Direct Detection and Quantification of Transition Metal Ions in Human Atherosclerotic Plaques: Evidence for the Presence of Elevated Levels of Iron and Copper

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Objective—The involvement of transition metals in atherosclerosis is controversial. Some epidemiological studies have reported a relationship between iron (Fe) and cardiovascular disease, whereas others have not. Experimental studies have reported elevated levels of iron and copper (Cu) in diseased human arteries but have often used methods that release metal ions from proteins.

Methods and Results—In this study, we have used the minimally invasive technique of electron paramagnetic resonance (EPR) spectroscopy and inductively coupled plasma mass spectroscopy (ICPMS) to quantify iron and copper in ex vivo healthy human arteries and carotid lesions. The EPR spectra detected are characteristic of nonheme Fe(III) complexes. Statistically elevated levels of iron were detected in the intima of lesions compared with healthy controls (0.370 versus 0.022 nmol/mg tissue for EPR, 0.525 versus 0.168 nmol/mg tissue by ICPMS, \( P < 0.05 \) in each cases). Elevated levels of copper were also detected (7.51 versus 2.01 pmol/mg tissue, lesion versus healthy control, respectively, \( P < 0.05 \)). Iron levels did not correlate with the gender or age of the donor, or tissue protein or calcium levels, but cholesterol levels correlated positively with iron accumulation, as measured by EPR.

Conclusions—These data support the hypothesis that iron accumulates in human lesions and may contribute to disease progression. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: iron ■ copper ■ atherosclerosis ■ oxidation ■ free radicals ■ EPR

Atherosclerosis is a multifactorial disease, and defining the role of individual risk factors has proven problematic. Oxidation, particularly oxidative modification of low-density lipoproteins (LDL) within the artery wall and its subsequent unregulated uptake by macrophages, has been postulated to be an important in disease development. A range of species can oxidize LDL in vitro, but the nature of the oxidants in vivo is controversial.

Evidence has been presented for a role for lipoxygenase, peroxynitrite, hypochlorous acid, and metal ion-mediated oxygen–radical formation. Products arising from the reactions of HOCl (3-chlorotyrosine), peroxynitrite (3-nitrotyrosine), lipoxygenase (oxidized lipids), and metal ions (hydroxylated amino acids) have been detected in human plaques. Plaques have been reported to contain hypochlorite-oxidized proteins, active myeloperoxidase (the enzymatic source of HOCl), lipoxygenase protein, mRNA, and redox-active metal ions. However, 3-nitrotyrosine can be generated by species other than peroxynitrite. The participation of (cytosolic) lipoxygenase in extracellular processes has been questioned, as has the presence of metal ions on the basis of the invasive methods used to detect these species.

The present study focuses on the quantification of transition metal ions in lesions, because recent studies have demonstrated the presence of elevated levels of specific protein oxidation products in advanced human lesions and identified metal ions as possible catalysts for these species.

Epidemiological studies have sought to link various measures of iron (Fe) and copper (Cu) with the incidence of cardiovascular disease, after the suggestion that the development of disease is linked to iron stores, with iron-deficiency offering protection. These data are equivocal, with positive associations detected in some studies but not others. Decreasing body iron levels have been reported to be protective, and iron administration can be detrimental. Thus, some data support an association between high levels of iron and copper and disease incidence.

The presence of metal ions in human plaques has been examined using a number of approaches, but these techniques are often invasive and destructive (eg, homogenisation and digestion), preventing information on the nature and reactivity of the metal ion from being obtained. The capacity of lesion materials to catalyze damage has been used to implicate metal ions but mechanical disruption (homogeni-
EPR detection and quantification of metal ions in arterial samples

Electron Paramagnetic Resonance Spectroscopy

Tissue samples (150 to 300 mg) were blotted dry, inserted in open-ended EPR tubes, frozen in liquid N₂, and inserted into a liquid N₂ Dewar within the EPR spectrometer cavity (EMX X-band spectrometer; Bruker Biospin GmbH, Rheinstetten, Germany) equipped with 100-KHz modulation and a cylindrical ER 4103TM cavity. Typical acquisition parameters were: gain 1 × 10⁷, modulation amplitude 2g, time constant 164 ms, scan time 844 seconds, conversion time 82 ms, resolution 1024 points, power 2.5 mW, and frequency 9.43 GHz, with 20 scans averaged. Multiple spectra were acquired for each sample and for empty tubes. The spectra were baseline- and background-corrected using the Bruker WINEREP program. The resulting data were imported in Origin 7.0 (OriginLab Corporation, Mass), double-integrated, and quantified, as previously mentioned, by comparison with standard curves generated using known concentrations of Fe(III)-desferrioxamine (1:1 complex, generated from the addition of known concentrations of FeCl₃ to desferrioxamine) under identical conditions.

