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Proteomic approach to coronary atherosclerosis shows ferritin light chain as a significant marker: evidence consistent with iron hypothesis in atherosclerosis

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You, Sun-Ah, Stephen R. Archacki, George Angheloiu, Christine S. Moravec, Shaoqi Rao, Michael Kinter, Eric J. Topol, and Qing Wang. Proteomic approach to coronary atherosclerosis shows ferritin light chain as a significant marker: evidence consistent with iron hypothesis in atherosclerosis. *Physiol Genomics* 13: 25–30, 2003; 10.1152/physiolgenomics.00124.2002.—Coronary artery disease (CAD) is the leading cause of mortality and morbidity in developed nations. We hypothesized that CAD is associated with distinct patterns of protein expression in the coronary arteries, and we have begun to employ proteomics to identify differentially expressed proteins in diseased coronary arteries. Two-dimensional (2-D) gel electrophoresis of proteins and subsequent mass spectrometric analysis identified the ferritin light chain as differentially expressed between 10 coronary arteries from patients with CAD and 7 coronary arteries from normal individuals. Western blot analysis indicated significantly increased expression of the ferritin light chain in the diseased coronary arteries (1.41 vs. 0.75; $P = 0.01$). Quantitative real-time PCR analysis showed that expression of ferritin light chain mRNA was decreased in diseased tissues (0.70 vs. 1.17; $P = 0.013$), suggesting that increased expression of ferritin light chain in CAD coronary arteries may be related to increased protein stability or upregulation of expression at the posttranscriptional level in the diseased tissues. Ferritin light chain protein mediates storage of iron in cells. We speculate that increased expression of the ferritin light chain may contribute to pathogenesis of CAD by modulating oxidation of lipids within the vessel wall through the generation of reactive oxygen species. Our results provide in situ proteomic evidence consistent with the "iron hypothesis," which proposes an association between excessive iron storage and a high risk of CAD. However, it is also possible that the increased ferritin expression in diseased coronary arteries is a consequence, rather than a cause, of CAD.

coronary artery disease; sudden death; atherosclerosis; proteomics

ALMOST 13 MILLION PEOPLE in the United States have coronary artery disease (CAD), which is the leading cause of death and disability in this country. In attempting to better understand CAD, we hypothesized that CAD is associated with distinct patterns of protein expression in the coronary arteries. The main goal of this study was to identify proteins associated with CAD. The approach we employed was proteomics, the analysis of a proteome, which can detect proteins that are associated with a disease by measuring their levels of expression between control and disease states. To our knowledge, this is the first application of proteomics to study CAD.

Ferritin is composed of two subunits: a 21-kDa heavy (H) chain and a 19-kDa light (L) chain. The major function of ferritin is intracellular iron storage. In mammalian cells, functional ferritin (relative mass of ~450 kDa) consists of 24 subunits of 2 species, the H and L subunits (22). The shape of ferritin is a hollow shell with an 80-Å diameter cavity, permitting the storage of up to 4,500 Fe^{3+} atoms held as ferric hydroxide phosphate complexes. H subunits catalyze the first step in iron storage, the oxidation of Fe^{2+}, whereas L subunits help mineralize the hydrous ferric oxide core within the protein shell interior (22). The ratio of H and L subunits varies between species and tissue type. Ferritin in the human liver and spleen, known as iron storage organs, are composed predominantly of the L subunit, whereas organs with low iron content, such as the heart and pancreas, are composed mainly of the H subunit (22). In this study we demonstrate that expression of the ferritin light chain in coronary arteries is significantly increased in patients with CAD.

MATERIAL AND METHODS

Sample preparation. In this study, we used 10 diseased and 7 normal coronary arteries, which were obtained either from the Cleveland Clinic Foundation Heart Transplant Program or autopsies. The Cleveland Clinic Foundation Institutional Review Board approved all tissue procurement. The normal arteries were free of stenosis, whereas the lumens in the diseased coronary arteries were stenosed by 80% to 100%. Samples were prepared through protein delipidation and precipitation (11). Coronary arteries were homogenized in 1 ml of 8 M urea, 1% Triton, 0.1 M dithiothreitol, 0.1 M NaCl, 0.045 M Tris (pH 7.4), and 4% "complete" protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN),

