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ORIGINAL

Relation of plasma protein oxidation parameters and paraoxonase activity in the ageing population

Ufuk Çakatay · Refik Kayali · Hafize Uzun

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Abstract The incidence of atherosclerosis increases with age. Oxidative changes in proteins and lipids are considered to be among the molecular mechanisms leading to endothelial dysfunction. Paraoxonase (PON1) is exclusively associated with high-density lipoprotein (HDL) and protects both HDL and low-density lipoprotein (LDL) from oxidation. PON1 has two cysteine residues for its antioxidant function. We investigated the relation between PON1 activity and protein oxidation parameters such as protein hydroperoxides (P-OOH), protein carbonyl (PCO), total thiol (T-SH) and advanced oxidation protein products (AOPP). Our study also covered other oxidative stress parameters such as oxidised LDL (oxLDL) and superoxide dismutase activity in the plasma of young, middle-aged and elderly individuals. PON1 activity of elderly and middle-aged individuals was decreased significantly compared with that in the young group. oxLDL levels of elderly individuals were increased significantly compared with those of both the young and middle-aged individuals. P-OOH, PCO and AOPP levels of the elderly and middle aged individuals were higher compared with those of the young. On the other hand, T-SH levels of the elderly and middle-aged individuals were lower compared with those of the young. Side by side with the decrease in the T-SH levels in the middle-aged and elderly groups as compared to the young, the increase we have observed in other protein oxidation parameters in the groups leading to decreasing PON1 activity might, we think, create a predisposition to atherosclerosis.

Keywords Protein oxidation · Thiol · Paraoxonase · Protein carbonyl · Protein hydroperoxide · Ageing

U. Çakatay (✉)
Şenesenevler Abdülezel Paşa Sk.
Çiçek Apt. No:9/7 Bostancı
Istanbul, Turkey
e-mail: cakatay@yahoo.com

U. Çakatay
Istanbul University
Istanbul Faculty of Medicine
Central Laboratory of Clinical Biochemistry
Çapa, 34390 Istanbul, Turkey

R. Kayali
Istanbul University
Cerrahpasa Medical Faculty
Department of Pediatrics
Laboratory of Biochemistry
34303 Istanbul, Turkey

H. Uzun
Istanbul University
Cerrahpasa Medical Faculty
Department of Biochemistry
34303 Istanbul, Turkey

Introduction

The incidence of cardiovascular diseases increases with age [1]. The underlying histopathological process is atherosclerosis [1, 2]. One of the most recognised initiating factors is hypercholesterolaemia, which is particularly associated with an increased susceptibility to oxidation of low-density lipoproteins (LDL) [3]. Oxidised LDL (oxLDL) causes alteration in membrane proteins and in phospholipids, and increased expression of signalling molecules [4]. Membrane damage caused by oxLDL impairs endothelial function. Paraoxonase (PON1) is

associated with high-density lipoprotein (HDL). PON1 functions in preventing lipid oxidation not only of LDL, but also of HDL itself [5]. This protection is most probably related to the PON1 hydrolysing activity of some activated phospholipids [6] and/or lipid peroxide [7] products. Human PON1 (aryldialkylphosphatase, EC 3.1.8.1) is a 43-kDa esterase associated with apolipoprotein A1 (apoA-I) and clusterin (apolipoprotein J) in HDL. On the other hand, PON1 was shown to possess peroxidase-like activity that can contribute to its protective effect against lipoprotein oxidation, as well as a homocysteine-thiolactonase activity that may be linked with its anti-atherogenic properties [7, 8].

