Short Communication

In vitro fatty acid enrichment of macrophages alters inflammatory response and net cholesterol accumulation

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Dietary long-chain PUFA, both n-3 and n-6, have unique benefits with respect to CVD risk. The aim of the present study was to determine the mechanisms by which n-3 PUFA (EPA, DHA) and n-6 PUFA (linoleic acid (LA), arachidonic acid (AA)) relative to SFA (myristic acid (MA), palmitic acid (PA)) alter markers of inflammation and cholesterol accumulation in macrophages (Mφ). Cells treated with AA and EPA elicited significantly less inflammatory response than control cells or those treated with MA, PA and LA, with intermediate effects for DHA, as indicated by lower levels of mRNA and secretion of TNFα, IL-6 and monocyte chemoattractant protein-1. Differences in cholesterol accumulation after exposure to minimally modified LDL were modest. AA and EPA resulted in significantly lower Mφ scavenger receptor 1 mRNA levels relative to control or MA-, PA- and LA-treated cells. These data suggest changes in the rate of bidirectional cellular cholesterol flux. In summary, individual long-chain PUFA have differential effects on inflammatory response and markers of cholesterol flux in Mφ which are not related to the n position of the first double bond, chain length or degree of saturation.

n-6 Fatty acids: n-3 Fatty acids: Macrophages: THP-1 cells: Inflammation: Minimally modified LDL-induced cholesterol accumulation

Dietary fatty acids are thought to affect atherosclerotic lesion progression, in part, through altering macrophage (Mφ) behaviour. With respect to long-chain PUFA, α-linolenic acid (18 : 3n-3) can be converted to EPA (20 : 5n-3) and DHA (22 : 6n-3) which are precursors of the 3-series eicosanoids. Linoleic acid (LA; 18 : 2n-6) can be converted to γ-linolenic acid (18 : 3n-6) and arachidonic acid (AA; 20 : 4n-6) which are precursors of the 2-series eicosanoids. The 3-series eicosanoids are less pro-inflammatory than their 2-series counterparts. The effect of dietary n-6 PUFA, including LA and AA, relative to the very-long-chain n-3 PUFA, EPA and DHA, on inflammatory biomarkers and CVD risk remains controversial\textsuperscript{(3)}.

In the aortic wall, Mφ play roles in both inflammation and cholesterol accumulation\textsuperscript{(2)}. Mφ express scavenger receptors that uptake modified lipoproteins through membrane-bound Mφ scavenger receptor 1 (MSR1) and cluster of differentiation 36 (CD36)\textsuperscript{(5)}. Increased expression of MSR1 and CD36 results in increased uptake of modified lipoproteins\textsuperscript{(4)}.

Two important Mφ membrane receptors involved in cholesterol efflux are ATP-binding cassette A1 (ABCA1) and scavenger receptor B class 1 (SR-B1). When Mφ cholesterol influx is greater than efflux, cholesterol homeostasis in Mφ is disturbed and cholesterol accumulates in the Mφ. Elevated levels of albumin-bound NEFA are positively associated with esterified cholesterol (EC) accumulation\textsuperscript{(5)}.

IL-6 and TNFα are major pro-inflammatory factors. Plasma IL-6 and TNFα concentrations are positively associated with CVD risk\textsuperscript{(6)}. Overexpression of the chemokine monocyte chemotactic protein-1 (MCP-1) has been positively associated with monocyte recruitment in fatty streaks\textsuperscript{(7)}. TNFα, IL-6 and MCP-1 have been used as biomarkers for CVD risk. Some studies have shown that n-3 PUFA decrease inflammatory response through binding and regulating NF-κB activity. In contrast, SFA do not bind to NF-κB\textsuperscript{(8)}. There is limited information on the impact of individual fatty acids on these biomarkers.

Abbreviations: AA, arachidonic acid; ABCA1, ATP-binding cassette A1; CD36, cluster of differentiation 36; EC, esterified cholesterol; LA, linoleic acid; Mφ, macrophage; MA, myristic acid; MCP-1, monocyte chemotactic protein-1; MSR1, macrophage scavenger receptor 1; PA, palmitic acid; SR-B1, scavenger receptor B class 1.

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The aim of the present study was to determine the effect of n-3 PUFA (EPA (20 : 5) and DHA (22 : 6)) and n-6 PUFA (LA (18 : 2) and AA (20 : 4)) relative to two SFA, myristic acid (MA; 14 : 0) and palmitic acid (PA; 16 : 0), on inflammatory response and cholesterol accumulation in MΦ differentiated from THP-1 cells.

Materials and methods

Cell culture

Human monocytic THP-1 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured as previously described(9). Exogenous fatty acids complexed to albumin at 100 μM were added in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% lipoprotein-deficient fetal bovine serum to cells and incubated for 24 h. This concentration mimics the physiological plasma concentration of MA, PA and LA(10), but it is somewhat higher than AA, EPA and DHA(11) normally observed in humans. Cell viability was determined by trypan blue exclusion. Cellular protein concentration was measured by the bicinchoninic acid method (Pierce Inc., Rockford, IL, USA). Each experiment was performed in three independent cell cultures.

