Oxidation of low density lipoprotein by iron or copper at acidic pH

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Abstract Oxidized low density lipoprotein (LDL) may play a significant role in atherosclerosis. We have investigated the effect of pH on the oxidation of LDL by iron or copper. When LDL was oxidized by iron in the presence of cysteine in either Hanks' balanced salt solution (HBSS) or Ham's F-10 medium, an acidic pH greatly decreased the lag period and increased the rate of formation of hydroperoxides and thiobarbituric acid-reactive substances (TBARS), and increased its uptake by macrophages. There was a dose-dependent increase of LDL oxidation at acidic pH in the presence of increasing concentrations of cysteine. When LDL was oxidized by copper in HBSS, an acidic pH increased the lag phase before the rapid formation of conjugated dienes, hydroperoxides, and TBARS, but increased its uptake by macrophages. Similar results were obtained using Ham's F-10 medium. Cysteine (100 μM) inhibited the modification of LDL by copper in HBSS at both pH 7.4 and 5.5. As atherosclerotic lesions may be acidic, these observations may help to explain why LDL oxidation occurs locally at these sites.—**Morgan, J., and D. S. Leake.** Oxidation of low density lipoprotein by iron or copper at acidic pH. *J. Lipid Res.* 1995. **36:** 2504-2512.

Supplementary key words atherosclerosis • low density lipoprotein • macrophage • oxidation • lipid peroxides

Atherosclerotic lesions are often characterized by the presence of large numbers of foam cells filled with lipid droplets consisting largely of cholesteryl esters. Many of these foam cells are derived from macrophages (1–4). Macrophages in culture take up low density lipoprotein (LDL) only slowly by their LDL receptors, which are present in relatively small numbers (5), but they take up oxidized LDL rapidly by means of their scavanger receptor(s) (6).

LDL can be oxidized in vitro under appropriate conditions, in the presence of low levels of transition metal ions, by a number of cell types including endothelial cells (6), smooth muscle cells (7), macrophages (8, 9), and lymphocytes (10). LDL can be oxidized by copper ions (11–13), iron ions (9, 12, 14), ferryl myoglobin (15), or ferryl hemoglobin (16) in the absence of cells. This oxidation is associated with free radical-mediated peroxidation of polyunsaturated lipids within the LDL. The decomposition of these peroxides results in the formation of short or medium chain aldehydes which are then capable of reacting with lysyl or other residues within apolipoprotein B-100 (the protein moiety of LDL), resulting in recognition by oxidized LDL receptors and internalization of the LDL by macrophages (11, 17, 18).

It has been proposed that the role of cells in the oxidation of LDL is limited to the release of thiols, mainly cysteine, which can react with iron or copper to generate reduced transition metal ions or reactive oxygen or sulfur-centered free radicals (19, 20). Furthermore, it has been shown that thiols in the presence of low levels of iron ions can oxidize LDL in the absence of cells (21).

We have previously shown, using an adaptation of Hanks' balanced salt solution (HBSS) containing 6 μM $FeSO_4$, that increasing the acidity of the medium increases the oxidation of LDL by macrophages as measured by thiobarbituric acid-reactive substances (TBARS) or macrophage uptake (22). A similar effect of pH was also seen when high concentrations of iron ions alone were used to catalyze the oxidation of LDL, as measured by conjugated diene formation (22).

In this study, we have further investigated the effect of pH on LDL oxidation by iron or copper ions alone in HBSS or Ham's F-10 medium. We used HBSS as well as the widely used Ham's F-10 medium because conjugated dienes can be measured by the technique we use in HBSS but not in Ham's F-10 medium. We have also looked at the effects of cysteine in these systems.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances.

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MATERIALS AND METHODS

Isolation and radioiodination of LDL

LDL (d 1.019–1.063 g/ml) was isolated from normal human blood by sequential density ultracentrifugation in KBr solutions at 4°C as described elsewhere (23) and radiolabeled with $Na^{125}I$ using iodine monochloride (9). The ^{125}I-labeled LDL was dialyzed extensively at 4°C in air against a buffer containing 154 mM NaCl, 16.7 mM NaH_2PO_4, 21.1 mM Na_2HPO_4, and 100 μM Na_2EDTA (pH 7.4), to remove non-incorporated radioiodide ions, and sterilized by membrane filtration (0.2 μm pore size, Sartorius). It was mixed with nonlabeled LDL to obtain a specific activity of 20–60 cpm/ng protein and diluted to 2 mg protein/ml with the above buffer, so as to standardize the amount of EDTA that would be present during the incubations with iron or copper, and stored at 4°C. It was no longer used 1 month after isolation.

Iron-mediated or copper-mediated oxidation of LDL

^{125}I-labeled LDL (100 μg protein/ml) was incubated with either 6 μM $FeSO_4$ or 10 μM $CuSO_4$ in tissue culture wells (22-mm multiwell dishes, Costar) for up to 24 h at 37°C in triplicate at the indicated pH value. This gave a final concentration of EDTA of 5 μM and a net concentration of $CuSO_4$ of 5 μM (taking into account the EDTA concentration in the ^{125}I-labeled LDL preparation and that EDTA–copper complexes are inactive as regards LDL oxidation in simple phosphate buffers (24)). Low concentrations of EDTA do not inactivate $FeSO_4$ in either Ham's F-10 medium (24) or simple phosphate buffers (D. J. Lamb and D. S. Leake, unpublished observations). Where indicated, the incubation medium was a modified HBSS and was prepared by adding 10 mM Na_2HPO_4 or 10 mM KH_2PO_4 (or NaH_2PO_4) to stock solutions of 113 mM NaCl, 5.36 mM KCl, 1.26 mM $CaCl_2$, and 0.81 mM $MgSO_4$, and then mixing the two solutions together to give the desired pH. Ham's F-10 medium (Gibco), specially formulated to be deficient in $FeSO_4$, $CuSO_4$, and $ZnSO_4$ was also used. Where appropriate, freshly dissolved L-cysteine was added.

