Direct Copper Reduction by Macrophages
ITS ROLE IN LOW DENSITY LIPOPROTEIN OXIDATION*

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Oxidation of low density lipoprotein (LDL) results in changes to the lipoprotein that are potentially atherogenic. Numerous studies have shown that macrophages cultured in vitro can promote LDL oxidation via a transition metal-dependent process, yet the exact mechanisms that are responsible for macrophage-mediated LDL oxidation are not understood. One contributing mechanism may be the ability of macrophages to reduce transition metals. Reduced metals (such as Fe(II) or Cu(I)) rapidly react with lipid hydroperoxides, leading to the formation of reactive lipid radicals and conversion of the reduced metal to its oxidized form. We demonstrate here the ability of macrophages to reduce extracellular iron and copper and identify a contributing mechanism. Evidence is provided that a proportion of cell-mediated metal reduction is due to direct transplasma membrane electron transport. Glucagon suppressed both macrophage-mediated metal reduction and LDL oxidation. Although metal reduction was augmented when cells were provided with a substrate for thiol production, thiol export was not a strict requirement for cell-mediated metal reduction. Similarly, while the metal-dependent acceleration of LDL oxidation by macrophages was augmented by thiol production, macrophages could still promote LDL oxidation when thiol export was minimized (by substrate limitation). This study identifies a novel mechanism that may contribute to macrophage-mediated LDL oxidation and may also reveal potential new strategies for the inhibition of this process.

The oxidative modification of LDL results in numerous changes to the lipoprotein that are potentially atherogenic (1, 2). In vitro copper-oxidized LDL can promote the accumulation of cholesterol in macrophages (3, 4) and stimulate monocyte recruitment (5) and adhesion (6) to endothelial cells and be cytotoxic (7). Most of the cell types present in the intima of arteries (including macrophages) can stimulate the oxidation of LDL in vitro (8–12), and there is evidence for the presence of oxidized LDL in atherosclerotic plaque (13, 14). The presence of transition metals (either deliberately added or adventitious) in the culture medium appears to be an absolute requirement for cell-mediated oxidation of LDL in vitro, indicating that the activity of cells is to accelerate ongoing metal-dependent oxidation (12, 15). There is also evidence for the presence of transition metals in plaque (16, 17), and it is known that physiologically relevant forms of both iron (e.g. heme and ferritin) and copper (e.g. ceruloplasmin) can promote LDL oxidation in vitro, particularly under conditions related to inflammation (17–19). These studies indicate (but do not prove) that metal-catalyzed LDL oxidation could be one contributing factor in the generation of oxidized LDL during atherosclerosis. It is therefore important to define the mechanisms that underlie the metal-dependent acceleration of LDL oxidation by macrophages, quantitatively one of the most important cell types present in the developing atherosclerotic lesion (20), to more completely understand the etiology of this disease.

Several cellular mechanisms have been proposed to contribute to the oxidative modification of LDL (12, 21). One potential mechanism is the cell-mediated reduction of transition metals, which might facilitate lipid hydroperoxide (L-OOH) decomposition and chain peroxidation (22) (Equations 1 and 2).

\[
M^{n+} + e^{-} (\text{cell-derived}) \rightarrow M^{(n-1)+} \quad \text{(Eq. 1)}
\]

\[
L-\text{OOH} + M^{(n-1)+} \rightarrow L-\text{O} + \text{OH}^- + M^{n+} \quad \text{(Eq. 2)}
\]

Such lipid hydroperoxides are present in atherosclerotic plaque (23). However, mechanisms of macrophage-mediated transition metal reduction have not been studied. One possible cell-derived extracellular reductant is O$_2^-$, but O$_2^-$ is clearly not a rate-limiting species for the accelerated oxidation of LDL by murine macrophages (24) or human monocyte-derived macrophages (MDM) (25). Several studies have highlighted the importance of cellular thiol production in the acceleration of LDL oxidation (26–29), and it has been suggested that thiol-derived and oxygen-derived free radicals are responsible for these effects (30). An alternative hypothesis could be that cell-derived thiols maintain transition metals in a reduced form, i.e., a state that confers high reactivity with lipid hydroperoxides. Such reduction of copper and iron by thiols (31) and other molecules (32) is well known to promote lipid peroxidation (up to a critical reductant concentration beyond which inhibition of peroxidation often occurs) (27, 31).