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then incubated for 15 min at 34°C. After cooling on ice for 10 min, DNase I (1 mg/ml) and RNase II (10 mg/ml; Sigma, St. Louis, MO) in Tris stock buffer, pH 7.8 (0.5M Tris and 50 mM $MgCl_2$), were added to the homogenate and incubated on ice for 10 min. The homogenate was then centrifuged at 4°C for 15 min at 10,000 *g*, and the aqueous phase between the upper lipid phase and lower cellular debris phase was extracted. The collected aqueous phase was mixed with 14 ml of ice-cold tri-*n*-butylphosphate:acetone:methanol (1:12:1), incubated at 4°C for 90 min, then centrifuged at 2,800 *g* for 15 min. Pellets were washed sequentially with 1 ml of tri-*n*-butylphosphate, acetone, and methanol, then air-dried. Precipitates were boiled in 0.1 ml of 0.325 M dithiothreitol, 4% CHAPS, and 0.045 M Tris (pH 7.4) for 5 min, then cooled to room temperature. The cooled samples were diluted in 1.5 ml of 8 M urea, 4% CHAPS, 0.1 M dithiothreitol, and 0.045 M Tris (pH 7.4) and incubated at 34°C for 15 min. Total proteins were quantified using the modified Bio-Rad protein assay.

Two-dimensional gel electrophoresis. Immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA) were rehydrated with 150 μg of protein at 50 V for 12 to 16 h. Isoelectric focusing electrophoresis was conducted at 20°C and 300 V for 3 h, followed by 1,500 V for 3 h, and finally 3,000 V for 18 h. After isoelectric focusing electrophoresis, the IPG strips were equilibrated with reducing reagent (6 M urea, 2% SDS, 20% glycerol, 0.38 M Tris, pH 8.8, and 0.1 M dithiothreitol) for 15 min followed by incubation for 15 min with alkylating reagent (6 M urea, 2% SDS, 20% glycerol, 0.38 M Tris, pH 8.8, and 0.1 M iodoacetamide). The two-dimensional (2-D) gel separation was performed on 12.5% Criterion precast gels (Bio-Rad, Hercules, CA). Electrophoresis was conducted at 200 V for 70 min. Gels were stained with silver staining followed by destaining with 50% methanol and 5% acetic acid in deionized water.

Protein sequencing and identification. Silver-stained 2-D protein gels for the patient tissues were compared with those for control tissues by direct visualization to identify the protein spots that show consistently higher (or lower) expression in diseased tissues. The protein spot of interest was cut from the gel and analyzed in the Lerner Research Institute Mass Spectrometry Laboratory for Protein Sequencing. Briefly, the destained gel piece was dehydrated in acetonitrile, then dried by vacuum centrifugation. The protease trypsin was driven into the gel piece by reswelling in 50 μl of trypsin (20 ng/μl) in 50 mM ammonium bicarbonate before overnight digestion at room temperature. The peptides formed by the digestion were extracted from the polyacrylamide gel with two 30-μl aliquots of 50% acetonitrile in 5% formic acid, and these extracts were evaporated to <20 μl for LC-MS and LC-tandem MS analysis using a Finnigan ion trap mass spectrometry system in the data-dependent mode. The protein identification program TurboSequest (Thermo Finnigan, San Jose, CA) was used to search the National Center for Biotechnology Information (NCBI) nonredundant database and identify the proteins.

Western blot analysis. Fifty micrograms of protein extracts was separated in a 12% SDS-polyacrylamide gel and electrophoretically transferred onto a PDVF membrane (Millipore, Etten-Leur, Netherlands). Membranes were blocked overnight with 5% nonfat dried milk and incubated for 2 h with an anti-ferritin antibody (Innogenex, San Ramon, CA) or an β-actin antibody (Sigma, St. Louis, MO). After washing with TBST (10 mM Tris, pH 8.0, 150 mM NaCl and 0.1% Tween-20), the membranes were incubated for 1 h with horseradish peroxidase-linked anti-mouse antibody (Santa Cruz Biotech, Santa Cruz, CA). The membranes were washed again with TBST, and the proteins were visualized using ECL Chemiluminescence (Amersham Life Sciences, Piscataway, NJ). Autography films were scanned and proteins were quantified with Scion Image Beta software.

