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ORIGINAL CONTRIBUTION

Danielle I. Shaw
Wendy L. Hall
Natasha R. Jeffs
Christine M. Williams

Comparative effects of fatty acids on endothelial inflammatory gene expression

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D.I. Shaw (✉) · N.R. Jeffs
C.M. Williams
Hugh Sinclair Unit of Human Nutrition
School of Food Biosciences
University of Reading
Reading RG6 6AP, UK

W.L. Hall
Nutritional Sciences Research Division
King's College London
London SE1 9NH, UK

Abstract *Background* Endothelial dysfunction may be related to adverse effects of some dietary fatty acids (FAs). Although *in vitro* studies have failed to show consistent findings, this may reflect the diverse experimental protocols employed and the limited range of FAs and end points studied. *Aims* To investigate the effect of dietary FA type (saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids), concentration, incubation time and cell stimulation state, on a broad spectrum of endothelial inflammatory gene expression. *Methods* Using human umbilical vein endothelial cells, with and without stimulation (±10 ng/ml TNFα), the effects of arachidonic (AA), docosahexaenoic (DHA), eicosapentaenoic (EPA), linoleic (LA), oleic (OA) and palmitic acids (PA) (10, 25 and 100 μM), on the expression of genes encoding a number of inflammatory proteins and transcription factors were assessed by quantitative real time RT-PCR. *Results* Individual FAs differentially affect endothelial inflammatory gene expression in a gene-specific manner. EPA, LA and OA significantly up-regulated MCP-1 gene expression compared to AA (p = 0.001, 0.013, 0.008, respectively) and DHA (p < 0.0005, = 0.004, 0.002, respectively). Furthermore, cell stimulation state and FA incubation time significantly influenced reported FA effects on gene expression. *Conclusion* The comparative effects of saturated, monounsaturated, n-6 and n-3 polyunsaturated FAs on endothelial gene expression depend on the specific FA investigated, its length of incubation, cell stimulation state and the gene investigated. These findings may explain existing disparity in the literature. This work was funded by the EC, Framework Programme 6 via the LIPGENE project (FOOD-CT-2003-505944).

Key words endothelium – fatty acid – gene expression – inflammation

Introduction

The endothelium is a monolayer of cells lining the blood vessel wall. It plays a critical role in the regulation of vascular tone, permeability and the maintenance of vascular homeostasis [18]. Endothelial dysfunction is characterized by chronic activation of the endothelium with increased production of inflammatory chemokines, e.g. monocyte chemoattractant protein-1 (MCP-1), cytokines, including

tumour necrosis factor α (TNFα), and cellular adhesion molecules (CAMs), such as vascular cellular adhesion molecule-1 (VCAM-1).

Although the role of diet in the aetiology of endothelial dysfunction is not fully understood, human epidemiological studies provide some evidence that dietary fat quality may influence the function of the endothelium [28, 36]. Human intervention studies conducted to date have evaluated only a small number of fatty acid (FA) subclasses and most are limited by low subject numbers and short duration of dietary intervention. In general, saturated fatty acids have been shown to have a negative effect on endothelial function, monounsaturated fatty acids a neutral or modestly beneficial effect, whilst the reported effects of long chain n-3 polyunsaturated fatty acids, on markers of endothelial function, are inconclusive [11–13, 33].

The composition of circulating free fatty acids reflects dietary FA composition to some extent. Therefore, dietary FA intake directly influences the FA profile to which endothelial cells are exposed. A number of *in vitro* studies have compared the effects of different FAs on markers of endothelial function. However, there is considerable disparity in these findings and, as with the human intervention studies, clear conclusions regarding the impact of individual FAs on markers of endothelial activation cannot be drawn at present. For example, linoleic acid (LA) has been reported to increase [34, 37], decrease [27], or have neutral effects on intracellular adhesion molecule-1 (ICAM-1) expression [15, 30]. Similarly, oleic acid (OA) has been found to increase [23], decrease [4, 21], or have no effect on NFκB activation [14, 34], whilst palmitic acid (PA) has reportedly increased [15], decreased [15], or had no effect on VCAM-1 expression [4, 9, 27]. No studies to date have clearly identified these inconsistencies and there has been little effort to explain current disparity.

