Polyunsaturated and monounsaturated fatty acids increase neutral lipid accumulation, caspase activation and apoptosis in a neutrophil-like, differentiated HL-60 cell line

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ABSTRACT

We report here that monounsaturated fatty acids and polyunsaturated fatty acids (PUFAs) provoke the accumulation of neutral lipids and apoptosis in retinoic acid-treated HL-60 cells in a concentration- and time-dependent manner. The PUFAs (arachidonic acid, docosahexanoic acid and eicosapentaenoic acid) provoked higher levels of HL-60 apoptosis compared with the monounsaturated oleic acid or the saturated palmitic acid. Cell size and granularity were also altered by fatty acid treatment. The PUFA-induced apoptosis was correlated with increased activity of caspase 3 and caspase 9. Lipid peroxidation was also increased in the presence of PUFAs, but was not responsible for activating cell apoptosis. Lipid derived metabolites may be responsible for activation of caspases and induction of cell apoptosis.

INTRODUCTION

It is now generally accepted that nutritional status is an important determinant of host immune responses. Rapid advances in nutritional and biochemical research have shown that lipid metabolism (specifically fatty acid metabolism) plays a significant role in immune-cell function. In particular, 20-carbon polyunsaturated fatty acids (PUFAs), derived from essential fatty acids, are precursors of eicosanoid signalling molecules that play vital roles in immune function and inflammatory processes [1]. In addition to eicosanoid-mediated effects, dietary fatty acids may also modulate immune function via the regulation of gene expression, their effects on membrane fluidity and cell metabolism and alterations in the rates of cellular apoptosis. Dietary intervention has attracted much attention as a means of beneficially modulating the structure and function of a variety of immune cells, including neutrophils. The neutrophil is the first line of defence against invading micro-organisms. Upon activation, neutrophil responses include adherence to endothelial cells, migration into inflamed tissue (chemotaxis), phagocytosis of microbes and production of superoxide radicals [2,3]. These responses are facilitated by a delay in the rate of apoptosis to allow accumulation of neutrophils at the site of infection [4]. The tight regulation of apoptosis is important for the resolution of neutrophil inflammatory responses, thereby limiting host tissue damage. Disorders such as cystic fibrosis [5], acute respiratory distress syndrome [6], acute pancreatitis [7] and inflammatory bowel disease [8] have been associated with delayed neutrophil apoptosis.

Although the effects of long-chain fatty acids (particularly the n-3 class of PUFAs) on neutrophil functional

Key words: apoptosis, fatty acid, lipid metabolism, oxidative metabolism, neutrophil.

Abbreviations: AA, arachidonic acid; DHA, docosahexanoic acid; EPA, eicosapentaenoic acid; FCS, foetal calf serum; OA, oleic acid; PA, palmitic acid; PUFA, polyunsaturated fatty acid; RA, retinoic acid; ROS, reactive oxygen species; TAG, triacylglycerol; TBARS, thiobarbituric-acid-reactive substance.

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parameters such as superoxide production and chemotaxis have been investigated extensively in numerous ex vivo studies [9–14], to our knowledge no studies have addressed adequately the effects of such fatty acids on neutrophil apoptosis. In the work presented here, retinoic acid (RA)-differentiated HL-60 cells were used as a model for human neutrophils. HL-60 cells can be induced to differentiate into a neutrophil-like cell after incubation with compounds including RA, DMSO, phorbol esters, and analogues of cAMP [15,16]. The morphological and functional changes in the HL-60 cells upon differentiation include a more vigorous respiratory burst upon activation, and a higher rate of apoptosis [17] due to loss of Bcl-2 expression [4]. Differentiated HL-60 cells also acquire a neutrophil-like sensitivity to Fas-induced apoptosis, in contrast with undifferentiated HL-60s that are resistant to Fas-mediated death signals [18]. Primary human neutrophils were not used in this study because they do not remain viable ex vivo for periods much longer than 12 h, and certainly less than the longest period of fatty acid incubation utilized in this study.