Determination of oxidized LDL uptake by J774 macrophage-like cells

Modified or control ^{125}I-labeled LDL was diluted to 10 μg protein/ml in culture medium (DMEM containing 20% (v/v) fetal calf serum, 10 i.u. penicillin/ml, 10 μg streptomycin/ml, and 1 μg amphotericin B/ml). The presence of serum in the growth medium should have prevented any further oxidation from taking place, as serum is a potent inhibitor of LDL oxidation (9). The pH of the culture medium under 5% CO_2 was not affected by the addition of the modified HBSS of various pH values, when added in a ratio of 1 volume of HBSS to 9 volumes of culture medium. The LDL was then

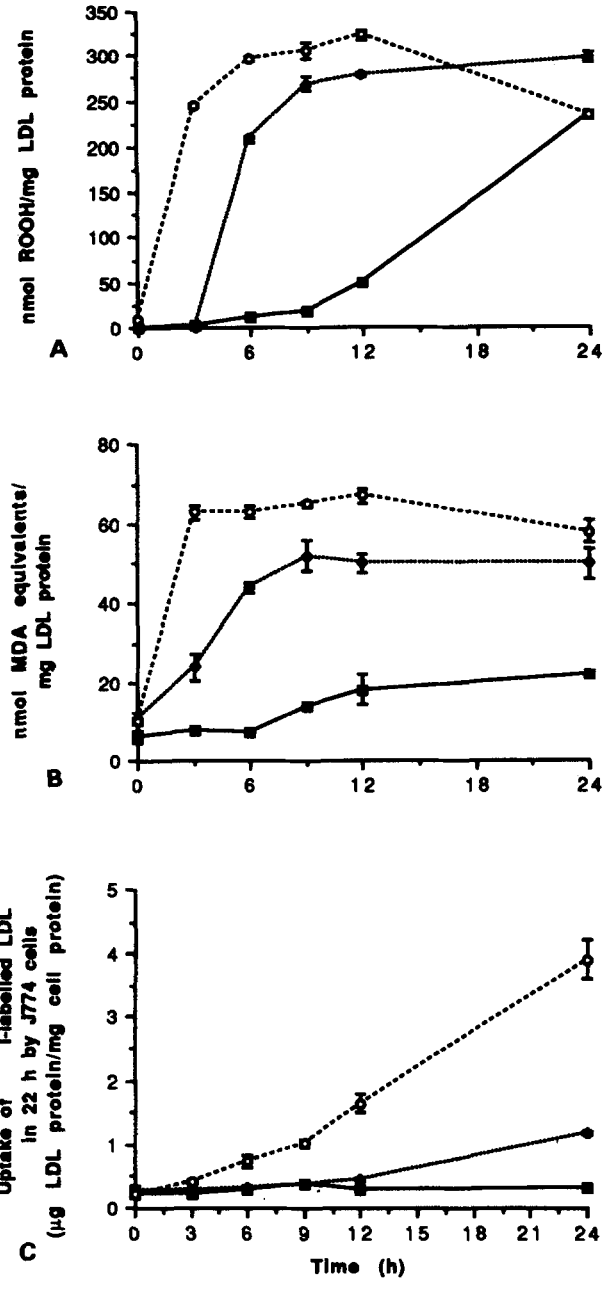

Fig. 1. Time course of LDL oxidation by iron/cysteine in HBSS as measured by hydroperoxides, TBARS, and macrophage uptake. ^{125}I-labeled LDL (100 μg protein/ml) was incubated with 6 μM $FeSO_4$ and 100 μM L-cysteine in HBSS at various pH values for up to 24 h at 37°C. Samples were collected into 20 μM BHT and 100 μM EDTA at appropriate time points to prevent further oxidation and assayed for (A) hydroperoxides, (B) TBARS, and (C) rate of uptake of the LDL by J774 cells. The rate of uptake of native ^{125}I-labeled LDL by J774 cells was 0.3 ± 0.02 μg protein/mg cell protein in 22 h. Each point represents the mean ± SEM of triplicate observations. The results were confirmed by two other experiments. pH 7.0 (-⊡-); pH 6.5 (···◆···); pH 5.5 (- -○- -).