Most *in vitro* studies have compared the effects of two or three FAs in relation to their impact on a limited number of markers of endothelial function. The most commonly employed markers have been cell surface VCAM-1, ICAM-1 and E-selectin protein expression. LA, OA, docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) have been investigated most frequently [4, 6, 34]. Many experimental variables, such as the type and concentration of FA used, the use (or not) of inflammatory stimuli, its concentration and duration of stimulant exposure, differ considerably between studies, making comparisons difficult. The major aim of the present study was to provide the first systematic investigation of the comparative effects of a wide range of FAs on a broad spectrum of end point measures, using a well established endothelial cell model. The present work was designed to assess effects of FAs under a range of methodological conditions, including various FA concentrations, cell stimulation states and FA incubation times. This study has focused on the effects of FAs on mRNA expression for genes involved in inflammation. Although there have been a limited number of studies which have investigated the impact of FAs on pro-inflammatory gene expression (mainly VCAM-1) and transcription factor activity (NFκB), the majority of previous work has focused on protein expression [8, 15, 27, 31].

Methods

Endothelial cell culture

Clonetics™ Human Umbilical Vein Endothelial Cells (HUVEC) were supplied by Cambrex Bio Science, Wokingham, England. The suppliers conducted routine characterisation of the HUVEC through morphological observations. The cultures, sourced from mixed donors and generated from cryopreserved primaries, were developed and maintained using Clonetics endothelial cell basal medium (EBM-2) (Cambrex Bio Science, Wokingham, England) supplemented with hydrocortisone, human fibroblast growth factor (hFGF-B), vascular endothelial growth factor (VEGF), insulin like growth factor-1 (IGF-1), ascorbic acid, heparin, fetal bovine serum (FBS) (2%) and human epidermal growth factor (hEGF). Cells were maintained in a 37°C, 5% humidified atmosphere. Cultures were seeded at a density of 5,000 cells/cm^2 and passages 2–6 were used in experiments.

Experimental protocol

HUVEC were grown to 90% confluence in 6 well plates and pre-incubated for 24 h in EBM-2 culture medium supplemented with 2% delipidised fetal calf serum (FCS) (Cambrex Bio Science, Wokingham, England). Subsequently, HUVEC were incubated for 24 h or 6 h with FAs (complexed to bovine serum albumin (BSA)) at 10, 25 and 100 μM. Control treatments contained BSA only (concentration used was an average of the amount present in the 10–100 μM FA-BSA complexes). To investigate the effect of cell stimulation state, 10 ng/ml recombinant human TNFα (RND Systems, Oxon, UK) was added for the last 4 h of the FA incubation period. Endothelial nitric oxide synthase (eNOS) gene expression was measured after 24 h FA incubation only. Each experiment was performed four times with the exception of peroxisome proliferator activated receptor α (PPARα) and PPARγ in which three experiments were carried out.

Fatty acid-BSA complexes

Sodium salts of arachidonic acid (AA), DHA, EPA, LA, OA and PA (Sigma-Aldrich Company Ltd., UK), were each dissolved in distilled water at a concentration of 50 mg/ml, at room temperature, to form a FA solution. A stock solution of each FA–BSA complex was prepared at molar ratio 2.5:1, FA:BSA, in EBM-2 medium (supplemented with 2% delipidised FCS). Stock solutions were mixed and warmed to 40°C, sterile filtered using a 0.2 μm syringe filter (Sartorius, Epsom, UK) and stored at −20°C. Stock solutions were defrosted and diluted appropriately with EBM-2 medium (2% delipidised FCS), to obtain the desired FA concentrations (10 μM, 25 μM and 100 μM), before addition to cell cultures.

Assessment of cell viability

CytoTox-ONE Homogenous Membrane Integrity Assay (Promega UK Ltd.) was used to assess cell viability. The level of resorufin was measured fluorometrically (emission wavelength 590 nm). This is indicative of lactate dehydrogenase release, a marker of cell membrane integrity.

RNA extraction, cDNA synthesis

Total cell RNA was extracted from HUVEC, cultured in 6 well plates, using the RNeasy Mini Kit (Qiagen Ltd, Crawley, West Sussex). cDNA was generated from RNA at 42°C for 50 min (reaction volume 20 μl) with oligo (dT) (Invitrogen, Paisley, UK) and reverse transcriptase (Superscript II, Invitrogen, Paisley, UK) using protocols recommended by the manufacturer.

