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REACTIVE CARBONYL FORMATION BY OXIDATIVE AND NON-OXIDATIVE PATHWAYS

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1. ABSTRACT

The spectrophotometric protein carbonyl assay is used as an indicator of protein damage by free radical reactions *in vitro* and in a variety of pathologies. We investigated model proteins and a variety of oxidative and non-oxidative reactions, as well as what effects hemoglobin, myoglobin, and cytochrome c might have on levels of protein carbonyls. We show that oxidative as well as non-oxidative mechanisms introduce carbonyl groups into proteins, providing a moiety for quantification with 2,4-dinitrophenylhydrazine (DNPH). Bovine serum albumin exposed to oxidative scenarios, such as hypochlorous acid, peroxynitrite, and metal-catalyzed oxidation exhibited variable, but increased levels of carbonyls. Other non-oxidative modification systems, in which proteins are incubated with various aldehydes, such as malondialdehyde, acrolein, glycolaldehyde, and glyoxal also generated significant amounts of carbonyls. Furthermore, purified myoglobin, hemoglobin, and cytochrome c show high absorbance at the same wavelengths as DNPH. The high levels observed are due to the innate absorbance of hemoglobin, myoglobin, and cytochrome c near the assay spectra of DNPH. These studies show that carbonyl content could be due to oxidative as well as non-oxidative mechanisms and that heme-containing compounds may effect carbonyl quantification.

2. INTRODUCTION

Oxygen free radicals are produced as byproducts of many endogenous and exogenous sources including UV light, radiation, neutrophil activity, and metabolism (1-4). Oxidative stress often leads to lipid, nucleic acid, carbohydrate, and protein modifications. Protein oxidation and modification have been shown to increase during aging and exacerbate many pathological processes, such as atherosclerosis and human cataracts (1, 3-5). Oxidation of proteins can lead to the formation of oxidized amino acids, such as dityrosine, 3-nitrotyrosine, 3-chlorotyrosine, oxohistidine, and altered amino acid side chains containing reactive carbonyls (2, 6-12). Oxidation of proteins can result in the loss of catalytic function, increased sensitivity to denaturation, and increased susceptibility to proteolysis (1-3, 13).

The accumulation of oxidized proteins is often measured by the content of reactive carbonyls (3, 14). Spectrophotometric assays for the appearance of carbonyl groups on proteins have been developed to quantify oxidative protein damage using 2,4-dinitrophenylhydrazine (DNPH) (15-17). Moreover, protein carbonyls can be measured by antibodies to DNPH by immunoblot analysis (18, 19). However, the spectrophotometric methods using DNPH still remain one of the primary ways to identify reactive carbonyl groups on proteins (15, 16) since this assay is convenient, fast, and inexpensive.

One major pathway believed to generate protein carbonyls *in vivo* is the metal-catalyzed protein oxidation pathway (3, 5, 14). However, it is still unclear whether this pathway has relevance in biological oxidation *in vivo* (20, 21). Metal-catalyzed oxidation *in vitro* is a source for carbonyl formation, a post-translational covalent modification of proteins (3, 5, 14), but this is only one pathway out of many generating carbonyls. Stadtman *et al* propose that metal ions such as copper and iron bind to

site-specific binding sites on amino-acid residues and react with oxidants, transforming side-chains of the amino acids into carbonyls (3, 5, 14).

Recently, it has become evident that metal-catalyzed oxidation is only one of many pathways generating carbonyls. Colleagues found that oxidative as well as non-oxidative pathways can generate carbonyls in proteins, through primary and secondary modification reactions (6, 17, 22-27). Primary modification results in alteration of the original structure of the amino acid from metal-catalyzed oxidation, radiation-mediated oxidation, oxidation by ozone or nitrogen oxides, and other strong oxidants, such as hypochlorous acid, and peroxynitrite. Secondary modification occurs when specific products progressively and covalently react with proteins integrating a moiety which is reactive with DNPH. Carbonyl groups may be introduced into proteins by the aforementioned reactions through enzyme glycosylation, non-enzymatic glycosylation, and ketoamines (2, 5, 13, 15). In addition, enzymatic modification of proteins has also been recognized as a major contributor to protein damage and possibly carbonyl formation (2, 8, 15).

In the current study, we investigated several biologically relevant oxidative and non-oxidative pathways that could potentially generate protein carbonyls, *providing* a moiety which could react with DNPH. We used several well-characterized model proteins, such as bovine serum albumin, ribonuclease A, and low-density lipoproteins to determine the extent of carbonyl formation. We modified these proteins by metal-catalyzed oxidation, aldehyde incorporation, hypochlorous acid oxidation, and peroxynitrite oxidation. By using the DNPH assay we found that all model proteins exposed to both oxidative and non-oxidative pathways formed reactive carbonyls. Carbonyl levels in isolated proteins showed extensive variation with both oxidative and non-oxidative pathways. In addition, heme-containing proteins, such as myoglobin, hemoglobin, and cytochrome c show a very similar absorbance spectrum compared to DNPH, and this may effect protein carbonyl determination.

