

## Chapter 2

# Transition Metals and Other Forms of Oxidative Protein Damage in Renal Disease

Vincent M. Monnier, Ina Nemet, David R. Sell, and Miriam F. Weiss

**Abstract** Oxidative and carbonyl stresses are dramatically increased in chronic renal disease, whereby an inverse relationship usually exists between renal clearance and the accumulation of low molecular weight compounds ultimately responsible for the damage to plasma constituents. Damage to proteins results from primary attack to protein residues by reactive oxygen species with or without metal catalyst, or via myeloperoxidase and hypochlorous acid. Secondary, indirect forms of damage result from oxoaldehydes and lipid peroxidation products involved in glycation and glycoxidation reactions with nucleophilic residues. The chemical oxidative pathways responsible for protein damage and its biological and clinical significance are discussed, emphasizing end stage renal disease. Interventions that improve or worsen oxidant stress, such as intravenous iron therapy, are reviewed.

**Keywords** Oxidative stress · Carbonyl stress · Glycation · Catalytic metals · Myeloperoxidase · Methylglyoxal · Ascorbic acid

## 1 Introduction

Oxidative modifications of proteins are strongly associated with renal disease. The presence of high levels of protein carbonyl, advanced oxidation protein products (AOPP), metal catalyzed oxidation products, and other oxidative modifications has been understood to be a sign of oxidant stress markers, a vague term that provides little information regarding mechanisms of formation. In addition to metal catalyzed oxidation (MCO), oxidative modifications in renal disease can involve hypochlorous acid, peroxide, peroxynitrite, hydrogen peroxide, and hydroxyl radicals.

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V.M. Monnier (✉)

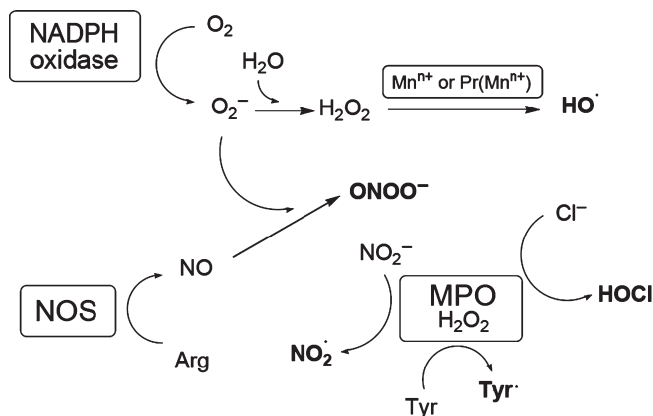
Department of Pathology and Biochemistry, Case Western Reserve University,  
Wolstein Research Bldg., 2103 Cornell Road, Cleveland, OH 44106-7288, USA  
e-mail: vmm3@cwru.edu

To clarify these diverse mechanisms, the first part of this chapter will be devoted to the chemistry of protein oxidation with the ultimate question of which modifications should be measured in order to unequivocally implicate a particular pathway of protein damage. It is useful to distinguish between *primary* damage to proteins (i.e., oxidative modifications of amino acid residues and peptide backbone fragmentation reactions) vs. *secondary* forms of damage involving modifications of nucleophilic amino acids (such as lysine, arginine, and cysteine) by carbonyls from lipoxidation or the Maillard reaction. Thus, *carbonyl* and *oxidative* stress are often tightly linked. However, protein modifications resulting from the latter, and in particular those from the Maillard reaction, will be reviewed elsewhere in this book.

The second part of this chapter will be devoted to clinical, biological, and structural aspects of metal catalyzed oxidation, focusing primarily on advanced oxidation products that are common to MCO and related mechanisms of oxidation.

## 2 Chemical Pathways of Protein Damage by Oxidation

A number of biomarkers for protein oxidation have been identified in recent years that demonstrate not only the extent of oxidative injury but also provide evidence for the source of the oxidant. Knowledge of the latter is essential for developing adequate interventions to stop or slow down damage. Figure 1 summarizes the major sources of protein oxidants *in vivo* (i.e., hypochlorous acid [HOCl], peroxynitrite [ONOO<sup>-</sup>], and free radicals), resulting from a combination of different enzymatic and nonenzymatic reactions.



**Fig. 1** Major pathways leading to formation of reactive oxidants via the myeloperoxidase (MPO), NADPH oxidase, nitric oxide synthase (NOS), and redox active metal ions — free ( $Mn^{n+}$ ) and protein bound metals ( $Pr(Mn^{n+})$ )

Sulfur containing amino acids, Cys and Met, are the major sites of oxidation within proteins. They can be oxidized easily by radical-mediated or two-electron pathways, typically involving HOCl, peroxides, peroxynitrite, or singlet oxygen. The resulting products are primarily disulfides (cystine or mixed disulfides) and methionine sulfoxide (MetSOX), respectively, regardless of the nature of the oxidant. Cysteine reacts with a thiyl radical ( $RS\bullet$ ) that dimerize with another cysteine or sulfhydryl residue to form the disulfide. The thiyl radical, in a competitive reaction with oxygen, gives a peroxy radical, which usually forms oxyacids ( $RSO_2H$  and  $RSO_3H$ ). The same products are formed in the two-electron mediated oxidation process where the initial adduct species (e.g.,  $RSCl$ ,  $RSOH$ ,  $RSNO$ ) form from HOCl, peroxides, and nitric oxide ( $NO^+$ ), respectively, and then react with another thiol group to produce disulfides or hydrolyze into the oxyacids. These intermediate compounds are usually too labile and complex for quantification in vivo.