Macrophage fatty acid analysis

MΦ lipid extraction and fatty acid analysis were performed as previously described(12).

Secretion of inflammatory factors

Cells were treated with fatty acids in combination with Escherichia coli lipopolysaccharide (Sigma, St Louis, MO, USA) as previously described(13). TNFα, IL-6 and MCP-1 protein concentrations in the culture media were determined using DuoSet® ELISA kits (R&D Systems, Minneapolis, MN, USA).

Minimally modified low-density lipoprotein preparation

LDL was isolated from human plasma by sequential ultracentrifugation(14). Minimally modified LDL was prepared by exposing human LDL to 2 μM-CuSO₄ for 5 h, and oxidation was confirmed by measuring thiobarbituric acid-reactive substances. The standard protocol was to incubate MΦ with 40 μg protein/ml minimally modified-LDL and 100 μM of individual fatty acids for 24 h. Cellular lipid extraction, non-esterified cholesterol and total cholesterol measurement were performed as previously described(15). EC was calculated as the difference between total cholesterol and non-esterified cholesterol.

Real-time polymerase chain reaction

RNA was extracted from MΦ using an RNeasy mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesised from RNA using SuperScript™ II RT according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA). β-Actin was used as an endogenous control. cDNA levels for the genes of interest were measured by using power SYBR green master mix on real-time PCR 7300 (Applied Biosystems, Foster City, CA, USA). mRNA-fold change was calculated using the 2^-△△C_T method(16).

Protein extraction and Western blot

MΦ protein was extracted using radio-immunoprecipitation assay (RIPA) kits (Santa Cruz Biotecchnology, Inc., Santa Cruz, CA, USA). Western blots were performed as previously described(17) using cell lysate with the following primary antibodies, MSR1 (Serotec, Raleigh, NC, USA), SR-B1 (Novus Biologicals, Littleton, CO, USA), ABCA1 (Novus Biologicals, Littleton, CO, USA) and β-actin (Sigma, St Louis, MO, USA). Signals were visualised by chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) and quantified using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA).

Statistical methods

ANOVA (PROC GLM) followed by Tukey’s post hoc test was performed to compare multiple group means (SAS version 9.1; SAS Institute Inc., Cary, NC, USA). Differences were considered significant at P<0.05. Results are presented as mean values and standard deviations.

Results

Cell viability and fatty acid profile

Cell viability was greater than 91% for all fatty acids at 100 μM (data not shown). The fatty acid profile of the MΦ reflected that of the incubation medium, confirming that the supplemental fatty acid was incorporated into the THP-1 cells (Table 1).

Effect of fatty acids on cholesterol accumulation and expression of genes involved in cholesterol flux in macrophages

All fatty acids significantly increased the EC content in MΦ compared with control cells (Table 1). EC accumulation was highest in the EPA- and AA-treated cells relative to the other fatty acid-treated MΦ. Nonetheless, the differences in the EC component of cells were modest, ranging from 15 to 25% of the total cholesterol. No significant effect of fatty acid treatment on MΦ total or non-esterified cholesterol content was observed.

mRNA levels of both MSR1 and ABCA1 were 2- to 3-fold lower in the cells treated with AA and EPA compared with control, MA- or PA-treated cells. This pattern was similar in LA- and DHA-treated cells, although to a lesser extent. The response of CD36 and SR-B1 was more modest than MSR1 and ABCA1 to the individual fatty acids. In contrast, exposure of MΦ to PUFA did not significantly alter the amount of SR-B1, CD36 or MSR1 protein compared with control or SFA, and only slightly lowered ABCA1 protein compared with MA- and PA-treated cells (data not shown). These data suggest that the effect of exposing MΦ to minimally modified...
There has been a lack of consistency in the literature as to the Discussion consistent for cells treated with AA and EPA relative to the other their ability to modulate the inflammatory response and as well as the individual fatty acids within each family, on (Mean values and standard deviations)

### Table 1. Fatty acid composition (mol %) and cholesterol content (mg/100 mg protein) of macrophages differentiated from THP-1 cells*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>MA</th>
<th>PA</th>
<th>LA</th>
<th>AA</th>
<th>EPA</th>
<th>DHA</th>
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<td>3·35</td>
<td>59·64a</td>
<td>1·16</td>
<td>54·46b</td>
<td>1·73</td>
<td>31·95±o</td>
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<td>0·07</td>
<td>0·54c,b</td>
<td>0·08</td>
<td>0·31c</td>
<td>0·06</td>
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<td>8‘ 0‘ 1‘ 6‘ a,b,c</td>
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<td>7‘ 26a</td>
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<td>0·19</td>
<td>1·22a</td>
<td>0·34</td>
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<td>0·18b</td>
<td>0·03</td>
<td>0·15a,b</td>
</tr>
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</table>

MA, myristic acid; PA, palmitic acid; LA, linoleic acid; AA, arachidonic acid; TC, total cholesterol; FC, non-esterified cholesterol; EC, esterified cholesterol.

* Mean values within a row with unlike superscript letters were significantly different (P<0·05).

* Each experiment was performed in three independent cell cultures.