The Student's *t*-test was used to compare protein expression data between the diseased and normal arteries. To avoid the bias, the data were further analyzed after adjusting for the age, sex, and sampling methods of the coronary arteries. Values are expressed as mean ± SD, and $P < 0.05$ was considered statistically significant.

Quantitative real-time PCR. Quantitative real-time PCR (rt-PCR) was used to examine changes in levels of ferritin light chain mRNA with total RNA prepared from the coronary arteries. It was performed in an ABI Prizm 7700 Sequence Detection System using SYBR green (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Primers for quantitative rt-PCR were designed based on sequences from the GenBank as follows: forward primer, 5′-TTCAACAGTGTTTGGACGGA-3′; reverse primer, 5′-ATTTGGTCCAAGGCTTGTT-3′. The β2-microglobulin gene was used as an internal control. The relative mRNA expression level of the ferritin light chain gene in each sample was calculated using the comparative expression level $2^{-\Delta\Delta CT}$ method (CT, threshold cycle or the fractional cycle number at which the amount of amplified target reaches a fixed threshold) (10). Briefly, the target PCR CT value is normalized to the β2-microglobulin PCR CT value by subtracting the β2-microglobulin CT value from the target PCR CT value, which gives the ΔCT value. The ΔCT value for each subject is adjusted to the mean ΔCT level of the normal tissues, yielding a ΔΔCT value. The relative mRNA expression level for each subject is then calculated using the following equation: relative mRNA expression = $2^{-\Delta\Delta CT}$.

For statistical analysis of quantitative rt-PCR data, the relative gene expression value (RE or $2^{-\Delta\Delta CT}$) for each subject was calibrated separately for each of three experiments (10), i.e., the measurement for each subject was adjusted to the mean level of the normal tissues in the corresponding experiment. Prior to the statistical testing for the differential expression between CAD and normal tissues, exploratory data analysis was conducted. Tests of normality for RE values showed that these values significantly depart from the normality assumption (skewness = 1.7; kurtosis = 4.2), indicating that data transformation is required. After several transformations were tried, a log transformation was used to obtain the log of RE values (skewness = 0.42; kurtosis = −0.17). Then, a repeated measures ANOVA F test using the SAS GLM procedure was performed to test against the null hypothesis of no difference in the log transformed RE values between CAD and normal tissues.

RESULTS

Our proteomics strategy used 2-D gel electrophoresis to separate the complete coronary artery protein complement by pI and molecular weight. Figure 1*A* shows a representative 2-D gel map of a normal coronary artery. The 2-D gel maps created for the 10 diseased and 7 normal coronary arteries consistently reproduced this overall pattern (data not shown).

Analysis of the 2-D gel maps by direct visualization identified one protein spot with a consistently higher level of expression in the diseased arteries (Fig. 1*B*). Protein sequencing experiments using mass spectrometry identified this protein spot as ferritin light chain (molecular mass, 20.0 kDa; pI, 5.5) by detecting and

