The nature of diene conjugation in human serum, bile and duodenal juice

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Diene-conjugated lipids have been located by HPLC in serum, bile and duodenal juice. Whether esterified or not the same predominant fatty acid is responsible for most of the diene conjugation in all of these biological fluids. Initial attempts to generate this fatty acid in pure lipid by classical lipid peroxidation in vitro were unsuccessful. Ultraviolet irradiation of free fatty acids in the presence of protein produced diene-conjugated lipids similar to those found in vivo. The predominant diene-conjugated fatty acid in vivo is an isomerised C18:2 compound.

Diene conjugation *Lipid peroxidation* *Free radical* *Serum* *Bile* *Duodenal juice*

1. INTRODUCTION

While several recent studies have underlined the possible importance of free-radical processes in human disease [1–3], they have also shown up the need for reliable free-radical 'markers' in accessible clinical material. Diene conjugation (DC) in chloroform extracts has been interpreted in the past as a measure of in vivo lipid peroxidation [4–6]; but the value of this measurement has remained severely limited by a persistent failure to generate in vitro the diene-conjugated products present in biological fluids and consequently to establish their source, derivation and identity. This work was undertaken to explore and overcome this difficulty.

2. MATERIALS AND METHODS

Duodenal juice and bile were collected from patients under investigation for pancreatic disease as in [7]. Blood for serum was collected from normal volunteers. Samples were stored at −20°C.

HPLC grade solvents and other chemicals were obtained from BDH Chemicals (Poole, Dorset). Lipid standards, free fatty acids, soybean lipoxidase, amino acids, ADP, glutathione, xanthine and xanthine oxidase were obtained from Sigma (Poole, Dorset). Glutathione peroxidase was obtained from Boehringer Corp. (Lewes, Sussex).

Lipids were extracted by vortex mixing, 1 ml sample with 8 ml chloroform/methanol (2:1, v/v) for 2 min. After centrifugation 5 ml of the chloroform layer was washed with 2 ml water to separate the two phases. DC was measured as absorbance at 240 nm in the chloroform phase read against an extracted water blank.

Isocratic HPLC analysis was performed on Laboratory Data Control (Stone, Staffordshire) apparatus comprising a Constametric III pump, computing integrator 308 and Constametric III variable wavelength ultraviolet (UV) detector. A Rheodyne injection valve (Corati CA) was used with a 20 μl sample loop. Flow rate was 2 ml/min throughout. Columns were 250 × 4 mm with 5 μm particles. Cholesteryl esters and triglycerides were analysed by normal-phase HPLC using a Hibar LiChrosorb Si60 column (Merck, Darmstadt) with a mobile phase of hexane/propan-2-ol (997:3,

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v/v). Phospholipids were analysed as in [8] using the same column with a mobile phase of acetonitrile/methanol/85% orthophosphoric acid (130:5:1.5, by vol.). Free fatty acids were analysed by reverse-phase HPLC using a Hibar LiChrosorb RP18 column with a mobile phase of acetonitrile/water/acetic acid (70:30:0.1, by vol.). Samples were prepared by extracting with chloroform/methanol as above. The chloroform layer was dried under a stream of nitrogen and resuspended in hexane for cholesteryl ester and triglyceride analysis, or in methanol for phospholipid or free fatty acid analysis. Serum and bile were hydrolysed by incubation at 37°C for 1 h with an equal volume of a saturated solution of 'Pancrex V' pancreatic extract (Paines and Byrne Ltd., Greenford, Middlesex) made up in phosphate-buffered saline (PBS) (50 mmol/l K_2HPO_4/KH_2PO_4, 150 mmol/l NaCl, pH 7.4). Serum cholesteryl esters, triglycerides and phospholipids were isolated by HPLC, reextracted with chloroform, dried under nitrogen and resuspended in a small volume of methanol and hydrolysed with pancreatic extract. After hydrolysis the free fatty acids were extracted and analysed by HPLC.

UV irradiation was carried out on 3 ml samples in quartz cuvettes (1 cm^2 in cross section) at a distance of 4 cm from a 254 nm light source (G8T5 8 W bulb with an average light intensity of 10.5 $\mu W/cm^2$ at 1 m).

In vitro lipid peroxidation was induced in polyunsaturated free fatty acids (linoleic acid, linolenic acid and arachidonic acid, 3.6 mmol/l) by the following methods:

(i) Autoxidation by exposing the fatty acid to air at 37°C for 48 h in water/methanol (1:1, v/v).
(ii) Incubation with soybean lipoxidase (10000 units/ml in PBS/methanol 19:1, v/v) for 1 h at 37°C.
(iii) Iron/ADP and xanthine/xanthine oxidase methods adapted from [9].
(iv) UV irradiation in PBS/methanol (19:1, v/v) of pure lipid emulsions.
(v) UV irradiation as in (iv) but with human albumin or gamma globulin (10 g/l) added to the lipid emulsion.
(vi) UV irradiation as in (iv) but with added tryptophan, arginine, histidine or lysine (10 mmol/l amino acid to 3.6 mmol/l fatty acid in PBS/methanol 1:1, v/v), or glutathione, oxidised or reduced (1–100 mmol/l).

After peroxidation, 1 ml aliquots were extracted with chloroform/methanol. Total DC was measured in the chloroform phase. The chloroform phase was then dried under a stream of nitrogen; the lipids were resuspended in 100 μl methanol; and 20 μl was used for HPLC analysis.