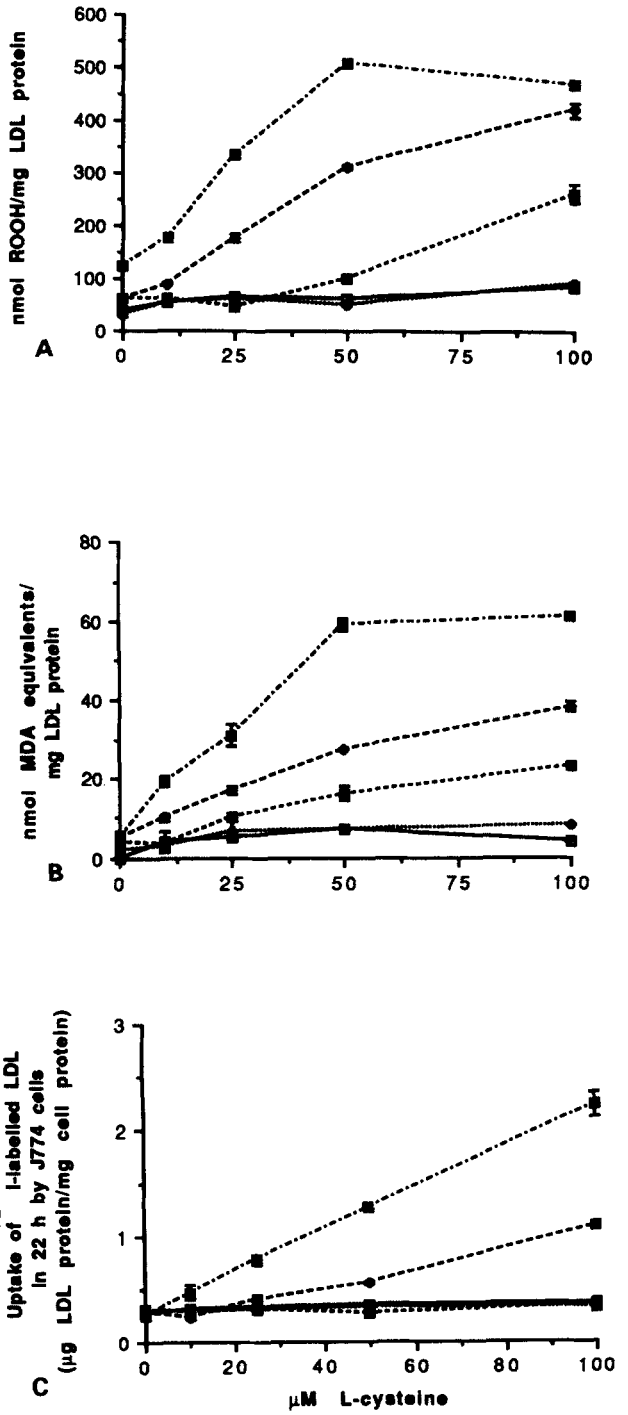

Fig. 2. Effect of increasing concentrations of L-cysteine on LDL oxidation by iron as measured by hydroperoxides, TBARS, and macrophage uptake. ^{125}I-labeled LDL (100 μg protein/ml) was incubated at 37°C for 18 jh with 6 μM $FeSO_4$ and increasing concentrations of L-cysteine in HBSS at various pH values and assayed for (A) hydroperoxides, (B) TBARS, and (C) rate of uptake of the LDL by J774 cells. The rate of degradation of native ^{125}I-labeled LDL by J774 cells was 0.4 ± 0.03 μg protein/mg cell protein in 22 h. Each point represents the mean ± SEM of triplicate observations. These results were confirmed by two other experiments. pH 7.4 (-□-); pH 7.0 (···◆···); pH 6.5 (-·□·-), pH 6.0 (- - ◆- -); pH 5.5 (··□··).

incubated for 18–24 h with 2×10^5 J774 cells (plated the day before) (kindly provided by Drs. J. Eckersley and D. T. Hart of the Life Sciences Division, King's College London) or in cell-free wells, with 1 ml per well. The radioactive noniodide, trichloroacetic acid-soluble degradation products released into the medium were measured as described previously (23). Degradation products in the cell-free wells were subtracted from those in the wells containing macrophages. The cells were washed in PBS containing Ca^{2+} and Mg^{2+}, lysed in 0.2 M NaOH as described elsewhere (23), and assayed for protein by a modified Lowry procedure (25). The radioactivity of the macrophage lysate (after subtracting the small amounts of radioactivity from the cell-free wells) was added to that of the degradation products to calculate total LDL uptake.

Thiobarbituric acid-reactive substances assay

Samples of modified LDL (250 μl of 100 μg protein/ml) were taken and BHT (20 μM) and EDTA (100 μM) were added (unless they were already present). Three ml of 0.335% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid was added to each sample and incubated at 95°C for 15 min. The absorbance was read at 535 nm (26). Standards of tetramethoxypropane (Sigma), up to 5 nmol per tube, were prepared in modified HBSS (pH 7.4) or Ham's F-10 where appropriate. The results were expressed as nmol of malondialdehyde (MDA) equivalents per mg of LDL protein.

Lipid hydroperoxide assay

Samples of modified LDL (250 μl of 100 μg protein/ml) were taken and BHT (20 μM) and EDTA (100 μM) were added (unless they were already present). The color reagent (1 ml) of the commercially available kit for the enzymatic determination of cholesterol (CHOD-iodide, Merck, Darmstadt, Germany) was added and incubated for 30 min in the dark (27). The absorbance of the tri-iodide ions produced was read at 365 nm. Standards of H_2O_2, up to 20 nmol per tube, were prepared in modified HBSS (pH 7.4).