Since previous studies demonstrated extracellular transition metal reduction by a direct trans-plasma membrane electron transport (TPMET) system in a variety of mammalian cells (33), we here sought evidence for such a system in macrophages. This system, which has been previously characterized by its ability to reduce extracellular ferricyanide, utilizes internal NADH as an electron donor (34), and transport of electrons out of the cell is accompanied by proton movement (35).
absorbance at 482 nm after determined in samples (after the initial reading of cell-mediated copper wells was subtracted from each of the corresponding values measured copper concentration. The absorbance from parallel cell-free control BCS. The molar ratio of 1:2.5 for copper/BCS was maintained at each (mPM) were harvested from QSmice (36) and adhered to 22-mm diameter tissue culture wells (Falcon, Lincoln Park, NJ) and cultured in RPMI 1640 medium plus 10% (v/v) heat-inactivated penicillin, and 100 that the elutriation medium contained 0.2% fetal calf serum instead of that Murine resident peritoneal macrophages (mPM) were harvested from Q5 mice (36) and adhered to 22-mm diameter (or 55-mm diameter where indicated) wells at 4 x 106 cells/well. Murine macrophage-like J774A.1 cells (American Type Culture Collection 67-TIB, batch F-10089) were seeded at a concentration of 0.5 x 107-22-mm diameter well and cultured for 48 h in medium A before use. All cells were cultured in 5% CO2 in air at 37 °C and were near confluence at the time of use in experiments. Cell protein was measured after washing cells twice with PBS at 37 °C, digestion in 0.2 M NaOH, and assay using the bicinchoninic acid method (Sigma) with bovine serum albumin as a standard. All cell preparations yielded 150–200 μg of protein/well when used in experiments. Cell viability was assessed using trypan blue exclusion.

Cell-mediated Iron and Copper Reduction—Monocyte-derived macrophages, or mPM, were washed three times with PBS at 37 °C and subsequently incubated at 37 °C in 1 ml of Hanks’ balanced salt solution (HBSS) routinely containing 50 μM CuCl2 and 125 μM BCS (unless indicated otherwise). At various times, the supernatants were removed and centrifuged for 60 s at 16,000 x g in an Eppendorf 5415C centrifuge, and the absorbance of a 700-ul aliquot of the supernatant was measured at 482 nm using a Hitachi U-1100 spectrophotometer. The concentration of copper reduced by the cells was calculated using an experimentally determined extinction coefficient of ε482 nm = 12,154 M -1 cm -1 for the Cu(I)-BCS complex (in agreement with a previous report (37)) after correction for the amount of absorbance in cell-free control wells (19 ± 8%; mean ± S.D., n = 21 experiments with incubations ranging from 0.5 to 5 h). The extinction coefficient and λmax (482 nm) were determined by addition of reductants (ascorbate, cysteine, and glutathione) to Cu(II)-BCS in HBSS. These parameters were identical for all reductants, and the dose responses were all linear over the range used.

The K0.5 for copper reduction by macrophages was determined by measuring the initial rate of copper reduction by cells using a range of Cu(II) concentrations, routinely from 1 to 100 μM, in the presence of BCS. The molar ratio of 12.5 for copper/BCS was maintained at each copper concentration. The absorbance from parallel cell-free control wells was subtracted from each of the corresponding values measured in the presence of cells. The total recovery of copper from cells was determined in samples (after the initial reading of cell-mediated copper reduction at 482 nm) by adding excess ascorbate and measuring the absorbance at 482 nm after ~5 min at 22 °C. The ability of cells to reduce ferricyanide was assessed after the cells were washed as described above and then exposed to 200 μM K3Fe(CN)6 in HBSS for the indicated times. The supernatants were then collected and centrifuged as described above, and the absorbance at 420 nm was measured. The amount of iron reduced (i.e. ferricyanide produced) was calculated using the absorbance at 420 nm and an extinction coefficient of ε420 nm = 1000 M -1 cm -1 for ferricyanide (33). Standard curves were produced using ascorbate and cysteine as reductants.

Thiol Assay and Reducing Activity of Cell Supernatants—Cells were washed three times with PBS at 37 °C and subsequently incubated at 37 °C in 1 ml of HBSS. At the indicated times, the supernatants were removed and centrifuged for 60 s at 16,000 x g in an Eppendorf 5415C centrifuge, and the supernatants were analyzed immediately for thiol content (38). A 200-ul aliquot of the supernatant was combined with 750 μl of 200 mM NaHPO4·12H2O (BDH) containing 20 mM EDTA, pH 8.0. Standards of t-cysteine (in HBSS with 20 mM EDTA) were prepared for each assay. Fifty μl of 4 mM DTNB in 50 mM sodium phosphate buffer, pH 7.0, was added to each sample or standard and incubated at 37 °C for 30 min, after which the absorbance at 412 nm was measured using a Hitachi U-1100 spectrophotometer. A sample with no DTNB added was used as a blank. The sensitivity limit of this assay was 0.2 nmol of thiol (equivalent to a concentration of 1 μM in the cell supernatants). Aliquots (600 μl) of cell-conditioned HBSS were also taken to assess their copper reducing capacity. The sample was made up to 50 μM CuCl2 and 125 μM BCS in a final volume of 700 μl, and after 5 min at 22 °C, the absorbance at 482 nm was measured.