Inductively Coupled Plasma Mass Spectroscopy

Tissue sections were dissected, blotted, weighed, and digested (24 hours, room temperature) in 500 µL HNO₃ (69%) in washed Eppendorf tubes. Samples were then diluted to 10 mL with Milli Q water; control samples were prepared in an identical manner. All glassware used was acid-washed in HNO₃ before use, and all plastic-ware was washed with Milli Q water. Analysis of Fe (⁵⁷Fe) and/or ⁶⁵Fe; both isopes gave values that were not statistically different, Cu (⁶⁵Cu), and Ca (⁴⁰Ca) was performed by Dr Jim Keegan (University of Technology, Sydney) using a Perkin Elmer SCIEX ELAN 5000 apparatus (Thornhill, Ontario, Canada) equipped with a concentric nebulizer and a conical spray chamber (Glass Expansion Ltd., Australia). Instrumental operation conditions were: plasma flow 15 L/min, nebulizer flow 0.95 L/min, auxiliary flow 0.8 L/min, RF power 1200 W, and sample uptake 0.8 mL/min.

Cholesterol Quantification

Cholesterol analysis was performed as described previously. Tissues were thawed, blotted dry, weighed, minced, and homogenized in sodium carbonate buffer at 4°C in Ultra-Turrax T8 homogenizer (3000 to 5000 rpm, 2 to 3 minutes; IKA Labortechnik, Janke and Kunkel GmbH, Staufen, Germany). Aliquots were extracted with 1 mL of methanol and 5 mL of hexane, vortexed, then centrifuged (1000g, 4°C, 5 minutes). Then 4 mL of the hexane layer was removed, dried, and resuspended in isopropanol for high-performance liquid chromatography analysis.

Protein Quantification

Protein concentrations were determined on homogenates (as mentioned) using the biocinchoninic acid assay with bovine serum albumin as standard, according to the manufacturers instructions, except with 30-minute incubation at 60°C.

Statistics

Student t test, or one-way ANOVA with Tukey multiple comparison tests (for analysis of data from >2 groups) were used (Prism Version 4 for Macintosh; GraphPad Software Inc., San Diego, Calif). Correlations were calculated using the linear least-squares function in Microsoft Excel. P < 0.05 was considered significant.

Results

Iron and copper concentrations were measured in intimal samples from healthy arteries and advanced human carotid atherosclerotic lesions by EPR spectroscopy and ICPMS. The former technique has been performed on intimal sections at liquid nitrogen temperatures, whereas ICPMS was performed on acid-digested tissue.

EPR Detection and Quantification of Metal Ions in Artery Samples

Typical EPR spectra obtained from intimal sections of a carotid plaque and a healthy artery are shown in Figure 1A. The intense absorption peak detected in the plaque at gca.4 is characteristic of the presence of high-spin, rhombic, mononuclear Fe(III) complexes. This signal was not present in the empty EPR tubes and was only detected at low levels in healthy human intima samples (Figure 1A). Similar spectra were detected with healthy intima and smooth muscle samples from pig arteries (data not shown). These absorption peaks are distinct from those observed from Fe(III) in heme.
proteins, which typically give absorption peaks at gca.6.26 No significant absorptions from Cu(II) (gca.2.1 26 ) were detected. Similar behavior was observed with pig artery samples (data not shown). Additional EPR absorption lines were detected at gca.2, which are characteristic of organic radicals or iron–sulfur clusters.26 These absorptions were only detected in the tissue samples, but the nature and concentration of these species have not been investigated further. Similar species have been detected in other tissues.27,28 The gca.4 Fe(III) species was quantified, and although the levels varied (Figure 1B), the mean value for all the plaques examined is significantly elevated over that detected in healthy intima samples when expressed per milligram of tissue (Mann-Whitney test, P=0.0001; Figure 1B). C, Correlation of EPR-detectable iron levels with crude lesion classification. Samples were graded (see text) into healthy intima (controls, mean iron value 0.022±0.021 nmol/mg tissue), “clean lesions” (mean iron value 0.088±0.048 nmol/mg tissue), “calcified lesions” (mean iron value 0.585±0.503 nmol/mg tissue), and “complex lesions” (mean iron value 0.489±0.284 nmol/mg tissue). Statistical significance was assessed by one-way ANOVA with Tukey multiple comparison test. Healthy intima intima values versus clean plaques, P<0.05; healthy intima versus calcified plaques, P<0.01; healthy intima versus complex plaques, P=0.05; clean plaques versus calcified plaques, P<0.05; clean plaques versus complex plaques, P=0.05; calcified plaques versus complex plaques, P>0.05.

