct light exposure, all represent important practical measures in order to avoid artifactual formation/loss of RSNO, whatever the method employed for RSNO determination is. The use of inhibitors of gamma-glutamyltransferase, as will be detailed in the following sections, has comparable importance (see below).

Critical factors in analytical procedures

Difficulties and artifacts associated with the various techniques employed to measure RSNO have been extensively discussed (Giustarini et al., 2007; Gow et al., 2007). At a general level, it is known e.g. that the agent selected for cleavage of the S-NO bond (photolysis, $HgCl_2$, $HgCl_2$/V (III), KI/I_2, Cys/KI/Cu(I), Cu(I)/Cys, Cu(I)/KI/I_2, CO/Cu(I)/Cys, DTT), the specific analyte being detected (NO, nitrite or thiol) and the revelation system employed (chromophores, fluorophores, ozone) represent as many critical steps for an accurate and reliable measurement (MacArthur et al., 2007). Since analytes are mostly investigated in complex biological matrices (e.g. plasma), then the possible interactions of the reagents employed with matrix constituents should always be carefully considered (Rogers et al., 2005). Several direct and indirect detection methods have been implemented.

Direct methods

The UV-spectrophotometric revelation at 334 nm, often coupled to separation procedures (HPLC or capillary zone electrophoresis), is a frequently used direct technique. Direct methods have the advantage of avoiding derivatization procedure, but – due to the rather low molar absorption coefficient of the *S*-nitroso grouping – UV-spectrophotometry however is restricted to detection of micromolar levels (Stamler and Loscalzo, 1992; Matthews and Kerr, 1993).

Mass spectrometry (MS) can be utilized both for determination of RSNO concentration and for the identification of individual molecular species, provided that sample preparation procedures can safeguard the integrity of the S-NO bond. Identification is made by the selective analysis of an appropriate mass/charge ratio (m/z) of the molecular species. When MS analysis is coupled to liquid chromatography (LC-MS) and the eluting phase is acid, it is important to exclude artifactual formation of RSNO from injected thiols and nitrite possibly present in the column. The analysis of standard ^{15}N-labeled RSNO, as well as of samples previously subjected to photolysis or treated with $HgCl_2$ (negative controls) is also necessary (Palmer et al., 2007). LC-MS systems have been used for determination of *S*-nitroso-glutathione (GSNO) in plasma (Tsikas et al., 2002), but the method has not yet been validated. MS can also be coupled to gas chromatography (GC-MS) for the analysis of low molecular weight RSNO, but the procedures require transformation of RSNO in nitrite followed by its derivatization with pentafluorbenzyle (Tsikas et al., 2002).

Direct detection of RSNO has been also achieved by means of planar amperometric sensors modified for NO (Cha et al., 2005). As far as immuno-based techniques, poly- and monoclonal antibodies recognizing the SNO grouping have been employed for the *in situ* revelation of RSNO in immunohistochemistry studies (Gow et al., 2004). The preparation of adequate positive controls is recommended, by treating tissue sections with nitrite and HCl. Negative controls can be prepared by treating tissue sections with $HgCl_2$, though this reagent may not efficiently decompose the S-NO bond *in situ*; more hydrophobic *p*-hydroxy-mercurybenzoate can be then used instead. The use of antibodies for immunoblot applications is anyway unfeasible, for stability of the S-NO bond during protein electrophoresis cannot be assured.

Indirect methods

Determination and quantification of RSNO can be indirectly obtained after cleavage of the S-NO bond and analysis of released metabolites. Decomposition of RSNO is obtained by chemical reduction or photolysis, and products of decomposition (•NO or nitrite) can be determined by various techniques (spectrophotometry, chemiluminescence, fluorometry, electrochemical revelation, electron spin resonance, mass spectrometry); details of the individual procedures can be found elsewhere (Gow et al., 2007; Giustarini et al., 2007). In general, the advantage of indirect methods lies in their good sensitivity, though several drawbacks have to be taken into account. These are usually related to length and complexity of procedures increasing the likelihood of artifacts, to interferences by contaminating nitrites and metals, to low sensitivity in some instances. A specific weak point of many of these assays lies in the employment of $HgCl_2$ for cleavage of the S–NO bond. $HgCl_2$ is to be used at mM concentrations, since part of it will be sequestered by (protein) thiols present in the sample, and the recognized toxicity of this compound poses safety issues both for operators and for the environment.

Complementary to detection of metabolites originating from the NO moiety of RSNO, other methods are aimed at determination of the thiol moieties after convenient derivatization. In this way e.g. glutathione obtained from reduction of GSNO by β-mercaptoethanol can be quantified by a complex procedure of derivatization with fluorescent ortho-phthalaldehyde (Tsikas et al., 1999). For the identification of individual *S*-nitrosylated proteins, the so-called 'biotin-switch' assay has been fruitfully applied (Forrester et al., 2009). Several variants of this procedure have been proposed. The assay generally includes (i) chemical blockade of free thiols, (ii) reduction of RSNO by ascorbate, (iii) labelling of the released thiols with a biotinylated reagent and (iv) revelation by immunoblotting, or alternatively, purification by streptavidin affinity chromatography.