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Lipid oxidation predominates over protein hydroperoxide formation in human monocyte-derived macrophages exposed to aqueous peroxyl radicals

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Abstract

In U937 and mouse myeloma cells, protein hydroperoxides are the predominant hydroperoxide formed during exposure to AAPH or gamma irradiation. In lipid-rich human monocyte-derived macrophages (HMDMs), we have found the opposite situation. Hydroperoxide measurements by the FOX assay showed the majority of hydroperoxides formed during AAPH incubation were lipid hydroperoxides. Lipid hydroperoxide formation began after a four hour lag period and was closely correlated with loss of cell viability. The macrophage pterin 7,8-dihydroneopterin has previously been shown to be a potent scavenger of peroxyl radicals, preventing oxidative damage in U937 cells, protein and lipoprotein. However, when given to HMDM cells, 7,8-dihydroneopterin failed to inhibit the AAPH-mediated cellular damage. The lack of interaction between 7,8-dihydroneopterin and AAPH peroxyl radicals suggests that they localize to separate cellular sites in HMDM cells. Our data shows that lipid peroxidation is the predominant reaction occurring in HMDMs, possibly due to the high lipid content of the cells.

Keywords: *Macrophage, protein oxidation, lipid oxidation, neopterin, antioxidant*

Introduction

Reactive oxygen species are known to adversely affect cellular function by causing damage to a range of biomolecules. Identifying the initial targets of oxidative damage within the cell provides a greater understanding of the cellular antioxidant defence mechanisms. It has recently been shown that proteins are a major target of both hydroxyl and peroxyl radical damage in U937 and mouse myeloma Sp2/0-Ag14 cells [1–3]. Protein hydroperoxide formation was found to be an early event, and occurred well before any significant lipid peroxidation or DNA fragmentation. Inhibition of the protein oxidation using the water soluble antioxidant, 7,8-dihydroneopterin, has highlighted a clear link between protein hydroperoxide formation and cell death [4]. Protein hydroperoxides have also been shown to form readily on low density lipoprotein (LDL), in parallel with lipid hydroperoxides [5]. Evidence for their formation *in vivo* arises from the measurement of both leucine and valine hydroperoxide break-down products in atherosclerotic plaque [6].

Protein hydroperoxide formation is clearly a significant secondary stress within the cells [7]. Though relatively stable [3], protein hydroperoxides possess sufficient reactivity to promote additional cellular damage. This damage includes the oxidation of nucleotides [8], formation of protein-DNA cross-links [9] and enzyme inactivation of caspases [10] and

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glyceraldehyde-3-phosphate dehydrogenase [10,11]. Protein fragmentation may be driven by hydroperoxide degradation, specifically when the hydroperoxide is located at the α-carbon position of the peptide backbone [12]. Additionally, protein hydroperoxides can reduce the cellular antioxidant capacity by consuming thiols [13] and antioxidants like glutathione and ascorbate [14,15].

Hydroperoxide formation has been even more extensively studied on lipid, particularly using substrates like LDL that are high in polyunsaturated fatty acids (PUFAs). Their formation is commonly associated with a self-propagating chain reaction that greatly amplifies the initial damage [16]. Evidence exists that under certain circumstances peroxidizing lipids may even damage protein by causing the loss of amino acids and protein solubility [17] as well as promoting protein polymerization [18], carbonyl formation [19,20] and protein peroxidation [5]. Numerous studies have also directly highlighted the cytotoxicity of lipid peroxidation by noting a loss of cellular viability upon incubation with lipid hydroperoxides and their aldehyde break-down products [21–26].

Direct cellular defence against protein hydroperoxides appears limited, as peroxidases are generally inefficient at degrading protein hydroperoxides, particularly when those hydroperoxides are associated with larger proteins [27]. A transplasma membrane electron transport reducing mechanism has been identified in THP-1 cells but it is also inactive against large protein-bound hydroperoxides [28]. By contrast, the antioxidant 7,8-dihydroneopterin has been shown to be a potent inhibitor of protein hydroperoxide formation during studies exposing U937 cells, lipoproteins and bovine serum albumin to a range of oxidative insults [4,5,29]. 7,8-Dihydroneopterin is synthesized by interferon-γ-stimulated macrophages as part of the inflammatory response [30,31]. Although its exact function *in vivo* remains uncertain, detailed kinetic analyses of LDL oxidation have demonstrated that 7,8-dihydroneopterin is an effective scavenger of lipid peroxyl radicals [5,32]. Spin trap studies have further confirmed 7,8-dihydroneopterin's peroxyl radical scavenging ability [33]. In addition to inhibiting protein hydroperoxide formation in U937 cells, 7,8-dihydroneopterin also prevents a loss of cellular thiols and viability caused by exposure of the cells to AAPH [4] or oxLDL [34]. These observations have led to the suggestion that macrophages may synthesize 7,8-dihydroneopterin to protect themselves from oxidant-induced damage at sites of inflammation [4,32]. This hypothesis has been complicated by the finding that, in contrast to U937 cells, 7,8-dihydroneopterin has only a very limited ability to protect THP-1 cells from loss of viability due to AAPH and provides no protection against oxLDL-induced THP-1 viability loss [34].