For many years lipid oxidation has been the focus of investigation, but due to their relatively high abundance it is now recognised that proteins are the main targets for oxidants. Recently, however, the number of reports supporting the idea that protein and lipid oxidation are coupled processes has increased. On the other hand, the interaction between lipids and proteins during oxidation is not well understood. Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cell function, therefore structural changes in proteins are considered to be among the molecular mechanisms leading to endothelial dysfunction [9–11]. It is well established that exposure of proteins to reactive oxygen species (ROS) can alter the physical and chemical structure of the target causing consequent oxidation of side-chain groups, protein scission, backbone fragmentation, cross-linking, unfolding and formation of new reactive groups. The latter include oxidation of hydrophobic amino acyl residues to hydroxy and hydroperoxy (P-OOH) derivatives, protein carbonylation (PCO), oxidation of thiol (T-SH) groups, dityrosine formation and many others. The conformational changes that result from this complex of reactions lead to the decrease or loss of protein biological function. These processes have been widely reviewed in ref. [12]. Recently, a new marker of protein oxidation, advanced oxidation protein products (AOPP), has begun to attract the attention of various investigators [9–11, 13]. AOPP are proteins, predominantly albumin and its aggregates damaged by oxidative stress [14]. They contain abundant dityrosines, which allow cross-linking, disulphide bridges and carbonyl groups [15] and are formed mainly by chlorinated oxidants – hypochloric acid and chloramines resulting from myeloperoxidase activity [14, 15]. Protein oxidation products mediated by chlorinated species generated by an enzyme myeloperoxidase were found in the extracellular matrix of human atherosclerotic plaques [9] and increased levels of AOPP were described as an independent risk factor for coronary artery disease [10].

This study aims to evaluate whether decreased PON1 activity might be associated with increased levels of oxidised proteins in the plasma of elderly people. We further assessed the activity of PON1 to maintain redox status of plasma proteins and investigated the effect of protein oxidation on its antioxidant activity.

Materials and methods

Subjects

The study population consisted of 87 healthy people not taking medication, who either attended a routine health check at a general practice or were members, and their relatives, of the staff of the Istanbul Faculty of Medicine, Central Laboratory of Clinical Biochemistry. The subjects in our study were definitely voluntary participants and all gave informed consent. The sample groups consisted of 29 young, 26 middle-aged and 32 elderly participants. The subjects were all healthy non-smokers and non-drinkers. Subjects who took drugs and food products that affect lipid metabolism and were judged as inappropriate for the study by the physician were excluded. The elderly subjects were living at home, functionally independent and cognitively intact. The age limits of the study groups and grouping of the various age limits were based on previously published literature [1, 3]. Demographic (gender, age), anthropometric (weight, height) and biochemical details of the populations studied are given in Table 1. The ethical procedures were performed according to the World Medical Association Declaration of Helsinki [16].

Sample collection

All samples were taken in the morning to avoid the confounding effect of diurnal variation of oxidative stress parameters as reported previously [17]. Venous blood samples were drawn in the fasting state and processed within 1 h of collection. Blood samples were collected in tubes containing lithium heparin, EDTA or no additive depending on the analysis. For protein oxidation parameters, plasma samples containing lithium heparin were stored at –70°C until analysis; all other parameters were determined on the same day of collection [13].

Analytical methods

Apparatus

Plasma P-OOH, PCO, T-SH, AOPP levels and PON1 activity were measured by a spectrophotometer (Heraeus 400, Kendro Laboratory Product, Osterode, Germany). Plasma oxLDL and superoxide dismutase (SOD) activity

Table 1 Demographic and anthropometric data, and the serum endogenous antioxidant levels of the participating subjects in the present study

	Young (n = 29) (22–45 years)	Middle-aged (n = 26) (46–65 years)	Elderly (n = 32) (66–89 years)
Subjects (n) (M/F)	15/14	12/14	13/19
Height (m)	1.66 ± 0.07	1.63 ± 0.07	1.61 ± 0.08
Weight (kg)	67.88 ± 11.99	73.71 ± 9.20	66.00 ± 9.83[a]
Albumin (g/l)	44.1 ± 0.28	42.7 ± 0.34	42.2 ± 0.30[b]
Uric acid (mg/dl)	4.11 ± 1.46	4.54 ± 0.70	4.95 ± 1.65
Total bilirubin (mg/dl)	0.75 ± 0.37	0.69 ± 0.34	0.72 ± 0.31
Conjugated bilirubin (mg/dl)	0.22 ± 0.10	0.19 ± 0.07	0.23 ± 0.10
Unconjugated bilirubin (mg/dl)	0.54 ± 0.27	0.52 ± 0.28	0.51 ± 0.22
Unsaturated iron binding capacity (μg/dl)	263.94 ± 68.58	258.93 ± 44.46	237 ± 64.62