HPLC Analysis of Ascorbate—Cells were rinsed with PBS as described above and then directly lysed and scraped into 250 μl of ice-cold methanol/H2O (60:40) containing 1 mM EDTA. This extract was briefly centrifuged (16,000 x g), flushed with argon, and placed on dry ice before HPLC analysis for ascorbate with electrochemical detection as described previously (39, 40). Cell-conditioned HBSS (4 h, 600 μl) was also assayed by this method.

LDL Isolation and Cellular Modification—LDL (ρ 1.020–1.050 g/ml; which excludes Lp(a) contamination) was isolated from fasted normal lipemic human plasma by sequential density gradient ultracentrifugation and dialyzed against Chelex-treated PBS as described previously (41). LDL samples were filter-sterilized (0.22 μM) and 100 μl of LDL containing 10 μM CuCl2 and 125 μM BCS were incubated for 2 h at 37 °C. The LDL containing LDL was then removed from wells to tubes containing EDTA and butylated hydroxytoluene (6 μl each; final concentrations of 2 mM and 20 μg/ml, respectively).

HPLC Analysis of LDL α-Tocopherol, Neutral Lipid, and Cholesteryl Ester Hydroperoxides—Three-hundred μl of the supernatants containing LDL was extracted into 2 ml of methanol and 10 ml of hexane as described previously (41). LDL (200 μl) was filter-sterilized (0.22 μM) and the hexane phase were evaporated under vacuum and redissolved in isopropl alcohol; in each case, 50 μl was injected onto an LC-18 column (25 x 0.46 cm, with 5-cm guard column; Supelco Inc., Bellefonte, PA) for HPLC analysis. Cholesteryl esters, α-tocopherol, and cholesteryl ester hydroperoxides were detected using UV, electrochemical, and chemiluminescence detection, respectively, as described previously (41). Cholesteryl ester hydroperoxides were quantified using CE18:2 hydroperoxide standards prepared as described previously (42).

Statistical Analysis—Statistical significance was determined using the two-tailed Student’s t test. A p value < 0.05 was considered significant.

RESULTS

Macrophase-mediated LDL Oxidation and Antioxidation and Influence of Transition Metals and Cystine—To examine the role of transition metals in cell-mediated LDL oxidation, LDL oxidation was assessed in a simple buffered salt solution (HBSS) by measurement of the consumption of polyunsaturated cholesteryl esters α-tocopherol and the generation of cholesteryl ester hydroperoxides (Fig. 1). Before incubation, LDL contained only 1 molecule of cholesteryl ester hydroperoxide/43 LDL particles (determined by chemiluminescence) (25). At more advanced stages of oxidation, cholesteryl ester hydroperoxide levels actually decline (8, 25), and the consump-
tion of oxidizable substrates becomes a more accurate measure of the degree of oxidation (25, 43). For these reasons, the cholesteryl ester hydroperoxide detected at late stages of incubation is not stoichiometric with cholesteryl ester loss; hence, both parameters are presented here.

Human MDM did not oxidize LDL in unsupplemented HBSS, although some oxidation did occur in this medium in control cell-free incubations (Fig. 1). The cell-free oxidation was completely suppressed if HBSS was prewashed with Chelex 100 (data not shown), indicating that contaminating trace amounts of redox-active metals were present and responsible for this lipid peroxidation. Inductively coupled plasma atomic emission spectroscopy indicated routine contamination of HBSS with iron (0–1 μM) and copper (0–0.2 μM). Under these conditions, MDM were clearly anti- rather than pro-oxidant, as evidenced by the lesser oxidation measured when cells were present (Fig. 1, no additions, MDM versus cell-free). Under these relatively mild oxidizing conditions, cells may inhibit LDL oxidation by sequestration of the contaminating metals or by selective removal of cholesteryl ester hydroperoxides from LDL (44). This was not investigated further. In contrast, when HBSS was supplemented with 3 μM iron and 0.01 μM copper (equal to their concentrations in Ham’s F-10 medium, which is permissive for cell-mediated LDL oxidation (8)), MDM produced a greater degree of LDL oxidation than in equivalent cell-free incubations. Similar murine macrophage-mediated oxidation of LDL in iron- and copper-supplemented HBSS has recently been reported (45).