The disparity between the response of these two cell lines to oxidative stress and 7,8-dihydroneopterin protection has made it difficult to determine which cell line provides the most representative model of monocyte/macrophage behaviour. To clarify this issue, and gain a greater understanding of the role of protein oxidation *in vivo*, this study has examined the effect of AAPH peroxyl radicals and 7,8-dihydroneopterin on human monocyte-derived macrophage (HMDMs).

Materials and methods

Materials

Chemicals and reagents were AR grade or better and, unless otherwise stated, were obtained from either Sigma Chemical Company (USA) or BDH Chemicals New Zealand Ltd. 7,8-Dihydroneopterin was supplied by Schirck's Laboratories (Switzerland); 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) from Aldrich Chemical Company (Sydney, Australia); Lymphoprep from Axis-Shield PoC AS (Norway); chloroform from Asia Pacific Speciality Chemicals Ltd (NZ); and chelex-100 resin from Bio-Rad Laboratories (NZ). Falcon brand tissue culture plasticware was supplied by BD Biosciences Ltd. 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.

Cell culture

Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and, unless otherwise stated, were maintained in RPMI 1640 supplemented with 10% heat-inactivated human serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Human monocyte-derived macrophages (HMDMs) were prepared using unlinked blood donated by haemochromatosis patients at the NZ Blood Bank (Christchurch). Monocyte isolation was achieved by density gradient centrifugation of the blood in the presence of Lymphoprep as described by the manufacturer. Cells were then washed four times in Hanks' Balanced Salt Solution (HBSS) before resuspension in RPMI 1640 (containing only 5 μl/ml of heat-inactivated human serum) and incubation at a concentration of 5×10^6 cells/ml in non-adherent plates for 40 h [56]. The monocytes were subsequently resuspended in fresh RPMI 1640 (containing 10% heat-inactivated human serum) and seeded at a concentration of 5×10^6 cells/ml in 12 well adherent plates. Media was replaced every 2–3 days,

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and differentiation to HMDMs occurred 10–20 days after the initial isolation.

Immediately prior to an experiment, the cells were washed three times with PBS, and Earle's Balanced Salt Solution (EBSS) was then added to each well. When required, cells were incubated with 7,8-dihydroneopterin for ten minutes prior to the addition of AAPH. Stock solutions of AAPH (250 mM) and 7,8-dihydroneopterin (2 mM) were each prepared fresh in degassed EBSS and sterilized via filtration through a 0.22 μm membrane filter.

Cell viability

Cell viability was measured by trypan blue exclusion staining [35] and the MTT reduction assay, as described by Mosmann,[36] but using 10% w/v sodium dodecyl sulphate (SDS, final concentration) to lyse the cells and solubilize the insoluble MTT-formazan salt.

Total cellular thiol content

Total cellular thiol concentration was analyzed using a modification of the Boyne and Ellman [37] protocol. HMDMs were washed in cold PBS, dislodged from the plate and lysed in SDS (10% w/v final concentration). The cell lysate was subsequently incubated with 30 μM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, final concentration) for 30 min at room temperature and the absorbance measured at 412 nm, using an extinction co-efficient of 13,600 M^{-1} cm^{-1} [37].

Hydroperoxide assays

Protein hydroperoxides were measured using two variants of the FOX assay. All required the HMDMs to be washed in PBS and lysed by scraping and sonication in 1 ml of cold water. The FOX-1 assay is modified from Gay and Gebicki [38], with cellular proteins precipitated using trichloroacetic acid (TCA) before washing twice in either ice cold 5% TCA or a range of solvents. Depending on the type of analysis required, solvent combinations included a wash in methanol followed by hexane, methanol followed by chloroform, or acetone followed by diethyl ether. The protein pellets were dried under mild vacuum, resuspended in 900 μl of 25 mM sulphuric acid and then 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, before centrifugation to remove cellular debris, and absorbance measurement at 560 nm against a water blank. Absorbances were converted to concentrations using an extinction co-efficient of 35,500 M^{-1} cm^{-1} [38].