Values are mean ± SD
[a] Elderly *vs*. middle-aged, $p < 0.05$
[b] Elderly *vs*. young, $p < 0.05$

measurements were performed by an ELISA system (Organon Teknika, Boseind, the Netherlands). Serum total protein, albumin, uric acid, total bilirubin, conjugated bilirubin, unconjugated bilirubin, unsaturated iron binding capacity, total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol and very low density lipoprotein (VLDL)-cholesterol levels were determined on a Modular Analytics DPP discrete autoanalyser (Roche Diagnostics, Switzerland). VLDL-cholesterol concentration was calculated using the Modular analyser software and the equation:

VLDL-cholesterol = total cholesterol–(HDL cholesterol +LDL-cholesterol)

HDL-cholesterol concentration was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups.

Assay of paraoxonase activity

Plasma PON1 activity was assayed using synthetic paraoxon (diethyl-*p*-nitrophenyl phosphate) as substrate. PON1 activity was determined by measuring the initial rate of substrate hydrolysis to *p*-nitrophenol, the absorbance of which was monitored at 412 nm in the assay mixture containing 2.0 mM paraoxon, 2.0 mM $CaCl_2$ and 20 μl of plasma in 100 mM Tris–HCl buffer (pH 8.0). The blank sample containing incubation mixture without plasma was run simultaneously to correct for spontaneous substrate breakdown. Enzymatic activity was calculated from the molar extinction coefficient 18.290 $M^{-1}cm^{-1}$ and is expressed as units per millilitre. One unit of PON1 activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions [18, 19]. The intra-assay and inter-assay coefficients of variation were 6.7% (n = 20) and 3.4% (n = 26), respectively.

Assay of arylesterase activity

Arylesterase activity was also measured spectrophotometrically [20, 21]. The assay contained 1 mM phenylacetate in 20 mM Tris–HCl (pH 8.0). The reaction was started by the addition of plasma, and the increase in absorbance was read at 270 nm. Blanks were included to correct the spontaneous hydrolysis of phenylacetate. Enzyme activity was calculated using the molar extinction coefficient 1310 $M^{-1}cm^{-1}$. Arylesterase activity was expressed in units per litre. One unit is defined as 1 μmol phenylacetate hydrolysed per minute.

Assay of oxidised low-density lipoprotein

The concentration of oxLDL in plasma was measured by a sandwich ELISA procedure using the murine monoclonal antibody, mAb-4E6, as capture antibody bound to microtitration wells and a peroxidase-conjugated anti-apolipoprotein B antibody recognising oxLDL bound to the solid phase (Mercodia Oxidized LDL ELISA, Uppsala, Sweden). The coefficients of intra- and inter-assay variations for ELISA assay were 5.2% (n = 8) and 7.3% (n = 8), respectively.

Assay of protein hydroperoxide

P-OOHs were measured by the guanidine-perchloric acid-ferric-xylenol orange method (G-PCA-FOX) [22, 23]. The assay is based on the oxidation of Fe^{2+} by peroxides in the presence of the dye xylenol orange that gives a coloured complex with the Fe^{3+} generated. The Fe-XO complex can be measured in the visible absorbance range (560 nm). Proteins were precipitated from 100 μl of plasma by the addition of 500 μl of 0.2 M ice-cold PCA. Samples were kept on ice for 5 min and

centrifuged at 6500 g. The precipitated proteins were dissolved in 1100 µl of 6 M guanidine hydrochloride. The protein solution was then washed twice with chloroform containing 4 mM butylated hydroxytoluene. After mixing the washed protein solution, xylenol orange and ferrous solutions, the absorbance at 560 nm was read after 60 min against a blank containing 6 M guanidine hydrochloride instead of the protein solution. The molar concentration of P-OOHs in the final medium was calculated with the equation $c = A_s/\varepsilon$ using the molar absorption coefficient value of 37 000 $M^{-1}cm^{-1}$ [22]. The coefficients of intra- and inter-assay variations for P-OOH assay were 4.2% (n = 10) and 8.1% (n = 10), respectively.

