Detection of specific nitrotyrosine-modified proteins as a marker of oxidative stress in cardiovascular disease

Serena Viappiani and Richard Schulz
Cardiovascular Research Group, Departments of Pediatrics and Pharmacology, University of Alberta, Edmonton, Alberta, Canada

THE OXIDATIVE MODIFICATION of proteins is a natural consequence of aerobic life and is also recognized to play a crucial role in the pathological response of cells to increased oxidative stress. Several oxidative modifications of a protein can occur as a result of oxidative stress. These can range from the facile oxidation of cysteine residues to changes caused by higher levels of oxidative stress, resulting in covalent crosslinking with other proteins (whether by S-S linkage or by 2-2'-biphenyl crosslink of 2 tyrosyl radicals), or the formation of noncovalent aggregates or even formation of adducts of proteins with other lipid, carbohydrate, or nucleic acid radicals.

Peroxy nitrite (ONOO⁻), the reaction product of nitric oxide and superoxide, is implicated as a key oxidant species in several pathologies and is well known to oxidize proteins. The range of possible modifications begins from the mild oxidation of susceptible cysteine sulphydryls (resulting in their S-nitrosylation and S-glutathiolation) to what is likely the result of a higher concentration and/or longer duration of exposure to ONOO⁻ to cause direct nitration of tyrosine residues. The nitration of proteins by oxidant species other than ONOO⁻ is also recognized under certain conditions (6). These modifications often result in the alteration of protein function or structure and, usually, inhibition of enzyme function. Proteins containing nitrotyrosine residues have been detected in different pathologies, including diabetes, hypertension, and atherosclerosis, all associated with enhanced oxidative stress, including that caused by increased production of ONOO⁻.

The quest to identify nitrated proteins as a reliable in vivo marker of oxidative stress, particularly to that caused by ONOO⁻, has proceeded with gusto. Early attempts utilized a polyclonal anti-nitrotyrosine antibody raised originally against nitration of keyhole limpet hemocyanin. This antibody detects several proteins containing tyrosine residues modified in position 3 of the phenyl ring by a nitro group (3). This antibody is most sensitive in its ability to detect nitrated proteins in situ by immunohistochemistry but has also been used to detect specific nitrated proteins isolated from cells by immunoblotting. Recently, a proteomic approach using this pan-nitrotyrosine antibody showed that a total of 48 putative proteins containing nitrotyrosine were identified in whole heart homogenates of aged rats (8). Two such proteins, a key mitochondrial antioxidant enzyme, Mn³⁺ superoxide dismutase (MnSOD), or the sarcoplasmic reticulum calcium ATPase type 2 (SERCA2), are nitrated at one or more tyrosine residues in disease states, resulting in a loss of activity. MnSOD is nitrated by ONOO⁻ in its active catalytic site and inactivated by nitration of a single tyrosine, Y-34 (10).

SERCA2 has been shown to be nitrated at two adjacent tyrosine residues (Y-294, Y-295) in skeletal and cardiac muscle from aged animals (9, 15), and tyrosine nitration of this enzyme results in decreased activity (1). Because these enzymes regulate critical components of the cell’s ability to deal with oxidative stress and calcium handling, it is likely that an inactivation of these enzymes will contribute to the pathogenesis of disease and aging. Indeed, a study in this issue of the American Journal of Physiology-Heart and Circulatory Physiology by Xu et al. (18) shows that both MnSOD and SERCA2 containing nitrotyrosine were found in different pathological conditions, including atherosclerosis, diabetes, and ANG II-induced hypertension, as well as aging.

The novelty of the study of Xu et al. (18) relies on the development of antibodies against site-specific nitrotyrosine-modified SERCA2 and MnSOD. Polyclonal and affinity-purified antibodies were raised against two specific tyrosine-nitrated peptides, a 16-amino acid sequence containing nitrated Y-34 of MnSOD and a 20-amino acid peptide containing nitrated Y-294 and Y-295 of SERCA2. Their ability to detect these modified proteins was evaluated by immunohistochemistry. First of all, this study shows that specific nitrotyrosine modifications of these proteins that were only predicted by in vitro studies are confirmed to take place in pathologies involving oxidative stress, not only in animal models but also in human disease. From a cellular point of view, the availability of these antibodies should now be exploited in confocal or immunogold electron microscopy analyses to elucidate the subcellular distribution of such modified proteins. Because posttranslationally modified proteins are characterized not only by different activity but often also by different distribution and rate of degradation, it should now be possible to add new knowledge about the compartmentalization and fate of these oxidized proteins. Indeed, the subtle posttranslational modification of a single amino acid residue of a protein causes a significant increase in its susceptibility to proteolytic degradation (4). For example, when the related cytoplasmic Cu,ZnSOD is exposed to nitrating agents, it becomes nitrated on tyrosine 108 and is consequently degraded by the proteasomal pathway at twice the rate (14). On the other hand, an enzymatic repair mechanism to specifically repair nitrated proteins has also been described (7), suggesting that protein nitration may also be part of a signaling pathway in some cases.