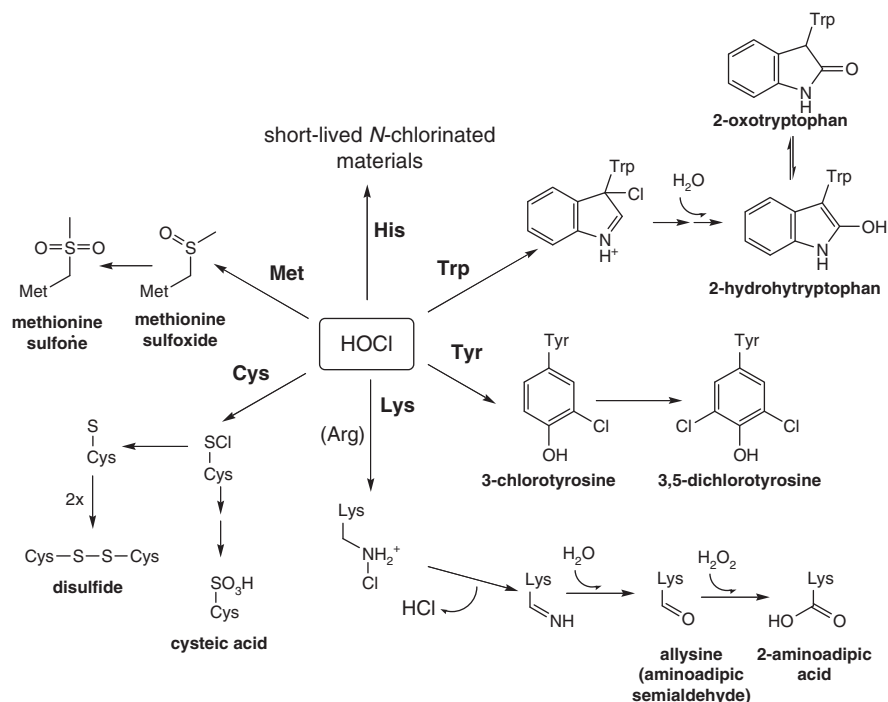
Methionine invariably gets oxidized into MetSOX, regardless of whether it is oxidized by free radicals, peroxide, or peroxynitrite [1]. The latter rapidly reacts with Met residues and gives MetSOX, however, in the presence of physiological levels of  $CO_2$  an  $ONOOOCO_2^-$  adduct is formed that oxidizes Met into MetSOX at a much slower rate than  $ONOO^-$ , suggesting that the extent of Met oxidation observed in vivo will be modulated to a major extent by local  $CO_2$  levels. MetSOX can be subjected to further oxidation and give of sulfone, however, this reaction occurs to a much lesser extent [1, 2].

In summary, the major end products of Cys and Met oxidation expected in vivo are cystine and methionine sulfoxide. Further oxidation of cystine into cysteic acid requires much more drastic conditions that are not easily achieved in vivo, except by the HOCl system.

## 2.1 HOCl Mediated Pathways

In vivo HOCl is formed from hydrogen peroxide and chloride ions ( $Cl^-$ ) in a reaction catalyzed by myeloperoxidase (see Fig. 1). In reactions with protein residues, HOCl generates oxidized (disulfide, MetSOX, carbonyls, and dityrosine) and chlorinated products (chloramines, 3-chlorotyrosine, and 3,5-dichlorotyrosine) (Fig. 2).

Oxidative reactions by HOCl are several orders of magnitude faster than chlorination reactions and therefore more common under physiological conditions. However, the oxidative products can also be generated by other oxidants (Figs. 3 and 4), making chlorinated products specific biomarkers for HOCl mediated protein modifications. The most favored chlorination reaction of HOCl is with amine groups. However, formed chloramines with typical half-lives of about 10 min at  $37^\circ C$  are readily reduced back to parent amines by biological reductants (thiols, ascorbate, or methionine) or spontaneously broken down to aldehydes [3]. Chlorination of tyrosyl residues, although less favored than chlorination of amines,



**Fig. 2** Products of amino acids residues oxidation by hypochlorous acid (HOCl)

produce stable end products: 3-chlorotyrosine and to a much lesser extent 3,5-dichlorotyrosine, which can be used as biomarkers of HOCl generation [4].

## 2.2 Free Radicals Oxidation

Highly reactive free radicals, such as HO•, initiate oxidation by abstracting a hydrogen atom from an amino acid residue and generating carbon-centered radicals. These radicals react further with O<sub>2</sub>, producing peroxy radicals (see Fig. 3). If the peroxy radical is formed on an α-carbon it undergoes decomposition and forms an amino derivative, which spontaneously hydrolyses and yields two peptide fragments. Peroxy radicals formed on amino-acid side chains rearrange, through incompletely defined reaction pathways, into alcohols or aldehydes. Exposure of tryptophan to HO• results in indole ring opening and kynurenine formation, while histidine generates 2-oxohistidine, asparagine, and aspartate. In a reaction with tyrosyl residues free radicals generate tyrosyl phenoxy radicals. The major fate of these structures is self-dimerization and formation of di-tyrosine crosslinks. Oxidation of phenylalanine, on the other hand, produces *ortho*- (2-hydroxyphenylalanine) and *meta*-tyrosine (3-hydroxyphenylalanine) as well as tyrosine.

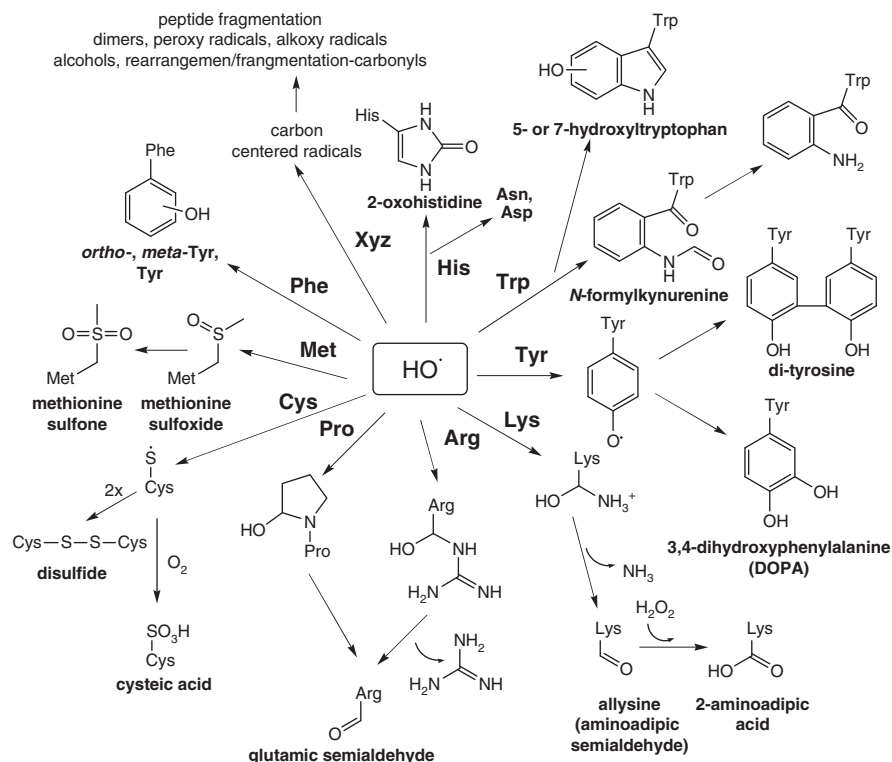


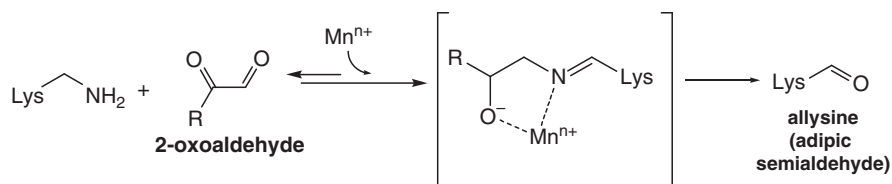
Fig. 3 Products of amino acids residues oxidation by hydroxyl radicals ( $\text{HO}^\bullet$ )

Stable modifications that are commonly measured *in vivo* include kynurenine, 2-oxohistidine, di-tyrosine, and o-tyrosine.