The second variant of the protein hydroperoxide FOX assay, G-PCA-FOX, is described by Gay and Gebicki [39]. Briefly, washed and lysed cells were precipitated with perchloric acid (PCA) and washed once with 0.2 M PCA. The resulting pellet was dissolved in 6 M guanidine hydrochloride (GuHCl) and mixed with an equal volume of chloroform (containing 4 mM butylated hydroxytoluene; BHT). The upper aqueous phase was isolated, washed with chloroform, and isolated once more. A 700 μl volume was then mixed with 40 μl of 0.5 M PCA, 25 μl water, 25 μl of 5 mM xylenol orange (in 110 mM PCA) and 10 μl of ferrous ammonium sulphate (in 110 mM PCA). Absorbances were measured at 560 nm, against blanks, after a 60 min incubation at room temperature in the dark. Protein hydroperoxide concentrations were determined using an extinction co-efficient of 35,900 M^{-1} cm^{-1} [39].

Lipid hydroperoxides were analyzed using the L-PCA-FOX method of Gay and Gebicki [39], with the lipid from washed and lysed HMDMs extracted into a 2:1 chloroform:methanol solution (containining 4 mM BHT). The solvent was subsequently dried under nitrogen, and the resulting residue dissolved in 250 μl chloroform and 460 μl methanol (both containing 4 mM BHT). Samples were incubated in the dark at room temperature for 60 min with 41 μl of 2M PCA, 30 μl of 5 mM xylenol orange (in 110 mM PCA) and 20 μl of ferrous ammonium sulphate (in 110 mM PCA). Absorbances were recorded at 560 nm, against blanks, and an extinction co-efficient of 51,200 M^{-1} cm^{-1} was used to calculate lipid hydroperoxide concentrations [39].

TBARS assay

Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS), by derivatisation with thiobarbituric acid (TBA), and analysis using reverse phase high performance liquid chromatography (HPLC) with fluorescence detection [40]. Further oxidation during analysis was prevented by the addition of BHT (in methanol) to all samples [41].

Statistical analysis

Data were analyzed using the Prism software package, supplied by Graphpad Software Inc. Statistical significance was confirmed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Where appropriate, significance is indicated on figures as $***p < 0.001$; $**p < 0.01$; $*p < 0.05$. Results shown are from single experiments, representative of a minimum of three, and are expressed as mean ± SEM of triplicate treatments.

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Results

Incubation of HMDMs with AAPH caused a concentration-dependent loss of viability, as measured by a decline in MTT reducing ability ($r^2 = 0.90$) and trypan blue exclusion staining ($r^2 = 0.99$) (Figure 1A). After a 12 h incubation with 20 mM AAPH, approximately 50% of the cells had lost viability. This result was reproducible, though donor variability accounts for a few exceptions of both limited and high viability loss (data not shown). All subsequent experiments investigating the effect of oxidative stress on HMDMs were therefore conducted using 20 mM AAPH.

Three day old human monocytes were found to be significantly more sensitive to peroxyl radicals than the 14 day old human macrophages, with approximately 50% of monocytes dead after a 12 h exposure to only 10 mM AAPH (Figure 1B). By 20 mM AAPH, the percentage of dead blood-derived monocytes was almost 90%.

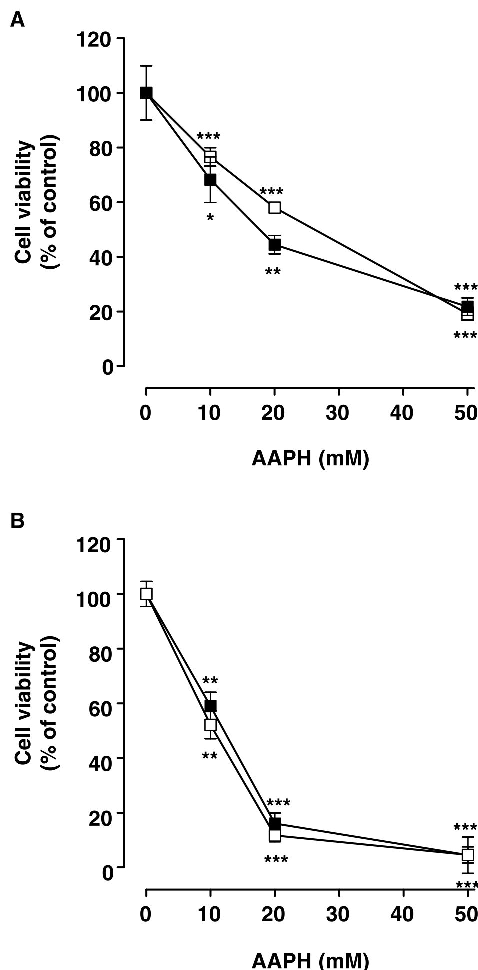

Figure 1. Loss of cellular viability with increasing concentrations of AAPH. (A) HMDMs and (B) 3 day old human monocytes, each at 5×10^6 cells/ml, were incubated in EBSS at 37°C with increasing concentrations of AAPH. After 12 h, cells were analyzed for viability via the MTT reduction assay (solid square) and trypan blue exclusion staining (open square). Significance is indicated from the 0 mM AAPH control. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

