Feed-Back Inhibition of Oxidative Stress by Oxidized Lipid/Amino Acid Reaction Products†

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ABSTRACT: Three oxidized lipid/amino acid reaction products (OLAARPs): 1-methyl-4-pentyl-1,4-dihydropyridine-3,5-dicarbaldehyde, 1-(5-amino-1-carboxypentyl)pyrrole, and *N*-(carbobenzyloxy)-1(3)-[1-(formylmethyl)hexyl]-L-histidine dihydrate, were prepared and tested for antioxidative activity in a microsomal system in order to investigate the effect that OLAARP formation may be playing in the oxidative stress process. The microsomal system consisted of freshly prepared trout muscle microsomes, which were oxidized in the presence of 5 μM Cu^{2+}, 1 mM Fe^{3+}/5 mM ascorbate, or 1 mM Cu^{2+}/10 mM H_2O_2, and the compound to be tested as antioxidant added at 50 μM. At different periods of time, samples were tested for lipid peroxidation, assessed by the formation of thiobarbituric acid reactive substances (TBARS), and protein damage, which was evaluated by the formation of protein carbonyls and amino acid analysis. The three OLAARPs and butylated hydroxytoluene significantly ($p < 0.05$) protected against lipid peroxidation and protein damage for the three systems assayed. On the contrary, neither the amino acids used in the preparation of OLAARPs nor α-tocopherol, mannitol, aminoguanidine, or 4,5-dihydroxy-1,3-benzenedisulfonic acid exhibited this constant protection. Because OLAARPs were produced at inhibitory levels during microsomal lipid peroxidation, these results suggest that OLAARP formation may be an antioxidative defense mechanism by which oxidative stress is feed-back-inhibited, delaying the damage caused by reactive oxygen species.

The importance of reactive oxygen species (ROS)[1] and antioxidants in health and disease is now recognized by every branch of medicine and biological science. Overwhelming evidence indicates that free radicals play a role in most major health problems of the industrialized world, including cardiovascular diseases, cancer, neurological diseases, and aging, and that antioxidants play a critical role in wellness, health maintenance, and the prevention of chronic and degenerative diseases (*1*−*6*).

Peroxidation of lipids is a well-recognized pathway of oxidant injury, and it is always accompanied in biological systems with the formation of aldehydes (*7*, *8*). Unlike reactive free radicals, aldehydes are long-lived and can therefore diffuse from the side of their origin and reach and attack targets intra- or extracellularly, which are distant from the initial free radical event (*9*). Modification of proteins and other biomolecules by lipid peroxidation products is believed to play a central role in many of the above pathophysiological conditions associated with free radical damage (*10*−*12*).

Among the many different aldehydes which can be formed during lipid peroxidation and that modify proteins, the most intensively studied have been malondialdehyde and 4-hydroxy-2-alkenals, and, more recently, 4,5-epoxy-2-alkenals. Malondialdehyde in fresh or peroxidized biological samples results from the oxidative degradation of polyunsaturated fatty acids with more than two methylene-interrupted double bonds, and it is able to react with the amino groups of amines, amino acids, and proteins, producing Schiff's bases (*13*) or dihydropyridines (*14*). 4-Hydroxy-2-alkenals are produced by an oxidative pathway originated in 3-alkenals (*15*), and are also able to react with amino, imidazole, and thiol groups on amino acid side chains on proteins, thus altering their charge and nature (*16*, *17*). 4,5-Epoxy-2-alkenals result from the decomposition of intermediate epoxyhydroperoxides (*18*) and are supposed to be intermediates in the formation of 4,5-dihydroxy-2-alkenals produced in the peroxidation of liver microsomal lipids (*19*). The reaction of 4,5-epoxy-2-alkenals with amines, amino acids, and proteins is very rapid, and produces pyrrole derivatives, which are responsible for the color and fluorescence formed in these reactions by means of a polymerization process (*20*, *21*). This last polymerization reaction, which has been characterized, has been suggested as an alternative mechanism for production in living beings of brown macromolecular pigments with fluorescent lipofuscin-like characteristics (*22*).

Although the importance and relative contributions of the above reactions are not yet well understood, nowadays there are increasing evidences suggesting that oxidized lipid/amino acid reaction products (OLAARPs) are produced naturally

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[1] Abbreviations: ACPP, 1-(5-amino-1-carboxypentyl)pyrrole; AG, aminoguanidine; BHT, butylated hydroxytoluene; DHBSA, 4,5-dihydroxy-1,3-benzenedisulfonic acid; DHP, 1-methyl-4-pentyl-1,4-dihydropyridine-3,5-dicarbaldehyde; HPLC, high-performance liquid chromatography; KRPB, Krebs−Ringer phosphate buffer; MAN, mannitol; OLAARPs, oxidized lipid/amino acid reaction products; PI, protection index; Pnl, ϵ-*N*-pyrrolylnorleucine; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TOC, α-tocopherol; ZH, *N*-(carbobenzyloxy)-L-histidine; ZHO, *N*-(carbobenzyloxy)-1(3)-[1-(formylmethyl)hexyl]-L-histidine dihydrate.