Conjugated diene measurement in copper-oxidized LDL

^{125}I-labeled LDL was dialyzed against several changes of buffer containing 154 mM NaCl, 16.7 mM NaH_2PO_4, and 21.1 mM Na_2HPO_4 (pH 7.4) at 4°C to remove EDTA. The radioactivity of the ^{125}I-labeled LDL was then determined and its concentration after dialysis was calculated (because its concentration decreased during dialysis, presumably due to binding to the dialysis membrane). The LDL was oxidized at 100 μg protein/ml using 10 μM $CuSO_4$ at various pH values in the modified

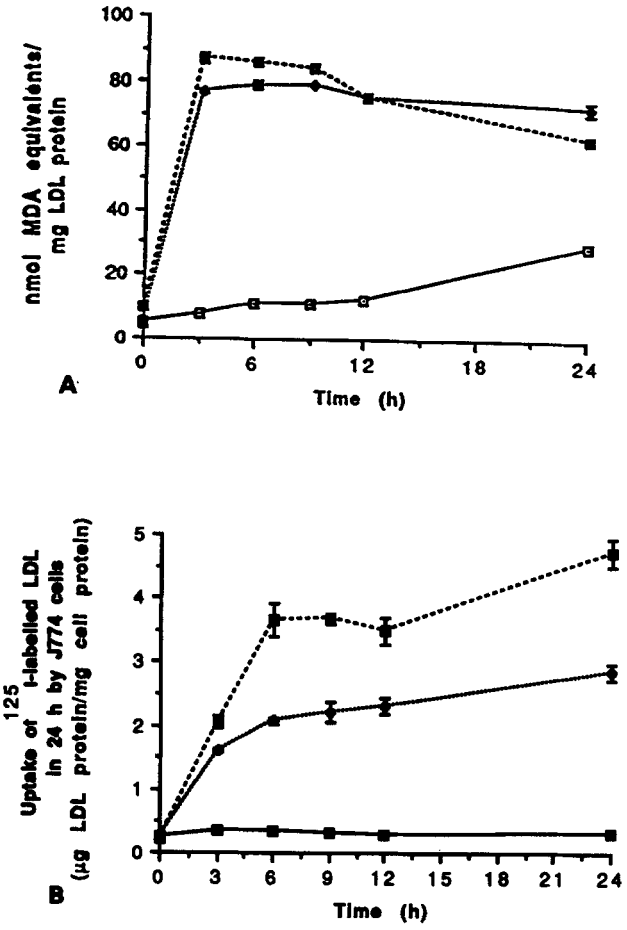

Fig. 3. Time course of LDL oxidation by iron in Ham's F-10 medium as measured by TBARS and macrophage uptake. ^{125}I-labeled LDL (100 μg protein/ml) was incubated at 37°C for up to 24 h with 6 μM $FeSO_4$ in F-10 medium (specially formulated to be deficient in $FeSO_4$, $CuSO_4$, $ZnSO_4$, and cysteine) containing freshly added cysteine (180 μM) at various pH values. Samples were collected into 20 μM BHT and 100 μM EDTA at appropriate time points and assayed for (A) TBARS and (B) rate of uptake of the LDL by J774 cells. The rate of uptake of native LDL by J774 cells was 0.28 ± 0.03 μg protein/mg cell protein in 24 h. Each point represents the mean ± SEM of triplicate observations. These results were confirmed by two other experiments. pH 7.4 (-▣-); pH 6.5 (···◆···); pH 5.5 (- - ■ - -).

HBSS at ambient temperature and monitored at 234 nm in a spectrophotometer (13). Preliminary experiments ruled out the possibility that the observed changes in absorbance were an artefact due to an effect of pH on the molar absorption coefficient of conjugated dienes, and this agrees with the findings of Malbrouk and Dugan (28) that pH does not affect the molar absorption coefficient of conjugated dienes in purified lipids.

RESULTS

By an adaptation of HBSS, we were able to produce a wide pH range in order to study the effect of pH on LDL oxidation by iron or copper ions. A time course was carried out in the presence of 6 μM $FeSO_4$ and 100 μM L-cysteine, as cysteine greatly increases the oxidation of LDL by iron (21). At pH 7.0 there was a lag phase of about 6–9 h and then the oxidation of LDL began to increase more rapidly, as judged by the formation of hydroperoxides (**Fig. 1A**) and TBARS (Fig. 1B). When the rate of uptake of these LDLs by J774 cells was determined, there was no increased uptake of LDL previously incubated at pH 7.0 with iron/cysteine (Fig. 1C). The lag phase before rapid hydroperoxide generation was reduced to about 3 h at pH 6.5 and even less at pH 5.5 (Fig. 1A). The rate of hydroperoxide formation was much greater at acidic pH than at pH 7.0. The lag phase before TBARS formation was also decreased by acidity and it appeared that the maximum values of the TBARS produced were greater at acidic pH than at pH 7.0 (Fig. 1B). There was a moderate increase in macrophage uptake of ^{125}I-labeled LDL that had previously been incubated at pH 6.5 for 24 h. ^{125}I-labeled LDL that had been incubated at pH 5.5, however, showed a much larger increase in uptake after a much shorter lag period. The rapid uptake of oxidized LDL occurred much later than the formation of hydroperoxides or TBARS.