Previous studies have suggested that cell-mediated LDL oxidation is dependent on the presence of extracellular cysteine, which can be generated from cystine by a cell-dependent process (26, 28, 30). While the above data demonstrate that this is not always the case, the influence of cystine on LDL oxidation in HBSS was also studied. The concentration used (52 μM) is that present in Ham’s F-10 medium and comparable to human plasma levels (~42 μM) (46). Cystine in the absence of added metals did not stimulate LDL oxidation in either the presence or absence of cells (Fig. 1). In the presence of iron and copper, cell-mediated LDL oxidation was enhanced by cystine; in the absence of cells, it was sometimes, but not always, slightly enhanced (e.g. Fig. 1). In summary, human MDM-mediated LDL oxidation is absolutely dependent on the presence of transition metals and will proceed in the absence of a source for thiol export, although addition of cystine does accelerate it.

**Macrophage-mediated Metal Reduction and LDL Oxidation**—Cells might accelerate LDL oxidation in metal-supplemented buffer without exogenous thiols by their direct reduction of transition metals. Macrophages (human MDM, J774A.1 cells, and murine peritoneal macrophages) were therefore assessed for their ability to reduce iron and copper in HBSS. Higher concentrations of iron and copper were used for this assay than are normally present in media permissive for LDL oxidation, both because of the limits of sensitivity of the methods and to measure the maximum activity of the cells under conditions of substrate excess.

Iron reduction was measured using K₃Fe(CN)₆, which is an impermeant acceptor of electrons donated by cell (and isolated) plasma membranes (33, 35). Macrophages reduced iron in a nonlinear but time-dependent manner for at least 30 min (Fig. 2). During longer incubations, the apparent reduction of iron reached a plateau or slightly decreased (data not shown). This has previously been observed and ascribed to a ferrocyanide oxidase activity also associated with cellular plasma membranes catalyzing reoxidation of ferrocyanide back to the ferric form rather than to cellular ferrocyanide uptake (47). Our kinetic data are consistent with previous kinetic studies with
other cell types (33).

We have previously described mPM-mediated copper reduction over periods of up to 2 h and in the absence of added thiols (45), using neocuproine to trap reduced copper as a stable chelate. Because neocuproine was toxic over longer incubations, copper reduction by human MDM was measured by trapping the Cu(I) generated as a stable chelate with BCS, which was not toxic for at least 5 h (95% viable after 5 h). Macrophage-mediated copper reduction followed biphasic kinetics, with an initial fast rate (0–15 min) followed by a slower but sustained rate of reduction for up to at least 5 h (Fig. 3). Murine peritoneal macrophages, J774A.1 cells, and human MDM (150–200 μg of protein/well) all routinely reduced ~5 nmol of copper in the first 2 h of incubation. The rate of copper reduction (per 1 × 10⁶ cells) by J774A.1 cells was 5.4 ± 1.4 and 2.1 ± 1.0 nmol/h (mean ± S.D., n = 13) for the fast and slow phases, respectively, and 7.9 ± 3.3 and 2.1 ± 1.0 nmol/h (mean ± S.D., n = 7), respectively, for MDM. Copper reduction was dependent on macrophage cell number and was also detected with human umbilical vein endothelial cells and human skin fibroblasts (data not shown). Copper reduction (per μg of cell protein) was quite similar for all these cell types. When MDM were incubated with LDL (100 μg/ml in HBSS containing iron (3 μM) and copper (0.01 μM) for 0, 4, 10, or 24 h and then washed and incubated for a further 2 h in copper (50 μM)/BCS (125 μM), their ability to reduce copper was unchanged (data not shown). Thus, the capacity of cells to reduce copper is not affected by their previous participation in LDL oxidation.

Total recovery of copper from the system after exposure to cells was assessed by addition of excess ascorbate at the end of the incubation to convert all metal to Cu(I)-BCS. 93 ± 4% (mean ± S.D., n = 8) was recovered, indicating no significant changes in copper availability during the incubations.

The direct reduction of copper by LDL has been reported in several studies (for example, Ref. 48), which may be due to components of LDL such as α-tocopherol. In this study, a small amount of cell-free copper reduction was measured even in a simple buffer system (Fig. 3, cell-free). Addition of LDL to cell-free HBSS caused a small increase in Cu(I) formation (1.1 ± 0.1 nmol/2 h in the absence versus 1.7 ± 0.4 nmol/2 h in the presence of 100 μg/ml LDL). A similar small difference in copper reduction by mPM in the absence or presence of LDL (6.2 ± 0.5 versus 7.2 ± 0.2 nmol/4 × 10⁶ cells/2 h, respectively) was observed. Thus, (a) under the conditions used for cell-mediated LDL oxidation, LDL-dependent copper reduction is minor compared with cell-mediated copper reduction; and (b) LDL does not stimulate cell-mediated copper reduction.

