Ceruloplasmin Copper Induces Oxidant Damage by a Redox Process Utilizing Cell-Derived Superoxide as Reductant†

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ABSTRACT: Oxidative damage by transition metals bound to proteins may be an important pathogenic mechanism. Ceruloplasmin (Cp) is a Cu-containing plasma protein thought to be involved in oxidative modification of lipoproteins. We have previously shown that Cp increased cell-mediated low-density lipoprotein (LDL) oxidation by a process requiring cell-derived superoxide, but the underlying chemical mechanism(s) is (are) unknown. We now show that superoxide reduction of Cp Cu is a critical reaction in cellular LDL oxidation. By bathocuproine disulfonate (BCS) binding and by superoxide utilization, we showed that exogenous superoxide reduces a single Cp Cu atom, the same Cu required for LDL oxidation. The Cu atom remained bound to Cp during the redox cycle. Three avenues of evidence showed that vascular cells reduce Cp Cu by a superoxide-dependent process. The 2-fold higher rate of Cp Cu reduction by smooth muscle cells (SMC) compared to endothelial cells (EC) was consistent with their relative rates of superoxide release. Furthermore, Cp Cu reduction by cells was blocked by Cu,Zn superoxide dismutase (SOD1). Finally, the level of superoxide produced by EC and SMC was sufficient to cause the amount of Cu reduction observed. An important role of Cp Cu reduction in LDL oxidation was suggested by results showing that SOD1 inhibited Cp Cu reduction and LDL oxidation by SMC with equal potency, while tumor necrosis factor-α stimulated both processes. In summary, these results show that superoxide is a critical cellular reductant of divalent transition metals involved in oxidation, and that protein-bound Cu is a substrate for this reaction. The role of these mechanisms in oxidative processes in vivo has yet to be defined.

The transition metal ions Cu and Fe are effective promoters of oxidation reactions, and can cause the oxidative modification of proteins, lipids, and DNA in vitro (*1*, *2*). The involvement of free metal ions in oxidation processes in vivo has been questioned since most of these ions are linked to prosthetic groups or are tightly sequestered by binding proteins and thus the level of free Cu (and Fe) in plasma and interstitial fluids is extremely low (*3*). In view of these findings, recent attention has focused on protein-bound metals (*4*−*6*), and on enzymatic mechanisms of oxidation not involving free metals, e.g., peroxidases (*7*, *8*). Cu,Zn superoxide dismutase (SOD1)[1] is one such metalloprotein that under some circumstances may cause oxidative damage. Neuropathologic damage caused by SOD1 mutants in familial amyotrophic lateral sclerosis was first thought to be due solely to an inactive dismutase. However, recent evidence indicates that the Cu atoms in certain SOD1 "gain-of-function" mutants have abnormally high surface exposure and may oxidatively modify inappropriate substrates (*9*−*11*). Likewise, Cu^{2+} bound to amyloid precursor protein may contribute to neurodegeneration in Alzheimer's disease (*12*). Recent studies show that the normal cellular form of prion protein binds Cu^{2+}, and suggest that tissue damage by the pathogenic form may be due to enhanced oxidative activity of the prion protein−Cu complex (*13*, *14*). Alternatively, injury may result from a decreased ability of the pathogenic form to sequester (and inactivate) Cu. A similar mechanism has been suggested for tissue protection by apolipoprotein E; the high susceptibility to Alzheimer's (and cardiovascular) disease in populations with a high prevalence of the E4 allele of apolipoprotein E (*15*) may be due to the weak Cu binding and low antioxidant activity of E4 compared to other isoforms (*16*). Much remains to be understood of the specific protein structures that permit oxidative injury by bound metals, the mechanism(s) underlying the damage, and the physiological significance of the reactions.

Human ceruloplasmin (Cp) is an acute-phase, plasma glycoprotein that contains approximately 95% of the total plasma Cu [see (*17*, *18*) for a review]. Studies by us (*19*−*21*) and others (*22*−*24*) show that Cp can cause oxidative damage to macromolecules; in particular, it is a potent promoter of LDL oxidation under a range of experimental conditions in vitro. Oxidized LDL influences multiple in vitro processes of vascular cells including motility, proliferation, and death, and many of these events are consistent with a role of the modified lipoprotein in atherogenesis [see (*2*, *25*, *26*) for a review]. Purified Cp contains six integral Cu atoms and a seventh which can be removed by solid-phase

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[1] Abbreviations: BCS, bathocuproine disulfonate; Cp, ceruloplasmin; EC, endothelial cells; LDL, low-density lipoprotein; MDA, malondialdehyde; PBS, phosphate-buffered saline; SMC, smooth muscle cells; SOD1, Cu,Zn superoxide dismutase; TBARS, thiobarbituric acid-reacting substances; TNF-α, tumor necrosis factor-α.

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chelators (*21*, *27*); removal of this single Cu by Chelex-100 completely blocks prooxidant activity (*21*). Site-directed mutagenesis studies suggest that the removable, prooxidant Cu resides, partly exposed, in a narrow valley above Cp His^{426} (*28*).

The mechanism(s) by which Cp (or free metal ions) promotes LDL oxidation is (are) incompletely understood. An insight into the mechanism of Cp was provided by experiments showing that the oxidant activity of Cp was blocked by proteins and by culture medium components (*20*, *21*). Oxidation was restored in the presence of either endothelial cells (EC) or smooth muscle cells (SMC), vessel wall cells that oxidize LDL in the presence of free transition metals in vitro (*29*−*31*). The cellular component required for Cp-stimulated LDL oxidation was shown to be superoxide by the (i) correlation between superoxide production and LDL oxidation, (ii) inhibition of cell-mediated LDL oxidation by SOD1, and (iii) reconstitution of SMC-mediated LDL oxidation under cell-free conditions by continuous addition of superoxide in the presence of Cp (*20*). Cell-derived superoxide was also required for free metal ion-dependent oxidation of LDL by SMC (*32*) and EC (*33*).

