Human serum, cysteine and histidine inhibit the oxidation of low density lipoprotein less at acidic pH

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Abstract Low concentrations of serum or interstitial fluid have been shown to inhibit the oxidation of low density lipoprotein (LDL) catalysed by copper or iron, and may therefore protect against the development of atherosclerosis. As atherosclerotic lesions may have an acidic extracellular pH, we have investigated the effect of pH on the inhibition of LDL oxidation by serum and certain components of serum. Human serum (0.5%, v/v), lipoprotein-deficient human serum at an equivalent concentration and the amino acids L-cysteine (25 μM) and L-histidine (25 μM), but not L-alanine (25 μM), inhibited effectively the oxidation of LDL by copper at pH 7.4, as measured by the formation of conjugated dienes. The antioxidant protection was reduced considerably at pH 6.5, and was decreased further at pH 6.0. These observations may help to explain why LDL becomes oxidised locally in atherosclerotic lesions in the presence of the strong antioxidant protection offered by extracellular fluid.

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Key words: Acidic pH; Antioxidant; Atherosclerosis; Copper; Low density lipoprotein; Oxidized low density lipoprotein

1. Introduction

Oxidised low density lipoprotein (LDL) may be involved in the pathogenesis of atherosclerosis in numerous ways [1,2]. Serum is a potent antioxidant and can protect LDL against oxidation by macrophages [3,4]. Albumin, the most abundant plasma protein, also inhibits LDL oxidation [5]. Amino acids, for example, cysteine [6–9] and histidine [10], can also protect LDL against oxidation. Plasma may thus protect LDL from oxidation in the circulation and this may help to explain why extensively oxidised LDL is probably not formed in the bloodstream. Dabbagh and Frei [4] reported the presence of significant concentrations of antioxidants within suction blister interstitial fluid (which is considered analogous to normal interstitial fluid) from humans and this fluid inhibited LDL oxidation catalysed by iron or copper effectively. Evidence does exist, however, for the presence of oxidised LDL within atherosclerotic lesions [11].

Atherosclerotic lesions may have an acidic extracellular pH [12,13]. These lesions show similarities to inflammatory sites, and inflammatory sites are known to have a decreased extracellular pH [14]. The deeper layers of the arterial wall are ischaemic [15] and anaerobic glycolysis may therefore be required for energy production with the resultant generation of lactic acid. Smith [16] has calculated that significant production of lactic acid may occur within the deeper regions of the human aortic intima and this may contribute to a localised lactic acidosis. Macrophages in atherosclerotic lesions are in a state of partial activation [17] and activated macrophages in vitro are capable of acidifying their immediate environment to a pH as low as 3.6 [18].

In this study, we have investigated the effect of pH on the antioxidant activity towards LDL oxidation of whole serum and lipoprotein-deficient serum and of cysteine, histidine and alanine, as examples of the components of extracellular fluids. All the antioxidant activities (except of alanine) were diminished substantially at acidic pH. This may help to explain why LDL oxidation occurs in atherosclerotic lesions in the presence of the presumably substantial antioxidant activity of the interstitial fluid of these lesions.

2. Materials and methods

2.1. LDL isolation

LDL (1.019–1.063 g/ml) was isolated in the presence of EDTA by sequential density ultracentrifugation from blood donated by healthy volunteers, as described previously [19], and stored aseptically at 4°C for up to 4 weeks in 140 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 and 100 μM EDTA-Na_2, pH 7.4.

2.2. Preparation of serum and lipoprotein-deficient serum

Blood from healthy volunteers was allowed to coagulate and form a blood clot at room temperature. The blood was then centrifuged at 4°C for 15 min at $250 \times g$ and the serum was removed and recentrifuged at 4°C for a further 10 min at $250 \times g$, to remove any remaining fragments of the clot.

Liposorb (Calbiochem-Novabiochem, Nottingham, UK) was used to prepare lipoprotein-deficient serum. 12.5 ml of 150 mM NaCl (pH 7.4) was added to 1 g of dry Liposorb and allowed to equilibrate and swell for 15 min. The mixture was then boiled briefly for 10 s at full power in a 600 W microwave oven, to form larger aggregates, and allowed to settle. Prior to use the Liposorb was centrifuged at $9447 \times g$ for 10 min to remove any excess fluid.

