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Background—Several lines of evidence support a pathophysiological role of immunity in atherosclerosis. Tyrosine-nitrated proteins, a footprint of oxygen- and nitrogen-derived oxidants generated by cells of the immune system, are enriched in atheromatous lesions and in circulation of patients with coronary artery disease (CAD). However, the consequences of possible immune reactions triggered by the presence of nitrated proteins in subjects with clinically documented atherosclerosis have not been explored.

Methods and Results—Specific immunoglobulins that recognize 3-nitrotyrosine epitopes were identified in human lesions, as well as in circulation of patients with CAD. The levels of circulating immunoglobulins against 3-nitrotyrosine epitopes were quantified in patients with CAD (n=374) and subjects without CAD (non-CAD controls, n=313). A 10-fold increase in the mean level of circulating immunoglobulins against protein-bound 3-nitrotyrosine was documented in patients with CAD (3.75±1.8 μg antibody Eq/mL plasma versus 0.36±0.8 μg antibody Eq/mL plasma), and was strongly associated with angiographic evidence of significant CAD.

Conclusions—The results of this cross-sectional study suggest that posttranslational modification of proteins via nitration within atherosclerotic plaque-laden arteries and in circulation serve as neo-epitopes for the elaboration of immunoglobulins, thereby providing an association between oxidant production and the activation of the immune system in CAD.

Key Words: atherosclerosis, immune system, antibodies, antigens, nitric oxide, free radicals

Chronic inflammation, oxidative processes, and the activation of the immune system are implicated in the pathogenesis of atherosclerosis.[1–3] The detection and quantification of tyrosine-nitrated proteins in human and animal models of atherosclerosis has provided one of the mechanistic links between chronic inflammation and oxidative processes in coronary artery disease (CAD). Tyrosine nitration is a covalent posttranslational modification of proteins that arises from the reaction of protein tyrosine residues with nitric oxide-derived oxidants.[4–6] Using mass spectrometry-based proteomic approaches, site-specific nitration of proteins with pathobiological relevance to CAD have been revealed,[7,8] whereas quantification of 3-nitrotyrosine in plasma using stable isotope dilution liquid chromatography tandem mass spectrometry demonstrated its association with CAD.[9] Despite the potential utility of 3-nitrotyrosine as a biomarker, at the individual protein level, it remains unclear whether protein nitration is responsible for alterations in cellular function that imparts an increased risk for disease development or unfavorable outcomes. Emerging data implicate tyrosine nitration as an activator of immune responses, including the elaboration of immunoglobulins, and in inflammatory diseases such as acute lung injury, osteoarthritis, rheumatoid arthritis, and systemic lupus erythematosus.[10–12] Moreover, a possible role of immune modulation by protein nitration can be evoked in other prevalent vascular diseases, such as angiotensin II-induced hypertension in which oxidants, T cells, and infiltrating monocytes contribute to the disease pathogenesis.[13,14] In this study, we provide clinical data demonstrating that circulating immunoglobulins that recognize protein-bound 3-nitrotyrosine are increased in patient with CAD and are associated with enhanced risk of angiographic evidence of significant CAD.

Table 1. Baseline Subject Characteristics by Coronary Artery Disease Status

Characteristic	All Subjects (n=687)	Non-CAD Subjects (n=313)	Subjects With CAD (n=374)	*P*
Demographics and cardiovascular risk factors				
Age (y)	68±10	61±8	70±9	<0.001
Male (%)	67	37	81	<0.001
Diabetes mellitus (%)	37	17	46	<0.001
Hypertension (%)	73	56	80	<0.001
Smoker (former/current, %)	67	52	73	<0.001
Prior myocardial infarction (%)	32	0	48	<0.001
Laboratory data				
LDL cholesterol (mg/dL)	96 (79–119)	110 (90–130)	91 (76–112)	<0.001
HDL cholesterol (mg/dL)	35 (28–41)	39 (34–50)	32 (27–39)	<0.001
Triglycerides (mg/dL)	115 (86–159)	104 (79–143)	119 (90–169)	0.006
Total leukocyte count (WBC/hpf)	6.12 (5.11–7.37)	5.87 (4.61–6.95)	6.24 (5.21–7.57)	0.003
Uric acid (mg/dL)	6.3 (5.1–7.3)	5.6 (4.7–6.7)	6.6 (5.5–7.6)	0.003
hsCRP (mg/L)	2.53 (1.07–6.66)	1.94 (0.85–5.29)	2.83 (1.24–7.08)	0.004
Myeloperoxidase (pg/mL)	114 (74–246)	104 (70–167)	124 (80–286)	0.001
Creatinine clearance (mL/min per 1.73 m)	88 (66–110)	97 (83–122)	79 (62–106)	<0.001
Baseline medications				
Aspirin	74	60	81	<0.001
β-adrenergic blockers	58	47	62	0.002
Angiotensin-converting enzyme inhibitors	53	34	61	<0.001
Statin therapy	54	26	67	<0.001

Values expressed in mean±standard deviation or median (interquartile range). LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; WBC/hpf, white blood cells per high power field; and hsCRP, high-sensitivity C-reactive protein.

Materials and Methods

Subjects

Stable subjects undergoing elective diagnostic cardiac evaluation with coronary angiography were recruited prospectively to participate. All blood draws were from fasting subjects, venous sampling into EDTA (lavender top) Vacutainers prior to heparin administration from sequential consenting subjects at the time of cardiac catheterization. Blood was placed immediately either on ice or in a refrigerator, and centrifuged subsequently for 15 minutes at 10 000 *g* at 4°C. Plasma was maintained between 0°C and 4°C, and aliquots were frozen at −80°C within 2 hours of blood draw. Subjects who experienced acute coronary syndrome within 30 days of sample collection or had significant end-organ dysfunction were excluded. All subjects included in the current studies were confirmed to be troponin I (Abbott Architect assay) negative. Angiographic evidence of significant CAD was defined by the presence of at least 50% stenosis in one or more major coronary vessel as judged by at least 2 cardiologists blinded to patient identification and history. The demographic data for the subjects are reported in Table 1. Subjects with no history of CAD, significant angiographic (using ≥50% stenosis as the cutoff) evidence of cardiovascular disease, peripheral artery disease, or other significant end-organ dysfunction were considered non-CAD subjects. The reasons for cardiac catheterization in the study cohort (subjects can have 1 or more reasons) included history of positive or indeterminate stress test (46%), evaluation for possible ischemic causes of symptoms (69%), preoperative evaluation (14%), and history of cardiomyopathy (3%). All study participants gave informed consent, and the study was approved by the Cleveland Clinic Institutional Review Board.

Materials

Free 3-nitrotyrosine and the synthetic octapeptide containing 2 3-nitrotyrosine residues (CGnitroYGGGnitroYG) (CPC Scientific, San Jose, CA) were conjugated to horseradish peroxidase (HRP) following a previously published 1-step procedure.[10] Affinity-purified polyclonal antinitrotyrosine antibodies were generated and characterized extensively,[8,15,16] and used as reference immunoglobulin. Synthetic peptides comprising amino acids 213 to 219 of human apolipoprotein A-I sequence (^{213}LAEYHAK^{219}) with either tyrosine in position 216 or 3-nitrotyrosine (^{213}LAEnitroYHAK^{219}) were used for ligand competition. Human fibrinogen (America Diagnostica, Stamford, CT), fibronectin, and ceruloplasmin were nitrated by adding 3 consecutive bolus additions of peroxynitrite (final concentration, 100 μmol/L) in 0.1 mol/L phosphate buffer containing 100 μmol/L diethylene triamine pentaacetic acid and 25 mmol/L bicarbonate, pH 7.2. These proteins were nitrated chemically and used to screen for the circulating immunoglobulin isotype.

Immunohistochemistry

To probe for tyrosine-nitrated proteins, sections from human carotid atheromatous lesions were fixed in cold methanol–acetone solution for 20 minutes at −20°C. Staining for tyrosine-nitrated proteins was performed using rabbit polyclonal affinity-purified antinitrotyrosine antibodies (7 μg/mL) raised against the synthetic peptide described previously.[15,16] A goat antirabbit secondary antibody conjugated to HRP (Biorad, Hercules, CA) was used for signal detection. As control for the specificity of the antibody binding, the anti-3-nitrotyrosine antibody was preincubated with 250 μmol/L 3-nitrotyrosine octapeptide or with 250 μmol/L of the corresponding tyrosine octapeptide for 1 hour at room temperature. Other control experiments included preincubation with 2.5 mmol/L free 3-nitrotyrosine, reduction of tissue nitrotyrosine to aminotyrosine using 0.5 mol/L dithionite in 0.1 N sodium hydroxide for 20 minutes at room temperature, as well as omission of the primary antibody. Oil

red-O, trichrome staining, and 3,3′-diaminobenzidine-chromogenic detection of immunoglobulins was performed in human lesions fixed in 4% paraformaldehyde as described previously.[8,15,16]

Affinity Purification of Immunoglobulins

Proteins that bind to 3-nitrotyrosine were affinity isolated using an Amino Link Plus Immobilization Kit (Pierce Biotechnology, Inc, Rockford, IL), as described previously with minor modifications.[8] Briefly, 10 mg plasma proteins (combining plasma from 3 patients with CAD) were diluted in binding buffer (50 mmol/L potassium phosphate, 400 mmol/L NaCl, pH 7.5), applied to the column, and incubated for 1 hour at room temperature. Unbound protein fractions were eluted by centrifugation with binding buffer. Bound proteins were eluted with 0.1 mol/L citrate, pH 2.5, and neutralized with 1 mol/L Tris, pH 9. Bound fractions were concentrated and buffer exchanged using Millipore 10-kDa centrifugal filter units (Millipore Corporation, Billerica, MA). The protein fractions were separated on 10% SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA), at 100 V and 4°C. After blocking with 3% BSA in Tris buffered saline with 0.05% tween-20, the membranes were incubated with either goat antihuman immunoglobulin G (IgG)-IRDye 800 CW (Rockland Immunochemicals, Inc, Boyertown, PA) or mouse antihuman IgM (1:2500; Sigma, St. Louis, MO) plus goat antimouse IgG conjugated to Alexa Fluor 680 (1:5000; Invitrogen, Eugene, OR) in blocking buffer. Images were obtained with an Odyssey near-infrared imaging system (LI-COR, Inc, Lincoln, NE) at 700 nm and 800 nm.