The reducing activity of superoxide and the strong prooxidant activity of reduced metal ions suggest a mechanism in which cell-derived superoxide reduces Cu^{2+} to Cu^{1+} which can drive lipid oxidation by several proposed mechanisms including the Fenton reaction. Alternatively, the reduction process itself, rather than the presence of Cu^{1+}, may be the force driving lipid and lipoprotein oxidation. The importance of reduced metal ions in the oxidation of polyunsaturated lipids has long been appreciated. A requirement for Cu^{2+} reduction in LDL oxidation was suggested by Maiorino et al. (*34*), who showed that phospholipid emulsions containing α-tocopherol, a major component of LDL, caused reduction of free Cu^{2+} and phospholipid oxidation. Likewise, LDL-bound α-tocopherol reduced free Cu^{2+} with consequent formation of α-tocopheroxyl radical (*35*, *36*). Cu^{2+} reduction by LDL was confirmed by Lynch and Frei (*37*), but their data suggested that the reductant was not α-tocopherol. In none of these studies was (were) the cell-derived reductant(s) identified, nor was the reducibility of Cu (or Fe) containing proteins shown. The following studies were designed to investigate the importance of superoxide-mediated reductive mechanisms in Cp-stimulated LDL oxidation by cultured cells.

EXPERIMENTAL PROCEDURES

Materials. Cp either was purified from human plasma as described (*38*) or was obtained from Calbiochem (La Jolla, CA). Cp homogeneity was verified by an absorbance ratio (610 nm/280 nm) greater than 0.045. Cp was primarily in the intact, 132 kDa form (85−90% intact by Coomassie staining and Western blot analysis), but it also contained the 115 and 19 kDa proteolytic fragments present in serum. Tumor necrosis factor-α (TNF-α) was obtained from GIBCO−BRL, Grand Island, NY. Chelex-100 was from Bio-Rad, Richmond, CA. Bathocuproine disulfonate (BCS), xanthine, xanthine oxidase, SOD1 (5 units/μg of protein), catalase, and all assay reagents were from Sigma. Human LDL (density = 1.019−1.063 g/mL) was prepared by sequential ultracentrifugation of freshly drawn, citrated, normolipemic plasma to which EDTA was added before centrifugation (*39*). Immediately before use, LDL was extensively dialyzed at 4 °C against 0.9% NaCl. LDL isolated from multiple donors gave essentially identical results.

Cell Culture. EC were isolated from adult bovine aortas (*40*), subcultured by trypsinization, and grown to confluence in Dulbecco's modified Eagle's medium and Ham's F12 medium containing 5% fetal calf serum (Hyclone Laboratories, Logan, UT). SMC were isolated by an explant method using intima-media of adult bovine aortas (*41*). The cells were made quiescent in Dulbecco's modified Eagle's medium containing 1 mg/mL gelatin for at least 24 h. Human aortic EC cultured on a fibronectin-coated (1 μg/cm^2) surface (*42*) and human aortic SMC (*41*) were used in some experiments. All experiments with cells were done at 37 °C in an atmosphere containing 95% air, 5% CO_2.

Other Methods. Superoxide production by cells, or by a superoxide-generating system containing xanthine and xanthine oxidase, was measured spectrophotometrically as SOD1-inhibitible reduction of cytochrome *c* (*43*). Cultured cells were incubated in RPMI 1640 medium (without phenol red dye) with 3×10^{-5} M cytochrome *c*. Superoxide-specific reduction of cytochrome *c* was determined as the difference in absorbance at 550 nm between dishes incubated in the presence and absence of SOD1 (100 units/mL). Cell-free dishes were used as background controls. Cu reduction was measured using BCS, which specifically interacts with Cu^{1+} to form a stable complex with an absorbance maximum at 480 nm and an extinction coefficient of 9058 M^{-1} cm^{-1} (*37*). In oxidation studies, LDL was incubated with cell cultures in serum- and transition metal-free RPMI 1640 medium at 37 °C. LDL oxidation was determined as thiobarbituric acid-reacting substances (TBARS) in the conditioned medium and expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of LDL cholesterol (*44*). The formation of 2-oxohistidine in Cp was measured by reverse-phase chromatography with precolumn derivatization by *o*-phthaldialdehyde as described by Lewisch and Levine (*45*). All of the data shown are from representative experiments done 3 or more times. The results from duplicate or triplicate measurements are given as the mean and standard error of the mean.

RESULTS

Superoxide-Dependent Reduction of Ceruloplasmin Copper. Studies by others have shown that LDL and its constituents can reduce free Cu^{2+} (*34*−*37*). To investigate the relevance to protein-bound metals, we tested whether LDL can reduce Cp-bound Cu^{2+}. When coincubated in phosphate-buffered saline (PBS), LDL effectively reduced Cp Cu as measured by the change in absorbance of BCS, a specific indicator of reduced Cu^{1+} (Table 1, experiment 1). An exogenous superoxide-generating system consisting of xanthine and xanthine oxidase also reduced Cp Cu, with an extent of reduction essentially equal to that by LDL. In contrast to the results in PBS, Cu^{2+} reduction by LDL in RPMI 1640 cell culture medium was very low (Table 1, experiment 2). However, the inhibition by culture medium (presumably due to inhibition by amino acids or other