### 2.3 Metal-Catalyzed Oxidation (MCO)

Redox-active metal ions, such as Fe(III) and Cu(II), catalyze oxidative damage of proteins in the presence of oxygen and an electron donor or by generating hydroxyl or alkoxy radicals through Haber-Weiss and Fenton-type chemistry from hydrogen or organic peroxides, respectively. Highly reactive radicals, as described above, nonselectively abstract hydrogen. Basically, this reaction can occur at all available sites, though His, Arg, Lys, Pro, and to a lesser extent Met, and Cys residues are the most common. On the other hand, aromatic amino acid residues, Trp, Tyr, and Phe, are not the major targets for this oxidation system, most probably because these residues are not commonly present at metal-binding sites of proteins [5].

As discussed above, carbonyls can also be generated by Lys and Arg oxidation with HOCl. However, the overall yield of carbonyls through HOCl oxidation



**Fig. 4** Suyama's pathway of lysine deamination by  $\alpha$ -oxoaldehydes

represents a relatively low percentage of the total [6]. Taken together, protein-bound carbonyls are the major products of oxidation catalyzed by metal ions. Therefore, protein-bound carbonyls can be considered important markers of the MCO, with one notable exception. Allsine can be formed *in vivo* by lysine oxidation with lysyl-oxidase (particularly in collagen), as well as through  $\alpha$ -oxoaldehydes mediated Cu(II) catalyzed lysine deamination (Suyama's pathway) [7] (see Fig. 4). This latter possibility must be recognized in biologic settings characterized by significant participation of dicarbonyls, as in end stage renal disease (ESRD). The extent to which lysyl oxidase can oxidize proteins other than nascent collagen is unclear, if not doubtful [8].

A number of assays have been developed for the measurement of total carbonyl compounds in proteins. Although suitable for quantification of the carbonyls in simple oxidation systems, the assays are not accurate for proteins isolated from complex biological samples because of possible interference with protein-bound carbonyls generated by glycation/lipoxidation.

A better approach includes reduction of the carbonyls with  $\text{NaBH}_4$  into corresponding alcohols followed by protein hydrolysis and analysis by isotope dilution gas chromatography-mass spectrometry (GC/MS) [9, 10]. However, the carbonyl compounds are not the final oxidation products (AOPPs) since they are prone to further oxidation into corresponding carboxylic acids, either by auto-oxidation or by reaction with  $\text{H}_2\text{O}_2$  or  $\text{HOCl}$ . Thus, for the complete oxidation profile, parallel measurements of the carboxylic acid, such as 2-aminoadipic acid, should also be included [10].

## 2.4 Oxidation by Peroxynitrite ( $\text{ONOO}^-/\text{HONOO}$ )

The weak oxidants nitric oxide and superoxide, which are produced by several cell types, rapidly combine to form a strong oxidant – peroxynitrite. Due to nitric oxide's longer biological half-life and facile diffusion in comparison with superoxide, peroxynitrite generation will predominantly occur nearer to sites of  $\text{O}_2^-$  formation [11]. Peroxynitrite anion is relatively stable, but its acid, peroxynitrous acid ( $\text{HONOO}$ ), quickly rearranges to form nitrate. Two major mechanisms of oxidation by peroxynitrite and its acid are accepted. The first mechanism includes

homolysis of peroxynitrite and formation of  $\text{HO}\cdot$  and  $\cdot\text{NO}_2$ . This pair of radicals can either diffuse apart, giving free radicals that can perform oxidations, or react together to form nitrate or to reform peroxynitrite. Because hydroxyl-radical scavengers do not completely block peroxynitrite reactions, it is thought that the peroxide bond O-O in peroxynitrous acid ( $\text{HO}-\text{O}-\text{N}=\text{O}$ ) can result in a transfer of an  $\text{HO}^-$  or an oxygen atom by electron donors [12].

Peroxynitrite also promotes nitration of amino acid residues Fig. 5. Most notably, protein tyrosine residues represent the major targets for peroxynitrite-mediated nitrations where 3-nitrotyrosine represents a marker of such modifications. Other mechanisms of biological nitrations exist (such  $\text{H}_2\text{O}_2/\text{NO}_2^-$  heme peroxidase, reaction of  $\cdot\text{NO}_2$  and NO nitration with concomitant oxidation). However, these alternative reactions are more restricted than the nitration reactions mediated by peroxynitrite. Peroxynitrite reacts with carbon dioxide (ubiquitous in biological systems) to form  $\text{ONOOCO}_2^-$  (nitroso-peroxocarbonate). The latter homolyses further into a relatively strong one-electron oxidant carbonate radical and nitrogen dioxide – a moderate oxidant and nitrating agent. The net consequence of the reaction of  $\text{CO}_2$  on peroxynitrite is to reduce peroxynitrite half-life and distance of diffusion, effects that reduce one-electron oxidation and enhance nitration [11].

From a practical viewpoint, one of the most frequently determined markers of peroxynitrite activity is 3-nitrotyrosine [13]. Not considered here is the broad field of sulfhydryl nitrosylation, which has regulatory effects on protein function as well as stochastic effects [14].

In summary, several oxidative mechanisms lead to protein damage. It is clear that metal catalyzed oxidation cannot be considered entirely separately from other