To look at the effect of L-cysteine at various pH values in more detail, ^{125}I-labeled LDL was incubated in HBSS in the presence of 6 μM $FeSO_4$ with increasing concentrations of L-cysteine. In the absence of L-cysteine, the levels of hydroperoxides and TBARS were low at all pH values but tended to increase slightly at acidic pH (**Fig. 2AB**). At pH 7.4 and 7.0, in this particular experiment, there was little increase in the levels of hydroperoxides as the L-cysteine concentration was increased up to 100 μM (Fig. 2A). For pHs 6.5, 6.0, and 5.5, there was a large and progressive increase in the levels of hydroperoxides

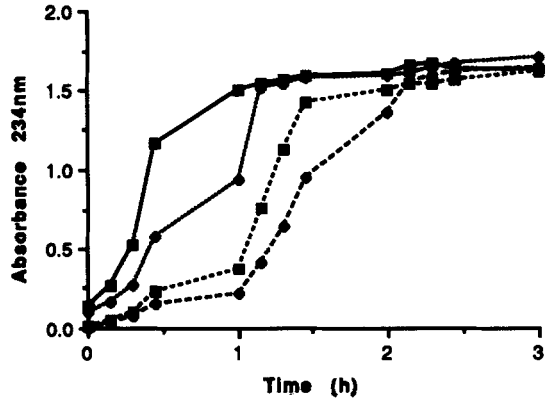

Fig. 4. Effect of pH on the oxidation of LDL by copper ions in HBSS as measured by conjugated diene absorbance. After dialysis to remove EDTA, LDL (100 μg protein/ml) was oxidized in the presence of 10 μM $CuSO_4$ at pH 7.4 (-▣-), 6.5 (···◆···), 6.0 (- ·■· -), or 5.5 (- - ◇ - -) in modified HBSS at ambient temperature and monitored at 234 nm in a spectrophotometer. The results shown are representative of four independent experiments.

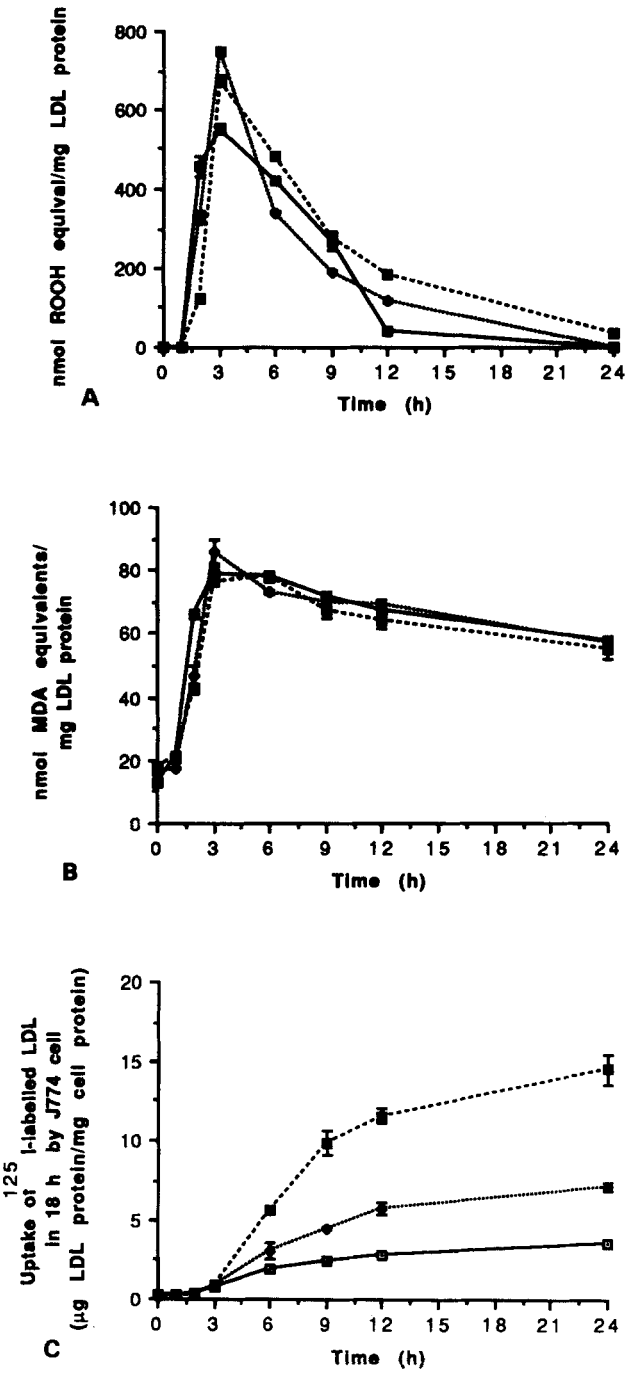

Fig. 5. Time course of oxidation of LDL by copper in HBSS as measured by hydroperoxides, TBARS, and macrophage uptake. ^{125}I-labeled LDL (100 µg protein/ml) was incubated at 37°C for up to 24 h with a net concentration of 5 µM $CuSO_4$ in HBSS at various pH values. Samples were collected into 20 µM BHT and 100 µM EDTA at the appropriate time point to prevent further oxidation and assayed for (A) lipid hydroperoxides, (B) TBARS, and (C) rate of uptake of the LDL by J774 cells. The rate of uptake of native ^{125}I-labeled LDL by J774 cells was 0.27 ± 0.04 µg protein/mg cell protein in 18 h. Each point represents the mean ± SEM of triplicate observations (sometimes the SEM cannot be seen when it is smaller than the symbols). The results of this experiment were confirmed by three other experiments. pH 7.4 (-⊡-); pH 6.5 (···◆···); pH 5.5 (- -◘- -).

as the L-cysteine concentration was increased. The levels of the hydroperoxides increased as the pH decreased. A very similar pattern was observed for TBARS production (Fig. 2B). There was no detectable increase in the rate of uptake by J774 cells of the LDL incubated with iron and L-cysteine at pH 7.4, 7.0 and 6.5 (Fig. 2C). An increase in the rate of uptake of LDL incubated at pH 6.0 occurred with the higher L-cysteine concentrations. This effect was more pronounced with LDL that had been incubated at pH 5.5, with the rate of uptake of the LDL increasing linearly with the concentration of L-cysteine.