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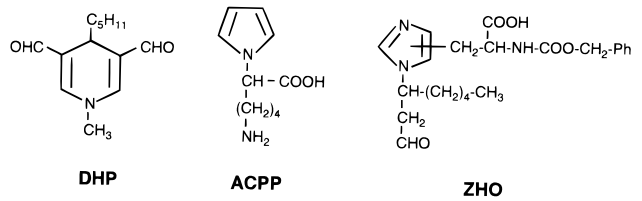

FIGURE 1: Chemical structures of OLAARPs tested as antioxidants.

in vivo. Thus, lipid hydroperoxides are present in the human plasma in the range 2−5 μM (*23*), and the presence of the OLAARP malondialdehyde-modified low-density lipoprotein has been detected in the sera of healthy individuals (*24*). In addition, other OLAARPs have been detected, for example, in urine (*25*), atherosclerotic lesions (*26*), oxidized hepatocytes (*27*), and oxidized mitochondria (*28*).

The objective of this study was to investigate the effect that OLAARP formation is playing in the oxidative stress process. Previous research from this laboratory has shown that most OLAARPs exhibited antioxidative activities against lipid peroxidation when tested in edible oils (*29*). If OLAARPs are able to protect against oxidative stress, OLAARP formation might be an antioxidative defense mechanism, and oxidative stress could be controlled, in some extent, by a feed-back inhibition, analogously to many enzymatic reactions.

EXPERIMENTAL PROCEDURES

Materials. α-Tocopherol (TOC) was purchased from Aldrich Chemical Co. (Milwaukee, WI). D-Mannitol (MAN), aminoguanidine (AG), 4,5-dihydroxy-1,3-benzenedisulfonic acid (DHBSA), butylated hydroxytoluene (BHT), *N*-(carbobenzyloxy)-L-histidine (ZH), and L-lysine were purchased from Sigma Chemicals Co. (St. Louis, MO). Other reagents and solvents used were analytical grade and were purchased from reliable commercial sources.

OLAARP Synthesis. Three OLAARPs were selected to be tested as potential oxidative stress inhibitors. They were representative of three major derivatives that are produced in oxidized lipid/amino acid reactions: dihydropyridines, produced between malondialdehyde and amino groups (*14*); pyrroles, produced in the reaction of amino groups with 4,5-epoxy-2-alkenals (*22*), 4-hydroxy-2-alkenals (*30*), or unsaturated epoxyoxo fatty acids (*31*); and Michael adducts, produced between histidine and α,β-unsaturated aldehydes (*17*). The compounds prepared were 1-methyl-4-pentyl-1,4-dihydropyridine-3,5-dicarbaldehyde (DHP), 1-(5-amino-1-carboxypentyl)pyrrole (ACPP), and *N*-(carbobenzyloxy)-1(3)-[1-(formylmethyl)hexyl]-L-histidine dihydrate (ZHO), respectively. Preparation of DHP, ACPP, and ZHO was carried out according to previously described procedures (see references *32*, *20*, and *33*, respectively). Obtained compounds were chromatographically pure, and their structures were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry. Structures for these compounds are given in Figure 1.

Preparation of Trout Muscle Microsomes. Muscle microsomes from freshly killed rainbow trout (*Salmo gairdnerii*) were prepared by differential centrifugation according to a procedure of Parkin and Hultin (*34*) as described previously (*35*). Washed microsomes were dispersed in Krebs−Ringer phosphate buffer (KRPB) (*36*), and protein concentration, measured according to Bradford's method using bovine serum albumin as standard (*37*), was adjusted to 3.0 mg/mL for experiments.

Exposure of Microsomes to ROS. Microsomes (50 μL of the stock solution) were suspended in 5 mL of KRPB and incubated at 37 °C in the presence of the ROS generating system and the compound to be tested as antioxidant added at 50 μM. Three different procedures were used to generate ROS. Procedure 1 consisted of treatment with 5 μM $CuCl_2$. Procedure 2 consisted of treatment with 1 mM $FeCl_3$ and 5 mM ascorbic acid. Procedure 3 consisted of treatment with 1 mM $CuCl_2$ and 10 mM H_2O_2. These three systems have been frequently used in the literature (*38, 39*), and they have been shown to be very efficient as nonenzymatic metal-catalyzed oxidative systems.

OLAARP Determination in Incubated Microsomes. The levels of OLAARPs produced in incubated samples were estimated by determining the OLAARP product ϵ-*N*-pyrrolylnorleucine (Pnl) by high-performance capillary electrophoresis (*35*). Briefly, incubated samples (15 mL) were centrifuged at 100000*g* for 1 h, and the resulting pellets, which contained the oxidized microsomes, were suspended in 1 mL of deionized water and submitted to basic hydrolysis with 2 N NaOH at 120 °C for 18 h. Pnl was determined by capillary electrophoresis after derivatization with diethyl ethoxymethylenemalonate and using homoarginine as internal standard.