3. MATERIALS AND METHODS

3.1. Materials, reagents, and isolation of proteins

Hemoglobin (from bovine blood), myglobin (from horse heart), cytochrome c (from bovine heart), bovine serum albumin (BSA; fatty acid free) and Ribonuclease A (Rnase; bovine pancreas) were obtained from Sigma (St. Louis, MO). Unless otherwise indicated, all other reagents were obtained from either Sigma or Aldrich Chemical (Milwaukee, WI) and all organic solvents were HPLC grade. Hemoglobin was isolated from the red blood cells of a healthy volunteer. Whole blood was immediately centrifuged at 3,000 g for 5 min and the plasma was extracted and retained on ice. Red blood cells were immediately frozen at –80ºC for 10 min and then allowed to thaw. The lysed blood cells were re-spun at 3,000 g for 5 min. The supernatant, which contains hemoglobin, was extracted and retained on ice. The protein concentration of the lysed cell supernatant and plasma protein were determined using the Bradford assay. Low density lipoprotein (LDL) (d = 1.018-1.063 g/ml) was isolated by sequential density ultracentrifugation from plasma (1 mg/ml EDTA) prepared from normolipidemic, healthy volunteers and extensively dialyzed against phosphate buffer (50 mM phosphate, 0.1 mM diethylenetriaminepentaacetic acid (DTPA), pH 7.4) before the experiments (11).

3.2. Metal-catalyzed protein oxidation

Proteins were incubated at 37ºC in buffer A (50 mM phosphate buffer pH 7.4) supplemented with 0.2 mM $CuSO_4$ and hydrogen peroxide (H_2O_2). The final volume in all experiments was 1 mL, the concentration of oxidants is indicated in each experiment, and the protein concentration was 1 mg/mL in each experiment. The concentration of H_2O_2 was determined spectrophotometrically at 240 nm (molar absorptivity$_{240}$ = 0.0394 mM^{-1} cm^{-1}). Reactions were stopped after 2h by the addition of 1 microliter of 50 mM DTPA and 1 microliter of 300 nM catalase to 1 mL of reaction mixture followed by acid precipitation of protein with ice-cold trichloroacetic acid (TCA; final concentration 10% v/v).

3.3. Protein modification by aldehydes

BSA was incubated with acrolein, glyoxal, glycoaldehyde, formaldehyde, or malondialdehyde (MDA) for 24 hours at 37ºC in Buffer A. Experiments were stopped by ice-cold TCA precipitation (final concentration 10% v/v).

3.4. Protein oxidation by hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$)

Proteins were incubated at 37ºC in buffer A with HOCl or with $ONOO^-$ alone or with HOCl and nitrite. Hypochlorous acid (pK_a = 7.5) is present as both hypochlorite (OCl^-) and hypochlorous acid at physiological pH. The concentration of (OCl^-/HOCl) acid in a dilution of sodium hypochlorite (NaOCl 4-6% purified grade) solution was determined spectrophotometrically at 290 nm (pH 12, molar absorptivity$_{290}$ = 350 M^{-1} cm^{-1}). Following addition of HOCl, samples were immediately vortexed. In nitrite/HOCl experiments, nitrite was added first, immediately followed by HOCl in equal molar concentration. $ONOO^-$ was synthesized from 2-ethoxyethyl nitrite and H_2O_2 as described previously (12, 28). The $ONOO^-$ stock prepared in 0.1 M NaOH (stored at -80ºC) was thawed just before the experiment and the concentration was measured spectrophotometrically at 302 nm (molar absorptivity$_{302}$ = 1,670 M^{-1} cm^{-1}). Experiments were stopped by ice-cold TCA precipitation (final concentration 10% v/v).

3.5. Determination of reactive carbonyls in proteins

Carbonyls were analyzed according to Levine *et al* (15) with slight modifications. Protein was precipitated with ice-cold trichloroacetic acid (final concentration 10% v/v). After a 10-min incubation period at 4 ^{0}C, samples were centrifuged at 11,000 g for 3 min. The protein pellet was re-suspended in 0.5 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) / 2 M HCL. Samples were placed in a sample holder and vortexed continuously at

