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Review for Themed Issue on Oxidative Toxicology: Role of ROS in Oxidative Stress

Biomarkers of nitro-oxidation and oxidative stress

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Abbreviations:

ACR: acrolein

ALE: aldehydic end-products

Cu,Zn-SOD: copper,zinc-superoxide dismutase

DNP: 2,4-dinitrophenylhydrazine DNP

FRR: free radical reaction

4-HNE: trans-4-hydroxy-2-nonenal

HRV: heart rate variability

$LO^{\bullet}$:lipid alkoxyl radical

$LOO^{\bullet}$: lipid peroxyl radical

LOOH: lipid hydroperoxide

LPO: lipid peroxidation

MDA: malondialdehyde

($^{\bullet}NO$): nitric oxide

N_2O_3: dinitrogen trioxide

nitro-OS: nitro-oxidative stress

NO^{+}: nitrosonium ion

Nrf2: Nuclear factor (erythroid-derived 2)-like 2

$^{\bullet}O_2^{-}$: superoxide ion

OMP: oxidatively modified protein

4-ONE: trans-4-oxo-2-nonenal

$ONOO^-$: peroxynitrite

OS: oxidative stress

PERK: Protein kinase RNA-like endoplasmic reticulum kinase

PUFA: polyunsaturated fatty acids

RNS: reactive nitrogen species

ROS: reactive oxygen species

TBARS: thiobarbituric acid reactive substances

UPR: unfolded protein response

Abstract

Macromolecule oxidation in response to reactive oxygen species (ROS) is associated with a variety of diseases. The recognition of NO as a key regulator of redox signalling has more recently led to the discovery that reactive nitrogen species (RNS) also elicit modifications of macromolecules which are also involved in pathophysiological processes.

This article provides an overview of key biomarkers used to assess oxidation, peroxidation and nitration/nitrosation signaling, stressing the necessity to analyse multiple biomarkers to understand the redox mechanisms involved in biological responses.

Introduction

Thirty years ago, Sies and Cadenas [1,2] introduced oxidative stress (OS) as a concept which associates oxidative chemistry with biological stress responses in redox biology and medicine.

This complex field of biochemistry which plays a key role in homeostasis through the regulation of a variety of enzymes involved in essential signalling pathways, is now often referred to as "redox signalling"[3] (Fig. 1) and has become a major area of investigation in chemistry, life sciences and medicine.

An aspect which is often neglected when addressing redox signalling or OS, is the role of nitric oxide ($^{\bullet}NO$) which is the primary substrate of the original reactive oxygen species (ROS), superoxide ($^{\bullet}O_2^-$). Indeed, the reaction rate of $^{\bullet}NO$ with $^{\bullet}O_2^-$, estimated at 9×10^9 $mol^{-1}.s^{-1}$, is 3 to 4 times faster than its catalysis by Cu,Zn-SOD (2.4×10^9 $mol^{-1}.s^{-1}$) and 2 to 4 orders of magnitude faster than its reaction with macromolecules such as aminoacids ($\approx 10^6 - 10^7$ $mol^{-1}.s^{-1}$), proteins ($\approx 5 \times 10^6$ $mol^{-1}.s^{-1}$ for albumin), lipids ($\approx 10^6$ $mol^{-1}.s^{-1}$ for palmitate) and DNA ($\approx 5 \times 10^5$ $mol^{-1}.s^{-1}$)[4–6]. This reaction generates peroxynitrite ($ONOO^-$), a highly reactive nitrogen species (RNS) responsible among others for nitration and nitrosation (figure 1) which play important pathophysiological roles [3,7,8]. It should be noted that *in vivo*, protein nitrosation essentially affects thiol groups of cysteine residues and is referred to as S-nitrosation. *In vivo* N-nitrosation of proteins has not been conclusively demonstrated except in the gastrointestinal tract by the bacterial flora. S-, N-, C- and O-

