Prooxidant and antioxidant properties of human serum ultrafiltrates
toward low density lipoprotein: Important role of uric acid

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Running footline: Prooxidant activity of serum ultrafiltrates toward LDL

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Abbreviations: LDL, low density lipoprotein; HBSS, Hanks’ balanced salt solution; BHT, butylated hydroxytoluene; HPODE, 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid.
Abstract

Oxidised low density lipoprotein (LDL) is present within atherosclerotic lesions, demonstrating a failure of antioxidant protection. A normal human serum ultrafiltrate of Mr below 500 was prepared as a model for the low Mr components of interstitial fluid and its effects on LDL oxidation were investigated. The ultrafiltrate (0.3%, v/v) was a potent antioxidant for native LDL, but was a strong prooxidant for mildly-oxidised LDL when copper, but not a water-soluble azo initiator, was used to oxidise LDL. Adding a lipid hydroperoxide to native LDL induced the antioxidant to prooxidant switch of the ultrafiltrate. Uric acid was identified, using uricase and add-back experiments, as both the major antioxidant and prooxidant within the ultrafiltrate for LDL. The ultrafiltrate or uric acid rapidly reduced Cu$^{2+}$ to Cu$. The reduction of Cu$^{2+}$ to Cu$^+$ may help to explain both the antioxidant and pro-oxidant effects observed. The decreased concentration of Cu$^{2+}$ would inhibit tocopherol-mediated peroxidation in native LDL and the generation of Cu$^+$ would promote the rapid breakdown of lipid hydroperoxides in mildly-oxidised LDL into lipid radicals. The net effect of the low Mr serum components would therefore depend on the pre-existing levels of lipid hydroperoxides in LDL. These findings may help to explain why LDL oxidation occurs in atherosclerotic lesions in the presence of compounds that are usually considered to be antioxidants.

Supplementary key words atherosclerosis • oxidized low density lipoprotein • oxidised low density lipoprotein • lipid hydroperoxide
INTRODUCTION
The oxidation of low density lipoprotein (LDL) may be an important event in the development of atherosclerosis (1). The mechanisms of LDL oxidation in atherosclerotic lesions are uncertain, but there is evidence that catalytically active transition metal ions are present in human lesions (2-4). There is also a suggestion that oxidation may be catalysed by metal ions in advanced atherosclerotic lesions (which cause clinical problems), but not early ones, based on the levels of protein-bound o-tyrosine in human atherosclerotic lesions (5).

In vitro, low concentrations of serum (6, 7) or interstitial fluid (8) can protect LDL against oxidation. Under normal circumstances, the antioxidant protection offered by interstitial fluid should be sufficient to protect LDL against oxidation in the arterial wall. Indeed, antibodies against oxidised LDL do not detect the presence of oxidised LDL in the normal arterial wall (9, 10), but oxidised LDL has been detected in atherosclerotic lesions (9, 10) implying that LDL oxidation occurs in diseased arteries.

We reported that ascorbate switched from being an antioxidant for native (nonoxidised) LDL to become a prooxidant toward partially-oxidised LDL (11). Antioxidant to prooxidant switches have since been reported for a number of different compounds including dehydroascorbate (12), flavonoids (13), caffeic and chlorogenic acid (14), catecholestrogens (15), ferulic acid (16), Trolox C (a water-soluble analogue of vitamin E) (17), aminoguanidine (18) and uric acid (19, 20).

Here we report that a human serum ultrafiltrate of M<sub>r</sub> below 500, prepared as a model for the low M<sub>r</sub> components of interstitial fluid, is antioxidant toward native LDL,
but prooxidant toward mildly-oxidised LDL. We have identified the component of the ultrafiltrate responsible for both its antioxidant and prooxidant activity as uric acid and discuss the possible mechanisms involved.
**EXPERIMENTAL**

**Isolation of LDL**

LDL was isolated by a modification of the method of Vieira et al. (21). Normal human plasma containing 3mM EDTA was adjusted to a density of 1.21g/ml by the addition of solid KBr, and overlayed with a KBr solution of density 1.006g/ml containing 300µM EDTA. Following centrifugation in a near vertical rotor at 365,000 g for 50 min at 4°C, the LDL band was removed and its density was adjusted to 1.15g/ml. The LDL was overlayed with a KBr solution (containing 300µM EDTA) of 1.063g/ml, and centrifuged as above for 3h. KBr and EDTA were removed by rapid filtration through two disposable desalting columns (PD 10, Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.) using Dulbecco’s phosphate buffered saline (PBS) pretreated with washed Chelex-100 (Sigma-Aldrich, Poole, Dorset, U.K.) containing 10µM EDTA. LDL was sterilised by membrane filtration (Minisart-plus, Sartorius, Goettingen, Germany) and was stored aseptically under argon in the dark for up to 4 days.

