

Nitrated and Oxidized Plasma Proteins in Smokers and Lung Cancer Patients¹

Brigitte Pignatelli, Chun-Qi Li,² Paolo Boffetta, Qiping Chen, Wolfgang Ahrens, Fredrik Nyberg, Anush Mukeria, Irene Bruske-Hohlfeld, Cristina Fortes, Vali Constantinescu, Harry Ischiropoulos, and Hiroshi Ohshima³

International Agency for Research on Cancer, F-69372 Lyon Cedex 08, France [B. P., C.-Q. L., P. B., H. O.]; The Stokes Research Institute and Department of Pediatrics, Neonatology Division, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19140 [Q. C., H. I.]; Bremen Institute for Prevention Research and Social Medicine (BIPS), 28199 Bremen, Germany [W. A.]; Institute of Environmental Medicine, Karolinska Institute, 17177 Stockholm, Sweden [F. N.]; Institute of Carcinogenesis, 115478 Moscow, Russia [A. M.]; National Research Center for Environment and Health (GSF), 85758 Munich, Germany [I. B.-H.]; Regional Epidemiological Unit, 00198 Rome, Italy [C. F.]; and National Institute of Public Health, 76256 Bucharest, Romania [V. C.]

ABSTRACT

Cigarette smoking is a cause of lung cancer and other respiratory diseases. Oxidants either present in cigarette smoke and/or formed in the lung of smokers may trigger oxidative and nitrative damage to DNA and cellular components, contributing to carcinogenesis. We have used immunodot and Western blot analyses to measure nitrated (nitrotyrosine-containing) and oxidized (carbonyl-containing) proteins in plasma samples collected from 52 lung cancer patients and 43 control subjects (heavy and light smokers, nonsmokers with or without exposure to environmental tobacco smoke). The levels of nitrated proteins were significantly higher in lung cancer patients than in controls ($P = 0.003$). On the other hand, the levels of oxidized proteins were significantly higher in smokers than in nonsmokers ($P < 0.001$). Western-blot analyses showed the presence of two to five nitrated proteins and one oxidized protein. Using immunoprecipitation and Western-blot analyses with eight different antibodies against human plasma proteins, we identified fibrinogen, transferrin, plasminogen, and ceruloplasmin as nitrated proteins and fibrinogen as the only oxidized protein present in human plasma of lung cancer patients and smokers. Our results indicate that cigarette smoking increases oxidative stress and that during lung cancer development, formation of reactive nitrogen species results in nitration and oxidation of plasma proteins.

INTRODUCTION

Lung cancer is one of the most commonly diagnosed malignancies and remains the main cause of cancer mortality, with >900,000 estimated deaths worldwide per year (700,000 in men and 230,000 in women) in 1990 (1, 2). Cigarette smoking is an established cause of human cancer in a variety of organs including the lung, larynx, oral cavity, and esophagus (3). The mechanisms of lung carcinogenesis through which tobacco smoke operates are not completely clear. In particular, cigarette smoke is divided into gas phase and particulate matter, both of which contain various oxidants and free radicals, including nitric oxide in the gas phase and a quinone/hydroquinone complex held in a tarry matrix (4). These oxidants can induce lipid peroxidation *in vitro* and *in vivo* (5–7) and cause DNA single-strand breaks as well as modifications of DNA bases, including formation of 8-hydroxyguanine, xanthine, and hypoxanthine (4, 8). Cigarette smoking also causes an acute inflammatory reaction in the lung characterized by the accumulation and activation of leukocytes *in vivo*, producing reactive oxygen and nitrogen species in high concen-

trations (9, 10). These reactive species could be responsible for most tissue injuries and disease states associated with inflammation. Increased oxidative DNA damage (8-hydroxyguanine formation) in human peripheral leukocytes (11–14) and in lung tissue (15) has been observed in smokers, compared with nonsmokers.

Proteins are major targets for oxidative and nitrative damage *in vivo* (16–18). Exposure of proteins to reactive oxygen or nitrogen species results in modification of amino acid residues, altering the protein structure and function (17, 18). Free and protein-bound NTYR,⁴ a stable product of nitration of tyrosine residues, has been measured as a biomarker of protein damage induced by peroxynitrite and other reactive nitrogen species (18). Increased levels of free NTYR in the plasma of cigarette smokers and of protein-bound NTYR in bronchoalveolar lavage fluids from patients with acute respiratory distress syndrome have been reported (19–21). Moreover, a recent study identified ceruloplasmin, transferrin, α 1-antichymotrypsin, α 1-protease inhibitor, and the β -chain of fibrinogen as the major nitrated plasma proteins in patients with acute respiratory distress syndrome (21). Similarly, carbonyl groups in proteins, determined as DNPH derivatives, have been analyzed as a biomarker of oxidative damage of proteins (17). Increased carbonyl levels in peripheral blood globin of smokers, compared with nonsmokers, have been reported (22). Modified forms of proteins accumulate during aging, oxidative stress, and some pathological conditions (17, 18).