Release of Soluble Reductants by Macrophages—Cell-mediated metal reduction could be due to direct electron transfer at the plasma membrane or to the release of soluble reductants from the cells. Therefore, we measured the release of several potential cell-derived reductants during incubation of macrophages in HBSS. There was no detectable (<1 nmol/ml) release of thiol-containing compounds from any of the cells studied during the first 2 h of incubation. Small quantities of thiol were detected in supernatants of mPM or J774A.1 cells after ~4 h, while release of thiol from MDM under these conditions was not routinely detected (Table I). The presence of LDL (50 μg/ml) did not affect thiol production by mPM cultured in HBSS (2.2 ± 0.3 versus 2.9 ± 0.6 nmol/4 × 10⁶ cells/h when LDL was present (mean ± S.E., n = 6 and n = 5, respectively)).

The majority of thiol released from mPM was of low molecular weight (Table I). It was calculated (assuming 1:1 stoichiometry) that not more than 20–30% of the copper reduction by mPM or J774A.1 cells between 2 and 4 h could be accounted for by extracellular thiol, and an even smaller proportion in the first 2 h. Assuming that MDM release thiols at a rate that yields an extracellular concentration after 5 h at the limits of sensitivity for the assay, then <10% of the copper reduction by MDM could be due to thiol release. This might be an underestimate if any released thiols were unstable in the culture conditions. When 50 μM cysteine was incubated in HBSS in the absence of cells, it was lost with a half-life of ~18 h, but the presence of cells stabilized it significantly (data not shown). Even using the shorter half-life to correct the estimate of cell-released thiols, it can be deduced that <13% of the copper reduction by MDM over the 5-h period could be due to thiol release.

Human MDM were pretreated for 15 min at 37 °C with the cell-impermeant sulphydryl-blocking agent p-chloromercuri-phenylsulfonate (10–500 μM) to assess the possible role of cell-surface thiols as copper reductants. The cells were washed and subsequently exposed to copper/BCS to measure cell-mediated copper reduction. Preincubation of cells with 50–500 μM p-chloromercuri-phenylsulfonate did not inhibit the initial
TABLE I

Macrophage-mediated thiol export in HBSS

| Time (h) | Low M<sub>c</sub> total | J774A.1, total | MDM, total |
|---------|------------------------|---------------|------------|
| 0.25    | 0.5 µM                 | <1.0          |            |
| 1.5     | 2.2 ± 0.6              | 2.9 ± 1.2     | <1.0       |
| 4       | 4.2 ± 0.9              | 3.0 ± 0.4     | 3.7 ± 1.7  |
| 8       | 6.1 ± 0.7              | 7.8 ± 1.7     |            |
| 11      | 7.0 ± 3.8              | 4.2 ± 0.6     | 6.9 ± 0.2  |
| 16      | 6.9 ± 0.2              | 4.6 ± 1.5     | <1.0       |
| 18      | 5.1 ± 2.1              |               |            |

*<i>4</i> × 10<sup>6</sup> cells, i.e. 150–200 µg of cell protein/well; data compiled from six experiments.

<sup>a</sup> Cell supernatants were mixed with an equal volume of 5% (v/v) metaphosphoric acid and centrifuged at 16,000 × g for 2 min to remove precipitated proteins. The supernatant was then assessed for thiol as described under "Experimental Procedures." Values are the means ± S.D. of independent experiments. Each experiment was performed in triplicate.

Fig. 4. Ability of cell-conditioned HBSS to reduce copper. MDM (●) and cell-free wells (○) were incubated with 50 µM copper and 125 µM BCS in HBSS; MDM were also incubated in HBSS alone (■). At the times indicated, media were removed and briefly centrifuged, and the supernatants were made up to 50 µM copper and 125 µM BCS. In all cases, the absorbance at 482 nm was measured to determine the amount of reduced copper as described under "Experimental Procedures." Values are means ± range of duplicates and are representative of two independent experiments.

The ability of "cell-conditioned" HBSS to reduce copper was also assessed by removing the HBSS from cells after various times of exposure and adding it immediately to copper/BCS. Fig. 4 shows that while substantial amounts of copper were reduced in the presence of cells over 60 min, the cell-conditioned HBSS removed at 30 or 60 min did not support significant copper reduction. That removed at the earliest time (10 min) did support a small amount of copper reduction (observed in two independent experiments) and could account for up to 20–40% of the initial fast phase of copper reduction. However, this was still only a small proportion (0.4 nmol of copper, <10%) of the total copper reduced during 2 h of incubation with cells. Taken together, these data indicate that macrophage-mediated transition metal reduction is predominantly dependent on the continued presence of cells or on the release of very unstable reductants.