nitrosation can also affect small molecules such as ethanol or compounds containing thiol, phenol, indole, amino and amido groups, *in* vivo and mainly in the gastrointestinal tract. Among these, N-nitroso compounds (NOC), especially nitrosamines and nitrosamides have been extensively investigated because of their carcinogenic potential (Kobayashi, 2017). Besides triggering nitration and nitrosation, $ONOO^-$ which is also a strong oxidant, can also induce DNA deamination and oxidation [9], methionine sulfoxidation [10], zinc finger oxidation [11] and lipid oxidation and peroxidation [12]. Peroxynitrite has also been reported to oxidize glutathione thereby leading to glutathiolation of selected cysteine residues [13]. There is thus a $^{\bullet}NO/^{\bullet}O_2^-$ system [3] which plays an important role in cell homeostasis through redox signalling mechanisms (figure 1).

The $^{\bullet}NO$ concentrations being three orders of magnitude higher under physiological conditions, it serves as a "sink" for $^{\bullet}O_2^-$, thereby preventing oxidative mechanisms. When under pathophysiological conditions, $^{\bullet}O_2^-$ and $^{\bullet}NO$ concentrations rise, nitrosation, nitration and potentially nitrative stress can occur, preceding OS [14,15]. OS appears when $^{\bullet}O_2^-$ reaches equimolar or higher concentrations than $^{\bullet}NO$. OS and nitro-OS which have a negative connotation and should in our view only be used in pathological conditions.
Since nitro-OS and OS are involved in a large variety of disorders encompassing most chronic as well as some acute diseases, it is of primary interest to be able not only to detect them but also to follow them in order to monitor the evolution of the disease and the efficiency of treatment.

Whereas over seventy OS biomarker assays have been developed [16,17], very few have yet been proposed and validated for monitoring nitrosation and nitration [18,19].

Biomarkers of protein S-nitrosation and nitration

The major RNS in eukaryotes are peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), nitrogen dioxide ($^{\bullet}NO_2$) and probably the elusive nitrosonium ion (NO^+). Daiber and Ullrich [14] elegantly demonstrated that the posttranslational modifications elicited by RNS depend on the relative fluxes of $^{\bullet}NO$ and $^{\bullet}O_2^-$. Thus, as long as the $^{\bullet}NO$ concentration remains 3-fold higher than the $^{\bullet}O_2^-$ concentration, $^{\bullet}NO$ reacts with the generated $ONOO^-$ to yield N_2O_3 which, through the formation of NO^+, S-nitrosates (R-SNO) defined cysteine residues. This modification is rapidly reversible by reduction and therefore depends on the redox status of the biological system. It is therefore essentially a physiological event which may exert protective actions. Indeed, S-nitrosation of homocysteine has been reported to prevent its conversion to its toxic thiolactone metabolite [20]. Deleterious effects of S-nitrosation only seem to appear when the R-SNO groups cannot be reduced to R-SH.

As the $^{\bullet}O_2^-$ generation further increases to equimolar concentrations of $^{\bullet}NO$, most of the latter is oxidized to $ONOO^-$ and can no longer generate N_2O_3. The main RNS are now $ONOO^-$ and its reduction product $^{\bullet}NO_2$. $ONOO^-$ induces nitration ($R\text{-}NO_2$) of selected tyrosine residues, a covalent modification which appears to be enzymatically reversible. Nitration can lead to nitrative stress if it exceeds the cells' or organism's ability to denitrate the target proteins. $^{\bullet}NO_2$ is a potent toxic oxidant which irreversibly peroxidizes lipids, carbonylates proteins and forms dityrosines often leading to cell death.

Detection of S-nitrosation

As indicated above, S-nitrosation is a rapidly reversible process which solely depends on the redox status. In addition the low dissociation energy of the RS-NO bond makes S-nitrosothiols unstable rendering the

assessment of the potential S-nitrosation of cysteine residues in biological samples uncertain with a serious risk of being artefactually generated or reversed during sample preparation due to changes in redox potential.