Human arterial tissue was obtained from the National Disease Resource Institute (Philadelphia, PA) or the Cleveland Clinic. Tissues (approximately 1 g) were cleaned of connective tissue and blood, snap-frozen in liquid nitrogen, and pulverized in the frozen state and stored at −80°C until use. Frozen tissue powder was thawed in 5 mL extraction buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO), and macerated further with a Tissue-Tek (Cincinnati, OH) homogenizer operated on full power for 10 s. This procedure was repeated 4 times per tissue sample. Tissue extracts were centrifuged at 700 *g* for 5 minutes and 21 000 *g* twice for 10 minutes to remove particulate material. Supernatants were combined and loaded to appropriate columns for affinity enrichment.

Isotype Determination

ELISA methodologies were developed to determine the isotype and the subisotype of the circulating immunoglobulin that recognize protein-bound 3-nitrotyrosine. First, to differentiate between IgG and IgM, 100 μL of 10 μg/mL of the synthetic 3-nitrotyrosine octapeptide described earlier was diluted in 50 mmol/L bicarbonate buffer, pH 9, and added in the wells of 96 well plates (MAXI-SORP, Nunc, Roskilde, Denmark). After overnight incubation at 4°C, the plates were washed and incubated in the presence of 100 μL/well 3% BSA in Tris buffered saline with 0.05% tween-20 (working buffer) for 2 hours at room temperature. Human plasma from patients with CAD (5 mg plasma protein/mL) were added to each well and incubated for 2 hours at room temperature. Antihuman IgG and antihuman IgM HRP-conjugated antibodies (Sigma) diluted to 1:5000 in working buffer were added to appropriate series of wells. Peroxidase activity bound to each well was determined with 3,3′,5,5′ tetramethylbenzidine (Pierce) substrate.

To determine the IgG subclass of the antibody, microplates were coated with 100 μL of 5 μg/mL solution of nitrated and native ceruloplasmin, fibronectin, and fibrinogen. The plates were incubated overnight at 4°C. After blocking, 100 μL human plasma (diluted 1:40), alone or in the presence of 10 μmol/L synthetic 3-nitrotyrosine octapeptide (specificity control) in blocking buffer, was added to each well and incubated for 1 hour, followed by the addition of 100 μL/well 1:2000 dilutions of monoclonal HRP-conjugated antihuman IgG 1, 2, 3, and 4 (Sigma). Absorbance was measured at 450 nm on a microplate reader (Spectra-Max 250; Molecular Devices Corporation, Sunnyvale, CA). The background absorbance obtained with unmodified proteins was subtracted from the absorbance of the samples that reacted with the nitrated protein antigens.

Ligand Competition ELISA

A competition ELISA described previously was implemented to determine the levels and specificity of circulating immunoglobulins that recognize 3-nitrotyrosine in human plasma.[10] Briefly, each well of a 96-well microtiter plate (MAXI-SORP) was coated with 50 μL of serial dilutions of human plasma (0.02–10 mg protein/mL) in 50 mmol/L sodium bicarbonate buffer, pH 9. After blocking, 75 nmol/L HRP-conjugated 3-nitrotyrosine or an HRP-conjugated synthetic octapeptide containing 2 3-nitrotyrosine residues (CGnitroYGGGnitroYG) were added in 0.1% BSA in Tris buffered saline with 0.05% tween-20 and were incubated for 1 hour. Peroxidase activity bound to each well was determined using the H_2O_2-peroxidase-mediated oxidation of tetramethylbenzidine measuring absorbance at 450 nm. To test the specificity of detection, the absorbance at 450 nm was competed in each sample by including excess free, unlabeled 3-nitrotyrosine (2.5 mmol/L). In some experiments, the CGnitroYGGGnitroYG octapeptide was used as the competitor.

A polyclonal rabbit antinitrotyrosine IgG antibody (0.08–40 μg protein/mL), characterized in detail previously,[15,16] was run each day along with plasma samples. The antibody titer in each sample was calculated using the absorbance at 450 nm of the samples ($Abs_{450\ nm}$), and the average of the parameters of the dose–response curve obtained with the polyclonal antinitrotyrosine antibody using the following equation:

$$Antibody\ titer = 200C\left(\frac{A-D}{Abs_{450\ nm}-D}-1\right)^{1/B}$$

where 200 represents the sample dilution, and the average values for the parameters *A*, *B*, *C*, and *D* were 0.07, 2.21, 0.04, and 1.57, respectively, denoting minimal absorbance, slope at the linear part of the curve, the dose at the central point, and the maximal absorbance, respectively. Antibody titers in human plasma are therefore expressed as microgram equivalents of the polyclonal antibody per milliliter of plasma (μg Ab Eq/mL plasma).

Isolation of Nitrated Proteins for Mass Spectrometry

Proteins bearing the 3-nitrotyrosine epitope were affinity captured from human CAD plasma by using the polyclonal anti-3-nitrotyrosine antibody conjugated to a recombinant protein A column (Pierce). Pooled plasma from 6 patients with CAD (14 mg total protein) was loaded into the column in 0.1 mol/L HEPES, pH 7.4 (binding buffer), and was incubated overnight at 4°C. The column was washed successively with 10 mL of the following solutions: binding buffer, 0.5 mol/L NaCl, and binding buffer again. Bound fractions were eluted with 10 mL of 0.1 mol/L glycine, pH 2.5, containing 0.15 mol/L NaCl. After concentration and buffer exchange using Centriprep YM-10 filters (Millipore Corporation, Billerica, MA), proteins were separated on 10% SDS-PAGE, and stained with colloidal blue (Invitrogen, Carlsbad, CA). An identical gel was transferred to the polyvinylidene difluoride membrane for immunodetection of protein 3-nitrotyrosine using the polyclonal antinitrotyrosine antibody.

Gel–Liquid Chromatography Tandem Mass Spectrometry Analysis

Colloidal blue-stained gels were cut into 11×2-mm slices and in-gel digested with trypsin as described previously.[8] Tryptic peptide digests were analyzed by an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) coupled to an Eksigent 2-dimensional liquid chromatography system (Eksigent Technologies, Livermore, CA) and autosampler. Buffers A and B were 0.1% formic acid/1% methanol and 80% acetonitrile/0.1% formic acid/1% methanol, respectively. Peptides were loaded isocratically onto a C18 trap column (75 μm ID×25 mm; New Objective Proteopep 2 [New Objective, Inc., Woburn, MA]) at a flow rate of 1 μL/min in 2% B. Peptides were then eluted onto a C18 analytic column (75 μm ID×150 mm; New Objective Proteopep 2). A linear gradient was

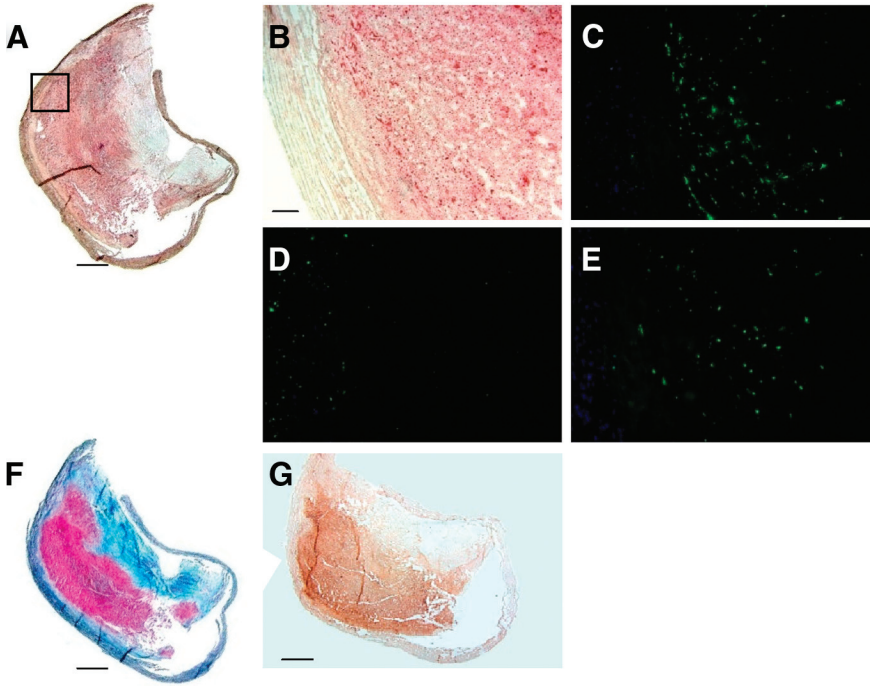

Figure 1. Immunohistochemical detection of nitrated proteins in human atherosclerotic lesions. (**A** and **B**) Oil red-O staining of the atherosclerotic lesion in human carotid at low magnification (1.25×) (**A**) and at high magnification (10×) (**B**). (**C**–**E**) Serial cross-sections were incubated with polyclonal anti-3-nitrotyrosine antibody (**C**). The immunoreactive nitrated proteins were visualized in the lesions by green fluorescence after incubation with secondary antirabbit/Alexa Fluor 488 antibody. Immunoreactivity was reduced by competition of the primary anti-3-nitrotyrosine antibody binding with the 250 μmol/L 3-nitrotyrosine-containing octapeptide (**D**), but not by 250 μmol/L tyrosine-containing octapeptide (**E**). Nuclei are stained blue with 4′,-6-diamidino-2-phenylindole. The results are representative of staining 2 different human lesions. (**F** and **G**) Trichrome staining of the carotid lesion (**F**) immunostaining with antihuman immunoglobulin G antibody revealed the presence of immunoglobulin G immunoglobulins within and around the lesion (**G**). Immunoreactivity (brown) was developed with 3,3′-diaminobenzidine. Bars indicate 100 μm for **B** through **E** and 1 mm for panels **A**, **F**, and **G**.

then initiated at a flow rate of 300 nL/min for 90 minutes from 3% to 40% B. The mass spectrometer was set to scan m/z repetitively from 375 nm to 1600 nm followed by data-dependent tandem mass spectrometry scans on the 5 most abundant ions with dynamic exclusion enabled.

