Conjugated linoleic acids modulate UVR-induced IL-8 and PGE2 in human skin cells: potential of CLA isomers in nutritional photoprotection

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Conjugated linoleic acids (CLA), derivatives of linoleic acid found in food products, inhibit chemically induced skin cancers in mice. However, their potential photoprotective properties remain unexplored. We examined whether CLA may modulate ultraviolet radiation (UVR)-induced secretion of interleukin (IL)-8 and prostaglandin E2 (PGE2), mediators implicated in UVR-induced inflammation and carcinogenesis, in human skin cells. Since tumour necrosis factor (TNF)-α is an early mediator of UVR effects, we also examined influence of CLA on TNF-α-induced mediator release. HaCaT keratinocytes were supplemented with CLA isomers cis-9-trans-11, (9,11-CLA; ≥90%), trans-10-cis-12 (10c,12-CLA; ≥90%) or all trans-cis-trans isomers (tt-CLA; 23.7%) in tetrahydrofuran/fetal calf serum (THF/FCS) or THF/FCS control. Supplementation of keratinocytes with c9,11-CLA reduced Ultraviolet B(UVB)-induced IL-8 from 37 113 ± 2063 pg/mg protein (P < 0.001). Similarly, t10c,12-CLA reduced UVB-induced IL-8 to 9786 ± 1291.5 pg/mg protein (P < 0.001). Additionally, t10c,12-CLA and tt-CLA inhibited TNF-α-induced IL-8 from 11 692 ± 1692 pg/mg protein in control cells to 5540 ± 191 (P < 0.001) and 8082 ± 1298 pg/mg (P < 0.01) protein, respectively, UVB-induced PGE2 release was reduced by tt-CLA supplementation, from 4.8 ± 1.2 to 1.6 ± 0.8 pg/mg protein (P < 0.01), but increased by t10c,12-CLA to 8.8 ± 1 pg/mg protein (P < 0.001). Influence of CLA on UBV-induced PGE2 release was further explored in CDD922SK dermal fibroblasts. CLA isomers reduced UVB-induced PGE2 in fibroblasts, reaching significance with c9,11-CLA (98 ± 5 falling to 0 pg/mg protein, P < 0.05). Hence, CLA isomers differentially modulate UBV effects on skin cells in vitro. CLA-containing foods have potential in photoprotection; the cutaneous effects of individual isomers warrant clinical study.

Introduction

Conjugated linoleic acids (CLA) are a diverse array of structural and geometric isomers derived from linoleic acid (LA). They can be generated by bacterial biohydrogenation in ruminant animals, industrial hydrogenation or by heat treatment of foods derived from ruminant animals, such as cooked meats or processed dairy products (1–3). Two isomers are predominant in animal tissue: cis-9-trans-11 (c9,t11)-CLA, (18:2n-7), comprising 80–90% of CLA found in animal fat, and trans-10-cis-12 (t10,c12)-CLA, (18:2n-6), whereas the other isomers, such as trans-trans-CLA (tt-CLA), are generally not detected in significant quantities in vivo (2,3). While the roles of the individual isomers appear to differ (4), CLA are reported to modulate a range of immune and inflammatory responses (5–11). They inhibit both the initiation and promotion stages of chemically induced skin cancer in mouse carcinogenesis models (2,12,13) and their anti-carcinogenic properties have been confirmed in several other models of cancer, including the mammary gland, stomach, colon and prostate (14). Furthermore, it has been suggested that the anti-carcinogenic properties of CLA may stem from their anti-inflammatory properties (15). However, their effects on ultraviolet radiation (UVR)-induced skin damage are currently unexplored.