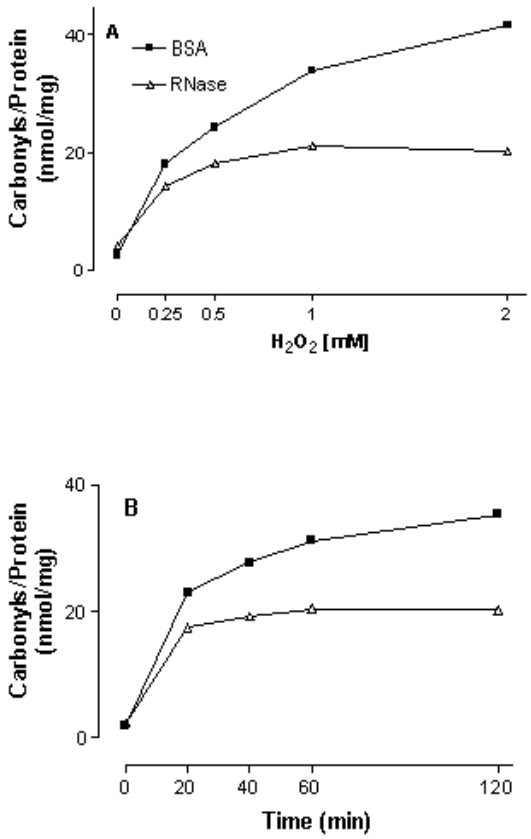

Figure 1. Carbonyl formation in BSA oxidized by $H_2O_2/CuSO_4$. (**A**) BSA (1mg/mL) was incubated with $CuSO_4$ (0.2 mM) and the indicated concentrations of H_2O_2 (0-2 mM) for 2h at 37^0C. (**B**) BSA was incubated with H_2O_2 (2mM) and $CuSO_4$ (0.2mM) at 37°C in 50 mM phosphate buffer pH 7.4 for the indicated time (0-2h). The reaction was terminated using DTPA (100 µM), catalase (300nM), and by TCA (10% v/v) precipitation. Protein carbonyls were then determined as described in Methods. The same pattern of oxidation was produced in each of at least two independent experiments using triplicate samples.

room temperature for 1 hour. The samples were precipitated with 0.5 mL of 20% TCA, centrifuged at 11,000 g for 3 min. The pellet was washed with 1 mL of ethanol-ethyl acetate (1:1;v/v) to remove free DNPH reagent, and allowed to stand for 10 min. The sample was centrifuged for 5 min at 11,000 g and the supernatant was discarded. The washing procedure was repeated two times for a total of three washes. The resulting protein pellet was resuspended in 1.0 mL of 6 M guanidine with 2 mM potassium phosphate buffer (pH 2.3, adjusted with trifluoroacetic acid). The samples were incubated at 37°C for 15-30 min to aid dissolution of the protein. Samples having difficulties going into solutions were briefly sonicated and incubated at an increased temperature of up to 70 °C. All samples were then centrifuged to remove any insoluble material remaining in suspension. The concentration of DNPH was determined at its maximum wavelength (360 nm) and the molar absorption coefficient of 22, 000 M^{-1} cm^{-1} was used to quantify the levels of protein carbonyls. Samples were spectrophotometrically analyzed against a blank of 1 mL of guanidine solution (6 M guanidine with 2mM potassium phosphate). Protein concentration was determined in the samples by determining the absorbance at 280 nm. Protein carbonyl content was expressed as nmol/mg.

4. RESULTS

4.1. Metal-catalyzed oxidation

Initially we used a well-characterized metal-catalyzed oxidation system ($CuSO_4/H_2O_2$) to show the yield of reactive protein carbonyl formation with our assay. This system has been extensively used and raises carbonyl levels significantly (14). As figure 1A shows, the metal-catalyzed oxidation system using $CuSO_4$ (0.2 mM) and the indicated final concentration of H_2O_2 generated reactive carbonyls in BSA. In contrast, the same amount of oxidant generated significantly lower levels of carbonyls in RNase. Thus, RNase exhibited greater resistance to modification by the metal-catalyzed oxidation system, as well as to other chemical modification experiments (see figure 2). Carbonyl formation was concentration and time dependent in both BSA and RNase (figure 1B). Metal-catalyzed oxidation of carbonyl formation was rapid within the first 15 minutes with both proteins and reached a plateau after 120 min.

4.2. Carbonyl formation by non-oxidative mechanisms

Next, we used commercially available aldehydes to test their ability to generate carbonyls in proteins. We used several biologically relevant aldehydes, e.g., malondialdehyde, acrolein, glyoxal, and glycoaldehydes to generate carbonyls (figures 2 and 3). We first investigated malondialdehyde (MDA), a well-characterized end product of lipid peroxidation. MDA at several concentrations generated significant amounts of reactive carbonyls in BSA after 24 hours (figure 2A), but significantly less in RNAse. Furthermore, we showed that MDA modification of BSA occurred within minutes (figure 2B), while MDA modification of RNAse was not significantly changed after 3 hours. The levels of carbonyls formed using MDA (30 mM) as compared to the MCO system (2 mM H_2O_2 and $CuSO_4$ 0.2 mM) after 2 hours was 2-fold greater.

