

Conjugated linoleic acid, unlike other unsaturated fatty acids, strongly induces glutathione synthesis without any lipoperoxidation

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Enhancement of the redox status of cells is a cytoprotective strategy against oxidative damage. We recently showed that DHA upregulates glutathione (GSH) content via an induction of its related enzymes γ -glutamylcysteine ligase and glutathione reductase. In the present study, we investigated the effects of eight other fatty acids on the redox status and lipid peroxidation of human fibroblasts. After 48 h, only arachidonic acid and conjugated linoleic acid (CLA) enhanced GSH content through an induction of γ -glutamylcysteine ligase. CLA was more potent than arachidonic acid in inducing GSH synthesis. For all the fatty acids tested, lipoperoxidation, estimated by cell malondialdehyde measurement, did not differ from that of controls at 48 h but dramatically increased at 7 d, except for CLA. Lipoperoxidation is associated at 7 d with a high level of reactive oxygen species and with increased haemoxygenase-1 and cyclooxygenase-2 mRNA expression. As demonstrated by a tert-butylhydroperoxide cytotoxicity test, the GSH synthesis obtained with arachidonic acid is not sufficient to protect the cells, whereas this protective effect was obvious with CLA at 48 h as well as at 7 d. The present results show that CLA is the only PUFA able to induce GSH synthesis without any change in oxidative balance, whereas an upregulation of cyclooxygenase-2 by other PUFA is concomitant with an overproduction of malondialdehyde and reactive oxygen species. The particular hairpin conformation obtained for CLA by molecular modelling could account for this specific biological effect.

PUFA: Conjugated linoleic acid: Redox status: Antioxidant response: Glutathione: Cyclooxygenase-2: Haemoxygenase-1: Reactive oxygen species: Lipid peroxidation

An increased dietary consumption of *n*-3 PUFA elevates their incorporation into membrane phospholipids of cells or tissues. Thus, *n*-3 PUFA are claimed to exert protection against CVD and cancer. The molecular mechanisms of these effects are, however, still under study (Jump, 2002).

The potential for preventive actions in CVD was recently bolstered by the finding that PUFA, especially *n*-3 PUFA, have a protective effect on ischaemia–reperfusion in neuroprotection and an anti-inflammatory action (Pepe & McLennan, 2002; Mukherjee *et al.* 2004). EPA (C20:5*n*-3) and DHA (C22:6*n*-3) are usually consumed in small quantities and are therefore found in relatively low proportions in plasma and tissue lipids. An increased consumption of these fatty acids is, however, associated with their increased proportions in various blood and tissue lipid pools (Chan *et al.* 2003; Laidlaw & Holub, 2003). Paradoxically, enrichment with *n*-3 PUFA is thought to enhance the susceptibility of tissues to oxidation by free radicals. The susceptibility of a particular tissue to oxidation is influenced by its PUFA and antioxidant content. Some studies have investigated the effects of dietary or supplemented PUFA on the

susceptibility of lipoproteins or cells to oxidation, and have demonstrated a marked reduction in the lag phase for oxidation by PUFA (Oostenbrug *et al.* 1994; Song *et al.* 2000). Not all data, however, agree that *n*-3 PUFA increase overall susceptibility to oxidation, and it is not clear whether the two major *n*-3 PUFA in fish oil, EPA and DHA, are equally potent in decreasing oxidation (Higdon *et al.* 2000; Mori *et al.* 2003).

Lipid peroxidation generates highly reactive aldehydes, such as 4-hydroxynonenal and malondialdehyde (MDA), which are involved in some pathophysiological processes. 4-Hydroxynonenal could modulate protein activity by addition reactions to cysteinyl residues, a mechanism that may explain some gene regulations via lipid peroxidation (Levonen *et al.* 2004). PUFA serve as substrates for several enzymes in eicosanoid metabolism, and their products play huge roles in the homeostasis of peroxide. Three isoforms of cyclooxygenase (COX), also named prostaglandin endoperoxide H synthase, exist in mammalian tissues, and these enzymes oxygenate arachidonic acid (AA; 20:4*n*-6) to form a substrate required for prostaglandin synthesis. COX-1 is constitutive and generates

Abbreviations: AA, arachidonic acid; CLA, conjugated linoleic acid; COX, cyclooxygenase; γ -GCL, γ -glutamylcysteine ligase; GSH, glutathione; H₂DCF-DA; 2',7'-dihydrodichlorofluorescein diacetate; HO-1, haemoxygenase-1; LA, linoleic acid; LLA; α -linolenic acid; GLA, γ -linolenic acid; MDA, malondialdehyde; OA, oleic acid; RA, arachidic acid; ROS, reactive oxygen species; t-BOOH, tert-butylhydroperoxide.

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prostaglandins in response to hormone stimulation, whereas COX-2 is an inducible enzyme, expressed in response to stress stimuli such as cytokines and tumour promoters. The induction of COX-2 is consequently implicated in cellular damage and in several inflammatory diseases; another isoform, COX-3, which was recently discovered, is still under study (Smith *et al.* 2000; Ringborn *et al.* 2001).