Table 1: Reduction of Cp Cu in Vitro[a]

	reduced copper ($\Delta A_{480\ nm}$)	Cu^{1+}/Cp (or Cu) (mol/mol)
experiment 1 (in PBS)		
LDL	0.000	0.00
Cp	0.001	0.02
Cp + LDL	0.038	0.84
Cp + X/XO	0.041	0.91
experiment 2 (in RPMI 1640)		
LDL	0.001	0.02
Cp + LDL	0.007	0.15
Cp + LDL + X/XO	0.046	1.02
experiment 3 (in PBS)		
Cp	0.005	0.11
Cp + X/XO	0.043	0.97
Cu	0.007	0.15
Cu + X/XO	0.048	1.06
experiment 4 (in PBS)		
Cp	0.007	0.15
Cp + X/XO	0.048	1.06
$Cp_{Chelex-100}$ + X/XO	0.009	0.20

[a] Purified human Cp (5 μM) was incubated with LDL (500 μg of cholesterol/mL) and BCS (360 μM) for 2 h at 37 °C in 1 mL of PBS (experiment 1) or RPMI 1640 medium (experiment 2). Superoxide was generated by a xanthine (1 mM) and xanthine oxidase (0.15 milliunit) generating system (X/XO) that produced about 2 nmol of superoxide in 2 h. Cu reduction was determined as the increase in absorbance at 480 nm, and the molar amount of reduced Cu was calculated using an extinction coefficient of 9058 M^{-1} cm^{-1} for the BCS−Cu^{1+} complex. Cp Cu reduction by X/XO was compared to reduction of 5 μM $CuSO_4$ (experiment 3) and to 5 μM Cp that was pretreated with Chelex-100 ($Cp_{Chelex-100}$, experiment 4) under the same conditions as in experiment 1.

medium components) was completely overcome by the addition of the superoxide-generating system. These results were consistent with a previous observation that Cp by itself oxidized LDL in PBS, but not in RPMI 1640 medium or in the presence of amino acid mixtures, and also that exogenous superoxide restored the prooxidant activity in RPMI 1640 medium (*20*, *21*).

The use of BCS to measure Cu^{2+} reduction has been scrutinized recently; under some conditions, BCS can complex with Cu^{2+} to form a stronger oxidizing agent than Cu^{2+} alone, as measured by oxidation of amino groups in HEPES and other small tertiary amines (*46*). If BCS likewise interacts with and activates Cp, then the reduction of Cp-Cu^{2+} by superoxide, in the presence of BCS, does not prove that reduction would occur in the absence of BCS. We therefore measured the effect of BCS on Cp-Cu^{2+} oxidation of HEPES amine groups as described by Wang and Sayre (*47*). In agreement with their results, BCS in the presence of free Cu^{2+} caused substantial HEPES oxidation. However, BCS in the presence of Cp did not oxidize HEPES, indicating that BCS did not artifactually increase the oxidizing potential of Cp (data not shown). Since the artifactual increase in oxidation potential requires binding of BCS to Cu^{2+}, we measured whether BCS binds to Cp-Cu^{2+}. In the absence of the superoxide as reductant, a Cp-Cu^{2+}−BCS complex was not formed (as measured by retention after ultrafiltration assay, data not shown), verifying the use of BCS in measurement of Cp-Cu^{2+} reduction.

A Single Ceruloplasmin Copper Is Reducible by Superoxide. The number of superoxide-reducible Cu atoms (calculated using 9058 M^{-1} cm^{-1} as the extinction coefficient of the Cu^{2+}−BCS complex) was approximately 0.9 mol of superoxide-reducible (and BCS-detectable) Cu (Table 1, experiment 1). The molar ratio was verified by showing equimolar reduction of Cp Cu and free Cu ion (in $CuSO_4$) by superoxide (Table 1, experiment 3). Purified human Cp contains six tightly bound Cu atoms and a single Cu that is rapidly removed by chelation with Chelex-100 (*21*). To examine which of the Cp Cu atoms were reducible by superoxide, Cp was incubated with Chelex-100 to remove the specific Cu previously shown to be responsible for LDL oxidation (*20*, *21*). The low amount of redox-active Cu indicated that the chelatable Cu was reduced by superoxide (Table 1, experiment 4) and suggested the identity of the reducible Cu and the prooxidant Cu in Cp (*20*, *21*).

The possibility remained that other Cu^{2+} atoms were reduced by superoxide but were not detected by BCS due to limited access of the reagent into the protein interior (*46*); therefore, a BCS-independent method that measured superoxide utilization was used. Superoxide was generated by xanthine and xanthine oxidase, in the presence of Cp, and superoxide was measured as SOD1-inhibitable cytochrome *c* reduction. In the presence of 1.5 nmol of Cp, superoxide generation decreased from 8.6 ± 0.3 nmol to 1.1 ± 0.2 nmol, indicating utilization of 7.5 nmol of superoxide. When the prooxidant Cu was first removed from Cp with Chelex-100, 8.4 ± 0.3 nmol of superoxide was generated, indicating essentially no utilization of superoxide. This experiment was critical because it showed superoxide-mediated Cp Cu^{2+} reduction (albeit indirectly) by a method independent of BCS and the potential confounding issues associated with this reagent. The experiment also confirmed that the prooxidant Cu was required for superoxide scavenging, and was the reductive target of superoxide. Finally, the excess molar utilization of superoxide, compared to the amount of Cp, indicated that the reaction was catalytic rather than stoichiometric, and that Cu^{2+}/Cu^{1+} recycling occurred.