The negative effects of UVR on human skin include the acute effects of sunburn (inflammation), photosensitivity rashes and immunosuppression and the longer term damage of photocarcinogenesis and photoaging (16). UVR is a complete carcinogen, both initiating the DNA damage that if unrepaird can lead to mutagenesis and promoting carcinogenesis principally through immunosuppression (17,18). Skin cancer is a major clinical problem continuing to rise in incidence and now presenting as the commonest cancer in white populations (19). Topical photoprotection has a number of drawbacks, and is often inadequately applied; hence, a dietary means for protection could provide a useful adjunctive measure, with potential application both to susceptible patients and the healthy population (16). As far as we are aware, we now report the first study designed to examine whether CLA may modulate UVR-induced effects and hence their potential activity as photoprotective agents.

Interleukin (IL)-8, an inflammatory cytokine and potent chemokine, is up-regulated in human skin following UVR exposure (20). It plays a pivotal role in the acute response of the skin to UBV, attracting neutrophils into the dermis where they mediate inflammation and cause tissue damage secondary to the release of reactive oxygen species. Prostaglandin E2 (PGE2) is another mediator important in the acute inflammatory response to UBV, responsible for the clinical erythema evident in the sunburn response (21,22), and capable of mediating the release of other inflammatory mediators including IL-6 and IL-8 in some, but not all, experimental models (23,24). The sunburn response and UBV-induced carcinogenesis are closely linked, and both IL-8 and PGE2 are also implicated in the latter. Whereas IL-8 is a mediator of angiogenesis, and is associated with tumour growth including development of malignant melanoma (25,26), PGE2 is a mediator of UBV-induced immunosuppression (27) and promotes skin carcinogenesis in mouse models (28). Furthermore, cyclooxygenase-2 inhibitors partially block photocarcinogenesis and this is believed to be mediated principally through reduction of PGE2 production (29).

The aims of this study were to examine the effects of purified forms of the two principal dietary CLA isomers, i.e. c9,t11-CLA and t10,c12-CLA, and a mixture of all tt-CLA isomers on UVR-induced secretion of IL-8 and PGE2. Since tumour necrosis factor (TNF)-α shows early up-regulation by UVR and is a key mediator in the orchestration of the acute UVR response, the effects of CLA isomers on TNF-α-induced IL-8 secretion were also examined. Studies were performed in human skin cells, principally keratinocytes, while the influence of CLA on UVB-induced PGE2 was further examined in fibroblasts.

Materials and methods

Cell culture

HaCaT keratinocytes [derived from the periphery of a melanoma on the non-extensively sun-exposed back of a 62-year-old male donor, considered immortal but not tumorigenic, obtained from Dr Petra Boukamp, German Cancer Research Centre, Heidelberg, Germany (30)] and CDD922SK fibroblasts (derived from normal human breast skin of a 22-year-old female donor, American Type Culture Collection, No. CRL 1828, MD) were cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal calf serum (FCS), 2 mM glutamine and 40 U/ml penicillin and 40 μg/ml streptomycin.

Supplementation of fatty acids to cells

Twenty-four hours prior to experimental use, FCS-containing medium was aspirated from subconfluent cells and the cells were washed twice with

Abbreviations: CLA, conjugated linoleic acid; c9,t11, cis-9-trans-11; FA, fatty acids; FCS, fetal calf serum; IL, interleukin; LA, linoleic acid; OA, oleic acid; PGE2, prostaglandin E2; t10,c12, trans-10-cis-12; THF, tetrahydrofuran; TNF, tumour necrosis factor; tt, trans-trans; UVR, ultraviolet radiation.
phosphate-buffered saline and re-immersed in defined serum-free media. AIMV® (Gibco BRL™ Life Technologies Ltd, Paisley, Scotland) containing 2 mM glutamine. Free fatty acid (FA) (5 μl) was dissolved in 235 μl of FCS, vortexed thoroughly to ensure complete emulsion and then 10 μl of tetrahydrofuran (THF) was added. The final mixture consisted of 2/49/94% FA/THF/ FCS (v/v/v). Stock solutions were then diluted with 49/96% THF/FCS (v/v) to produce substock solutions of 5 mM. This was diluted 1:100 with FCS-free culture media and supplemented to cells, providing a final dietary oil concentration of 50 μM. FAs used were c9,11-CLA (18:2n-7; 93.8% purity), t10,c12-CLA (98% purity, 18:2n-6), n-CLA (23.7% purity; all CLA isomers were provided by Unilever Research Laboratories, Bedford, UK) and oleic acid (OA, 18:1n-9; 95% purity; Sigma, Poole, UK). Cell cultures were supplemented with FAs or THF/FCS vehicle control for 4.5 days before UVB exposure.

