Measurement and meaning of markers of reactive species of oxygen, nitrogen and sulfur in healthy human subjects and patients with inflammatory joint disease

Paul G. Winyard1, Brent Ryan2, Paul Eggleton3, Ahuva Nissim†, Emma Taylor6, Maria Letizia Lo Faro*, Torsten Burkholz5, Katalin E. Szabó-Taylor*, Bridget Fox*, Nick Viner*, Richard C. Haigh*, Nigel Benjamin*, Andrew M. Jones‡ and Matthew Whiteman*

†Peninsula Medical School, University of Exeter, St. Luke’s Campus, Exeter EX1 2LU, U.K., †William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ, U.K., and ‡School of Sport and Health Sciences, University of Exeter, St. Luke’s Campus, Exeter EX1 2LU, U.K.

Abstract

Reactive species of oxygen, nitrogen and sulfur play cell signalling roles in human health, e.g. recent studies have shown that increased dietary nitrate, which is a source of RNS (reactive nitrogen species), lowers resting blood pressure and the oxygen cost of exercise. In such studies, plasma nitrite and nitrate are readily determined by chemiluminescence. At sites of inflammation, such as the joints of RA (rheumatoid arthritis) patients, the generation of ROS (reactive oxygen species) and RNS overwhelms antioxidant defences and one consequence is oxidative/nitrative damage to proteins. For example, in the inflamed joint, increased RNS-mediated protein damage has been detected in the form of a biomarker, 3-nitrotyrosine, by immunohistochemistry, Western blotting, ELISAs and MS. In addition to NO*, another cell-signalling gas produced in the inflamed joint is H2S (hydrogen sulfide), an RSS (reactive sulfur species). This gas is generated by inflammatory induction of H2S-synthesizing enzymes. Using zinc-trap spectrophotometry, we detected high (micromolar) concentrations of H2S in RA synovial fluid and levels correlated with clinical scores of inflammation and disease activity. What might be the consequences of the inflammatory generation of reactive species? Effects on inflammatory cell-signalling pathways certainly appear to be crucial, but in the current review we highlight the concept that ROS/RNS-mediated protein damage creates neoepitopes, resulting in autoantibody formation against proteins, e.g. type-II collagen and the complement component, C1q. These autoantibodies have been detected in inflammatory autoimmune diseases.

Introduction

RA (rheumatoid arthritis) is an inflammatory autoimmune disease affecting approximately 1–2% of the population worldwide [1]. A key feature of the disease is chronic inflammation of the synovial joints (with superimposed episodes of acute inflammation), and this appears to be linked to dysfunctional apoptosis [2] and oxidative/nitrative stress [1]. Oxidative or nitrative stress arises when the production of ROS (reactive oxygen species) or RNS (reactive nitrogen species) exceeds their removal by scavenger systems, resulting in the accumulation of end-products of the reactions of these agents with biomolecules [3].

In inflammatory diseases, such as inflammatory joint disease and inflammatory bowel disease, excessive ROS/RNS arise from the activation, within inflammatory cells, of NADPH oxidase and iNOS (inducible nitric oxide synthase) respectively [1]. Other mechanisms of ROS/RNS generation during the inflammatory response are clearly important, and have been reviewed elsewhere [4]. Likewise, the reader is referred to a recent review of enzymic antioxidants [e.g. SOD (superoxide dismutase), catalase, glutathione peroxidase and peroxiredoxin] and non-enzymic antioxidants (e.g. ascorbic acid and α-tocopherol) in inflammatory joint disease [1].

Over the last 30 years or so a plethora of papers describing the measurement of the products generated by ROS/RNS have appeared in the literature, in relation to biofluids from RA patients [1,4]. There is no doubt that, in many instances, the products of free radical reactions with lipids, proteins, DNA and carbohydrates have been successfully and convincingly detected in tissues from patients with RA. Furthermore, it is clear that the tissue concentrations of these

Key words: advanced glycation end-product (AGE), reactive nitrogen species (RNS), reactive oxygen species (ROS), rheumatoid arthritis (RA).