MATERIALS AND METHODS

Chemicals and reagents

RPMI 1640, foetal calf serum (FCS), L-glutamine and penicillin/streptomycin solution were purchased from Gibco-Life Technologies (Paisley, U.K.). Ac-DEVD-AMC (caspase 3) and Ac-LEHD-AFC (caspase 9) fluorogenic substrates were purchased from BioMol, Affinity Research Products (Exeter, U.K.). Nile Red was purchased from Molecular Probes (Leiden, The Netherlands). All other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, U.K.).

Cell and culture conditions

HL-60 cells were cultured in RPMI 1640 medium supplemented with 20% FCS (v/v), 3 mM L-glutamine, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. HL-60 cells were differentiated into neutrophil-like cells by treating with 1 μM all-trans RA for 3–4 days. Fatty acids were added to the cell culture medium to give final concentrations of 25, 50 or 75 μM, and it was ensured that the final concentration of ethanol was always less than 0.05% (so as not to affect the cell cycle or differentiation [19]).

Quantification of apoptosis

Apoptosis of RA-treated HL-60 cells was quantified, by flow cytometry, as the percentage of cells with hypodiploid DNA as described previously [4]. A Becton Dickinson FACStar Plus Flow Cytometer equipped with CellQuest software (Becton Dickinson, Dublin, Ireland) was used for data acquisition and analysis. Laser excitation was set at 488 nm and propidium iodide fluorescence was collected using a 530/30 nm band pass filter. A total of 10000 events were collected per sample and these were gated on the basis of forward (cell size) and side (cell granularity) light scatter. The proportion of necrotic neutrophil-like cells determined by this method was negligible.

Cell size and granularity

Changes in the light-scattering properties of the cells were determined as described previously [20] using a Becton Dickinson FACStar Plus equipped with CellQuest software. Light scattered in the forward direction is roughly proportional to cell size, whereas light scattered at a 90° angle is proportional to cell granularity [21]. The mean of the heights of the signals received at the forward-scatter and side-scatter detectors were recorded and used as indices of cell size and granularity respectively.

Cell morphology

Cells were centrifuged at 400 g for 5 min on to glass slides using a Shandon Cytospin 2 centrifuge (Shandon Inc., Pittsburgh, PA, U.S.A.). The cells were fixed by immersion in methanol and then stained with Giemsa. The cells were examined by phase-contrast microscopy, using a JVC high-resolution digital camera (KY-F55BE) attached to a Nikon TMS phase-contrast microscope.

Measurement of intracellular neutral lipids

Nile Red is a selective fluorescent stain for intracellular lipid droplets when an excitation wavelength of between 450 and 500 nm and an emission wavelength of greater than 528 nm are used [22]. After staining with Nile Red, the intracellular lipid content of neutrophil-like HL-60 cells was analysed using a Becton Dickinson FACStar Plus Flow Cytometer as described previously [23]. Laser excitation was set at 488 nm and Nile Red fluorescence was collected through a 530/30 nm band pass filter. A total of 10000 events were collected per sample.

Caspase activity assay

Caspase activities were determined as described previously [24], using Ac-DEVD-AMC and Ac-LEHD-AFC as fluorogenic substrates for caspase 3 and caspase 9 respectively.

Measurement of intracellular thiobarbituric-acid-reactive substances (TBARS)

After the resuspension of cells in 20% (w/v) trichloroacetic acid, the intracellular TBARS concentration was
Effects of unsaturated fatty acids on HL-60 cells
determined as described previously [25]. The TBARS
congestion in each sample was calculated using an
extinction coefficient of 1.56 × 10^5 M^{-1} cm^{-1} [25], and
then expressed as nmol TBARS/mg of protein. Vitamin E supplementation (10 μM α-tocopherol) was in-
cluded in some incubations, where indicated.

Measurement of reactive oxygen species (ROS) production
Cells (1 × 10^6) were resuspended in Hanks balanced salt
solution containing the fluorescent probe lucigenin
(0.5 mM bis-N-methylacridinium nitrate). After the addi-
tion of 100 ng/ml of PMA, the generation of ROS was
assessed by the reduction of lucigenin and the results
expressed as the increase in chemiluminescence relative
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Statistical analysis
Statistical analysis was carried out using Student’s t test
and ANOVA with Student–Newman correction. A
P value of < 0.05 was considered to be statistically
significant.