Generation and Evaluation of SEQUEST Peptide Assignments

DTA files were generated from the tandem mass spectrometry spectra extracted from RAW data files (intensity threshold, 1000; minimum ion count, 50) and processed by the ZSA and Correction algorithms of the SEQUEST Browser program. DTA files were submitted to Sorcerer-SEQUEST (ver. 3.11, rev 11; Sagen Research, San Jose, CA) using the following parameters: Database searching was performed against a Uniprot database containing *Homo sapiens* sequences from Swiss-Prot plus common contaminants, which were then reversed and appended to the forward sequences (91 522 entries). The database was indexed with the following parameters: mass range of 600 to 3500 atomic mass units, tryptic cleavages with a maximum of 1 missed cleavage, and static modifications of cysteine by carboxyamidomethylation (+57 amu). The DTA files were searched with a 2.0-amu peptide mass tolerance, 1.0-amu fragment ion mass tolerance, and variable modification of methionine (+16 amu). Potential sequence-to-spectrum peptide assignments generated by Sorcerer-SEQUEST were loaded into Scaffold (version 2.2; Proteome Software, Portland, OR) to validate protein identifications and perform manual inspection of tandem mass spectrometry spectra containing 3-nitrotyrosine. Protein identifications were accepted at a threshold of ≥99% protein confidence with ≥2 unique peptides at ≥80% confidence. From these proteins, manual inspection of 3-nitrotyrosine-containing tandem mass spectrometry spectra were performed using the following criteria: (1) assignment of the majority of fragment ion abundance, (2) 3-nitrotyrosine (+45 amu) modification supported by either y- or b-ions series (≥5 consecutive fragments), and (3) correctly assigned charge state and diagnostic markers, such as N-terminal proline, C-terminal aliphatic amino acids, and loss of H_2O/ammonia consistent with amino acid sequence.

Statistical Analysis

The Student t test or Wilcoxon rank-sum test for continuous variables and χ^2 test for categorical variables were used to examine the difference between the groups (CAD versus non-CAD). The relationship between plasma immunoglobulin levels and risk for having CAD was determined by calculating the odds ratio and 95% confidence intervals using multiple logistic regression treating immunoglobulin levels against protein-bound nitrotyrosine as a continuous variable. Models adjusted for traditional risk factors (age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking) or traditional risk factors plus history of myocardial infarction, and medications (aspirin, angiotensin-converting enzyme, statin, β-blockers). Cubic spline curves with 95% confidence intervals were constructed to illustrate the relationship between antinitrotyrosine immunoglobulin levels and the odds ratio of angiographic evidence of significant CAD. All analyses were performed using R 2.13.1 (Foundation for Statistical Computing, Vienna, Austria), and probability values <0.05 were considered statistically significant.

Results

Nitrated Proteins in Human Atherosclerotic Lesions and Plasma

The presence of nitrated proteins in atherosclerotic lesions or plasma of patients with CAD could serve as neo-epitopes, triggering an immune response and the elaboration of immunoglobulins. Therefore, we first sought to document the presence of nitrated proteins by immunohistochemistry in human lesions and by mass spectrometry in lesion extracts as well as plasma of subjects with CAD.

A typical atherosclerotic lesion in human carotid artery stained with Oil red-O is depicted in Figure 1A and 1B. The immunofluorescence images after staining for nitrated protein epitopes is depicted in Figure 1C through 1E, and indicate the presence of nitrated proteins within the lesion, consistent with previous studies in human atherosclerotic lesions.[6] The specificity of the antinitrotyrosine antibodies used for staining was confirmed by competition experiments with excess of the nitrated octapeptide (CGnitroYGGGnitroYG) (Figure 1D). Moreover, staining with antihuman IgG also revealed the presence of

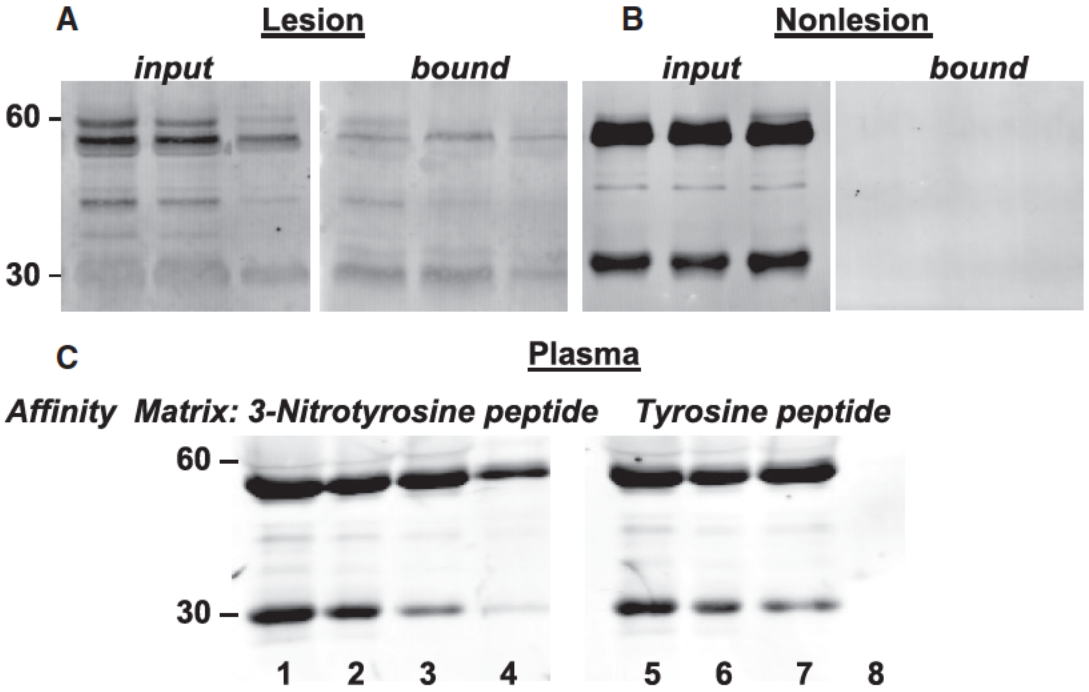

Figure 2. Affinity detection and isotyping of antinitrotyrosine immunoglobulins from aortic lesions and CAD plasma. (**A**) Representative Western blots from 3 different atherosclerotic lesion extracts (input) and bound fractions eluted from a 3-nitrotyrosine affinity column using antihuman immunoglobulin G antibodies. (**B**) Representative Western blots from the same vessels as in **A** using the equivalent protein extracts from the same tissue without visible lesion. Immunoreactivity is observed only in the fractions originating from lesion areas. (**C**) Affinity-captured plasma proteins using either immobilized 3-nitrotyrosine-containing octapeptide (lanes 1–4) or tyrosine-containing octapeptide (lanes 5–8) probed with polyclonal antihuman immunoglobulin G. Lanes 1 and 5 indicate unfractionated input plasma; lanes 2 and 6, unbound fraction; lanes 3 and 7, wash fractions; and lanes 4 and 8, bound fractions eluted with 0.1 mol/L citrate buffer, pH 2.5. Data are representative of 6 independent experiments with similar results.

immunoglobulins in the lesions (Figure 1F and 1G). Nitrated proteins extracted from atherosclerotic lesions and plasma were affinity enriched using the previously characterized polyclonal antinitrotyrosine antibody.[15,16] As a control, lesion extracts were incubated with a nonspecific rabbit immunoglobulin under the same conditions (representative Western blots confirming selective enrichment are depicted in the online-only Data Supplement Figure). The bound fractions were separated by 1-dimensional SDS-PAGE and were analyzed by liquid chromatography tandem mass spectrometry. Only proteins that were identified in the bound fraction of antinitrotyrosine-enriched samples are reported (online-only Data Supplement Table I). Within the lesion, several proteins that have been reported previously to be modified by tyrosine nitration, such as apolipoprotein AI (apoA-I), β chain of fibrinogen, α1-antitrypsin, and complement, were identified.[7,17–19] A similar analysis in human plasma revealed for the first time the presence of specific peptide sequences that contained the nitrated tyrosine residue. Several of these sequences belong to immunoglobulins (online-only Data Supplement Table II). Interestingly, 1 specific tyrosine nitrated peptide [(K)SGTASVVCLLNNFnitroYPR(E)] from human immunoglobulin was also identified in the plasma of atherosclerosis rodent models; specifically, the low-density lipoprotein receptor and apolipoprotein B mRNA-editing enzyme 1 null double-knockout mice, as well as the low-density lipoprotein receptor/apolipoprotein B mRNA-editing enzyme 1 knockout mice lacking apoA-I.[8] Collectively, the data indicate that nitrated proteins are present in human atherosclerotic lesions as well as plasma of subjects with CAD and could serve as antigenic neo-epitopes to trigger the activation of the immune system.

Affinity Chromatography Identified Immunoglobulins That Recognize 3-Nitrotyrosine and Nitrated Proteins

Protein extracts of human aorta atherosclerotic lesions were allowed to bind to affinity columns conjugated with 3-nitrotyrosine. After binding and exhaustive washing, the bound fractions were eluted, separated by SDS-PAGE, and analyzed by Western blot using antihuman IgG antibodies (Figure 2A). The presence of immunoglobulins that bound to 3-nitroyrosine was documented in the lesion tissue extract. As controls, tissue from the same aorta free of lesion was used and analyzed in parallel (Figure 2B). Equivalent loading of extracts derived from aorta segments from the same tissue, but without visible atherosclerotic lesions, did not show immunoreactive bands for IgG in the bound fraction eluted from the 3-nitrotyrosine affinity column (Figure 2A).