We further investigated other aldehydes and their ability to generate carbonyls (figure 3). Acrolein was the most reactive, increasing protein carbonyl levels by 3-fold as compared to other aldehydes using 1 mM concentrations. In addition, carbonyl formation due to aldehyde incorporation was extremely efficient and for some aldehydes, significantly greater than that due to metal-catalyzed oxidation. In other experiment, we have shown that increases in carbonyls with both acrolein and malondialdehyde can occur within minutes, whereas modification with glyoxal, formaldehyde, and glycoaldehyde takes hours (data not shown).

4.3. Carbonyl formation by the strong oxidants hypochlorous acid and peroxynitrite

We found that both hypochlorous (HOCl) acid and peroxynitrite ($ONOO^-$) oxidation caused increases in

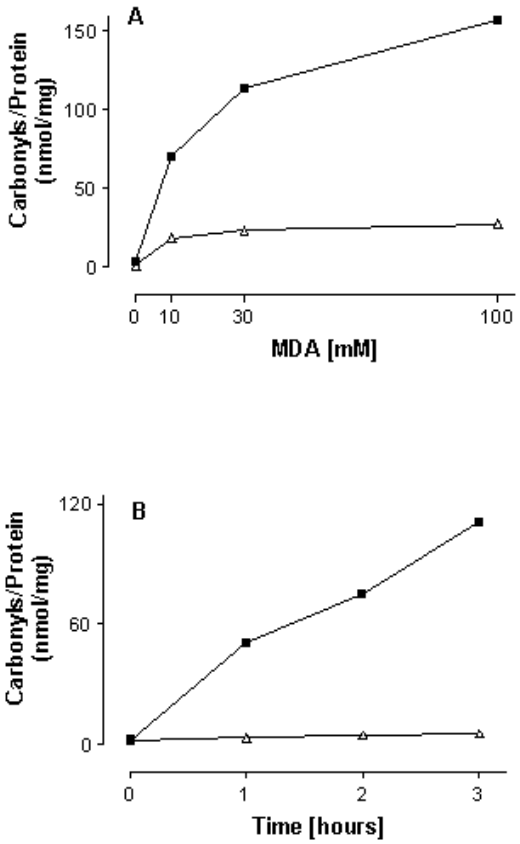

Figure 2. The effect of malondialdehyde on protein modification in BSA and RNase. (**A**) BSA and RNase (1 mg/mL) were incubated with indicated concentrations of malondialdehyde (MDA) for 24 h. (**B**) BSA and RNase were incubated with 30mM of MDA at 37°C in 50 mM phosphate buffer pH 7.4 for 0-3h. After the indicated time, the reaction was terminated with the addition of TCA (10% v/v). Protein carbonyls were analyzed as described in Methods. The same pattern of carbonyl formation was produced in each of at least two independent experiments using triplicate samples.

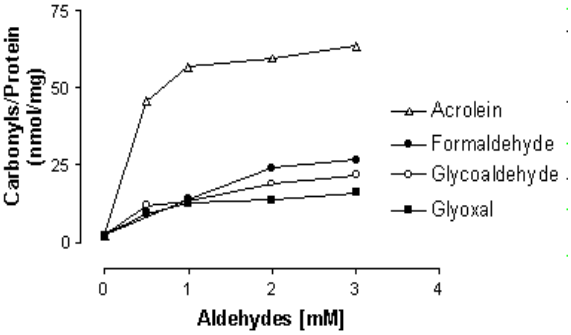

Figure 3. Reactive aldehydes generate carbonyls in protein. BSA (1 mg/mL) was incubated with the indicated concentration of aldehydes at 37°C for 24h in 50 mM Phosphate buffer pH 7.4. Protein carbonyls were analyzed as described in Methods. The same pattern of carbonyl formation was produced in each of at least two independent experiments using triplicate samples.

protein carbonyl levels in BSA and low density lipoproteins (LDL; Figure 4). HOCl increased carbonyl levels significantly (Figure 4A), but concentrations greater than 2 mM did not result in a further increase. This effect is most likely due to the decline in the quantity of available amino acids to react with HOCl. We found no difference in carbonyl levels using equal molar concentration of nitrite in addition to HOCl (figure 4A). In other experiments we have shown that HOCl reacts extremely fast to form carbonyls. In these experiments we saw no increase in protein carbonyl content after 10 seconds (data not shown). Peroxynitrite also generated reactive carbonyls in proteins very rapidly. Peroxynitrite increased the levels of reactive carbonyls in both BSA and low-density lipoproteins (figure 4B). Carbonyl generation in LDL demonstrated a lag phase whereas BSA plateaus around 0.3 mM, possibly due to the reaction with the lipid components of the LDL particle (figure 4B). Furthermore, we exposed LDL to a variety of well-known chemical modification systems that play a role in arteriosclerosis and we determine the amount of protein carbonyl formation (figure 5). Copper sulfate ($CuSO_4$), copper/H_2O_2-system, hemin, peroxynitrite, and HOCl all generated significant amounts of reactive carbonyls (figure 5).