RESULTS
RA-induced differentiation of HL-60 cells into neutrophil-like cells
RA treatment resulted in a time-dependent increase in
the percentage of HL-60 cells undergoing spontaneous
apoptosis, from approx. 2% prior to treatment with RA
to approx. 10% and 60% after 3 and 10 days of treatment
respectively (Figure 1a). RA treatment of HL-60 cells
also increased their PMA-induced rate of superoxide
production in a time-dependent manner (Figure 1b), in

Figure 1 Effects of RA on (a) spontaneous apoptosis and (b)
PMA-stimulated ROS production in HL-60 cells
HL-60 cells were differentiated into neutrophil-like cells by treating them with RA
(1 μM) over a 10-day period. HL-60 cell differentiation was determined at various
time-points by monitoring the percentage of apoptosis and ROS production after
stimulation with PMA (100 ng/ml). (a) Apoptosis was assessed using flow
cytometry to measure the binding of propidium iodide to DNA. (b) HL-60 cells
(1 × 10^6) were suspended in Hanks balanced salt solution containing 0.5 mM
lucigenin and the rate of ROS production was determined as described in the
Materials and methods section. RLU, relative light unit.

Figure 2 Effects of fatty acid supplementation on the size
and granularity of RA-treated HL-60 cells
Neutrophil-like HL-60 cells were treated with various fatty acids for 24 h. Using
flow cytometry, the forward light scatter and side light scatter of the laser beam
were used as indices of cell size and granularity respectively, as described in the
Materials and methods section. Representative dot plots are shown for untreated
(control) cells and those treated for 24 h with (a) No supplementation, or 75 μM
of the following fatty acids (b) AA, (c) DHA, (d) EPA, (e) OA and (f) PA. Each dot
plot represents 10 000 events (cells).
agreement with a previous report [26]. Taken together, these results demonstrate that RA treatment of HL-60 cells resulted in their differentiation into cells that displayed neutrophil-like characteristics.

Accumulation of cellular lipid following fatty acid supplementation

Based on the light scatter of control (non-supplemented) cells, a gate (region 1, R1) representing the main population of cells in the CellQuest software-derived dot plot was formed (Figure 2a). Analysis of the cells in R1 of the dot plots indicated that there was a general increase in both the forward scatter and side scatter of the cells following treatment with arachidonic acid (AA), docosahexanoic acid (DHA), eicosapentaenoic acid (EPA) or oleic acid (OA), which indicates an increase in the size and granularity of the cells (Figure 2). Supplementation with palmitic acid (PA) also resulted in an increase in cell size and granularity, but to a lesser extent (Figure 2f). Although an increase in cell size is usually associated with necrotic cell death [27], morphological examination of the cells by phase-contrast microscopy after supplementation with fatty acids did not provide evidence of necrosis (results not shown); however, it was noticeable that cells that had been incubated with AA, DHA, EPA or OA for 8 h or more contained numerous intracellular inclusions that were not observed in control cells or those supplemented with PA (Figure 3).

In order to determine whether the intracellular inclusions observed in the fatty acid-supplemented cells were accumulations of fatty acids in the form of neutral lipid, e.g. triacylglycerol (TAG), the cells were treated with Nile Red. Nile Red has been shown previously to selectively stain intracellular neutral lipid when an
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Figure 5  Effect of fatty acids on the apoptosis of RA-treated HL-60 cells

Neutrophil-like HL-60 cells (treated with 1 μM RA for 4 days) were supplemented with one of a number of fatty acids (AA, DHA, EPA, OA or PA). At various time-points, the percentage of apoptotic cells was assessed by the binding of propidium iodide to DNA using flow cytometry, as described previously [44]. Results shown are means ± S.D. of four separate experiments.

Excitation wavelength between 450 and 500 nm and an emission wavelength greater than 528 nm are used [22]. The Nile Red fluorescence of neutrophil-like HL-60 cells treated with AA, DHA, EPA or OA was clearly greater than that of the non-supplemented cells (Figure 4), which indicates an increase in intracellular neutral lipid content likely to be as a result of fatty acid uptake. In the case of cells supplemented with PA, no increase in Nile Red fluorescence was evident compared with control cells (Figure 4e).