ICPMS Quantification of Metal Ions in Artery Samples
Because EPR spectroscopy does not detect all iron ions [eg, signals are not detected from some Fe(II) complexes, or multinuclear Fe(III) complexes, such as ferritin26], ICPMS was used to quantify total iron levels; total copper and
calcium were also assessed in some cases. Statistically elevated levels of total iron were detected in diseased intima when compared with healthy intima and healthy smooth muscle samples (Figure 2A). Statistically elevated levels of total copper were detected, although at lower levels than for total iron, in diseased intima samples when compared with healthy intima (7.51 versus 2.01 pmol/mg tissue, respectively), but not healthy smooth muscle (5.17 pmol/mg tissue).

Comparison of Iron Levels Detected by EPR Spectroscopy and ICMPS

Because EPR spectroscopy is nondestructive, some samples were analyzed by both techniques. The levels of Fe(III) detected by EPR spectroscopy were ~70% of the total iron value detected by ICPMS, and a good correlation was observed between the 2 techniques ($r=0.26$; Figure 3).}

Correlation of Metal Ion Levels With Lesion Parameters

No significant differences were detected in the levels of EPR-detectable Fe(III) or total (ICPMS) iron with sex or age of the donors (Figure 4). Insufficient data were obtained to allow a similar analysis for copper.

The iron and copper levels in the lesions did not correlate with total protein concentration (which might include iron- and copper-binding species) in the intimal samples (Figure 5A). The level of EPR-detectable Fe(III) correlated positively with cholesterol levels measured in the same samples (Figure 5B; $r=0.2233$). When a single outlying point (indicated in Figure 5B) is removed from the analysis, a much stronger correlation is observed ($r=0.5444$). The total iron levels detected by ICPMS did not correlate with cholesterol levels (Figure 5C).
As some ligands (e.g., carboxylic acids) bind both calcium and iron, and both metal ions were quantified in some samples, but no correlation was detected between these parameters (Figure 5D).

Discussion

Metal ions have been proposed as causative agents in a number of diseases, including atherosclerosis.18 Although some epidemiological studies have reported positive correlations between iron levels and cardiovascular disease, others have been negative.16–18 Previous experimental data on metal ion levels in human lesions have been criticized as a result of the methods used to quantify these materials.20,29 In the current study, we used a novel minimally invasive method, EPR spectroscopy, to quantify metal ion levels in washed, but otherwise intact, intimal samples from healthy and diseased arteries ex vivo. Because this methodology is nondestructive, and because the samples are kept at −196°C under an atmosphere of nitrogen during the experimental measurements (thereby preventing sample deterioration), both metal ion and cholesterol measurements could be made on the same samples, thereby eliminating tissue inhomogeneity as a confounding factor.

Statistically elevated levels of iron were detected in advanced human carotid lesions when compared with normal healthy intima samples by both techniques. These 2 measurements correlate well. The values obtained by EPR, which measures only Fe$^{2+}$ complexes and not multinuclear complexes such as ferritin and hemosiderin, accounted for ≈70% of the total iron present. The elevation in iron levels is

Figure 4. Correlation of EPR- and ICPMS-detectable iron levels in intimal samples from advanced human atherosclerotic plaque samples, with gender and age of donors. No significant difference was observed between EPR- (A, $P=0.6784$) and ICPMS-detectable levels of iron (B, $P=0.21$) and patient gender, or between the EPR- (C, $r=0.0067$), and ICPMS-detectable levels of iron (D, $r=0.0074$) and donor age.