Serum (250 μl) was incubated with Liposorb (250 mg wet weight) at 4°C overnight to remove all the lipoproteins. It was then centrifuged at $9447 \times g$ for 10 min and the supernatant was recentrifuged under the same conditions to remove all the Liposorb. Agarose gel electrophoresis (Paragon Gels, Beckman, USA) showed the treatment removed the lipoprotein bands seen in whole serum. All the cholesterol was removed from the serum, as measured by a cholesterol kit (Boehringer Mannheim, Lewes, East Sussex, UK). The treatment decreased the protein content of serum by 45–50% as measured by a modified Lowry assay [20], which was accounted for by the dilution of the serum by the NaCl solution used to swell the Liposorb prior to use.

2.3. Measurement of conjugated dienes

LDL (50 μg protein/ml) was incubated in quartz cuvettes (1 cm light path) at 37°C with $CuSO_4$ (5 μM above the final concentration of EDTA carried over from the LDL preparation, which was less than 1 μM) in modified Hanks' balanced salt solution (HBSS). HBSS was prepared from 5 mM H_3PO_4, 113 mM NaCl, 5.36 mM KCl, 0.81 mM $MgSO_4$ and 1.26 mM $CaCl_2$ and adjusted to pH 7.4, 6.5 or 6 with NaOH in the presence of Chelex-100 (1 g/l) (Sigma; pre-washed with distilled water to remove any antioxidant [21]), to remove any con-

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taminating transition metal ions. Freshly dissolved L-cysteine, L-histidine or L-alanine (Sigma) was added (to give a final concentration of 25 μM), where appropriate. The absorbance at 234 nm was recorded in a Perkin-Elmer Lambda 2 split beam UV/visible spectrophotometer at regular intervals, as a measure of the accumulation of conjugated dienes [22].

The formation of conjugated dienes in LDL in the presence of serum or lipoprotein-deficient serum was recorded at 245 nm, as described by Schnitzer et al. [23]. Serum and lipoprotein-deficient serum have a substantial absorbance at 234 nm, but the ratio of absorbance due to these and that due to conjugated dienes is considerably less at 245 nm than at 234 nm [23]. The addition of serum, cysteine, histidine or alanine at the above concentrations did not affect the pH of the buffers at pH 7.4, 6.5 or 6.0.

Reference cuvettes were prepared in the same manner as the test cuvettes, but without LDL, and their absorbance was automatically subtracted by the spectrophotometer from that of the test cuvettes. The lag period was taken as the intercept between the tangent to the curve during the propagation phase of lipid peroxidation and the abscissa. The rate of propagation of lipid peroxidation was calculated as the gradient of the tangent to the curve during the propagation phase [22].

3. Results

3.1. The effect of pH on LDL oxidation by copper

LDL oxidation by copper, monitored by following the formation of conjugated dienes, exhibited the typical pattern described by Esterbauer et al. [22], with a lag phase, a propagation phase in which the conjugated dienes increased rapidly and a decomposition phase (Fig. 1). Decreasing the pH from 7.4 to 6.5 or 6.0 increased progressively the length of the lag phase and decreased progressively the rate of propagation (Fig. 1 and Table 1). The maximum levels of conjugated dienes were similar at the various pH values.