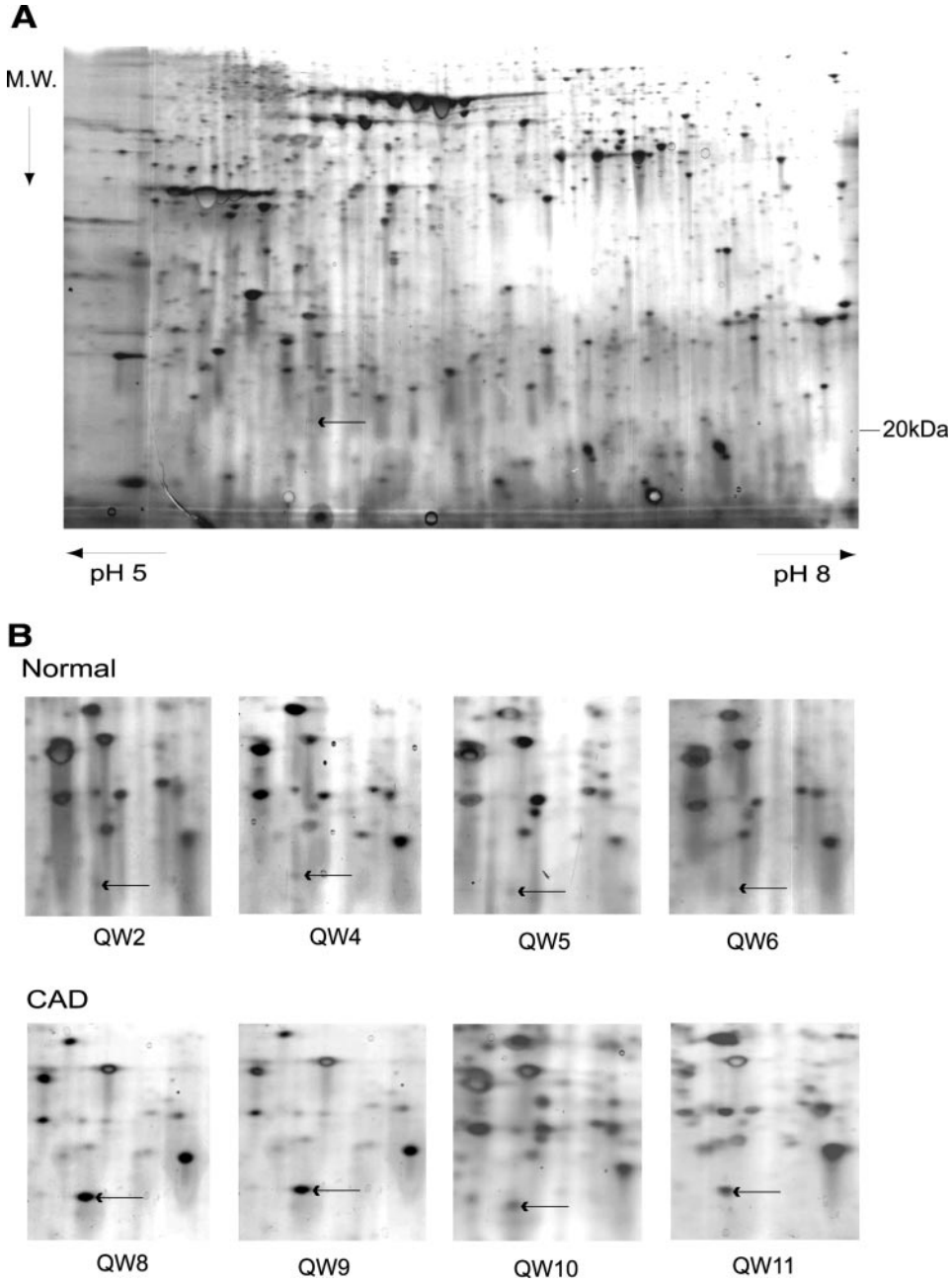

Fig. 1. Two-dimensional (2-D) map of human coronary artery proteins. *A*: representative gel from a normal human coronary artery (QW4). Reproducible 2-D patterns were obtained from other normal coronary arteries and CAD coronary arteries. The resolution of protein spots was optimized using pH 5–8 IPG strips. *B*: enlarged areas from 2-D gel images of normal individuals (QW2, QW4, QW5, QW6; *top*) and CAD patients (QW8, QW9, QW10, QW11; *bottom*). The protein spot indicated by the arrow shows a higher level of expression in CAD than in their normal counterparts. The spot has pI of 5.5 and molecular mass of about 20 kDa. CAD, coronary artery disease.

sequencing nine peptides covering 55.4% of the protein sequence. As a result, this proteomic experiment suggests that increased ferritin light chain expression is associated with CAD.

To confirm the 2-D gel results, we performed Western blot analysis using an equal amount of protein extracts from the normal and diseased coronary arteries (Fig. 2*A*). The total protein load was verified from the β-actin expression level. Western blot analysis was performed using a polyclonal antibody for the ferritin light chain. As shown in Fig. 2*A*, the expression level of this protein was higher in the CAD arteries, which confirmed the 2-D gel analysis results.

Statistical analysis determined that the ferritin light chain expression in the diseased arteries was significantly higher than that in the normal arteries. Using Scion Image Beta software, the ferritin light chain expression level was calibrated with that of the corresponding β-actin level. As shown in Fig. 2*B*, ferritin light chain expression in the diseased arteries was significantly higher than that in the normal counterparts (1.41 vs. 0.75; $P = 0.01$).