When a time course experiment was carried out in Ham's F-10 medium containing 6 µM $FeSO_4$, a similar pattern of oxidation was seen to that in HBSS, whereby acidic pH greatly increased TBARS formation (**Fig. 3**). The TBARS increased slowly over 24 h at pH 7.4 whereas at both pH 6.5 and 5.5 the TBARS levels were maximal by 3 h. This effect on TBARS was mirrored in macrophage uptake, in that no increase in uptake was seen with LDL previously incubated at pH 7.4 but there was a large and rapid increase with LDL previously incubated at pH 6.5 and an even greater one at pH 5.5.

When the extent of LDL oxidation by $CuSO_4$ (10 µM) was measured by conjugated diene formation, we found that there was a progressive increase in the lag phase and a slight decrease in the rate of the propagation phase when the pH was reduced from 7.4 to 5.5 (**Fig. 4**). There was no effect on the peak absorbance.

A time course was used to investigate the effect of pH on LDL oxidation by copper in modified HBSS. The levels of hydroperoxides in the LDL rose sharply after a lag phase of about 1 h at pH 7.4, 6.5, or 5.5, peaking at nearly the same levels at around the 3 h time point (**Fig. 5A**). The increase was fastest at pH 7.4, however, slower at pH 6.5, and slower still at pH 5.5. After 3 h the levels of hydroperoxides decreased so that by the 24-h time point there were few if any detectable hydroperoxides present. TBARS production also had a lag phase of about 1 h and then increased to a maximum at the 3-h time point (Fig. 5B). The increase was again slower at acidic pH. The TBARS decreased slowly thereafter at a similar rate for all the pH values. After a lag of about 2 h, there was a progressive increase in the uptake by macrophages of ^{125}I-labeled LDL oxidized at pH 7.4 so that it was taken up much faster than the unmodified LDL (Fig. 5C). The modification of LDL, as measured by macrophage uptake, was considerably greater even at the mildly acidic pH of 6.5 and with oxidation at pH 5.5 for 9–24 h the uptake by macrophages was about 4 times as much as with oxidation at pH 7.4.

When Ham's F-10 medium was substituted for HBSS and a detailed time course was carried out during the early phase of the oxidation with copper, a similar effect

was seen, whereby there was a longer lag period before TBARS formation at pH 5.5 than at pH 7.4 (**Fig. 6**). The uptake of the LDL by J774 cells was greater after oxidation at acidic pH (results not shown).

A time course of LDL oxidation by copper in HBSS at pH 7.4 or 5.5 was carried out in the presence or absence of 100 μM L-cysteine. The presence of L-cysteine resulted in a much longer lag phase before the rapid formation of TBARS at pH 7.4 (**Fig. 7A**). In the experiment shown in Fig. 7A, TBARS formation at pH 5.5 was also slowed down by cysteine (but this was not always the case at this pH). The presence of L-cysteine prolonged the lag period prior to increased macrophage uptake from about 1 h to 6 h at both pH 7.4 and 5.5 (Fig. 7B). After 24 h, however, the uptake of the LDL was not greatly different in the absence or presence of cysteine, but was greater at pH 5.5 than at pH 7.4.

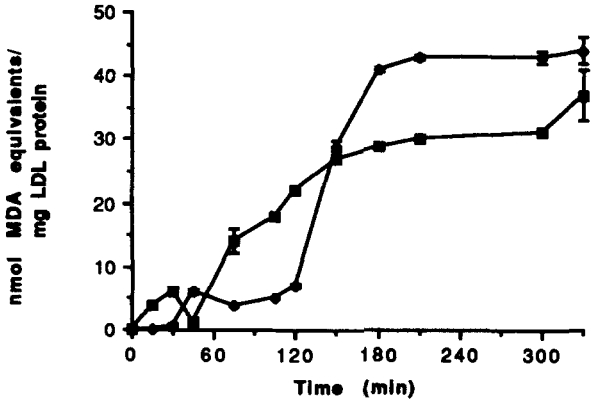

Fig. 6. Detailed time course of LDL oxidation by copper in Ham's F-10 medium as measured by TBARS. LDL (100 μg protein/ml) was incubated at 37°C for up to 7 h with $CuSO_4$ at a net concentration of 5 μM in Ham's F-10 medium containing 180 μM cysteine (specially formulated to be deficient in $CuSO_4$, $FeSO_4$, and ZnSO4) at various pH values. Samples are collected into 20 μM BHT and 100 μM EDTA at appropriate time points to prevent further oxidation and assayed for thiobarbituric acid–reactive substances (TBARS). Each point represents the mean ± SEM of triplicate observations. These data are representaive of three experiments; pH 7.4 (-◘-); pH 5.5 (◆).

DISCUSSION

We have shown previously that macrophages oxidize LDL faster in iron-containing buffer when the pH is low (22). In the present study, we have shown that when LDL was oxidized by $FeSO_4$ plus L-cysteine, there was a large decrease in the lag phase (which is due to the presence of antioxidants in LDL) and an increase in the rate of formation of hydroperoxides, TBARS, and in macrophage uptake as the acidity was increased, even modestly to pH 6.5.