Redox reactions at the His-containing Cu binding site in human growth hormone (*48*) and SOD1 (*49*) cause formation of 2-oxo-His at the active site, resulting in enzyme inactivation and Cu release. We used amino acid analysis to measure 2-oxo-His formation (*45*) in Cp (5 μM) after continuous addition of superoxide (2 nmol/h using a xanthine/xanthine oxidase system). We did not detect any 2-oxo-His formation, or His loss, after 2 or 24 h of treatment with superoxide. As a positive control, incubation of Cu,Zn superoxide dismutase with 5 mM H_2O_2 for 30 min at 37 °C (*45*) was accompanied by substantial 2-oxo-His formation and disappearance of His. This result was consistent with our finding that Cp Cu induced LDL oxidation (and presumably redox cycling) for up to 24 h (*21*).

The Superoxide-Reducible Copper Remains Bound to Ceruloplasmin. A plausible mechanism explaining how reduced Cp Cu can oxidize LDL involves superoxide-mediated release of reduced Cu from Cp, analogous to the release of Fe^{2+} from ferritin (*50*), and subsequent LDL oxidation by free Cu. To examine Cu release, Cp was reduced by incubation with the superoxide-generating system and subjected to ultrafiltration to separate free Cu from Cp. Bound Cu on the retained protein was measured by treatment with superoxide and subsequent detection with BCS, and by its ability to oxidize LDL. Both methods showed that the prooxidant Cu remained tightly bound to Cp (Table 2). Control experiments with $CuSO_4$ showed that free Cu was

Table 2: Effect of Superoxide on Cu Release from Cp[a]

	reducible copper (μM)		LDL oxidation (nmol of MDA/mg of cholesterol)	
	retentate	filtrate	retentate	filtrate
Cp alone	4.2 ± 0.4	0.2 ± 0.1	13.4 ± 1.1	1.7 ± 0.2
Cp + X/XO	4.3 ± 0.4	0.2 ± 0.1	12.6 ± 0.9	1.9 ± 0.2
Cp + XO	4.3 ± 0.4	0.2 ± 0.1	12.3 ± 1.0	1.9 ± 0.2
$CuSO_4$	0.1 ± 0.1	4.7 ± 0.2	2.2 ± 0.3	18.7 ± 1.1

[a] Purified human Cp (5 μM) was incubated in the absence (Cp alone) or presence (Cp + X/XO) of a superoxide-generating system containing 1 mM xanthine and 0.3 milliunit of xanthine oxidase (generating about 2 nmol of superoxide per hour) in 0.5 mL of RPMI 1640 medium. After 1 h, the samples were subjected to ultrafiltration using a Microcon 10 filter. As a control, Cp was similarly incubated with 0.3 milliunit of xanthine oxidase alone (Cp + XO). An additional control with $CuSO_4$ (5 μM) was done to show that free Cu was not retained by the filter. The retentates and filtrates were separately reincubated with the complete superoxide-generating system (2 nmol/h) in the presence of BCS (360 μM), and copper reduction was measured as increased absorbance at 480 nm. In parallel wells, similarly treated Cp (1 μM) or $CuSO_4$ (0.7 μM) was incubated for 24 h with LDL (0.5 mg of cholesterol/mL) in the presence of the superoxide-generating system, and LDL oxidation was measured as TBARS.

not retained by the filter under these conditions. To confirm that bound Cu was susceptible to redox cycling while the metal is bound to Cp, we measured reoxidation of Cu^{1+}. BCS was added 3 h after incubation of Cp with the superoxide-generating system (when generation of superoxide was zero). No Cu^{1+} was detected, indicating complete reoxidation to Cu^{2+} (data not shown).

To confirm that Cu was not released from Cp, we examined the effect of albumin, a protein that binds Cu with high affinity, on Cp-stimulated LDL oxidation. Bovine serum albumin only weakly inhibited superoxide-dependent, Cp (0.7 μM)-mediated LDL oxidation, with an apparent K_i > 5 mg/mL. At the same concentration of free Cu salt (causing the same level of LDL oxidation), albumin was a very potent inhibitor of LDL oxidation with an apparent K_i of about 0.8 mg/mL. This result clearly showed that the oxidation mechanism of Cp Cu was not the same as that of free Cu, and supported the conclusion that Cu was not released from Cp during the oxidation process.

Superoxide-Dependent Reduction of Ceruloplasmin Copper by Cells. To begin to study the role of cellular reduction processes in LDL oxidation, we measured the ability of cultured cells to reduce Cp Cu. Confluent bovine aortic EC and SMC cultures both caused significant Cp Cu reduction compared to cell-free controls (Figure 1A). The initial rate of reduction by SMC was nearly twice that by EC, consistent with the nearly 2-fold higher rate of superoxide production (Figure 1B), and LDL oxidation (*20*), by SMC. BCS did not have a cytotoxic effect since it did not alter cell morphology or decrease the rate of superoxide production (not shown). To directly test the role of cell-derived superoxide, Cp Cu reduction was measured in the presence of SOD1. SOD1, but not heat-inactivated SOD1, completely blocked Cu reduction by bovine SMC (Table 3). Hydrogen peroxide and hydroxyl radical did not appear to be required since their respective scavengers, catalase and mannitol, were completely ineffective; however, the high reactivity of hydroxyl radical and the relatively low efficiency of its scavengers make the latter conclusion uncertain. Similar SOD1-inhibitable reduction of Cp Cu was observed in separate studies using bovine aortic EC, and human aortic SMC and EC (not shown). The inhibition of cell reduction

