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Redistribution of metal ions to control low density lipoprotein oxidation in Ham's F10 medium

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Abstract
The study of cell-mediated low density lipoprotein (LDL) oxidation has traditionally been undertaken using Ham's F10 media due to its high metal content and low levels of antioxidants. Although there has been no acknowledged change to this media in recent years by the suppliers, Ham's F10 medium has been found to be extremely inconsistent in its promotion of LDL oxidation in the absence of cells. This variability contrasts with the relatively consistent rates of THP-1 cell-mediated LDL oxidation. This study has now shown that the variability in cell-free LDL oxidation is medium-dependent and not an artefact of experimental protocol. It presents evidence that suggests the variable rates of cell-free LDL oxidation are caused by iron auto-oxidation during storage of the Ham's F10 medium. The medium can be standardized by removal of all transition metals, by treatment with Chelex, before the addition of known amounts of iron or copper. This treatment generates a cell culture medium that only allows very slow LDL oxidation in the absence of cells.

Keywords: *Low-density-lipoprotein, oxidation, cell-free, Ham's-F10, iron*

Introduction

Ham's F10 has been the traditional medium of choice when studying the cell-mediated oxidation of low density lipoprotein (LDL) [1–8]. It has several advantages when compared to other types of media, being more nutrient-rich than minimal media like Hanks' Balanced Salt Solution (HBSS) and Earle's Balanced Salt Solution (EBSS), but less enriched than RPMI. This balance enables Ham's F10 to effectively maintain cells in a healthy state for several days while limiting the level of any compounds that could potentially inhibit the pro-oxidant activity of the incubating cells [9]. For this reason, Ham's F10 requires supplementation with a lower level of transition metal ions than RPMI 1640 to achieve an equivalent level of cell-mediated LDL oxidation [10]. It is also less likely than RPMI to interfere with the antioxidant activity of any compounds studied [9].

Although the suppliers (both Gibco BRL (Auckland, New Zealand) and Sigma (St Louis, USA)) have recorded no change in the chemical composition of the Ham's F10 from one batch of medium to the next, we have found major inconsistencies in the rate of cell-free LDL oxidation, both within and between batches of medium. This difference from one preparation to the next ranged from no oxidation to oxidation occurring at rates faster than in the presence of cells. In contrast, the cell-mediated rates of LDL oxidation appeared to remain relatively constant.

The cell-free (LDL only) control is important because a low basal level of LDL oxidation is normal

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in the presence of transition metals. Cells have previously been reported to enhance this oxidation by either increasing the level of reduced metal ions or providing additional oxidants [11–14]. Unpredictable changes in the rate of cell-free LDL oxidation have made it difficult to study cell-mediated LDL oxidation in a systematic manner and to draw firm conclusions on the various potential processes. This paper characterizes the variation seen during cell-free LDL oxidation in Ham's F10 medium and provides an effective means of removing the cause of the inconsistency.

Materials and methods

Materials

Chemicals and reagents were AR grade or better and, unless otherwise stated, were obtained from either Sigma Chemical Company (St Louis, USA) or BDH Chemicals supplied by Biolab (Auckland, New Zealand). Chelex-100 resin was supplied by Bio-Rad Laboratories (Auckland, NZ) and Falcon brand tissue culture plasticware was supplied by BD Biosciences Ltd (Auckland, New Zealand). The Ham's F10 medium was supplied by Gibco BRL through Invitrogen NZ Ltd (Auckland, New Zealand), Sigma Chemical Co (St Louis, USA) and prepared as specified by the manufacturers. All solutions were prepared with high purity water from a NANOpure ultrapure water system, supplied by Barnstead/Thermolyne (Iowa, USA). Phosphate buffered saline (PBS) solution consisted of 150 mM sodium chloride and 10 mM sodium phosphate, pH 7.4.