There is increasing evidence that thiols may play an important role in LDL oxidation (19–21). When LDL was incubated with iron at acidic pH, but not at pH 7.4, the formation of hydroperoxides and TBARS and the uptake by macrophages were increased greatly as the cysteine concentration was increased. Our observations are supported by Parthasarathy (21), who showed that thiols promoted the oxidation of LDL by iron ions. Cysteine may increase LDL oxidation by iron by reducing Fe(III) to Fe(II), which would decompose lipid hydroperoxides faster than Fe(III) (29), by binding iron ions and keeping them in a more soluble form or by reducing the α-tocopheroxyl radicals in oxidized LDL back to α-tocopherol. The oxidation of cysteine to cystine may also generate oxygen- or sulfur-centered free radicals (30), which may attack LDL.

There are many reasons why acidity may increase LDL oxidation by iron. It may possibly affect the redox potential or solubility of iron or, if superoxide ions are involved, they would be converted to hydroperoxyl radicals more at acidic pH, the reaction $O_2^{\bullet-} + H^+ = HO_2^\bullet$ having a pKa of 4.8 (31). Bedwell, Dean, and Jessup (32) have shown that hydroperoxyl radicals generated by a ^{60}Co source are much more active than superoxide anions in oxidizing LDL. Superoxide anions dismutate spontaneously to H_2O_2 faster at acidic pH. If the H_2O_2 and superoxide anions were then to react together in an iron-catalyzed Haber-Weiss reaction, this would form hydroxyl radicals, which are very reactive towards LDL in the presence of oxygen (32). In addition, the effect of pH may be explained by considering the following set of reactions (33, 34):

$RSH \rightleftharpoons RS^- + H^+$ (pKa 8.4)

$RS^- + Fe^{3+} \rightarrow RS^\bullet + Fe^{2+}$ (reaction 1)

$RS^\bullet + LH \rightarrow RSH + L^\bullet$ (reaction 2)

$RS^\bullet + RS^- \rightarrow RSSR^{\bullet-}$ (reaction 3)

$RSSR^{\bullet-} + O_2 \rightarrow RSSR + O_2^{\bullet-}$ (reaction 4)

$O_2^{\bullet-} + H^+ \rightleftharpoons HO_2^\bullet$ (reaction 5)

$RSH + HO_2^\bullet \rightarrow RS^\bullet + H_2O_2$ (reaction 6)

Cysteine oxidizing in the presence of transition metal ions would form thiyl radicals (reaction 1), which might initiate lipid peroxidation within the LDL particle (reaction 2). Alternatively, the thiyl radical can react with the thiolate anion to form the disulfide anion radical (reaction 3) which is oxidized by molecular oxygen to yield the disulfide and superoxide (reaction 4). Less RS^- would be present at acidic pH and therefore reaction 3 would be slower and the concentration of $RS^\bullet$ may be increased, thereby possibly leading to an increase in LDL oxidation. In addition, reactions 5 and 6 may be increased at acidic pH.

When LDL was incubated with $CuSO_4$, its initial oxidation was actually slowed down at acidic pH, as judged by the formation of conjugated dienes, hydroperoxides, and TBARS. LDL oxidation by copper had an increased lag phase at acidic pH. The reason for this is unknown but copper ions are known to bind to LDL (28, 35–37), possibly directly to the histidine residues in apolipoprotein B-100 (38), and acidity may decrease this

binding, (the imidazole ring of histidine residues has a typical pKa of about 6.5).

There was little difference in the peak levels of hydroperoxides or TBARS or their rate of decay with copper at different pH values. Despite this, a significant increase was seen in the uptake by macrophages of LDL that had been modified at acidic pH. It has very recently been shown that malondialdehyde modifies LDL faster at acidic pH in terms of electrophoretic mobility (which measures net negative charge) or the percentage of lysyl residues blocked (39). In addition, even at equivalent extents of modification as judged by these two criteria, the LDL was degraded faster by J774 cells when it had been treated with malondialdehyde at pH 6.4 rather than at pH 7.4. This may explain why we saw an increase in the uptake of the LDLs modified at acidic pH.

When L-cysteine was added to the LDL and copper, there was a significant delay in the formation TBARS, at least at pH 7.4. This increased lag period was also seen in terms of macrophage uptake at both pH 7.4 and 5.5. In contrast, cysteine greatly increased the oxidation of LDL by iron. There are several possibilities to explain how cysteine increased the lag phase with copper. It is possible that cysteine may have bound the copper ions and removed them from the LDL, scavenged free radicals, or reacted with the aldehydes from oxidizing LDL. In contrast to our findings, Heinecke et al. (40) found that cysteine increased LDL oxidation by copper in terms of TBARS. The reason for this difference in our results may, in part, be explained by the fact that Heinecke et al. (40) oxidized LDL with copper in the presence of a molar excess of EDTA, whereas we used a molar excess of copper (D. J. Lamb, B. Gale, R. Patterson, E. Rees, and D. S. Leake, unpublished results). Our work is supported by the finding of Graham et al. (41) that LDL oxidation by copper was inhibited by cysteine in phosphate-buffered saline.

Lipid hydroperoxide decomposition in oxidizing LDL was much slower with iron than copper. It is known that transition metals in their lower valency state are more effective in decomposing lipid hydroperoxides (28). It is therefore possible that Cu(I) is formed from Cu(II) more readily than Fe(II) is from Fe(III) in these systems or that Cu(I) may be better than Fe(II) in decomposing lipid hydroperoxides. It may be of interest to note that Lynch and Frei (42) have shown that LDL is capable of reducing Cu^{2+} to Cu^{+} but not Fe^{3+} to Fe^{2+}.