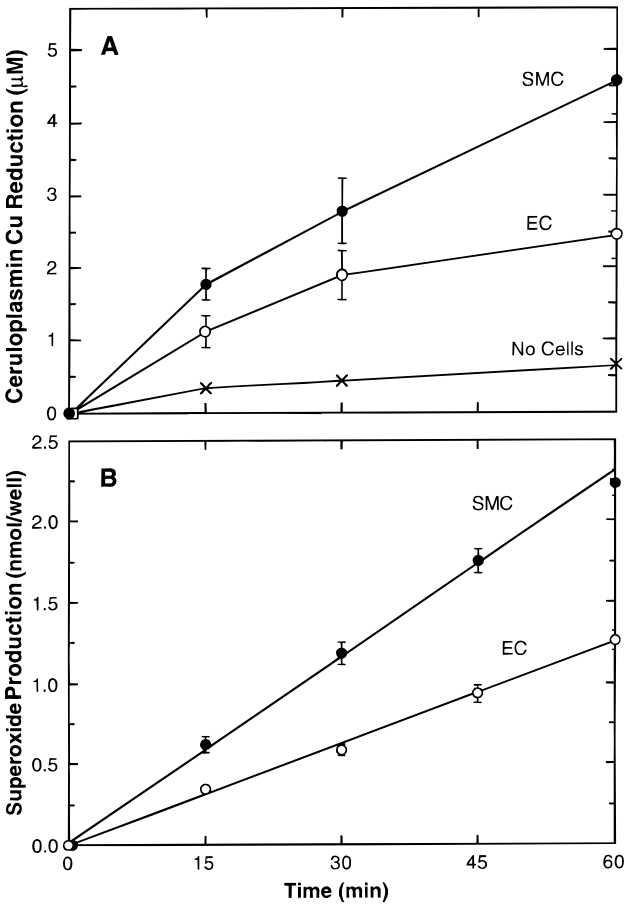

FIGURE 1: Cp Cu reduction and superoxide production by aortic SMC and EC. (A) To measure Cp Cu reduction, confluent cultures of bovine aortic SMC (●) and EC (○), or cell-free wells (×), were incubated with purified human Cp (5 μM) and BCS (360 μM) for 1 h in a total volume of 0.5 mL of RPMI 1640 medium. Cu reduction was determined as the change in absorbance at 480 nm resulting from BCS−Cu^{1+} complex formation. (B) To determine the rate of superoxide production, confluent cultures of SMC (●) and EC (○) were washed and incubated with cytochrome *c* (3×10^{-5} M) in RPMI 1640 medium (without phenol red dye). Superoxide was calculated by the reduction of cytochrome *c* as determined by the difference in absorbance at 550 nm between dishes incubated in the presence and absence of SOD1 (100 units/mL).

Table 3: Inhibition of Cell-Mediated Reduction of Cp Cu by Scavengers of Reactive Oxygen Species[a]

	reduced copper (μM)	inhibition (%)
no cells	0.77 ± 0.11	−
smooth muscle cells alone	4.86 ± 0.44	−
+SOD1	0.88 ± 0.22	97
+SOD1 (heated)	5.10 ± 0.44	−6
+catalase	4.53 ± 0.55	8
+mannitol	4.75 ± 0.44	3

[a] Confluent bovine aortic SMC were incubated with purified human Cp (5 μM) and BCS (360 μM) for 1 h in 0.5 mL of RPMI 1640 medium in the presence of SOD1 (100 units/mL), heat-inactivated SOD1 (equivalent to 100 units/mL), catalase (1000 units/mL), and mannitol (20 mM). SOD1 was inactivated by incubation at 90−100 °C for 10 min, and loss of activity was verified by the lack of inhibition of a superoxide-generating system containing xanthine and xanthine oxidase. Cp Cu reduction was measured as the change in absorbance of BCS at 480 nm.

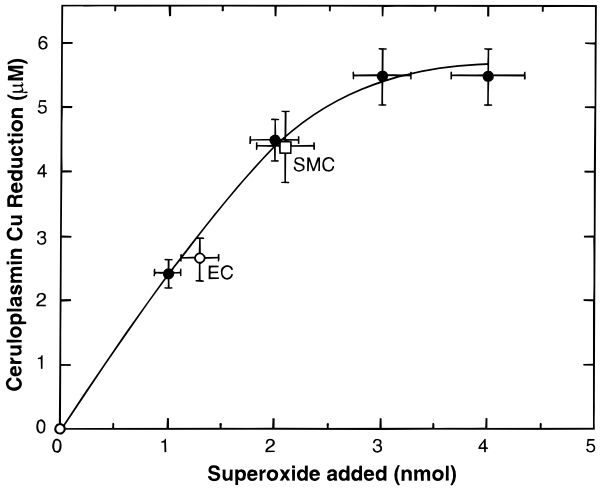

FIGURE 2: Reduction of Cp Cu by superoxide and by vascular cells. A superoxide-generating system consisting of xanthine (1 mM) and xanthine oxidase (from 0.15 to 0.60 milliunit) was incubated with Cp (5 μM) and BCS (360 μM) for 1 h at 37 °C in 0.5 mL of RPMI 1640 medium. The amount of superoxide produced was measured as SOD1-inhibitable reduction of cytochrome *c*. Cp Cu reduction (●) by superoxide was measured as the change in absorbance at 480 nm. To parallel wells containing bovine aortic SMC (□) and EC (○) were added Cp and BCS for 1 h, and superoxide production and Cp Cu reduction were measured as above.

of Cp Cu by SOD1, and the correlation between cellular superoxide production and Cp Cu reduction (Figure 1), suggests an important role of superoxide in Cp Cu reduction.