**LDL oxidation**

LDL was incubated at 37°C in modified Hanks’ balanced salt solution (HBSS) containing CuSO₄ (usually 5μM net above the concentration of EDTA carried over from the storage buffer, which was below 1µM), with or without the addition of the ultrafiltrates (0.3%, v/v), as indicated in the figure legends. CuSO₄ was the last component to be added (except when the ultrafiltrates or uric acid were added after the oxidation was already underway). The HBSS was pretreated with Chelex-100 (washed
prior to use with distilled H₂O to remove any contaminating antioxidant activity (22)), and consisted of 113mM NaCl, 5.36mM KCl, 5mM H₃PO₄, 1.26mM CaCl₂ and 0.81mM MgSO₄ and adjusted to pH 7.4 by the addition of NaOH. LDL oxidation was monitored continuously by following the formation of conjugated dienes (23) and the lag phase determined by extrapolating the tangent to the most rapid part of the propagation phase to the x-axis. The oxidation of LDL was also measured noncontinuously after halting oxidation with EDTA (1mM) and butylated hydroxytolulene (BHT; 20µM from a stock solution of 2mM in ethanol) using a tri-iodide lipid hydroperoxide assay (24).

LDL was oxidised by 1mM 2,2’-azo-bis(2-amidinopropane) dihydrochloride (AAPH; Polysciences, Warrington, PA, USA) at 37°C in the above buffer and conjugated dienes measured. AAPH was also added to the reference cuvettes and its absorbance subtracted automatically from that of the test cuvettes.

**Preparation of serum ultrafiltrates**

Normal human serum was centrifuged at 9,774g for 1 h at 4°C through a filtration unit with a membrane selecting for components of Mᵣ below 100,000 (Whatman International Ltd, Maidstone, Kent, U.K.) to remove large serum proteins. The ultrafiltrate of Mᵣ below 100,000 was then refiltered by centrifugation at 2,000g overnight at 4°C through a filtration unit fitted with a membrane to select for components of Mᵣ below 500 (Micropartition kit, Amicon, Beverly, MA, USA).
Uric acid (Sigma) was dissolved in 1 or 5M NaOH and the pH value adjusted to about pH 7.4 using HCl.

**Uricase and catalase treatment and uric acid analysis of the serum ultrafiltrate**

The serum ultrafiltrate was incubated for 15 min at room temperature with uricase (Type V from porcine liver; Sigma) at a concentration of 2mg/ml (equivalent to 0.066 units of uricase/ml) and/or catalase (450ng/ml, equivalent to 5 units/ml; thymol-free, derived from bovine liver; Sigma). To separate the ultrafiltrate from the uricase and/or catalase, the samples were then loaded into filtration units fitted with a membrane selecting for components of $M_r$ below 5,000 (Whatman) and centrifuged at 9,774g for 30 min at 4°C. The uric acid content of the ultrafiltrates was analysed by UV absorbance at 292nm, and compared to a series of standards prepared from uric acid in 700mM glycine of pH 9.

**Copper reduction by serum ultrafiltrates**

The reduction of Cu$^{2+}$ to Cu$^{+}$ was measured using bathocuproine disulphonic acid, which binds Cu$^{+}$ to give a complex with an absorbance at 480nm (25). A serum ultrafiltrate (0.3%, v/v), uric acid (1µM), or LDL (50µg protein/ml) were incubated in modified HBSS at 37°C with bathocuproine disulphonic acid (360µM) in the presence of CuSO$_4$ (5µM). The absorbance at 480nm was recorded prior to the addition of CuSO$_4$ and was subtracted from subsequent readings. The $A_{480}$ was recorded every 30 seconds and the concentration of Cu$^{+}$ was calculated using a molar absorption coefficient for the Cu$^{+}$-bathocuproine disulphonic complex of 12200 M$^{-1}$ cm$^{-1}$, which was calculated from a standard plot prepared from CuSO$_4$ reduced by excess ascorbic acid (1mM; Sigma) to
form Cu⁺. To investigate copper reduction following uricase treatment of the ultrafiltrate or uric acid, the ultrafiltrate (0.3%, v/v) or uric acid (1µM) was added to CuSO₄ (5µM) in modified HBSS, and bathocuproine disulphonic acid (360µM) was added immediately and the absorbance was measured at 480nm. The absorbance of the appropriate mixture in the absence of CuSO₄ was subtracted and the concentration of Cu⁺ was calculated from its molar absorption coefficient. The ultrafiltrate did not contain any copper detectable by this method, either with or without the addition of ascorbic acid.