4.4. Hemoglobin, myoglobin, and cytochrome c have a similar absorbance spectrum to DNPH

When examining the levels of carbonyls in a variety of abundant biological proteins we discovered that certain proteins, e.g., hemoglobin, myoglobin, and cytochrome c exhibited high absorbance readings at 360 nm; this is the same wavelength used in the DNPH-assay to measure the amounts of reactive carbonyls. From the full spectrum (figure 6) it is clear that these high readings were due to the strong absorbance of these heme-compounds around 360 nm. We compared levels of absorbance in purified BSA, hemoglobin, myoglobin, and cytochrome c, as well as proteins freshly isolated from human red blood cells and plasma proteins and calculated the carbonyl content without using a protein blank (table 1). We found that absorbance levels are very low in both plasma and BSA. In striking contrast, we found very high absorbance readings and therefore calculated carbonyl levels in hemoglobin, myoglobin, and cytochrome c. The high levels of hemoglobin present in red-blood-cell lysate explain the high absorbance readings and calculated carbonyl levels in red-blood-cell lysate. When not using a blank (protein only) these biomolecules and other heme-containing compounds could significantly affect the levels of protein carbonyls calculated.

We further examined how metal-catalyzed oxidation would affect the levels of cabonyls in these proteins (table 1). We found significant increases in carbonyl levels in bovine serum albumin and plasma following metal-catalyzed oxidation. In striking contrast, metal-catalyzed oxidation decreased the absorbance levels in red-blood-cell lysate, hemoglobin, and cytochrome c. These changes could be due to heme oxidations and a consequential shift in the absorbance spectrum of the heme structures in these biomolecules.

Table 1 Comparison of calculated carbonyls in bovine serum albumin, human plasma, human red-blood-cell lysate, hemoglobin, myoglobin, and cytochrome c using a standard spectrophotometric assay

	Control	$H_2O_2/CuSO_4$
Bovine serum albumin	3.50 ± 0.85	35.50 ± 0.61*
Plasma	1.83 ± 0.40	27.30 ± 2.52*
RBC Lysate	38.23 ± 1.11	24.30 ± 1.08*
Hemoglobin	26.6 ± 2.1	24.00 ± 0.59*
Myoglobin	39.2 ± 0.60	37.5 ± 0.40*
Cytochrome c	50.9 ± 1.47*	39.43 ± 0.45*

Proteins (1mg protein/mL) were incubated without or with H_2O_2 (2 mM) and $CuSO_4$ (0.2 mM) in phosphate buffer pH 7.4 for 2 hours at 37 ^{0}C. Results reflect the mean with standard error (n=3). The same pattern of oxidation was produced in each of at least two independent experiments using triplicate samples. The concentration of carbonyls was read at 360 nm and calculated using a molar absorption coefficient of 22, 000 M^{-1} cm^{-1}. Protein concentration was determined in the samples by determining the absorbance at 280 nm and carbonyl content was expressed as nmol/mg protein. *p < 0.05 different from control.

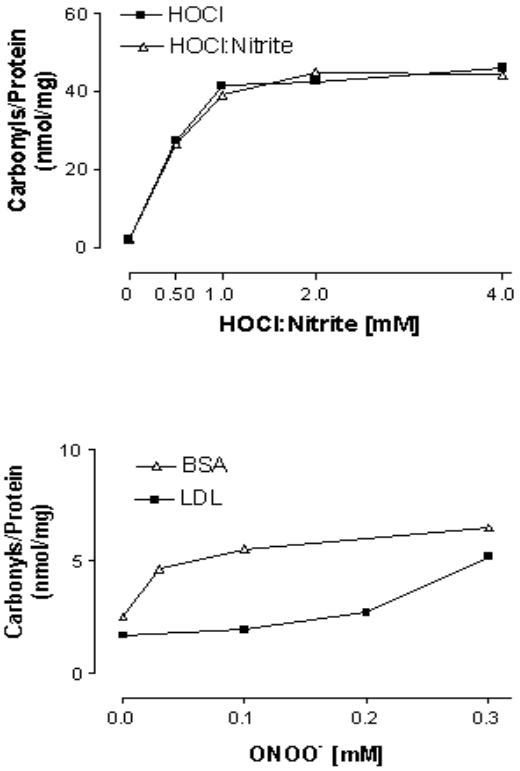

Figure 4. Concentration of protein carbonyls by HOCl and peroxynitrite. (**A**) Hypochlorous acid or peroxynitrite (**B**) were added to BSA or LDL (1 mg/mL) in 50 mM phosphate buffer (pH 7.4) and rapidly vortexed. Samples were then incubated for 5 min at 37°C and acid precipitated with ice-cold TCA (10% v/v). Protein carbonyls were analyzed as described in Methods. The same pattern of oxidation was produced in each of at least two independent experiments using triplicate samples.