Abbreviations used: AGE, advanced glycation end-product; CBS, cystathionine γ-synthase; CSE, cystathionine-γ-lyase; 8-oxoG, 8-oxo-7,8-dihydro-2’-deoxyguanosine; HNE, hydroxynonenal; H2S, hydrogen sulfide; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; MDA, malondialdehyde; MS/MS, tandem MS; NO*, nitric oxide; O2, oxygen; RNS, reactive nitrogen species; RSNO, S-nitrosothiol; SLE, systemic lupus erythematosus; TNF-α, tumour necrosis factor-α.

1To whom correspondence should be addressed (email paul.winyard@pms.ac.uk).

2Present address: Department of Physiology, Anatomy and Genetics, University of Oxford, Le Gros Clark Building, Oxford, OX1 3QX, U.K.
The measurement of oxidative damage to DNA has been used as a method to define the extent of ROS activity in blood cells and biofluids from patients with inflammatory joint disease and comparative healthy tissues, as well as the end-products of free radical reactions in both inflammatory and comparative healthy tissues. This measurement was the concept that these products were associated with biomolecular damage which, in and of itself, played a direct role in cellular and ECM (extracellular matrix) destruction [5]. More recently, evidence has been found that the inflammatory generation of reactive species also plays an indirect, but crucial, pathogenic role, through at least three mechanisms. First, ROS/RNS (and/or the products of their reactions) may modulate cell-signalling pathways such as transcription factor activation [e.g. NF-κB (nuclear factor-κB) and Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2)] [6] and apoptosis PCD (programmed cell death) [2]. A failure of inflammatory cells to undergo apoptosis has been implicated in persistence of the inflammatory response [2], while chronic exposure to oxidative stress is associated with resistance to apoptosis [7]. Secondly, ROS/RNS may induce somatic mutations, which result in altered protein function and/or immunogenicity [4,8,9]. Thirdly, ROS/RNS-mediated damage may create neoepitopes, resulting in the production of autoantibodies against proteins, e.g. type-II collagen and the complement component, C1q. Of note, the three above types of pathways provide mechanisms for the amplification of the effects of a single damaging ‘hit’ on a biomolecule, via the ‘apoptotic cascade’, DNA replication or the immune response respectively.

The present review will focus on the measurement of end-products of free radical reactions in both inflammatory joint disease and comparative healthy tissues, as well as the implications of such damage for the immune response.

Detection of the reaction products of oxygen-centred species in peripheral blood cells and biofluids from patients with inflammatory joint disease

The measurement of oxidative damage to DNA has been a key method in defining the extent of ROS activity in human tissues, in relation to toxic environmental exposures, oxidative stress resulting from inflammatory autoimmune diseases and many other situations. One of the key products that is commonly measured is 8-oxodG (8-oxo-7,8-dihydro-2′-deoxyguanosine), which is formed as a result of the attack of the OH* (hydroxyl radical) on guanine bases within DNA [10]. The main techniques for measurement of 8-oxodG are HPLC–ESI (electrospray ionization)–MS/MS (tandem MS) or HPLC with electrochemical detection. We have previously used HPLC with electrochemical detection to measure 8-oxodG in the peripheral blood cells of patients with a range of inflammatory autoimmune diseases, including RA, and demonstrated increased levels of DNA damage [11]. This observation is consistent with the idea that these base modifications lead to somatic mutations, which may play a role in autoimmune inflammatory diseases [8,9].

In relation to oxidative post-translational modifications of the amino acid side chains of proteins, the oxidation of methionine (Figure 1A) and cysteine residues (Figure 1B) occurs relatively readily. Oxidation of the cysteine thiol (-SH) group to cysteine sulfinic, sulfenic and sulfonic acid derivatives occurs in the presence of oxidants such as H2O2. The cysteine thiol group can also be oxidized, to form a disulfide (RS-SR) with another free thiol. Similarly, methionine can be oxidized to methionine sulfoxide and further to methionine sulfone [12]. Other oxidizable amino acids include tryptophan (Figure 1C), phenylalanine (Figure 1D) and tyrosine (Figure 1E). Attack by OH* results in the hydroxylation of phenylalanine, with the formation of o-tyrosine, m-tyrosine and 3,4-dihydroxyphenylalanine. The OH* radical, along with other reactive species, induces formation of the tyrosyl radical. This radical can readily form dityrosine by reacting with another tyrosyl radical, forming stable inter- or intramolecular cross-links. The action of ROS on tryptophan causes hydroxylation of the indole ring system or the degradation of the pyrrole ring to form the fluorescent products, N-formylkynurenine or kynurenine [12].