Statistics

A Student’s t test was used to detect differences between conditions. A difference was deemed significant if the P value was below 0.05.
RESULTS

Antioxidant activity of the serum ultrafiltrate toward native LDL oxidised by copper

LDL (50µg protein/ml) was oxidised by 5µM copper ions and the accumulation of conjugated dienes was measured. There was a lag phase prior to the rapid propagation phase, as expected (23) (Fig. 1). An ultrafiltrate containing components of M_r below 500 was prepared from normal human serum and tested for antioxidant activity toward LDL oxidation by copper. The ultrafiltrate potently inhibited the oxidation of native LDL, with substantial inhibition being obtained even with 0.3% (v/v) of the ultrafiltrate (P<0.005; n=10 experiments). In the example shown in figure 1, the lag phase was increased from 32 min to 94 min without significantly changing the rate of oxidation during the propagation phase. A similar effect was seen when lipid hydroperoxides were measured (a LDL concentration of 100µg protein/ml, rather than 50µg protein/ml, was used for this experiment because of the lower sensitivity of the lipid hydroperoxide assay than the conjugated diene assay) (Fig. 2).

An antioxidant effect of the ultrafiltrate was also seen when LDL was oxidised by 10µM copper and conjugated dienes were measured (Fig. 3). No antioxidant effect was observed, however, when 1µM copper was used to oxidise the LDL (Fig. 3).

Pro-oxidant activity of the serum ultrafiltrate toward mildly-oxidised LDL in the presence of copper

The ultrafiltrate, at the same concentration (0.3%, v/v) that had previously resulted in antioxidant activity toward native LDL with a copper concentration of 5µM or above,
was also added to LDL in an early stage of oxidation by 5µM copper (which contained 4% ± 0.5 (mean ± S.E.M for 10 independent experiments) of the maximum levels of conjugated dienes) (Fig. 1). The ultrafiltrate immediately increased the rate of oxidation of the mildly-oxidised LDL (Fig. 1). A similar result was obtained when lipid hydroperoxide formation was measured (Fig. 2). Similar antioxidant and prooxidant effects toward native and mildly-oxidised LDL, respectively, were seen with ultrafiltrates of M_r below 100,000, 30,000, 20,000, 12,000 or 5,000, and with serum components of M_r below 12,000-14,000 prepared by dialysis of normal human serum (data not shown).

A similar rapid prooxidant effect was also seen when the ultrafiltrate was added to partially-oxidised LDL in the presence of 10µM copper and conjugated dienes were measured, but the pro-oxidant effect was less apparent when 1µM copper was used (Fig. 3).

**Identification of the component responsible for the prooxidant and antioxidant activities as uric acid**

To identify the active component in the ultrafiltrate responsible for these observations, the effects of cysteine, ascorbate and uric acid towards LDL oxidation by copper were investigated. At the concentration predicted to be present in the ultrafiltrate (0.3%, v/v), neither cysteine nor ascorbate (0.1µM; approximately 0.3% of their serum concentrations (26, 27) were able to cause the antioxidant/prooxidant effects shown by the ultrafiltrate (data not shown). Uric acid is present in serum at a concentration of around 300µM (28), and would be present at approximately 1µM in the ultrafiltrate at
0.3% (v/v). Uric acid (1µM) had antioxidant and prooxidant activities towards the oxidation of native and mildly-oxidised LDL, respectively (Fig. 4). These observations agree with the findings of Abuja (20) and Bagnati et al. (19).

Pre-treatment of the ultrafiltrate with uricase (an enzyme that catalyses the conversion of urate into allantoin (reaction 1)) completely abolished both the antioxidant effect of the ultrafiltrate towards native LDL and the prooxidant effect toward mildly-oxidised LDL (Fig. 5).

\[
\text{uricase} \\
\text{uric acid} + 2\text{H}_2\text{O} + \text{O}_2 \rightarrow \text{allantoin} + \text{CO}_2 + \text{H}_2\text{O}_2 \quad \text{(reaction 1)}
\]

The ultrafiltrate was analysed for uric acid content before and after uricase treatment. The uric acid concentration of the ultrafiltrates varied between donors over a range of 235 to 300µM and was decreased to 4.5 ± 2 µM (mean ± S.E.M. of 9 independent experiments) following uricase treatment. The addition of uric acid (1µM; the concentration predicted to be present in the ultrafiltrate (0.3%, v/v)) to the uricase-treated ultrafiltrate confirmed that uric acid was responsible for the antioxidant and prooxidant effects, as this restored the antioxidant and pro-oxidant effects of the uricase-treated ultrafiltrate (Fig. 5).