Analytical Measurements. Lipid peroxidation during incubations at 37 °C was assessed by the formation of thiobarbituric acid reactive substances (TBARS) using a previously described procedure (*40*), which was modified. Incubated samples (150 μL) were diluted with water (560 μL) and treated with 1.1 mL of acetic acid (20% solution, pH 3.5), 1.3 mL of thiobarbituric acid (0.71% solution), and 40 μL of BHT (0.8% solution in acetic acid). Solutions were heated at 100 °C for 60 min, then cooled, and, finally, extracted with 3 mL of *n*-butyl alcohol. Organic layers were separated by centrifugation, and TBARS determined by fluorescence using λ_{ex} 535 nm and λ_{em} 550 nm.

Protein damage during incubations at 37 °C was evaluated by the formation of protein carbonyls using a procedure of Levine *et al.* (*41*). Briefly, 2 mL microsomal aliquots were treated with 2 mL of 12.5 mM 2,4-dinitrophenylhydrazine in 2.5 M HCl, and the mixture was incubated for 1 h at room temperature. After that time, proteins were precipitated with 2 mL of 30% (w/v) trichloroacetic acid at 0 °C for 1 h, and, then, centrifuged at 2200*g* for 15 min. The pellet produced was washed 4 times with a 1:1 mixture of ethanol/ethyl acetate, then dissolved in 2 mL of 6 M guanidine hydrochloride with 20 mM phosphate buffer/trifluoroacetic acid, pH 2.3, and, finally, left for 30 min at 37 °C with vortexing. Any insoluble materials were removed by centrifugation, and the reactive carbonyl content was calculated from its peak absorption at 370 nm using a molar absorption coefficient (ϵ) of 22 000 M^{-1}.

At the end of the incubation period, incubated samples (1.8 mL) were hydrolyzed with 6 N HCl for 20 h (110 °C), and, then, submitted to amino acid analysis by high-performance liquid chromatography (HPLC) using a previously described procedure (*42*).

For comparison purposes, and when differences were significant, a protection index (PI) was calculated, as described previously (*43*), according to the equation:

$$PI = 100 \times [1 - ((x - y)/(a - b))]$$

where a is the control with ROS, b is the control without ROS, x is the sample with antioxidant treated with ROS, and y is the untreated sample with the antioxidant added. For most experiments, the results obtained for the control without ROS and the sample with antioxidant but without ROS were the same. Therefore, in these cases the above formula could be simplified to

$$PI = 100 \times [1 - ((x - b)/(a - b))]$$

Statistical Analysis. All results are expressed as mean values of three experiments unless otherwise indicated. Statistical comparisons between two groups were made using Student's *t*-test. With several groups, ANOVA was used. When significant *F* values were obtained, group differences were evaluated by the Student−Newman−Keuls test (*44*). All statistical procedures were carried out using *Primer of Biostatistics: The Program* (McGraw-Hill, Inc., New York). Significance level is $p < 0.05$ unless otherwise indicated.

RESULTS

Exposure of Microsomes to ROS. Exposure of microsomes to the different ROS generating systems produced increased lipid peroxidation, measured by the formation of TBARS, and protein damage, which was determined by protein carbonyl formation and amino acid analysis. Figure 2 shows the TBARS production in microsomes incubated with different nonenzymatic metal-catalyzed oxidative systems as a function of incubation time. Lipid peroxidation slowly increased with time in control microsomes. However, addition of 5 μM Cu^{2+} produced a higher TBARS production, which was significant after 1 h and continued increasing for the next 24 h (Figure 2A). TBARS production increased very rapidly when the Fe^{3+}/ascorbate system was used (Figure 2B). In this case, significant lipid peroxidation was observed after only 15 s, and TBARS increased for the next 3 h. At the end of this period, the highest TBARS values were obtained, and these values did not increase significantly afterward (data not shown). TBARS production with the Cu^{2+}/H_2O_2 system was similar to the Fe^{3+}/ascorbate system for the first hour of incubation (Figure 2C). However, TBARS values decreased afterward (data not shown), more likely as a consequence of TBARS degradation by the ROS system. According to these results, the incubation times used for testing inhibition of lipid peroxidation with the three ROS generating systems were 24 h, 3 h, and 1 h for Cu^{2+}, Fe^{3+}/ascorbate, and Cu^{2+}/H_2O_2 systems, respectively.