The approach taken by Xu et al. (18) showed that nitrated forms of MnSOD and SERCA2 can be readily and specifically identified by immunohistochemical analysis in kidneys of ANG II-infused rats, atherosclerotic rabbit aorta, cardiac sarcoplasmic reticulum and skeletal muscle from aged rats, and blood vessels and atrium obtained from a patient with atherosclerosis and diabetes. Staining was reduced in appropriate

Address for reprint requests and other correspondence: R. Schulz, Cardiovascular Research Group, Unv. of Alberta, 4–62 HMRC, T6G 2S2 Edmonton, Alberta, Canada (e-mail: richard.schulz@ualberta.ca).
controls, including preabsorption with the specific nitrotyrosine-containing peptides or reduction of nitrotyrosine by pretreatment of sections with sodium dithionite to reduce nitrotyrosine to aminotyrosine. These nitrotyrosine-specific peptide-based antibodies will be useful additions to our toolbox for the detection of oxidatively modified proteins, which are supplemented by biochemical and mass spectrometric techniques. A particular advantage is their sensitivity in immunohistochemistry, allowing the detection of their nitrated proteins in small biopsy samples. However, several issues remain, including their lack of sensitivity for use in Western blotting in bulk tissue homogenate (begging the question as to what is the proportion of these nitrated proteins in disease processes), the need for comparison to matched healthy controls in each of the disease models, the subcellular localization of the affected proteins, and whether this has an impact on their distribution compared with the native proteins.

Although it is difficult to generalize about posttranslational modifications affecting protein function, reversible reactions (i.e., S-nitrosylation and S-glutathiolation) have more frequently been associated with an increase of enzymatic activity (2). In a very elegant study, Cohen’s group (1) previously reported that the activation of SERCA2 is mediated by low micromolar concentrations of ONOO\(^{-}\) and glutathione found at a normal concentration within cells. The reversible modification that takes place leads to S-glutathiolation of SERCA2 and results in the increase in \(\text{Ca}^{2+}\)-ATPase and pump activity, essential for the physiological regulation of vascular tone. However, in pathological conditions such as in the atherosclerotic blood vessel, SERCA2 shows a more severe state of oxidation, mainly due to the irreversible and higher oxidation states of critical cysteine sulfhydryl residues that causes an uncontrolled loss of enzyme activity. Another important class of enzymes activated by ONOO\(^{-}\) during mild states of oxidative stress is represented by the family of matrix metalloproteinases (MMPs), whose activity has been shown to be increased by S-nitrosylation (for MMP-1, -8 and -9) (11) or S-nitrosylation (for MMP-9) (5) of a critical cysteine in the regulatory prodomain of the protein. It appears that an important target of the early phase of ONOO\(^{-}\)-induced oxidative stress injury in the heart is activation of MMP-2 and its resultant proteolysis of novel intracellular targets, including troponin I (17) and myosin light chain 1 (13).

The findings by Xu et al. (18) support the concept that oxidative stress is an important component in the development of several chronic cardiovascular diseases. To what extent this is a marker or a mediator and how early these alterations occur in the progress of the disease are questions that remain to be answered. If we agree that changes in protein function due to oxidative stress are not merely an index of a disease but a cause of further tissue damage and loss of function, then it is appropriate to plan simple and effective strategies to increase the antioxidant capacity of the human body. This could be achieved simply through regular physical activity and by means of a healthier balanced diet that provides appropriate levels of dietary antioxidants and reduces pathways generating enhanced oxidative stress, such as with the Mediterranean diet and/or supplementation with antioxidants (12, 16).

REFERENCES