Real time reverse transcriptase polymerase chain reaction (RT-PCR)

Primer sequences were designed across an exon-exon junction from the published full length mRNA sequences (www.ncbi.nlm.nih.gov) using the Primer Express software 2.0 (Applied Biosystems, Warrington, UK). Others were sourced from previous publications. The sequences and their source are stated; PPARγ forward primer 5′-TCT CTC CGT AAT GGA AGA CC-3′ reverse primer: 5′-GCA TTC TGA GAC ATC CCC AC-3′ [29, 32]; PPARα forward primer: 5′-ACT TAT CCT GTG GTC CCC CGG-3′, reverse primer: 5′-CCG ACA GAA AGG CAC TTG TGA-3′ [19]; eNOS forward: 5′-AAG GCA GGA GAC AGT GGA TGG A-3′, reverse: 5′-CCC AGT CAA TCC CTT TGG TGC TCA-3′ [22]. The following primers were designed using Primer Express 2.0, NFκB forward: 5′-GGC TAC ACC GAA GCA ATT GAA-3′, reverse: 5′-GTC TCG GAG CTC GTC TAT TTG-3′; MCP-1 forward: 5′-TTC TCA AAC TGA AGC TCG CAC TCT CGC C-3′, reverse: 5′-TGT GGA GTG AGT GTT CAA GTC TTC GGA GTT-3′; β-actin forward: 5′-GTG GGG CGC CCC AGG CAC CA-3′, reverse: 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′; E-selectin forward: 5′-CCG AGC GAG GCT ACA TGA ATT GAT-3′, reverse: 5′GAG CAC ACC TCA CCA AAC CCT TCG-3′; VCAM-1 forward: 5′-GAC TGG GAG CTC TGT CAC TGT AAG C-3′, reverse: 5′-GAC CAA GAC GGT TGT ATC TCT GGG-3′. Real time RT-PCR was performed using Absolute QPCR SYBR Green ROX Mix (ABgene, Epsom, UK) using a 7300 Real time PCR system (Applied Biosystems). The PCR was run for 15 min at 95°C followed by 40 cycles of 30 s at 94°C, 60 s at 55°C and 30 s at 72°C. After amplification, melting curve analysis was performed to verify specificity of the reactions. The end point used in the real time RT-PCR quantification, Ct, was defined as the PCR cycle number at which threshold fluorescence was reached, and the levels of relative gene expression were calculated using the Pfaffl method [25]. The expression of each target gene was normalized to β-actin expression, and the data for each FA represents fold change in mRNA expression relative to the appropriate control, unstimulated or stimulated, i.e. BSA only or BSA with 10 ng/ml TNFα, respectively. The relative expression fold change was arbitrarily set at 1.

Statistics

Data were analysed using SPSS version 14 (SPSS, Inc., Chicago, IL). Results are presented in the text and figures as means ± SEM.

A four factor analysis of variance was used to assess the effect of FA type, concentration, cell stimulation state and incubation time on relative gene expression. Significant findings were further explored using the least significant difference (LSD) post hoc test. In the case of eNOS, a three factor analysis of variance was used as the effect of incubation time was not investigated.

Results

Toxicity studies

Compared to normal EBM-2 supplemented medium there was an absence of any significant cytotoxic effects of the individual FAs at concentrations up to 100 μM when incubated for 24 h.

Comparison of effects of fatty acids on the gene expression of inflammatory proteins, eNOS and transcription factors

The effects of six FAs on the gene expression of CAMs, MCP-1, eNOS and transcription factors are shown in Figure 1 a–b. These data represent the overall effect of the FAs when investigated at three concentrations (10, 25, 100 μM), after incubation for 6 and 24 h, in both TNFα stimulated and unstimulated HUVEC.

There were significantly different responses to individual FAs for MCP-1 gene expression ($p < 0.0005$). Post hoc analysis showed EPA, LA and OA significantly up-regulated MCP-1 gene expression compared to AA ($p = 0.001, 0.013, 0.008$, respectively) and DHA ($p < 0.0005, = 0.004, 0.002$, respectively). EPA also significantly up-regulated MCP-1 gene expression compared to PA ($p = 0.016$). Although there appeared to be a tendency for AA and DHA to cause down-regulation in VCAM-1, E-selectin and eNOS expression compared to the other FAs (which all tended to up-regulate expression), differences did not reach statistical significance. There were no significant differential effects of any of the FA exposures on the expression of PPARs or NFκB.