To bridge the gap between laboratory experiments and the population-based epidemiological study, the present transitional study (23) was performed. We have used immunodot blot and WB assays to determine levels of both nitrated and oxidized proteins in human plasma samples from cases of lung cancer and controls stratified according to cigarette smoking and exposure to ETS. We found that levels of nitrated proteins are significantly elevated in plasma samples from lung cancer patients compared with healthy controls, and that levels of oxidized protein are higher in smokers than nonsmokers. Furthermore, we identified some of these modified proteins present in human plasma using immunoprecipitation and WB analyses with specific antibodies to human plasma proteins.

MATERIALS AND METHODS

Subjects. During 1994–1996, we enrolled histologically confirmed cases of lung cancer and controls in eight countries: Sweden, France, Germany, Italy, Poland, Russia, Romania, and Brazil. These subjects were part of a larger study aimed at assessing the role of markers of individual susceptibility to lung cancer among nonsmokers (24). In Sweden and Germany, controls were selected from the general population, whereas in the other countries they were selected among healthy individuals or patients admitted to the same hospitals as the cases. Patients admitted to the hospitals for tobacco-related diseases were excluded as controls.

Cases and controls were administered a validated and standardized questionnaire (25, 26) that included detailed sections on active smoking, ETS exposure during childhood, ETS exposure during adulthood from the spouse

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² C.-Q. L. is currently at the Massachusetts Institute of Technology, Division of Bioengineering and Environmental Health, Cambridge, MA 02139.

³ To whom requests for reprints should be addressed, at the International Agency for Research on Cancer, 150 Cours Albert Thomas, 69372 Lyon, Cedex 08, France. Fax: 33-4-72-73-80-88; E-mail: ohshima@iarc.fr.

⁴ The abbreviations used are: NTYR, nitrotyrosine; DNP, dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; ETS, environmental tobacco smoke; WB, Western blot.

Table 1 Selected characteristics of study subjects, by smoking habit and case-control status

	Cases			Controls		
	M/F ^a	Age ^b	Tobacco ^c	M/F ^a	Age ^b	Tobacco ^c
Ever smokers						
Heavy smokers	8/8	64 ± 9	37 ± 17	4/11	72 ± 11	39 ± 32
Light smokers	1/7	64 ± 15	2.2 ± 1.4	1/2	67 ± 12	2.5 ± 1.6
Never smokers						
ETS exposure	0/15	68 ± 10		1/14	67 ± 10	
No ETS exposure	1/12	62 ± 19		2/8	60 ± 16	

^a Number of male and female subjects.^b Average and SD.^c Cumulative tobacco smoking among smokers expressed in pack-years (average and SD).

and at the workplace, occupational exposures, and family history of cancer. The section on ETS exposure had been used in a previous study conducted in Europe (25) and previously validated against urinary cotinine measurements (26).

We defined as nonsmokers those cases and controls who had smoked less than 400 cigarettes, or the equivalent amount of tobacco as cigars, cigarillos or pipe tobacco, during their life time. This corresponds to about one cigarette per day for 1 year. Nonsmokers were classified as exposed or unexposed to ETS from the spouse or at the workplace. Smokers were classified according to cumulative consumption of tobacco, expressed as pack-years. Two groups of smokers were formed, one of heavy smokers (range 21–143 pack-years) and one of light smokers (range 0.3–4.3 pack-years). Ex-smokers were defined as smokers who had stopped smoking for at least 1 year before the start of the study.

We included 52 lung cancer cases and 43 controls, of whom 24 were from the general population and 19 from hospitals. Eighty-five % of the subjects were enrolled at six centers in Sweden, Germany, Italy, Romania, and Russia. Table 1 reports selected characteristics of study subjects. Cases and controls were similar with respect to sex, age, and tobacco consumption. However, there were more women among nonsmokers than among smokers (*P* of the difference, 0.001). Histopathological types of cancer include squamous cell carcinoma (15 cases, of whom 4 were nonsmokers), small cell carcinoma (8 cases, of whom 4 were nonsmokers), adenocarcinoma (21 cases, of whom 17 were nonsmokers), and other and mixed types (8 cases, of whom 3 were nonsmokers). Informed consent was obtained from all of the patients and the protocol was approved by the local and IARC Ethical Committees.

A 15-ml blood sample was collected from each case and control at the time of interview. Aliquots of plasma, WBCs, and RBCs, were separated shortly after collection and stored at -80°C until shipment to IARC, where they were stored under similar conditions until analysis. Total storage time was in the range of 2–4 years. Protein concentration in plasma was measured with a protein assay (Bio-Rad Laboratories, Hercules, CA) according to the method described in the reagent instructions. Mean plasma protein concentration was $16.7 \pm 5.72 \mu\text{g}/\mu\text{l}$ (range, 5.5–26.8 $\mu\text{g}/\mu\text{l}$). All of the biochemical analyses were carried out blindly for case/control and smoking status in duplicate and were repeated at least twice.