Pooled plasma from subjects with CAD was allowed to bind to affinity columns that were made by conjugating either the nitrated tyrosine octapeptide (CGnitroYGGGnitroYG) or the equivalent tyrosine peptide (CGYGGGYG). The data in Figure 2C indicate the presence of IgG in the human plasma of subjects with CAD that bind the nitrated tyrosine peptide specifically but not the tyrosine peptide. Plasma proteins recovered in the bound fraction from the nitrated tyrosine octapeptide affinity column also showed immunoreactivity toward human IgM antibodies. By using ELISA in which the

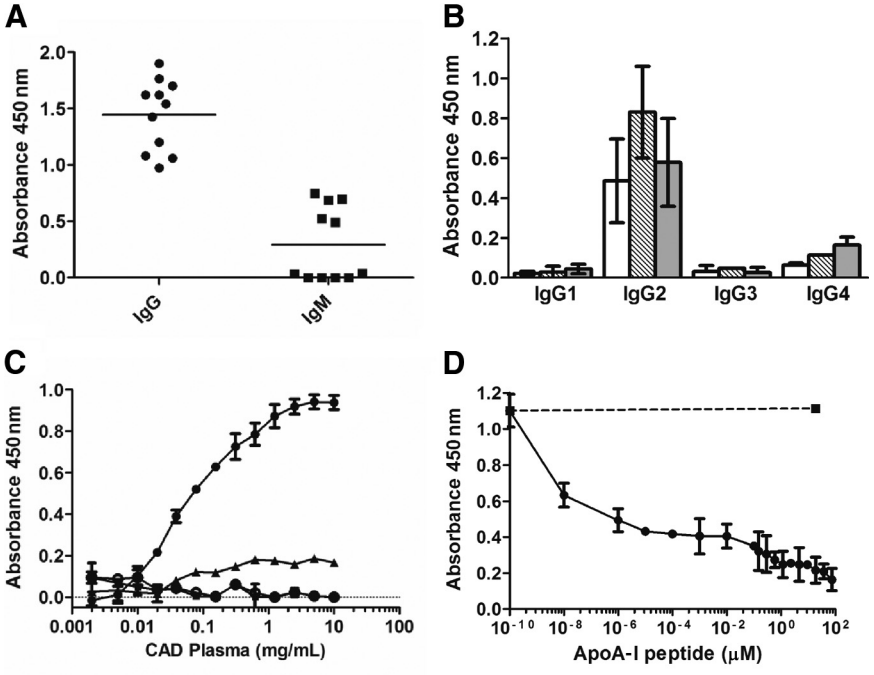

Figure 3. Screening for circulating immunoglobulins that recognize 3-nitrotyrosine. (**A**) Immunoglobulin (Ig) G and IgM levels in human plasma were measured by ELISA in the plasma of 10 randomly selected subjects with coronary artery disease using the 3-nitrotyrosine octapeptide to bind the immunoglobulins and the respective antihuman immunoglobulin for detection. (**B**) IgG subclass was determined by ELISA in 3 independent coronary artery disease plasma samples using nitrated ceruloplasmin (white bars), nitrated fibronectin (dashed bars), and nitrated fibrinogen (gray bars) as capturing antigen, and horseradish peroxidase (HRP)-conjugated mouse antihuman IgG1, IgG2, IgG3, and IgG4 for detection. (**C**) Typical antigen–antibody binding curves from 3 randomly selected subjects with coronary artery disease each performed in triplicate using as ligand the 3-nitrotyrosine-octapeptide conjugated to HRP (●). No binding was observed using the same tyrosine-containing octapeptide conjugated to HRP (○). The binding of the 3-nitrotyrosine octapeptide conjugated to HRP was eliminated with 75 μmol/L unlabeled 3-nitrotyrosine-octapeptide (▲) or by 250 μmol/L 3-nitrotyrosine (■). (**D**) The binding of the 3-nitrotyrosine octapeptide conjugated to HRP to 3 different plasma samples, each performed in triplicate, was also eliminated with increasing concentrations of apolipoprotein A-I peptide 213 to 219 (^{213}LAEnitroYHAK^{219}), which contained 3-nitrotyrosine in position 216 (●) but not by the same peptide that contained tyrosine in position 216 (■). Data are mean±standard deviation. CAD indicates coronary artery disease.

3-nitrotyrosine octapeptide was used to bind the immunoglobulins and the respective antihuman immunoglobulin to develop, we quantified the relative levels of IgG and IgM in the plasma of subjects with CAD. Plasma immunoglobulins that recognize the nitrotyrosine modification were predominantly IgG, because the signal was nearly 5-fold higher for IgG than IgM (1.4±0.3 and 0.3±0.3 average absorbance respectively, Figure 3A). Therefore, subsequent studies focused on quantifying the levels of circulating IgG immunoglobulin isoform in subjects. The IgG present in human plasma of subjects with CAD was characterized further by isotyping using an ELISA in which potential endogenous 3-nitrotyrosine-containing proteins—nitrated ceruloplasmin, nitrated fibronectin, and nitrated fibrinogen—were used as antigens. The predominant isotype that recognized preferentially the nitrated proteins was IgG2 (Figure 3B).

ELISA Development to Quantify Plasma Levels of Antinitrotyrosine Immunoglobulins in Subjects With and Without CAD

We next sought to determine whether plasma immunoglobulins that recognize the neo-epitope 3-nitrotyrosine are more prevalent in subjects with CAD. For these studies, we modified a competition ELISA described previously for use in human plasma and mouse models of atherosclerosis.[8,10] Typical antigen–antibody binding curves using the nitrotyrosine-containing octapeptide conjugated to HRP are shown in Figure 3C. No binding was observed using the same tyrosine-containing octapeptide. The specificity of binding was confirmed by competing the binding of the 3-nitrotyrosine peptide-conjugated HRP with excess free 3-nitrotyrosine (2.5 mmol/L), as well as unconjugated nitrotyrosine peptide (75 μmol/L). In all subsequent analyses, the difference between the absorbance in the absence versus presence of excess 3-nitrotyrosine (2.5 mmol/L), considered as background, was used to calculate the titer of circulating anti-3-nitrotyrosine immunoglobulins. Furthermore, the binding of immunoglobulins to the HRP-conjugated nitrotyrosine peptide was competed by a peptide derived from apoA-I (^{213}LAEnitro YHAK^{219}) containing 3-nitrotyrosine in place of tyrosine in position 216. Maximal inhibition was obtained at 1 μmol/L ^{213}LAEnitroYHAK^{219} (Figure 3D). At the same concentration, the unmodified apoA-I peptide, ^{213}LAEYHAK^{219}, was unable to compete (Figure 3D), confirming the specificity of the immunoglobulins toward protein tyrosine-nitrated epitopes.

Plasma Levels of Antibodies That Recognize Protein Nitrotyrosine Are Increased in Subjects With CAD

Plasma levels of anti-3-nitrotyrosine immunoglobulins were quantified in subjects with and without CAD as documented

during elective cardiac evaluations, as described in Methods. Subjects with clinically documented CAD had 10 times higher levels of anti-3-nitrotyrosine immunoglobulins (3.75±1.8 μg Ab Eq/mL plasma) than subjects without CAD (0.36±0.8 μg Eq/mL plasma; $P<0.001$ for comparison with CAD) (Figure 4A). Interestingly, subjects with angiographic evidence of significantly obstructed (≥50% stenosis) CAD showed increased plasma anti-3-nitrotyrosine immunoglobulin titers compared with subjects without CAD, regardless of the number of coronary arteries affected by CAD (Figure 4B). A striking dose-dependent positive association was observed between plasma levels of antinitrotyrosine IgG and angiographic evidence of significant CAD; in fact, because the non-CAD cohort had low levels of anti-3-nitrotyrosine immunoglobulins (undetectable in 76% of the samples), a remarkably large odds ratio was observed for anti-3-nitrotyrosine IgG titer versus the presence of significant angiographic evidence of CAD (Figure 4C). Multilogistic regression analyses indicated that the robust association of immunoglobulin levels against protein 3-nitrotyrosine with cardiovascular risk remains significant even after adjustments for multiple traditional risk factors, including age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, history of myocardial infarction, and medications (aspirin, angiotensin-converting enzyme inhibitors, statin, β-blockers; Table 2).

Discussion

Clinical evidence and data from animal models have indentified various forms of oxidized lipoproteins and heat shock proteins as immunogens that induce adaptive immune responses, including antibody elaboration, and that modulate atherosclerotic lesion formation in experimental models of atherosclerosis.[20–29] Most studies have focused predominantly on covalent adducts formed by the addition of electrophilic lipid peroxidation products to proteins and their relationship to the development and progression of atherosclerosis.[20–26] Moreover, the levels of circulating IgM that recognized oxidized forms of low-density lipoprotein with covalently adducted lipid oxidation products have been reported to correlate with severity of atherosclerosis in mouse models and in some studies with the progression of atherosclerosis in coronary or carotid vascular beds, or with risk for myocardial infarction and death in humans.[25] Collectively, these data highlight the critical importance of understanding the immune system responses in CAD and raise the possibility of novel therapeutic treatments for atherosclerosis.[30,31] The data also underscore the need for identifying antigens and immune responses that are linked mechanistically to the underlying pathophysiology of cardiovascular diseases.