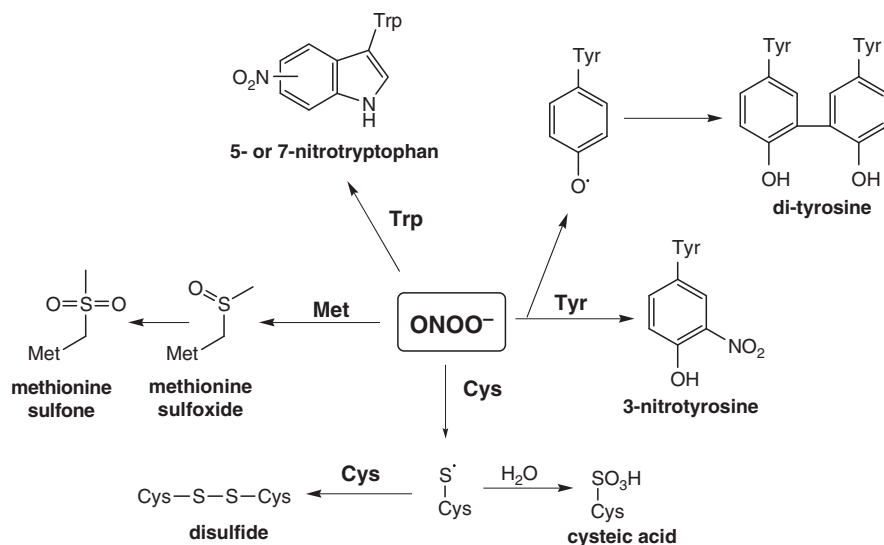


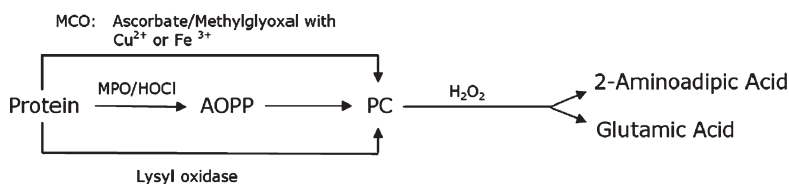
Fig. 5 Products of amino acids residues oxidation by peroxynitrite ( $\text{ONOO}^-$ )

oxidative mechanisms. Below, we present selected human and animal *in vivo* studies that help shape the “oxidative landscape” in renal disorders.

### 3 Protein Carbonyls (PCs) and Advanced Oxidation Products (AOPPs) in Renal Disorders

Much of the understanding of the role of AOPPs in renal disease comes from the pioneering work of Witko-Sarsat et al. [15]. The first assay was based on the notion that in renal disease lysines in serum proteins have been oxidized into chloramines by HOCl, which upon reaction with potassium iodide at acidic pH generates yellow tri-iodide,  $I_3^-$ . Therefore, a standard curve is produced using chloramine T, a widely used reagent for the radio labeling of proteins with the 125 or 131 isotopes of iodine. Since chloramines are not very stable and tend to decompose into protein carbonyls (see Figs. 2 and 6), it is not exactly clear what the assay measures nor is the exact nature of the color generated known, since multiple secondary reaction products might be expected. In the aggregate, however, yellow color is measured photometrically at 340 nm immediately after the reaction. This assay has been adapted for microtiter plates [15].

The “protein carbonyls” that are assayed in most publications reviewed below are all based on the reaction of protein with 2,4-dinitrophenylhydrazine (DNP). The resulting hydrazone is quantitated spectrophotometrically or using Western blotting with an anti-DNP antibody. Several methods have been developed based on this reaction [17]. Similar to the assay for AOPP, these methods are generally not specific, as they measure carbonyls from both oxidation and glycation. Mass spectrometry methods involving reduction of the protein with sodium borohydride to



**Fig. 6** Relationship between advanced oxidation protein products (AOPPs) and protein carbonyls (PCs). AOPPs are difficult to quantitate by chromatographic methods because they easily decompose into PCs. PCs can be assayed by the hydrazine method, which is not specific. The two major protein PCs formed during protein oxidation by metals are adipic semialdehyde (allysine) and glutamic semialdehyde. These compounds spontaneously oxidize into 2-amino adipic acid (2-AAA) and glutamic acid, respectively. (Note: glutamic acid is not a product of myeloperoxidase [MPO] or lysyl oxidation.) In highly oxidizing environments, PC levels may remain low because of rapid oxidation to 2-AAA and glutamic acid, respectively [16]. The presence of 2-AAA and the borohydride reduction products of allysine PC (i.e., 6-hydroxynorleucine) are evidence that lysine oxidation occurred. The relative importance of MCO, myeloperoxidase, and lysyl oxidase (if at all) in renal disease and ESRD remains to be determined. In contrast the finding of the borohydride reduction product of glutamyl semialdehyde (i.e., 5-hydroxy-2-aminovaleric acid) is specific for MCO

measure the alcohols of adipic semialdehyde (allysine) and glutamic semialdehyde have greater specificity [9, 10].

In the sections that follow, we will use the terms AOPPs and protein carbonyls (PCs), as they are used by the authors, without going into technical details as to what assay was used and which chemical entity was measured. The relationship between these products is summarized below:

### **3.1 Clinical Studies**

#### **3.1.1 Oxidation Markers in End Stage Renal Failure and Earlier Stages of Renal Disease**

Numerous compounds accumulate when kidneys fail, disturbing the function of multiple organ systems [18]. At the top of the list of organs systems affected by uremia is the heart and vasculature. Cardiovascular disease is the leading cause of death in all age groups of patients with ESRD [19]. In uremia, the association between traditional risk factors, such as high cholesterol and accelerated heart disease, is weak. Instead, cardiovascular complications correlate with markers of inflammation and malnutrition [20, 21] and the presence of a markedly increased oxidative burden [22]. In an otherwise puzzling pathogenetic process, oxidative stress has been proposed as the key evidence linking uremia to cardiovascular complications [23].

Direct measurements have been made of increased oxidative activity in the circulation of patients treated by hemodialysis using electron-spin resonance spectroscopy (ESR) and spin-traps. ESR activity is localized to the middle molecular weight range (~3,000 Da) of dialyzable toxins and is reduced significantly by dialysis [24]. Reactive oxidants have short half-lives, and direct measurements are difficult and cumbersome. Therefore, the devastation caused by oxidative stress is evidenced by extensive protein damage (i.e., thiol oxidation, amine oxidation, and carbonyl modification) (Table 1) present on circulating, cellular [30], and tissue proteins as well as lipids [22]. Because reactive oxidants have short half-lives, oxidative stress is better measured clinically as stable oxidized compounds, such as advanced oxidation (AOPPs) and glycation end products (AGEs), which some workers believe represent a form of uremic toxin [31, 32].