Glutathione (GSH) is the main cellular antioxidant. As such, GSH is critical for maintaining cellular functions; it provides protection against increases in the level of reactive oxygen species (ROS; Meister, 1983). GSH is involved in detoxification processes via the conjugation of xenobiotics and lipid peroxidation products with γ -glutamyl S transferase. Its level is maintained by both a recycling of oxidised GSH by glutathione reductase and a *de novo* synthesis by γ -glutamylcysteine ligase (γ -GCL), the rate-limiting enzyme in this synthesis. The transcription of the two subunits of γ -GCL seems to be regulated by dimeric transcription factors such as small Maf proteins and other bZIP family members, including Nrf 1 and 2. In addition, these transcription factors upregulate cytoprotective genes such as haemoxygenase-1 (HO-1; Wild *et al.* 1999; Leung *et al.* 2003). Another regulation by NF κ B is involved in the transactivation of γ -GCL during induced stress (Urata *et al.* 1996). The involvement of these transcription factors in GSH homeostasis suggests that they are self-sensitive to the cell redox environment (Wu *et al.* 1996).

In the present report, we have investigated, on the one hand, the effects of fatty acids on the thiol redox status of cells and their protective effects against oxidative damage, and on the other hand, the involvement of lipid peroxidation in enhancing this damage.

Materials and methods

Chemicals

Chemicals were obtained as follows: arachidic acid (AA; 20:0), oleic acid (OA; 18:1*n*-9), linoleic acid (LA; 18:2*n*-6), α -linolenic acid (LLA, 18:3*n*-3), γ -linolenic acid (GLA; 18:3*n*-6), AA (20:4*n*-6), conjugated LA (CLA; 18:2 [9Z, 11E]), EPA (20:5*n*-3) and 4-hydroxynonenal were purchased from Cayman Chemical (Interchin, Montluçon, France); PBS, 2, α -aminobutyrate and L-glutamate from Sigma-Aldrich (Saint Quentin Fallavier, France); reduced GSH, ATP, phosphoenolpyruvate and pyruvate kinase from Roche (Meylan, France); 2',7'-dihydrodichlorofluorescein diacetate (H₂DCF-DA) from Fluoroprobe (Interchin); and Meth-Prep II from Alltech (Interchin). All other reagents were of analytical grade.

Fatty acid stock solutions (150 mmol/l) were prepared in C₂H₅OH, divided into aliquots and stored under N₂ in brown glass vials at -20°C . All concentrations are given in final concentrations in incubation or culture medium.

Cell culture

Human skin fibroblasts were a gift from Dr Damour of the Tissue and Cell Bank HCL, Hopital Edouard Herriot, Lyon, France. The human fibroblasts were grown in DMEM containing 10% FCS (Gibco; Pontoise, France) and 2.5 μg fungizone/ml, 98 IU penicillin/ml and 98 μg streptomycin/ml (Cambrex, Emerainville, France). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂.

The culture medium was supplemented with each fatty acid at 30 $\mu\text{mol/l}$: the fatty acid stock solution was dissolved 1:5000 in culture medium, with the appropriate controls. Cells were grown

for different periods of time, up to 7 d, and the culture medium was changed every day. At 48 h or 7 d, cells were harvested by trypsinisation and washed twice with PBS 1X. Cells were collected in 500 μl Tris-HCl 50 mmol/l, pH 7.4 and Tween20 0.2%, and 50 μl were processed immediately to measure oxidised and reduced GSH, as previously described (Steghens *et al.* 2003). For other tests, the cells were lysed with two successive freezing and thawing operations with 15 s sonication. The cell lysates were stored at -20°C until analysis.

For fatty acid analysis, cells were collected after washing and lysed by repeated freezing and thawing in 500 μl Tris-HCl 50 mmol/l, pH 7.4. Cell lysates were stored at -80°C until analysis.

Cell lysate proteins were measured by bichromatic spectrophotometry (570 and 700 nm) with bicinchonic acid (Pierce, Interchin) at 37°C on a Kone analyser (Thermo Electron, La Garenne-Colombes, France) with human albumin as standard.

Fatty acid analysis

Lipids were extracted from 200 μl cell extract (approximately 10^6 cells) with chloroform-methanol (2:1 v/v), with di-palmitoyl-d31-phosphatidylcholine as an internal standard. Fatty acid methyl esters were formed after hydrolysis by transmethylation of the lipids with Meth-Prep II. Analyses were carried out on an HP 5980 gas chromatograph (Agilent, Massy, France) equipped with a flame ionisation detector maintained at 220°C , an HP 6980 auto injector (Agilent), a 1 m silanised precolumn and a 30 m \times 0.32 mm \times 0.2 μm SP2340 fused silica capillary column (Supelco, Saint Quentin Fallavier, France). Results are expressed as μmol /million cells.

Thiol redox status

Oxidized and reduced glutathione. GSH was measured by LC-MS as previously described (Steghens *et al.* 2003). The processed sample was diluted 1:5 (v/v) in distilled water and subjected to LC-MS analysis.

γ -Glutamyl-cysteine ligase activity. The activity of γ -GCL was determined by a new kinetic LC-MS method (Chik *et al.* 2005). Briefly, after incubating the cell extract (three aliquots of 10 μl each) at 37°C in 70 μl of the first reagent (Tris 25 mmol/l, KCl 150 mmol/l, MgCl₂ 20 mmol/l, EDTA 2 mmol/l, L-glutamate 40 mmol/l, Na₂ATP 5.6 mmol/l, phosphoenolpyruvate 2.2 mmol/l, pyruvate kinase 3.4 IU/ml, pH 8.6), the reaction was triggered by 20 μl 2, α -aminobutyrate 20 mmol/l and then stopped after 2, 4 or 6 min (depending on the aliquot) by adding sulfo-5-salicylic acid. The slope of production of γ -glutamyl aminobutyrate, measured by LC-MS, corresponded to the catalytic activity, and the results were expressed as IU/mg protein.