AAPH-induced damage to HMDMs can be monitored not only by loss of viability but also by hydroperoxide formation. The general TCA precipitation protocol, described by the FOX-1 assay, lacks a lipid extraction step but has been successfully used to measure protein hydroperoxides in U937s due to the absence of significant lipid hydroperoxide formation in this cell line after AAPH treatment [3]. By contrast, the FOX-1 assay appears unsuitable for measuring protein hydroperoxides on HMDMs. The general TCA precipitation certainly enabled detection of hydroperoxides on HMDMs after a 16 h incubation with 20 mM AAPH, but washing these 72% TCA-precipitated pellets in a range of solvents both eliminated lipids and significantly reduced hydroperoxide levels (Table I). This drop occurred to a similar extent whether washing in methanol followed by hexane, methanol followed by chloroform or acetone followed by diethyl ether. The decrease in absorbance is therefore unlikely to be due to the use of a specific solvent combination. The inclusion of a 5% TCA wash to remove trace solvent following the other wash steps also did not prevent the sudden drop in measured hydroperoxides (data not shown).

These results were further confirmed using the G-PCA-FOX assay (Table II). A general PCA precipitation yielded a hydroperoxide concentration not significantly different from the result obtained when using TCA. The inclusion of chloroform wash steps (once the PCA-precipitated hydroperoxides were dissolved in GuHCl) significantly reduced the hydroperoxide content to 0.66 ± 0.36 μM. This concentration is not significantly different from the levels detected after solvent washes in the FOX-1 assay, and is attributed to the formation of protein hydroperoxides on HMDMs. Another variation of the FOX assay, the L-PCA-FOX, provided a measure of lipid

Table I. Detection of hydroperoxide formation on HMDM cells using TCA precipitation and sulphuric acid solubilization before FOX analysis.

	Hydroperoxide formation (μM)
General acid precipitation	
5% TCA	1.47 ± 0.02
Solvent washes—protein hydroperoxides	
Methanol/hexane	0.39 ± 0.16
Methanol/chloroform	0.27 ± 0.02
Acetone/diethyl ether	0.27 ± 0.13

HMDMs, at 5×10^6 cells/ml, were incubated in EBSS at 37°C with 20 mM AAPH. After 16 h, HMDMs were harvested and all samples precipitated with TCA before being subject to one of four treatments—washes with 5% TCA; methanol followed by hexane; methanol followed by chloroform; or acetone followed by diethyl ether. The washed pellets were then resuspended in sulphuric acid, with hydroperoxides detected at 560 nm after reaction with the FOX reagents and subtraction against controls incubated in the absence of AAPH. Results are displayed as mean ± SEM of triplicates.

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Table II. Detection of hydroperoxide formation on HMDM cells using lipid extraction or PCA precipitation and solubilization in GuHCl before FOX analysis.

	Hydroperoxide formation (μM)
General acid precipitation	
5% PCA	1.66 ± 0.23
Solvent washes—protein hydroperoxides	
Chloroform/chloroform	0.66 ± 0.36
Lipid extraction—lipid hydroperoxides	
2:1 Chloroform:methanol	2.46 ± 0.54

HMDMs, at 5×10^6 cells/ml, were incubated in EBSS at 37°C with 20 mM AAPH. After 16 h, HMDMs were harvested and samples subjected to one of three treatments—a PCA-induced precipitation followed by washes in 5% PCA and resuspension in GuHCl; a PCA-induced precipitation followed by resuspension in GuHCl and washes in chloroform; extraction into 2:1 chloroform:methanol followed by evaporation and resuspension of the residue in 25:46 chloroform:methanol. Suspensions from all three treatments were then acidified in PCA, with hydroperoxides detected at 560 nm after reaction with the FOX reagents and subtraction against controls incubated in the absence of AAPH. Results are displayed as mean ± SEM of triplicates.

hydroperoxide content via extraction of the HMDM lipids into a 2:1 chloroform:methanol solution before analysis. Under such conditions, AAPH-induced lipid hydroperoxides were detected on HMDMs at a concentration of 2.46 ± 0.54 μM. A comparison to the protein hydroperoxides detected by the G-PCA-FOX indicates that lipid hydroperoxides account for 79% of all hydroperoxides formed on HMDMs exposed to AAPH. Due to the generation of these high levels of lipid hydroperoxides, the PCA-FOX assays were used for all subsequent analyses.