Attack on unsaturated double bonds in lipids, by species such as HO2• results in the formation of lipid RO2• (peroxyl radicals). These radicals then act on other unsaturated lipids to propagate lipid peroxidation. The process of lipid peroxidation provides yet another mechanism for the amplification of the initial damage to one molecule. Lipid peroxidation disrupts lipid membranes, leading to apoptosis or necrosis of cells and also to production of other reactive species. Two major reactive species produced by lipid peroxidation are MDA (malondialdehyde) and 4-HNE (4-hydroxynonenal). These products react with nucleophilic amino acid residues in proteins, including lysine, arginine, methionine, tyrosine and histidine through Schiff base and Michael addition reactions [12,13] (see also Figure 1 and accompanying review by Spickett and Fauzi [13a]).

In addition to RA, another inflammatory connective tissue disease in which oxidative stress appears to be an important pathogenic feature is SLE (systemic lupus erythaematosus). Davies and co-workers (see [14] and citations therein) measured the concentrations of modified amino acids in serum proteins from SLE patients by HPLC coupled with fluorescence and UV detection. There were significant increases in serum methionine sulfoxide and 3-nitrotyrosine (see below) concentrations in SLE patients compared with healthy control subjects, with the methionine sulfoxide levels correlating with disease activity in SLE patients. A significant decrease in phenylalanine concentrations was observed and was accompanied by non-significant increases in o-tyrosine and 3,4-dihydroxyphenylalanine levels. 3-Chlorotyrosine (Figure 1E; formed through a neutrophil myeloperoxidase-catalysed reaction) and dityrosine levels were not significantly different in the two groups. Other markers of oxidative stress were also observed in SLE patients, such as increased protein carbonyl groups as measured by ELISA, and decreased
free protein thiols as assessed using the Ellman’s test (based on DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)]). These results suggested that oxidative stress is prevalent in SLE patients, and results in modification to specific amino acid residues in serum proteins [14].

Other recent studies demonstrated by ELISA that SLE patients had raised serum levels of MDA- and HNE-modified proteins [15–17], and suggested that these chemical modifications constitute neoepitopes that give rise to antigenicity (see below). Antibody titres against MDA- and HNE-modified ovalbumin were raised in SLE patients, and correlated with a clinical index of disease activity (the so-called ‘SLEDAI’ score) [16]. SLE patients were also found to have raised levels of IgG and IgM antibodies to MDA-modified LDL (low-density lipoprotein) compared with healthy subjects [18]. Furthermore, IgG anti-MDA-LDL levels were higher in SLE patients with CVD (cardiovascular disease) than those without [18]. These observations suggest the systemic presence of MDA in SLE patients and that MDA modification of LDL may be an important route of LDL modification in atherosclerosis.

The reactive species glyoxal, methylglyoxal and pentosidine are formed from the oxidation of glucose, a process that is increased under oxidative stress. The reaction of glyoxal and methylglyoxal with proteins results in the formation of AGEs (advanced glycation end-products), which are associated with a number of pathologies [19]. AGEs are formed through Maillard reactions between reactive carbonyl groups and basic amino acid side chains such as lysine and arginine (see Figure 1F). Serum levels of pentosidine were found to be increased in both RA and SLE patients [20]. Moreover, using LC-MS/MS, Ahmed et al. [21] showed an increase in specific AGEs (glyoxal-derived hydroimidazolone and methylglyoxal-derived hydroimidazolone), as well as methionine sulfoxide, in RA plasma compared with healthy plasma. Both ROS-modified IgG and glycated IgG appear to be targets for IgM isotype antibodies in RF (rheumatoid factor)-positive RA and SLE patients [22,23].