Effect of fatty acids on apoptosis of neutrophil-like HL-60 cells

Flow cytometric analysis of DNA fragmentation

Analysis of DNA fragmentation demonstrated that fatty acid supplementation (25–75 μM) induced apoptosis in RA-treated HL-60 cells in a time- and concentration-dependent manner (Figure 5). PUFAs had the strongest pro-apoptotic effect, that of PA was the weakest. These findings were confirmed by analysis of cell morphology, where an increased percentage of fatty acid-supplemented cells exhibited characteristic features of apoptosis including cell shrinkage and indistinct nuclei (Figure 6).

Effects of fatty acids on caspase activities

Caspases are the central executioners of the apoptotic process, and the effect of fatty acid supplementation for 4 h on the caspase 3 and caspase 9 activities of RA-treated HL-60 cells was examined after supplementation. Caspase 3 and caspase 9 activities were increased significantly (P < 0.05) after 4 h of supplementation with AA, DHA or EPA, compared with pre-supplementation values (Table 1). The mean caspase 3 and caspase 9 activities of the cells tended to be increased by OA and decreased by PA, but these effects were not statistically significant (P > 0.10) (Table 1).

Effect of fatty acid supplementation on lipid peroxidation products

The effect of fatty acid supplementation on cellular lipid peroxidation was determined by measuring the intracellular TBARS concentration. Supplementation of neutrophil-like HL-60 cells with PUFAs significantly (P < 0.05) increased the intracellular TBARS concent
Table 1  Effects of 4 h of fatty acid supplementation on the caspase 3 and caspase 9 activity of neutrophil-like HL-60 cells

HL-60 cells that had been treated with RA (1 µM, for 4 days) were incubated with 75 µM fatty acid (AA, DHA, EPA, OA or PA) for 4 h. Cell lysates were prepared from $10 \times 10^6$ cells and the caspase 3 and caspase 9 activities were assessed by the increased fluorescence intensity of free fluorescent AMC cleaved from Ac-DEVD-AMC and Ac-LEHD-AFC respectively, as described previously [24]. Results are means ± S.D. from four separate experiments. * $P < 0.05$ compared with value in absence of fatty acid supplementation.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Caspase 3 (relative fluorescence units)</th>
<th>Caspase 9 (relative fluorescence units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.02 ± 3.81</td>
<td>11.04 ± 3.26</td>
</tr>
<tr>
<td>AA</td>
<td>18.89 ± 4.64*</td>
<td>16.64 ± 3.02*</td>
</tr>
<tr>
<td>DHA</td>
<td>17.96 ± 4.19*</td>
<td>15.07 ± 2.83*</td>
</tr>
<tr>
<td>EPA</td>
<td>17.03 ± 3.68*</td>
<td>15.35 ± 2.94*</td>
</tr>
<tr>
<td>OA</td>
<td>15.84 ± 3.40</td>
<td>13.09 ± 2.11</td>
</tr>
<tr>
<td>PA</td>
<td>9.87 ± 2.53</td>
<td>9.07 ± 1.99</td>
</tr>
</tbody>
</table>

Figure 7  TBARS levels in fatty acid-supplemented, RA-treated HL-60 cells

Neutrophil-like HL-60 cells (treated with 1 µM RA for 4 days) were supplemented with one of various fatty acids (AA, DHA, EPA, OA or PA). At various time-points, the intracellular TBARS levels were determined as described previously [2], while the percentage of apoptotic cells was assessed, using flow cytometry, by the binding of propidium iodide to DNA, as previously described [4]. Results are means ± S.D. from five separate experiments.

Fatty acid-induced apoptosis occurs independently of lipid peroxidation

Owing to the fact that the fatty acid-induced apoptosis of RA-treated HL-60 cells was associated with a concomitant increase in intracellular lipid peroxidation products, we wanted to determine whether lipid peroxidation was involved in the pro-apoptotic mechanism. To do this, 10 µM vitamin E was included in some incubations, since this concentration has been shown previously to protect against intracellular increases in lipid peroxidation products induced by supplementation with PUFAs [28].