5. DISCUSSION

The spectrophotometric assay for carbonyl detection using 2,4-dinitrophenylhydrazine (DNPH) is frequently used to measure protein oxidation (3, 14, 15, 17, 22, 23, 29, 30). In this study, we point out that carbonyls are increased using different oxidative and non-oxidative chemical pathways. Using metal-catalyzed oxidation systems, we showed that this chemistry effectively increases the levels of carbonyls in proteins. The same amount of oxidant generated significantly lower levels of carbonyls in RNase possibly due to 1) fewer reactive binding sites for Cu^{2+}, 2) possibly less-susceptible amino-acid residues for metal-catalyzed oxidation and 3) less accessible amino acids. Furthermore, we found that oxidants such as hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) generated carbonyls into several proteins (figures 4 and 5). HOCl is an inflammatory mediator and a strong oxidizing and chlorinating compound, which can form additional reactive metabolites in the presence of nitrite, such as nitryl chloride (NO_2Cl) and nitrogen dioxide ($NO_2^{\bullet}$) (2). Peroxynitrite is formed during the interaction of nitric oxide ($NO^{\bullet}$) with superoxide ($O_2^{\bullet-}$) and could be a relevant oxidant in a variety of pathological conditions. Protonated peroxynitrite rapidly decomposes to generate several other reactive nitrogen species, including the nitronium ion (NO_2^+) and $NO_2^{\bullet}$. These oxidants have been implicated to play a role in atherosclerosis by modifying LDL particles (11, 12). Nitrite is found in millimolar concentrations in most biological fluids and we therefore tested whether reactions with HOCl and equal molar concentrations of nitrite result in a different amount of carbonyl formation. We found no differences in carbonyl formation using HOCl alone or in the presence of ntrite.

Furthermore, non-oxidative mechanisms can increase carbonyl levels in proteins as effectively as oxidative mechanisms. For example, we found that several well-known aldehydes increase carbonyl levels in bovine serum albumin (figures 2 and 3). The non-oxidative addition of aldehydes may be expected, since for example acrolein and other alpha-beta unsaturated aldehydes are strong nucleophiles that react with many molecules via a "Michaels addition". In addition, the presence of malondialdehyde probably reflects a Schiff condensation forming amines. Others found similar increases in the levels of carbonyls when adding MDA to proteins (17). They attributed the increases to MDA addition to primary amines forming enamine-adducts with an amino acid. These findings could have implications in aging research when quantifying carbonyl levels in biological tissues. For example, MDA bound to protein could contribute significantly to the increased levels of carbonyls seen in aging animals and humans. Therefore, not all increases in carbonyls in free radical biology are due to protein modification.

Next we discovered that purified hemoglobin, myoglobin, and cytochrome c from red blood cells showed high absorbance readings at the same wavelength as DNPH

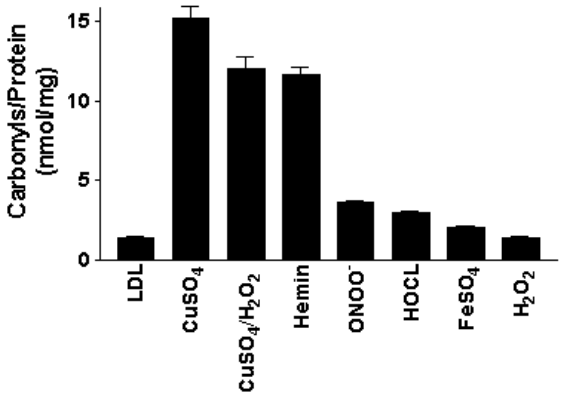

Figure 5. Formation of carbonyls in low-density lipoproteins by different oxidation systems. LDL (512 µg protein/ml) was incubated at 37°C in phosphate buffered saline (50 mM sodium phosphate, 100 mM NaCl, pH 7.4) alone (LDL) or with the indicated modification system. LDL was exposed for 24 hours to $CuSO_4$ (0.1 mM), $CuSO_4/H_2O_2$ (0.1 mM/2 mM), H_2O_2 (2 mM) alone, and hemin (0.1 mM). LDL was incubated for 1 hour at 37°C with $ONOO^-$ (0.1 mM) and HOCl (0.1 mM). Values are the means of two independent experiments performed in duplicate measurements. The same pattern of oxidation was produced in each of at least two independent experiments using triplicate samples.