Kinetic analysis revealed that the incubation of HMDMs with 20 mM AAPH was associated with a loss of viability only after an initial 4 h lag phase (Figure 2). In contrast to the almost complete survival of control cells (particularly when monitored by trypan blue exclusion staining), virtually 100% of AAPH-treated cells were dead after 24 h of exposure.

A lag phase of approximately 4 h was also observed during the AAPH-induced formation of lipid hydroperoxides (Figure 3) and TBARS (data not shown). This lag was followed by a rapid increase in both lipid peroxidation parameters until oxidation levels plateaued after 18 h. Oxidation proceeded at a rate of 0.15 μM lipid hydroperoxides per hour during the propagation phase, but peroxyl radicals were generated at $3.19 \times 10^{-7} \times$ [AAPH] $M s^{-1}$ at 37°C [42]. In the presence of 20 mM AAPH, this equates to a steady rate of 22.97 μM peroxyl radicals per hour, with only one lipid hydroperoxide being formed per 152 peroxyl radicals. This ratio is an approximation, and may be a slight underestimate, because any hydroperoxides detected do represent a balance between formation and degradation.

Lipid hydroperoxide concentrations were generally 20 times greater than TBARS, but this latter assay measures mainly malondialdehyde formation, a significant but late product of arachidonate oxidation. Despite this, TBARS also became significantly elevated above baseline levels within 12 h ($p < 0.01$ for lipid hydroperoxides and $p < 0.001$ for TBARS). Protein hydroperoxides did not increase significantly above the zero hour measurement, even though protein hydroperoxide formation showed similarity to the trend observed for lipid peroxidation (Figure 3).

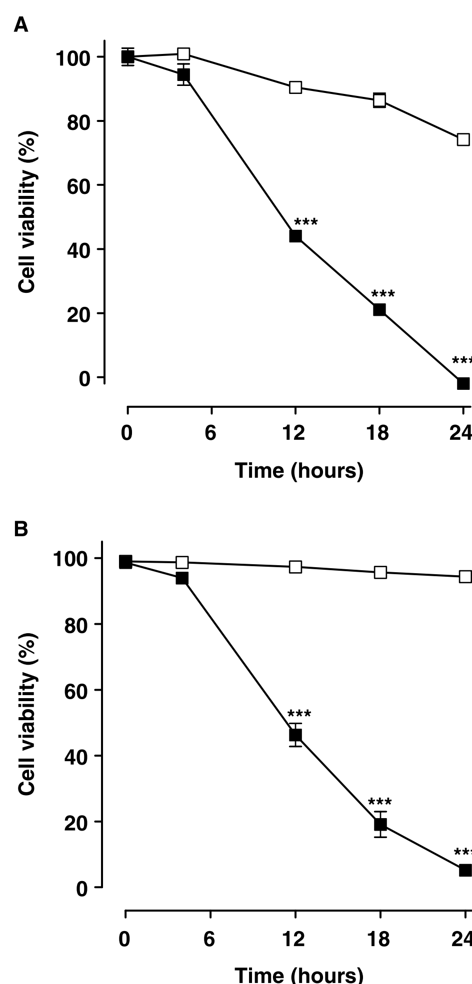

Figure 2. Time course of AAPH-induced loss of HMDM viability. HMDMs, at 5×10^6 cells/ml, were incubated in EBSS at 37°C in the presence (solid square) and absence (open square) of 20 mM AAPH. At various times HMDMs were analyzed for cell viability by both the (A) MTT reduction assay and (B) trypan blue exclusion staining, with data expressed as a percentage of zero hour levels. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

7,8-Dihydroneopterin has previously been shown to inhibit AAPH- and oxLDL-induced cell death and thiol loss in the U937 monocyte cell line [4,34]. However, incubation of HMDMs with up to 200 μM 7,8-dihydroneopterin for 12 h failed to prevent the loss of viability caused by 20 mM AAPH (Figure 4).