Characterization of Macrophage-mediated Copper Reduction—The K<sub>m</sub> for copper reduction by cells in HBSS was determined and found to be 6.0 ± 1.6 µM (mean ± S.D., n = 4) for J774A.1 cells and 6.9 µM for MDM (Fig. 7). Note that while copper reduction was a saturable process, the time course studies show that copper reduction does not follow Michaelis-Menten kinetics (Fig. 3). The data above showed that release of cellular reductants could only account for ~40% of macro-

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*<sup>b</sup> 1 × 10<sup>6</sup> cells, i.e. ~200 µg of cell protein/well; data compiled from three experiments.

*<sup>c</sup> 1 × 10<sup>6</sup> cells, i.e. ~200 µg of cell protein/well; data compiled from two experiments.

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*<sup>d</sup> B. Garner, D. van Reyk, R. T. Dean, and W. Jessup, unpublished observation.
phage-mediated copper reduction (after subtraction of the cell-free copper reduction observed). Therefore, another process(es) must account for the remaining 60% of macrophage-mediated copper reduction. The contribution of a TPMET system to macrophage-mediated copper reduction was assessed. Removal of iron from the plasma membrane of a fibroblast cell line (CCl39) significantly inhibited TPMET activity (51); human MDM were therefore pretreated for 90 min with 1 mM BPS in PBS (to remove and chelate iron) and then washed, and their ability to reduce copper was assessed. The mass of iron removed was $27 \text{ ng/mg of cell protein}$ ($\varepsilon_{535}\text{nm} = 22,140 \text{ M}^{-1} \text{ cm}^{-1}$ (37)), which is comparable to that recovered from CCl39 cells ($8 \text{ ng of iron/10^6 cells/h}$ (51)). Table II shows that there was a fall (30%) in the extent of copper reduction after this treatment that was maintained over at least 160 min (data not shown), consistent with the involvement of a TPMET system in cellular copper reduction.

Several compounds known to modulate TPMET activity in other cell types were also assessed as modulators of macrophage-mediated copper reduction. The protonophoric uncoupling agent FCCP (TPMET stimulus (52)) also stimulated macrophage-mediated copper reduction (Table II) at concentrations down to 1 $\mu$M for >2 h (data not shown). Pretreatment of cells with glucagon (0.1 IU/ml for 18 h), which inhibits ferricyanide reduction by rat liver cells (53), suppressed macrophage-mediated copper reduction (assessed after removal of glucagon and subsequent exposure of MDM to copper/BCS) by $15\%$ (Table II), and this was sustained for at least 5 h (data not shown).

![Fig. 5. Addition of cystine to HBSS increases cell-mediated copper reduction.](image)

**FIG. 5.** Addition of cystine to HBSS increases cell-mediated copper reduction. Human MDM (A) or J774A.1 cells (B) were incubated for the times indicated in 1 ml of HBSS containing $50 \mu$M CuCl$_2$ and $125 \mu$M BCS in either the absence (○) or presence (£) of 100 $\mu$M cystine. At the times indicated, media were removed and briefly centrifuged, and the supernatants were assessed for Cu(I)-BCS complex formation by measuring the absorbance at 482 nm. Values are means ± range of two experiments (A) or duplicate determinations (B).

When glucagon was continuously present, macrophage-mediated copper reduction was suppressed by 47% (Table II).

Thus, compounds that modulate TPMET activity also modulate macrophage-mediated copper reduction, consistent with a contribution of the TPMET system to this reduction. Repeated 90-min exposures of the same set of macrophages to fresh aliquots of copper/BCS resulted in copper reduction during the second and third exposures that was 85–90% of that observed during the first exposure, indicating that HBSS (which contains 5.55 mM glucose) was adequate to maintain cellular levels of reducing equivalents.

**Parallel Inhibition of Macrophage-mediated LDL Oxidation and Copper Reduction**—Compounds that modulated macrophage-mediated copper reduction were tested for their effects on cell-mediated LDL oxidation. FCCP was excluded because it had cell-independent antioxidant properties for LDL (data not shown). Glucagon suppressed cell-mediated LDL oxidation both in HBSS with metal added (Table III) and in thiol-containing Ham’s F-10 medium (data not shown). Glucagon suppressed cell-mediated LDL oxidation by 47% (Table III) and in thiol-containing Ham’s F-10 medium (data not shown). In other experiments, we demonstrated that glucagon treatment had no effect on thiol export by MDM or recovery of extracellular copper (data not shown). The action of glucagon on macrophage-mediated LDL oxidation can be most readily explained by its capacity to inhibit direct reduction of copper by the cells. The degree of suppression of macrophage-mediated copper reduction achieved in the presence of glucagon (47% out of a possible maximum of $60\%$), together with a similar extent of suppression of LDL oxidation, indicates that TPMET activity is a major component of thiol-independent copper reduction and macrophage-mediated LDL oxidation.
DISCUSSION

This study demonstrated the ability of macrophages to reduce transition metals. Furthermore, we have attempted to delineate the mechanisms that underlie this process and their relationship to macrophage-mediated LDL oxidation.