Real-time PCR mRNA quantification

Total RNA was isolated from fibroblasts by the total RNA isolation kit (Rneasy mini kit; Qiagen, Courtaboeuf, France). Reverse transcription was performed with 1 μg total RNA for each condition with a first-strand cDNA synthesis kit (Amersham, Orsay, France). Human-specific primers were used for β -actin, the catalytic subunit of γ -GCL, HO-1 and COX-2. Primer sequences, gene references and fragment sizes are shown in Table 1. The specificity of the products

Table 1. DNA sequences of sense and antisense primers used for quantitative PCR analysis

Name	Primers sense/antisense	Amplicon size (bp)	GenBank accession number
β -Actin	5'-TCG-TGC-GTG-ACA-TTA-AGG-AG-3' 5'-AGC-ACT-GTG-TTG-GCG-TAC-AG-3'	262	NM:001101
Catalytic subunit of γ -glutamylcysteine ligase	5'-GAA-CTG-GCT-TGT-ACA-TTT-T-3' 5'-CAA-AGG-AAA-ATT-AAC-GAG-AG-3'	204	NM:001498
Cyclooxygenase-2	5'-TCT-CCT-TGA-AAG-GAC-TTA-TG-3' 5'-CAT-TGA-TGG-TGA-CTG-TTT-TA-3'	198	NM:000963
Haemoxygenase-1	5'-ACA-GTT-GCT-GTA-GGG-CTT-TA-3' 5'-CTC-TGA-AGT-TTA-GGC-CAT-TG-3'	247	NM:002133

was demonstrated for each fragment by melting curve analysis and gel electrophoresis.

The following thermocycler program was used for quantitative PCR: 10 min preincubation at 95°C, followed by 55 cycles of 95°C for 15 s, and then 53°C for HO-1, 56°C for γ -GCL, 61°C for COX-2 for 5 s, and 72°C for 10 s. β -Actin was used to standardise the total amount of cDNA. qPCR was carried out on a Light Cycler (Roche) by normalising all cDNA to β -actin.

Flow cytometry analysis of reactive oxygen species production

H₂DCF-DA, after deacetylation inside the cell, becomes a probe sensitive to ROS such as H₂O₂, the superoxide anion (O₂⁻) and hydroxyl radicals (OH). The procedure for its use was adapted from that of Carter *et al.* (1994). After 48 h and 7 d of culture, cells were washed twice with PBS, incubated with 2 μ mol H₂DCF-DA/l in PBS at 37°C for 30 min, and subjected to fluorescent flow cytometry analysis (Vantage; Becton Dickinson, Le Pont-de-Claix, France); the oxidation of H₂DCF was measured, with the excitation set at 488 nm, as an increase in fluorescence at 530 nm on a log scale for 10 000 events (cell counts).

Lipid peroxidation

After 48 h and 7 d of cell culture, intracellular MDA was measured by LC-MS using a method based on diaminonaphthalene derivatisation, as previously described (Peiro *et al.* 2005). The quantification was carried out with a dideuterated MDA internal standard, and the derivatives of MDA and d₂-MDA were detected at *m/z* 195.2 and 197.2, respectively. The chromatographic mobile phase (ammonium acetate 5 mmol/l, adjusted at pH 1.8 with formic acid containing 15% (v/v) of a methanol-acetonitrile (1:1) mix) was adjusted to enable full separation of acetaldehyde (*m/z* = 183.2) and MDA derivatives.

In order to exclude the effect of contaminating lipoperoxides in fatty acid preparations, we measured lipoperoxides in cell culture media, alone as a control or supplemented with 30 μ mol/l fatty acids, as previously described (Arab & Steghens, 2004).

Cell viability

Fibroblasts were grown with different fatty acids for 48 h and 7 d, and cells were harvested in DMEM-based incubation media at a density of 1×10^6 cells/well in a standard 96-well plate. The cells were then incubated at 37°C for 4 h with different concentrations of tert-butylhydroperoxide (t-BOOH), followed by incubation at 37°C for 3 h with the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). Cell viability was

measured as a whole by the tetrazolium salt assay (Mosmann, 1983), according to the protocol proposed by Thermo Electron with its kit assay. Metabolically active cells reduced the dye to purple formazan. Formazan crystals were dissolved in isopropanol with 0.4 M-HCl. Absorbance was measured bichromatically at 570 and 600 nm using a multiwell plate reader (SpectraMax Model 190; Molecular Devices, Saint Grégoire, France).

Molecular modelling of fatty acids

Conformation studies were achieved on a PC workstation using the software package Sybyl 7.0 for Linux (Tripos Associates Inc., St Louis, MO, USA). A conformational grid search was performed on each PUFA by varying key torsion angles adjacent to double bonds. All the resulting conformations were then classified according to the increasing order of energy values. Conformational analysis of PUFA was achieved by calculating the energy of conformations obtained by relative variation of key torsion angles ω_1 and ω_2 for CLA (1296 conformations), ω_1 - ω_3 for LA (1728 conformations), ω_1 - ω_4 for LLA and GLA (1296 conformations), ω_1 - ω_5 for AA and EPA (7776 conformations) and ω_1 - ω_6 for DHA (46 656 conformations). Conformations with the lowest energy were finally minimised using the Tripos force field to give the representative preferential conformation for each PUFA.