Preparation of Standard Nitrated and Oxidized Human Plasma Proteins. Human plasma proteins (albumin, transferrin, and fibrinogen; Sigma, St. Louis, MO) were incubated at 1 mg/ml with 1 mM peroxynitrite in 0.5 M phosphate buffer (pH 7.4). Peroxynitrite was synthesized in a quenched flow reactor, and excess hydrogen peroxide was destroyed by granular manganese dioxide (27). Peroxynitrite-treated transferrin (1 mg/ml) was incubated for 16 h at 50°C with 1 mg of dialyzed Pronase E. Concentrations of NTYR were measured by high-performance liquid chromatography using a postseparation on-line reduction column and an electrochemical detector, as reported previously (28). The concentration of protein carbonyls in human fibrinogen oxidized by peroxynitrite was determined spectrophotometrically after formation of the DNPH derivative, as previously described (29). Peroxynitrite-treated transferrin and fibrinogen, which contained 8 nmol of NTYR/mg of protein and 400 nmol of carbonyls/mg of protein, respectively, were used as standards.

Preparation of DNPH-derivatized Proteins for SDS-gel Electrophoresis and Immunodot Blot Analysis. DNPH was prepared as a 20-mM solution in 10% trifluoroacetic acid (30, 31). A 20- μg aliquot of plasma proteins was placed in a 1.5-ml tube, dried under vacuum, and then resuspended in 3 μl of

H_2O . The sample was mixed with 3 μl of 12% SDS and 6 μl of the DNPH solution and incubated for 20 min at room temperature. The solution was neutralized by addition of 4.5 μl of 2 M Tris base/30% glycerol (v/v), giving final concentrations of 0.54 M and 8.2% (v/v), respectively. The derivatized and neutralized samples were applied to 7.5% SDS PAGE or dot-blotted onto a membrane.

Immunodot Blot Analysis of NTYR and Oxidized Proteins. Twenty μg of plasma proteins were used either directly for NTYR analysis or after DNPH-derivatization for analysis of oxidized proteins using previously published methods (32, 33) that were modified as follows: proteins were diluted in 200 μl of PBS and dotted onto a Millipore Immobilon-P membrane using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membrane was blocked using 5% nonfat dried milk for 1 h at room temperature and incubated with either a mouse anti-NTYR monoclonal IgG (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:2000 or a rabbit anti-DNP antibody (Oxyblot; Oncor, Gaithersburg, MD) at a dilution of 1:300 overnight at 4°C , followed by either a goat antimouse secondary antibody conjugated to horseradish peroxidase (1:4000 dilution) or a horseradish peroxidase-conjugated goat antirabbit secondary antibody (1:400) for 2 h at room temperature. The membrane was then examined by chemiluminescence using a Covalab kit (Dako, Trappes, France) and exposed to hyperfilm (Amersham Pharmacia Biotech, Orsay, France), and the films were exposed for 3 min. Transferrin and fibrinogen treated with peroxynitrite were used as standards for NTYR and oxidized proteins, respectively. Blot densities were measured by scanning the films using a GS-670 Imaging Densitometer (Bio-Rad). Concentrations of nitrated and oxidized plasma proteins were calculated using calibration curves drawn with different concentrations of standard proteins (see typical examples in Fig. 1). The variation between duplicate measurements was 10–15%. Detection limits for the analyses of nitrated and oxidized proteins were 0.08 and 0.5 nmol/mg protein, respectively.

WB Analyses of NTYR and Oxidized Proteins. The DNPH-derivatives of oxidized proteins are heat-sensitive, and we also noted very frequently that bands corresponding to NTYR-containing proteins disappeared if a sample was heated in Laemmli buffer at 95°C for 5 min before separation on SDS-PAGE. This latter observation is in accordance with that of Balabanli *et al.* (34), who recently reported that NTYR is easily reduced to aminotyrosine by heating in thiol-containing Laemmli buffer. Therefore, 20 μg of plasma protein or DNPH-derivatized plasma proteins were mixed with Laemmli buffer, loaded without heating, separated by 7.5% SDS-PAGE under reducing conditions, and transferred to Millipore Immobilon-P membrane with a Mini-

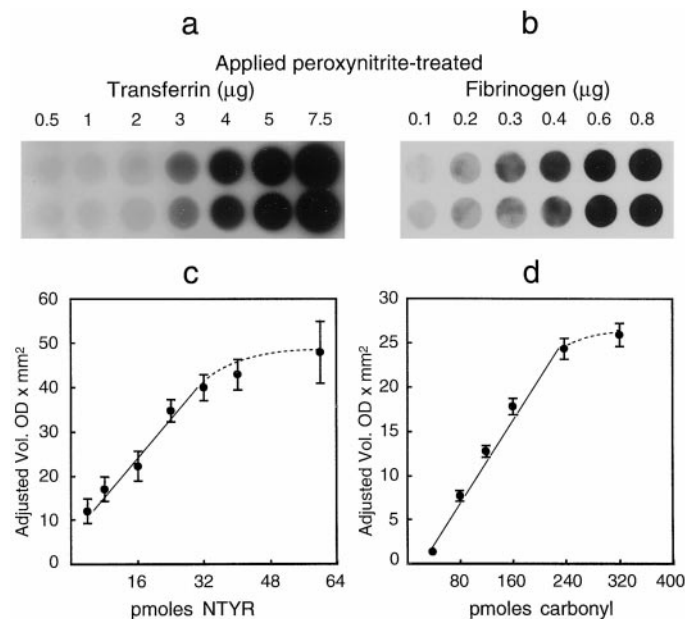


Fig. 1. Analyses of NTYR (a, c) and carbonyl (b, d) contents of peroxynitrite-treated human standard proteins. Immunoblots from peroxynitrite-treated transferrin (a), peroxynitrite-treated fibrinogen (b), and corresponding calibration curves generated by densitometric evaluation (c and d).