Untreated HMDMs contained approximately 35 nmole of thiols per well and showed no significant

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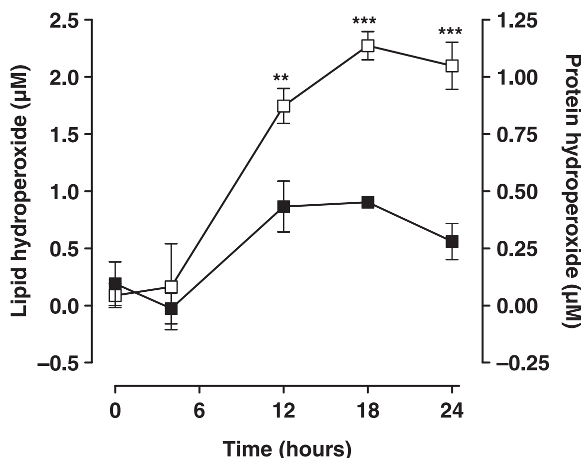

Figure 3. Time course of AAPH-induced hydroperoxide formation on HMDMs. HMDMs, at 5×10^6 cells/ml, were incubated with 20 mM AAPH in EBSS at 37°C. Controls were conducted in the absence of AAPH. At various times HMDMs were analyzed for lipid hydroperoxide (open square) and protein hydroperoxide (solid square) formation, using lipid and protein FOX assays, respectively, with controls subtracted from the AAPH-treated samples. The average protein concentration of the HMDMs was 113 ± 4.57 ug/ml in the wells. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

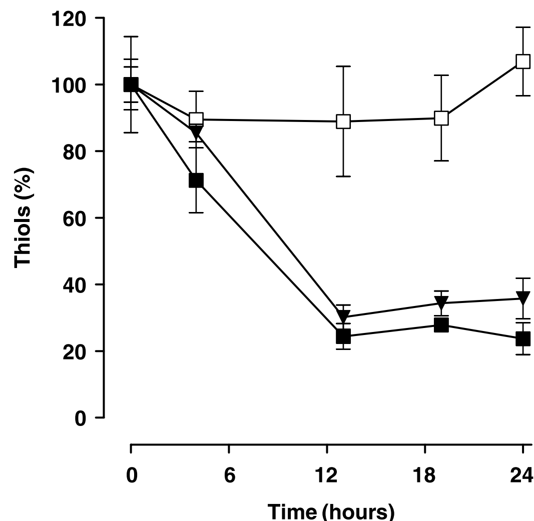

Figure 5. 7,8-Dihydroneopterin does not prevent AAPH-induced loss of HMDM thiols. HMDMs, at 5×10^6 cells/ml, were incubated with 20 mM AAPH in EBSS at 37°C in the presence (closed triangle) or absence (closed square) of 200 μM 7,8-dihydroneopterin. Controls were conducted in the absence of AAPH (open square). At various time points HMDMs were analyzed for total cellular thiol content, with data expressed as a percentage of zero hour levels. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

change during the course of the incubation (Figure 5). Thiol loss in the presence of 20 mM AAPH became significantly different from the control after 4 h. This loss continued until a plateau was reached, at 25% of the untreated cells, between 12 and 24 h. The addition of 200 μM 7,8-dihydroneopterin to the AAPH incubation failed to cause any significant change in the loss of the cellular thiols. 7,8-Dihydroneopterin was also unable to protect HMDMs from AAPH-induced lipid hydroperoxide formation (Figure 6).

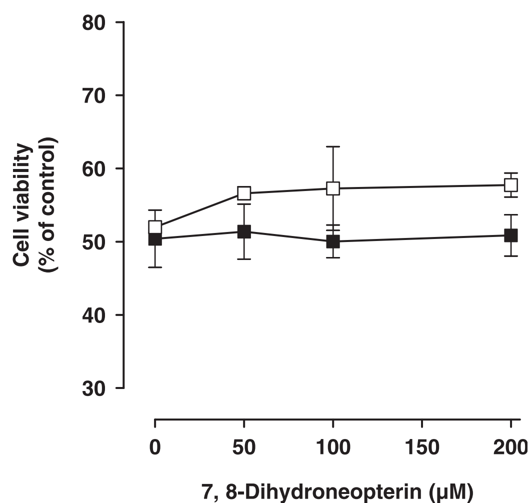

Figure 4. 7,8-Dihydroneopterin does not prevent AAPH-induced loss of HMDM viability. HMDMs, at 5×10^6 cells/ml, were incubated with 20 mM AAPH in EBSS at 37°C and increasing concentrations of 7,8-dihydroneopterin. Controls were conducted in the absence of AAPH. After 12 h, HMDMs were analyzed for cell viability by both the MTT reduction assay (solid square) and trypan blue exclusion staining (open square). ANOVA analysis revealed no statistical significance from 0 μM 7,8-dihydroneopterin. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