**Detection of the reaction products of nitrogen-centred species in biofluids and tissues from patients with inflammatory joint disease**

Nitric oxide (NO•) is important not only in human physiological processes but also in many inflammatory diseases, including RA [24,25]. Human NOS (nitric oxide
is a homodimeric enzyme which catalyses the generation of NO\(^{\cdot}\) and L-citrulline from L-arginine. Other substrates and products are involved in this reaction [24]. In RA, the production of NO\(^{\cdot}\) by iNOS is increased in macrophages by the pro-inflammatory cytokines IL-1 (interleukin-1), TNF\(\alpha\) (tumour necrosis factor \(\alpha\)) and IFN\(\gamma\) (interferon \(\gamma\)). NO\(^{\cdot}\) reacts rapidly with superoxide (rate constant approximately \(10^{10}\) M\(^{-1}\) s\(^{-1}\)) producing the potent oxidant, peroxynitrite (ONOO\(^{\cdot}\)) which, in turn, causes biomolecular damage such as protein nitration, as discussed later.

Because NO\(^{\cdot}\) has a short t\(_{1/2}\) in biological samples, stable end-products of the reactions of NO\(^{\cdot}\) are usually measured, as markers of this radical species. Most commonly, these markers are NO\(_2\)\(^{\cdot}\) (nitrite) and NO\(_3\)\(^{\cdot}\) (nitrate). One of the best-accepted methods for the determination of NO\(_2\)\(^{\cdot}\) and NO\(_3\)\(^{\cdot}\) in biofluids is based on the measurement of chemiluminescence generated by the interaction of NO\(^{\cdot}\) with ozone (O\(_3\)) [26]. Oxidation products of NO\(^{\cdot}\), such as NO\(_2^{\cdot}\) and NO\(_3^{\cdot}\), must be converted back into NO\(^{\cdot}\) before analysis. In this assay system, nitrite is converted into NO\(^{\cdot}\) using iodide and acid, according to the following equation:

\[
2\text{I}^{-} + 2\text{NO}_2^{\cdot} + 4\text{H}^{+} \rightarrow 2\text{NO}^{\cdot} + \text{I}_2 + 2\text{H}_2\text{O}
\]

NO\(_2^{\cdot}\) in biological samples is reduced to NO\(^{\cdot}\) using a saturated vanadium (III) chloride solution in 1 M hydrochloric acid at high temperature, according to the following equation:

\[
2\text{NO}_3^{-} + 3\text{V}^{3+} + 2\text{H}_2\text{O} \rightarrow 2\text{NO}^{\cdot} + 3\text{VO}_2^{+} + 4\text{H}^{+}
\]

However, NO\(_2^{\cdot}\) is also reduced to NO\(^{\cdot}\) by this method, so vanadium (III) reduction followed by chemiluminescence generates a value for both NO\(_2^{\cdot}\) and NO\(_3^{\cdot}\) combined (NO\(_2\)\(^{\cdot}\)). Farrell et al. [27] and Ueki et al. [28] detected increased NO\(_2^{\cdot}\) concentrations in the serum and knee-joint synovial fluid from patients with RA compared with healthy control subjects.

NO\(^{\cdot}\) may also form RSNOs (S-nitrosothiols) by the NO\(^{\cdot}\)-dependent S-nitrosation of thiol-containing proteins and peptides [29]. Increased concentrations of RSNOs in RA plasma and synovial fluid, compared with healthy plasma, have been reported, as determined by both a chemiluminescence-based method [30] and by EPR spectrometry in combination with spin trapping [31].

Tyrosine (either within the polypeptide backbone of proteins or as the free amino acid) may be nitrated by two distinct mechanisms to form 3-nitrotyrosine [32]. Peroxynitrite nitrates the aromatic ring of free or protein tyrosine via a nitronium ion (NO\(_2^{\cdot}\)) intermediate. Alternatively, tyrosine nitration can occur through NO\(_2\) (nitrogen dioxide) generated by the oxidation of nitrite by peroxidases, e.g. myeloperoxidase from activated leucocytes [32]. Mapp et al. [25] detected 3-nitrotyrosine in inflamed rheumatoid knee-joint synovial tissue by immunohistochemistry. Staining predominated in the VSMCs (vascular smooth muscle cells) and macrophages. Several groups have measured 3-nitrotyrosine concentrations in RA synovial fluid, serum and urine, as well as in SLE serum, using a variety of methods including HPLC, GC-MS/MS and ELISA-based methods. The reported concentration values span a large range, and several methodological issues have been identified [33]. Nevertheless, urinary excretion of 3-nitrotyrosine was reported as significantly elevated in a group of patients with inflammatory rheumatic diseases compared with healthy subjects (1.2 ± 0.3 against 0.39 ± 0.16 nmol/mmol of creatinine) [33].