This risk is further increased by the biotin-switch technique [21] currently used to detect the S-nitrosated cysteines as the multiple steps involved, increase the risk of RSNO hydrolysis. Moreover the lack of specificity of the nitrosocysteine reduction step by ascorbic acid can result in the labelling of sulfenic acids (RSHO) and disulfides as well. A recent modification involving derivatization with NEM called d-switch, allows simultaneous detection and most importantly, identification of both S-nitrosated and non-nitrosated cysteines by MS [22].

Chemiluminescence-based assays like the tri-iodide and the 3C methods carry the same risk of artefactual cysteine-SNO generation and hydrolysis. In addition, these methods do not allow the identification of the S-nitrosated residues.

Recently, the direct detection of cysteine-SNO residues by MS allowing simultaneous determination of the redox status of defined proteins has been described for thioredoxin [23]. Unfortunately, this type of approach can obviously not be applied to complex biological samples.

The current research on novel RSNO chemistry and direct labelling techniques is however progressing. Indeed, triarylphosphines, which react with organic RSNOs to yield S-substituted aza-ylides ($RS\text{-}N{=}PR_3$) without reacting with disulphide bonds are of real potential interest as they should allow direct labelling and detections of cysteine-SNOs without the risk of artificial modifications [24]. This method also allows the differential labelling of nitroxyl (HNO) groups [25].

Very recently, Daiber's team has developed an assay using salicylaldehyde as a probe to measure very low fluxes of peroxynitrite generation compatible with *in vivo* concentrations [19]. This approach opens the way to a novel method allowing the determination of peroxynitrite generation in vivo.

Detection of nitration

As opposed to S-nitrosation, nitration can be investigated much more easily being a stable covalent modification. In eukaryotes nitration mainly affects tyrosine residues yielding 3-nitrotyrosine (3-NT) [8], but it can apparently also modify tryptophan residues, giving rise essentially to 6-nitrotryptophan residues under certain pathophysiological conditions [26].

Although tyrosine nitration is not direct enzymatically driven, tyrosine residues are not randomly nitrated under pathophysiological conditions, i.e. when peroxynitrite fluxes remain at µM levels [27,28].

The easiest way to detect protein nitration in cells or tissues is by immunoblotting and immunohistochemistry provided the antibodies used are specific for nitrotyrosine residues. In order to exclude cross-reactivity with 3-aminotyrosine, 3-chlorotyrosine, orthophosphotyrosine or nitrotryptophan, controls should be performed by reducing nitrotyrosine to aminotyrosine with thiosulfate which should result in total disappearance of the signal. This obviously precludes the use of sulphur reducing conditions for SDS-PAGE.

Nitrotyrosine residues can now also be identified by mass spectrometry but this requires prior derivatization of the 3-NT residues and subsequent enrichment of the nitropeptides as only a very reduced fraction of the proteins of interest are usually nitrated [29,30].

The most common biomarker for studying "nitrative stress" used to be free 3-nitrotyrosine [31,32], but since most of the circulating free nitrotyrosine comes from nitration of dietary proteins in the gastrointestinal tract [33,34] it cannot serve as a reliable indicator of endogenous systemic nitration. For the same reason, this is also true for peptide-bound nitrotyrosine which, like free nitrotyrosine, can be measured by HPLC. In addition, artificial nitration during the assay [35,36] is another serious drawback of this approach. The same holds for the 3-nitrotyrosine metabolite

Another biomarker, 3-nitro-4-hydroxyphenylacetic acid which has been proposed is no better as it is not only a metabolite of 3-nitrotyrosine but also the nitration product of para-hydroxyphenylacetic acid, a metabolite of tyrosine [37,38].