Exhaustion of antioxidant defense systems is a distinctive feature of uremia. For example, in patients with ESRD treated by hemodialysis, levels of the antioxidant ascorbate are low, and the ratio of reduced to oxidized ascorbate is several-fold greater than normal [33–35]. In addition to massive oxidant stress there is massive carbonyl stress [36], resulting in a five- to tenfold increase in dicarbonyl adducts to plasma proteins, such as those of glyoxal, methylglyoxal and 3-deoxyglucosone [37, 38]. Thus ascorbate oxidation and accumulation of metabolites such as

**Table 1** Representative plasma and human skin collagen levels of oxidation products

| Protein modification                       | Fluid           | Normal            | Diabetes              | ESRD              | Comments                            | References |
|--------------------------------------------|-----------------|-------------------|-----------------------|-------------------|-------------------------------------|------------|
| AOPP ( $\mu\text{mol/L}$ )                 | Plasma          | $29.4 \pm 4.9$    | –                     | $137.6 \pm 11.1$  | No correl. w. prot. conc.           | [15]       |
| AOPP ( $\mu\text{mol/L}$ )                 | Plasma          | $0.76 \pm 0.51$   | CRF: $13.73 \pm 4.45$ | $16.95 \pm 2.62$  |                                     | [25]       |
| 3-Chloro-tyrosine $\mu\text{mol/mol tyr.}$ | Plasma          | N.D.              | –                     | $3.5 \pm 0.8$     |                                     | [26]       |
| PC (nmol/mg)                               | Plasma          | $2.06 \pm 0.33$   | $2.96 \pm 0.48$       | $2.51 \pm 0.18$   |                                     | [27]       |
| PC (nmol/mg)                               | Plasma          | $0.093 \pm 0.014$ | $0.099 \pm 0.014$     | $0.16 \pm 0.050$  | Inversely related with PON1 in ESRD | [28]       |
| PC (nmol/mg)                               | Plasma          | $0.35 \pm 0.04$   | –                     | $1.23 \pm 0.50$   |                                     | [29]       |
| Alkanals (nM)                              | Plasma          | $1,407 \pm 286$   | –                     | $2,088 \pm 831$   |                                     | [29]       |
| Alkenals (nM)                              | Plasma          | $825 \pm 142$     | –                     | $2,453 \pm 821$   |                                     | [29]       |
| 4-OH-alkenals (nM)                         | Plasma          | $460 \pm 102$     | –                     | $1,732 \pm 598$   |                                     | [29]       |
| Free SH nmol/mg                            | Plasma          | $4.48 \pm 1.12$   | –                     | $2.69 \pm 1.23$   |                                     | [29]       |
| Prot-NH <sub>2</sub> (AU/mg prot)          | Plasma          | $287 \pm 163$     | –                     | $188 \pm 106$     |                                     |            |
| Allysine (mmol/mol lys)                    | Collagen (skin) | $0.05 \pm 0.3$    | $0.04\text{--}0.3$    | $0.8\text{--}0.4$ | Not age-dependent                   | [10]       |
| 2-Aminoadipic acid (mmol/mol lys)          | Collagen (skin) | $0.2\text{--}0.8$ | $0.2\text{--}2.0$     | $0.5\text{--}7.0$ | Strongly age-dependent              | [10]       |

dehydroascorbic acid and diketogulonic acid contribute to carbonyl stress and further increase the generation of AGEs [39].

Even patients with mild kidney disease show evidence of increased protein damage, i.e., decreased free sulfhydryl groups on proteins [25], oxidized low-density lipoprotein (LDL) [40], the buildup of advanced oxidation protein products (AOPPs) [41], and AGEs [42–44]. AGE-modified proteins are removed by glomerular filtration followed by tubular reabsorption and degradation [45–47]. Thus impaired renal function itself is an important mechanism of accumulation in renal disease. Mild chronic kidney failure is associated with impaired antioxidant defense systems as well [48]. A strong proof that systemic oxidant stress (and formation of AGEs) is dependent on renal function is that protein carbonyls and other plasma markers of oxidant and carbonyl stress increased following development of chronic allograft nephropathy, but decreased in complication-free transplant patients [49]. Similarly, in the Project to Improve Care in Acute Renal Disease (PICARD) study, plasma PCs and proinflammatory cytokines were increased in patients with acute renal failure (ARF), and protein carbonyls were higher in ARF than in critically ill and ESRD patients and controls [50].

In the presence of renal disease, oxidation reactions form one part of a vicious circle [51]. In turn, modified proteins induce oxidative stress through several mechanisms. At the cellular level, AGEs bind to specific receptors, activate intracellular signals (such as nuclear factor  $\kappa$ B [NF $\kappa$ B]) and stimulate cytokine release and inflammation [52, 53]. Neutrophils and other inflammatory cells produce numerous reactive oxygen species, including superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorous acid [54]. Thus oxidized proteins and lipids interact with inflammatory cells to generate reactive oxidants, particularly in the vascular endothelium [22].

In renal disease oxidized proteins are both the result of oxidative stress, and one of its major causes, but patterns of protein modifications differ in different disease settings. In a Chinese study of type 2 diabetes, erythrocyte antioxidants stress enzymes and plasma vitamin C were decreased while plasma indices of lipid peroxidation and AOPPs were increased, and were much higher in presence of nephropathy where they correlated with each other [55]. Conversely, while plasma protein carbonyls were found increased in ESRD, they were not in complication-free diabetes (see Table 1) [28]. This suggests that diabetes per se does not increase MCO. However, the more specific MCO marker 2-aminoadipic acid Fig. 6 was elevated in skin collagen from diabetic individuals even in the absence of renal failure, suggesting increased metal catalyzed oxidation in diabetes [10].

Likewise, serum levels of oxidized proteins were elevated in systemic lupus erythematosus (SLE), but not related to renal involvement (i.e., lupus nephritis) [56]. Thus, both in lupus and diabetes there may be an independent systemic effect, one linked to an autoimmune process and hyperglycemia, respectively, the other linked to severity of the renal disease. In further support of a dual mechanism for oxidation, increased serum levels of AOPPs and AGEs were found in patients with amyloid A-related rheumatoid arthritis compared to rheumatoid arthritis patients without amyloidosis, whereby AGE levels were related to kidney function but

AOPPs were not [57]. In another study, protein carbonyl formation was strongly dependent on renal function, but not on essential hypertension alone [58]. This surprising dichotomy may mean that protein carbonyl formation in renal disease proceeds primarily via MCO rather than via MPO and AOPP (Fig. 6). However, when ESRD is reached, both MCO and MPO are expected to contribute to protein PCs and 2-aminoadipic acid, whereby  $H_2O_2$  is a dramatic catalyst for the latter [16].

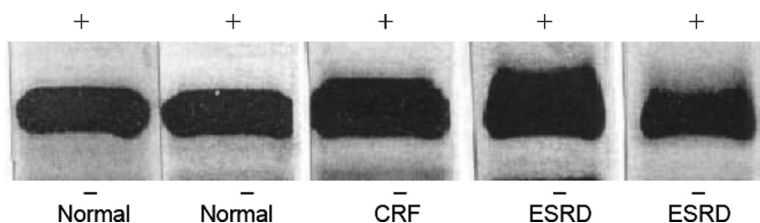
### 3.1.2 Relationship Between Oxidation and Complications in ESRD

Patients with ESRD experience an increased risk of cardiac complications, more than 10–20 times the incidence of the general population [59]. Although many patients start dialysis with conditions that predispose to atherosclerosis (such as diabetes mellitus, hypertension, or advanced age), these classic cardiac risk factors are insufficient to explain the extraordinary prevalence of cardiovascular disease and death in uremia [60].