Co-incubation with 10 µM vitamin E significantly attenuated ($P < 0.05$) the PUFA-induced increase in the duration of supplementation and the concentration of fatty acid used (Figure 7).
in intracellular TBARS concentration in RA-treated HL-60 cells. After 4 h of supplementation, the TBARS levels measured in the presence of vitamin E were between 45% and 75% of the values observed in the absence of vitamin E (results not shown). At the 24-h time-point, the intracellular TBARS levels in cells that were incubated with AA, DHA or EPA in the presence of vitamin E were 34%, 31% and 31%, respectively, of the levels measured in the absence of vitamin E (Figure 8a). Vitamin E did not affect the TBARS levels of control cells or those supplemented with PA (Figure 8a). Despite its protective effect against PUFA-induced increases in intracellular TBARS levels, vitamin E did not significantly (P > 0.15) alter the PUFA-induced increase in apoptosis of the RA-treated HL-60 cells (Figure 8b).

**DISCUSSION**

The aim of the work presented here was to determine whether supplementation of a neutrophil-like cell line with one of a variety of unsaturated or saturated fatty acids could alter its rate of apoptosis. HL-60 cells that had been differentiated with RA were used as a model for human neutrophils. RA-induced differentiation of HL-60 cells has been extensively studied. Increased superoxide production upon stimulation with PMA and induction of apoptosis are two markers that increase during the differentiation process [26]. Primary human neutrophils have a limited life span (8–24 h) and would not be of use in studies in which a prolonged period of incubation is necessary (as described here).

The total concentration of fatty acids added to the incubation medium in this study was in the range 25–75 μM, which is lower than the total *in vivo* fatty acid concentration that is typically found in human serum (approx. 0.3–1.0 mM) [29]; however, most serum fatty acid is bound to albumin, and only a small percentage of the total amount remains as unbound fatty acid that can interact directly with cells [30]. Similarly, a substantial percentage of the fatty acid added to the medium used in this study would have been bound with albumin provided by FCS (which constituted 20% of total medium volume). The exact concentration of unsaturated fatty acids in the incubation medium was not determined, but may have been higher than the *in vivo* concentration, which has been reported to be as low as 10 nM [29].

A major finding of the work presented here is that fatty acid supplementation of a neutrophil-like cell line resulted in fatty acid uptake and accumulation as neutral lipid in cytoplasmic inclusions (Figure 3). This response to *in vitro* fatty acid supplementation has previously been reported for U937-1 cells [30]. The appearance of intracellular lipid stores (perhaps in the form of lipid bodies) reported here was associated, perhaps not surprisingly, with an increase in the size and granularity of the RA-treated HL-60 cells (Figure 2). Similar increases in cell size and granularity following vitamin E formation have previously been reported for fibroblasts [23]. The *in vitro* ‘lipid body’ formation observed in this study may be particularly relevant to sites of inflammation in *in vivo*, where a high fatty acid concentration in serum may be created by the actions of secretory phospholipase A₂ [31]; leucocytes from the joints of patients with arthritis [32] and the airways of patients with acute respiratory distress syndrome [33] characteristically contain lipid-rich cytoplasmic inclusions.

This study demonstrates that total fatty acid concentrations of between 25 and 75 μM induce apoptosis in RA-treated HL-60 cells in a time- and concentration-dependent manner, as assessed by analysis of DNA fragmentation (Figure 5). The results obtained by the flow cytometric analysis of cellular DNA were confirmed by morphological examination of the cells for characteristic features of apoptosis, including chromatin condensation and cell shrinkage (Figure 6). The additional confirmatory method of apoptosis detection was employed in the light of several reports of a type of cell death that resembles apoptosis, but which lacks the characteristic DNA fragmentation [34,35]. In addition to the effects on RA-treated HL-60 cells reported here, PUFA supplementation has been shown to induce apoptosis in a number of other cell types, including human neutrophils [36]. This raises the possibility that fatty acid concentration *in vivo* may impact upon the rate of neutrophil apoptosis and thus contribute to the resolution of inflammation.

