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Comparative effect of dairy fatty acids on cell adhesion molecules, nitric oxide and relative gene expression in healthy and diabetic human aortic endothelial cells

K.M. Livingstone [a,b,*], D.I. Givens [a], K.G. Jackson [b,c], J.A. Lovegrove [b,c]

[a] Food Production and Quality Research Division, Faculty of Life Sciences, The University of Reading, Reading RG6 6AR, UK
[b] Department of Food and Nutritional Sciences, University of Reading, Reading RG6 6AR, UK
[c] Institute for Cardiovascular and Metabolic Research, University of Reading, Reading, RG6 6AR, UK

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ABSTRACT

Objective: Dairy intake, despite its high saturated fatty acid (SFA) content, is associated with a lower risk of cardiovascular disease and diabetes. This *in vitro* study determined the effect of individual fatty acids (FA) found in dairy, and FA mixtures representative of a high SFA and a low SFA dairy lipid on markers of endothelial function in healthy and type II diabetic aortic endothelial cells.
Methods: Cells were incubated for 24 h with FA mixtures (400 μM) and individual FA: oleic acid (OA; 150 μM); palmitic acid (PA; 150 μM); stearic acid (SA: 40 μM); *trans*-palmitelaidic acid (*trans*-PA; 20 μM); *trans*-vaccenic acid (*trans*-VA; 20 μM); α-linolenic acid (ALA; 20 μM) and linoleic acid (LA; 20 μM). Cellular adhesion molecules (sICAM-1, sVCAM-1 and sE-selectin) and nitric oxide (NO) were measured using ELISA and a chemiluminescent-based assay, respectively. Relative gene expression of these markers, including the insulin receptor, was performed using real-time PCR as well as FA compositions of cell pellets by gas chromatography.
Results: FA mixtures affected sE-selectin concentrations ($P = 0.008$), with concentrations lower following the high SFA compared to the low SFA mixture ($P = 0.004$), while NO concentrations were higher in diabetic compared to healthy cells ($P = 0.029$). Individual FA affected NO ($P = 0.007$) and sE-selectin ($P = 0.040$) concentrations with an increase following PA incubation relative to all other FA treatments ($P < 0.05$). PA increased sE-selectin compared with other FA treatments ($P < 0.05$). sE-selectin concentrations were also higher in healthy compared to diabetic cells ($P = 0.023$). Expression of ICAM-1 and insulin receptor was up-regulated in healthy compared to diabetic cells ($P = 0.014$ and $P = 0.006$ respectively).
Conclusions: Healthy and type II diabetic cells respond differently to incubation with FA treatments. Overall, physiological concentrations of dairy FA, but not dairy FA mixtures, substantially affected markers of endothelial function.

1. Introduction

With CVD now a formidable social and economic burden in the UK, EU and worldwide [1–3], the role of modifiable risk factors, such as diet, is becoming increasingly important. Despite its high SFA content, milk has been highlighted as a cardio-protective agent via its effects on blood pressure [4,5], and more recently arterial stiffness [6,7], as well as end point CVD events [8]. In order to improve the benefits of milk intake further, research in dairy cows has shown partial replacement of milk SFA with mono and poly-unsaturated FA (MUFA and PUFA), which could have implications on vascular health [9]. As summarised in a recent review [10], limited research has investigated the effectiveness of this strategy on CVD risk factors using *in vitro* and *in vivo* studies.

Evidence from epidemiological studies suggests that SFA intake is detrimental to CVD risk, while *cis*-MUFA and *cis*-PUFA intake is protective [11], yet *in vivo* [12] and *in vitro* [13,14] evidence is limited and inconsistent. *In vitro* studies have reported variable effects of individual FA on markers of endothelial dysfunction, including soluble E-selectin (sE-selectin), inter-cellular (sICAM-1) and vascular (sVCAM-1) adhesion molecule concentrations and suppression of endothelial NO synthase (eNOS) [15]. *In vitro* studies using physiological FA mixtures and concentrations are limited.

* Corresponding author. Tel.: +44 (0)118 378 5469; fax: +44 (0)118 378 6595.
E-mail addresses: katherinemlivingstone@gmail.com (K.M. Livingstone), d.i.givens@reading.ac.uk (D.I. Givens), k.g.jackson@reading.ac.uk (K.G. Jackson), j.a.lovegrove@reading.ac.uk (J.A. Lovegrove).

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One of the major risk factors for CVD is type II diabetes, the prevalence of which has been doubling in some European countries over the past decade [1] and is projected to increase further [16]. The need for dietary strategies, such as modification of dairy product FA composition, to minimise the burden of this preventable disease is paramount. The present study aimed to compare the response of human aortic endothelial cells (HAEC) derived from healthy and type II diabetic individuals to incubation with physiological concentrations of dairy FA that are representative of those found in milk and dairy products: PA; OA; SA; *trans*-PA); *trans*-VA; ALA; LA and FA mixtures derived from a typical, high SFA (high SFA, low MUFA/PUFA) dairy lipid and a modified, low SFA (low SFA, high MUFA/PUFA) dairy lipid. Analytes included gene expression and production of NO, ICAM-1, VCAM-1 and E-selectin, as well as gene expression of insulin receptor.