The immunological similarity of 3-nitrotyrosine and a synthetic hapten, 4-hydroxy-3-nitrophénylacetyl, has been demonstrated in mice. Anti-3-nitrotyrosine antibodies, which cross-react with ssDNA (single-stranded DNA), were induced by both the synthetic hapten and autologous, nitrated, IgG [34]. In addition, it has been shown that the immune system is not able to tolerate peptides bearing 3-nitrotyrosine when presented in association with the MHC-II [35]. This results in a prolonged immune response against 3-nitrotyrosine-containing peptides, suggesting that 3-nitrotyrosine has the potential to play an important role in eliciting autoantibody responses against specific proteins (see below and Figure 2).

Although, as described above, plasma NO\(_2^{\cdot}\) and NO\(_3^{\cdot}\) concentrations are frequently measured as an index of NO\(^{\cdot}\) production, it is important to remember that the levels of both these compounds in human plasma may be influenced by dietary NO\(_3^{\cdot}\). Leafy vegetables (e.g. spinach and lettuce) and beetroot [36] contain high levels of NO\(_3^{\cdot}\) that can be absorbed from the human stomach and small intestine into the plasma. The NO\(_3^{\cdot}\) is then concentrated in the salivary glands before being excreted into saliva, increasing the concentration to at least 10 times that of plasma [36]. Some of the NO\(_3^{\cdot}\) is then converted into NO\(_2^{\cdot}\) by bacteria present in the oral cavity. NO\(_2^{\cdot}\) can then be either absorbed from the stomach or further reduced to NO\(^{\cdot}\). Once NO\(_2^{\cdot}\) reaches the blood and tissues, it may be reduced to NO\(^{\cdot}\) by a variety of potential mechanisms, including the action of xanthine oxidase, deoxyhaemoglobin and mitochondrial enzymes [36–38].

Not only can dietary NO\(_3^{\cdot}\) contribute to plasma concentrations of both NO\(_3^{\cdot}\) and NO\(_2^{\cdot}\), but dietary intake of NO\(_3^{\cdot}\) is associated with human physiological responses. NO\(_2^{\cdot}\) is thought to act as a circulating reservoir of NO\(^{\cdot}\) within the vasculature [38], allowing vasodilation to occur under hypoxic conditions. Moreover, in human studies, there have been recent demonstrations of a reduction in diastolic and/or systolic blood pressure (see [39] and citations therein; [40–42]) and a lowering of the oxygen-cost of exercise [39–42], in response to dietary NO\(_3^{\cdot}\). These effects appear to be, most likely, mediated through the formation of NO\(^{\cdot}\), although there is evidence that NO\(_2^{\cdot}\) itself may signal directly without NO\(^{\cdot}\) formation [39]. It is conceivable that dietary NO\(_3^{\cdot}\), by virtue of \(\text{in vivo} \) NO\(_2^{\cdot}\)/NO\(^{\cdot}\) generation, might also have cytoprotective and anti-inflammatory effects in diseases involving inflammatory and tissue damage, but these ideas remain to be tested experimentally.
Figure 2 | Schematic representation of B- and T-cell activation, showing how the oxidative modification of proteins, thereby generating ‘non-self’ or ‘danger’ proteins, may give rise to autoimmunity

1. A native protein is endocytosed and presented on the MHC-II molecule. However, in the absence of antigen-presenting cell (APC) activation and reactive B-/T-cells, no immune response occurs. (2) B-cells can act as APCs and present antigenic peptides on the MHC-II molecule to CD4+ T-cells. The T-cell interaction causes cytokine release and B-cell activation and differentiation. (3) T-cells are activated by interaction with the MHC-II peptide complex on the APC surfaces, through T-cell receptor signalling and through receiving co-stimulation from the APC. IL-4, interleukin 4.