Thus only circulating proteins nitrated endogenously outside the gastrointestinal tract an be considered reliable biomarkers for exploring systemic nitration. Several plasma nitroproteins have been identified [39] but so far only a quantitative ELISA for nitroalbumin has been analytically and clinically validated [15,40]. Plasma nitroalbumin has been shown to correlate with the severity of neonatal encephalopathy [40] in perinatal asphyxia, a condition reported to be associated with increased protein nitration in the human brain [41,42]. In neonatal hypoglycaemia, it was shown to correlate with the number and severity of hypoglycemic events [15] which have been reported to potentially impair psychomotor development.

Lipid peroxidation

Lipid peroxidation (LPO) is an autocatalytic reaction cascade producing reactive aldehydes such as 4-hydroxynonenal (HNE), malondialdehyde (MDA) and acrolein [43,44]. These aldehydes further react with other molecules thereby regulating redox signaling or, if present in high concentrations, irreversibly damaging (extra)cellular macromolecules resulting in protein carbonyls [45]. Consequently, these products emerged as possible biomarkers of different pathologies [17,46].

Detection methods for oxidatively modified proteins include carbonyls and hydroperoxides which have great diversity and are therefore, detected not so specifically, while other methods measure single LPO product bound to proteins, or even more specifically to specific protein side chain. To demonstrate, one of the methods for MDA was the popular TBARS (thiobarbituric acid) assay [47]. TBARS assay was further improved by HPLC, for which the inter-laboratory study from 2010 showed to be the method of choice for LPO measurements [48]. Still, good detection method does not indicate the relevance of the measured product [48]. In addition, the mentioned inter-laboratory study also included GC-MS analysis of isoprostanes (F2-IsoPs) and non-commercial ELISA for HNE-protein adducts which was developed for cell culture research [48,49] and gave high inter-laboratory variation indicating need to adjustments for plasma measurements.

4-HNE-His ELISA

In 2013, the modifications the 4-HNE-His ELISA genuine using genuine non-commercial and also commercially available monoclonal antibodies were described for human plasma and sera samples showing that commercially available antibodies were less sensitive [50]. Nevertheless, in both cases the 50% increase of the HNE-His products in the blood was detected for apparently healthy obese young to mid-aged men [50].

It is also important to mention that further work introduced modifications of the 4-HNE-His ELISA enabling to measure HNE in murine tissue homogenates which had 50% increase in 4-HNE-His adducts in liver if rats were fed by PUFA enriched diet [51].

The Importance of 4-HNE-His adducts as biomarker of OS is shown by Japanese researchers whose labeling with the fluorescent probe 2-aminopyridine found that HNE-His adducts were dominant in Cu^{2+}-oxidized human LDL [52]. The genuine 4-HNE-His monoclonal antibodies are applicable in broad spectrum of samples, from cell cultures (Figure 2), tissue homogenates, to plasma and sera, taking important part in LPO research. Consequently, 4-HNE-His adducts are often reffered as the major biomarkers of OS, as was recently verified by a serious of clinical trials combining HNE-His immunochemistry with metabolomics/lipidomics in patients with rheumatoid arthritis, encephalitis, neuroborreliosis, metabolic syndrome and liver cancer [53–58], while in patients with lung malignancies it was verified by lipidomics and by PET/CT [59,60].

Qualitative analysis of 4-HNE-His adducts

The advantage of HNE-His adducts in addition to visualization by immunohistochemistry lies in the stability of the complex in formalin-fixed samples, which last for years. The advantages, disadvanages and technical features of all the methods for LPO biomarker detection were recently detail reviewed in several review papers [17,61,62], while the comprehensive update reviewing benefits of immunohistochemistry for HNE-His adducts in clinical studies has just been published by Zarkovic K. et al. [63], who summarized these benefits briefly as follows:

- Immunohistochemistry specific for HNE-protein adducts, mostly for HNE-histidine epitopes, is valuable qualitative and semi-quantitative method to study pathophysiology of oxidative stress and lipid peroxidation.
- Immunohistochemical studies confirm association of HNE with major human diseases.
- Formation of HNE-protein adducts is not irreversible, but correlates with the age of patients and the severity of respective degenerative, metabolic or inflammatory diseases.
- In case of carcinogenesis HNE can play undesirable, pro-carcinogenic role, but it could also cause the desirable decay of cancer.
- Non-malignant stromal cells and the cells in the vicinity of cancer can generate HNE to defend the organism from cancer invasion.