We also investigated the mRNA expression level of ferritin light chain in both CAD and normal coronary arteries using quantitative rt-PCR. Three independent quantitative rt-PCR experiments showed that mRNA expression of ferritin light chain in the diseased coro-

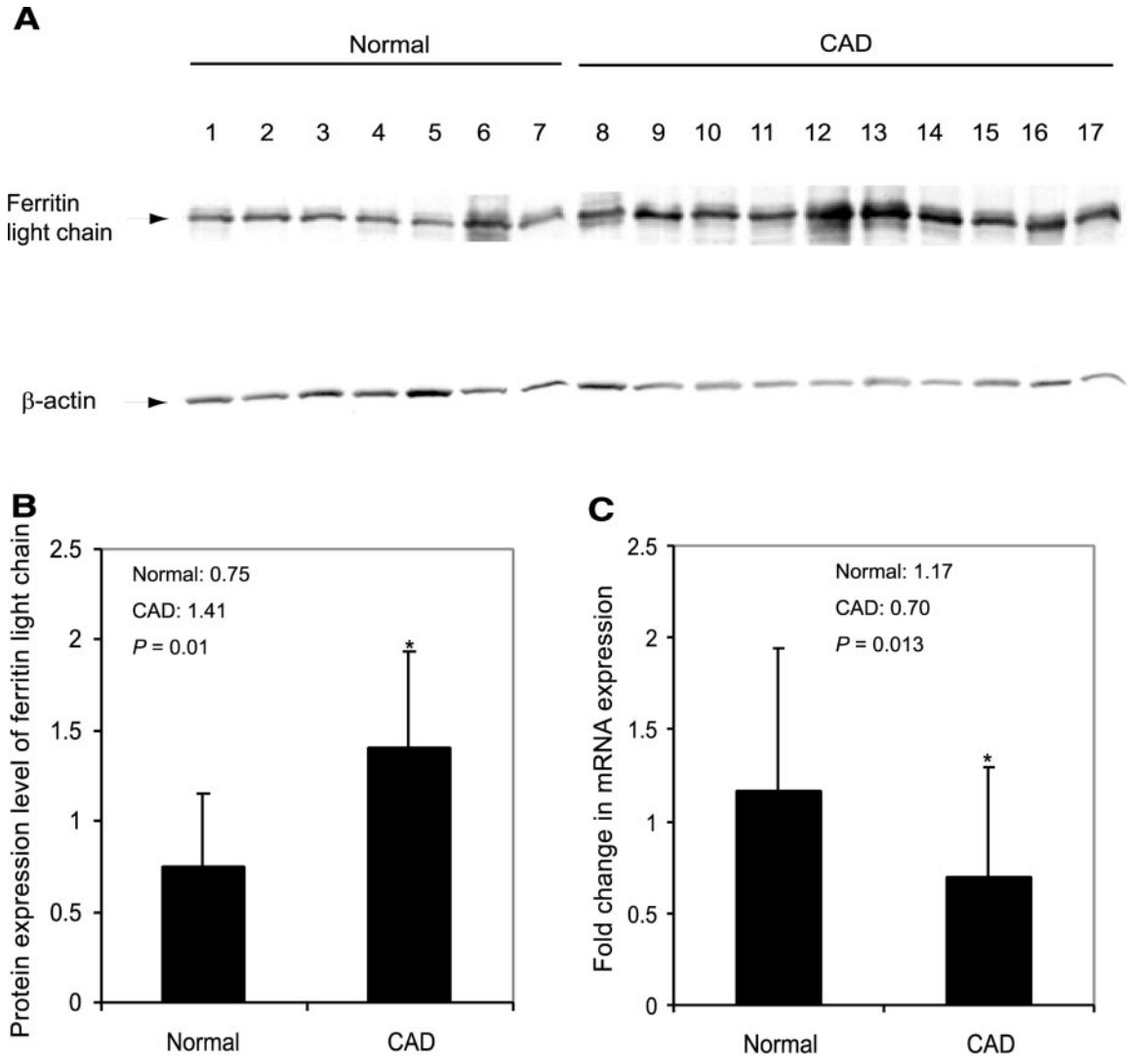

Fig. 2. *A*: Western blot analysis of the ferritin light chain. The protein extracts used for 2-D gel analysis were analyzed using Western blot for ferritin and β-actin expression. Normal coronary arteries are in *lanes 1* to *7* (QW1 to QW7) and CAD coronary arteries are in *lanes 8* to *17* (QW8 to QW17). CAD coronary arteries expressed high levels of ferritin compared with normal ones. *B*: significant association of increased expression of the ferritin light chain with CAD. The expression level of the ferritin light chain was calibrated by the corresponding β-actin value from the same tissue. CAD coronary arteries expressed about 2-fold greater ferritin light chain than the normal ones ($P = 0.01$). *C*: mRNA expression of ferritin light chain in CAD ($n = 8$) and normal ($n = 7$) coronary arteries. The vertical axis (*y*-axis) shows the mean fold change in ferritin light chain mRNA expression (mean ± SD). A repeated measures ANOVA F test yielded a *P* value of 0.013 on null hypothesis of no difference in gene expression between CAD and normal tissues, suggesting that CAD coronary arteries expressed less ferritin light chain mRNA than the normal ones.

nary arteries was slightly decreased than that in the normal coronary arteries (the mean fold change in gene expression: 0.70 for CAD vs. 1.17 for normal; $P = 0.013$) (Fig. 2*C*).

Our study was carried out for 10 CAD and 7 normal coronary arteries. The normal group includes 4 autopsy and 3 transplant tissues, and the CAD group has 8 autopsy and 2 transplant tissues. When expression of ferritin light chain was analyzed for autopsy or transplant tissues separately, significant differences were also observed between the normal and CAD coronary arteries. For autopsy tissues, the expression difference between the normal and CAD groups is statistically significant and approximately twofold (normal:CAD = 0.64:1.33, ~2.0-fold, $P = 0.0022$). For transplant tissues, the difference is 1.96-fold (normal:CAD = 0.9:1.77, 1.96-fold, $P = 0.0187$). Therefore, a bias in the sampling is unlikely to cause the difference observed between normal and CAD coronary arteries.

The difference in ferritin light chain expression between CAD and normal arteries was increased significantly after adjusting for gender and age. Our normal