UVB exposure

Cells were exposed to single doses of UVB using a filtered broadband source from 25 to 100 mJ/cm². Cell viability was determined using the trypan blue exclusion assay. There was no effect of UVB on HaCaT cell viability at doses of 25, 50 or 75 mJ/cm² but after an exposure of 100 mJ/cm² there was a significant decrease to 86.7% (Figure 1). RIA kit NEN Life Science Products, Hounslow, UK. Since cell supernatants contain agents that facilitate PGE2 synthesis. Briefly, cells were cultured in serum-containing medium, the NEN [125I]-RIA assay was adapted for the PGE2 assay was for 30 min at 4°C and the pellet counted for 1 min in a gamma counter. The sensitivity limit for the PGE2 assay was <0.44 pg/0.1 ml; cross-reactivity was 30% with PGE2, and ≤1% with other prostaglandins.

Cell viability and protein analysis

Cell viability was determined using the trypan blue exclusion assay. There was no effect of UVB on HaCaT cell viability at doses of 25, 50 or 75 mJ/cm² but after an exposure of 100 mJ/cm² there was a significant decrease to 86.7%, P < 0.05. There was no significant difference in cell viability after supplementation of any of the FAs in HaCaT cells. Protein content, determined by the bicinchoninic acid method, was used to standardize IL-8 measurements (31).

Bioavailability of FAs

Cellular FA content was analyzed by gas chromatography. Lipid was extracted from cells using chloroform:methanol:0.9% aqueous NaCl, 2:1:0.9 (v/v/v), with a 20:1 (v/v) solvent to cellular extract ratio. Butylated hydroxytoluene (BHT) was added as an antioxidant at a final concentration of 0.005%. FAs used were arachidonic acid and OA, in either cell line. OA supplements had no significant impact on keratinocyte content of other cellular FAs other than a lowering of LA in unexposed cells relative to THF/FCS controls, with 10.5 ± 0.4 and 8.3 ± 0.4%, respectively, P < 0.01. This may be attributable to displacement of membrane FAs and indicates that OA cannot generally be assumed to be an inactive control supplemental FA.

Effect of CLA isomers on keratinocyte IL-8 production in basal and UVB-exposed cells

UVB exposure of keratinocytes at doses ranging from 25 to 100 mJ/cm² produced a UVB dose-related increase in supernatant IL-8 level, P < 0.001, with optimal induction at 48 h after exposure. Hence, IL-8 was assayed at 48 h following a single dose of 100 mJ/cm² UVB in subsequent experiments. It was found that basal, i.e. non-UVB exposed, levels of IL-8 were not significantly altered by the CLA isomers. UVB significantly induced IL-8 in the THF/FCS control group, levels increasing from 7279.4 ± 1088.7 to 37 113.0 ± 2903.3 pg/ng protein (P < 0.001), and the findings were similar in the OA-supplemented cells. Both c9,11-CLA and t10,c12-CLA isomers significantly reduced UVB-induced IL-8, from 37 113.0 ± 2903.3 to 14.167 ± 2063.2 pg/ng protein (P < 0.001) by c9,11-CLA and to 4470.5 ± 1291.5 pg/ng protein (P < 0.001) by t10,c12-CLA, at 48 h following 100 mJ/cm² UVB (Figure 1). t10,c12-CLA quenched IL-8 production.