The degradation of uric acid by uricase produces \( \text{H}_2\text{O}_2 \) (reaction 1), which may possibly have been prooxidant toward LDL. To ensure that any prooxidant activity of \( \text{H}_2\text{O}_2 \) did not mask any antioxidant activity of other molecules within the uricase-treated
ultrafiltrate, the ultrafiltrate was treated with uricase plus catalase to decompose \( \text{H}_2\text{O}_2 \). The ultrafiltrate treated with both uricase and catalase was not significantly different in its antioxidant and prooxidant effects towards LDL compared to the ultrafiltrate treated with uricase alone (data not shown).

**Copper reduction by the serum ultrafiltrate**

We investigated the ability of the serum ultrafiltrates of M, below 500 to reduce \( \text{Cu}^{2+} \) to \( \text{Cu}^{+} \). In the absence of any addition, the reduction of \( \text{Cu}^{2+} \) to \( \text{Cu}^{+} \) was slow (Fig. 6a). Addition of the ultrafiltrate, however, resulted in a rapid reduction of \( \text{Cu}^{2+} \) to \( \text{Cu}^{+} \), and within the first minute of addition, the ultrafiltrate reduced 2.4\( \mu \text{M} \) of the available 5\( \mu \text{M} \) \( \text{Cu}^{2+} \) to \( \text{Cu}^{+} \). Similarly, uric acid (1\( \mu \text{M} \)) reduced 2.3\( \mu \text{M} \) \( \text{Cu}^{2+} \) to \( \text{Cu}^{+} \) within the first minute of addition. The concentration of \( \text{Cu}^{+} \) increased only very slowly after 1min. Taking into account the reagent blank, the stoichiometry of \( \text{Cu}^{2+} \) reduction was 2 copper ions reduced per molecule of uric acid. LDL (50\( \mu \text{g} \) protein/ml), at the same concentration as used in the oxidation experiments, reduced \( \text{Cu}^{2+} \) to \( \text{Cu}^{+} \) at a slower rate than the ultrafiltrate or uric acid, taking about 4 minutes until the rate of \( \text{Cu}^{2+} \) reduction became very low.

To demonstrate the importance of uric acid within the ultrafiltrate toward copper reduction, the uricase-treated ultrafiltrates were also tested for their copper reducing activity (Fig. 6b). Treatment of the ultrafiltrates with uricase greatly diminished their ability to reduce \( \text{Cu}^{2+} \) to \( \text{Cu}^{+} \), implying that uric acid was the main reductant of \( \text{Cu}^{2+} \) within the ultrafiltrate.
Antioxidant to prooxidant switch and lipid hydroperoxide availability

The switch from antioxidant to prooxidant activity may have been dependent on the availability of lipid hydroperoxides. To test this, we added a lipid hydroperoxide in the form of 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (HPODE; 30nmol/mg LDL protein (about 4% of the maximum content of lipid hydroperoxides in oxidised LDL (see Fig. 2))) to native LDL in the presence or absence of the ultrafiltrate (0.3%, v/v) (Fig. 7). The addition of HPODE to native LDL shortened the lag phase compared to control LDL, as expected (29). The lag phase in the presence of added lipid hydroperoxides was further decreased upon addition of the ultrafiltrate. In the absence of added lipid hydroperoxides, however, adding the ultrafiltrate to native LDL resulted in a much longer lag phase.

Effects of the presence of serum

The effects of adding human whole serum to the system was investigated because interstitial fluid contains proteins, as well as low M_r components (8). Human whole serum at a concentration of 0.3 or 1% (v/v) added at the start of oxidation dose-dependently inhibited the oxidation of LDL, whereas serum at a concentration of 0.1% (v/v) had little effect (Fig. 8). Whole serum at 0.3% (v/v) inhibited the oxidation less than the serum ultrafiltrate at the same concentration, suggesting that the whole serum contained some prooxidant activity or some high M_r factor(s) that prevented the antioxidant activity of the uric acid. Whole serum at 0.1 or 0.3% (v/v) greatly lessened the inhibition of LDL oxidation by the ultrafiltrates (0.3%, v/v). Effective inhibition of LDL oxidation by 1% (v/v) serum, with or without the ultrafiltrate, was observed.
throughout the duration of the experiments. Whole serum at 0.1 or 0.3% also greatly lessened the inhibition of LDL oxidation by 1µM uric acid (results not shown).

Whole serum at 0.3% (v/v) modestly decreased the propagation rate of LDL oxidation when added after the oxidation was already underway, whereas 1% (v/v) serum had a much large antioxidant effect (Fig. 9). Serum at a concentration of 0.1% (v/v) did not inhibit the oxidation. As expected, the addition of serum ultrafiltrates at 0.3% (v/v) accelerated the oxidation. Serum at 0.3 or 1% (v/v), but not at 0.1% (v/v), prevented the prooxidant effect of the ultrafiltrate when they were added together.