Analogous results were obtained when protein carbonyls were determined (Figure 2). Addition of Cu^{2+} to the microsomal system significantly increased protein carbonyls for the first hour, and this difference continued increasing for the next 24 h. Protein carbonyls increased much more rapidly when the Fe^{3+}/ascorbate system was used, and also for the Cu^{2+}/H_2O_2 system. The same incubation times selected for testing inhibition of lipid peroxidation with the three ROS generating systems were also used for testing inhibition of protein carbonyl formation: 24 h, 3 h, and 1 h for Cu^{2+}, Fe^{3+}/ascorbate, and Cu^{2+}/H_2O_2 systems, respectively.

The effect of the different ROS generating systems on the amino acid composition of microsomes is shown in Table

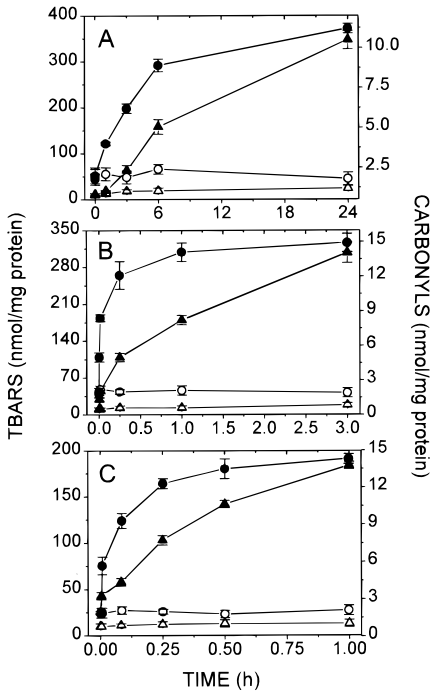

FIGURE 2: Production of TBARS and protein carbonyls in microsomes incubated with ROS. Microsomes were incubated in the presence of (A) 5 μM Cu^{2+}, (B) 1 mM Fe^{3+}/5 mM ascorbate, or (C) 1 mM Cu^{2+}/10 mM H_2O_2. At different incubation times, microsomal aliquots were taken from the incubation mixtures and tested for TBARS (▲) and protein carbonyls (●) as described under Experimental Procedures. Control microsomes were also tested for TBARS (△) and protein carbonyls (○).

Table 1: Modification of Amino Acid Composition of Microsomes Incubated with Different ROS Generating Systems[a]

	oxidative system			
amino acid	none ($n = 35$)	Cu^{2+} (5 μM) ($n = 12$)	Fe^{3+} (1 mM)/ ascorbate (5 mM) ($n = 9$)	Cu^{2+} (1 mM)/ H_2O_2 (10 mM) ($n = 9$)
Ala	2.54 ± 0.13^b	2.56 ± 0.08^b	2.43 ± 0.18^b	1.80 ± 0.16^c
Arg	1.16 ± 0.06^b	1.11 ± 0.03^c	0.97 ± 0.11^d	0.67 ± 0.08^e
Asx[f]	3.84 ± 0.20^b	3.97 ± 0.14^b	3.63 ± 0.19^c	2.82 ± 0.29^d
cystine	0.15 ± 0.04^b	0.09 ± 0.01^c	0.12 ± 0.02^d	0.00 ± 0.00^e
Glx[f]	3.00 ± 0.16^b	3.01 ± 0.11^b	2.79 ± 0.14^c	2.23 ± 0.25^d
Gly	2.42 ± 0.17^b	2.45 ± 0.11^b	2.19 ± 0.18^c	1.66 ± 0.28^d
His	0.53 ± 0.07^b	0.35 ± 0.06^c	0.27 ± 0.07^d	0.16 ± 0.04^e
Ile	1.74 ± 0.10^b	1.74 ± 0.06^b	1.54 ± 0.14^c	1.12 ± 0.15^d
Leu	2.50 ± 0.13^b	2.52 ± 0.07^b	2.27 ± 0.19^c	1.44 ± 0.21^d
Lys	2.00 ± 0.10^b	1.65 ± 0.06^c	1.52 ± 0.10^d	0.96 ± 0.21^e
Met	0.90 ± 0.19^b	0.82 ± 0.15^b	0.74 ± 0.15^b	0.26 ± 0.22^c
Phe	1.19 ± 0.06^b	1.18 ± 0.04^b	1.02 ± 0.10^c	0.02 ± 0.02^d
Ser	1.69 ± 0.08^b	1.71 ± 0.06^b	1.53 ± 0.13^c	1.05 ± 0.16^d
Thr	1.80 ± 0.15^b	1.82 ± 0.05^b	1.54 ± 0.14^c	1.21 ± 0.20^d
Tyr	0.68 ± 0.03^b	0.59 ± 0.02^c	0.54 ± 0.05^d	0.00 ± 0.00^e
Val	2.50 ± 0.13^b	2.49 ± 0.08^b	2.27 ± 0.18^c	1.79 ± 0.22^d

[a] Microsomes were incubated at 37 °C for 24 h in the presence of the ROS generating system and, then, submitted to amino acid analysis after acid hydrolysis. [b−e] Means in the same row with different superscripts are significantly different ($p < 0.05$). [f] Asx, aspartic acid+asparagine; Glx, glutamic acid+glutamine.