To determine whether the amount of superoxide produced by EC and SMC cells was quantitatively sufficient to reduce Cp Cu to the extent observed, we compared Cp Cu reduction by cells to reduction by exogenous superoxide generated under cell-free conditions. Superoxide generated by xanthine and xanthine oxidase reduced Cp Cu in a dose-dependent manner with half-maximal reduction at about 1.5 nmol/h of superoxide, and with maximal reduction occurring at about 3 nmol/h (Figure 2). The rate of superoxide production by cultured EC and SMC was measured, and the ability of the cells to reduce Cp Cu was compared to that of exogenous superoxide. As shown in Figure 2, the cell culture data points lie on the curve formed by exogenous superoxide, showing that the amount of superoxide produced by these cells completely accounts for their reduction of Cp Cu at the rate observed. This finding confirms that superoxide is the principal cellular reductant of Cp Cu.

Role of Ceruloplasmin Copper Reduction in LDL Oxidation. If Cp Cu reduction is required for LDL oxidation, then a correlation between these processes should be observed. This possibility was first examined under cell-free conditions. LDL was incubated with a constant amount of Cp, and with an exogenous superoxide generating system at amounts designed to produce amounts of superoxide spanning a wide range. LDL oxidation almost precisely correlated with Cp Cu reduction (Figure 3A). Interestingly, LDL oxidation increased linearly in the part of the curve characteristic of superoxide production by EC and SMC (about 1−3 nmol/h), suggesting the possibility of physiologic regulation of LDL oxidation by superoxide. To confirm this finding, LDL was incubated with a constant amount of exogenous superoxide and with a range of Cp concentrations. The reduction of Cp Cu again paralleled LDL oxidation (Figure 3B). The

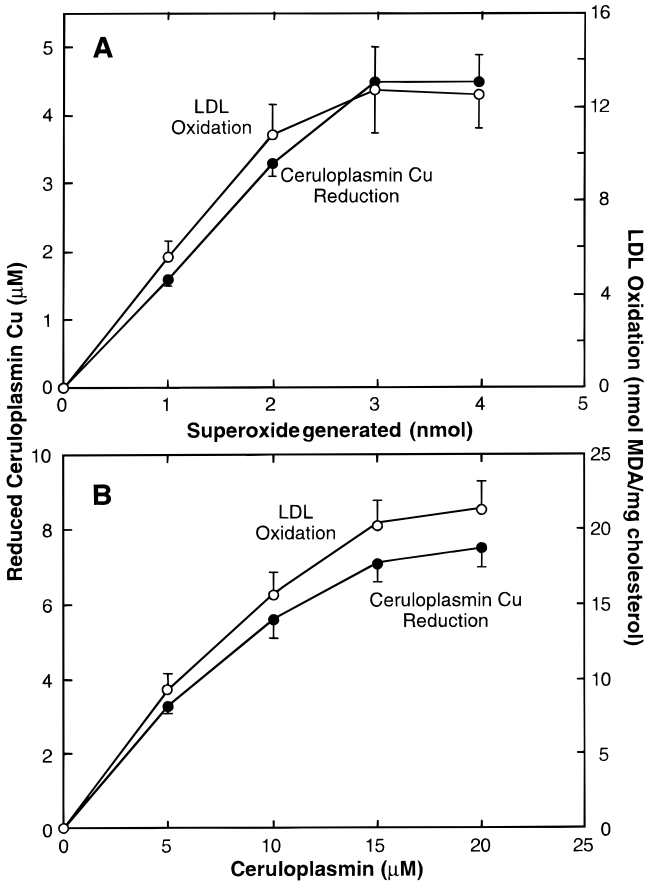

FIGURE 3: Correlation between Cp Cu reduction and LDL oxidation. (A) Cp Cu reduction and LDL oxidation were compared as a function of amount of superoxide at a constant, limiting amount of Cp. Cp (5 μM) was incubated with LDL (100 μg/mL) and BCS (360 μM) in the presence of xanthine (1 mM) and xanthine oxidase (from 0.15 to 0.60 milliunit) for 1 h at 37 °C in 0.5 mL of RPMI 1640 medium; superoxide generation was measured as SOD1-inhibitable reduction of cytochrome *c*. After 1 h, Cp Cu reduction was measured as the change in absorbance at 480 nm (●). In parallel wells, Cp was incubated with LDL and xanthine and xanthine oxidase for 24 h as above, but in the absence of BCS. LDL oxidation was measured as TBARS and expressed as nanomoles of MDA-equivalents per milligram of LDL cholesterol (○). (B) Cp Cu reduction and LDL oxidation were compared as a function of Cp concentration at a constant, limiting amount of superoxide (about 2 nmol). The procedure was otherwise the same as in (A).

increase in LDL oxidation is linear at Cp concentrations spanning the physiological range, about 2 μM in plasma of healthy adults and about 6 μM during extreme acute-phase reactions. These results provide correlative evidence that Cp Cu reduction is important for LDL oxidation. The results also suggest that both processes may be physiologically regulated by changes in Cp concentration or superoxide production.

To test the requirement for Cp Cu reduction in cell-mediated LDL oxidation, BCS was used to bind Cu^{1+} in redox-inactive complexes (*37*). BCS almost completely inhibited Cp-stimulated LDL oxidation by both SMC and EC (Figure 4). As a control, we showed that the inhibitory activity of BCS did not occur by a nonspecific mechanism not involving Cp Cu, since oxidation of LDL by iron citrate in the presence of xanthine and xanthine oxidase was not inhibited by BCS (not shown). To further show the importance of Cp Cu reduction in SMC-mediated LDL oxidation, the relative susceptibility of both processes to