Table I. Effect of supplements on IL-8 production in basal and UVB-exposed HaCaT keratinocytes

<table>
<thead>
<tr>
<th>Supplements</th>
<th>THF/FCS</th>
<th>OA</th>
<th>c9,11-CLA</th>
<th>t10,c12-CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ml/cm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>45.2 ± 1.9</td>
<td>46.7 ± 3.4</td>
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<td>34.4 ± 2.4</td>
</tr>
<tr>
<td>C9t11</td>
<td>0</td>
<td>0</td>
<td>2.2 ± 0.2***</td>
<td>0</td>
</tr>
<tr>
<td>T10c12</td>
<td>0</td>
<td>0</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>100 ml/cm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>37.6 ± 1.6**</td>
<td>40.1 ± 1.9***</td>
<td>43.6 ± 3.3</td>
<td>34.0 ± 4.1</td>
</tr>
<tr>
<td>C9t11</td>
<td>0</td>
<td>0</td>
<td>1.3 ± 0.2***</td>
<td>+++</td>
</tr>
<tr>
<td>T10c12</td>
<td>0</td>
<td>0</td>
<td>0.5 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard error of mean, n = 4 (except for t10,c12-CLA supplements, where n = 2); **P < 0.01 and +++P < 0.001 comparing supplemented groups with THF/FCS vehicle control; *P < 0.05 and **P < 0.01 comparing UVB-irradiated with non-irradiated cells. %FA is the % FA/total cellular FAs.
levels so effectively that the UVB-induced IL-8 level was no longer significantly different from the basal IL-8 levels.

**Effect of CLA isomers on TNF-α-induced IL-8 production by keratinocytes**

Figure 2 illustrates that 20 ng/ml TNF-α induced a significant increase in IL-8 production in the THF/FCS control cells. r10,c12-CLA and t-t-CLA supplements significantly reduced TNF-α-induced IL-8 compared with the THF/FCS vehicle control group. Levels decreased from 11 669.3 ± 1692.1 pg/mg protein in control cells to 5540.5 ± 191.2 pg/mg protein (P < 0.001) after r10,c12-CLA and to 8082.2 ± 1297.9 pg/mg protein (P < 0.01) after t-t-CLA. OA and c9,t11-CLA supplements did not reduce cytokine-stimulated IL-8 production and similar levels were seen in vehicle control cells.

**Effect of CLA isomers on basal and UVB-induced PGE2 release by keratinocytes**

Figure 3 shows the effect of the FAs on PGE2 production by HaCaT cells cultured with serum-free medium. A single dose of 100 mJ/cm² UVB resulted in a significant increase in PGE2 production in all groups, except for the t-t-CLA-supplemented groups. The vehicle control group supplemented with THF/FCS produced equivalent PGE2 levels to the unsupplemented cells. The t-t-CLA supplementation significantly increased basal PGE2 synthesis from below the limit of detection to 4.33 ± 0.55 pg/mg protein (P < 0.001), while it reduced UVB-induced PGE2 from 4.83 ± 1.24 to 1.62 ± 0.84 pg/mg protein (P < 0.01). The r10,c12-CLA supplementation significantly increased both basal and UVB-induced PGE2 synthesis from 0 to 2.04 ± 0.97 pg/mg protein (P < 0.05) and 4.83 ± 1.24 to 8.78 ± 0.99 pg/mg protein (P < 0.001), respectively.

**Effect of CLA isomers on basal and UVB-induced PGE2 release by fibroblasts**

In CCD922SK cells, there was a significant increase in PGE2 synthesis after exposure to 100 mJ/cm² UVB in the unsupplemented group. There was an increase in PGE2 following UVB in the THF/FCS-treated cells, significant at the P < 0.05 level by paired t-test, but significance was lost when subjected to more multiple comparison (analysis of variance) analysis (Figure 4). Levels secreted by THF/FCS-supplemented control cells were not significantly different from unsupplemented cells. c9,t11-CLA and t-t-CLA supplements decreased basal PGE2 synthesis so effectively that values were below the levels of detection. c9,t11-CLA also inhibited UVB-induced PGE2 synthesis from 97.5 ± 5.1 pg/mg protein to a value that was below the level of detection.