Protein tyrosine nitration is considered to be a posttranslational modification reflective of inflammatory-mediated oxidative processes involving nitric oxide-derived oxidants. Although the ability of nitrated proteins to induce the generation of specific antibodies in experimental animals has long been known,[15,16,32–36] the contribution of this posttranslational protein modification as a trigger of immune reactions in pathology has just recently been explored.[10–12] In this study we demonstrated for the first time the association of CAD, the

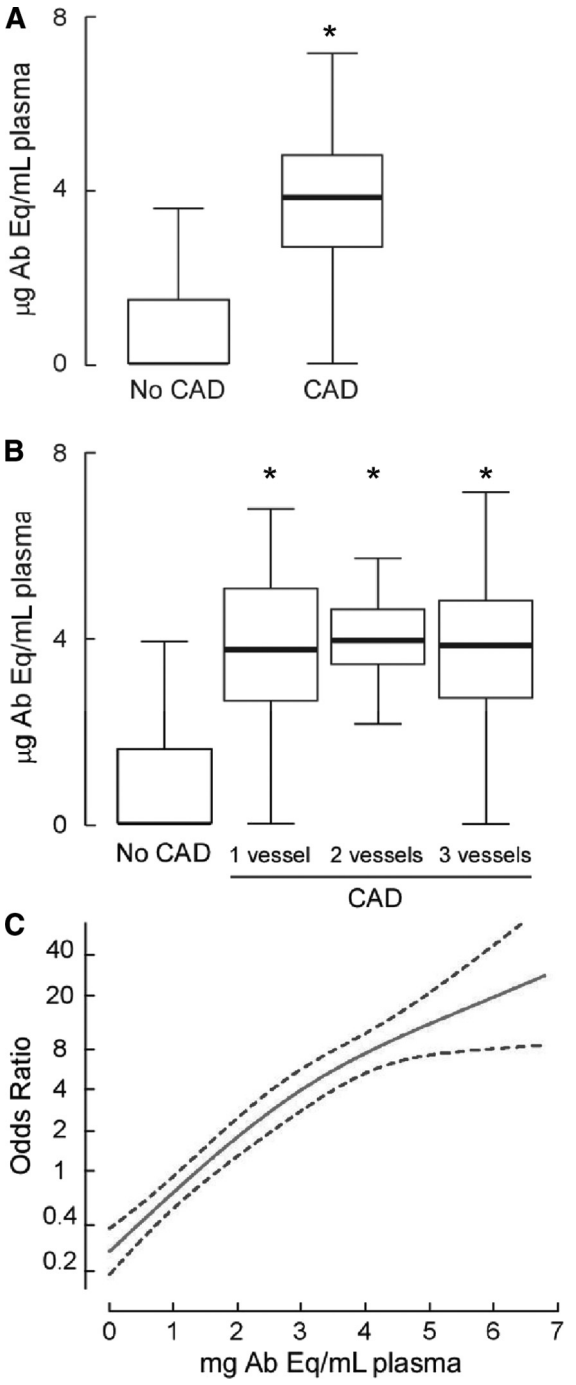

Figure 4. Antinitrotyrosine circulating immunoglobulins in coronary artery disease (CAD). (**A**) Plasma levels of anti-3-nitrotyrosine circulating immunoglobulins were quantified in clinically documented subjects with CAD versus subjects without significant angiographic evidence of CAD. Antibody equivalents were calculated from standard curves performed with a polyclonal antinitrotyrosine antibody. (**A** and **B**) Box-whisker plots of immunoglobulin levels encompass the 25th and 75th percentiles, and lines within boxes represent median values. Bars represent the 2.5th and 97.5th percentiles. Statistically significant differences in immunoglobulin content were found between CAD and non-CAD ($P<0.0001$, Kruskal-Wallis test). (**C**) Odds ratio and 95% confidence interval (dotted lines) for the relationship between plasma levels of antinitrotyrosine immunoglobulins and angiographic evidence of significant CAD were calculated as described under Methods. Ab indicates antibody.

Table 2. Relationship Between Immunoglobulin Against 3-Nitrotyrosine and Prevalence of Coronary Artery Disease

	μg Ab Eq/mL Plasma
CAD	
Unadjusted OR	12.45 (9.14–16.97)*
Adjusted OR (1)†	12.43 (7.57–20.42)*
Adjusted OR (2)‡	14.18 (8.02–25.06)*

CAD indicates coronary artery disease; OR, odds ratio; and Ab, antibody.

Results of fitting 1 univariate and 2 multivariable logistic regression models treating analyte (immunoglobulin against 3-nitrotyrosine) as a continuous variable. Shown are adjusted odds ratio and 95% confidence interval for prevalent CAD per standard deviation change in log-transformed analyte measured.

*$P<0.001$.

†Model 1 was adjusted for traditional risk factors, including age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, and diabetes.

‡Model 2 was adjusted for traditional risk factors, including age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, diabetes mellitus, history of myocardial infarction, aspirin, angiotensin-converting enzyme, statin, and β-blockers.

most common and life-threatening cardiovascular condition, with increased plasma levels of immunoglobulins reactive against proteins modified by nitration. The binding of plasma immunoglobulins to target 3-nitrotyrosine-containing bait (HRP conjugated to 3-nitrotyrosine or 3-nitrotyrosine containing octapeptide) served as a competition-based ELISA to quantify autoantibody titers for 3-nitrotyrosine-recognizing immunoglobulins within subjects' plasma. The specificity of the methodology was confirmed by numerous approaches, including demonstration that the 3-nitrotyrosine binding activity in plasma was outcompeted specifically by free 3-nitrotyrosine as well as by 3-nitrotyrosine-modified peptides and proteins, ruling out nonspecific binding. In particular, a nitrated peptide reflecting an endogenous nitrated tyrosine residue identified in apoA-I recovered from human atherosclerotic plaque,[7] but not the native tyrosine-containing peptide, was able to outcompete the binding of the 3-nitrotyrosine-coupled HRP bait to immunoglobulins. Moreover, the circulating immunoglobulins were able to recognize nitrated fibrinogen and other nitrated plasma proteins but not the native counterparts (Figure 3B). These data support a possible specific antigenic role of those endogenous-nitrated proteins as the neo-epitope that triggers antinitrotyrosine immunoglobulins. It is remarkable that both of these proteins (apoA-I and fibrinogen) have been reported previously to be nitrated selectively in plasma from patients with CAD relative to healthy control subjects, and that nitration of these particular proteins may have a potential functional role in cardiovascular events. In the case of fibrinogen, nitration increases the rate as well as the extent of fibrin clot formation,[17,18] leading potentially to enhanced thrombotic risk and adverse cardiovascular events. On the other hand, 3-nitrotyrosine-modified apoA-I was found in serum and human atherosclerotic lesions. Furthermore, exposure of apoA-I to myeloperoxidase, an enzymatic source of nitric oxide-derived oxidants, creates a dysfunctional form of high-density lipoprotein with diminished ATP-binding cassette sub-family A1-dependent cholesterol efflux capacity and reduced lecithin cholesterol acyl transferase activity.[7,37] The molecular mechanisms for the production of immunoglobulins with specific and selective epitope recognition for 3-nitrotyrosine have been explored in mouse models.[35,36] Termination of self-tolerance and escaping of negative selection after active immunization with nitrated peptides was documented.[35,38] Moreover, monoclonal antibodies that recognize only nitrated α-synuclein (including stereospecific clones that recognized 1 of the 4 nitrated tyrosine residues in α-synuclein) but not the unmodified protein or other tyrosine-nitrated proteins,[32] as well as antibodies recognizing only nitrated Mn superoxide dismutase[33] and only nitrated tau[34] have been reported. In one model of mouse neurodegeneration, the specific immune response to nitrated α-synuclein produced a vigorous immunoinflammatory response that led to the degeneration of dopamine-producing neurons.[39]

The pathophysiological relevance of circulating immunoglobulins targeting protein 3-nitrotyrosine remains to be determined. Indeed, despite the strikingly elevated levels of anti-3-nitrotyrosine antibodies in subjects with CAD, it is possible that the antibody response within atherosclerotic plaque may promote a protective role, with immunoglobulins targeting proteins bearing potentially threatening modifications for destruction. Furthermore, the relatively high-risk cohort of subjects examined (those undergoing elective diagnostic cardiac evaluations) makes the results of the current study of unclear relevance when translated to a community-based screen. It also remains to be determined whether elevated levels of 3-nitrotyrosine-targeted antibodies are increased in subjects prior to clinically overt development of atherosclerotic CAD, and whether increased levels may serve as a sentinel of increased risk of major adverse cardiac events. Further studies are needed to both identify the role, if any, of 3-nitrotyrosine immunoglobulins in CAD pathophysiology, as well as whether anti-3-nitrotyrosine immunoglobulin titer carries prognostic value and is modulated by CAD-targeting therapies.

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Disclosures

Dr Tang reports having received research grant support from Abbott Laboratories, and served as consultants for Medtronic Inc and St. Jude Medical. Dr Hazen reports being named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to

cardiovascular diagnostics; having been paid as a consultant by Cleveland Heart Laboratory, Inc, Esperion, Liposciences, Inc, Merck & Co., Inc, and Pfizer, Inc; having received research funds from Abbott, Cleveland Heart Laboratory, Esperion, and Liposciences; and having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics from Abbott Laboratories, Cleveland Heart Laboratory, Frantz Biomarkers, Liposciences, and Siemens.