PROTEAN II electrophoresis cell (Bio-Rad) for analysis of NTYR or oxidized proteins. The membrane was blocked using 5% nonfat dried milk. NTYR and oxidized proteins were detected by incubation of blots with either a mouse anti-NTYR monoclonal antibody or a rabbit anti-DNP polyclonal antibody, respectively, overnight at 4°C, followed by a secondary goat antimouse or antirabbit IgG conjugated to horseradish peroxidase, and determination of chemiluminescence. Peroxynitrite-treated human plasma proteins (fibrinogen and transferrin) with or without derivatization with DNPH were used as positive controls for oxidized proteins and NTYR residues in proteins, respectively. Human plasma proteins treated only with peroxynitrite or only with DNPH or with neither were used as negative controls. Films exposed for 7 min were used for analyses. The intensity of the WB bands was compared with positive standards. Intensities of bands with 1 and 3 μg peroxynitrite-treated transferrin were designed as attributed values of 2 and 4, respectively, for nitrated proteins. Similarly, the intensities of bands with 0.5 and 0.8 μg peroxynitrite-treated fibrinogen were designed as attributed values of 2 and 4, respectively, for oxidized proteins. Intensity of bands found in human plasma samples were evaluated as: 0, null; and 1–4 by comparison with positive standards. The levels of modified proteins were estimated from the sum of the intensities of each band.

Identification of Nitrated and Oxidized Plasma Proteins by Immunoprecipitation and WB Analyses. An aliquot of 125 μg plasma proteins was used to perform immunoprecipitation experiments for identification of the nitrated proteins as described previously (21). The sample volume was adjusted to 500 μl with immunoprecipitation buffer [20 mM Tris-base (pH 7.4), 140 mM NaCl, 10% glycerol, 1% Triton X-100, 4 mM EGTA, supplemented with 10 $\mu\text{g}/\text{ml}$ aprotinin and 1 mM phenylmethylsulfonyl fluoride in methanol] and precleared by incubation with 15 μl of protein G (Amersham Pharmacia Biotech) at 4°C for 1 h. After centrifugation at $10,000 \times g$ for 3 min, the supernatant was transferred to a new tube and incubated with 2 μl (1 $\mu\text{g}/\mu\text{l}$) of mouse anti-NTYR monoclonal antibody overnight at 4°C, followed by incubation with 30 μl of protein G at 4°C for 1 h. After centrifugation at $10,000 \times g$ for 2 min, the pellet was washed with 0.5 ml of immunoprecipitation buffer. This procedure was repeated four times. After the last centrifugation, the pellet was resuspended in 30 μl of 2.5 \times Laemmli buffer, heated for 8 min at 100°C, electrophoresed through a 12% SDS-PAGE, and transferred to Millipore Immobilon-P membrane. The membrane was blocked for 2 h at room temperature with 10% nonfat dried milk and incubated with one of the following seven rabbit polyclonal antibodies against human plasma proteins: ferritin (1:8,000 dilution), fibrinogen (1:4,000 dilution), albumin (1:5,000 dilution), plasminogen (1:5,000 dilution), transferrin (1:5,000 dilution), ceruloplasmin (1:5,000 dilution), antichymotrypsin (1:5,000 dilution); (all of the above protein antibodies from Dako), and one mouse monoclonal antibody against human α_1 -antitrypsin (1:5,000 dilution; Calbiochem, La Jolla, CA). The membrane was further incubated with a secondary goat antirabbit (1:3,000 dilution) or antimouse (1:3,000 dilution) antibody conjugated to horseradish peroxidase (Dako). The membrane was then observed by supersignal ultrachemiluminescence (Pierce, Rockford, IL).

Similarly, an aliquot of 125 μg plasma protein was derivatized with DNPH, and the DNPH-derivatized proteins were immunoprecipitated with 3 μl (0.5 $\mu\text{g}/\mu\text{l}$) of rabbit anti-DNP antibody and examined by WB analyses using the above antibodies to identify oxidized plasma proteins.

The results from immunoprecipitation and WB analyses were confirmed with reverse immunoprecipitation experiments. The plasma proteins were first immunoprecipitated with one of the above-mentioned antihuman plasma protein antibodies and then analyzed by WB analyses using either mouse anti-NTYR monoclonal antibody or rabbit anti-DNP antibody.