Although fatty acid supplementation of RA-treated HL-60 cells induced both formation of lipid bodies and apoptosis, a potential role for lipid bodies in the apoptotic pathway was not investigated further. Lipid bodies are thought to be important modulators of immune-cell function, and a correlation between lipid body formation and the inflammatory response of neutrophils has been reported [37]. Lipid bodies may exert their effects on leucocyte functions through the actions of inflammatory mediators such as eicosanoids, since cyclo-oxygenase has been found in lipid bodies from human mast cells, macrophages, neutrophils and eosinophils [38,39]. No direct evidence was obtained in this study to support ‘lipid body’ involvement in fatty acid-induced apoptosis of RA-treated HL-60 cells, and lipid body formation may have occurred only as a consequence of an early, biochemically active phase of the apoptotic process [40]; however, an association between lipid body formation and apoptosis has been reported for several cell types. For example, the postseptation apoptosis of rat lung fibroblasts occurs primarily in lipid body-containing cells and not in those lacking lipid bodies [23]. In addition, reduced proliferation of the human gastric cell line HGT has been shown to result from a shift of AA from the phospholipid to the TAG lipid fraction [41].
while it appears that the reduction in U937-1 cell numbers caused by incubation with EPA may be due to the accumulation of TAG-rich lipid droplets containing EPA [30]. It should be noted, however, that a recent paper dealing with lipid body formation in β-cells suggests that intracellular TAG accumulation may in fact be a mechanism of protection against free fatty acid-induced apoptosis [42]. Maximum accumulation of fatty acids in lipid body TAG stores may lead to a rise in the cellular free fatty acid content and subsequent apoptosis through the increased synthesis of ceramide [43]. What is clear is that further work is required to fully elucidate the roles played by lipid bodies in the apoptotic and functional responses of immune cells.

Stimulation of caspase activity is critical for the initiation of the apoptotic process. It is clear from our results that the activities of caspase 3 and caspase 9 are activated after treatment of neutrophil-like HL-60 cells with unsaturated fatty acids (Table 1). It is possible that fatty acid-derived metabolites can interact with and activate the caspase cascade (see below).

Supplementation of the neutrophil-like HL-60 cells with unsaturated fatty acids resulted in a significant increase in intracellular levels of lipid peroxidation products, whereas PA had no effect (Figure 7). Such increases in intracellular levels of lipid peroxidation products following PUFA supplementation have been reported for other cell types [28,44,45]. In addition, it has been shown that supplementation of the human diet with relatively high levels of PUFAs increases indices of oxidative stress in plasma [46,47] and urine [48], and increases the concentration of oxidized glutathione in whole blood [48]. It appears, from the work presented here, that the pro-apoptotic effects of unsaturated fatty acids on RA-treated HL-60 cells may be mediated largely via a lipid peroxidation-independent mechanism, as indicated by the lack of effect of vitamin E on the rate of fatty acid-induced apoptosis (Figure 8b), despite significant inhibition of lipid peroxidation (Figure 8a).

To conclude, high extracellular concentrations of free fatty acids induce lipid accumulation and apoptosis in RA-differentiated (neutrophil-like) HL-60 cells. The fatty acid-induced apoptosis appeared to occur mainly via a mechanism that was independent of TBARS formation, and was associated with increased activities of caspase 3 and caspase 9. The ability of the neutrophil-like cells to accumulate cytoplasmic TAG may act as a protective mechanism against free fatty acid-induced apoptosis by preventing a cellular rise in free fatty acyl moieties, as recently hypothesized in the case of the pancreatic β-cell [42]. If this proves to be the case, hormonal or pharmacological stimulators or inhibitors of TAG synthesis may thus have the potential to modulate levels of apoptosis in neutrophils and other cell types. This may benefit disease resolution in neutrophil-mediated inflammatory conditions; for example, disorders such as cystic fibrosis, acute respiratory distress syndrome, acute pancreatitis and inflammatory bowel disease have been associated with delayed neutrophil apoptosis which leads to their persistence and associated damage of the lungs and other organs owing to inflammatory mediators such as reactive oxygen intermediates, elastase, collagenase, myeloperoxidase and lysozyme.

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