Protein carbonyls

Mild oxidative stress is stimulative, while high levels cause molecular and cellular damage [64], oxidizing proteins and forming carbonyl groups (aldehyde and ketone) on variety of amino acid side chains [65,66]. Carbonylation often occurs due to reaction with reactive aldehydes produced by lipid peroxidation. These aldehydes bind to lysine, histidine and cysteine side chains and forming advanced lipoxidation end products (ALEs) [43]. Other amino acid side chains, such as proline, methionine, valine, threonine, alanine aspartate, thryptophane and isoleucine side chains can be directly attacted by ROS also forming protein carbonyls [65]. Glycation is also possible mechanism of protein carbonylation, which oocurs via reaction of side chains which glucose of fructose, which is also succeptible for ROS attact with resulting advanced glycoxidation end products (AGE) [67]. Protein carbonyls are considered to be irreversible [68], and as such change protein structure and function. Due to different mechanisms of production and realtively high levels makes prtoein carbonyls often used as a measure of oxidative stress [69], but these facts also reduce their relevance in their use as biomarkers of oxidative stress [70].

Protein carbonyls are detected by immunological techniques, spectrofotometric/fluorimetric and chromatographic tehniques and by mass spectrometry (Figure 3) [68]. Immunological and spectrophotometric/fluorimetric assays are based on raction of derivatization with different agents such as 2,4-dinitrophenylhydrazine (DNP), 7-hydrazino-4-nitrobenzo-2,1,3-oxadiazole (NBDH) [65,71]. The drawback of these methods are the indirect measurements of the target molecules, being work-intensive and time consuming. Due to complicated procedures with numerous washing steps these methods are not convenient for high throughtput measurements. In addition, nucleic acid could also provide signals as they may contain carbonyl groups [65]. HLPC and mass spectrometry may overcome some of these problems but still these methods are expensive and time consuming. Currently, newly developed procedures for mass spectrometry seem promising in the research of protein carbonyls but the diversity in protocoles and labeling conditions are too wide to combine and adequately compare results from different studies [68].

Conclusions and perspectives

In conclusion, redox signalling is vital for the maintenance of homeostasis from the organelle to the organism. The reactive species are merely composed of oxygen, nitrogen and hydrogen and their generation and degradation are regulated by a very limited number of enzymes which affect most of the essential cell functions from proliferation, metabolism and differentiation to death. Thus these mechanisms, which have long been considered as pathological, clearly play essential physiological roles. This has confirmed by several interventional studies using various “antioxidants” aimed at preventing various diseases and which at best have shown little or no effects at low doses and deleterious effects at higher doses [72–77].

It is therefore crucial to better understand the molecular mechanisms of action not only of ROS and RNS, but also of the many "antioxidants" whose prescription and customary consumption has been dramatically increasing during the last years.

Biomarkers should be useful to monitor the real redox potential of these compounds in vivo in order to better evaluate their potential benefit, but also whether their beneficial or detrimental effects are directly linked to their redox potential or to distinct mechanisms.

There is still a desperate need for validated biomarkers to study nitro-oxidative status and stress which precede oxidation in most pathological conditions.

At a time where nutritional supplements have become trendy to improve health and ageing despite a cruel and desperate lack of scientifically proven benefit [78,79], this area of research obviously appears to be of primary importance.