Dialysis patients appear to be chronically in higher inflammatory states, compared to normal subjects, as suggested by high serum levels of C-reactive protein (CRP) [61] and pro-inflammatory cytokines [62, 63]. While inflammation is a source of oxidative stress [64] and high serum CRP levels have been associated with higher mortality in dialysis patients (including those without documented ischemic heart disease) [65], it is unclear which inflammatory mechanisms are involved and how they are related to specific types of clinical cardiovascular disease (CVD) (e.g., coronary artery disease vs. cardiomyopathy).

Specific protein modifications have been associated with the pathophysiology of uremic complications, including arterial calcification [44, 66, 67], carotid atherosclerosis [68], and cardiac hypertrophy [68, 69]. In ESRD, plasma protein carbonyls and oxidant stress markers were elevated in symptomatic chronic heart disease (CHD) patients compared to those without [70]. Carotid artery intima-media thickness (CMT), serum PCs, and markers of oxidant stress are significantly increased in hemodialysis patients, but only serum thiobarbiturate reactive substances (TBARS) and serum nitrite/nitrate levels actually correlated with CMT [71]. These observations suggest that MCO in the blood poorly reflects the process in the artery.

Albumin is a major protein target of oxidative activity in uremic plasma as well as a critical defense against oxidative stress [72]. When inflammation or malnutrition leads to low serum albumin levels in renal disease, this protection is impaired or lost [73]. Patients with low albumin levels develop marked increases in the peroxidation of erythrocyte membrane lipids [30]. How sick albumin is in ESRD is exemplified in Fig. 7, which depicts a simple serum protein electrophoresis in which the albumin band is thickened and blurry because of damage by oxidation and glycation. Such albumin from ESRD patients has impaired drug-binding properties [74]. Albumin from hemodialysis (HD) patients was found to have not only decreased antioxidant activity, to be more oxidized, negatively charged, and conformationally altered, but also triggered oxidative burst when incubated with



**Fig. 7** Appearance of the albumin band from patients with chronic renal failure or end stage renal disease (ESRD) in non-denaturing serum protein electrophoresis in agarose gel. The band is thickened and streaks toward the anode, indicating increased negative charge due to oxidative deamination, CML formation, and loss of arginines from modification by dicarbonyl compounds

human neutrophils [27]. Also, some of the compounds that impair albumin activity appear to be extractable with charcoal [75]. A comprehensive review of the impact of albumin oxidation and other modifications on its function is available elsewhere [76].

From the above, it is not surprising that mortality risk is inversely related to albumin levels [77]. In that regard, plasma PCs and inflammatory stress markers are worse in hypo- than normo-albuminemic patients on HD [78], and it is the combined oxidant and carbonyl stress that is thought to be an important event in the pathogenesis of atherosclerosis [79, 80], the leading cause of morbidity and mortality in uremic patients [81].

Finally, only few efforts have been made to map the target amino acids of oxidative plasma protein damage in patients on hemodialysis. Besides the studies on albumin mentioned above, Alhamdani et al. found elevated levels of plasma alkanals and other lipid peroxidation products [29]. Using GC/MS these authors succeeded in identifying and quantifying up to 11 alkanals, alkenals, and hydroxyl-alkenals in plasma, in addition to the other already known modifications (see Table 1).

### 3.1.3 Interventions That Decrease Plasma Oxidation Products in Humans

#### Dialysis Therapy

Multiple compounds accumulate when kidneys fail. Because dialysis is not selective, uremic toxins are classified commonly by their molecular weight [18, 82]. Dialysis removes solutes by diffusion, convection, or adsorption onto the dialyzer membrane surface. Blood levels of markers could be affected directly by any of these mechanisms, with measurable changes occurring during the hemodialysis session, or indirectly by removal of other solutes that are precursors of their metabolic pathways (e.g., glycation pathways) [37, 83]. These indirect effects could occur during the HD session or over longer intervals, depending on the kinetics of chemical reactions and biological events that affect biomarker levels.

Beta-2 microglobulin ( $\beta_2\text{M}$ ) (11,800 Da) is a convenient marker of molecular size because it can be readily measured and has relevance in the pathogenesis of dialysis-related amyloidosis [84, 85]. Numerous studies over the past decade have shown that high-flux dialysis membranes are associated with higher clearances of  $\beta_2\text{M}$  than low-flux membranes [84, 86]. Importantly,  $\beta_2\text{M}$  is rendered more toxic by oxidative modification to form AGE-modified  $\beta_2\text{M}$  [87].

Predialysis circulating levels of protein-bound AGEs have been reported to be lower in patients treated with high-flux HD than those treated with low-flux HD [37, 88, 89]. In a cross-over study, protein-bound pentosidine (by high-performance liquid chromatography [HPLC]) and AGE-modified  $\beta_2\text{M}$  levels were reduced by long-term HD ( $\geq 6$  months) with high-flux membranes. High flux (F60S, Fresenius) caused a slight to moderate decrease, in contrast to the more pronounced effect of superflux F800S [90]. A recent study used protein-leaking dialyzers (MW cut-off  $> 70$  kDa) and demonstrated further decreases in albumin-bound pentosidine levels. Interestingly, these decreases were correlated with significant improvements in levels of inflammatory cytokines (interleukin [IL]-1 $\beta$ , IL-6, tumor necrosis factor [TNF]  $\alpha$ ), oxidative markers (AOPP, protein carbonyls), and immune-modulating cytokines (IL-10 and interferon [INF]  $\gamma$ ) [91].

AGEs are difficult to study in clinical settings because (a) they are chemically and biologically diverse [92, 93], and (b) AGE assays by immunologic methods (e. g., enzyme-linked immunosorbent assay [ELISA]) are not directly comparable to analytic methods (HPLC, liquid chromatography-mass spectrometry [LC/MS]) [88]. In addition, after gastrointestinal digestion, AGEs and glycation adducts present on proteins in food are absorbed as low molecular weight (LMW) or free adducts, which can be removed by HD [94, 95]. Because of the variability in epitope structure and antibody binding characteristics, it is likely that ELISA more readily detects dietary free and LMW AGEs, while underestimating the total AGE content of serum proteins [88]. Thus, while ELISA methods are widely used, analytic chemistry methods are considered the standard of accuracy for detecting AGE proteins [88].