group has 7 males, and no females. The CAD group has 2 females and 8 males. When ferritin light chain expression was compared between the 7 normal males and 8 CAD males, we found that the difference was highly significant (normal:CAD = 0.76:1.51, ~2-fold, $P = 0.0046$). Furthermore, we found that female CAD tissues showed lower expression than male CAD samples (female:male = 1.04:1.51, $P = 0.0041$), although it is important to note that only 2 females samples were compared with 8 male samples.

It should be noted that the average age for the CAD group is greater than that for the normal group (52.4 years vs. 39.4 years). However, when we analyzed our data for age-matched samples, the expression level of ferritin light chain in the CAD group was 2.9-fold higher than that in the normal group (age, 51 yr for CAD vs. 50.8 yr for control; expression, 1.51:0.52; ~2.9-fold; $P = 0.0008$). Thus the age difference cannot account for the expression difference of ferritin light chain observed between normal and CAD coronary arteries.

DISCUSSION

In this study, we demonstrated that the protein expression level of ferritin light chain was about 1.9-fold higher in diseased coronary arteries than in normal ones. This difference remains highly significant when the data were analyzed for gender- and age-matched samples or when expression of ferritin light chain was analyzed for autopsy or transplant tissues separately. Our data are consistent with the "iron hypothesis" for CAD that was first proposed by Sullivan in 1981 (21). Sullivan found that men and postmenopausal women had a higher risk of CAD than premenopausal women because these former two groups have higher amounts of iron stored in their bodies. Later, Salonen et al. (18) found that high levels of iron in the body, measured through serum ferritin concentration, is a risk factor for myocardial infarction (MI) in middle-aged eastern Finnish men. In that study, men with ferritin levels ≥200 μg/l possessed a 2.2-fold increased risk of developing an acute MI. In addition, several epidemiological studies have supported the theory that high levels of stored iron increase the risk of CAD (8, 9, 12, 23). Kiechl et al. (8) reported that serum ferritin concentration was highly significant correlated with pathological carotid artery wall thickening in a longitudinal cohort study, and Haidari et al. (6) recently demonstrated that serum ferritin is significantly associated with an increased risk of CAD in Iranian men.