Hams F10 Chelex treatment

In a 50 ml centrifuge tube, 1 g of Chelex-100 resin was added to 50 ml of Ham's F10 medium. The Chelex resin had been previously washed with water. The centrifuge tube was placed on a rotary mixer at 10 rpm for 4 h at 4°C. The Chelex was subsequently pelleted by centrifugation and the Ham's F10 was filter-sterilized through a 0.22 μM membrane filter.

LDL preparation

Whole blood was collected from healthy, fasting volunteers into tubes containing 0.1% EDTA (pH 7.4). Plasma was isolated by centrifugation before being pooled from multiple donors and stored in 0.6% sucrose at −80°C [15]. LDL was subsequently purified from this pooled plasma by a 22 h ultracentrifugation, with a four step discontinuous gradient, in a Beckman SW41 rotor [15]. The purified LDL was desalted by dialysis for 24 h against four changes of nitrogen-gassed Chelex-treated PBS (pH 7.4). An LDL concentration was calculated using a CHOL reagent kit (Roche Chemicals, NZ), assuming cholesterol accounts for 31.6% of the LDL particle by weight and the molecular weight of LDL is 2.5 MDa [15].

Cell culture

THP-1 cells were maintained in suspension in RPMI 1640, supplemented with 5% heat-inactivated foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO_2. The THP-1 monocytes were converted to adherent macrophage-like cells by incubating 1×10^5 cells/ml with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) in standard six well plates for 7 days [16].

Immediately prior to an experiment, the cells were washed three times with PBS and Ham's F10 medium (Chelexed or non-Chelexed) was added to each well. Depending on experimental requirements, each well was then supplemented with ferrous sulphate, ferric chloride and/or copper chloride and a pre-incubation period of 5 min was allowed before the inclusion of filter sterilized LDL (final concentration, 40 nM). Cell-free controls, lacking cells but containing all other reagents, were also prepared. Samples were incubated for various times before analysis of the thiobarbituric acid reactive substances (TBARS), α-tocopherol or protein hydroperoxide content of the LDL.

TBARS assay

Lipid peroxidation was measured as TBARS, by derivatization with thiobarbituric acid (TBA) and analysis using reverse phase high performance liquid chromatography (HPLC) with fluorescence detection [17]. Further oxidation during analysis was prevented by the addition of BHT (in methanol) to all samples [18].

α-Tocopherol assay

α-Tocopherol levels were monitored at selected time points by removing 100 μl of medium and halting the oxidation reaction in 400 μl of water, containing 10 μl of 100 mg/ml ethylenediaminetetraacetic acid (EDTA, pH 7.4) and 25 μl of 20 mg/ml butylated hydroxytoluene (BHT, in methanol). The α-tocopherol was subsequently extracted into hexane and dried under oxygen-free nitrogen gas. The resulting residue was dissolved in methanol and detected fluorometrically by HPLC, using excitation and emission wavelengths of 292 nm and 335 nm, respectively, and a mobile phase of 100% methanol [19].

FOX assay

Protein hydroperoxide formation on LDL was measured as described by Gieseg et al. [20]. Briefly,

proteins were precipitated from the medium using trichloroacetic acid (TCA). The resulting pellet was vortexed in 1 ml of 1:1 chloroform:methanol, centrifuged and air dried at room temperature. Once dry, the pellet was resuspended in 900 µl of 50% $^{v}/_{v}$ glacial acetic acid and mixed with 50 µl each of xylenol orange and ferrous ammonium sulphate (both 5 mM in 25 mM sulphuric acid). Samples were incubated at room temperature in the dark for 30 min and the absorbance subsequently measured at 560 nm against a water blank. An extinction co-efficient of 48 000 M^{-1} cm^{-1} was used to calculate the protein hydroperoxide concentration [21].

A basic FOX assay enabled quantification of iron(II) and iron(III) levels in the Ham's F10 [21]. The medium was adjusted to pH 1.7–1.8 with sulphuric acid before being mixed with 50 µl of 5 mM xylenol orange (in 25 mM sulphuric acid) and 50 µl of either water or 1 mM hydrogen peroxide. (Hydrogen peroxide was used to oxidize any iron(II) to iron(III) in the media.) Samples were incubated in the dark for 30 min before measuring peak absorbance at 560 nm against a blank, using an extinction co-efficient of 44 000 M^{-1} cm^{-1} [21]. Iron(II) levels were calculated as the difference between absorbances in the presence and absence of hydrogen peroxide.