Plasma samples from healthy humans, which contained no detectable level of nitrated or oxidized proteins (<0.08 and <0.5 nmol/mg protein, respectively) were used as negative specimens. Several control experiments were also carried out, including immunoprecipitation without anti-NTYR or anti-DNP antibody, or WB analyses without any specific plasma protein antibodies. All of the experiments were repeated at least two or three times to confirm results.

Statistical Analyses. We compared the mean levels of oxidized and nitrated proteins according to smoking habits and according to case/control status, using Student's *t* test. In addition, we conducted a multiple linear regression analysis of the association between levels of oxidized and nitrated proteins and smoking habit and case/control status, after controlling for potential confounding factors (sex, age, and center). We also conducted a

multiple logistic regression analysis, again with adjustment for sex, age, and center; in this case, elevated levels of oxidized and nitrated proteins were treated as the exposure variables, and smoking habit and case/control status were the outcome variables (35).

RESULTS

Immunodot Blot Analyses of Modified Plasma Proteins. Typical immunodot blot analyses of nitrated and oxidized proteins in plasma from lung cancer patients and smokers are shown in Fig. 2, *a* and *b*. Human transferrin and fibrinogen treated with peroxynitrite were used as standards to quantify nitrated and oxidized proteins, respectively. Table 2 presents the levels of nitrated and oxidized proteins determined by immunodot blot analyses in human plasma of lung cancer patients and controls according to their smoking status. In positive plasma samples, concentrations of nitrated proteins were in the range of 0.10–1.0 nmol of NTYR/mg of protein, and those of oxidized proteins were from 1.0 to 8.0 nmol of carbonyls/mg protein.

WB Analyses of Modified Plasma Proteins. The results of the WB analyses of modified proteins in 95 plasma samples from lung cancer patients and corresponding controls are shown in Table 2 and Fig. 2, *c* and *d*. Some plasma samples contained up to five bands for NTYR-containing proteins, but the number of bands varied between samples (Table 2), and many NTYR-positive samples contained three major bands, with relative molecular masses of $M_r \sim 205,000$, 110,000, and 90,000 (Fig. 2*c*). Only one carbonyl-containing protein band with a relative molecular mass of $M_r \sim 60,000$ was found (Fig. 2*d*). The levels of both nitrated and oxidized plasma proteins determined on the basis of the number and intensity of bands detected by WB analyses correlated well with those determined by immunodot blot analyses.

Multivariate Statistical Analysis. Table 3 presents the results of the multivariate linear regression analysis based on WB analysis. After adjustment for potential confounders, smoking status was associated with elevated levels of oxidized proteins but not of nitrated proteins. No difference between heavy and light smokers was seen for either type of protein modification. Among nonsmokers, ETS exposure was not associated with elevated levels of oxidized proteins and was inversely associated with the level of nitrated proteins.

In the whole study population, a difference between cases and controls was evident with respect to nitrated but not oxidized proteins (Table 3). This difference, however, was present among smokers but not among nonsmokers.

Similar results on the effect of tobacco smoking, ETS exposure, and case/control status were obtained when the analysis was based on results of immunodot blot analysis rather than WB results (not shown in detail). Similarly, the results of the logistic regression analysis provided evidence of an association between smoking status and elevated levels of oxidized proteins (odds ratio for WB analysis, 4.0; 95% confidence interval, 1.4–11) and between case status and elevated levels of nitrated proteins (odds ratio for WB analysis 2.5; 95% confidence interval, 1.0–5.9).

Identification of Specific Nitrated and Oxidized Human Plasma Proteins. To identify nitrated and oxidized proteins present in human plasma from lung cancer patients and smokers, we immunoprecipitated modified proteins using anti-NTYR and anti-DNP antibodies. The immunoprecipitated proteins were examined by WB analyses using eight different antibodies specific for human plasma proteins, which were selected on the basis of their abundance in human plasma and molecular mass. Typical results are shown in Fig. 3*a* for nitrated proteins. The plasma proteins immunoprecipitated with the anti-NTYR antibody were analyzed by WB using an array of antibodies against human proteins. When the immunoprecipitates with the anti-