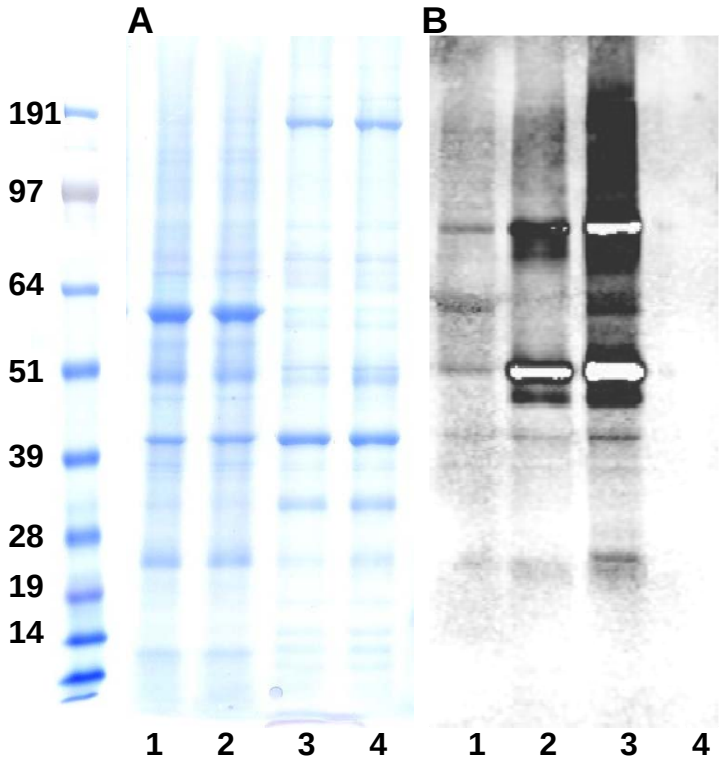
References

1. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317–325.
2. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol*. 2006;6:508–519.
3. Binder CJ, Chang M-K, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired immunity in atherogenesis. *Nature Med*. 2002;8:1218–1226.
4. Ischiropoulos H, Zhu L, Chen J, Tsai J-HM, Martin JC, Smith CD, Beckman JS. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys*. 1992;298:431–437.
5. Hazen SL, Zhang R, Shen Z, Wu W, Podrez EA, MacPherson JC, Schmitt D, Mitra SN, Mukhopadhyay C, Chen Y, Cohen PA, Hoff HF, Abu-Soud HM. Formation of nitric oxide-derived oxidants by myeloperoxidase in monocytes: pathways for monocyte-mediated protein nitration and lipid peroxidation in vivo. *Circ Res*. 1999;85:950–958.
6. Beckman JS, Ye YZ, Anderson PG, Chen J, Accavitti MA, Tarpey MM, White CR. Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe Seyler*. 1994; 375:81–88.
7. Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, Hazen SL. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest*. 2004;114:529–541.
8. Parastatidis I, Thomson L, Fries DM, Moore RE, Tohyama J, Fu X, Hazen SL, Heijnen HF, Dennehy MK, Liebler DC, Rader DJ, Ischiropoulos H. Increased protein nitration burden in the atherosclerotic lesions and plasma of apolipoprotein A-I deficient mice. *Circ Res*. 2007;101: 368–376.
9. Shishehbor MH, Aviles RJ, Brennan ML, Fu X, Goormastic M, Pearce GL, Gokce N, Keaney JF Jr, Penn MS, Sprecher DL, Vita JA, Hazen SL. Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *JAMA*. 2003;289:1675–1680.
10. Thomson LM, Christie J, Vadseth C, Lanken PN, Fu X, Hazen SL, Ischiropoulos H. Identification of immunoglobulins that recognize 3-nitrotyrosine in patients with acute lung injury following major trauma. *Am J Respir Cell Mol Biol*. 2007;36:152–157.
11. Khan F, Siddiqui AA. Prevalence of anti-3-nitrotyrosine antibodies in the joint synovial fluid of patients with rheumatoid arthritis, osteoarthritis and systemic lupus erythematosus. *Clin Chim Acta*. 2006;370:100–107.
12. Khan F, Siddiqui AA, Ali R. Measurement and significance of 3-nitrotyrosine in systemic lupus erythematosus. *Scand J Immunol*. 2006; 64:507–514.
13. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C, Harrison DG. Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J Exp Med*. 2007;204:2449–2460.
14. Wenzel P, Knorr M, Kossmann S, Stratmann J, Hausding M, Schuhmacher S, Karbach SH, Schwenk M, Yogev N, Schulz E, Oelze M, Grabbe S, Jonuleit H, Becker C, Daiber A, Waisman A, Münzel T. Lysozyme M-positive monocytes mediate angiotensin II-induced arterial hypertension and vascular dysfunction. *Circulation*. 2011;124: 1370–1381.
15. Fries DM, Paxinou E, Themistocleous M, Swanberg E, Griendling KK, Salvemini D, Slot JW, Heijnen HF, Hazen SL, Ischiropoulos H. Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells: role of reactive oxygen species. *J Biol Chem*. 2003;278:22901–22907.
16. Heijnen HF, van Donselaar E, Slot JW, Fries DM, Blachard-Fillion B, Hodara R, Lightfoot R, Polydoro M, Spielberg D, Thomson L, Regan EA, Crapo J, Ischiropoulos H. Subcellular localization of tyrosine-nitrated proteins is dictated by reactive oxygen species generating enzymes and by proximity to nitric oxide synthase. *Free Rad Biol Med*. 2006;40: 1903–1913.
17. Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, Torbet J, Vilaire G, Bennett JS, Murciano JC, Muzykantov V, Penn MS, Hazen SL, Weisel JW, Ischiropoulos H. Pro-thrombotic state induced by post translational modification of fibrinogen by reactive nitrogen species. *J Biol Chem*. 2004;279:8820–8826.
18. Parastatidis I, Thomson L, Burke A, Chernysh I, Nagaswami C, Visser J, Stamer S, Liebler DC, Koliakos G, Heijnen HF, Fitzgerald GA, Weisel JW, Ischiropoulos H. Fibrinogen β-chain tyrosine nitration is a prothrombotic risk factor. *J Biol Chem*. 2008;283:33846–33853.
19. Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF III, Finkel B, Lanken PN, Ischiropoulos H. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am J Physiol Lung Mol Cell Physiol.* 2000;278:L961–L967.
20. Salonen JT, Ylä-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssönen K, Palinski W, Witztum JL. Autoantibody against oxidized LDL and progression of carotid atherosclerosis. *Lancet*. 1992;339: 883–887.
21. Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, Chen PP, Witztum JL. Human-derived anti-oxidized LDL autoantibody blocks uptake of oxidized LDL by macrophages and localizes to atherosclerotic lesions in vivo. *Arterioscler Thromb Vasc Biol*. 2001;21: 1333–1339.
22. Zhu J, Quyyumi AA, Rott D, Csako G, Wu H, Halcox J, Epstein SE. Antibodies to human heat-shock protein 60 are associated with the presence and severity of coronary artery disease: evidence for an autoimmune component of atherogenesis. *Circulation*. 2001;103:1071–1075.
23. Hulthe J, Wiklund O, Hurt-Camejo E, Bondjers G. Antibodies to oxidized LDL in relation to carotid atherosclerosis, cell adhesion molecules, and phospholipase A(2). *Arterioscler Thromb Vasc Biol*. 2001;21:269–274.
24. Fredrikson GN, Hedblad B, Berglund G, Alm R, Ares M, Cercek B, Chyu KY, Shah PK, Nilsson J. Identification of immune responses against aldehyde-modified peptide sequences in apoB associated with cardiovascular disease. *Arterioscler Thromb Vasc Biol*. 2003;23:872–878.
25. Karvonen J, Päivansalo M, Kesäniemi YA, Hörkkö S. Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. *Circulation*. 2003;108: 2107–2112.
26. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, Bäckhed F, Miller YI, Hörkkö S, Corr M, Witztum JL, Binder CJ. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest*. 2009;119: 1335–1349.
27. George J, Afek A, Gilburd B, Blank M, Levy Y, Aron-Maor A, Levkovit H, Shaish A, Goldberg I, Kopolovic J, Harats D, Shoenfeld Y. Induction of early atherosclerosis in LDL-receptor-deficient mice immunized with beta2-glycoprotein I. *Circulation*. 1998;98:1108–1115.
28. Schiopu A, Bengtsson J, Söderberg I, Janciauskiene S, Lindgren S, Ares MP, Shah PK, Carlsson R, Nilsson J, Fredrikson GN. Recombinant human antibodies against aldehyde-modified apolipoprotein B-100 peptide sequences inhibit atherosclerosis. *Circulation*. 2004;110: 2047–2052.
29. Foteinos G, Afzal AR, Mandal k, Jahangiri M, Xu Q. Anti-heat shock protein 60 autoantibodies induce atherosclerosis in apolipoprotein E-deficient mice via endothelial damage. *Circulation*. 2005;112: 1206–1213.
30. Nilsson J, Hansson GK. Autoimmunity in atherosclerosis: a protective response losing control? *J Intern Med*. 2008;263:464–478.
31. Nilsson J, Kovanen PT. Will autoantibodies help to determine severity and progression of atherosclerosis? *Curr Opin Lipidol*. 2004;15:499–503.
32. Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, Lee VM. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science*. 2000;290:985–989.
33. Xu S, Ying J, Jiang B, Guo W, Adachi T, Sharov V, Lazar H, Menzoian J, Knyushko TV, Bigelow D, Schöneich C, Cohen RA. Detection of sequence-specific tyrosine nitration of manganese SOD and SERCA in cardiovascular disease and aging. *Am J Physiol Heart Circ Physiol*. 2006;290:2220–2227.
34. Reyes JF, Fu Y, Vana L, Kanaan NM, Binder LI. Tyrosine nitration within the proline-rich region of tau in Alzheimer's disease. *Am J Pathol*. 2011;178:2275–2285.
35. Birnboim HC, Lemay AM, Lam DK, Goldstein R, Webb JR. MHC class II-restricted peptides containing the inflammation-associated marker 3-nitrotyrosine evade central tolerance and elicit a robust cell-mediated immune response. *J Immunol*. 2003;171:528–532.

36. Herzog J, Maekawa Y, Cirrito TP, Illian BS, Unanue ER. Activated antigen-presenting cells select and present chemically modified peptides recognized by unique CD4 T cells. *Proc Natl Acad Sci U S A*. 2005;102: 7928–7933.
37. Zheng L, Settle M, Brubaker G, Schmitt D, Hazen SL, Smith JD, Kinter M. Localization of nitration and chlorination sites on apolipoprotein A-I catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in ABCA1-dependent cholesterol efflux from macrophages. *J Biol Chem*. 2005;280:38–47.
38. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J, Gabrilovich DI. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med*. 2007;13: 828–835.
39. Benner EJ, Banerjee R, Reynolds AD, Sherman S, Pisarev VM, Tsiperson C, Nemachek P, Ciborowski S, Przedborsk S, Mosley RL, Gendelman HE. Specific immune response to nitrated a-synuclein produced a vigorous immuno-inflammatory response in mice that led to the degeneration of dopamine-producing neurons. *PLoS ONE*. 2008;3:e1376.