Thiol assay

The thiol concentration in Ham's F10 was analysed by incubating medium with 30 µM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, final concentration) for 30 min at room temperature. The absorbance was then measured at 412 nm, against a blank, using an extinction co-efficient of 13 600 M^{-1} cm^{-1} [22].

Statistical analysis

Data were analysed using the Prism software package, supplied by Graphpad Software Inc (San Diego, CA, USA). Statistical significance was confirmed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Results shown are from single experiments, representative of a minimum of three and are expressed as mean ± SEM of triplicate treatments.

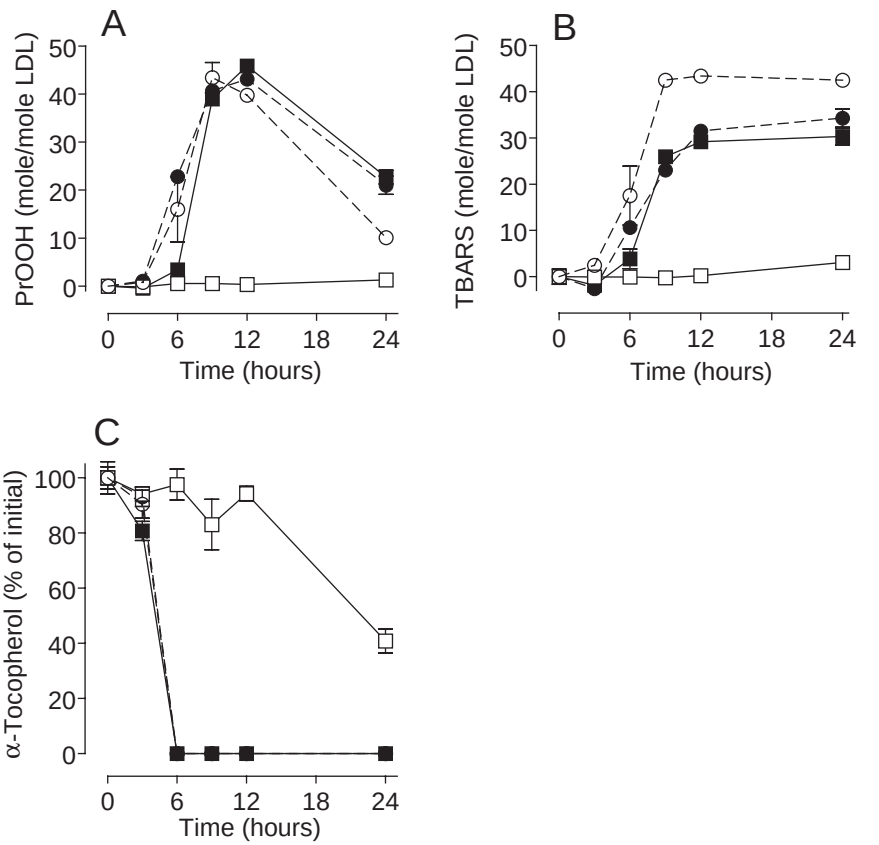

Figure 1. Variable rates of cell-free LDL oxidation in Ham's F10 medium. LDL (0.1 mg/ml) was incubated at 37°C in Ham's F10, supplemented with 25 mM HEPES and 1.5 µM $FeSO_4$, in the absence (square) or the presence (circle) of adherent THP-1 macrophage-like cells (1×10^5 cells/ml). Samples were analysed at various time points for an increase in (A) protein hydroperoxides (PrOOH) and (B) TBARS and (C) a loss of vitamin E. Medium 1 (closed shapes) and medium 2 (open shapes) were prepared from the same batch of Ham's F10 but stored in different bottles. Results are displayed as mean ± SEM of triplicates.