Statistical analysis

Statistical analysis was carried out with GraphPad software (version 2.1; GraphPad, Deltapoint, Monterey, CA, USA). All data were expressed as means and their standard errors. Comparisons between groups were performed by one-way ANOVA with 95% CI, followed with Dunnett's multiple range tests.

Results

Incorporation of fatty acids in cell lipids

To assess the role of fatty acids in the modulation of thiol redox status in fibroblasts, eight fatty acids representative of the main classes were used: RA, a saturated fatty acid (20:0), OA, a MUFA (18:1*n*-9), three *n*-6 PUFA (LA (18:2*n*-6), GLA (18:3*n*-6) and AA (20:4*n*-6)), two *n*-3 PUFA (LLA (18:3*n*-3) and EPA (20:5*n*-3)) and CLA (18:2[9Z, 11E]). Total fatty acids were analysed after 48 h culture in order to check that added fatty acids had been incorporated into cell lipids (Table 2). All fatty acids were highly incorporated, with an increase of at least 100%, except for RA. OA and *n*-6 PUFA displayed a systematic increase in

Table 2. Fatty acid analysis of human fibroblasts supplemented with of each fatty acid (30 $\mu\text{mol/l}$) for 48 h (Mean values with their standard errors for three determinations)

	Solvent control		γ -Linolenic acid (C20:3n-6) ($\mu\text{mol/l}$ per million cells)		Arachidonic acid (C20:4n-6) ($\mu\text{mol/l}$ per million cells)		α -Linolenic acid (C18:3n-3) ($\mu\text{mol/l}$ per million cells)		EPA (C20:5n-3) ($\mu\text{mol/l}$ per million cells)		Conjugated linoleic acid (C18:2[9Z,11E]) ($\mu\text{mol/l}$ per million cells)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
C14:0	8.5	0.02	10.2	1.42	19.4	5.16	17.3	0.66	8.6	1.18	7.5	0.21
C15:0	2.38	0.08	2.6	0.38	1.9	0.06	1.8	0.05	2.3	0.33	2.2	0.08
C16:0	86.5	4.28	94.1	14.95	89.2	5.46	75.3	1.53	81.8	11.62	85.2	2.81
C16:1n-7c	10.7	1.03	8.6	1.63	7.3	0.64	6.3	0.2	9.2	1.29	7.9	0.26
C17:0	3.5	0.5	4.1	0.86	3.1	0.23	2.8	0.17	3.9	0.55	4.0	0.13
C18:0	48.9	6.58	50.0	11.6	48.3	4.88	43.4	3.87	49.6	6.76	54.1	1.78
C18:1n-9c	69.9	11.61	55.7	13.13	59.8	9.03	54.4	3.44	62.7	8.13	58.3	1.79
C18:1n-7c	18.4	2.36	15.0	3.7	12.9	1.76	10.2	0.58	16.1	2.08	20.3	0.66
C18:2n-6	10.3	1.9	9.1	1.79	15.5	7.84	19.6	1.21	11.1	1.53	10.9	0.31
C18:3n-6	1.6	0.22	12.2	2.33	2.6	0.28	1.32	0.05	1.7	0.23	1.3	0.04
C20:0	0.5	0.16	0.3	0.07	0.25	0.04	0.22	0.01	0.2	0.04	0.4	0.1
C18:3n-3	0.22	0.02	0.2	0.04	0.17	0.01	13.6	0.84	0.4	0.05	0.2	0.02
C20:3n-6	10.4	1.87	34.4	8.46	8.6	1.46	7.4	0.6	8.3	1.14	8.9	0.29
C20:4n-6	33.6	6.2	42.5	8.63	48.2	5.26	25.1	2.05	27.3	3.36	34.6	1.47
C20:5n-3	9.8	3.92	0.9	0.29	1.6	0.41	13.0	0.55	18.4	2.27	6.1	2.56
C22:4n-6	2.5	0.94	6.9	2.07	11.3	3.23	1.3	0.2	2.5	0.36	3.7	0.21
C22:5n-6	1.18	0.19	1.6	0.36	2.8	0.4	0.8	0.03	0.8	0.09	1.2	0.06
C22:5n-3	7.3	3.04	6.3	2.13	6.1	2.55	8.6	0.91	24.3	3.1	11.0	0.48
C22:6n-3	14.3	3.57	10.6	2.69	8.6	2.13	6.5	0.57	9.8	1.29	15.7	0.52

their content and in that of their elongation products associated with a decrease in *n*-3 PUFA content.

For *n*-3 PUFA, LLA supplementation increased EPA content, whereas EPA supplementation had no effect on DHA. Furthermore, these results showed that PUFA *n*-6 elongation was more efficient than that of *n*-3 PUFA.

It is noteworthy that CLA supplementation did not give rise to any changes in fatty acid content.

Fatty acid supplementation and lipoperoxides

The concentrations of lipoperoxides in the cell culture medium alone or supplemented with each fatty acid were ($\mu\text{mol/l}$): 1.9 (SE 0.15) for controls, 1.9 (SE 0.15) for RA, 2.0 (SE 0.04) for oleic acid, 1.9 (SE 0.15) for LLA, 1.9 (SE 0.10) for LLA, 2.2 (SE 0.15) for GLA, 2.1 (SE 0.12) for AA, 2.0 (SE 0.18) for LLA, 2.2 (SE 0.16) for EPA and 2.1 (SE 0.12) for CLA (*n* 3). We can therefore exclude the hypothesis that lipoperoxides are responsible for the biological effects we have described.