**Discussion**

This study makes the novel observation that CLA isomers modulate UVR-induced effects, and suggests their potential as dietary photoprotective agents. Both of the isomers commonly found in human foods, i.e. c9,t11-CLA and r10,c12-CLA, were incorporated into human keratinocytes, where they profoundly reduced UVB-induced IL-8 secretion (P < 0.001), while having no or minimal effect on basal IL-8 levels. Keratinocytes are believed to be the major cellular source of UVB-induced IL-8 (33). IL-8 is a potent chemoattractant, important in the UVB-induced inflammatory (sunburn) response, recruiting neutrophils into the skin where they may then cause tissue damage through release of reactive oxygen species (20). IL-8 also induces keratinocyte proliferation and angiogenesis, and may promote...
the growth of a range of tumours, including malignant melanoma (25,26,34,35). Thus, inhibition of IL-8 is anticipated to convey significant protection against UVB-induced skin inflammation and the longer term complication of skin cancer. Keratinocyte exposure to the pro-inflammatory cytokine TNF-α also augmented IL-8 secretion, although not to the degree seen following UVB. While the t10,c12-CLA isomer, and also the t-CLA isomers, significantly inhibited TNF-α-induced IL-8 (P < 0.001 and P < 0.01, respectively), revealing a secondary route for photoprotection by CLA as well as potential protection of skin cells from other injurious stimuli, no effect was seen with the c9,t11-CLA isomer. This highlights that individual CLA may convey differential effects, and consequently the importance of examining the activities of the isomeric forms. In this study, however, the two common CLA isomers were seen to convey a comparable degree of protection against UVB-induced IL-8 secretion.

Examination of the influence of the CLA isomers on UVB-induced PGE₂ secretion by keratinocytes did not show the consistent pattern observed with UVB-induced IL-8. While c9,t11-CLA had no impact on PGE₂ levels, the t10,c12-CLA significantly increased the UVB-induced level, and only the all t-CLA reduced the UVB-induced PGE₂. Additionally, both the r10,c12-CLA and the all t-CLA elevated the basal (non-UVB exposed) PGE₂ level. This effect of r10,c12-CLA is consistent with reports that this isomer may increase PGE₂ secretion in other cell culture models (36). Interestingly, where detrimental effects of CLA are observed in animal studies, these have been reported to be associated with the t10,c12-CLA isomer, including procarcinogenic effects in some cancer models. Few previous studies have examined the molecular effects of t-CLA, with which to compare our experimental results. However, while they are generally less prevalent in foods, t-CLA can represent a substantial component of some food products, and our data indicate they should not be completely overlooked since they clearly can have significant effects on UVB-induced mediator production. In 12-0-tetradecanoylphorbol-13-acetate-stimulated keratinocytes of the cell line HEL-30, pre-treatment with CLA reduced the resultant PGE₂ levels (37), and in an animal model of 12-0-tetradecanoylphorbol-13-acetate-induced skin tumour promotion, dietary CLA reduced PGE₂ synthesis in the epidermis (38). In view of the disparate effects of CLA observed in keratinocytes, we further examined their effects on UVB-induced PGE₂ in cultured skin fibroblasts. Here, in contrast, we found that both the c9,t11 and t10,c12 isomers markedly reduced the UVB-induced PGE₂ levels and basal PGE₂. Hence, the modulatory effects of the CLA appear specific to cell type as well as isomer. Since a range of other dermal and epidermal skin cells, both resident cells e.g. endothelial cells and melanocytes, and infiltrating leucocytes may secrete PGE₂ in response to UVB, the overall impact of CLA supplementation on skin production of this mediator requires examination in vivo. In our studies, we removed cells from serum-containing media prior to experimentation since serum is an endogenous source of CLA. In keeping with other reports of analysis of PGE₂ in serum-free media (39,40), however, we found that the increases in this mediator induced by UVB were not of high magnitude, and this should be taken into consideration in the interpretation of these experiments. The mechanism of the effects of c9,t11 and t10,c12 CLA isomers in fibroblasts and all t-CLA in keratinocytes on PGE₂ secretion has not been elucidated in the present study, but could be similar to those proposed for another family of dietary FAs, the omega-3 FAs, which reduce UVB-induced PGE₂ levels in vitro and in vivo (41,42). Hence, they may be capable of competing with the omega-6 polyunsaturated FAs LA and arachidonic acid for incorporation into cell membranes, and/or release by phospholipases, and possibly for metabolism and subsequent eicosanoid synthesis (6,37,43). Potent omega-6 polyunsaturated FA metabolites might be replaced by less active CLA-derived products, although the ability of CLA to act in this manner has been questioned. Since cyclooxygenase requires a methylene group at carbon 13 in the hydrocarbon chain, it is thought to be unlikely to act on either of the 20-carbon products of CLA isomers (5). Moreover, as our experimental data indicate, the mechanisms of action of the CLA isomers and t forms cannot be assumed to be uniform, with both anti- and pro-inflammatory activities being seen with respect to PGE₂ synthesis.