LDL was to alter the rate of cholesterol flux with little effect on net accumulation.

**Effect of individual fatty acids on inflammatory factor secretion and mRNA levels in stimulated macrophages**

Relative to control, MA and PA, ΜΦ exposed to AA and EPA resulted in lower levels of TNFα, IL-6 and MCP-1 in the culture medium (Fig. 1). Of note, the relationship between inflammatory factor secretion and their mRNA levels was consistent for cells treated with AA and EPA relative to the other cells but not with DHA (Fig. 1).

**Discussion**

There has been a lack of consistency in the literature as to the nature and relative potencies of the n-6 and n-3 ΜΦA families, as well as the individual fatty acids within each family, on their ability to modulate the inflammatory response and aortic lesion formation (11). This is the first study to address this issue in an isolated cell system.

AA and EPA resulted in the lowest in vitro inflammatory response in ΜΦ relative to the other fatty acids assessed. The inflammatory factors IL-6 and TNFα and the chemokine MCP-1 have relatively short half-lives in plasma (19). Their sustained concentrations depend on new protein synthesis. In the present study we observed that the inhibitory effect of AA and EPA relative to the other fatty acids assessed on the secretion inflammatory factors was associated with lower mRNA levels of these inflammatory factors, suggesting that AA and EPA may have altered protein synthesis at the transcriptional level. Since some PUFA and their metabolites can regulate NF-κB activity, we speculate that the altered expression of these inflammatory factors may have been mediated by NF-κB (19).

In vivo, desaturases and elongases convert a fraction of dietary LA to γ-linolenic acid and AA. Both γ-linolenic acid and AA modulate the inflammatory state. In the present study, as suggested by the fatty acid profile of the ΜΦ, there was little conversion of LA to AA, which may explain why there was little effect of LA on IL-6 secretion. The fatty acid profile of the ΜΦ post-treatment also suggested little conversion of EPA to DHA and retro-conversion of DHA to EPA. This result is consistent with a previous report (13).

ΜΦ play a major role in the uptake of modified LDL and deposition in the intimal layer of the arterial wall. In response to exposure of the fatty acid-treated ΜΦ to modified LDL there were modest differences in EC accumulation but no net change in total cholesterol concentration. Nevertheless,
relative to MA and PA, AA and EPA, and to a lesser extent LA and DHA, significantly lowered the mRNA levels of MSR1 and ABCA1, and ABCA1 protein levels, suggesting alternations in cellular cholesterol flux. In addition to these findings, differential expression and activities of acyl-CoA:cholesterol acyltransferase and cholesteryl ester hydrolase may have led to the observed differences in MΦ EC accumulation. Furthermore, incubating MΦ with LDL enriched with different fatty acids vs. fatty acids bound to albumin has been shown to differentially affect EC hydrolysis and cellular cholesterol efflux\(^22\), which could also account for the present results.

The lack of clear influence of the position of the first double bond from the methyl end of the acyl chain on inflammatory factor release and mRNA expression was somewhat unexpected\(^22\). Previous work has demonstrated that fish oil, containing both EPA and DHA, reduced secretion of inflammatory factors in lipopolysaccharide-stimulated mononuclear cells\(^23\). Nevertheless, few studies have directly compared EPA with DHA. Although peritoneal MΦ isolated from C57BL/6 mice fed fish oil containing different ratios of EPA:DHA were reported to exhibit reduced secretion of TNFα and IL-6, and this reduction was greater in those mice fed fish oil containing the highest ratio of EPA:DHA\(^24\).

Fig. 1. Effect of individual fatty acids on the secretion (A, C, E; expressed as ng inflammatory factor/mg cell protein) and mRNA levels (B, D, F; expressed as fold change relative to control (Con)) of TNFα (A and B), IL-6 (C and D) and monocyte chemotactic protein-1 (MCP-1) (E and F) in macrophages (MΦ) differentiated from THP-1 cells. MΦ were pretreated with 100 μM-fatty acids for 2 h. Thereafter lipopolysaccharide was added at 1 μg/ml, and the cells were incubated for an additional 24 h. MA, myristic acid; PA, palmitic acid; LA, linoleic acid; AA, arachidonic acid. Values are the means of three independent experiments, with standard deviations represented by vertical bars. *a,b,c,d Mean values with unlike letters were significantly different (\(P < 0.05\)).
In summary, relative to control and SFA, PUFA had an inhibitory effect on transcriptional levels of inflammatory factors in and their secretion from MΦ differentiated from THP-1 monocytes. AA and EPA had a more pronounced effect than LA and DHA. These data suggest that individual long-chain PUFA have differential effects on lipopolysaccharide-stimulated inflammatory response and transporters of cholesterol flux in MΦ which are not related to the \( n \) position of the first double bond, chain length or degree of saturation.

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S. W. and A. H. L. planned the experiments. S. W. and K. L. H. performed the analytical work. D. W., S. L.-F. and N. R. M. provided scientific expertise. S. W. and A. H. L. wrote the manuscript, with D. W., S. L.-F. and N. R. M. providing input.

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The authors have no conflict of interest to declare.

References