Results

The rate of THP-1 macrophage-mediated LDL oxidation remained relatively constant between experiments (Figure 1). Inconsistency in the Ham's F10 only became apparent when monitoring the rate of cell-free LDL oxidation (squares), with the lag phase varying from anywhere between 3 h to over 24 h (Figure 1). In some experiments the cell-free oxidation proceeded so fast that no significant difference existed between incubations in the presence and absence of cells.

The variability in the rate of cell-free LDL oxidation was real and medium-dependent. It could be detected whether measuring LDL protein oxidation, lipid oxidation or α-tocopherol loss (Figure 1) and it remained even when the only difference between cell-free treatments was the source of the Ham's F10 (Table I). Contaminants in the Ham's F10 medium seemed unlikely to account for this variability as both the Sigma powder form and Gibco BRL liquid form of Ham's F10 were associated with inconsistent rates of cell-free LDL oxidation. The use of water from other purifiers on campus also failed to prevent the variation in oxidation rates in the absence of cells (data not shown).

Light-dependent oxidative reactions have previously been noted in the presence of HEPES and riboflavin, both constituents of Ham's F10 [23]. These reactions are of little relevance to the current study because inconsistency in the rate of cell-free LDL oxidation was not resolved by storing the medium in the dark at 4°C, with minimal handling, until required (data not shown). Similarly, neither the thiols (Table I) nor phenol red (Table II) in Ham's F10 had an ability to significantly stabilize the rate of cell-free LDL oxidation, as measured by protein hydroperoxide formation. Protein hydroperoxides are suitable markers of LDL oxidation as protein peroxidation has previously been shown to parallel lipid peroxidation on LDL [20].

The variability was generally less pronounced when using newer batches of media. N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (25 mM, HEPES) had little impact on the rate of cell-free LDL oxidation when added to older media immediately prior to an experiment, but supplementing the Ham's F10 with HEPES at the time of media preparation did have some effect. Although this ultimately failed to prevent the occurrence of variable LDL oxidation rates, such supplementation was often able to delay the onset of this variability by a couple of weeks (data not shown).

Iron chemistry can be quite complex and is further complicated by the tendency of iron(II) in Ham's F10 to autoxidize to the iron(III) state [4,24]. Autoxidation of iron during the storage of Ham's F10 provides a means by which iron could contribute to the variability in cell-free LDL oxidation because changes in the relative amounts of iron(II), iron(III) and lipid have been suggested to modulate the rate of cell-free LDL oxidation [25,26]. Despite iron(III) not being listed as a constituent of Ham's F10, iron levels in each of four sampled media generally yielded a ratio of one iron(II) per 2.2–2.9 iron(III) ions (Table III). Chelexing the medium removed all iron detectable by the FOX assay (Table III) and, accordingly, Chelexed medium was not permissive for either THP-1 macrophage-mediated or cell-free LDL oxidation even after resupplementation with a small quantity (1.5 μM) of ferrous sulphate (data not shown). Cell-mediated oxidation returned after supplementing the Chelexed Ham's F10 with 4.5 μM and 0.01 μM of freshly prepared ferrous sulphate and cupric chloride, respectively (Figure 2). These concentrations represent the transition metals normally present in Ham's F10, plus the supplemental 1.5 μM ferrous sulphate added immediately prior to every LDL oxidation experiment. Cell-free LDL oxidation was still limited under these conditions, with no protein hydroperoxide formation occurring and significant vitamin E loss ($p < 0.05$) beginning only at 24 h (Figure 2). Subsequent experiments indicated that some variability in the rate of cell-free LDL oxidation persisted, but this variability was considerably less pronounced and less frequent than that experienced when using non-Chelexed Ham's F10. Cell-free LDL oxidation in the latter medium

Table I. Comparison between thiol levels in Ham's F10 media and cell-free protein hydroperoxide formation on LDL. Thiol levels were measured in each of three batches of Ham's F10 media before supplementing with 1.5 μM $FeSO_4$ and incubating with 0.1 mg/ml LDL at 37°C. After 9 h, samples were analysed for an increase in protein hydroperoxides (PrOOH). All three media were prepared from Sigma Ham's F10 powder, but at intervals prior to the experiment of 5 months for medium 1, 4 months for medium 2 and 1 week for medium 3. Results are displayed as mean ± SEM of triplicates.