Arachidonic acid and conjugated linoleic acid induce glutathione synthesis

Thiol redox status was assessed by measuring cell GSH content and γ -GCL activity. OA, LA and RA were tested and had no effect on thiol redox status.

During the first 48 h, AA and CLA induced a strong antioxidant response by increasing intracellular GSH (more than 100% for CLA, Fig. 1), associated with an activation of γ -GCL (Fig. 2(A)). The activation and induction of the γ -GCL enzyme suggests a response against oxidative conditions, as confirmed by a 3-fold increase in mRNA expression

by AA and CLA. Other fatty acids had no effect on the antioxidant response (Fig. 2(B)).

After 7 d supplementation, AA and CLA maintained their effects by increasing GSH content and γ -GCL activity, but with a non-significant increase in the GSH level for CLA. GLA, LLA and EPA activated γ -GCL with no increase in intracellular GSH. Analysis of the mRNA expression level for the catalytic subunit of γ -GCL confirmed the induction (3–4-fold) for these three fatty acids (Fig. 2).

Measurement of intracellular reactive oxygen species

GSH is known as the main intracellular antioxidant, and it is often assumed that its content maintains the redox status of the cell; in this way, it is related to intracellular ROS levels. For this reason, intracellular ROS production was assessed in cells supplemented with fatty acids: OA, LA and RA were tested and had no influence.

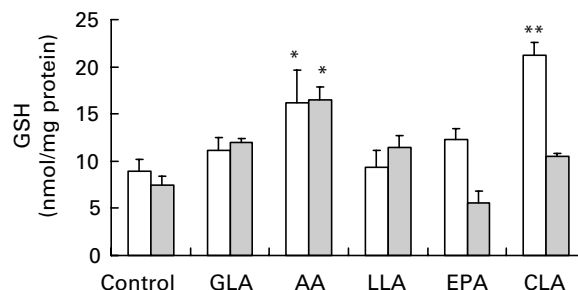


Fig. 1. Glutathione (GSH) content measured in total cell lysate after 48 h (□) and 7 d (■) of supplementation with different fatty acids. Data are expressed as means and with their standard errors indicated by vertical bars (*n* 4). GLA, γ -linolenic acid; AA, arachidonic acid; LLA, α -linolenic acid; CLA, conjugated linoleic acid. Mean values were significantly different from those of the control group: * $P < 0.05$, ** $P < 0.01$.

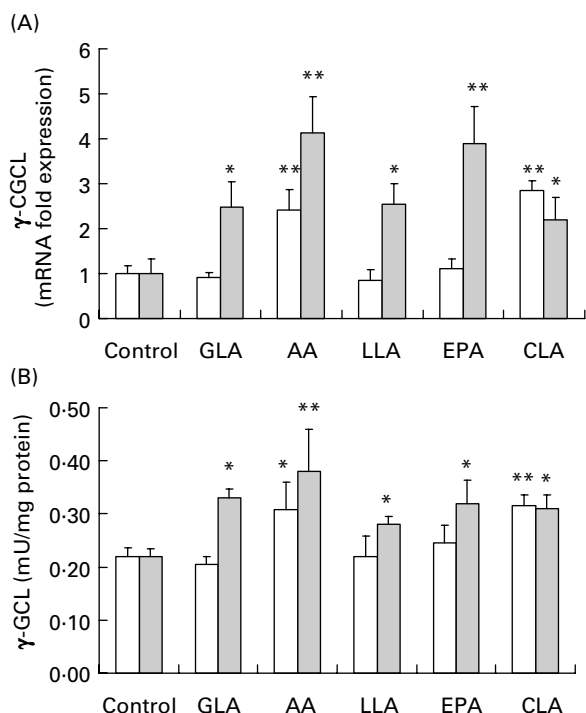


Fig. 2. mRNA expression level for the catalytic subunit of γ -glutamylcysteine ligase (γ -GCL) (A) and the catalytic activity of γ -GCL (B). Data are expressed as means with their standard errors shown by vertical bars (n 4). \square , 48h; \blacksquare , 7d. GLA, γ -linolenic acid; AA, arachidonic acid; LLA, α -linolenic acid; CLA, conjugated linoleic acid. Mean values were significantly different from those of the control group: * P <0.05, ** P <0.01.

At 48 h, flow cytometry analysis showed a 40% reduction in ROS level by AA, CLA and EPA (in comparison with the solvent control), associated with increased GSH, except for EPA. LLA and GLA had no effect. At 7 d, GSH induction failed to maintain a lowered H_2DCF oxidation in the cells supplemented with AA. With GLA, exceptionally high ROS production was detected (300%), although GLA was inactive during the first 48 h. Furthermore, LLA and EPA induced the same level of ROS as that of AA (Fig. 3).