2. Methods

2.1. Endothelial cell culture

Healthy and diabetic HAEC were supplied by Lonza (Basel, Switzerland). The healthy and type II diabetic donors were matched for age (50–55 years of age), gender (male), ethnicity (Caucasian), and alcohol intake (non-consumers). Cells were cultured using endothelial cell growth medium (EGM-2; Lonza Walkersville Inc., MD, USA) containing hydrocortisone, human fibroblast growth factor (hFGF-B), vascular endothelial growth factor (VEGF), insulin like growth factor-1 (IGF-1), ascorbic acid, heparin, foetal bovine serum (FBS; 2%) and human epidermal growth factor (hEGF). Cells were maintained in a 37 °C, 5% CO_2 humidified atmosphere. Based on recommendations by the manufacturer, cultures were seeded at a density of 5000 cell/cm^2 and passages 5–7 were used in experiments.

2.2. Experimental protocol

Healthy and diabetic HAEC were grown to 90% confluence in 6-well plates. Subsequently, healthy and diabetic HAEC were incubated for 24 h with OA (150 μM), PA (150 μM), SA (40 μM), *trans*-PA (20 μM), *trans*-VA (20 μM), ALA (20 μM), and LA (20 μM) and a high and low saturate FA mixture (400 μM). FA were complexed to bovine serum albumin (BSA). Large vessel endothelial cell growth medium (Caltag Medsystems Ltd., Buckingham, UK), supplemented with 2% foetal calf serum containing amphotericin B/gentamycin, was used for the initial 21 h of the incubations. A serum-free large vessel endothelial cell growth medium was used for the final 3 h of the incubations. Control treatments contained BSA only. Each experiment was carried out in triplicate. Cell supernatant was collected for analysis of NO and adhesion molecule production, while RNA and cell pellets were collected for gene expression and FA composition analysis respectively.

2.3. Sample preparation: production of modified dairy products and lipid extraction

Two mid-lactation multiparous cows were used to produce the milk as part of a separate study [17]. Briefly, each cow was fed for two weeks on one of two different diets – a control diet, typical of that fed on commercial farms, and a modified diet containing a rumen-protected *cis*-MUFA oil supplement. The milk from the control diets (high SFA, low MUFA/PUFA) and the milk from the supplemented diet (low SFA, high MUFA/PUFA) were used to produce a low and high SFA Cheddar cheese. Lipid was extracted from 3 g of both cheeses using a Soxflo apparatus – methods are described elsewhere [18].

2.4. Fatty acid sodium salts

150 mg of high and low SFA dairy lipid was pipetted into a screw-capped tube. Following addition of 25 ml of methanolic KOH, samples were warmed for 1 h at 80 °C in a water bath. After cooling, non-saponified lipids were extracted with two washings of 25 ml diethyl ether and samples were acidified with 6 M HCl (about 1.5 ml). FA were extracted with five washings of 25 ml hexane. Ethanol:diethyl ether mixture (1:1, 1.9 ml) was added to the free FA. Saponification values were calculated according to equations detailed elsewhere [19]. To saponify 150 mg of high and low SFA lipid, 21.5 mg and 20.6 mg of NaOH were added respectively. Samples were left at room temperature for 15 min. FA compositions of the FA-sodium salts are detailed in the Supplement.

2.5. Fatty acid–BSA complexes

Sodium salts of OA, PA, SA, *trans*-PA, *trans*-VA, ALA, LA (Sigma–Aldrich Company Ltd., UK) and FA mixtures were prepared according to methods detailed by Shaw et al. (2007). Briefly, sodium salts were dissolved in distilled water at a concentration of 50 mg/ml to form an FA solution. A stock solution of each FA–BSA complex was prepared at a molar ratio 2.5:1, FA: BSA, in EGM-2 medium (supplemented with 2% delipidized FBS). Stock solutions were mixed and warmed to 40 °C, sterile filtered using a 0.2 μm syringe filter and stored at −20 °C. NEFA concentrations of stock solutions were determined using an automated clinical chemistry analyser (ILAB 600) using kits supplied by Instrumentation Laboratory (Warrington, UK). Stock solutions were defrosted and diluted appropriately with large vessel endothelial cell growth medium with 2% FBS for 21 h and then large vessel endothelial cell growth medium serum free for 3 h to obtain the desired FA concentrations for both the individual FA and FA mixtures, before addition to cell cultures.

2.6. Assessment of cell viability

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability following 24 h FA incubations in both healthy and diabetic HAEC.

2.7. RNA extraction, cDNA synthesis

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

2.8. Real time PCR

Primer sequences were sourced from previous publications [20,21]. The specific primer for the insulin receptor was designed across an exon–exon junction from the published full-length mRNA sequence (www.ncbi.nlm.nih.gov) using Primer Express software version 2 (Applied Biosystems, Warrington, Cheshire, UK). The sequences are summarised in Table 1. Real time PCR was performed using absolute QPCR SYBR Green ROX Mix (ABgene, Epsom, UK) using a 7300 real time RT-PCR system (Applied Biosystems, Paisley, UK). The RT-PCR was run for 15 min at 95 °C followed by 40 cycles of 30 s at 92 °C and 60 s at 55 °C. Following amplification,