Cystine-independent Cell-mediated LDL Oxidation—Our studies show that macrophages are capable of promoting LDL oxidation in the absence of an extracellular substrate for thiol production. Careful assessment of data provided in previous reports that claimed a critical role for cell-derived thiols in LDL oxidation also showed that cell-mediated LDL oxidation was significantly greater than in parallel cell-free conditions even in thiol-free media. Thus, the monocytic THP-1 cell line (29) and the RECB4 endothelial cell line (28) promoted LDL oxidation significantly (versus cell-free controls) in the absence of cystine, but in the presence of transition metals (0.01 μM copper and 3.0 μM iron). Santanam and Parthasarathy (54) have also recently argued that the cellular cysteine generation is not important for LDL oxidation.

This study has confirmed the ability of macrophages to promote LDL oxidation in the presence of 0.01 μM copper and 3.0 μM iron. In addition, the cellular mechanisms underlying the apparently thiol-independent acceleration of LDL oxidation have not been previously addressed.

A role for cellular thiol production in cell-mediated LDL oxidation has been suggested to be due to the production of \( \text{O}_2^- \) by the extracellular oxidation of the thiol (26, 30). However, the inability of superoxide dismutase to prevent macrophage-mediated LDL oxidation argues against this as a predominant mechanism (24). Macrophage-mediated transition metal reduction was investigated to understand the thiol-independent ac-

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TABLE II

Modulation of macrophage-mediated copper reduction

Copper reduction was measured in all cases over a 45-min period in the presence of 50 μM CuCl2 and 125 μM BCS and in the absence or presence of the indicated agent. At the end of the incubation, cell supernatants were removed and assessed for Cu(I)-BCS complex formation as described under “Experimental Procedures,” with correction made for the small amount of reduction measured in cell-free controls. Data are for cells exposed to the appropriate agent and are expressed as a percentage of control (nontreated) cells incubated in parallel. The absorbance in cell-free controls has been subtracted and was <14% of the conditions containing cells in all cases.

| Compound   | Cu(I)/10^6 cells | Stimulation (±\(\psi\)) inhibition (–) |
|------------|------------------|---------------------------------------|
|            | Control | Treatment |                          |
| FCCP\(^a\) | 3.8     | 6.1       | +61                      |
| BPS\(^b\)  | 4.1     | 2.9       | -30                      |
| Glucagon   | 4.1     | 3.5       | -15                      |
| Continuous \(^c\) | 4.7 | 2.5       | -47                      |

\(^a\) J774A.1 cells were incubated in 1 ml of HBSS in the absence or presence of 1 μM FCCP. Values are the means of duplicates and are representative of two independent experiments.

\(^b\) Monocyte-derived macrophages were incubated in 1 ml of PBS containing 1 mM BPS or in PBS alone. Values are the means of duplicates.

\(^c\) Monocyte-derived macrophages were preincubated for 15 h with growth medium containing 0.1 unit/ml glucagon or growth medium alone. The cells were then washed, and copper reduction was measured in HBSS without glucagon. Values are the means of three independent experiments.

\(^d\) Conditions were the same as for Footnote c, except that glucagon was also present during the 45-min incubation with copper. Values are the means of two independent experiments.

FIG. 7. Macrophage-mediated copper reduction is a saturable process. The initial rate of copper reduction was measured using 1–100 μM copper for J774A.1 cells (A) and human monocyte-derived macrophages (B). Supernatants were removed and assayed for Cu(I)-BCS as described under “Experimental Procedures.” Data are representative of four independent experiments for J774A.1 cells and a single experiment for monocyte-derived macrophages. Double-reciprocal plots (C and D) were derived from data in A and B to calculate the \(K_m\) for copper reduction for J774A.1 and monocyte-derived macrophages, respectively.
celeration of LDL oxidation by these cells. A role for cell-derived thiols as enhancers of such cellular metal reduction was also examined. Cell-mediated metal reduction may stimulate LDL oxidation by reaction of reduced metal with lipoprotein hydroperoxides, causing propagation of lipid peroxidation.