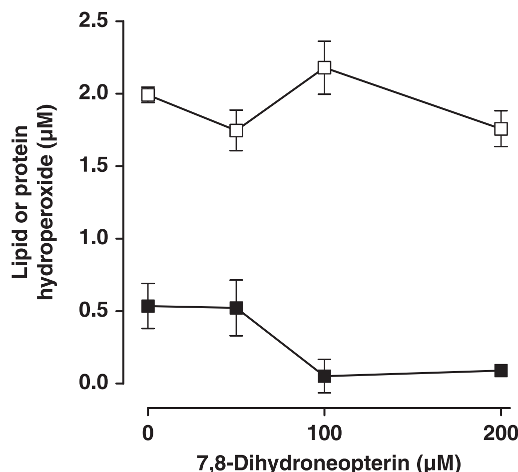

Figure 6. Effect of 7,8-dihydroneopterin on AAPH-induced hydroperoxide formation on HMDMs. HMDMs, at 5×10^6 cells/ml, were incubated in EBSS at 37°C with 20 mM AAPH and increasing concentrations of 7,8-dihydroneopterin. Controls were conducted in the absence of AAPH. After 12 h HMDMs were analyzed for lipid hydroperoxide (open square) and protein hydroperoxide (solid square) levels, using lipid and protein FOX assays, respectively, with controls subtracted from the AAPH-treated samples. ANOVA analysis revealed no statistical significance from 0 μM 7,8-dihydroneopterin. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

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The addition of 7,8-dihydroneopterin did prevent protein hydroperoxide formation but, due to the minimal levels of protein hydroperoxides being detected, this inhibition failed to reach significance (Figure 6).

It remained possible that the high radical flux generated by 20 mM AAPH overwhelmed any ability of 7,8-dihydroneopterin to inhibit cell death. A lower radical flux with 10 mM AAPH reduced the loss of HMDM viability to 70–80% after a 12 h incubation. However, 7,8-dihydroneopterin still failed to exert any protective effect against AAPH-mediated cell death (data not shown). Stimulating the HMDMs with interferon-γ to induce *de novo* synthesis of 7,8-dihydroneopterin also had no impact on the loss of viability and formation of hydroperoxides detected after incubation with 20 mM AAPH (data not shown).

Discussion

Peroxyl radical exposure to HMDMs resulted in large amounts of lipid rather than protein hydroperoxides. The majority of previous studies, using other monocyte/macrophage cell lines, have found protein oxidation to be the major event during AAPH or hydroxyl radical exposure [1–3]. Proteins are certainly excellent scavengers of these radicals, especially in systems of low PUFA content, and will outcompete lipids for hydroxyl radicals in liposomes, serum and mouse myeloma cells [2,7]. This is not the case with HMDMs, where the majority of hydroperoxides were observed to form on lipid rather than protein.

Generally, eukaryotic cells contain approximately 74% protein vs. 21% lipid and 4% DNA [1]. Just as critically, the $n-3$ and $n-6$ PUFA content of U937 cells is relatively low [43]. HMDMs, by contrast, are associated with large amounts of lipid as differentiation from blood-derived monocytes to macrophages causes a 77-fold increase in total neutral lipid mass [44]. Such a dramatic accumulation of lipid during the culture of HMDMs produces cells with a foam-like appearance and has been attributed to the triglyceride-rich lipoprotein fraction of human serum [45], particularly long chain PUFAs of the $n-3$ series [46].

The majority of studies that place a large emphasis on lipid oxidation have used the relatively inaccurate method of TBARS analysis, using only absorption measurements without the use of lipid oxidation inhibitors during the derivatization step. This is likely to lead to an over-estimation of the lipid oxidation occurring during cellular damage [3]. The more accurate FOX assay directly measures the reactivity of the lipid hydroperoxides and has been used in the current study to show that lipid oxidation is the predominant reaction occurring in HMDM cells.

Even though protein oxidation was considerably less pronounced than lipid oxidation, the kinetics of protein hydroperoxide formation paralleled that of lipid peroxidation. This correlation was similarly described during the oxidation of LDL, which also contains high levels of PUFA [5]. In that study, the lipid peroxyl radicals were shown to initiate protein hydroperoxide formation. If protein oxidation were to follow a similar mechanism for HMDMs, it must be occurring at a much lower rate than that observed for LDL.