Assay of protein carbonyl

Plasma PCO levels were measured spectrophotometrically using the method of Reznick and Packer [24]. PCO groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. DNPH was dissolved in HCl, and after the DNPH reaction proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with 4 ml of an ethanol/ethyl acetate mixture (1:1). Washings were achieved by mechanical disruption of pellets in the washing solution using a small spatula, and re-pelleting by centrifugation at 6000 g for 5 min. Finally, the precipitates were dissolved in 6 M guanidine–HCl solution and the absorbances were measured at 360 nm, using the molar extinction coefficient of DNPH, $e = 2.2 \times 10^4$ $M^{-1}cm^{-1}$. Protein contents were determined on the HCl blank pellets spectrophotometrically using a Folin kit (Sigma Diagnostics, St. Louis, MO, USA). The coefficients of intra- and inter-assay variations for carbonyl assay were 4.1% (n = 10) and 8.4% (n = 10), respectively.

Assay of total thiol

Plasma total thiol (T-SH) concentration was determined using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) as described by Hu [25]. The coefficients of intra- and inter-assay variations were 1.8% (n = 10) and 4.6% (n = 9), respectively.

Assay of advanced protein oxidation products

Spectrophotometric determination of AOPP levels was performed by modification of Witko-Sarsat's method [26]. Samples were prepared in the following way: 200 ml of plasma was diluted 1:5 in PBS, 10 ml of 1.16 M potassium iodide was then added to each tube, followed two minutes later by 20 µl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 2000 µl of PBS, 100 µl of KI and 200 µl of acetic acid. The coefficients of intra- and inter-assay variations were 1.6% (n = 10) and 2.4% (n = 10), respectively. The chloramine-T absorbance at 340 nm being linear within the range 0–100 µmol/l, AOPP concentrations were expressed as micromoles per litre of chloramine-T equivalents.

Assay of superoxide dismutase activity

We measured plasma SOD activity with a commercially available kit (Cayman Chemical Company, Michigan, USA). Actual activity of SOD in the samples was obtained from the linear regression of the standard curve substituting the linearised rate for each sample.

Statistical analysis

The computer program InStat was used for data analysis. Descriptive statistics were given as mean ± SD. None of the data were normally distributed. The plasma P-OOH, PCO, AOPP, T-SH, SOD, oxLDL, PON1, arylesterase and endogenous antioxidant parameters levels of aged populations were compared using the Kruskal–Wallis nonparametric analysis of variance (ANOVA) test. Spearman's rank correlation coefficients were used to investigate various oxidative stress markers. When not specified, $p < 0.05$ was considered significant.

Results

The demographic data, anthropometric data and serum endogenous antioxidant levels such as albumin, uric acid, total bilirubin, conjugated bilirubin, unconjugated bilirubin and unsaturated iron binding capacity in the study groups are given in Table 1. Among the serum endogenous antioxidant parameters, the levels of serum albumin within the normal range were significantly lower in older persons ($p < 0.05$), while the levels of the other endogenous antioxidant parameters were all found to be not different.

Serum lipid profile parameters, oxLDL, PON1 and arylesterase activity in the study groups are given in Table 2. Fasting serum lipid profile parameters except HDL-cholesterol in the elderly group were significantly different than the other groups. Serum oxLDL levels of the elderly subjects were significantly increased compared with those of the young subjects ($p < 0.001$), while oxLDL levels of young and middle-aged subjects were not different. On the other hand, serum oxLDL levels of the elderly subjects were significantly increased compared with those of the middle-aged subjects ($p < 0.05$). Serum PON1 activities of middle-aged and elderly subjects were significantly decreased compared

Table 2 Serum lipids, oxLDL, PON1 and arylesterase activity in the study groups

	Young (n = 29) (22–45 years)	Middle-aged (n = 26) (46–65 years)	Elderly (n = 32) (66–89 years)
Triglycerides (mg/dl)	102.48 ± 51.77	144.15 ± 89.40	130.84 ± 76.29^{a*b*}
Total cholesterol (mg/dl)	189.62 ± 35.02	229.42 ± 37.40	202.25 ± 38.47^{a***b*}
HDL-cholesterol (mg/dl)	49.43 ± 11.00	55.42 ± 13.40	54.56 ± 14.70
LDL-cholesterol (mg/dl)	116.71 ± 32.38	142.61 ± 29.53	143.34 ± 31.67^{a**b*}
VLDL-cholesterol (mg/dl)	22.93 ± 8.72	31.38 ± 15.77	26.34 ± 12.08^{a*b*}
oxLDL (U/ml)	52.87 ± 7.96	58.98 ± 9.51	67.03 ± 8.59^{b***c*}
PON1 (U/ml)	179.51 ± 50.36	105.79 ± 47.35	77.59 ± 24.87^{a***b***}
Arylesterase (U/ml)	78.19 ± 9.77	61.81 ± 9.00	52.05 ± 5.09^{a***b***c**}