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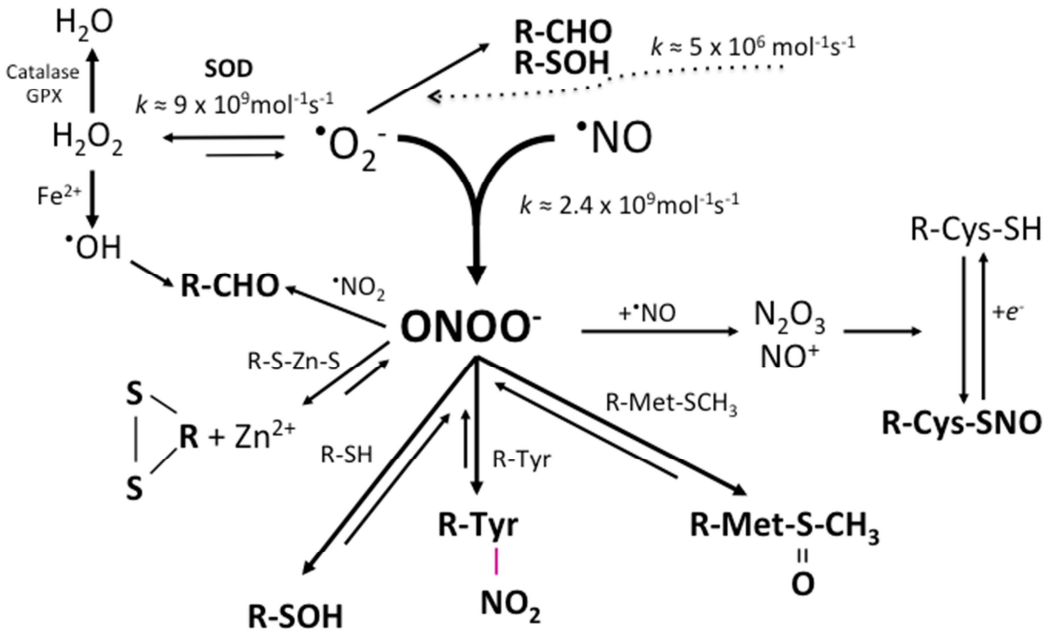
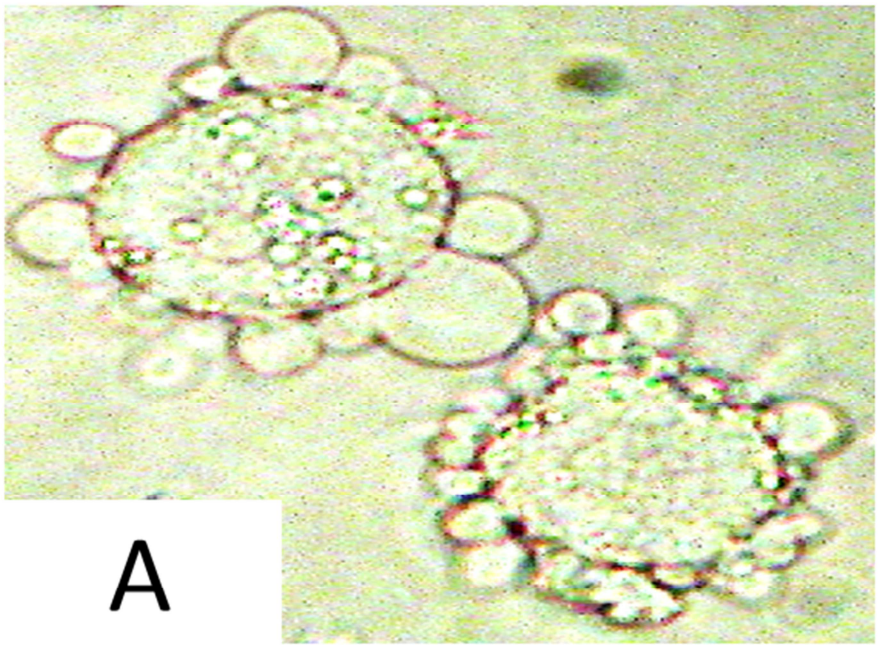
Legend to the figures

Figure 1. Nitro-oxidation reactions and posttranslational protein modifications.