3.2. Effect of pH on the inhibition of LDL oxidation by serum

A low concentration (0.5%, v/v) of normal human serum protected LDL effectively from oxidation by copper at pH 7.4 (Fig. 1 and Table 1). There was an initial increase in A_{245} in the presence of serum, which continued to increase at a lower rate prior to the rapid increase in conjugated dienes characteristic of the propagation phase. A similar pattern has been observed in the presence of albumin [23]. The lag phase before the propagation phase was increased over 10-fold and the rate of propagation was inhibited by 85% by serum. The antiox-
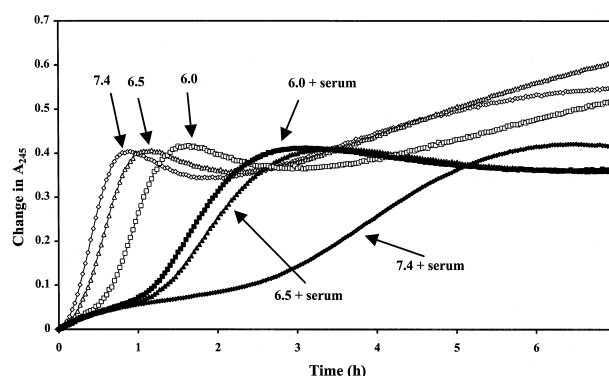

Fig. 1. Effect of pH and serum on LDL oxidation by copper ions as measured by conjugated dienes. LDL (50 μg protein/ml) was oxidised at 37°C in modified HBSS of various pH values in the presence of $CuSO_4$ (5 μM net), with (filled symbols) or without (open symbols) serum (0.5%, v/v). The absorbance at 245 nm was monitored against appropriate reference cuvettes without LDL every 2 min. The results shown are representative of five independent experiments.

idant effect of the serum was less at pH 6.5 than at pH 7.4. At pH 6.5, the increase in the lag phase due to serum was 3.8-fold, and the rate of the propagation phase was decreased less than at pH 7.4. Decreasing the pH to 6.0 resulted in an additional decrease in the antioxidant effect of the serum, the lag phase being increased by only 1.8-fold. The lag phase in the presence of serum was decreased and the rate of propagation was increased as the pH was lowered, reversing the pattern observed in the absence of serum.

We also investigated the effect of pH and lipoprotein-deficient serum on the oxidation of LDL, as serum would contain lipoproteins (albeit at low concentrations in 0.5% (v/v) serum). We used a concentration of 1% (v/v) of lipoprotein-deficient serum because, as described in the methods section, the protein concentration was reduced by 45–50% by the Liposorb used to remove the serum lipoproteins (probably due to the dilution of the serum proteins by the NaCl solution used to swell the Liposorb powder). The serum and lipoprotein-deficient serum would therefore have been used at about the same protein concentration. Lipoprotein-deficient serum inhibited LDL oxidation, but the inhibition was less at acidic pH

Table 1
The change in lag time, propagation rate and maximum increase in conjugated dienes in LDL oxidised by copper in the presence or absence of serum or lipoprotein-deficient serum at various pH values

pH	Addition of inhibitor	Lag (min)	Fold increase in lag phase due to inhibitor	Propagation rate (change in A_{245}/min)	Decrease in propagation rate with inhibitor (%)	Max increase in A_{245}
7.4	No addition	10 ± 2		0.0132 ± 0.0003		0.404 ± 0.006
	+Serum	107 ± 7	10.7	0.0020 ± 0.0001	85	0.410 ± 0.021
6.5	No addition	16 ± 2		0.0111 ± 0.0002		0.404 ± 0.006
	+Serum	61 ± 2	3.8	0.0039 ± 0.0002	65	0.417 ± 0.014
6.0	No addition	28 ± 2		0.0085 ± 0.0004		0.391 ± 0.010
	+Serum	49 ± 6	1.8	0.0047 ± 0.0004	45	0.403 ± 0.012
7.4	No addition	37 ± 2		0.0142 ± 0.0014		0.407 ± 0.011
	+LPDS	70 ± 1	1.9	0.0041 ± 0.0002	71	0.420 ± 0.023
6.5	No addition	41 ± 1		0.0104 ± 0.0001		0.402 ± 0.005
	+LPDS	50 ± 6	1.2	0.0063 ± 0.0003	39	0.403 ± 0.009
6.0	No addition	46 ± 2		0.0084 ± 0.0003		0.403 ± 0.011
	+LPDS	45 ± 2	1.0	0.0071 ± 0.0001	15	0.433 ± 0.010