These points are illustrated by two studies of HD patients that yielded apparently contradictory findings. In a Swedish study of 90 patients, pentosidine measured by HPLC positively correlated with inflammatory markers (CRP, fibrinogen, and IL-6), although there was no association between pentosidine and survival [96]. In a German study, baseline AGEs were measured using an ELISA, with the result that higher AGE levels were paradoxically associated with better survival [97]. A potential explanation of the results of the second study is that the intake of highly glycated (better-tasting) food leads to better nutrition and better survival. Food scientists were the first to take interest in the chemistry of AGE formation [98]. In general, foods cooked at high temperatures undergo more “browning” reactions [99], and tasty food has a high content of aromatic, flavorful AGE compounds. Foodstuffs high in AGEs and AGE precursors undergo proteolysis in the intestine and are absorbed as LMW fragments termed glycotoxins [99]. After a meal high in AGEs, an increase in circulating LMW AGEs is readily detected by ELISA [53, 99]. In the absence of kidney clearance, these products accumulate in the circulation

[100]. Thus, patients who have high dietary protein intake would also be expected to have higher circulating levels of LMW AGEs as detected by ELISA [97].

Although dialysis removes filterable low molecular compounds that may have toxicity, dialysis itself can trigger oxidant stress, therefore acting against the desirable effect. In one study protein carbonyls were not decreased immediately after dialysis in contrast to dialyzable molecules such as malonyldialdehyde [101]. Dursun et al. measured protein carbonyls in patients with diabetes and ESRD patients before and after hemodialysis [102]. Baseline plasma protein carbonyls were increased in diabetic patients and further increased in diabetic ESRD patients. Surprisingly, the dialysis procedure contributed to further increase in protein carbonyl levels. The highest levels were observed in patients with diabetes after dialysis. Simultaneously dialysis improved depressed protein-SH levels in patients treated by hemodialysis. Similar observations were made by Ward et al. who found that neutrophil reactive oxygen species (ROS) production was increased in the first 30 min of dialysis with high flux membranes, but then decreased postdialysis [103]. Though protein-SH normalized postdialysis, there was no change in PCs and AOPPs.

These results are best interpreted as follows: protein-SH oxidation is strongly dependent on ROS production and highly reversible. In contrast in a recent study, our laboratory showed that  $H_2O_2$  was not a requirement for protein carbonyl formation via the MCO [16]. However, oxidation of lysine by either MOP/HOCl or metals is quasi-irreversible. Thus, it is likely that dialysis decreases ROS species responsible for SH oxidation, but not those species inducing MCO, including peptide-bound redox active metals.

## Antioxidants and Other Drugs

Two small randomized trials respectively suggested the beneficial effect of antioxidants, vitamin E, and *N*-acetylcysteine on CVD outcomes in HD patients [104, 105], in contrast to the lack of benefit shown in large prospective studies in the general population [106, 107]. Thus proponents of antioxidant therapy argue that greater benefit might be found in the setting of abnormal levels of oxidative stress, such as in patients with ESRD [108]. Indeed, protein and DNA oxidation was decreased in hemo- and peritoneal dialysis patients treated with 300 mg vitamin E [109]. Yet, in hemodialysis patients treated with  $\alpha$ -tocopherol, circulating levels of anti-inflammatory  $\gamma$ -tocopherol decreased markedly. There were no net changes in oxidative protein modifications (including pentosidine, iso(4)-levuglandin E2 and (E)-4-oxo-2-nonenal) [110].

Further evidence of dissociation between effects of inflammation and oxidation was revealed when  $\gamma$ -tocopherol was administered together with docosahexaenoic acid to hemodialysis patients. Plasma IL-6, white blood cell (WBC), and neutrophil counts decreased. But CRP, F2 isoprostanes, and protein carbonyls remained unchanged. Moreover, hemodialysis per se did not influence plasma levels of protein carbonyls, CRP, IL-6, and IL-10 [111]. Similarly, in patients with advanced

diabetic nephropathy, the hypoglycemic agent and peroxisome proliferator-activated receptors (PPAR) agonist pioglitazone improved plasma inflammatory markers, such as  $\text{TNF}\alpha$ , but not protein carbonyls, suggesting that inflammation and oxidation pathways have independent effects [112].

We conclude that in patients with renal disease, the balance of oxidant/antioxidant systems is highly complex. Both chemical pathways and inflammatory components appear to be involved in the formation of glycoxidation and protein-bound lipid oxidation products [110].

### Transplantation

Rapid decreases in PC and F2 isoprostanes and levels of pro-inflammatory proteins IL-6,  $\text{TNF}\alpha$ , and CRP were observed upon renal transplantation in ESRD patients [113]. Yet surprisingly, in the 24-h period following renal transplantation, plasma protein carbonyls and lipoxidation markers increased due to ischemic stress. At 7 days' posttransplant, lipoxidation markers decreased while the protein carbonyls further increased [114]. Nevertheless, most studies show that renal transplantation is the most efficacious long-term intervention for decreasing AOPPs, AGEs, and proinflammatory markers, especially with normalization of renal function [115–118].

## 4 Metal Catalyzed Oxidant (MCO) Stress in End Stage Renal Disease and the Impact of Iron Supplementation

What is the source of increased oxidative activity in uremia? As reviewed above, current evidence points to the retention of oxidative compounds as renal function deteriorates. Once oxidative damage is extensive, cause and effect become much more difficult to distinguish from each other. The systemic response to oxidative damage results in accelerated atherosclerosis, and the resultant chronic inflammation leads to malnutrition [22, 23]. When malnutrition leads to low albumin levels, a critical defense against oxidative stress is lost [73].

To complicate the picture, dialysis activates production of reactive oxidants by inflammatory cells directly [119], and it leads to peroxidation of lipids [120]. Capeillere-Blandin et al. investigated whether their original data on AOPPs/protein carbonyl formation could be explained by myeloperoxidase [121]. Incubation of albumin with this enzyme in the presence of  $\text{H}_2\text{O}_2/\text{Cl}^-$  lead to chlorination of the protein, suggesting this oxidizing system could explain the data in ESRD patients. Myeloperoxidase, a hemoprotein stored in the azurophilic granules of neutrophils and macrophages, functions to catalyze the conversion of chloride and hydrogen peroxide to hypochlorite. Clinical studies have demonstrated the predictive value of directly measured myeloperoxidase for the presence of coronary artery disease in the general population [122].

Activation of neutrophils to release pro-oxidant molecules is an important concern [123]. However, it is generally accepted that the oxidative burst is transient in dialysis using biocompatible dialysis membranes [124]. Therefore, the net effect of dialysis is to reduce the production of oxidants [103] and to improve reversible biomarkers of oxidation (e.g., high-flux dialysis regenerates free sulfhydryl groups on albumin) [25, 103, 125]. These observations suggest that in the clinical setting dialyzable compounds with oxidative activity are the most important cause of oxidative stress in renal failure.