Figure 2. Immunocytochemical detection of HNE-His adducts. Upper panel: HeLa cells after HNE-treatment show membrane blebbing. Lower panel: immunocytochemical staining for HNE-His adducts.

Figure 3. Overview of protein carbonyl detection methods. Except for MS, all other methods for protein carbonyl detection need derivatization step in the process of measurement.





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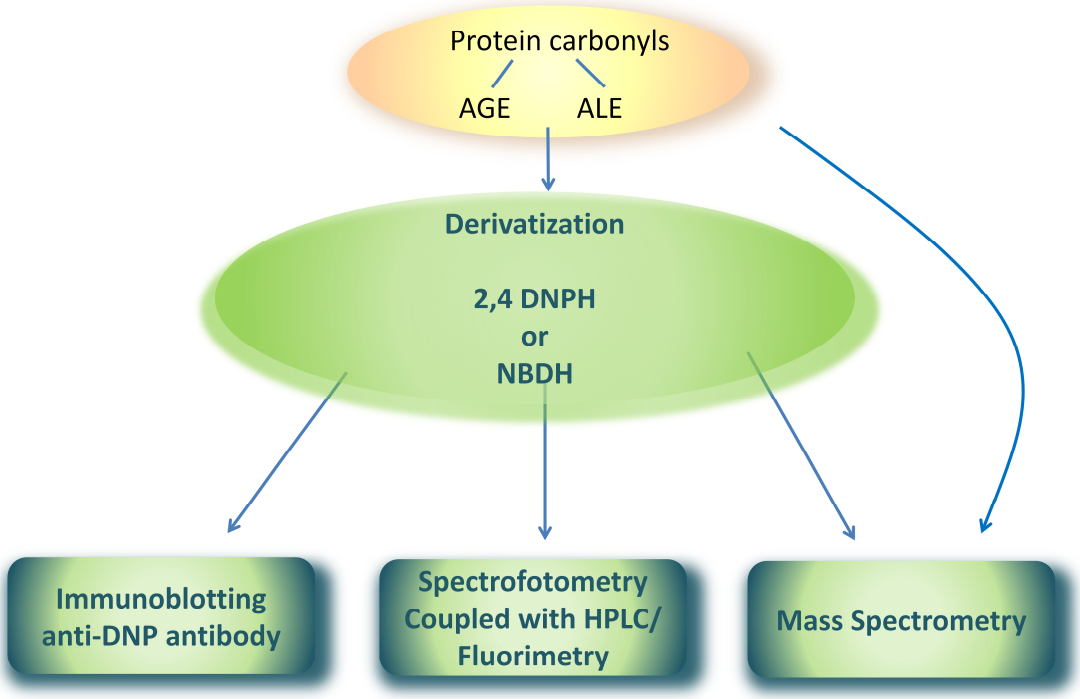

Highlights

In redox signalling, nitric oxide is the primary substrate of the original reactive oxygen species, superoxide.

The $^{\bullet}NO$ concentrations being three orders of magnitude higher under physiological conditions, it serves as a "sink" for $^{\bullet}O_2^-$, thereby preventing oxidative mechanisms. When under pathophysiological conditions, $^{\bullet}O_2^-$ and $^{\bullet}NO$ concentrations rise, peroxynitrite is generated and S-nitrosation and nitration occur, preceding oxidative stress.

Whereas over seventy oxidative stress biomarker assays have been developed, very few have yet been proposed and validated for monitoring S-nitrosation and nitration.

4-HNE-His adducts are often referred to as the major biomarkers of oxidative stress, as recently confirmed by a series of clinical trials combining HNE-His immunochemistry with metabolomics/lipidomics.

At a time where nutritional supplements have become trendy to improve health and ageing despite a cruel and desperate lack of scientifically proven benefit research in the area of redox signalling is of primary importance.