Values are mean ± SD
[a] Middle-aged *vs.* young; [b] elderly *vs.* young; [c] elderly *vs.* middle-aged. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

with those of the young subjects ($p < 0.001$, $p < 0.001$, respectively). There were no significant differences between middle-aged and elderly subjects for PON1 activity. Serum arylesterase activity of the middle-aged and elderly subjects were significantly decreased compared with those of the young subjects ($p<0.001$, $p < 0.001$, respectively). On the other hand, serum arylesterase activity of the elderly persons were significantly decreased compared with those of the middle-aged subjects ($p < 0.01$).

Protein oxidation markers in the study groups are given in Table 3. Serum P-OOH levels of middle-aged and elderly subjects were significantly increased compared with those of the young subjects ($p < 0.001$, $p < 0.001$, respectively). There was a trend toward higher P-OOH levels in elderly subjects compared to middle-aged subjects, but this did not reach statistical significance (Table 3). On the other hand, plasma PCO and AOPP levels of the elderly and middle aged individuals were higher than those of the young subjects ($p < 0.01$; $p < 0.05$, for both parameters respectively). Plasma T-SH levels of the elderly and middle-aged individuals were lower compared with those of the young subjects ($p < 0.001$; for both groups). In our study, we observed an age-dependent increase in SOD activity in elderly individuals *vs*. the middle-aged group.

Spearman's rank correlation coefficients between PON1, arylesterase, oxLDL and protein oxidation markers in the whole series of study groups are given in Table 4.

Discussion

The existence and the role of increased extra- and intracellular oxidative stress in ageing and aged-related disorders is currently under discussion. In a previous study, Seres et al. demonstrated a decrease in PON1 activity with ageing, which was not explained by a decrease in

Table 3 Parameters used to determine protein oxidation in plasma of the studied groups

	Young (n = 29) (22–45 years)	Middle-aged (n = 26) (46–65 years)	Elderly (n = 32) (66–89 years)
P-OOH (nmol/mg.pr)	1.22 ± 0.10	1.43 ± 0.21	1.51 ± 0.26^{a***b***}
PCO (nmol/mg.pr.)	0.60 ± 0.10	0.68 ± 0.11	0.71 ± 0.14^{a*b**}
T-SH (μmol/l)	420.64 ± 32.48	369.92 ± 37.74	334.46 ± 50.60^{a***b***}
AOPP (μmol/l of chloramine-T equivalents)	40.99 ± 9.52	62.02 ± 26.59	69.59 ± 16.90^{a*b**}
SOD (U/l)	2.183 ± 0.61	1.885 ± 0.38	2.231 ± 0.57^{c*}

Values are mean ± SD
[a] Middle-aged *vs.* young; [b] elderly *vs*. young; [c] elderly *vs.* middle-aged. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
pr protein

Table 4 Spearman's rank correlation coefficients between PON1, arylesterase, oxLDL and protein oxidation markers in the whole series of study groups

	P-OOH	PCO	T-SH	AOPP	SOD
PON1	–0.43***	–0.32**	0.49***	0.42***	–0.08
Arylesterase	–0.48***	–0.24*	0.49***	–0.05	–0.04
OxLDL	0.52***	0.34**	–0.25*	0.03	0.12

* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

the PON1's plasma concentration or by a change in the HDL circulating levels [1]. On the other hand, Seres et al.'s results suggested that increasing oxidative stress with ageing could explain, in part, the observed reduction in PON1 activity of the elderly. Ageing is accompanied by the development of oxidative protein damage as evaluated by an increase in the circulating oxidised proteins [9–11, 27]. That change could be related to an increased risk for endothelial damage. Indeed, these biochemical modifications could affect PON1 activity and as a result lead to an increased susceptibility of plasma proteins to protein oxidation in elderly individuals. Therefore, the reduction in the PON1 activity as well as in its antioxidant activity could negatively affect the atheroprotective properties of plasma proteins in the elderly.