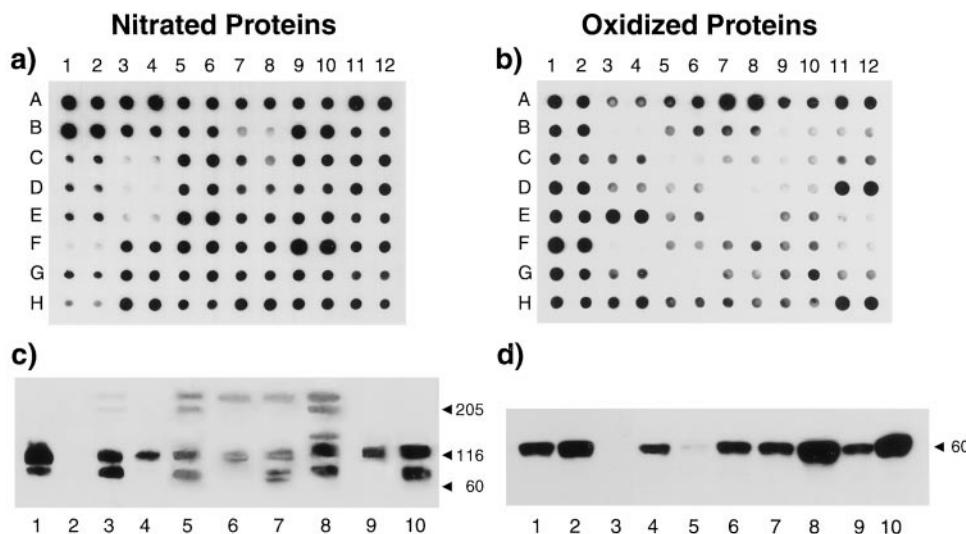


Fig. 2. Detection of nitrated and oxidized proteins by immunoassays. *a*, immunodot blot analyses of nitrated protein in duplicate. Transferrin treated with peroxynitrite (from A1 and A2 to D1 and D2: 3, 2, 1, and 0.5 μg , respectively), fibrinogen treated with peroxynitrite (G1 and G2, and H1 and H2: 0.1 and 0.05 μg , respectively), albumin treated with peroxynitrite (from A3 and A4 to C3 and C4: 3, 2, and 1 μg , respectively), blank (D3 and D4), and human plasma samples (all other positions, *i.e.*, A5 and A6; A7 and A8, and so forth, 20 μg each). *b*, immunodot blot analyses of oxidized proteins in duplicate. Fibrinogen treated with peroxynitrite (F1 and F2, G1 and G2, H1 and H2, and A3 and A4: 0.8, 0.6, 0.5, and 0.4 μg , respectively); blank (B3 and B4 and E7 and E8), and human plasma samples (all other positions, *i.e.*, A1 and A2, B1 and B2, and so forth, 20 μg each). *c*, WB analyses of nitrated proteins in human plasma samples. Lane 1, lung cancer patient exposed heavily to ETS; Lane 2, healthy subjects (nonsmoker, no exposure to ETS); Lanes 3, 5, 9, and 10, lung cancer patients (ex-heavy smokers); Lane 4, lung cancer patient (nonsmoker, no ETS); Lanes 6, 7, and 8, lung cancer patients (heavy active smokers). *d*, WB analyses of oxidized proteins. Lanes 1 and 6, healthy subjects (nonsmokers, no ETS); Lanes 2 and 7, healthy subjects (nonsmokers, exposed heavily to ETS); Lanes 3 and 4, lung cancer patients (ex-light smokers); Lane 5, lung cancer patient (light active smoker); Lanes 8, 9, and 10, lung cancer patients (heavy active smokers). For WB analyses, 20 μg of plasma proteins were subjected to SDS-PAGE.

NTYR antibody of human plasma, which contained nitrated proteins initially, were analyzed by WB, bands were observed at expected molecular weights with the following antibodies. Antibody against human fibrinogen showed two bands at M_r 55,000 and 65,000, which may correspond to the α and γ chains (Lane 1). Antibodies against transferrin, plasminogen, and ceruloplasmin showed one band at M_r 77,000 (Lane 3), one band at M_r 95,000 (Lane 5) and two bands at about M_r 140,000 (Lane 7), respectively. No bands were detected with antibodies against human albumin, ferritin, antichymotrypsin, and antitrypsin (data not shown). When a human plasma sample that did not contain nitrated proteins initially was examined in a similar manner, no bands corresponding to the specific proteins were detected by WB analyses with any of the eight antibodies (Lanes 2, 4, 6, and 8 in Fig. 3a).

Similarly, the immunoprecipitates with anti-DNP antibody of DNPH-derivatized proteins were analyzed by WB analyses using eight different antibodies. Two or three bands between M_r 55,000 and M_r 65,000 were detected with antifibrinogen antibody, probably corresponding to the α , β and γ -chains (Fig. 3b). No bands at expected molecular weights were detected with the other antibodies. When a human plasma sample which did not contain oxidized proteins initially was examined, no bands corresponding to the specific proteins were detected by WB analyses with any of the eight antibodies (Lanes 1, 7, and 8 in Fig. 3b).

Reverse immunoprecipitation experiments were carried out to confirm these results. The immunoprecipitates with antibodies against fibrinogen, transferrin, plasminogen, and ceruloplasmin showed the occurrence of nitrated proteins at expected molecular weights, which

Table 2 Immunodot blot and WB analyses of oxidized and nitrated proteins

Intensities of bands with 1 and 3 μg peroxynitrite-treated transferrin were designed as attributed values of 2 and 4, respectively, for nitrated proteins. Similarly, the intensities of bands with 0.5 and 0.8 μg peroxynitrite-treated fibrinogen were designed as attributed values of 2 and 4, respectively, for oxidized proteins. Intensity of bands found were evaluated as: 0, null; and 1 to 4 by comparison with positive standards. Mean values were calculated on the sum of the intensities of each band.