Serum was added at 0.5% (v/v) and the lipoprotein-deficient serum at 1% (v/v). The mean ± S.E.M. is shown for five individual experiments for serum and three for lipoprotein-deficient serum (LPDS). Different batches of LDL were used for the experiments involving serum and lipoprotein-deficient serum.
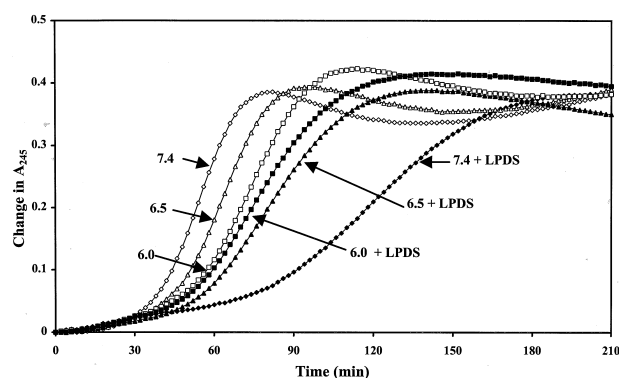
Fig. 2. Effect of pH and lipoprotein-deficient serum on LDL oxidation by copper ions as measured by conjugated dienes. LDL was incubated as described in Fig. 1 with or without lipoprotein-deficient serum (1% v/v, an approximately equivalent protein concentration to the serum used in Fig. 1). The results shown are representative of three independent experiments.
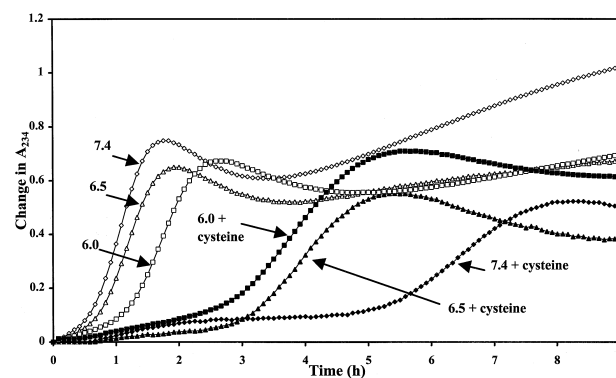

Fig. 3. Effect of pH and L-cysteine on LDL oxidation by copper ions as measured by conjugated dienes. LDL was incubated as described in Fig. 1, with or without freshly dissolved 25 μM cysteine. The absorbance at 234 nm was monitored against appropriate reference cuvettes without LDL every 5 min. The results shown are representative of three independent experiments.

than at pH 7.4 (Fig. 2 and Table 1), as was the case with serum. In the presence of lipoprotein-deficient serum, the oxidation was faster at acidic pH than at pH 7.4.

3.3. Effect of pH on the inhibition of LDL oxidation by the amino acids cysteine, histidine and alanine

Cysteine (25 μM) was an effective antioxidant against LDL oxidation by copper at pH 7.4 (Fig. 3). It resulted in about an eight-fold increase in the duration of the lag phase and a decrease in the rate of propagation of 77% (Table 2). At pH 6.5, cysteine increased the duration of the lag phase by only about four-fold and decreased the rate of propagation by 56%. The inhibition by cysteine of LDL oxidation decreased further at pH 6.0.

LDL oxidation by copper was inhibited more effectively by L-histidine (25 μM) than by an equimolar concentration of L-cysteine (Fig. 4 and Table 2). A similar effect of pH was

observed for histidine as for cysteine, in that histidine had a lower antioxidant activity at acidic pH than at pH 7.4. The oxidation of LDL was faster at acidic pH than at pH 7.4 in the presence of either histidine or cysteine.

In the presence of cysteine or histidine, there was an initial increase in A_{234} followed by a lower rate of increase before the propagation phase of oxidation of LDL was reached, which was similar to the effect observed at 245 nm with serum.

Alanine (25 μM), which lacks the functional groups of the side chains of cysteine or histidine, increased slightly the duration of the lag phase of LDL oxidation, but was a much less effective antioxidant than cysteine or histidine (Fig. 5 and Table 2). The formation of conjugated dienes in LDL by copper was slower at acidic pH than at pH 7.4 in the presence or absence of alanine, unlike the case with the other two amino acids.