Significant amounts of lipid hydroperoxides were formed, but only one lipid hydroperoxide was generated in the HMDMs per 152 peroxyl radicals. Lipid oxidation is therefore not the predominant mechanism for scavenging peroxyl radicals. Thiols represent another target of peroxyl radicals, with significant protein thiol loss on the HMDMs observed within the first 12 h of AAPH incubation (Figure 5). Protein thiol oxidation signifies the loss of key metabolic and regulatory enzymes, which require free thiols for full activity. As glutathione is a major intracellular antioxidant and reductant, the loss of these protein thiols represents the failure of the glutathione protection system. Glutathione is a potent scavenger of peroxyl radicals and is a co-factor in a number of peroxidases. Some cell lines have also been shown to expel glutathione as a mechanism of inducing apoptosis during cellular damage [47]. The importance of thiol loss in comparison to hydroperoxide formation is difficult to determine from our data. Neither thiol loss nor hydroperoxide formation occurred to a significant extent in the HMDM cells until after 4 h of incubation with AAPH. It is at this point that cell viability begins to drop, suggesting a link between these parameters and cell death. Protein thiol oxidation would result in the loss of significant ion regulators within the cell membrane [48], while lipid hydroperoxides and their aldehyde break-down products have been found to be cytotoxic [21–26]. Protein hydroperoxides are also reactive towards a range of biomolecules, including thiols [49], but their concentration is minimal in HMDM cells.

The dose-dependent decrease in cell viability induced by AAPH was considerably more pronounced for 3 day old monocytes than HMDMs (Figure 1). Differences between differentiated and undifferentiated cells have also been observed for the THP-1 cell line, with THP-1 macrophage-like cells consistently displaying an increased resistance towards oxidative stress compared to undifferentiated THP-1 monocytes when exposed to oxLDL, hydrogen peroxide or a nitric oxide donor [50,51]. Similarly, tissue macrophages are more resilient towards ionizing radiation than circulating monocytes [52]. The enhanced resistance of macrophages towards AAPH cytotoxicity when compared to monocytes is likely due to the increase in antioxidant activity that has previously been described upon monocyte differentiation [51,53,54].

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7,8-Dihydroneopterin is a potent scavenger of peroxyl radicals, inhibiting lipoprotein [5,32] and cellular oxidative damage [4,55]. Studies on lipoproteins, using both metal ions and AAPH, have shown that 7,8-dihydroneopterin can even effectively compete with α-tocopherol for the peroxyl radicals [32]. Despite such strong evidence for a peroxyl radical scavenging activity, the addition of up to 200 μM 7,8-dihydroneopterin failed to protect HMDMs from the cytotoxicity, lipid peroxidation and thiol loss induced by 20 mM AAPH (Figures 4 and 6). It has previously been noted that, in THP-1 cells, 7,8-dihydroneopterin is similarly ineffective against AAPH-induced thiol loss and offers only a limited protection against cell death [34]. 7,8-Dihydroneopterin is soluble in water and should therefore be a potent scavenger of the water soluble AAPH peroxyl radicals. Our previous studies exposing bovine serum albumin or U937 cells to AAPH certainly suggested this to be the case [4,29]. Why the scavenging does not occur in the presence of the HMDM cells, and to only a limited degree in the presence of THP-1s [34], is difficult to determine. The data suggests that 7,8-dihydroneopterin and/or AAPH are partitioning in such a way that they are incapable of interacting. Perhaps one or the other binds directly to, or is absorbed by, the HMDM cells. As attractive as this partitioning hypothesis is, our other cellular studies have failed to find evidence of any such interaction. By contrast, 7,8-dihydroneopterin has been shown to directly interact with lipid peroxyl radicals during the inhibition of LDL oxidation [5,32]. There was no indication of direct AAPH peroxyl radical scavenging in the aqueous phase in either of these studies. The concept of an interaction between 7,8-dihydroneopterin and the HMDMs therefore remains worthy of consideration.

In contrast with lipid peroxidation, 100 μM 7,8-dihydroneopterin appeared to inhibit AAPH-induced protein hydroperoxide formation in HMDMs (Figure 6). However the level of protein hydroperoxide formation was too low for these results to be significant which make it difficult to draw any conclusions.

In conclusion, lipid rather than protein is the predominant site of oxidative damage in HMDMs exposed to aqueous peroxyl radicals. This is in striking contrast to what has previously been observed in U937 and mouse myeloma Sp2/0-Ag14 cell lines, where protein was the primary scavenger of oxidative radicals [1–3]. We hypothesize that this difference is due to the accumulation of PUFA-rich lipid within the HMDM cells when cultured in 10% human serum. The water soluble macrophage synthesized antioxidant, 7,8-dihydroneopterin, was totally ineffective at preventing thiol loss, lipid hydroperoxide formation and loss of cell viability, though a protective effect was observed against the limited formation of protein hydroperoxides. The data indicates that 7,8-dihydroneopterin and/or AAPH interact with HMDM cells in such a way that they are unable to interact. This lack of 7,8-dihydroneopterin effect is similar to the result previously described for the THP-1 cell line but contrasts completely with the U937 cells.

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