Influence of cell stimulation state on endothelial gene expression

Under control conditions, stimulation of HUVEC with TNFα (10 ng/ml) for 4 h, caused up-regulation in VCAM, MCP-1, E-selectin and NFκB relative gene expression (74.54 ± 50.91, 22.46 ± 6.65, 234.45 ± 112.86, 9.62 ± 4.96, respectively). TNFα stimulation caused little up-regulation in the expression of PPARα, PPARγ and eNOS (1.18 ± 0.19, 1.08 ± 0.28, 1.53 ± 0.83, respectively).

Figure 2a shows the effect of FA incubation on endothelial gene expression in stimulated and unstimulated cells. Importantly, this figure displays the effect of FA incubation relative to the appropriate control (±TNFα), thereby controlling for the effects of stimulation. The data presented shows the overall effect of six FAs, investigated at three concentrations (10, 25, 100 μM), after 6 and 24 h FA incubation. FA incubation in cells stimulated with TNFα (10 ng/ml, 4 h) caused a statistically significant up-regulation of VCAM-1, NFκB and eNOS gene expression compared to FA incubation in unstimulated cells ($p < 0.0005, = 0.009, 0.035$, respectively). E-selectin gene expression also significantly increased after FA incubation in stimulated compared to unstimulated cells ($p = 0.001$) but this did not necessarily occur at all time points or FA concentrations as there were significant interaction effects noted between stimulation state and incubation time ($p = 0.01$) and stimulation state and FA concentration ($p = 0.01$) with this gene.

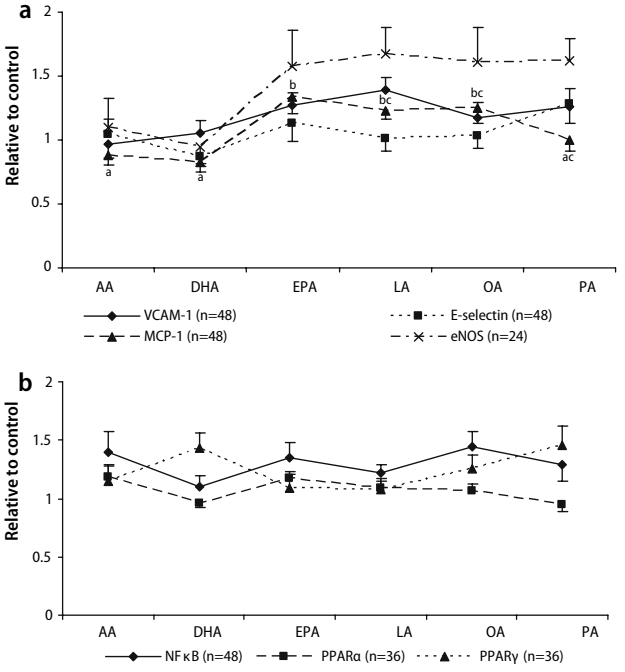

Fig. 1 (**a**–**b**) Individual FAs and gene expression of CAMs, eNOS and transcription factors. FAs (10–100 μM, 6–24 h, ±TNFα) incubated with HUVEC and gene expression measured by real time RT-PCR. Arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA). Fold change in gene expression displayed relative to BSA control, (arbitrarily set at 1). MCP-1, 4 factor ANOVA $p < 0.0005$. ^{abc} Differences in symbol subscripts denote statistical significance. Values expressed as means ± SEM

Influence of fatty acid incubation time on endothelial gene expression

Figure 2b illustrates the effect of FA incubation time on endothelial gene expression. These data represent the overall effect of incubation time when six FAs, at three concentrations (10, 25, 100 μM), ±TNFα stimulation were investigated. VCAM-1 and MCP-1 expression were significantly up-regulated after 24 h FA incubation compared to 6 h incubation ($p = 0.001$, $p = 0.019$). In contrast, E-selectin gene expression was significantly up-regulated at 6 h compared to 24 h FA incubation ($p = 0.002$), although this did not occur at all FA concentrations or stimulation states due to the previously noted interaction effects.