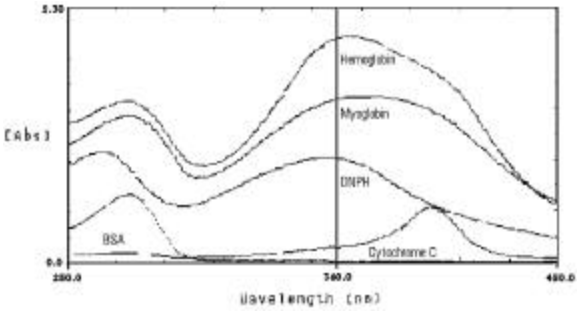

Figure 6. Hemoglobin, myoglobin and cytochrome c show high absorbance at the DNPH wavelength maximum absorbance of 360 nm. Hemoglobin, myoglobin, cytochrome c and bovine serum albumin (each 1mg/mL) were dissolved in (6 M guanidine with 2 mM potassium phosphate). Absorption spectra were obtained using a Beckman 600 spectrophotometer.

and that metal-catalyzed oxidation decreased the absorbance in most of these proteins. This finding raises the necessity to determine carbonyl content using appropriate blanks (protein only without DNPH). and also raises the possibility that biological samples containing high amounts of hemoglobin and/or myoglobin could show an increased variability and therefore mask true biological oxidative changes. One potential method to avoid possible interference by hemoglobin is by perfusing the animal using buffers containing antioxidants and metal chelators. This procedure may be important to reduce background readings and prevent other possible artifacts from hemoglobin contamination, such as oxidation by hemoglobin of other biological compounds. Anesthetized animals and/or specific organs can be perfused with ice-cold antioxidant buffer containing 0.1 mM DTPA (metal chelator), 1 mM butylated hydroxytoluene (inhibits lipid peroxidation), 1 % (vol/vol) ethanol, 10 mM 3-aminotriazole (nitric oxide synthase inhibitor), 50 mM $NaHPO_4$, pH 7.4 (10-12, 31-33). In addition, tissues need to be rinsed extensively with the antioxidant buffers to reduce the chance for *ex vivo* oxidation.

In summary, our data suggest that carbonyl quantification could depend on oxidative as well as non-oxidative mechanisms originating from lipid peroxidation and the generation of reactive low molecular weight aldehydes. Therefore, the spectrophotometric carbonyl assay measures reactive carbonyls in certain isolated proteins *in vitro* very well; however, the carbonyl assays may not accurately measure carbonyl levels due to protein oxidation *in vivo*. Further investigation on the origin of protein carbonyls *in vivo* is warranted. In addition, heme-containing proteins may contribute significantly to background absorbance and may explain the variability using the carbonyl assay when analyzing biological samples (29, 30).

6. ACKNOWLEDGEMENTS

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7. REFERENCES

1. Beckman, K.B., and B.N. Ames: The free radical theory of aging matures. *Physiol Rev* 78, 547-81 (1998)

2. Dean, R.T., S. Fu, R. Stocker, and M.J. Davies: Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324, 1-18 (1997)

3. Stadtman, E.R., P.E. Starke-Reed, C.N. Oliver, J.M. Carney, and R.A. Floyd: Protein modification in aging. *Exs* 62, 64-72 (1992)

4. Pollack, M., and C. Leeuwenburgh: Molecular mechanisms of oxidative stress and aging: Free radicals, aging, antioxidants, and disease. In: Handbook of oxidants and antioxidants in exercise. Eds: Sen, C.K., L. Packer, and O. Hanninen, Elsevier, Amsterdam, 881-926 (1999)

5. Stadtman, E.R: Protein oxidation and aging. *Science* 257, 1220-4 (1992)

6. Hazell, L.J., J.J. van den Berg, and R. Stocker: Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. *Biochem J* 302, 297-304 (1994)

7. Hazen, S.L., and J.W. Heinecke: 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* 99, 2075-81 (1997)

8. Heinecke, J.W., F.F. Hsu, J.R. Crowley, S.L. Hazen, C. Leeuwenburgh, D.M. Mueller, J.E. Rasmussen, and J. Turk: Detecting oxidative modification of biomolecules with isotope dilution mass spectrometry: sensitive and quantitative assays for oxidized amino acids in proteins and tissues. *Methods Enzymol* 300, 124-44 (1999)

9. Uchida, K., and S. Kawakishi: 2-Oxo-histidine as a novel biological marker for oxidatively modified proteins. *FEBS Lett* 332, 208-10 (1993)

10. Leeuwenburgh, C., P. Wagner, J.O. Holloszy, R.S. Sohal, and J.W. Heinecke: Caloric restriction attenuates dityrosine cross-linking of cardiac and skeletal muscle proteins in aging mice. *Arch Biochem Biophys* 346, 74-80 (1997)

11. Leeuwenburgh, C., J.E. Rasmussen, F.F. Hsu, D.M. Mueller, S. Pennathur, and J.W. Heinecke: Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *J Biol Chem* 272, 3520-6 (1997)