Detection of sulfur-centred species in RA knee-joint synovial fluid

H2S (hydrogen sulfide), like NO•, is a gas that is involved in crucial cell-signalling pathways. H2S is produced by the pyridoxal phosphate-dependent enzymes CSE (cystathionine-γ-lyase) and CBS (cystathionine-β-synthase), using the amino acid substrates cysteine, homocysteine and cystathionine. It has recently become clear that H2S regulates a growing list of physiological functions, such as blood pressure, pain and inflammation [43]. In relation to the inflammatory response, H2S synthesis by CBS and CSE is up-regulated by LPS (lipopolysaccharide) and inflammatory cytokines (e.g. TNFα). Several different analytical methods have been used for the detection of H2S in plasma, serum and tissues, but there are discrepancies between the values found [44]. This is mainly due to the lack of an H2S-specific detection system. An H2S-sensitive electrode has also been used to obtain measurements in vitro, but in complex biological matrices the electrode is prone to fouling because of non-specific adsorption of proteins on to the electrode’s sulfide-selective membrane, thereby lowering the sensitivity of the method. Despite the uncertainty around the ‘absolute’ H2S levels in tissues it is important to note that increased levels (compared with healthy controls) have been found in several inflammatory conditions. Using the zinc-trapping method, we have detected H2S at high concentrations in RA synovial fluid [45]. There is mounting evidence that H2S plays an anti-inflammatory role in such situations [43] and that H2S mediates its cell-signalling roles through protein S-sulfhydration (i.e. cysteine perthiol formation; see Figure 1) [46].

In the context of sites of autoimmune inflammation, what does the observed increase in oxidative protein modifications mean? Is this an explanation for the breakdown of tolerance in autoimmunity? If the constituent amino acids of a given protein are modified sufficiently (e.g. by oxidative modifications, glycation, methylation, phosphorylation, etc.) the immune system may fail to recognize the protein as ‘self’ and will mount an immune response against the neoepitope (reviewed by Eggleton et al. [47]; and see Figure 2). For example, citrullination is known to be the result of an enzymatic post-translational modification by PAD (peptidylarginine-deiminase). Autoantibodies to proteins containing citrulline residues, such as vimentin, fibrin and filaggrin, have been detected.
detected in RA serum samples [48]. Indeed, diagnostic tests that measure the levels of antibodies to cyclic synthetic peptides containing citrulline are now routinely used in the diagnosis of RA [9]. It therefore appears that there is an association between post-translational modifications and autoimmunity. This is exemplified, in the context of the reactive species discussed here, by the increased antigenicity of type-II collagen when modified with a range of oxidizing, nitrating or chlorinating species or when glycosylated. Significantly more RA patients recognized the modified forms of the protein than the unmodified form [49]. Another example of oxidative post-translational modifications giving rise to the breaking of immune tolerance is provided by the complement protein C1q. Antibodies to C1q are known to be clinically useful in the diagnosis of nephritis in SLE. Recently, it was demonstrated that the oxidative modification of C1q increased the antigenicity of C1q when testing serum from SLE patients [50].

In order to translate the above observations into medicine, we are investigating the diagnostic utility of oxidatively modified protein antigens. To this end, it will be important to identify the precise primary sequences, within proteins at inflammatory sites, which are modified by the oxygen-, nitrogen-, sulfur- and chlorine-centred species mentioned in this overview. The amino acid side chains within these peptide neoepitopes are predicted to contain many of the chemical modifications discussed above and shown in Figure 1. LC-MS/MS will be a key analytical tool in defining such modifications.

Acknowledgements

Owing to space limitations, we have been constrained to producing a ‘viewpoint’-style article. We apologize to the many colleagues in the field whose important contributions have had to be omitted.

Funding

This work was funded by the following agencies: the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement [grant number 215009], Arthritis Research UK, DAART (Devon Arthritis Appeal Research Trust), the Biotechnology and Biological Sciences Research Council, the Torbay Medical Research Fund, the Northcott Devon Medical Foundation and the Peninsula Knowledge Transfer Fund.

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