**Macrophage-mediated Transition Metal Reduction**—Macrophages were able to reduce both iron and copper in the absence of a substrate for cellular thiol production at rates that could not be substantially accounted for by reductants released into the cell supernatant. When cystine was added to cells, the apparent cell-mediated copper reduction was enhanced after \( \approx 1 \) h, and a larger proportion of this enhancement could be explained by exported thiols (\( \approx 85\% \) of the supernatant’s ability to reduce copper could be due to DTNB-detectable thiol). These results suggest that a proportion of cell-mediated metal reduction is due to direct electron transport and that, in the absence of a substrate for cell-derived thiols (i.e. when cystine is not supplied), this proportion is large. Evidence for copper reduction via a direct plasma membrane electron transport system was therefore sought by use of agents known to modulate similar systems (TPMET) in other cell types (33, 35, 51). Macrophage-mediated copper reduction shared striking similarities with previous reports in its sensitivity to depletion of plasma membrane iron (51, 55) and exposure to FCCP (52) or glucagon (53).

**Cell-mediated Metal Reduction Dominates over Other Mechanisms**—Our data show that in the absence of cystine, cell-mediated metal reduction is much more extensive than that induced by other components of the system, notably LDL. The reduction of copper by LDL, with or without cells, was an order of magnitude smaller than the cellular contribution. This quantity (\( \approx 0.6 \) nmol) is similar to the amount of tocopherol present in LDL, consistent with previous reports that tocopherol in lipid systems can reduce transition metals stoichiometrically (e.g. Ref. 56). Such data have also been obtained with isolated LDL (48). In contrast, Lynch and Frei (57) have claimed that LDL possesses a nonsaturable capacity to reduce copper in cell-free conditions, also measured by Cu(I)-BSC formation. This disparity may be explicable by two factors. First, the presence of selective reduced metal chelators can drive the metal toward the reduced chelated complexes (58, 59). Second, the extensive Cu(I)-BSC generation by LDL (57) may be due to the lipoprotein oxidation that occurred simultaneously. Many components of this pathway (e.g. hydroperoxides) reduce copper; thus, the metal reduction in this system may be part of a consequence of oxidation, rather than a determinant of it. In our study, LDL was not present during measurement of cellular metal reduction, so its oxidation could not contribute to the rates of copper reduction reported. It is possible that BCS could promote Cu(I) generation in the presence of MDM, leading to an overestimate of the rate and extent of cellular reduction. However, the low ratio of chelator to copper (2.5:1) used in this study and the saturaibility of cell-mediated copper reduction (Fig. 7) indicate that chelator-driven reduction was not a significant factor here.

**Involvement of Macrophage-mediated Transition Metal Reduction in LDL Oxidation**—The final objective of this work was to assess the impact of changes in cell-mediated metal reduction on cell-mediated LDL oxidation. Glucagon suppressed both macrophage-mediated copper reduction and LDL oxidation, consistent with our hypothesis that the cell’s ability to promote LDL oxidation is dependent on its capacity to reduce transition metals. Since glucagon also inhibited macrophage-mediated LDL oxidation in media that contained cystine, TPMET-mediated metal reduction may remain a rate-limiting mechanism in LDL oxidation even when thiol export is operational. In agreement with our conclusion that TPMET activity may be central to LDL oxidation, two recent studies have shown that other compounds that modulate its activity also modulate metal-dependent cell-mediated LDL oxidation. Thus, insulin increases ferricyanide reduction by HeLa cells (60, 61) and human erythrocytes (62) and accelerates LDL oxidation by peripheral blood mononuclear cells (63). Actinomycin D inhibits both cellular TPMET activity (64) and macrophage-mediated LDL oxidation (65). While agents that modulate cellular TPMET activity may have other effects that are relevant to the cells’ ability to promote LDL oxidation (for example, modulation of thiol export), the possibility that cellular TPMET capabilities are key to the process of cell-mediated LDL oxidation remains plausible. That all mammalian cells studied so far display TPMET activity (33) indicates that many cell types could promote the metal-dependent oxidation of LDL by a similar mechanism.

In conclusion, the results presented in this paper argue that macrophages can both accelerate the metal-dependent oxidation of LDL and reduce transition metals when cellular thiol production is minimized by omission of extracellular cystine. The cellular reducing activity shares many features with a TPMET system previously characterized in other cell types. Cellular TPMET activity appears to be amenable to modulation by hormones, growth factors, and drugs (33). Thus, controlling the processes that contribute to cell-mediated transition metal redox cycling may provide an opportunity for controlling the formation of copper-oxidized LDL.

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