Groups	n	Immunodot analysis (means \pm SD)		WB analyses (means \pm SD)		
		Oxidized proteins ^a (nmol carbonyls/mg protein)	Nitrated proteins ^b (nmol NTYR/mg protein)	Oxidized proteins ^a (band intensity) ^c	Nitrated proteins ^b	
					No. of bands	Band intensity ^c
Ever smokers						
Heavy smokers						
Cases	16	5.00 \pm 3.27	0.53 \pm 0.41	1.91 \pm 1.51	2.25 \pm 1.88	4.69 \pm 4.30
Controls	15	4.00 \pm 1.31	0.18 \pm 0.22	2.00 \pm 1.36	0.20 \pm 0.56	0.20 \pm 0.56
Light smokers						
Cases	8	3.50 \pm 3.34	0.47 \pm 0.31	1.37 \pm 1.51	1.50 \pm 0.93	2.63 \pm 2.13
Controls	3	1.33 \pm 2.31	0.08 \pm 0.14	0.33 \pm 0.58	0	0
Never smokers						
ETS exposure						
Cases	15	0.80 \pm 1.26	0.18 \pm 0.35	0.70 \pm 0.86	0.40 \pm 0.74	1.00 \pm 1.96
Controls	15	1.60 \pm 2.84	0.32 \pm 0.33	0.63 \pm 1.16	0.87 \pm 0.83	1.67 \pm 2.06
No ETS exposure						
Cases	13	1.84 \pm 3.00	0.54 \pm 0.42	0.77 \pm 1.13	1.77 \pm 1.74	2.85 \pm 3.39
Controls	10	0.80 \pm 1.94	0.17 \pm 0.33	0.75 \pm 1.14	1.00 \pm 1.49	1.80 \pm 3.22

^a Carbonyl-containing proteins.

^b NTYR-containing proteins.

^c The intensity of the WB bands was compared with positive standards.

Table 3 Results of multiple linear regression on oxidized and nitrated proteins and tobacco smoke and case-control status

	Oxidized proteins			Nitrated proteins		
	β	SE	P	β	SE	P
All subjects						
Case-control status	0.024	0.26	0.9	0.88	0.29	0.003
Smoking status	1.07	0.27	<0.001	0.27	0.30	0.4
Smokers						
Case-control status	0.17	0.55	0.8	1.99	0.48	<0.001
Heavy smoking	0.56	0.49	0.3	0.71	0.42	0.1
Nonsmokers						
Case-control status	-0.050	0.29	0.9	-0.001	0.34	1.0
ETS exposure	-0.23	0.30	0.5	-0.84	0.36	0.02

were detected with anti-NTYR antibody (data not shown). The immunoprecipitates of the DNPH-derivatized protein with antifibrinogen contained the protein, which was detected by anti-DNP antibody (data not shown). On the basis of these results, we conclude that the major nitrated proteins present in human plasma from lung cancer patients were fibrinogen, transferrin, plasminogen, and ceruloplasmin and that the oxidized protein found frequently in the plasma of smokers was fibrinogen.

DISCUSSION

We have developed a sensitive and specific method to analyze nitrated and oxidized proteins in human plasma samples using immunodot blot and WB methods. The methods have been validated using standard proteins, which were nitrated or oxidized *in vitro* by incubation with peroxyntirite or hydroxyl radical generated by a Fenton-type reaction.⁵ The methods have been used to analyze human plasma samples collected from lung cancer patients and healthy controls (smokers and nonsmokers). WB analyses were carried out to obtain qualitative data on the number and molecular weights of modified proteins. In general, the two assays (immunodot blot and WB analyses) gave similar results on the levels of modified proteins. In the present study, we detected nitrated proteins in the range from 0.1 to 1.0 nmol of NTYR/mg of protein and oxidized proteins from 1.0 to 8.0 nmol of carbonyls/mg of protein in positive plasma samples. There are very few reports of the concentrations of protein-bound NTYR in human plasma. Normal plasma proteins have been shown by a gas chromatography and mass spectrometry method to contain 0.012 ± 0.002 nmol of NTYR/mg of protein (36). The levels of NTYR in bronchoalveolar lavage fluid measured by high-performance liquid chromatography were 0.17 ± 0.08 nmol/mg protein and the level increased significantly to 2.65 ± 0.92 nmol/mg protein in patients with acute respiratory distress syndrome (37). Levels of carbonyl groups similar to those that we found have also been reported, ranging from 1 nmol/mg protein in many physiological tissue samples to 8 nmol/mg protein in some diseased brain samples (16).

Our major findings in this study were (a) elevated levels of nitrated proteins in lung cancer patients compared with those in controls; and (b) elevated levels of oxidized proteins in smokers compared with nonsmokers. Plasma samples from patients with lung cancer, especially those of smokers, showed high levels of nitrated proteins. These results suggest that during development of lung cancer, production of reactive nitrogen species is increased and that this mechanism might be involved in, but not limited to, tobacco-related lung cancer development. It has been reported that activities of nitric oxide synthase and expression of inducible nitric oxide synthase were increased in patients with lung cancer (38–41) and a variety of respiratory disorders (42, 43). In view of the expression of inducible nitric oxide synthase

in various cancerous and precancerous tissues in humans, it would be interesting to study whether levels of nitrated proteins are elevated in plasma of patients with other types of cancer, and whether they could be measured as diagnostic markers for early detection of tumors.