Influence of fatty acid concentration on endothelial inflammatory gene expression

Figure 3a–d shows the dose-response effects of six FAs on MCP-1, E-selectin, PPARα and PPARγ gene

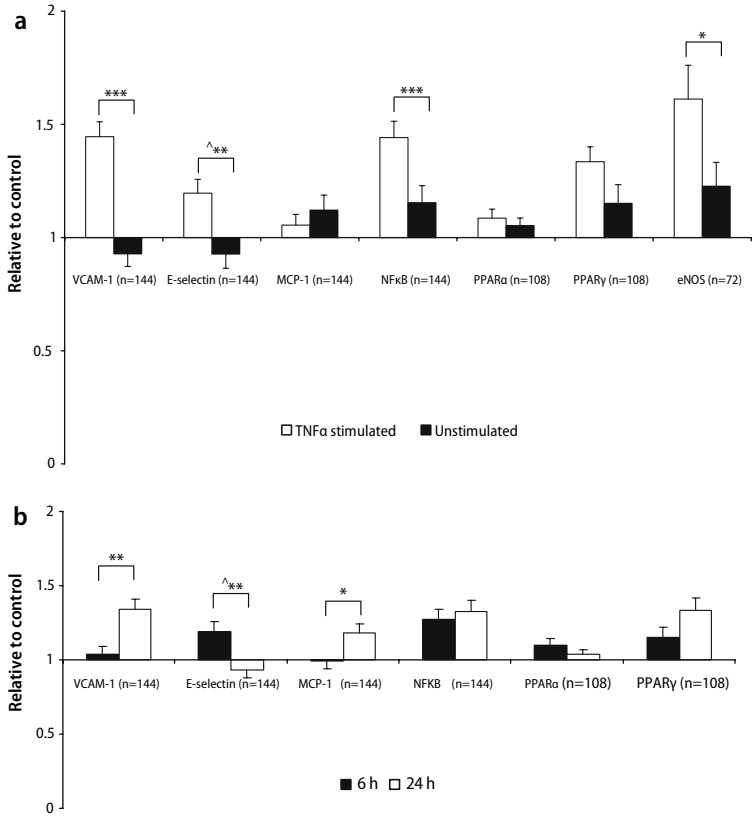

Fig. 2 (**a**–**b**) Influence of study methodology on FA induced gene expression. (**a**) Influence of HUVEC stimulation state on FA induced effects. White bars represent FA incubated with cells stimulated by TNFα 10 ng/ml; black bars represent data for HUVEC incubated with FAs in unstimulated cells. (**b**) Influence of FA incubation time on gene expression. Black bars represent data for 6 h incubation; white bars represent data for 24 h incubation. Data displayed as fold change from relevant control (BSA ± TNFα). Control arbitrarily set at 1. Values expressed as means, error bars represent SEM. Four factor analysis of variance used to assess effect of stimulation and incubation time on all genes with exception of eNOS when three factor analysis was used as eNOS measured at one time point only. *** $p < 0.0005$, ** $p < 0.01$, * $p < 0.05$, ^ interaction effect noted between stimulation state and FA concentration, and stimulation state and incubation time

expression in HUVEC. These data represent the overall effect of FA concentration in stimulated and unstimulated cells, incubated with FAs for 6 and 24 h. There was no significant effect of FA concentration on the expression of any of the genes investigated.

Discussion

This is the first systematic comparative study of the effects of a wide range of FAs, representing the major FA subclasses, on the expression of a broad spectrum of endothelial inflammatory genes and transcription factors. It is important to note that in addition to the use of physiologically relevant concentrations, the FAs used were complexed to BSA. FAs *in vivo* circulate bound to plasma proteins which influences their availability to cells. This is an important factor which has not been considered in the majority of previous work [4, 6, 10, 15, 26, 27, 31].

The present study has produced two main findings. Firstly, individual FAs differentially affect endothelial inflammatory gene expression. Secondly, cell stimulation state and FA incubation time are important variables influencing the effects of FAs on endothelial gene expression. Results indicate similar patterns of VCAM-1, MCP-1, E-selectin and eNOS gene expression following incubation of the six FAs tested, with significant differential FA effects noted in the case of the MCP-1 gene. AA and DHA consistently produced a neutral or down-regulatory effect on the expression of the CAMs, MCP-1 and eNOS. In contrast, EPA, LA, OA and PA predominantly caused up-regulation of the CAMs, MCP-1 and eNOS, although the extent of up-regulation differed between genes.

These findings suggest that the effects of a specific FA on the regulation of an endothelial activation-associated gene will generally be reflected by other markers of endothelial activation. However, LA and OA caused up-regulation of MCP-1, VCAM-1 and eNOS but had neutral effects on E-selectin. Therefore the broad categorisation of a single FA as being either pro- or anti-inflammatory should be applied with caution.