1. Oxidation with Cu^{2+} was the mildest of the assayed treatments. Only cyst(e)ine (with a loss of 40%), histidine (34%), lysine (18%), tyrosine (13%), and arginine (4%) were partially destroyed after 24 h incubation. The Fe^{3+}/ascorbate system was more destructive, and all amino acids decreased at least by 5−10%. In addition, basic, aromatic, and sulfur-containing amino acids were more sensitive. Thus, the highest decreases were observed in histidine (49%), lysine

(24%), tyrosine (21%), cyst(e)ine (20%), arginine (16%), and phenylalanine (14%). Finally, the treatment with the Cu^{2+}/H_2O_2 system was the strongest of the assayed treatments, all the amino acids were destroyed at least by 25−30%, and three of them [cyst(e)ine, phenylalanine, and tyrosine] were destroyed almost completely. In addition to these three amino acids, the most damaged amino acids upon Cu^{2+}/H_2O_2 treatment were methionine (71%), histidine (70%), lysine (52%), arginine (42%), and leucine (42%). Therefore, basic, aromatic, and sulfur-containing amino acids were more sensitive to oxidative damage independently of the ROS generating system employed.

OLAARP Formation in Microsomes Exposed to ROS. OLAARP production is the last step in the lipid peroxidation process, because most produced lipids are able to modify reactive groups in neighbor proteins. To estimate the levels of OLAARPs produced after exposition of microsomes to ROS, the OLAARP Pnl was determined in control microsomes incubated for 3 h and in microsomes incubated for the same time in the presence of Fe^{3+}/ascorbate. Pnl concentration in control microsomes was 0.5 ± 0.4 μM, and this concentration increased significantly ($p < 0.001$) when microsomes were incubated in the presence of the Fe^{3+}/ascorbate system. Pnl concentration in these oxidized microsomes was 2.3 ± 0.6 μM. Because the Pnl concentration is less than 10−15% of total pyrrolic OLAARPs produced (*35*), a minimum final concentration of 15−25 μM pyrrolic OLAARPs in treated microsomes may be estimated. But pyrrolic OLAARPs are only a part of OLAARPs produced. Therefore, a concentration of 50 μM was considered appropriate to test OLAARPs for antioxidant activity.

OLAARP Effect on Oxidative Damage Induced by ROS in Microsomes. Addition of OLAARPs to microsomes protected them against lipid peroxidation and protein damage. Figure 3 shows the TBARS values obtained in microsomes oxidized in the presence of the three OLAARPs (DHP, ACPP, and ZHO), the two amino acids used in the preparation of the OLAARPs (lysine and ZH), and several common antioxidants and radical scavengers added at 50 μM. The three OLAARPs assayed significantly decreased TBARS for the three ROS generating systems tested, and this protective effect was not observed, or it was much lower, for the two amino acids used in the preparation of OLAARPs. Antioxidative activity depended on the compound tested and the ROS generating system employed. Thus, antioxidative activities were ZHO ≈ ACPP > DHP for Cu^{2+}, and ACPP > DHP ≈ ZHO for both Fe^{3+}/ascorbate and Cu^{2+}/H_2O_2. Common antioxidants and radical scavengers showed diverse activities for the different systems. Thus, BHT was the best antioxidant for the three systems, and TOC only protected significantly with Cu^{2+}/H_2O_2. The effect of MAN, AG, and DHBSA depended on the ROS generating system used. Thus, MAN protected for Fe^{3+}/ascorbate and Cu^{2+}/H_2O_2, AG protected for Fe^{3+}/ascorbate, and DHBSA protected for the Cu^{2+} and Fe^{3+}/ascorbate systems. Table 2 collects the PI obtained for the tested compounds with the three systems assayed. BHT exhibited the highest PI (PI = 97−98), followed by ACPP (PI = 22−43), ZHO (PI = 24−26), and DHP (PI = 16−27). The behavior of DHBSA was different and exhibited a great dependence of the system employed to produce ROS (PI = 0−50).

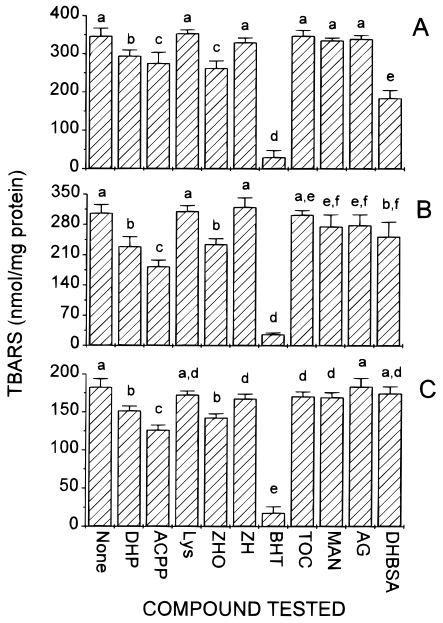

FIGURE 3: Effect of OLAARPs on TBARS production in microsomes incubated with different ROS generating systems. Microsomes were incubated in the presence of the compound to be tested as antioxidant added at 50 μM and (A) 5 μM Cu^{2+}, (B) 1 mM Fe^{3+}/5 mM ascorbate, or (C) 1 mM Cu^{2+}/10 mM H_2O_2. The incubation time depended on the ROS generating system used and was 24 h for Cu^{2+}, 3 h for Fe^{3+}/ascorbate, and 1 h for Cu^{2+}/H_2O_2. Bars with different letters are significantly different ($p < 0.05$).