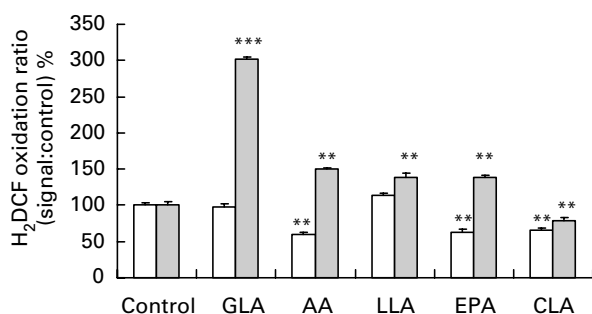


Fig. 3. Evaluation of reactive oxygen species by dichlorofluorescein (DCF) fluorescence flow cytometry analysis of cell suspensions after incubation with different fatty acids for 48 h (\square) and 7 d (\blacksquare). Flow cytometry analysis was carried out on 1×10^5 cells. In each experiment, conjugated linoleic acid (CLA) was the only fatty acid to induce a significant decrease in reactive oxygen species at 48 h and 7 d. Data are expressed as means, with their standard errors shown by vertical bars of the relative fluorescent cell number (signal:control %; (n 3). GLA, γ -linolenic acid; AA, arachidonic acid; LLA, α -linolenic acid. Mean values were significantly different from those of the control group: ** P <0.01, *** P <0.001.

Association of haemoxygenase-1 and cyclooxygenase-2 induction with lipoperoxidation

HO-1, formerly known as heat shock protein 32, is the inducible form of HO, implicated in the cell response against oxidative damage. It is also considered to be a good biomarker of the antioxidant response. Lipid peroxidation is a biomarker of oxidative damage. Thus, the association of this biomarker with HO-1 mRNA induction seems to confirm the increase in oxidative status. In addition, Uchida and colleagues reported that 4-hydroxynonenal, a lipid peroxidation byproduct, could be responsible for the elevation of COX-2 mRNA (Kumagai *et al.* 2004), and we could hypothesise that an induction of HO-1 and an increase in MDA are suitable conditions for COX-2 induction.

After 48 h, lipoperoxidation did not increase, whatever the fatty acid used, whereas after 7 d, EPA was the strongest inducer of lipid peroxidation (MDA 233 (SE 4.17) μ M/mg protein), with significant and similar, but lower, lipoperoxidation for AA, GLA and LLA (about 130 μ M/mg protein; Fig. 4). In this context, the quantification of the mRNA level of HO-1 showed a 3-fold induction for EPA and a lower but significant induction for GLA, AA and LLA (Fig. 5).

At 48 hours as well as at 7 d, CLA did not modify the MDA content or the HO-1 and COX-2 mRNA levels (Fig. 6). It should be noted that OA, LA and RA had no effect on either lipoperoxidation or HO-1 and COX-2 mRNA expression.

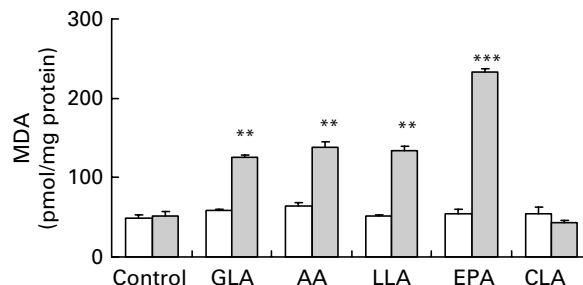


Fig. 4. Lipoperoxidation measured as true malondialdehyde (MDA) cell content after 48 h (\square) and 7 d (\blacksquare) of fatty acid supplementation. Data are expressed as means with their standard errors shown by vertical bars (n 3). GLA, γ -linolenic acid; AA, arachidonic acid; LLA, α -linolenic acid; CLA, conjugated linoleic acid. Mean values were significantly different from those of the control group: ** P <0.01, *** P <0.001.

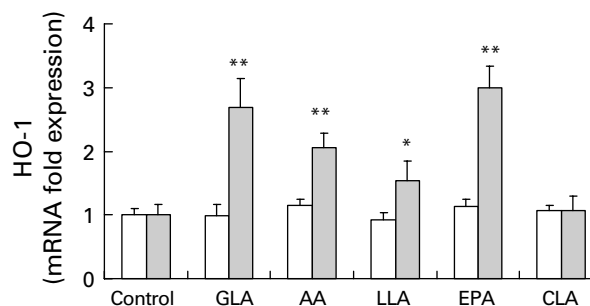


Fig. 5. Haemoxygenase-1 (HO-1) mRNA expression level. Data are expressed as means with their standard errors indicated by vertical bars (n 3). \square , 48h; \blacksquare , 7d. GLA, γ -linolenic acid; AA, arachidonic acid; LLA, α -linolenic acid; CLA, conjugated linoleic acid. Mean values were significantly different from those of the control group: * P <0.05, ** P <0.01.

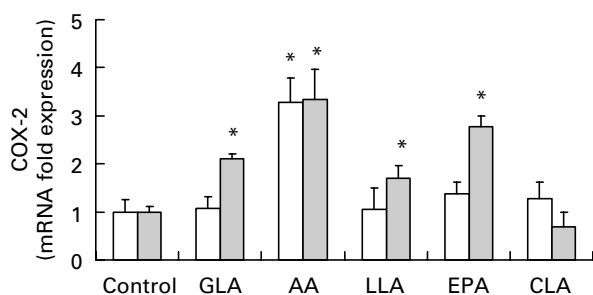


Fig. 6. mRNA expression level of cyclooxygenase-2 (COX-2) in cells supplemented with each fatty acid, at 48 h (□) and 7 d (▒). Data are expressed as means with their standard errors indicated by vertical bars (n 3). GLA, γ -linolenic acid; AA, arachidonic acid; LLA, α -linolenic acid; CLA, conjugated linoleic acid. Mean values were significantly different from those of the control group: * P < 0.05.