The previous studies investigating the iron hypothesis measured the serum ferritin level or iron stores in blood. In contrast, in this study we measured the cellular ferritin levels in coronary arteries, the target organ for CAD. Our results indicate that ferritin light chain expression in coronary arteries is significantly increased in CAD patients and are consistent with the iron hypothesis.

The main function of ferritin is intracellular iron storage. Several studies have concluded that ferritin protects cells from oxidative stress by maintaining iron homeostasis (3, 13, 24). These studies demonstrated that increased ferritin levels reduces the labile iron pool and limits Fe(II) available for participating in the generation of oxygen free radicals. Many contrasting observations, however, have shown that ferritin is a harmful iron donor (5, 15, 17). Reif (15) suggested that the superoxide anion, formed enzymatically, radiolytically, or through redox cycling xenobiotics, causes ferritin iron liberation ($O_2^{\cdot -} + Fe^{3+} - \text{ferritin} \rightarrow O_2 + Fe^{2+} + \text{apoferritin}$). Additionally, Sakaida et al. (17) demonstrated that autophagic ferritin degradation is a source of iron release from ferritin by oxidative stress in cultured hepatocytes. Augmented free irons may catalyze hydroxyl radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$), and these reactive oxygen species may promote oxidative modifications of low-density lipoprotein. Evidence for this proposal has been found in human cell lines, such as arterial smooth muscle cells and human atherosclerotic lesions (7, 20, 25). The present study might support the hypothesis that increased ferritin light chain expression in diseased coronary arteries serves as an iron donor, promoting the oxidation of low-density lipoprotein and subsequent arterial wall damage by oxidative stress, resulting in CAD.

Several prospective epidemiological studies have shown conflicting results about the association between iron overload and CAD (1, 14, 19). Moreover, Danesh and Appleby (4) showed that published prospective studies did not provide sufficient evidence to support the existence of strong epidemiological association between the iron status and coronary heart disease. More recently published data by Ascherio et al. (2) do not support the hypothesis that reduced body iron stores lower CAD risk. Thus the increased ferritin light chain expression in diseased coronary arteries found in this study may reflect a consequence of CAD. Atherosclerosis is now recognized as an inflammatory disease (16), and ferritin synthesis can be upregulated by proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-1α (22). Therefore, increased ferritin light chain expression may be a marker of inflammation. Future studies are needed to verify whether increased ferritin light chain expression is a cause, or a consequence, of coronary atherosclerosis.

It is interesting to note that expression of ferritin light chain is significantly increased in CAD coronary arteries on the protein level, but not on the mRNA level. It is known that in some cases mRNA expression may not correlate well with protein expression. The different results obtained on mRNA and protein expression in this study highlight the importance of studying disease-associated gene expression on the protein level using proteomics. Our results also support the hypothesis that the increased expression of ferritin light chain in CAD coronary arteries may be related to increased protein stability or upregulation of expression at the posttranscriptional level in the dis-

eased tissues. This hypothesis is consistent with the finding over the last 15 years that ferritin expression is largely regulated through posttranscriptional pathways by iron. When labile iron levels are high, iron regulatory proteins are released from the 5′-untranslated region of ferritin mRNA, and ferritin synthesis then starts. Conversely, when iron levels are low, ferritin synthesis is decreased through the binding of iron regulatory proteins to iron responsive elements located at the 5′-untranslated region of ferritin mRNA.

A limitation of the present study is the small sample size due to difficulties in obtaining coronary artery tissues. Future studies with an increased sample size of CAD and normal coronary arteries will further validate our conclusion. Another limitation is the detection limit of 2-D gel electrophoresis, which might make evident only the most obvious changes in highly expressed proteins while obscuring changes in other proteins with lower expression levels.

In summary, this study represents the first application of proteomics in analyzing the pathogenesis of CAD. Our results indicate that expression of the ferritin light chain is significantly higher in diseased coronary arteries than in normal arteries. Our finding is consistent with the hypothesis that high levels of stored iron increase the risk of CAD.