12. Leeuwenburgh, C., M.M. Hardy, S.L. Hazen, P. Wagner, S. Oh-ishi, U.P. Steinbrecher, and J.W. Heinecke: Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem* 272, 1433-6 (1997)

13. Grune, T., T. Reinheckel, and K.J. Davies: Degradation of oxidized proteins in mammalian cells. *Faseb J* 11, 526-34 (1997)

14. Stadtman, E.R., and C.N. Oliver: Metal-catalyzed oxidation of proteins. Physiological consequences. *J Biol Chem* 266, 2005-8 (1991)

15. Levine, R.L., J.A. Williams, E.R. Stadtman, and E. Shacter: Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* 233, 346-57 (1994)

16. Reznick, A.Z., and L. Packer: Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol* 233, 357-63 (1994)

17. Burcham, P.C., and Y.T. Kuhan: Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. *Biochem Biophys Res Commun* 220, 996-1001 (1996)

18. Nakamura, A., and S. Goto: Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. *J Biochem (Tokyo)* 119, 768-74 (1996)

19. Shacter, E., J.A. Williams, M. Lim, and R.L. Levine: Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. *Free Radic Biol Med* 17, 429-37 (1994)

20. Halliwell, B., and J.M. Gutteridge: Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 186, 1-85 (1990)

21. Halliwell, B., and J.M.C. Gutteridge. 1999. Free radicals in biology and medicine. Clarendon Press, Oxford University Press, Oxford, New York. xxxi, 936 pp (1999)

22.Blakeman, D.P., T.P. Ryan, R.A. Jolly, and T.W. Petry: Diquat-dependent protein carbonyl formation. Identification of lipid- dependent and lipid-independent pathways. *Biochem Pharmacol* 50, 929-35 (1995)

23. Eiserich, J.P., A. van der Vliet, G.J. Handelman, B. Halliwell, and C.E. Cross: Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. *Am J Clin Nutr* 62, 1490S-1500S (1995)

24. Cross, C.E., A.Z. Reznick, L. Packer, P.A. Davis, Y.J. Suzuki, and B. Halliwell: Oxidative damage to human plasma proteins by ozone. *Free Radic Res Commun* 15, 347-52 (1992)

25. O'Neill, C.A., B. Halliwell, A. van der Vliet, P.A. Davis, L. Packer, H. Tritschler, W.J. Strohman, T. Rieland, C.E. Cross, and A.Z. Reznick: Aldehyde-induced protein modifications in human plasma: protection by glutathione and dihydrolipoic acid. *J Lab Clin Med* 124,359-70 (1994)

26. Reznick, A.Z., C.E. Cross, M.L. Hu, Y.J. Suzuki, S. Khwaja, A. Safadi, P.A. Motchnik, L. Packer, and B. Halliwell: Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem J* 286, 607-11 (1992)

27. Ischiropoulos, H., and A.B. al-Mehdi: Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett* 364, 279-82 (1995)

28. Uppu, R.M., G.L. Squadrito, R. Cueto, and W.A. Pryor: Synthesis of peroxynitrite by azide-ozone reaction. *Methods Enzymol* 269, 311-21 (1996)

29. Cao, G., and R.G. Cutler: Protein oxidation and aging. I. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine. *Arch Biochem Biophys* 320, 106-14 (1995)

30. Lyras, L., P.J. Evans, P.J. Shaw, P.G. Ince, and B. Halliwell: Oxidative damage and motor neurone disease difficulties in the measurement of protein carbonyls in human brain tissue. *Free Radic Res* 24, 397-406 (1996)

31. Leeuwenburgh, C., and L.L. Ji: Alteration of glutathione and antioxidant status with exercise in unfed and refed rats. *J Nutr* 126, 1833-43 (1996)

32. Leeuwenburgh, C., P. Hansen, A. Shaish, J.O. Holloszy, and J.W. Heinecke: Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats. *Am J Physiol* 274, R453-61 (1998)

33. Leeuwenburgh, C., P.A. Hansen, J.O. Holloszy, and J.W. Heinecke: Hydroxyl radical generation during exercise increases mitochondrial protein oxidation and levels of urinary dityrosine. *Free Radic Biol Med* 27, 186-92 (1999)

Abbreviations: BSA, bovine serum albumin, $CuSO_4$, copper sulfate, DNPH, 2,4-dinitrophenylhydrazine, DTPA, diethylenetriaminepentaacetic acid, H_2O_2, hydrogen peroxide, HOCl, hypochlorous acid, LDL, low-density lipoprotein, MCO, metal catalyzed oxidation, MDA, malondialdehyde, $NO^{\bullet}$, nitric oxide, $NO_2^{\bullet}$, nitrogen dioxide NO_2^{+}, nitronium ion, $O_2^{\bullet -}$, superoxide, OCl^{-}, hypochlorite, $ONOO^{-}$, peroxynitrite, Rnase, ribonuclease A, TCA, trichloroacetic acid

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