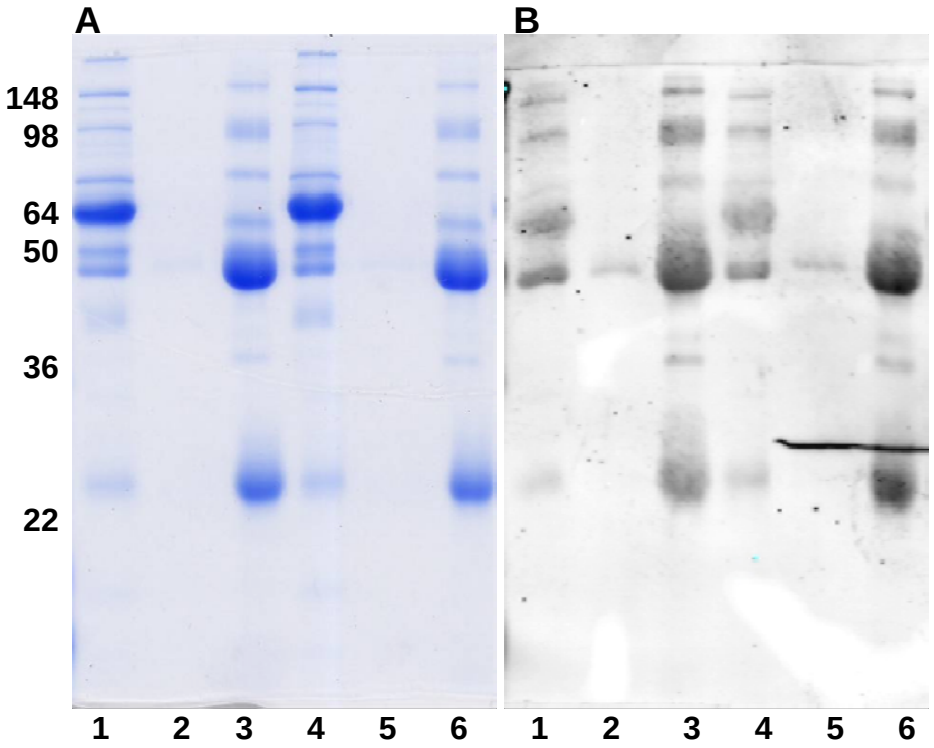
SUPPLEMENTAL MATERIAL

Thomson et al. Supplementary figure 1



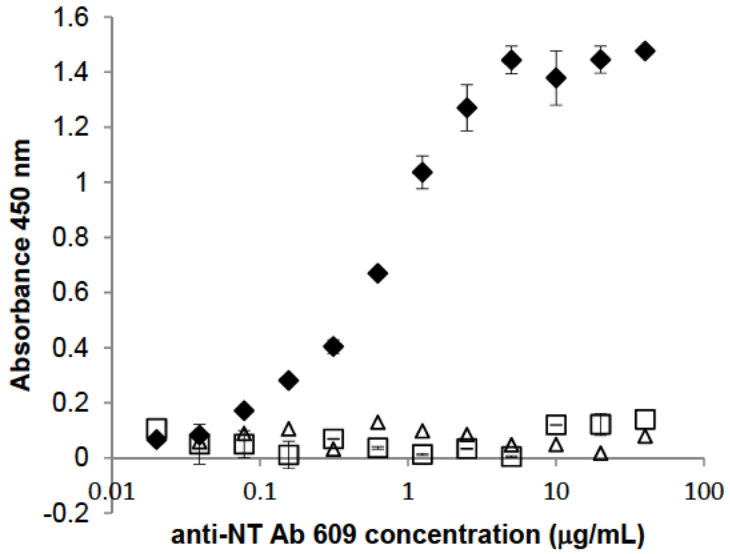
Affinity enrichment of nitrated proteins from human atherosclerotic lesions. Proteins extracted from atherosclerotic human lesions (0.84 mg of protein) were incubated with Dyna-beads/protein-A cross-linked to a polyclonal anti-nitrotyrosine antibody in lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM Hepes, pH 7.4, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1/100 Sigma protease inhibitor without metal chelators). Enrichment using the same beads cross-linked to a non-specific IgG were used as control. Bound fractions from the beads were eluted using 0.1 M citrate buffer, pH 2.5, and the fractions were separated in 10% SDS-PAGE, transferred to PVDF and probed with a polyclonal anti-nitrotyrosine antibody. (**A)** Colloidal blue stain and **(B)** western blot developed with an anti-nitrotyrosine polyclonal antibody. Lanes: 1) Input un-fractionated lesion protein extract; 2) Unbound fraction from the anti-nitrotyrosine antibody beads. 3) Bound fraction from the anti-nitrotyrosine antibody beads; 4) Bound fraction from the non-specific IgG beads. Representative data from 3 different human lesions.

Thomson et al. Supplementary figure 2



Affinity enrichment for nitrated proteins from human CAD plasma. Pooled plasma CAD subjects was applied to a agarose column that a polyclonal anti-nitrotyrosine antibody was ligated. After binding and extensive washing the bound proteins were eluted with 0.1 M glycine, pH 2.5, containing 0.15 M NaCl and separated in 10% SDS-PAGE. **(A)** Colloidal blue stain and **(B)** western blot developed with an anti-nitrotyrosine polyclonal antibody. Lanes 1, and 4, un-fractionated plasma (input); Lanes 2, and 5, last wash before elution; Lanes 3, and 6, eluted bound proteins.

Thomson et al. Supplementary figure 3



Typical binding curve and specificity of the reference anti-nitrotyrosine antibody. Polyclonal antibody 609 was generated using the nitrated tyrosine octapeptide (CGnitroYGGGnitroYG) as antigen as described in detail previously (15). The binding of the antibody to nitrated proteins (♦) was competed by the inclusion of 250 µM 3-nitrotyrosine (□) or 10 µM nitrated tyrosine octapeptide (⌂). Data reports mean ± standard deviation.

Supplementary Table 1. Proteins that bound to anti-nitrotyrosine antibodies in human atherosclerotic lesions.

Protein	Uniprot Accession Number	Molecular Weight (kDa)	Unique peptides
14-3-3 protein sigma	P31947	28	6
14-3-3 protein zeta/delta	P63104	28	4
78 kDa glucose-regulated protein	P11021	72	6
Actin-related protein 2/3 complex subunit 4	P59998	20	3
Adenylyl cyclase-associated protein 1	Q01518	52	5
Adipocyte plasma membrane-associated protein	Q9HDC9	46	3
ADP-ribosylation factor 3	P61204 (+1)	21	3
Alcohol dehydrogenase 1B	P00325	40	8
Aldehyde dehydrogenase X, mitochondrial	P30837	57	3
Aldehyde dehydrogenase, mitochondrial	P05091	56	5
Alpha-1-acid glycoprotein 1	P02763	24	5
Alpha-1-antichymotrypsin	P01011	48	9
Alpha-1-antitrypsin	P01009	47	24
Alpha-1B-glycoprotein	P04217	54	8
Alpha-2-antiplasmin	P08697	55	4
Alpha-actinin-1	P12814	103	23
Alpha-actinin-4	O43707	105	39
Alpha-enolase	P06733	47	14
Angiotensinogen	P01019	53	3
Annexin A1	P04083	39	6
Annexin A4	P09525	36	4
Annexin A5	P08758	36	8
Annexin A6	P08133	76	13
Antithrombin-III	P01008	53	9
Apolipoprotein A-I	P02647	31	13
Apolipoprotein A-IV	P06727	45	14
Apolipoprotein D	P05090	21	8
Apolipoprotein E	P02649	36	15
Asporin	Q9BXN1	43	3
ATP synthase subunit alpha, mitochondrial	P25705	60	10
ATP synthase subunit beta, mitochondrial	P06576	57	15
Basement membrane-specific heparan sulfate proteoglycan core protein	P98160	469	31
Beta-2-glycoprotein 1	P02749	38	7
C4b-binding protein alpha chain	P04003	67	12
Cadherin-13	P55290	78	7
Calmodulin-like protein 5	Q9NZT1	16	4
Calponin-1	P51911	33	6

Carbonic anhydrase 1	P00915	29	6
Catalase	P04040	60	12
Cathepsin D	P07339	45	6
CD109 antigen	Q6YHK3	162	3
CD5 antigen-like	O43866	38	3
Ceruloplasmin	P00450	122	13
Chloride intracellular channel protein 1	O00299	27	3
Clathrin heavy chain 1	Q00610	192	9
Clusterin	P10909	52	16
Coagulation factor X	P00742	55	3
Cofilin-1	P23528	19	5
Collagen alpha-1(VI) chain	P12109	109	14
Collagen alpha-1(XII) chain	Q99715	333	6
Collagen alpha-1(XIV) chain	Q05707	194	28
Collagen alpha-1(XV) chain	P39059	142	4
Collagen alpha-1(XVIII) chain	P39060	178	4
Collagen alpha-2(IV) chain	P08572	168	3
Collagen alpha-2(VI) chain 4	P12110	109	10
Collagen alpha-3(VI) chain	P12111	344	43
Complement C1s subcomponent	P09871	77	4
Complement C3	P01024	187	58
Complement C4-A	P0C0L4 (+1)	193	32
Complement C5	P01031	188	24
Complement component C6	P13671	105	8
Complement component C7	P10643	94	6
Complement component C8 gamma chain	P07360	22	3
Complement component C9	P02748	63	15
Complement factor B	P00751	86	8
Complement factor H	P08603	139	29
Complement factor H-related protein 1	Q03591	38	3
Cystatin-B	P04080	11	3
Cysteine and glycine-rich protein 1	P21291	21	4
Cytosol aminopeptidase	P28838	56	5
Cytosolic non-specific dipeptidase	Q96KP4	53	3
Decorin	P07585	40	4
Desmocollin-3	Q14574	100	3
Dihydropyrimidinase-related protein 2	Q16555	62	4
EGF-containing fibulin-like extracellular matrix protein 1	Q12805	55	9
Elongation factor 1-alpha 1	P68104 (+1)	50	5
Elongation factor 2	P13639	95	5
Endoplasmin	P14625	92	3
Epiplakin	P58107	556	11
Eukaryotic initiation factor 4A-I	P60842	46	3