Table 2: PI Obtained for OLAARPs and Other Antioxidants and Radical Scavengers on TBARS Production in Microsomes Incubated with Different ROS Generating Systems[a]

compound tested	oxidative system		
	Cu^{2+} (5 μM)	Fe^{3+} (1 mM)/ ascorbate (5 mM)	Cu^{2+} (1 mM)/ H_2O_2 (10 mM)
none	–	–	–
DHP	16	27	18
ACPP	22	43	33
Lys	–	–	–
ZHO	26	25	24
ZH	–	–	9
BHT	98	97	97
α-tocopherol	–	–	7
mannitol	–	11	8
AG	–	10	–
DHBSA	50	19	–

[a] PI were calculated as described under Experimental Procedures.

Addition of OLAARPs to microsomes also induced protection against protein damage as could be determined by protein carbonyl formation and amino acid analysis. Figure 4 shows protein carbonyls measured in microsomes oxidized in the presence of the three OLAARPs and the other tested compounds added at 50 μM. Analogously to TBARS, the three OLAARPs significantly decreased protein carbonyls for the three ROS generating systems assayed, and this protective effect was always higher than that observed for the two amino acids used in the preparation of OLAARPs. Once again, the antioxidative activity depended on the compound tested and the ROS generating system employed. Thus, antioxidative activities were ACPP > DHP ≈ ZHO for Cu^{2+} and Fe^{3+}/ascorbate, and ACPP ≈ DHP ≈ ZHO for Cu^{2+}/H_2O_2. The other antioxidants and radical scavengers assayed exhibited diverse activities for the different systems. Thus, BHT was a worse antioxidant for protein damage than

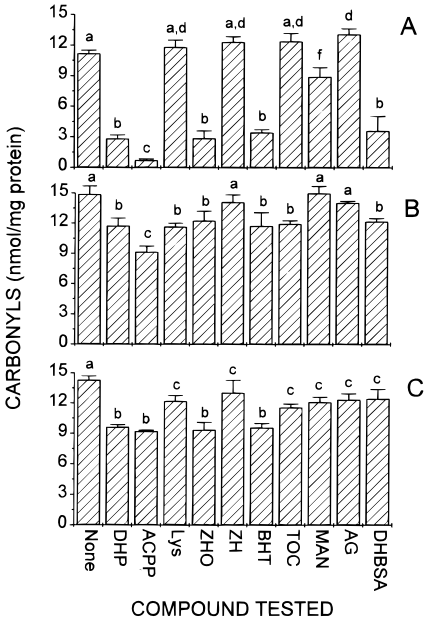

FIGURE 4: Effect of OLAARPs on protein carbonyls of microsomes incubated with different ROS generating systems. Microsomes were incubated in the presence of the compound to be tested as antioxidant added at 50 μM and (A) 5 μM Cu^{2+}, (B) 1 mM Fe^{3+}/5 mM ascorbate, or (C) 1 mM Cu^{2+}/10 mM H_2O_2. The incubation time depended on the ROS generating system used and was 24 h for Cu^{2+}, 3 h for Fe^{3+}/ascorbate, and 1 h for Cu^{2+}/H_2O_2. Bars with different letters are significantly different ($p < 0.05$).

Table 3: PI Obtained for OLAARPs and Other Antioxidants and Radical Scavengers on Protein Carbonyls of Microsomes Incubated with Different ROS Generating Systems[a]

compound tested	oxidative system		
	Cu^{2+} (5 μM)	Fe^{3+} (1 mM)/ ascorbate (5 mM)	Cu^{2+} (1 mM)/ H_2O_2 (10 mM)
none	–	–	–
DHP	89	24	38
ACPP	111	44	42
Lys	–	25	17
ZHO	89	20	40
ZH	–	–	10
BHT	82	24	39
α-tocopherol	–	23	22
mannitol	24	–	18
AG	−20	–	16
DHBSA	81	21	15

[a] PI were calculated as described under Experimental Procedures.

for lipid peroxidation, and the other compounds were less protective than BHT or the OLAARPs. Table 3 collects the PI obtained for the tested compounds with the three systems assayed. ACPP (PI = 42−111), DHP (PI = 24−89), ZHO (PI = 20−89), BHT (PI = 24−82), and DHBSA (PI = 15−81) protected for the three systems assayed. The other compounds showed smaller protective effects and only for some systems. A prooxidant effect (PI = −20) was observed for AG with the Cu^{2+} system.