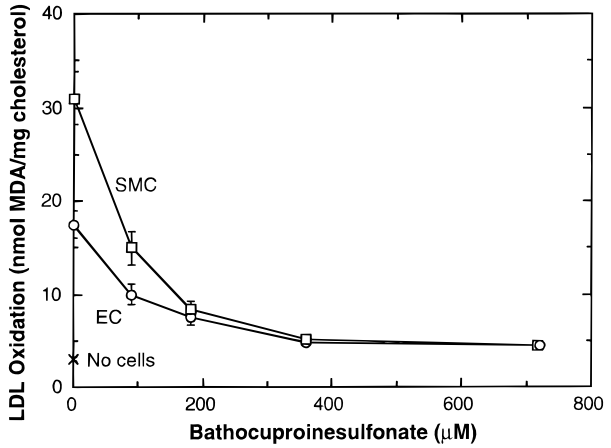

FIGURE 4: Inhibition of cell-mediated LDL oxidation by BCS. LDL (1 mg of cholesterol/mL) was incubated with confluent cultures of bovine aortic SMC (□) and EC (○) in the presence of Cp (1 μM) and BCS. In parallel control wells, LDL was incubated in the absence of cells (×). After 24 h, LDL oxidation was measured as TBARS and expressed as nanomoles of MDA-equivalents per milligram of LDL cholesterol.

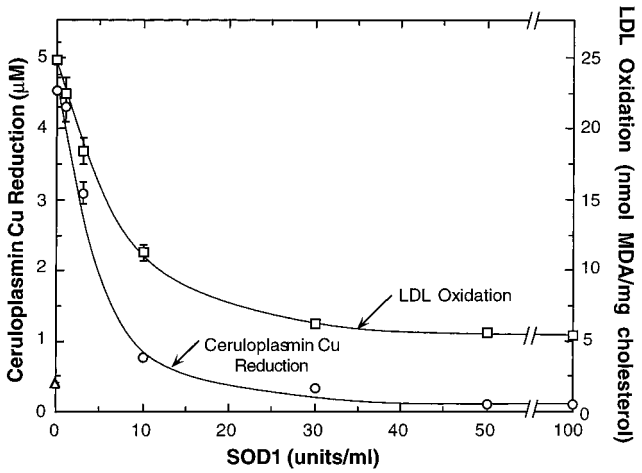

FIGURE 5: Parallel inhibition of Cp Cu reduction and LDL oxidation by SOD1. Confluent bovine aortic SMC cultures were incubated with purified human Cp (5 μM) and BCS (360 μM) for 1 h in 0.5 mL of RPMI 1640 medium in the presence of SOD1 as indicated. Cp Cu reduction was measured as the change in absorbance at 480 nm (○). In parallel wells, LDL (1 mg of cholesterol/mL) was incubated for 24 h with SMC in the presence of Cp (1 μM) and SOD1. LDL oxidation was measured as TBARS and expressed as nanomoles of MDA-equivalents per milligram of LDL cholesterol (□). The oxidation level of untreated LDL is shown (△).

inhibition by SOD1 was compared. Half-maximal inhibition of both processes was observed at about 5 units/mL SOD1, suggesting that the superoxide requirement is similar and that the processes may thus be causally related (Figure 5).

The role of Cu reduction during cytokine-stimulated LDL oxidation was examined. TNF-α, a monocyte/macrophage-derived cytokine, induces superoxide production in mouse and rat pulmonary artery EC (*51*, *52*), and also stimulates LDL oxidation by mouse EC (*52*). Treatment of bovine aortic EC with TNF-α increased the rate of superoxide production and LDL oxidation by 73% and 60%, respectively (Figure 6). TNF-α increased EC reduction of Cp Cu by 83%, showing that physiological agents regulate this process and providing further evidence for the relationship between cellular superoxide generation, reduction of Cp Cu, and LDL oxidation.

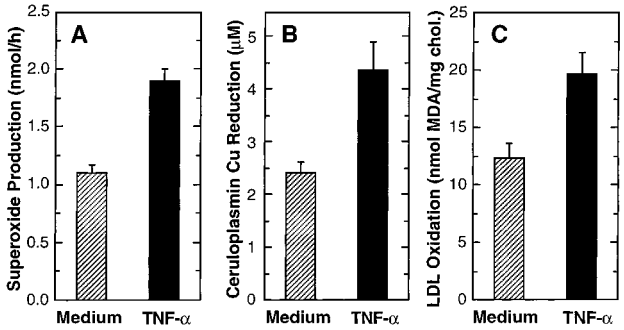

FIGURE 6: Regulation of EC superoxide production, Cp Cu reduction, and LDL oxidation by TNF-α. (A) Bovine aortic EC were pretreated in the absence (striped bars) or presence of 25 ng/mL TNF-α (black bars) for 2 h, washed with PBS, and incubated with cytochrome *c* (3×10^{-5} M) for 30 min in RPMI 1640 medium (minus phenol red dye). Superoxide production was measured as the difference in absorbance of cytochrome *c* at 550 nm between dishes incubated with or without SOD1 (100 units/mL). (B) Cultured EC were incubated with TNF-α as in (A), but in the presence of Cp (5 μM). Cp Cu reduction was measured as the change in absorbance of BCS at 480 nm after 1 h. (C) Cultured EC were incubated for 24 h with TNF-α as in (A), but in the presence of Cp (μM) and LDL (1 mg of cholesterol/mL). LDL oxidation was measured as TBARS.

DISCUSSION

Our results show that a single specific Cu atom at the surface of Cp is reducible by superoxide. The results are based on two independent methods: (i) by spectrophotometric measurement using BCS; and (ii) by superoxide utilization. In view of a recent report on artifactual increases in oxidation strength by BCS, we have verified that BCS does not alter the oxidative activity of Cp-Cu^{2+} (and thus Cp-Cu^{2+} reduction) by showing that a BCS−Cp-Cu^{2+} complex does not form and that susceptible amine groups in HEPES are not oxidized. These results are consistent with studies by others showing that Cu^{2+}-coordinating ligands, especially His-containing peptides, compete with BCS for Cu^{2+} binding (*46*). Our recent finding that the pro-oxidant Cu binds to Cp in the region above His^{426} (*28*) may explain the inactivity of BCS in our experiments. The superoxide utilization experiments are consistent with previous reports of substantial superoxide scavenging activity of Cp (*53*, *54*).