Effects of the serum ultrafiltrate toward LDL oxidation by AAPH

The serum ultrafiltrate had no prooxidant activity toward partially-oxidised LDL when the hydrophilic azo initiator AAPH was used to oxidise LDL, having instead a slight antioxidant effect (Fig. 10).
DISCUSSION

A low concentration of a human serum ultrafiltrate of Mr below 500 effectively inhibited the oxidation of native LDL by 5 or 10µM copper ions. This result agrees with the previous observation by Kalant and McCormick (7), who demonstrated that a human serum ultrafiltrate of Mr below 1,000 could inhibit LDL oxidation. In contrast to their effects on native LDL, our ultrafiltrates of below 500Da possessed prooxidant activity toward mildly-oxidised LDL. Similar observations have been reported for a number of different purified compounds (11-20), but this is the first report that a human biological fluid similar in some ways to that which may be present within the arterial wall, can either promote or inhibit LDL oxidation depending upon the oxidation state of the lipoprotein.

In accordance with the results of previous workers (28), the data presented here suggest that uric acid is the major low Mr antioxidant within human serum that protects LDL against oxidation by copper. Removal of uric acid from the ultrafiltrate prevented both the ability of the ultrafiltrate to protect LDL against oxidation by copper and the ability to promote the oxidation of LDL that was already mildly-oxidised.

Supplementation of LDL with HPODE converted the antioxidant effect of the ultrafiltrate toward native LDL into a prooxidant effect similar to that seen with mildly-oxidised LDL. The accumulation of lipid hydroperoxides within LDL may therefore be responsible for the switch from antioxidant to prooxidant activity of the serum ultrafiltrate. In agreement with the findings of Bagnati et al. (19), this change in
behaviour may not be due to the consumption of α-tocopherol, but due to the accumulation of lipid hydroperoxides within the LDL particle.

We propose the following mechanisms to explain the antioxidant effect of the serum ultrafiltrate toward native LDL and its prooxidant effect toward mildly-oxidised LDL. LDL reduces Cu$^{2+}$ to Cu$^{+}$ (fig. 6) (25, 30), maybe because Cu$^{2+}$ reacts with α-tocopherol to form Cu$^{+}$ and the α-tocopheroxyl radical (30).

$$\alpha\text{-tocOH} + \text{Cu}^{2+} \rightarrow \alpha\text{-tocO•} + \text{H}^+ + \text{Cu}^+$$  \hspace{1cm} (reaction 2)

The α-tocopheroxyl radical may then abstract a hydrogen atom from a polyunsaturated fatty acyl group (31), forming a lipid alkyl radical and then a lipid peroxyl radical.

$$\alpha\text{-tocO•} + \text{LH} \rightarrow \alpha\text{-tocOH} + \text{L•}$$  \hspace{1cm} (reaction 3)

$$\text{L•} + \text{O}_2 \rightarrow \text{LOO•}$$  \hspace{1cm} (reaction 4)

The lipid peroxyl radical may then abstract a hydrogen atom from another polyunsaturated fatty acyl group, leading to a chain reaction of lipid peroxidation.

$$\text{LOO•} + \text{L'H} \rightarrow \text{LOOH} + \text{L•}$$  \hspace{1cm} (reaction 5)

Cu$^{2+}$ was immediately reduced to Cu$^{+}$ by both the serum ultrafiltrate (reaction 6) and uric acid (reaction 7) (Fig. 6a). LDL itself could reduce Cu$^{2+}$, as has been observed before (25), but this was slower than Cu$^{2+}$ reduction by the serum ultrafiltrate or uric acid (Fig. 6a).
Ultrafiltrate$^-$ + Cu$^{2+}$ → Ultrafiltrate$^\bullet$ + Cu$^+$ (reaction 6)
Urate$^-$ + Cu$^{2+}$ → Urate$^\bullet$ + Cu$^+$ (reaction 7)

Urate anions (uric acid has a pK_a of 5.4 and therefore from the Henderson-Hasselbalch equation would be expected to be 99% ionised at pH 7.4) within the ultrafiltrate may donate an electron to Cu$^{2+}$, possibly forming a urate anion free radical (reaction 7), which may then donate an additional electron to another Cu$^{2+}$ ion.

Reaction 6 would decrease the concentration of Cu$^{2+}$ and may therefore decrease the rate of reaction 2, thus decreasing the number of $\alpha$-tocopheroxyl radicals produced. This may in turn decrease the rate of initiation of LDL oxidation (reactions 3 - 5). We propose that this mechanism may explain, at least in part, why serum ultrafiltrates (and other antioxidants that reduce Cu$^{2+}$ to Cu$^+$) inhibit the oxidation of native LDL.