The effect on amino acid composition of microsomes has been collected in Figure 5. Because many amino acids are involved and only some of them were lost to a high extent when microsomes were treated with the different ROS (see Table 1), only lysine, which was the amino acid that experienced the highest absolute losses for the three systems assayed, was studied for OLAARP protection. Figure 5 shows the values obtained for lysine in microsomes oxidized

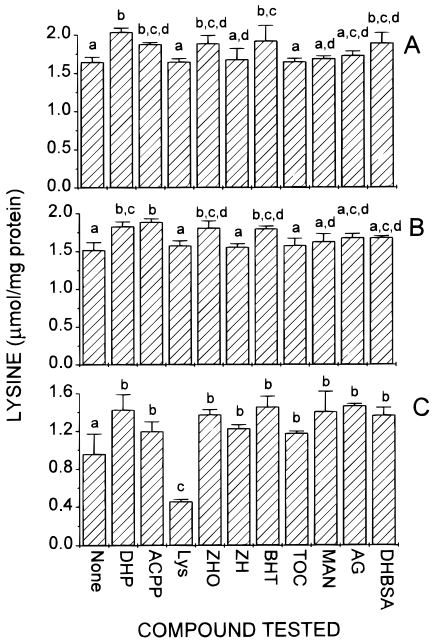

FIGURE 5: Effect of OLAARPs on lysine residues of microsomes incubated with different ROS generating systems. Microsomes were incubated in the presence of the compound to be tested as antioxidant added at 50 μM and (A) 5 μM Cu^{2+}, (B) 1 mM Fe^{3+}/5 mM ascorbate, or (C) 1 mM Cu^{2+}/10 mM H_2O_2. Values corresponding to ACPP and lysine were corrected by subtracting the contribution of ACPP and lysine to the peak of microsomal lysine in the chromatogram (see Results). Bars with different letters are significantly different ($p < 0.05$).

in the presence of the three OLAARPs (DHP, ACPP, and ZHO), the two amino acids used in the preparation of the OLAARPs (lysine and ZH), and several common antioxidants and radical scavengers added at 50 μM. As expected, both ACPP and lysine exhibited high recoveries of lysine after acid hydrolysis, which was a consequence of the contribution of the tested compounds to the peak of lysine in the chromatogram. In these cases, the lysine values obtained for control microsomes treated with 50 μM ACPP and lysine were 3.40 ± 0.04 and 4.11 ± 0.10 μmol of lysine/mg of protein, respectively. The values shown in Figure 5 for ACPP and lysine have been corrected by subtracting the contribution of lysine and ACPP to the microsomal lysine. Analogously to TBARS and protein carbonyls, the antioxidative protection observed for lysine residues depended on the OLAARPs tested and the ROS generating system employed, but it always was significant for the three OLAARPs and BHT, and these four compounds exhibited analogous activities for the three systems. PI are collected in Table 4 and were 45−111 for DHP, 23−77 for ACPP, 40−69 for ZHO, and 48−77 for BHT. DHBSA protected for Cu^{2+} and Cu^{2+}/H_2O_2 systems, and ZH, TOC, MAN, and AG protected for the Cu^{2+}/H_2O_2 system. On the contrary, lysine exhibited a prooxidant effect (PI = −48) for the Cu^{2+}/H_2O_2 system.

DISCUSSION

The results obtained in this study show that the three OLAARPs studied, which are representative of three major derivatives produced in oxidized lipid/amino acid reactions, exhibited antioxidative activities against both lipid peroxidation and protein damage when tested *in vitro* in a

Table 4: PI Obtained for OLAARPs and Other Antioxidants and Radical Scavengers on Lysine Residues of Microsomes Incubated with Different ROS Generating Systems[a]

compound tested	oxidative system		
	Cu^{2+} (5 μM)	Fe^{3+} (1 mM)/ ascorbate (5 mM)	Cu^{2+} (1 mM)/ H_2O_2 (10 mM)
none	−	−	−
DHP	111	65	45
ACPP	66	77	23
Lys	−	−	−48
ZHO	69	60	40
ZH	−	−	26
BHT	77	58	48
α-tocopherol	−	−	21
mannitol	−	−	43
AG	−	−	49
DHBSA	69	−	39

[a] PI were calculated as described under Experimental Procedures.

microsomal system and in the presence of three different ROS generating systems. The antioxidative activities obtained for the OLAARPs when lipid peroxidation was studied were lower than that exhibited by BHT. However, the antioxidative activities observed for the OLAARPs were analogous to BHT when the protection of protein damage was studied. These protective activities obtained for the OLAARPs were always higher than that of TOC.