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REFERENCES

1. **Aronow WS.** Serum ferritin is not a risk factor for coronary artery disease in men and woman aged ≥62 years. *Am J Cardiol* 72: 347–348, 1993.
2. **Ascherio A, Rimm EB, Giovannucci E, Willett WC, and Stampfer MJ.** Blood donations and risk of coronary heart disease in men. *Circulation* 103: 52–57, 2001.
3. **Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, and Vercellotti GM.** Ferritin: a cytoprotective antioxidant stratagem of endothelium. *J Biol Chem* 267: 18148–18153, 1992.
4. **Danesh J and Appleby P.** Coronary heart disease and iron status: meta-analyses of prospective studies. *Circulation* 99: 852–854, 1999.
5. **Dutra F, Knudsen FS, Curi D, and Bechara EJ.** Aerobic oxidation of aminoacetone, a threonine catabolite: iron catalysis and coupled iron release from ferritin. *Chem Res Toxicol* 14: 1323–1329, 2001.
6. **Haidari M, Javadi E, Sanati A, Hajilooi M, and Ghanbili J.** Association of increased ferritin with premature coronary stenosis in men. *Clin Chem* 47: 1666–1672, 2001.
7. **Heinecke JW, Rosen H, and Chait A.** Iron and copper promote modification of low-density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest* 74: 1890–1894, 1984.
8. **Kiechl S, Willeit J, Egger G, Poewe W, and Oberhollenzer F.** Body iron stores and the risk of carotid atherosclerosis: prospective results from the Bruneck study. *Circulation* 96: 3300–3307, 1997.
9. **Lauffer RB.** Iron stores and the international variation in mortality from coronary artery disease. *Med Hypotheses* 35: 96–102, 1991.
10. **Livak KJ and Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402–408, 2001.
11. **Mastro R and Hall M.** Protein delipidation and precipitation by tris-*n*-butylphosphate, acetone, and methanol treatment for isoelectric focusing and two-dimensional gel electrophoresis. *Anal Biochem* 273: 313–315, 1999.
12. **Morrison HI, Semenciw RM, Mao Y, and Wigle DT.** Serum iron and risk of fatal acute myocardial infarction. *Epidemiology* 5: 243–246, 1994.
13. **Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, and Rosenberg ME.** Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *J Clin Invest* 90: 267–270, 1992.
14. **Rauramaa R, Vaisanen S, Mercuri M, Rankinen T, Penttila I, and Bond MG.** Association of risk factors and body iron status to carotid atherosclerosis in middle-aged eastern Finnish men. *Eur Heart J* 15: 1020–1027, 1994.
15. **Reif DW.** Ferritin as a source of iron for oxidative damage. *Free Radic Biol Med* 12: 417–427, 1992.
16. **Ross R.** Atherosclerosis: an inflammatory disease. *N Engl J Med* 340: 115–126, 1999.
17. **Sakaida I, Kyle ME, and Farber JL.** Autophagic degradation of protein generates a pool of ferric iron required for the killing of cultured hepatocytes by an oxidative stress. *Mol Pharmacol* 37: 435–442, 1990.
18. **Salonen JT, Nyyssonen K, Korpela H, Tuomilehto J, Seppanen R, and Salonen R.** High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. *Circulation* 86: 803–811, 1992.
19. **Sempos CT, Looker AC, Gillum RF, and Makuc DM.** Body iron stores and the risk of coronary heart disease. *N Engl J Med* 330: 1119–1124, 1994.
20. **Smith C, Mitchinson MJ, Aruoma OI, and Halliwell B.** Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem J* 286: 901–905, 1992.
21. **Sullivan JL.** Iron and the sex difference in heart disease risk. *Lancet* 1: 1293–1294, 1981.
22. **Torti FM and Torti SV.** Regulation of ferritin genes and protein. *Blood* 99: 3505–3516, 2002.
23. **Tuomainen TP, Punnonen K, Nyyssönen K, and Salonen JT.** Association between body iron stores and the risk of acute myocardial infarction in men. *Circulation* 97: 1461–1466, 1998.
24. **Vile GF and Tyrrell RM.** Oxidative stress resulting from ultraviolet an irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. *J Biol Chem* 268: 14678–14681, 1993.
25. **Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, and Steinberg D.** Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 84: 1086–1095, 1989.