Atherosclerotic lesions are analogous in many ways to chronic inflammatory sites and as such may be expected to have a low pH. For instance, a pH of 6.8 has been recorded in pleural exudates in dogs and skin exudates in rabbits (43, 44). The pH of the bulk synovial fluid in rheumatoid arthritis falls from pH 7.4 to 7.2 and the pH decreases even further the nearer the fluid is to the

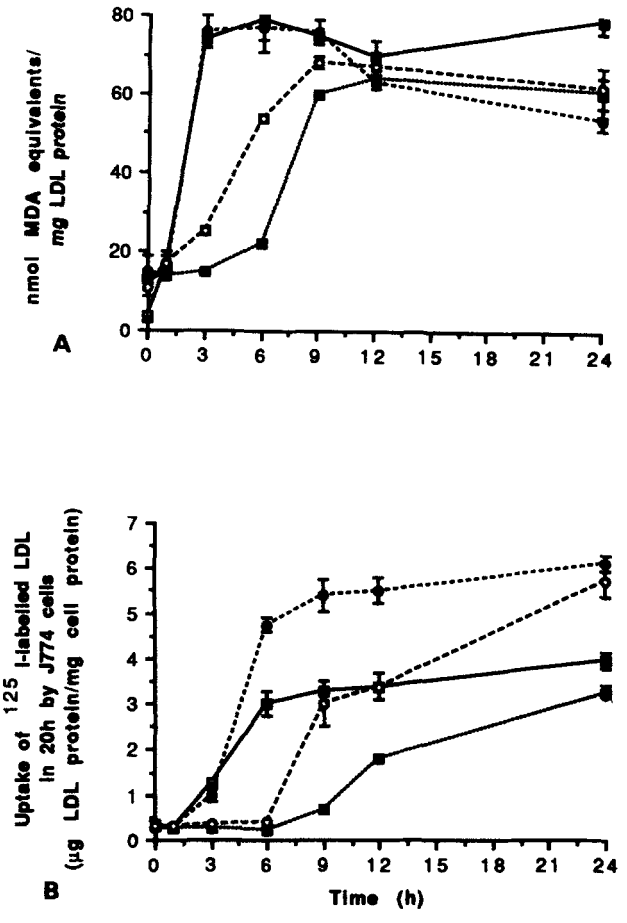

Fig. 7 Time course of the effect of L-cysteine on LDL oxidation by copper as measured by TBARS and macrophage uptake. ^{125}I-labeled LDL (100 mg protein/ml) was incubated at 37°C for up to 24 h with $CuSO_4$ at a net concentration of 5 µM in HBSS at either pH 7.4 or 5.5 in the presence or absence of 100 µM L-cysteine (cys). Samples were collected into 20 µM BHT and 100 µM EDTA at the appropriate time point to prevent further oxidation and assayed for (A) TBARS and (B) the rate of uptake of the LDL by J774 cells. The rate of uptake of native ^{125}I-labeled LDL by J774 cells was 0.32 ± 0.04 µg LDL protein/mg cell protein in 20 h. Each point represents the mean ± SEM of triplicate observations. The results of this experiment were confirmed by three other experiments. pH 7.4 (- cys) (-■-); pH 7.4 (+cys) (···□···); pH 5.5 (-cys) (- -●- -); pH 5.5 (+cys) (- -○- -).

synovial membrane (45). The mild media of arteries is known to be ischemic (46, 47) and the mid regions of atherosclerotic lesions in the intima may similarly be ischemic. Cells in atherosclerotic lesions may therefore use anerobic glycolysis to obtain ATP and in doing so produce lactic acid. This may diffuse away only slowly due to the poor perfusion of atherosclerotic lesions, thus causing lactic acid to build up and acidify the extracellular space. Indeed, it has been calculated that even in the normal human aortic intima significant lactate may be produced in the deeper regions (48). It has been shown that during complete brain ischemia in hyperglycemic rats, the pH may fall to 6.2 (49) and in

tumors in hyperglycemic animals the pH may fall to as low as 5.2 (50). The localized pH near to the surface of glycosaminoglycans may be acidic due to the electrostatic attraction of hydrogen ions (51). The extracellular space may also be acidified by macrophages extruding H^+ in part by a H^+-ATPase present in their plasma membranes and, in fact, macrophages have been shown to acidify their extracellular pH to 6.5 over 25 min in a weakly buffered medium (52). A pH as low as 3.6 (53) has been recorded at the surface of an activated macrophage and as macrophages in human atherosclerotic lesions are known to be selectively activated (54, 55) they may also acidify their extracellular space, especially as they tend to occur in clusters.

The finding that LDL oxidation by iron or copper ions leading to increased macrophage uptake takes place faster at acidic pH (although the initial oxidation by copper is somewhat slower at acidic pH) may help to explain why atherosclerotic lesions are apparently one of the very few sites in the body where LDL oxidation occurs to an extensive degree. In addition, acidic pH makes ceruloplasmin (the copper-carrying protein of plasma) catalyze LDL oxidation by macrophages more effectively (56) and acidic pH makes transferrin (the iron-carrying protein in plasma) capable of catalyzing LDL oxidation (57). It is quite conceivable that the acidic interstitial fluid within a cluster of macrophages in an atherosclerotic lesion may provide the ideal microenvironment for LDL oxidation to take place.

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