Protection of OLAARPs for both lipid peroxidation and protein damage was observed for the three ROS generating systems assayed, suggesting that the proportion in which the different ROS are produced was not a requisite for their antioxidative function. This constant protective effect was not observed for the other radical scavengers and inhibitors when they were assayed at 50 μM. Thus, MAN, an $OH^{\bullet}$ scavenger (*45*), only exhibited a small protection for lipid peroxidation when the Fe^{3+}/ascorbate and Cu^{2+}/H_2O_2 systems were used, and for protein damage when the Cu^{2+} and Cu^{2+}/H_2O_2 systems were used. DHBSA, an $O_2^{\bullet-}$ scavenger (*45*), exhibited a high protection (PI = 50−81) for both lipid peroxidation and protein damage when the Cu^{2+} system was used, but this protection was much lower or null for the Fe^{3+}/ascorbate or Cu^{2+}/H_2O_2 systems. Finally, AG, an inhibitor of advanced glycosylation (*46*), only exhibited an important protection for lysine recovery after acid hydrolysis when using the Cu^{2+}/H_2O_2 system.

The protections observed for the different OLAARPs were not observed for the corresponding amino acids from which the OLAARPs were derived, suggesting that OLAARPs should be produced to exhibit antioxidant properties. Thus, lysine did not exhibit any antioxidative properties for lipid peroxidation, and only a small activity for protecting protein carbonyl formation was observed when the Fe^{3+}/ascorbate system was used. The results obtained for protein damage using the Cu^{2+}/H_2O_2 system were contradictory. Thus, lysine exhibited a small protection for protein carbonyl formation but a prooxidant effect in lysine recovery after acid hydrolysis. This last result was likely a consequence of the null protection of lysine. Thus, the ROS generated by the Cu^{2+}/H_2O_2 system were able to degrade the lysine added to be tested in addition to the microsomal lysine. ZH also exhibited null protection for Cu^{2+} and Fe^{3+}/ascorbate, and only a small protection for the Cu^{2+}/H_2O_2 system.

The results obtained in this study are in agreement with a certain role of OLAARPs in the oxidative stress process.

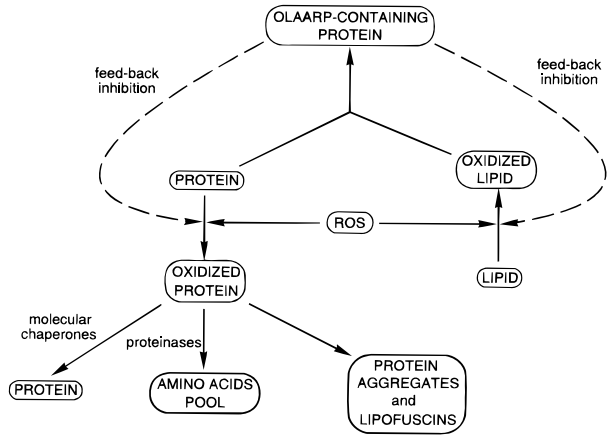

FIGURE 6: Proposed feed-back inhibition of oxidative stress by OLAARPs. ROS production initiates both lipid and protein oxidation. However, oxidized lipids are able to react with new proteins, producing OLAARP-containing proteins that delay the oxidative stress process. In addition, and because oxidative stress is not completely controlled, oxidized proteins can be restored to its native structure, or either degraded or accumulated. Among these accumulated proteins are likely included OLAARP-containing proteins.

This has been summarized in Figure 6. Thus, ROS are able to react with both lipids and proteins, producing oxidized derivatives. However, oxidized lipids are also able to react with reactive groups of proteins, producing modified proteins with OLAARP residues, and these residues have been shown to have antioxidative properties for both lipid peroxidation and protein damage at the same levels of concentration at which they are produced. In addition, these antioxidative properties have also been shown for OLAARP-containing proteins (F. J. Hidalgo, M. Alaiz, and R. Zamora, unpublished results). Therefore, OLAARP formation should be controlling in some extent the oxidative stress process, which would be feed-back-inhibited, analogously to many enzymatic reactions. Because oxidative stress is not completely controlled, these results are also in agreement with the proposed mechanisms for restoring or degrading damaged proteins. Thus, if proteins have been only slightly modified, they can be restored to their functional structure, a process that can be favored by molecular chaperones (*47*, *48*). More damaged proteins may be susceptible to proteolytic degradation by a variety of exogenous and endogenous proteinases (*49*), or to be accumulated as it is produced during animal aging (*50*) or under several pathological conditions (*51*, *52*). Among these accumulated proteins, OLAARP-containing proteins may also be included.

All these results suggest that modification of proteins by lipid peroxidation products is not necessarily a negative consequence of oxidative stress, but it may be a protective mechanism to block highly toxic aldehydes and to produce endogenous antioxidants that will delay oxidative stress. This might be another of the physiological functions of some broadly extended proteins, like albumin.

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