Oxoaldehydes like methylglyoxal are five- to tenfold elevated in ESRD and can oxidize lysine by the Suyama pathway [126]. This pathway involves complex formation between  $\text{Fe}^{3+}$  or  $\text{Cu}^{2+}$  and a methylglyoxal-lysine adduct leading to allysine formation. Moreover, the same damage results from the traditional Stadtman pathway of MCO. Previously, in patients with ESRD, we elucidated the mechanisms leading to accelerated formation of two distinct structurally identified AGEs: the glycooxidation products  $\text{N}^{\epsilon}$ (carboxymethyl)lysine (CML) and pentosidine [37]. Though formation of these two modifications proceeds by different pathways, it is markedly enhanced by metal catalyzed oxidation reactions. Conversely, proteins with high AGE content readily bind redox active metal in a dose-dependent fashion [51, 127, 128], taking up three to four times as much iron or copper as in the unglycated state [127]. In the healthy state, metal ions are tightly sequestered in the functional core of hemoglobin and enzymes, or stored and transported on specific binding proteins. In addition, a biologic defense exists against their action as hydroxyl radical generators through the Fenton reaction [129]. In contrast to physiologic iron-binding proteins like transferrin, “glycochelates” are highly oxidative, leading to a vicious circle of increased AGE formation via oxidative pathways [127, 130, 131].

Therapeutic infusion of iron plays an important role in the management of anemia in patients with ESRD. During and immediately after infusion, temporary oversaturation of transferrin is seen, resulting in transient release of redox-active labile iron [132–135]. Increased levels of free iron induce oxidative stress [136], causing oxidation of circulating and cellular lipids [137] and proteins [138], with an increase in plasma MDA, a decrease in nonprotein SH groups [139], and increased albumin protein carbonyl formation and sulfhydryl oxidation [140].

Tovbin et al. similarly reported that intravenous (IV) iron saccharate induced a 37% increase in AOPP level, which correlated positively with prehemodialysis CRP level and was  $\text{TNF}\alpha$  related [141]. Intravenous iron administration did not affect tetraethylammonium chloride (TEAC), thiol, dityrosine, or  $\text{TNF}\alpha$  levels. These authors concluded that intravenous iron administration induces an increase in protein oxidation (AOPP levels) that was related to the baseline degree of inflammation. At present the long-term dangers of iron infusion and the safest formulations, dosages, and strategies to minimize iron toxicity remain undefined in ESRD [142–144].

These observations raise the question of how to distinguish between the oxidative toxicity of metal catalyzed and myeloperoxidase pathways of oxidation. For example, in one study, peroxide concentrations significantly increased during HD but were not correlated to IV iron administration and seemed to result from

other sources of oxidative stress related to HD [145]. Cavdar et al. [146] observed that IV administered iron in 20 and 100 mg doses did not cause additional deteriorating effect on oxidant stress (plasma MDA) and EDEF (erythrocyte deformability) and was even improved by IV iron. Finally, Driss et al. conducted a study on the effects of intravenous polymaltose iron on oxidant stress and nontransferrin-bound iron (NTBI) in hemodialysis patients [147]. The serum iron and transferrin saturation index increased during iron infusion and NTBI transiently appeared in some patients, but markers of oxidative stress were not significantly affected. They concluded that although ESRD patients have a high prevalence of NTBI in their serum, no correlation could be established between the presence of NTBI and an increased oxidative stress. The slow infusion of maltofer did not promote a significant increase in the plasma concentration of oxidative stress markers.

The apparent quandary may be best explained based on differences in the concentration of infused iron. Michelis et al. determined carbonyls on fibrinogen in hemodialysis patients on maintenance iron [138]. During a dialysis session, carbonyls further increased when 125 mg but not when 62.5 mg or no iron gluconate was administered. The changes in fibrinogen-PC during dialysis showed a significant linear correlation with the calculated values of transferrin saturation and free transferrin.

## 5 Conclusions and Therapeutic Implications

The first and foremost impression emanating from this review is that plasma protein oxidation remains low if not negligible as long as renal function is not impaired, whether acutely or chronically. There may be some exceptions for diabetes and septicemia and any other massive ubiquitous systemic stress such as SLE and amyloidosis. However, even in these conditions minor impairment of renal function contributes to protein oxidation. It is also well recognized that chronic renal failure and end stage renal disease are associated with massive combined oxidant and carbonyl stress and various specific AGEs, and oxidation products have been shown to be markedly increased in ESRD (see Table 1).

The more difficult question raised by this review is the relative importance of two disparate chemical mechanisms. To what extent is the formation of AOPPs related to MPO/HOCl, and to what extent is the formation of protein carbonyls directly related to metal catalyzed oxidation or to MPO? There is not only a need to confirm the molecular nature of AOPP by mass spectrometric methods, but also to determine the relative roles of myeloperoxidase vs. redox active metals in the formation of PCs. A strong though circumstantial evidence for MCO is the fact that ESRD serum and dialysis fluid specimens easily glycoxidize *in vitro* [37]. However, myeloperoxidase activity is increased in all renal failure patients, and there is more activation and “priming” of leukocytes in patients treated

by hemodialysis [148]. In addition, there is an increase in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase dependent superoxide formation [149].

Some clues might come from the administration of vitamin E. Alpha-tocopherol is a known inhibitor of lipid peroxidation. However, there is a paradoxical effect in that both catalytic metals and lipid peroxides can enhance  $\alpha$ -tocopherol oxidation by a similar mechanism, possibly explaining the inability of  $\alpha$ -tocopherol to prevent secondary modification of serum proteins by iso[4]-levuglandin E2, 4-hydroxynonenal (HNE), and 4-oxo-2-nonenal (ONE) [110]. As for MPO  $\alpha$ -tocopherol was also ineffective against MPO-mediated oxidation and peroxynitrite. Kamgar et al. [150] treated HD patients with a cocktail of vitamins including vitamin E and observed no decrease in PCs. However, since they included 250 mg vitamin C, a classical substrate for MCO, they might have induced rather than suppressed MCO. Nevertheless, the results were similarly negative with  $\gamma$ -tocopherol [151]. In view of the fact that tocopherol is primarily an antioxidant in the lipid phase, while MCO occurs primarily in the aqueous, the most efficient way to differentiate between MPO and MCO will be to carry out interventions with chelating agents, such as desferrioxamine or iron. Although desferrioxamine has been used to treat aluminum overload in ESRD, we did not come across studies on the impact of chelators on PCs in ESRD. Such studies are urgently needed.

Thus far, currently the most efficient therapy to decrease oxidant stress, besides renal transplantation, appears to be the utilization of high flux dialysis membranes. The fact that they decrease levels of methylglyoxal [152], a cofactor for MCO via the Suyama pathway, provides support for this mechanism of protein damage in ESRD. However, current dialysis modalities are altogether still insufficient for the elimination of the AGE, PC, and AOPP burden that is in part responsible for the complications of uremia.

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