Levels of oxidized protein were also higher in smokers than in nonsmokers. However, among nonsmokers, ETS exposure was not associated with increased levels of oxidized proteins. Increased oxidative stress in smokers has been shown by measuring various biomarkers, including lipid peroxidation products in plasma (6, 7), oxidized DNA bases in human leukocytes DNA and urine (11–14, 44) and F2-isoprostanes in plasma and urine (5, 6). It would be worthwhile to further study the correlation between these biomarkers and levels of oxidized proteins in relation to smoking habits.

Using immunoprecipitation and WB analyses, we identified fibrinogen, transferrin, plasminogen, and ceruloplasmin as major nitrated proteins present in plasma from lung cancer patients and fibrinogen as the only oxidized protein found frequently in the plasma of smokers. In contrast, a major human plasma protein, albumin, was neither nitrated nor oxidized. It has been reported that transition metals can catalyze peroxyntirite-mediated nitration of phenolic compounds (45). Metalloproteins such as transferrin and ceruloplasmin can also interact with nitric oxide to form a metal-nitrosyl complex, which can generate nitrosonium cation (NO^+), an electrophilic nitrosating agent (46, 47), which may react with tyrosine residues to form 3-nitrosotyrosine, which is then converted to NTYR by further oxidation (48). Because levels of fibrinogen (49) and ceruloplasmin (50) are elevated in plasma of subjects with lung cancer, these plasma proteins could be better targets for nitration and oxidation when human plasma was exposed to oxidant(s) produced by the iron-ascorbic acid system, fibrinogen in plasma was much more susceptible to oxidative modification (carbonyl formation) than other major plasma proteins such as albumin, immunoglobulins, and transferrin (31). Oxidized fibrinogen inhibits thrombin-catalyzed clot formation, whereas nitrated fibrinogen appears to accelerate this process (51, 21). Many other proteins and enzymes change their conformation and lose their function after modification of amino acid residues by reactive oxygen and nitrogen species (17, 18). Additional studies are needed to elucidate the biological significance of nitration and oxidation of human plasma proteins, especially fibrinogen, transferrin, plasminogen, and ceruloplasmin, in relation to coagulation, fibrinolysis, and metal ion metabolism, as well as to tumor growth, invasion, and metastasis.

Although proteins are major targets for oxidative and nitrative

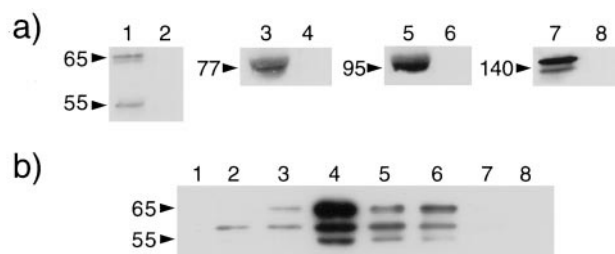


Fig. 3. Identification of nitrated and oxidized plasma proteins by immunoprecipitation and WB analyses. a, nitrated proteins. An aliquot of 125 μg of plasma proteins was immunoprecipitated with mouse anti-NTYR monoclonal antibody. The immunoprecipitates were analyzed by WB using rabbit polyclonal antibodies against human fibrinogen (Lanes 1 and 2), transferrin (Lanes 3 and 4), plasminogen (Lanes 5 and 6), and ceruloplasmin (Lanes 7 and 8). Human plasma samples that contained nitrated proteins (Lanes 1, 3, 5, and 7) and one sample that did not contain any nitrated proteins (Lanes 2, 4, 6, and 8) were analyzed. b, oxidized protein. An aliquot of 125 μg of plasma protein was derivatized with DNP, and the DNPH-derivatized proteins were immunoprecipitated with rabbit anti-DNP antibody and examined by WB analyses using rabbit polyclonal antibodies against human fibrinogen. Human plasma that contained oxidized proteins (Lanes 2–6) and those that did not contain oxidized proteins (Lanes 1, 7, and 8) were analyzed.

⁵ C-Q. Li, B. Pignatelli and H. Ohshima, manuscript in preparation.

damage *in vivo* (16–18), the modified proteins in human plasma have not until recently been extensively measured as possible biomarkers of oxidative stress in relation to human nutrition, disease status, life-style, and so forth. The immunoassays described in this study are sensitive and specific for oxidized and nitrated proteins. Only 20 μ l of plasma is needed for the analyses. In addition, modified proteins could be measured as better biomarkers than modified DNA bases because (a) target proteins occur at higher concentrations than DNA; (b) they may exist for a longer period than modified DNA bases because they are possibly not repaired efficiently; and (c) during the analytical process described in this work, proteins may be less susceptible to artifactual modification than DNA bases. Additional studies are in progress in our laboratory using these methods, especially to investigate effects of antioxidants and cessation of cigarette smoking on levels of nitrated and oxidized proteins in human plasma.

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