The concurrent protection conveyed by the CLA isomers against UVB-induced IL-8 production suggests that protection against the release of both IL-8 and PGE₂ might occur upstream, such as at the level of transcription factor activation. Although PGE₂ has been reported to release IL-8, this was not replicated in skin cells (23,24). UVR activates nuclear factor-kappa beta, and this may be mediated via UVR generation of reactive oxygen species or pro-inflammatory cytokines such as TNF-α. The up-regulation of a wide range of genes responsible for pro-inflammatory cytokines and enzymes such as cyclooxygenase results. CLA are capable of inhibiting nuclear factor-kappa beta activity (10), and this may occur through their antioxidant activity reported in some (32,44), although not all studies (45). Again, parallels exist with omega-3 FAs, which are similarly effective in reducing UVB-induced IL-8 secretion in vitro (46), and appear effective in reducing aspects of oxidative damage in human studies (47). Interestingly, CLA inhibition of the growth of human cancer cells can occur in a PGE₂-independent manner (48) and in association with a reduction in oxidative stress (1). There is also evidence that CLA may convey anti-carcinogenic properties through wide-ranging activities including modulation of the nuclear hormone receptor peroxisome proliferator-activated receptor gamma, inhibition of angiogenesis, inhibition of cell proliferation and induction of apoptosis (49–53).

In summary, both CLA isomers c9,t11-CLA and t10,c12-CLA significantly protect against UVB-induced IL-8 secretion by human keratinocytes, suggesting their potential in nutritional photoprotection. Moreover, the distinct action of t10,c12 in suppressing TNF-α-induced IL-8 suggests its potential for protection against other forms of cellular stress. The effects of CLA on UVB-induced PGE₂ secretion appear isomer and cell type specific; evidence of protection by the more prevalent c9,t11 isomer and augmentation by t10,c12 implies a protective effect in the proportions found in common food-stuffs, and is in keeping with the generally stronger evidence for the anti-carcinogenic properties of the c9,t11 isomer (4). Studies should be performed to examine the differential effects of these isomers in human skin in vivo, with a view to optimizing the composition of CLA supplements for use in human photoprotection trials.

Acknowledgements

We thank Gary Sassano and John Bosley (Unilever R&D, Colworth, Sharnbrook, Bedford, UK) for assistance with gas chromatographical analysis and Anne Pellow (Unilever R&D, Colworth, Sharnbrook, Bedford, UK) for preparation of CLA isomers. This research was supported by financial grants from the Medical Research Council, London, UK and Unilever R&D, Colworth, Sharnbrook, Bedford, UK.

Conflict of Interest Statement: None declared.

References

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