Table 2
The change in lag time, propagation rate and maximum increase in conjugated dienes of LDL oxidised by copper in the presence or absence of L-cysteine, L-histidine or L-alanine at various pH values

pH	Addition of amino acid	Lag (min)	Fold increase in lag phase due to amino acid	Propagation rate (change in A_{234}/min)	Decrease in propagation rate with amino acid (%)	Max increase in A_{234}
7.4	No addition	33 ± 3		0.0180 ± 0.0025		0.735 ± 0.006
	+Cysteine	275 ± 33	8.3	0.0042 ± 0.0005	77	0.639 ± 0.060
6.5	No addition	43 ± 2		0.0153 ± 0.0025		0.752 ± 0.059
	+Cysteine	174 ± 21	4.0	0.0068 ± 0.0013	56	0.727 ± 0.087
6.0	No addition	61 ± 6		0.0105 ± 0.0028		0.781 ± 0.054
	+Cysteine	174 ± 14	2.9	0.0056 ± 0.0008	47	0.751 ± 0.024
7.4	No addition	32 ± 4		0.0220 ± 0.0019		0.770 ± 0.002
	+Histidine	475 ± 83	14.7	0.0029 ± 0.0004	87	0.638 ± 0.090
6.5	No addition	37 ± 5		0.0182 ± 0.0004		0.784 ± 0.040
	+Histidine	267 ± 18	7.2	0.0050 ± 0.0004	73	0.756 ± 0.037
6.0	No addition	50 ± 3		0.0144 ± 0.0003		0.775 ± 0.021
	+Histidine	249 ± 29	5	0.0050 ± 0.0008	65	0.714 ± 0.085
7.4	No addition	23 ± 0.2		0.0282 ± 0.0044		0.925 ± 0.108
	+Alanine	29 ± 0.3	1.3	0.0277 ± 0.0045	2	0.977 ± 0.121
6.5	No addition	33 ± 1.0		0.0209 ± 0.0024		0.820 ± 0.028
	+Alanine	36 ± 1.0	1.1	0.0193 ± 0.0030	8	0.866 ± 0.098
6.0	No addition	48 ± 1.0		0.0157 ± 0.0018		0.816 ± 0.066
	+Alanine	53 ± 0.6	1.1	0.0154 ± 0.0020	2	0.857 ± 0.085

The amino acids were added at a concentration of 25 μM. The mean ± S.E.M. is shown for three individual experiments using different batches of LDL.

4. Discussion

As expected, the early stages of LDL oxidation by copper were slower at acidic pH than at pH 7.4, in agreement with previously published work [8,24]. Although the earlier stages of LDL oxidation are delayed at acidic pH, the later stages of oxidation leading to increased uptake of LDL by macrophages are potentiated at acidic pH [8].

It has previously been reported [3,4] that LDL oxidation is inhibited potently by serum in vitro, and the results presented here agree with this finding. A low concentration of serum or lipoprotein-deficient serum effectively delayed the onset of LDL oxidation by copper at pH 7.4. Cysteine (25 µM), at a near plasma concentration (33 µM) [25], also inhibited effectively the oxidation of LDL by copper, in agreement with previous findings [6–9]. Histidine is an effective chelator of copper (log stability constant 10.2 [26]) and at a concentration (25 µM) much below its plasma level (which is about 90 µM [25]) was an excellent antioxidant for LDL at pH 7.4 in this study.

In the presence of serum, cysteine or histidine, there was an initial increase in the A_{245} or A_{234} of LDL incubated with copper. The rate of oxidation then became less but the oxidation continued to increase slowly until the rate of oxidation increased rapidly during the propagation phase. Similar observations have been described previously for albumin [23] and the initial increase in oxidation ascribed to tocopherol-mediated peroxidation [23,27].

Alanine was included in this study to show that the antioxidant effect of cysteine and histidine involved the side chains of these amino acids. Alanine, with simply a methyl group as a side chain, was a poor antioxidant compared to cysteine, which has a methylene and thiol group as a side chain, and histidine, which has a methylene and imidazole group as its side chain.