Conjugated linoleic acid protects cells from oxidative stress

In order to compare the potential protection of different fatty acids against t-BOOH-induced oxidative stress, we assessed cell viability by the methyl tetrazolium salt test after 48 h and 7 d culture with different fatty acids. As shown in Table 3, CLA was the only PUFA able to enhance cell resistance against oxidative stress at 48 h as well as at 7 d; cell resistance increased to 130 %, compared with the control at 48 h and 7 d, and was associated with a high GSH cell content or GCL activity. Conversely, after 7 d, cell resistance was significantly decreased with GLA (20 %), AA (15 %) and EPA (27 %). Cell susceptibility to t-BOOH seems to be related to intracellular MDA cell content.

Discussion

In the present report, we investigated the effect of several fatty acids on the cellular antioxidant response and on lipoperoxidation. Fatty acid supplementation increased their concentration in the cells; the present results showed that the elongation products of fatty acids increased, except for EPA, which did not modify DHA content. Conversely, we have previously shown that DHA supplementation increased EPA cell content (Arab *et al.* 2006). The results obtained with GLA, LA and AA suggest that n -6 elongation is more efficient than n -3 PUFA elongation in human fibroblasts. It is worth noting that CLA supplementation

Table 3. Tert-butylhydroperoxide (t-BOOH) cytotoxicity test (EC_{50}) after incubation of cells with different fatty acids for 48 h and 7 d (Mean values with their standard errors for three separate experiments)

Time of fatty acid incubation	t-BOOH EC_{50} (mmol/l)			
	48 h		7 d	
	Mean	SE	Mean	SE
Control	0.64	0.01	0.63	0.01
γ -Linolenic acid	0.66	0.02	0.52*	0.02
Arachidonic acid	0.65	0.02	0.55*	0.02
α -Linolenic acid	0.67	0.01	0.71	0.01
EPA	0.69	0.01	0.47*	0.01
Conjugated linoleic acid	0.84*	0.02	0.84*	0.03

Mean values were significantly different from those of the control group: * P < 0.05. † EC_{50} , the concentration of a compound that is required to obtain 50% cell death.

led to a lack of change in fatty acid content, suggesting that this effect uses a different signalling pathway.

The modulation of redox status of the cells appears to be a cytoprotective strategy to reduce oxidative damage, and we showed that GSH synthesis is upregulated by AA and CLA, mainly through an induction of γ -GCL: an induction of γ -GCL is characteristic of an antioxidant response (Lee *et al.* 2003; Rahman, 2005), as previously demonstrated with different compounds such as DHA and sulforaphane (Gao *et al.* 2001; Kim *et al.* 2003; Arab *et al.* 2006). So we tested the hypothesis that other PUFA could induce an antioxidative response and could explain their beneficial effect in preventing oxidative damage (Manna *et al.* 1999; Suh *et al.* 2004). It is noteworthy that the results in Table 2 are for total cell fatty acids (i.e. mainly fatty acids in membrane phospholipids) and that GLA, a precursor of AA, unlike AA, is unable to induce GSH content and other protective enzymes. We may thus suppose that the fatty acids which efficiently induce an antioxidant response are in a 'free form'.

As a consequence of their chemical structure, which is very sensitive to free radicals, it is assumed that the oxidation of n -3 PUFA generates byproducts such as 4-hydroxynonenal and MDA, which seem to play a role in the exacerbation of oxidative damage. Conversely, EPA is often described as a potential cytoprotective agent against oxidative stress by downregulating proinflammatory genes, interleukin-8 and COX-2 (Obata *et al.* 1999; Storey *et al.* 2005). For EPA, in our model, the present results enable us to exclude the enhancement of thiol redox status to explain this cytoprotection as well as the downregulation of genes such as COX-2. However, the ability of EPA and other n -3 PUFA to induce apoptosis and tumour suppression through lipid peroxidation is well described (Lynch *et al.* 2003; Utomo *et al.* 2004; Ng *et al.* 2005) and is in excellent agreement with the large production of MDA detected in our human fibroblasts and in an MRC5 cell culture model (data not shown). We suggest that this lipoperoxidation may be a protective mechanism against malignancy and carcinogenesis (Maheo *et al.* 2005).

The presence of protein-bound lipoperoxidation products has been reported in many chronic diseases in man, including atherosclerosis and neurodegenerative disorders (Terasawa *et al.* 2000; Liu *et al.* 2005). On the one hand, lipoperoxidation is associated with an induction of HO-1, an association reported to be a defence mechanism during oxidative injury (Hill-Kapturczak *et al.* 2003; Kim *et al.* 2005). On the other hand, the involvement of COX-2 in lipoperoxidation has been demonstrated by several studies, but the effect of lipoperoxidation products on its expression is less known. In addition, some reports have indicated that 4-hydroxynonenal induces COX-2 mRNA and promotes oxidative damage (Nanji *et al.* 2001; Ishii *et al.* 2004; Kumagai *et al.* 2004). COX-2 might then, via lipoperoxidation, mediate the formation of DNA adducts and increase the risk of tumorigenesis (Lee *et al.* 2005). Taking these relations into account, we tested the hypothesis that lipoperoxidation products might be involved in the upregulation of COX-2 and compared the level of MDA, a terminal lipoperoxidation product, with ROS production and COX-2 and HO-1 induction. Except for CLA, the present results showed that a high production of MDA was associated with a high induction of HO-1, COX-2 and ROS production. The fact that COX-2 is suggested to be