Reaction 6 also produces Cu$^+$. Although the breakdown of lipid hydroperoxides is faster with Cu$^+$ (reaction 8) than Cu$^{2+}$ (reaction 9) (32), there will be only a low level of lipid hydroperoxides in native LDL and Cu$^+$ may not be prooxidant until a sufficiently high level of lipid hydroperoxides have accumulated in LDL.

LOOH + Cu$^+$ → LO$^\bullet$ + OH$^-$ + Cu$^{2+}$ (reaction 8)

LOOH + Cu$^{2+}$ → LOO$^\bullet$ + H$^+$ + Cu$^+$ (reaction 9)
Other mechanisms, in addition to the reduction of Cu\textsuperscript{2+} to Cu\textsuperscript{+} and the prevention of \( \alpha \)-tocopherol–mediated peroxidation, may contribute to the antioxidant effect of the ultrafiltrate. These may include conversion of \( \alpha \)-tocopheroxyl radicals back into \( \alpha \)-tocopherol (33) and copper binding by certain components of the ultrafiltrate including uric acid (34), although copper binding may not be the primary antioxidant mechanism due to the nature of the oxidation kinetics (35). Ziouzenkova \textit{et al.} (35) demonstrated two different types of kinetics of LDL oxidation with different concentrations of copper. At a relatively high concentration of copper, the kinetics of LDL oxidation follow the pattern originally described by Esterbauer \textit{et al.} (23). A decrease in the copper concentration to submicromolar levels changed the kinetics so that a lag phase and propagation phase occurred prior to a second lag and propagation phase. Copper chelation by an antioxidant would effectively decrease the concentration of copper available to oxidise LDL and thus the second type of oxidation kinetics may be expected. In the case of the ultrafiltrate, however, the oxidation kinetics are identical to those originally described by Esterbauer \textit{et al.} (23), implying that copper chelation is not the primary antioxidant mechanism. The ultrafiltrates may also have scavenged free radicals in the aqueous phase, as a slight scavenging effect was observed when the hydrophilic azo initiator AAPH was used to oxidise LDL.

The prooxidant activity of the ultrafiltrate towards mildly-oxidised LDL may also be explained by its copper reducing activity. Mildly-oxidised LDL already contains lipid hydroperoxides. The serum ultrafiltrate reduced Cu\textsuperscript{2+} to Cu\textsuperscript{+} (reaction 6) and Cu\textsuperscript{+} rapidly converts lipid hydroperoxides into lipid alkoxyl radicals (reaction 8) much faster than the conversion of lipid hydroperoxides into lipid peroxy radicals by Cu\textsuperscript{2+} (reaction 9)
(32). This may lead to a prooxidant effect as a result of the abstraction by lipid alkoxyl radicals of hydrogen atoms from other polyunsaturated lipids (reaction 10).

\[
\text{LO}^* + \text{L'H} \rightarrow \text{LOH} + \text{L}'^* \quad \text{(reaction 10)}
\]

Alternatively, the lipid alkoxyl radical may propagate lipid peroxidation after first becoming an epoxyperoxyl radical (36). The lipid alkoxyl radicals may also undergo \( \beta \)-scission to form aldehydes and allylic radicals (36, 37).

The antioxidant effect towards native LDL and the prooxidant effect towards partially oxidised LDL were readily observed with 5 or 10\( \mu \)M copper, but were less obvious with 1\( \mu \)M copper. The reasons for this are unknown, but may possibly relate to our finding that LDL has about 40 binding sites for copper per particle (38) and at 50\( \mu \)g of LDL protein/ml (about 0.1\( \mu \)M LDL) there would be insufficient copper at 1\( \mu \)M to bind to all the sites. Alternatively, the ratio of \( \text{Cu}^{2+} \) to \( \text{Cu}^+ \) may vary with the concentration of copper and this may affect the antioxidant/prooxidant activities observed.

As proteins are present in interstitial fluid (8), we investigated the effect of whole serum and serum ultrafiltrate in combination on LDL oxidation. When added to LDL at the start of oxidation in the absence of a serum ultrafiltrate, whole serum at 0.3 and 1\% (v/v), but not at 0.1\% (v/v), inhibited the oxidation. When added in combination, serum at 0.1 or 0.3\% (v/v) greatly lessened the inhibition of LDL oxidation by the ultrafiltrate (0.3\%, v/v). The reason for this is unknown, but it may possibly be due to the binding of uric acid or copper to the serum proteins. The binding of copper to serum proteins...
would lead to a decrease in the concentration of ‘free’ copper. In support of this, the antioxidant effect of the serum ultrafiltrates was less at 1µM copper than at higher concentrations of copper (Fig. 3).