This is the first study to investigate the effects of PA on E-selectin mRNA levels and our findings are consistent with those of Stentz et al. [31] who found PA increased E-selectin cell surface protein expression. However, other studies have demonstrated no effect of PA on cell surface E-selectin expression [15, 27]. These findings do not support the theory that the presence of a double bond within the structure of a FA is a necessary requirement for alteration in endothelial function, as suggested by Carluccio et al. [4]. Nevertheless,

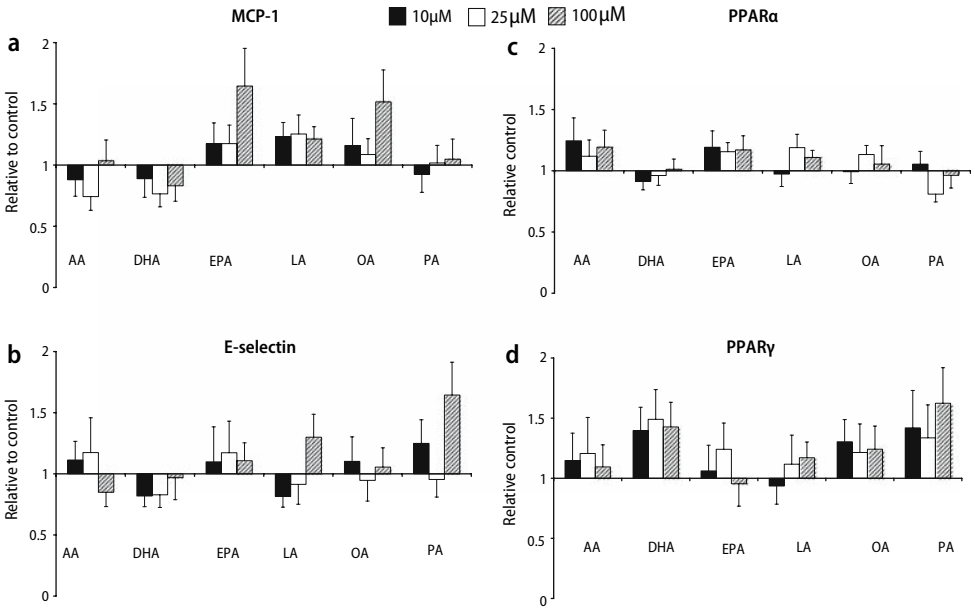

Fig. 3 Dose-response effects of FAs on relative gene expression. (**a**) MCP-1, (**b**) E-selectin, (**c**) PPARα, (**d**) PPARγ. Data displayed as fold change from relevant BSA control (arbitrarily set at 1). Arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA). Black bars represent 10 μM, white bars 25 μM, hatched bars 100 μM FA concentrations. Values expressed as means, error bars represent SEM. n per treatment = 16 for E-selectin and MCP-1, n = 12 for PPARα and PPARγ

we observed that exposure of cells to LA was consistently associated with pro-inflammatory effects, with increased up-regulation of VCAM-1 and MCP-1 relative gene expression, which is in agreement with previous *in vitro* work [10, 34]. Conversely, the results shown here for OA effects on mRNA are not in agreement with previous findings that have shown OA to cause a reduction or neutral effect on inflammatory markers such as ICAM-1, VCAM-1 and MCP-1 [4, 15, 34]. The present findings indicate that OA caused increased VCAM-1 and MCP-1 gene expression compared to control, DHA and AA. This is further supported by the reported up-regulation of NFκB expression after exposure to this FA. Interestingly, the only other study to date that has investigated the effects of BSA-bound OA, reported increased ET-1 production and increased NFκB activation [23]. Davda *et al.* [7] have reported inhibition of endothelium-dependent vasodilation in rabbit femoral artery rings after OA incubation and in human volunteers postprandial flow-mediated vasodilation was reduced by 31% following an OA rich meal [35]. These results suggest caution in attributing the beneficial effects of an olive oil-rich Mediterranean diet on endothelial function, to anti-inflammatory effects of OA [24].

Importantly, these data show that FAs within the same subclasses, e.g. AA and LA (n-6) and DHA and EPA (n-3), do not necessarily influence endothelial inflammatory gene expression in similar directions. Furthermore, although previous work has suggested the degree of FA unsaturation was positively associated with inhibition of cytokine-induced endothelial activation, as assessed by cell surface expression of VCAM-1 [9], our findings for effects on endothelial mRNA expression do not accord with this paradigm.