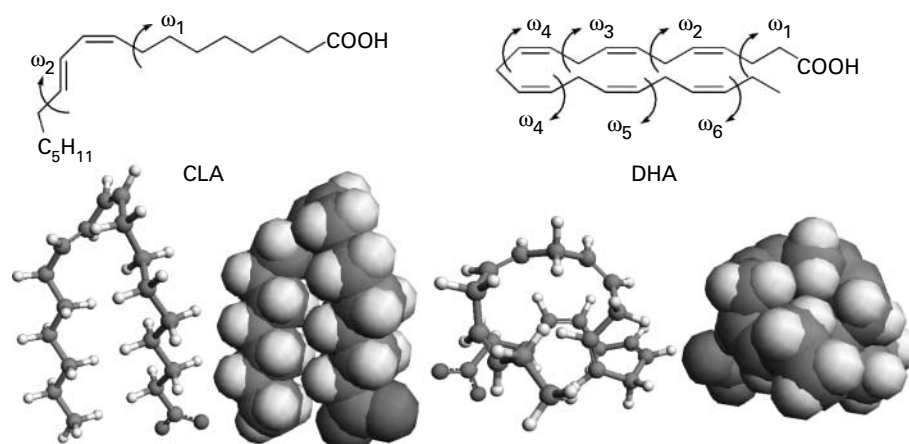


Fig. 7. Molecular modelling study of PUFA (not shown for arachidonic acid, EPA, linoleic acid, α -linolenic acid or γ -linolenic acid): key torsion angles ω_1 – ω_2 (conjugated linoleic acid; CLA). ω_1 – ω_6 (DHA) was employed for the conformational analysis and visualisation of the representative preferential conformations for the PUFA. CLA and DHA are shown on ball and stick and spacefill representations. Representative preferential conformations of PUFA can be described by the shape of the overall structure. CLA adopts a 'hairpin' conformation owing to the intramolecular hydrophobic interactions of the two alkyl chains on either side of the double bonds, whereas EPA and DHA adopt globular conformations. The preferential conformation of arachidonic acid is intermediate between a hairpin and a globular shape owing to a more folded structure compared with CLA.

auto-inducible by its preferential substrate AA could explain the elevation in COX-2 mRNA, detected as soon as 48 h, when cells are supplemented with AA.

The measurement of cell viability in the presence of t-BOOH was used as a further test to check cell protection: only CLA protected cells against oxidative damage induced by t-BOOH (Table 3). For CLA, this protection seems to be mediated by the high level of GSH; the same protective effect was found for DHA (EC_{50} 0.95 (SE 0.03) mmol/l; data not shown), which also induced a strong antioxidant response associated with a 4-fold increase in GSH at 7 d (Arab *et al.* 2006). The elevated MDA content and the induction of COX-2, associated with a lower increase in GSH in cells supplemented with AA, could explain the absence of a protective effect of GSH against oxidative damage. Cell viability might be dependent on a balance between thiol redox status and lipoperoxidation. The model with GLA and EPA is in agreement with this hypothesis, and further work is necessary to confirm whether there is a link between lipoperoxidation and COX-2 induction.

CLA (18:2[9Z, 11E]) is a member of the linoleic acid isomers, described in the 1990s as potent bioactive molecules against insulin resistance and recently as agonists of PPAR- γ (Ryder *et al.* 2001; Kuniyasu *et al.* 2006). We used CLA (18:2[9Z, 11E]) because it is the most abundant isomer in the diet of man. The study of the dose effect for AA and CLA showed that both were already significantly active at 6 μ mol/l (Supplementary Fig.) but with a different slope for each fatty acid. In addition, CLA induced GSH synthesis, without any induction of ROS production (even at 4 or 8 h; data not shown). This demonstrates that GSH synthesis was induced through a mechanism independent of ROS production. As the ability of CLA to enhance GSH content relies on γ -GCL induction, and the main inducers of this activity are ROS and PPAR- γ , our results suggest that the induction of γ -GCL by CLA, unlike other PUFA, could be mediated through PPAR- γ .

To explain this specific behaviour of CLA, a structure–activity relationship was attempted through a conformational study of PUFA. The molecular modelling study was carried out in order to delineate preferential conformations that PUFA would adopt in a biological medium. This conformation analysis of PUFA clearly shows the following:

1. Double bonds induce the presence of curved regions.
2. The packed conformation that allows intramolecular hydrophobic interactions increases with the degree of unsaturation of the PUFA. The position of the double bonds also enhances these interactions in GLA, for example, compared with LLA (two double bonds each).
3. Particular preferential conformations are remarkable for CLA (hairpin shape) and for AA, EPA and DHA (globular shape; Fig. 7).

Except for GLA, whose conformation is difficult to classify, this study suggests that the particular hairpin conformation of CLA (similar to that of the isomer *trans*-10, *cis*-12; data not shown) may allow specific hydrophobic interactions with proteins and could explain its specific interaction with PPAR- γ .

In the present report, we showed that, of the eight fatty acids tested, AA and CLA were the most potent inducers of GSH synthesis, and only CLA had a protective action, due to GSH synthesis without lipoperoxidation. The supposed relationships between fatty acid structures and their actions need to be explored to clarify the role of CLA and its isomers in modulating the redox status of the cell. The protective effect of a CLA-rich diet has already been described and could be related to the enhancement of the thiol redox status described here.

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