Albumin is the most abundant plasma protein and is an extracellular antioxidant. Albumin contains 17 disulphide bridges and has a single remaining cysteine residue and it is this residue that is responsible for the capacity of albumin to react with and neutralise peroxyl radicals. The measurement of T-SH is a good reflection of excess free radical generation, as the conformation of albumin is altered, allowing thiol (-SH) groups to be oxidised [27, 28]. In a previous studies, serum albumin levels have been shown to be decreased in elderly subjects [28]. We found that serum albumin levels of the elderly were significantly lower than those of the other groups. Decreased T-SH levels have been attributed to a decrease in serum albumin levels and increased oxidative stress in the elderly. AOPP are defined as dityrosine-containing cross-linked protein products and are considered as reliable markers to estimate the degree of protein oxidation [29]. Biochemical characterisation of AOPP in plasma revealed that both the high- and low-molecular-weight AOPP peaks contain oxidised albumin in aggregate-forming or monomeric form [26]. In the present study, the occurrence of protein oxidation in plasma of ageing individuals was also confirmed by a novel marker (AOPP assay) that provides information on the degree of oxidative damage to proteins, and the data obtained support those found with detection of P-OOH, PCO and T-SH. There was a trend toward similar SOD activity in elderly subjects compared to young subjects, but this did not reach statistical significance. Significantly higher plasma SOD activity was observed in the elderly group compared to the middle-aged group. Probably, increased plasma superoxide radical status makes elderly individuals more susceptible to protein and LDL oxidation. The increased activity of SOD might be an adaptive response, and it results in increased dismutation of superoxide to hydrogen peroxide. However, the exact mechanism is not known.

The apparent dominance of free radical scavenging by the cellular proteins results in the formation of reactive P-OOHs within the actual cells [30]. It is well established that the P-OOHs formed particularly on aliphatic amino acid residues (Val, Leu, Ile, Glu, Pro, Lys) are generated under the absolute requirement of the oxygen presence [31, 32]. The P-OOHs may be reductively detoxified to hydroxides but their decomposition can also result in the formation of further radicals that may propagate reaction chains [31, 32]. Unlike PCOs, P-OOHs are reasonably reactive and will cross-link with DNA [31] and oxidize cellular thiols [33]. PON1's free thiol group is supposed to be the active site for its antioxidant activity [8]. The thiol (-SH) moiety on the side chain of the amino acid cysteine is particularly sensitive to redox reactions and is an established redox sensor [34]. Protein thiols are also readily oxidised by P-OOHs with this potentially resulting in enzyme inactivation [35]. In the current study, significantly lower T-SH levels and PON1 activity were observed in elderly and middle-aged groups compared to young groups. On the other hand, our data show high P-OOH and PCO levels both in elderly and middle-aged groups: the two groups show the same susceptibility to protein oxidation because of the possible failure of the protective role of T-SH and PON1. To our knowledge, there is no correlation analysis between protein oxidation markers and PON1 activity in the ageing population in the literature. In our study, P-OOH, PCO, AOPP and T-SH levels in the whole series of study groups were significantly correlated with plasma PON1 activity. Decreased PON1 activity has been attributed to increased plasma oxidative protein damage in the ageing population. We also observe decreased arylesterase activity, which is less affected by genetic polymorphisms. Arylesterase activity shows a similar trend to that in the PON1 activity in our study groups.

There is evidence of a possible relationship between increased plasma protein oxidation parameters and decreased PON1 activity. Inactivation of PON1 itself may lead to a predisposition to atherosclerosis as well as increased markers of oxidative stress. It would be interesting to clarify whether the decrease in PON1 activity occurs selectively in preference to a range in other redox-sensitive enzymes with thiols in their active site, or whether there is a general diminution of enzyme activity. We hope to study this point in some future experiment or it may be taken up by other researchers before we tackle it. This hypothesis needs further investigation in order to confirm the relationship between these phenomena.

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