The antioxidant protection provided by whole serum, lipoprotein-deficient serum, cysteine and histidine was decreased considerably at acidic pH. In the absence of these antioxidants, the formation of conjugated dienes in LDL incubated with copper was slower at acidic pH, whereas in their presence the formation of the conjugated dienes was faster at acidic pH.

We propose that an acidic pH decreases the binding of

Fig. 5. Effect of pH and L-alanine on LDL oxidation by copper ions as measured by conjugated dienes. LDL was incubated as described in Fig. 1, with or without freshly dissolved 25 µM L-alanine. The absorbance was monitored at 234 nm every 2 min for the duration of the experiment. The results were confirmed by two other independent experiments.

copper to these antioxidants. The amount of copper available to catalyse LDL oxidation would therefore be increased at acidic pH. Less copper may bind to proteins, cysteine (or more likely, its oxidation products) or histidine at acidic pH, due to the protonation of the residues involved in copper binding. The imidazole group of histidine (as the free amino acid) has a pK_a of 6.0 and the Henderson-Hasselbach equation predicts that at pH 7.4 only about 4% of these groups would be present in the protonated form, whereas at pH 6.5 this increases to 24%, and at pH 6.0 to 50%. (It should be noted, however, that the pK_a values of the ionisable groups may be affected by their binding of copper.) The result of the protonation of such amino acids may be to decrease copper binding to them, due to electrostatic repulsion, and consequently more copper may be available to oxidise LDL. The thiol group of cysteine has a pK_a of 8.35 and if the negatively charged thiolate form were to bind copper ions more avidly than does the non-dissociated form, an acidic pH would be expected to decrease the binding of copper to cysteine. The thiol group of cysteine is rapidly oxidised by copper [28], however, and so this consideration may not apply. The effect of pH on the binding of copper to the oxidation products of cysteine is unclear.

In conclusion, atherosclerotic lesions may possibly have an acidic extracellular pH [13] and the decreased antioxidant protection at acidic pH by components of the interstitial fluid of these lesions may help to explain why LDL oxidation occurs at these sites.
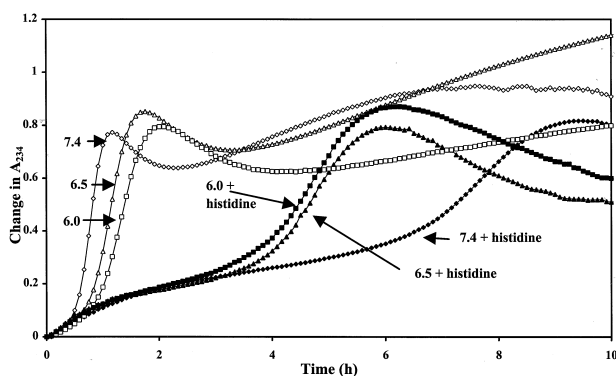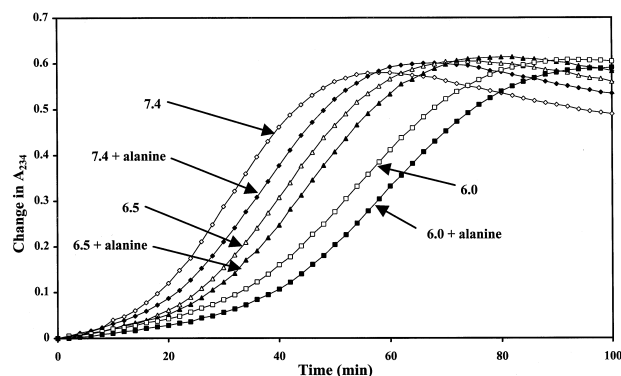
Fig. 4. Effect of pH and L-histidine on LDL oxidation by copper ions as measured by conjugated dienes. LDL was incubated as described in Fig. 1, with or without freshly dissolved 25 µM L-histidine. The absorbance was monitored at 234 nm every 5 min for the duration of the experiment. The results were confirmed in two other independent experiments.

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