	Medium 1	Medium 2	Medium 3
PrOOH (mole/mole LDL)	0.9 ± 0.2	5 ± 1	36.9 ± 0.3
Media thiols (μM)	1.79 ± 0.05	2.97 ± 0.02	2.84 ± 0.06

Table II. Effect of phenol red on cell free LDL oxidation. LDL (0.1 mg/ml) was incubated at 37°C in Ham's F10, containing 25 mM HEPES and either 1.3 mg/l or 12 mg/l phenol red. All media were supplemented with 1.5 μM $FeSO_4$. After both 6 and 9 h, samples were analysed for an increase in protein hydroperoxides (PrOOH) and a loss of vitamin E. Results are displayed as mean ± SEM of triplicates.

	1.3 mg/ml phenol red	12 mg/ml phenol red
PrOOH (mole/mole LDL)		
6 h	3.5 ± 1	8 ± 1
9 h	27 ± 3	37 ± 4
α-Tocopherol (% of initial)		
6 h	61 ± 17	14 ± 7
9 h	0.9 ± 0.6	0.14 ± 0.1

Table III. Iron concentrations in different bottles of Ham's F10 media. The FOX assay was used to measure the quantity of iron(II) and iron(III) levels in two bottles each of Gibco BRL Ham's F10 and Sigma Ham's F10. Samples were incubated with xylenol orange for 30 min in the presence or absence of hydrogen peroxide. Iron levels were also assessed in freshly Chelexed Sigma Ham's F10. Results are displayed as mean ± SEM of triplicates.

	Fe(II) concentration (μM)	Fe(III) concentration (μM)	Total concentration (μM)	Fe(II):Fe(III) ratio
Gibco BRL 1	0.56 ± 0.06	1.27 ± 0.04	1.83 ± 0.07	1:2.3
Gibco BRL 2	0.54 ± 0.06	1.19 ± 0.05	1.73 ± 0.08	1:2.2
Sigma 1	0.54 ± 0.05	1.50 ± 0.04	2.04 ± 0.06	1:2.8
Sigma 2	0.59 ± 0.06	1.68 ± 0.03	2.27 ± 0.07	1:2.9
Chelexed Sigma	0.00 ± 0.01	0.00 ± 0.02	0.00 ± 0.02	

produced lag phases ranging anywhere from 3–24 h. Detecting maximal levels of cell-free oxidation products by 12 h was also not uncommon. By contrast, when using Chelexed Ham's F10, a lag phase of less than 12 h was never observed and those of 18–24 h were common.

Discussion

Inconsistency in the rate of cell-free LDL oxidation was quickly attributed to some variable in the Ham's F10 medium. The addition of 25 mM HEPES provided a degree of stabilization, but the problem could be more comprehensively resolved by completely removing all transition metals in the Ham's F10 via Chelexing. The ability of Chelex to remove redox active metal ions from buffers has been well documented [27]. Immediately prior to an experiment, the medium could then be standardized by supplementing with a known concentration of free transition metals (4.5 μM ferrous sulphate and 0.01 μM cupric chloride).

This approach was required due to auto-oxidation of the predominant Ham's F10 transition metal, iron, during storage of the medium (Table III). Iron-mediated oxidation of LDL has been reported to occur at a significant rate only when both iron(II) and iron(III) are present. Some studies suggest a requirement for an, as yet unidentified, iron(II)–iron(III) complex [28–30]. Others have argued against this and instead suggested that all observations could be explained by the balance between iron(II)'s decomposition of pre-existing lipid hydroperoxides (reaction 1) and its scavenging of the resulting lipid alkoxyl and peroxyl radicals (reactions 2 and 3) [26]. Based on the above theory, the lipid peroxidation chain reaction (reactions 4 and 5) would begin only once iron(II) has been oxidized to such an extent that it can no longer effectively compete with lipid for the alkoxyl/peroxyl radicals (reactions 2 and 3).