Previously, only the effects of LA on eNOS expression have been investigated which reported up-regulation, in agreement with our results [26]. Increased eNOS levels have been associated with reduced inflammation, as this enzyme synthesizes nitric oxide (NO), an important vasodilator, which has also been shown to reduce VCAM-1 gene expression [17]. In contrast, our findings show that the same FAs induced up-regulation of CAMs and eNOS expression. This could be explained by the existence of a negative feedback mechanism, whereby an adaptive response is induced involving increased eNOS gene expression to reverse the effects of exposure to FAs which cause CAM up-regulation. In this context it is interesting to note that eNOS level/activity is reported to increase following myocardial infarction [1, 2], which would support the possibility of its up-regulation in response to a major inflammatory insult. Further studies on eNOS activity, NO production and its bioavailability are required to fully elucidate the overall differential effects of FAs on eNOS function. The data presented in this report highlight, for the first time, that eNOS mRNA levels, as for CAM mRNA, can be differentially expressed following exposure to different FAs.

The effects of various FAs on PPARα, PPARγ and NFκB gene expression were investigated. Although differential FA effects did not reach the level of statistical significance, these results provide an indication of possible differential effects of FAs on the mRNA expression of these important transcription factors. PPARα and NFκB followed the same FA response pattern. In contrast, the FA response pattern

for PPARγ differed from that of the other transcription factors investigated, mainly due to opposing responses following exposure to DHA and PA.

This study has shown the effect of FAs on endothelial gene expression depends on the stimulation state of the cells investigated. The FA induced increases in mRNA expression of VCAM-1, E-selectin, NFκB and eNOS were significantly greater when FAs were incubated in TNFα stimulated HUVEC compared to unstimulated HUVEC. If it is accepted that stimulation or non-stimulation of endothelial cells is a representative model for the activated diseased state or the non-activated healthy state, these results show that FAs may cause up-regulation of certain genes in an already activated endothelium compared to a normal, unstimulated endothelium. This work highlights the need for *in vivo* studies to investigate FA effects in both healthy and diseased individuals with the possibility that their response to dietary manipulation may differ.

This study has also found FA incubation time can significantly influence the reported effects of FAs on mRNA expression. In contrast to E-selectin, expression of VCAM-1 and MCP-1 was significantly greater after 24 h FA incubation compared to 6 h. Such time-dependent, gene specific differences may be explained with consideration of the function of the proteins these genes encode. E-selectin plays a key role in the earliest stages of monocyte recruitment at the endothelial surface with involvement in the rolling of monocytes across the endothelium. Following this, the expression of VCAM-1 on the surface of endothelial cells promotes the adherence of the monocytes to the endothelium and their transmigration through it [3, 16]. MCP-1 encourages the migration of monocytes to the endothelial surface and also plays a key role in the tight adhesion of monocytes to the endothelial surface prior to their transmigration [5, 20]. Therefore an earlier timed increase in E-selectin compared to VCAM-1 and MCP-1 after FA incubation might be expected.

Although no significant effects of FA concentration were reported, these data illustrate the degree of variation in response that is observed across this relatively narrow range of concentrations. Further investigation of the comparative effects of individual FAs at their specific physiologically relevant concentrations may be warranted.

The findings presented here show that variation in experimental conditions may explain some of the disparity present in current literature. Moreover, these findings emphasise the need to investigate the effects of FAs under various conditions if a full understanding of their overall impact is to be gained. The current work has met this demand and therefore the authors believe the overall effects of the FAs reported here are of great importance.

In summary, this work confirms that LA, OA and PA tended to have up-regulatory effects on the expression of some inflammatory genes, whereas DHA and AA resulted in either neutral or slight down-regulatory effects on genes associated with pro-inflammation. Furthermore, EPA and DHA tended to cause opposing effects. Importantly, this study has not found AA to up-regulate expression of these inflammatory genes in endothelial cells, although it may be pro-inflammatory via other mechanisms.

This work has confirmed that a wide range of FAs can cause transcriptional alterations in endothelial inflammatory gene expression and these effects are both FA-dependent and gene-specific. Methodological differences such as cell stimulation state and FA incubation time can influence reported FA-induced effects which may explain some of the disparity present in current literature. It is likely that various additional mechanisms, alongside transcriptional alteration, are involved in FA alteration of endothelial inflammatory gene expression if the complexity of their reported effects is considered.

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