Figure 5. Relationships between (A) total iron and protein concentrations, (B) EPR-detectable iron and free cholesterol concentrations, (C) total iron and free cholesterol concentrations, and (D) total iron and calcium concentrations, in intimal tissue from advanced human atherosclerotic plaques. Total iron and calcium were measured by ICPMS, EPR-detectable iron by EPR spectroscopy, protein levels by the BCA assay, and free cholesterol by high-performance liquid chromatography (see Methods). In (A) and (C), concentrations are expressed in nmol per mg wet-weight tissue, whereas in (B) and (C), iron and free cholesterol concentrations are in nmol per mg protein. The lines represent the linear least-squares fit of the data in each case; $r$ values as indicated.
localized to the intima, with only low levels of iron detected in smooth muscle cells from the medial layer. The levels of iron measured by ICPMS are similar in magnitude to those reported for other human artery samples. The increase in total iron levels detected in the current human study (3.3-fold) is lower than that detected in rabbits fed a high-fat diet to promote atherosclerosis (7- to 8-fold increase), with this iron deposition reported to occur at the onset of lesion formation. Elevated levels of total copper were also detected by ICPMS, although at much lower concentrations than iron. The absolute concentrations of copper are lower than, but of similar magnitude to, those detected in rabbits exposed to high copper levels. The difference in iron levels detected by EPR and ICPMS is ascribed to contributions from EPR-silent Fe(II) complexes (particularly heme proteins) and multinuclear iron complexes such as ferritin. The Fe(III) signals detected by EPR have been assigned to high-spin rhombic species and are not caused by typical heme proteins. These signals are similar to those reported for poorly defined low-molecular-weight iron complexes detected in yeast, bacteria, and mammalian tissues. This iron pool may arise from the presence of elevated heme protein levels in the lesions (although no evidence was obtained for this), with subsequent degradation or damage to these species and iron release, either as a result of oxidative events or through the action of heme oxygenase on released heme. Previous studies have reported iron release from oxidized heme proteins and for elevated levels of heme oxygenase mRNA and protein in lesions.

The greater increase in iron levels detected by EPR (16.7-fold) when compared with ICPMS (3.3-fold) may be of particular significance, because this reflects species that are not present within ferritin or hemosiderin (which do not give rise to EPR signals caused by exchange effects) or heme. The elevated levels of ferritin gene expression detected in lesions may be a response to these elevated iron levels. This nonferritin, nonheme iron pool may be redox-active and catalyze oxidative events within the artery wall, although this has not been investigated here. A recent report has suggested that iron may accumulate in lesions from intraplaque hemorrhage, and this would be consistent with the presence of these iron complexes in macrophage-derived cells, as proposed previously.

The correlation between the EPR-detectable iron and cholesterol levels in advanced lesions is consistent with the accumulation of these species being inter-related. Whether this elevated nonferritin, nonheme iron pool contributes to low-density lipoprotein oxidation and the formation of macrophage-derived foam (lipid-laden) cells requires further study; both iron and copper can promote such processes in vitro. Iron and copper can promote oxidative damage to extracellular matrix components. These elevated metal ions levels may therefore affect plaque stability and propensity to rupture. Such damage may occur independently of, or synergistically with, that induced by matrix-degrading enzymes (eg, matrix metalloproteinases). Metalloproteinases are released as inactive pro forms, and oxidation can activate these species and inactivate inhibitors. Previous oxidative damage can also enhance matrix degradation by proteolytic enzymes. Studies with rupture-prone lesion types (ie, those with thin fibrous caps, low levels of smooth muscle cells, large numbers of macrophages, and lipid-rich) would therefore seem warranted. The elevated metal ion levels are also consistent with the detection of elevated levels of protein oxidation products ascribed to metal ion catalyzed reactions detected in lesion proteins. Recent studies have shown that the majority of these species are present on matrix-associated materials, consistent with the mentioned hypothesis.

Overall, the data obtained in the current study are consistent with the hypothesis that high iron and copper levels may contribute to atherosclerosis and its sequelae as one factor in a multifactorial disease.

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References
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