Ferritin heavy chain	P02794	21	3
Ferritin light chain	P02792	20	9
Fibrillin-1	P35555	312	5
Fibrinogen alpha chain	P02671	95	13
Fibrinogen beta chain	P02675	56	26
Fibronectin	P02751	263	36
Fibulin-1	P23142	77	9
Filamin-A	P21333	281	57
Fructose-bisphosphate aldolase A	P04075	39	8
Galectin-1	P09382	15	3
Galectin-3	P17931	26	4
Galectin-3-binding protein	Q08380	65	9
Galectin-7	P47929	15	7
Gasdermin-A	Q96QA5	49	5
Gelsolin	P06396	86	17
Glucose-6-phosphate isomerase	P06744	63	8
Glutamate dehydrogenase 1, mitochondrial	P00367 (+1)	61	4
Glutathione S-transferase P	P09211	23	5
Glycogen phosphorylase, brain form	P11216	97	6
Guanine nucleotide-binding protein G(i) subunit alpha-2	P04899	40	3
Haptoglobin	P00738	45	16
Haptoglobin-related protein	P00739	39	3
Heat shock 70 kDa protein 1A/1B	P08107	70	6
Heat shock cognate 71 kDa protein	P11142	71	13
Heat shock protein beta-1	P04792	23	7
Hemoglobin subunit delta	P02042	16	3
Hemopexin	P02790	52	11
Heparin cofactor 2	P05546	57	3
Histidine ammonia-lyase	P42357	73	4
Histone H2A type 1	P0C0S8 (+5)	14	4
Histone H4	P62805	11	5
HLA class II histocompatibility antigen, DR alpha chain	P01903	29	3
Ig gamma-2 chain C region	P01859	36	6
Ig gamma-3 chain C region	P01860	41	6
Ig heavy chain V-III region BUT	P01767	12	4
Ig heavy chain V-III region GAL	P01781	13	6
Ig heavy chain V-III region TEI	P01777	13	3
Ig heavy chain V-III region VH26	P01764	13	3
Ig kappa chain V-I region AU	P01594	12	3
Ig kappa chain V-III region SIE	P01620 (+3)	12	3
Ig kappa chain V-IV region Len	P01625 (+3)	13	3
Ig lambda chain V-III region LOI	P80748	12	3
IgGFc-binding protein	Q9Y6R7	572	13

Immunoglobulin lambda-like polypeptide 5	B9A064	23	5
Integrin alpha-1	P56199	131	3
Integrin alpha-V	P06756	116	5
Integrin beta-1	P05556	88	11
Integrin beta-2	P05107	85	3
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	101	6
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	106	6
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	103	4
Interleukin-36 gamma	Q9NZH8	19	3
Involucrin	P07476	68	4
Kallistatin	P29622	49	4
Kininogen-1	P01042	72	4
Lactadherin	Q08431	43	16
Leukotriene A-4 hydrolase	P09960	69	4
Lipopolysaccharide-binding protein	P18428	53	4
Liver carboxylesterase 1	P23141	63	4
L-lactate dehydrogenase A chain	P00338	37	7
L-lactate dehydrogenase B chain	P07195	37	6
Lumican	P51884	38	10
Lysosome-associated membrane glycoprotein 1	P11279	45	4
Lysosome-associated membrane glycoprotein 2	P13473	45	3
Macrophage-capping protein	P40121	38	4
Major vault protein	Q14764	99	5
Malate dehydrogenase, cytoplasmic	P40925	36	3
Malate dehydrogenase, mitochondrial	P40926	36	3
Membrane primary amine oxidase	Q16853	85	9
Mimecan	P20774	34	5
Myoferlin	Q9NZM1	235	4
Myosin light polypeptide 6	P60660	17	6
Myosin-10	P35580	229	9
Myosin-11	P35749	227	24
Myosin-9	P35579	227	15
Neuroblast differentiation-associated protein AHNAK	Q09666	629	3
Peptidyl-prolyl cis-trans isomerase A	P62937	18	8
Periostin	Q15063	93	20
Peroxiredoxin-2	P32119	22	4
Peroxiredoxin-6	P30041	25	3
Phosphatidylinositol-glycan-specific phospholipase D	P80108	92	4
Phosphoglucomutase-1	P36871	61	4
Phosphoglucomutase-like protein 5	Q15124	62	14
Phosphoglycerate kinase 1	P00558	45	11
Phosphoglycerate mutase 1	P18669	29	4
Phospholipid transfer protein	P55058	55	10

Pigment epithelium-derived factor	P36955	46	5
Plasma protease C1 inhibitor	P05155	55	9
Plasminogen	P00747	91	6
Plastin-2	P13796	70	24
Plastin-3	P13797	71	16
Polymerase I and transcript release factor	Q6NZI2	43	3
Polyubiquitin-B	P0CG47 (+3)	26	3
Profilin-1	P07737	15	6
Prostacyclin synthase	Q16647	57	5
Protein disulfide-isomerase A3	P30101	57	5
Protein disulfide-isomerase	P07237	57	5
Protein POF1B	Q8WVV4	68	4
Protein S100-A7	P31151	11	8
Protein S100-A8	P05109	11	5
Protein S100-A9	P06702	13	9
Prothrombin	P00734	70	4
Pyruvate kinase isozymes M1/M2	P14618	58	25
Rab GDP dissociation inhibitor beta	P50395	51	5
Ras GTPase-activating-like protein IQGAP1	P46940	189	5
Ras-related protein Rab-1A	P62820 (+2)	23	7
Ras-related protein Rab-7a	P51149	23	4
Rho GTPase-activating protein 1	Q07960	50	3
Serine protease HTRA1	Q92743	51	4
Serotransferrin	P02787	77	35
Serpin B3	P29508	45	16
Serpin B4	P48594	45	5
Serpin B5	P36952	42	3
Serum paraoxonase/arylesterase 1	P27169	40	3
SH3 domain-binding glutamic acid-rich-like protein	O75368	13	4
Synaptic vesicle membrane protein VAT-1 homolog	Q99536	42	3
Tenascin	P24821	241	8
Tetranectin	P05452	23	3
Thioredoxin domain-containing protein 5	Q8NBS9	48	4
Thrombospondin-1	P07996	129	4
Thrombospondin-2	P35442	130	9
Thymidine phosphorylase	P19971	50	5
Transforming growth factor-beta-induced protein ig-h3	Q15582	75	9
Transgelin	Q01995	23	17
Transgelin-2	P37802	22	9
Transketolase	P29401	68	8
Transmembrane protein 43	Q9BTV4	45	3
Transthyretin	P02766	16	7
Triosephosphate isomerase	P60174	27	8

Tropomyosin alpha-1 chain	P09493	33	3
Tropomyosin beta chain	P07951	33	7
Tubulin alpha-1B chain	P68363 (+1)	50	9
Tubulin beta-2C chain	P68371	50	13
Tubulointerstitial nephritis antigen-like	Q9GZM7	52	3
Vimentin	P08670	54	31
Vinculin	P18206	124	33
Vitamin D-binding protein	P02774	53	6
Vitamin K-dependent protein S	P07225	75	5
WD repeat-containing protein 1	O75083	66	6
Zinc-alpha-2-glycoprotein	P25311	34	6

Sequence-to-spectrum assignments from three biological replicates were combined in Scaffold. The proteins listed above satisfied two criteria: (1) identified by at least one protein capture method (≥3 unique peptides), (2) not identified in the respective non-specific IgG column. Uniprot accession numbers correspond to the full-length unprocessed precursor when available (www.uniprot.org). Additional accessions were listed if the observed peptides could not distinguish between protein isoforms. Proteins were identified in three independent human lesion extracts analyzed at different times by LC-MS/MS as described in the Methods section.

Supplementary Table 2. Nitrated proteins and the corresponding modified peptides in human CAD plasma.

Protein	Accession	Sequence
Ig gamma-1 chain C region	P01857	(K)FNWY$_{161}$*VDGVEVHNAK(T)
		(K)TTPPVLDSDGSFFLY$_{290}$*SK(L)
Ig kappa chain C region	P01834	(K)SGTASVVCLLNNFY$_{32}$*PR(E)
		(K)VY$_{84}$*ACEVTHQGLSSPVTK(S)
Ig lambda chain C region	P01842	(R)SY$_{84}$*SCQVTHEGSTVEK(T)
Ig mu chain C region	P01871	(K)GVALHRPDVY$_{332}$*LLPPAR(E)
Ig alpha-1 chain C region	P01876	(K)Y$_{276}$*LTWASRQEPSQGTTTFAVTSILR(V)
Ig heavy chain V-III region WEA	P01763	(K)NSLY$_{80}$*LQMSSLR(A)
Ig heavy chain V-III region BUR	P01773	(R)TEDTAVYY$_{95}$*CAK(L)
Ig kappa chain V-III region B6	P01619	(R)ASQSLSGNY$_{33}$*LAWYQQKPGQAPR(L)
Zinc finger and BTB domain-containing protein 1	Q9Y2K1	(K)ISAECFDLILQFM(OH)Y$_{83}$*L(G)
Protein EFR3 homolog B	Q9Y2G0	(S)KISEVLGGSGY$_{669}$*NSDR(L)

Accession: Uniprot accession, which refers to the unprocessed precursor protein when available (www.uniprot.org). *Residue refers to the identified 3-nitrotyrosine residue. Amino acid modifications are designated as, M(OH), methionine oxidation (+16 amu) and Y*, tyrosine nitration (+45 amu). Putative Sequest peptide assignments containing 3-nitrotyrosine were accepted only if the following multiple selection criteria were met: (1) Peptide assignments contained a Tyr residue with +45 amu mass increase in the *y*- or *b*-ion series. (2) Manual inspection of the MS/MS spectra for a continuous *b*- or *y*-ion series of at least 5 fragment ions and assignment of the most intense fragment peaks to a *b*- or *y*-ion, a *b*- or *y*-ion resulting from a neutral loss of water/ammonia, or a multiply protonated fragment ion. These sites of tyrosine nitration were identified in two independent pooled CAD plasma analyzed at different times by LC-MS/MS as described in the Methods section.