When they were added together to LDL whose oxidation was already underway, whole serum at 0.3 or 1% (v/v) prevented the prooxidant effect of the ultrafiltrate. This may possibly have been due to the peroxidase activity of uric acid in the presence of albumin, as reported by Proudfoot et al. (39).

No prooxidant effect was observed when serum ultrafiltrates were added to LDL partially oxidised by the water-soluble azo initiator AAPH, suggesting that the prooxidant effect may be limited to systems involving transition metal ions.

In conclusion, we report the novel finding that low Mr components prepared from a human physiological fluid can either inhibit or increase LDL oxidation by copper in vitro, depending on the oxidation state of the LDL. Uric acid is the key component responsible for these activities. The components of the serum ultrafiltrates, including uric acid, should be present in the interstitial fluid of atherosclerotic lesions (40) and in direct contact with native or oxidised LDL. Once the lipid hydroperoxide levels within the LDL particle reach a certain threshold level, the low Mr components of the interstitial fluid have the potential to accelerate, rather than inhibit, LDL oxidation. Uric acid in interstitial fluid may protect native LDL against oxidation by transition metal ions, but it may tend to increase the oxidation of mildly-oxidised LDL. The epidemiology of plasma uric acid concentrations and coronary heart disease is
controversial, because plasma uric acid concentrations are related to many of the established risk factors for this disease (41). The question needs to be addressed of whether uric acid would offer antioxidant protection and protect against atherosclerosis or would it tend to act as a prooxidant and encourage the progression of atherosclerosis?
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REFERENCES


FIGURE LEGENDS

Fig. 1 The effect of serum components below 500Da on the oxidation of native or mildly-oxidised LDL. Native LDL (50µg protein/ml) was incubated at 37°C with CuSO₄ (5µM net) in modified HBSS (line 1; ◊). A serum ultrafiltrate of Mr below 500 (0.3%, v/v) was added at zero time (line 2; ■) or after 20 min (line 3; ▲) of oxidation by copper. To monitor conjugated diene formation, the absorbance at 234nm was monitored every 2 min against appropriate reference cuvettes without LDL. The results are expressed as the change in A₂₃₄ and were confirmed by nine other experiments.

Fig. 2 The effect of serum components of Mr below 500 on the oxidation of native or mildly-oxidised LDL. Native LDL (100µg protein/ml) was incubated in triplicate at 37°C with CuSO₄ (10µM net) in modified HBSS (line 1; ◊). Additions of ultrafiltrate were made at zero time (line 2; ■) and after 45 min (line 3; ▲). Oxidation was halted at each time point by the addition of EDTA (1mM) and BHT (20µM), and the lipid hydroperoxide content was measured. The mean ± S.E.M. for the triplicate samples is shown except where the error bar is smaller than the symbol. The results shown were confirmed by two other experiments. The ultrafiltrate (0.3%, v/v) did not have significant absorbance at 234nm and did not interfere with the lipid hydroperoxide assay.

Fig. 3. The effect of serum components of Mr below 500 on the oxidation of native or mildly-oxidised LDL by different concentrations of copper. Native LDL (50µg protein/ml) was incubated at 37°C with CuSO₄ at a net concentration of 1µM (open symbols) or 10µM (closed symbols) in modified HBSS (line 1, ◊ or line 2, ♦). A
serum ultrafiltrate of Mₜ below 500 (0.3%, v/v) was added at zero time (line 3, □ or line 4, □) or after 68 min (line 5; △) or 48 min (line 6, ▲) of oxidation by copper. (The conjugated dienes had increased to about the same levels at 68 and 48 min with 1 and 10µM copper, respectively.) To monitor conjugated diene formation, the absorbance at 234nm was monitored against appropriate reference cuvettes without LDL. The results are expressed as the change in A₂₃₄ and were confirmed by another experiment.

**Fig. 4** The effect of uric acid on the oxidation of native or mildly-oxidised LDL. Native LDL (50µg protein/ml) was incubated at 37°C with CuSO₄ (5µM net) in modified HBSS (line 1; ◇). Additions of uric acid (1µM) were made at zero time (line 2; □) or after 33 min (line 3; ▲). The absorbance at 234nm was monitored every 2 min against appropriate reference cuvettes without LDL. The results shown were confirmed by five other experiments.