We have previously shown that Cp enhances SMC- and EC-mediated LDL oxidation, and that the specific contribution of the cells is the production of superoxide (*20*). Our new results describe the underlying mechanism of this process, namely, that the single, specific prooxidant Cu atom of Cp is reduced by cell-derived superoxide, and that Cp-stimulated LDL oxidation depends on Cu^{2+} reduction. In a previous report, Garner et al. (*55*) have shown that mouse peritoneal macrophages under certain conditions reduce free Cu^{2+}; in this study, the reductant has not been identified, but the authors indicate that it is not superoxide. Our new results clarify several aspects of cellular oxidation processes; they show that vascular EC and SMC, like macrophages, reduce Cu^{2+}, that protein-bound copper is a target of cellular reduction, and that superoxide is the critical cell-derived reductant.

The importance of metal ion reduction in LDL oxidation under cell-free conditions has been shown previously (*34*−*37*). In most of these studies, the reduction of free Cu^{2+}

salt was examined. However, Yoshida et al. (*35*) investigated the reduction of protein-bound Cu. In contrast to our results, they reported that LDL reduced free Cu^{2+} but not Cu^{2+} bound to Cp (or to albumin) and ruled out Cp as the source of redox-active Cu ion for LDL oxidation. This apparent discrepancy may be due to the physical state of Cp. We have previously shown that when the intact, 132 kDa Cp was partially degraded by limited proteolysis, all prooxidant activity was lost (*21*). Most commercial preparations of Cp that we have tested, including several from Sigma, the source of the protein used by Yoshida et al. (*35*), were degraded and lacked prooxidant activity. When we used Cp from this source, we also failed to detect Cu reduction either by vascular cells or by the xanthine/xanthine oxidase superoxide-generating system (data not shown). Differences in Cp redox activity may also reflect the number of Cu atoms bound. The use of EDTA, or other chelating agents, during blood collection or protein purification would likely remove the reducible Cu.

The accessibility of the reducible Cp Cu to Chelex-100 (and to BCS) suggests that the Cu is located at or near the protein surface. Recent studies have shown that the prooxidant Cu site in Cp is near the bottom of a narrow valley at the protein surface above His^{426} (*28*). The ability of proteins with Cu binding sites at the surface to cause oxidative damage was first shown by Samuni et al. (*56*). Using penicillinase as a model, they showed oxidant damage consistent with induction by the superoxide-driven Fenton reaction. Our results are consistent with the concept of copper−protein complexes causing oxidative injury. However, our data do not show a role for the Fenton reaction since an inhibitor (catalase) of one of the substrates, H_2O_2, and a scavenger (mannitol) of the reactive product, OH•, did not inhibit Cu reduction (Table 3) or LDL oxidation (*20*). The actual radical that initiates Cp-stimulated LDL oxidation is also not known. Evidence for (*57*) and against (*58*) prooxidant activity of an $Fe^{2+}-O_2-Fe^{3+}$ complex has been described; the activity of the analogous $Cu^{1+}-O_2-Cu^{2+}$ complex is unlikely for Cp given the lack of release of free Cu from the protein. We have no evidence for the role of any Cu radicals analogous to other reactive iron complexes, e.g., percupryl radical ($Cu^{2+}O_2^-$) or cupryl ion (CuO_2^{1+}).

Other important mechanistic questions also remain unanswered. Our results show that reduction by superoxide does not release Cu from Cp for subsequent interaction with LDL. The possibility remains that Cp itself may bind to the lipoprotein substrate in a way that permits interaction of the reduced Cu with LDL. This interaction has not been reported, but in view of the known interaction of Cp with high-density lipoprotein (*22*, *59*), it is possible that interaction with other lipoproteins may also occur. The cellular source of superoxide has not been addressed in these studies. Possible sources include xanthine oxidase, NAD(P)H oxidase, or the sulfur-containing amino acids in the media, e.g., cystine (*32*), Finally, while the ability of superoxide to reduce Cp Cu has been clearly shown, the ability of other metal reductants including α-tocopherol (*35*, *36*) has not yet been examined.

Oxidation of LDL within the wall of susceptible arteries may play an important role in the onset and progression of atherosclerosis (*2*). For the mechanism of LDL oxidation described in this report to be relevant in vivo, colocalization of Cp and superoxide, most likely within the vessel wall itself, is required. Cultured macrophages, EC, and SMC all release superoxide, as do SMC (*60*) and EC (*61*) in aortic strips. Interestingly, conditions known to accelerate aortic lesion formation including hypercholesterolemia (*61*) and balloon injury (*62*) increase superoxide production. Detection of Cp in human atherosclerotic lesions has been reported (*63*, *64*). The source(s) of vessel wall Cp is (are) not known, but synthesis and secretion by vascular cells is a potential source. To our knowledge there are no reports of Cp production by vascular EC or SMC; however, two cytokines, TNF-α (*65*) and interferon-γ (*66*), stimulate Cp gene expression and protein synthesis in pulmonary macrophages and peripheral blood monocytes, respectively. Thus, given the current state of knowledge about superoxide production and the presence of Cp in the vessel wall, one would predict that these agents may be involved in LDL oxidation processes during atherogenesis. In vivo studies, possibly using transgenic animals, will be necessary to establish the physiological role of Cp/superoxide interactions in LDL oxidation and vessel wall diseases.

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