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\cdot} + OH^{-} \quad (1)$$

$$Fe^{2+} + LO^{\cdot} + H^{+} \rightarrow Fe^{3+} + LOH \quad (2)$$

$$Fe^{2+} + LOO^{\cdot} + H^{+} \rightarrow Fe^{3+} + LOOH \quad (3)$$

$$LO^{\cdot}/LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOH/LOOH \quad (4)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (5)$$

Whatever the precise mechanism, changes in the relative amounts of iron(II), iron(III) and lipid obviously have the potential to modulate the rate of cell-free LDL oxidation. Differences in these parameters from one batch of medium to the next can account for the variable rates observed both over time

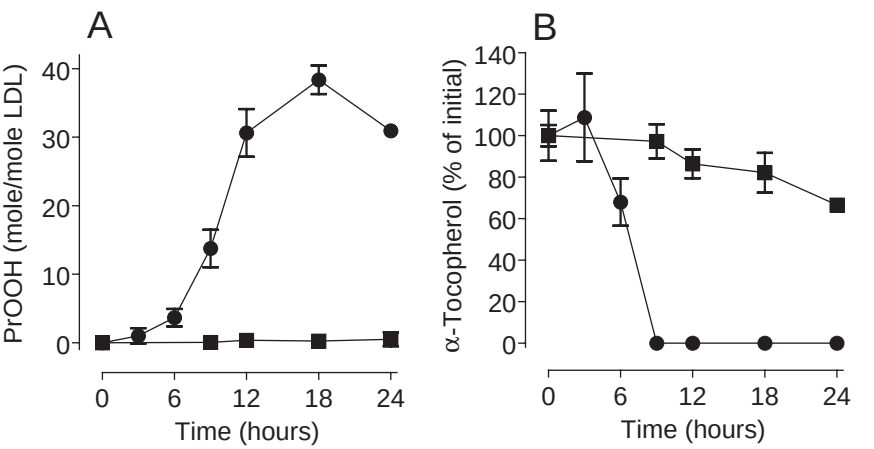

Figure 2. Cell-free LDL oxidation using Chelexed Ham's F10 with 4.5 μM $FeSO_4$ and 0.01 μM $CuCl_2$. LDL (0.1 mg/ml) was incubated in Ham's F10 at 37°C in the absence (square) or presence (circle) of adherent THP-1 macrophage-like cells (1×10^5 cells/ml). Ham's F10 contained 25 mM HEPES and was freshly Chelexed and supplemented with 4.5 μM $FeSO_4$ and 0.01 μM $CuCl_2$. Samples were analysed at various time points for an increase in (A) protein hydroperoxides (PrOOH) and (B) a loss of vitamin E. Results are displayed as mean ± SEM of triplicates.

and between bottles of media. Limiting the extent of such changes by Chelexing the medium and adding fresh ferrous sulphate immediately prior to an experiment provides an effective means of reducing the variability of cell-free LDL oxidation.

Throughout this entire study, the relative stability of cell-mediated LDL oxidation has been in direct contrast to the inconsistent rates of cell-free LDL oxidation. Such a discrepancy can also be explained by the iron auto-oxidation theory because cells possess an inherent ability to reduce iron to the iron(II) state [14,31]. This, in turn, minimizes the consequences of any iron autoxidation that occurs in the medium during storage.

The apparent ability of HEPES to delay the onset of variability can also be accounted for by the fact that iron auto-oxidation is significantly slower in HEPES buffer than in the phosphate and bicarbonate buffers that provide buffering capacity to Ham's F10 lacking HEPES [32].

In conclusion, the inconsistent rates of cell-free LDL oxidation associated with Ham's F10 can be prevented by first Chelexing the medium and then resupplementing with fresh transition metals immediately prior to an experiment. The addition of HEPES to Ham's F10 at the time of media preparation also affords some limited stabilization.

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