**Fig. 5** Identification of the component responsible for the antioxidant and prooxidant activity of serum ultrafiltrates towards native or mildly-oxidised LDL. Native LDL (50µg protein/ml) was incubated at 37°C with CuSO₄ (5µM net) in modified HBSS (line 1; ◇). Addition of 0.3% (v/v) ultrafiltrate of Mₜ below 500 (T=0, line 2, □; T=45, line 5, ▲), uricase-treated ultrafiltrate (T=0, line 3, +; T=45, line 6, ⊗) or uricase-treated ultrafiltrate plus 1µM uric acid (T=0, line 4, ●; T=45, line 7, ⧫) were made at zero time or after 45 min. The absorbance at 234nm was monitored every 2 min against appropriate reference cuvettes without LDL. The results are expressed as the change in A₂₃₄ and were confirmed by three other experiments.
Fig. 6  The reduction of Cu$^{2+}$ to Cu$^{+}$ by serum ultrafiltrates.  (a) CuSO$_4$ (5µM) was incubated alone (line 1; ◊), with LDL (50µg protein/ml; line 2; ▲), a serum ultrafiltrate (0.3%, v/v; line 3; ■) or uric acid (1µM; line 4; ●) at 37°C in the presence of bathocuproine disulphonic acid (360µM). The absorbance at 480nm was monitored against time, and the Cu$^{+}$ concentration was calculated from the molar absorption coefficient of Cu$^{+}$-bathocuproine disulphonic acid. Similar results were obtained in two other experiments.  (b) Cu$^{2+}$ (5µM) was added to serum ultrafiltrates (0.3%, v/v) or uric acid (1µM) or to ultrafiltrates or uric acid treated with uricase. Bathocuproine disulphonic acid was added immediately to quantify the amount of copper present as Cu$^{+}$. The mean ± S.E.M., for triplicate samples from 3 individual experiments using serum ultrafiltrates prepared from 3 different individuals are shown. P<0.05 for the control vs ultrafiltrate, control vs uric acid, ultrafiltrate vs uricase-treated ultrafiltrate, and for uric acid vs uricase-treated uric acid.

Fig. 7  Induction of the antioxidant to prooxidant switch of a serum ultrafiltrate by the addition of lipid hydroperoxides to native LDL. Native LDL was incubated as described in figure 1(a) in the absence (line 1, ◊) or presence (line 2, ■) of a serum ultrafiltrate of M$_r$ below 500 (0.3%, v/v). HPODE (Affinity, Exeter, Devon, U.K.) was added to give a final concentration of 30nmol/mg LDL protein from a stock solution of 160µM in ethanol in the absence (line 3, ○) or presence (line 4, ▲) of the ultrafiltrate (0.3%, v/v). Oxidation was monitored by following the formation of conjugated dienes, and values are expressed as the change in $A_{234}$. Ethanol at the final concentration of 0.975 % (v/v) did not affect the oxidation of LDL. Similar results were obtained in two other experiments.
Fig. 8. Effect of whole serum on the oxidation of native LDL in the presence of a serum ultrafiltrate. Native LDL (50µg protein/ml) was incubated at 37°C with 5µM CuSO₄ (5µM net) in modified HBSS (line 1; ◇). A serum ultrafiltrate of Mᵋ below 500 (0.3%, v/v) (line 2; □) and whole serum at 0.1% (line 3, ▲), 0.3% (line 4; △) or 1% (v/v) (line 5; ※) were added at zero time. Whole serum at 0.1% (line 6, ●), 0.3% (line 7, ◆) or 1% (v/v) (line 8; □) were also added at zero time in the presence of the serum ultrafiltrate (0.3%, v/v). The formation of conjugated dienes over time was measured. The reference cuvettes contained all the components except LDL. The results were confirmed by another experiment.

Fig. 9. Effect of whole serum on the oxidation of mildly-oxidised LDL in the presence of a serum ultrafiltrate. LDL (50µg protein/ml) was incubated at 37°C with 5µM CuSO₄ (5µM net) in modified HBSS and the formation of conjugated dienes over time was measured (line 1; ◇). At about 54min, a serum ultrafiltrate of Mᵋ below 500 (0.3%, v/v) (line 2; □) or whole serum at 0.1% (line 3, ▲), 0.3% (line 4; △) or 1% (v/v) (line 5; ※) were added. Whole serum at 0.1% (line 6, ●), 0.3% (line 7, ◆) or 1% (v/v) (line 8; □) was also added in the presence of the serum ultrafiltrate (0.3%, v/v). The reference cuvettes contained all the components except LDL. Due to the number of additions, not all the components could be added at exactly the same time. The results were confirmed by another experiment.

Fig. 10. Effect of serum components of Mᵋ below 500 on the oxidation of native or partially-oxidised LDL by AAPH. Native LDL (50µg protein/ml) was incubated at
37°C with AAPH (1mM) and the formation of conjugated dienes over time was measured (line 1; ◊). A serum ultrafiltrate of Mr below 500 (0.3%, v/v) was added at zero time (line 2; ■) or after 108min (line 3; ▲), 172min (line 4; ○) or 273min (line 5; △). The reference cuvettes contained all the components except LDL. The results were confirmed by two other experiments.