The diene-conjugated lipids in biological fluids and those produced in vitro (1 ml aliquots) were incubated with sodium borohydride (100 μl 2.5 mol/l in PBS) or with glutathione peroxidase (100 μl of 5 units/ml in PBS) and reduced glutathione (100 μl 7 mmol/l in PBS) for 30 min at 37°C, followed by lipid extraction and HPLC analysis.

Free sulphydryl group generation in the irradiated proteins was measured as in [10] and fluorescence generation monitored as in [11] using a Perkin Elmer MPF-3L fluorimeter (Beaconsfield, Bucks), ex. 360 nm, em. 454 nm.

Labelled [1-^{14}C]linolenic acid (Amersham International, Bucks) was incorporated in the UV irradiation with albumin experiment (procedure v). The specific activity was 97 MBq/mmol and the final concentration was 3.8 mmol/l. 1 ml aliquots were taken at times 0, 60 and 120 min, followed by lipid extraction. Each extract was dried under nitrogen and the whole extract analysed by gradient HPLC. The equipment comprised a Pye Unicam (Cambridge) LC 3XP pump with low-pressure solvent programmer, Pye Unicam LC UV detector and Spherisorb ODS2 250 × 5 mm column. Solvent A was acetonitrile/water/acetic acid (70:30:0.1, by vol.); solvent B was acetonitrile/water/acetic acid (90:10:0.088, by vol.). A linear gradient was run from 10–90% A in A + B over 20 min at 2 ml/min; 0.5 ml fractions were collected every 15 s into 4.5 ml scintillation fluid (United Technologies Packard Emulsifier Scintillator 299). The specimens were counted in a Packard Tri Carb 460 CD scintillation counter (Reading, Berks) using the preset values for ^{14}C. Quenching was uniform despite the changing solvent.

3. RESULTS

The diene-conjugated lipids investigated fall into

three broad categories:

(A) Diene-conjugated lipids present in biological fluids as esterified lipids or free fatty acids;

(B) Diene-conjugated products of in vitro peroxidation of pure lipids;

(C) Diene-conjugated products of in vitro peroxidation of lipids in the presence of protein.

Cholesteryl esters, triglycerides, phospholipids and free fatty acids in extracts from biological fluids were located using standard lipids and three HPLC systems. In serum recovery of the diene-conjugated lipids associated with cholesteryl esters, triglycerides and phospholipids after HPLC analysis confirmed that these three lipid fractions accounted for all DC measured in total chloroform extracts. The elution times of diene-conjugated and non-diene-conjugated esterified lipid fractions were identical.

In bile with a high total DC from a patient with chronic pancreatitis 90% of the DC was associated with phosphatidyl choline (fig.1B). The distribution was different in bile with a low total DC from a control subject (fig.1A). The clinical significance of the difference is discussed in [7].

Duodenal juice did not contain esterified lipids. All DC was associated with free fatty acids, one free fatty acid predominating (fig.2).

Hydrolysis of serum lipids or of bile yielded the same diene-conjugated fatty acid as the predominant diene-conjugated fatty acid in duodenal juice (fig.3). Hydrolysis of isolated cholesteryl esters, triglycerides or phospholipids yielded the same diene-conjugated fatty acid. The HPLC retention of this fatty acid was unaffected by treatment with sodium borohydride or glutathione peroxidase.

The peroxidation of pure lipids by procedures (i)–(iii) yielded a number of lipid hydroperoxides with DC. All of these were more polar than their parent fatty acids and more polar than the diene-conjugated fatty acid in biological fluids. Treatment with sodium borohydride or glutathione peroxidase reduced the peroxy to hydroxyl groups.

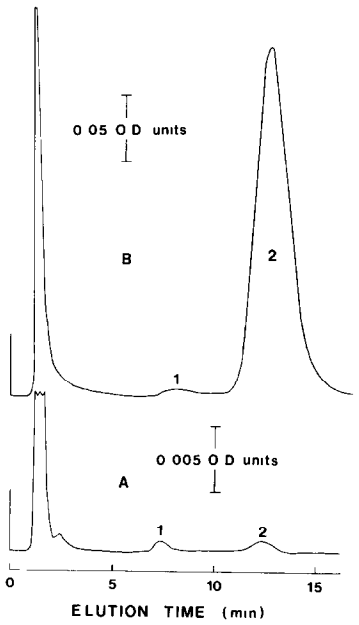

Fig.1. Normal-phase HPLC analysis of phospholipids with DC. Bile (0.5 ml) was extracted with chloroform/methanol (2:1, v/v). The chloroform phase was washed with water, dried under a stream of nitrogen and resuspended in 0.2 ml hexane; 20 μl was injected; (A) bile from a control patient; (B) bile from a patient with chronic pancreatitis; (1) phosphatidylethanolamine; (2) phosphatidylcholine; detector, 234 nm.

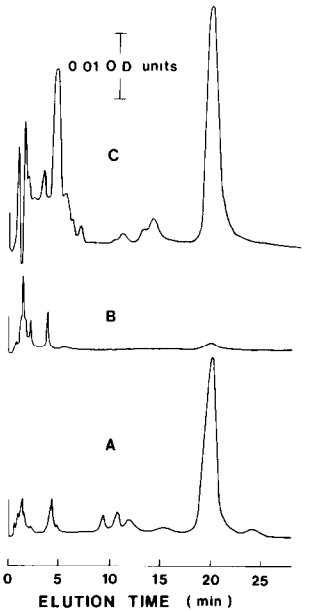

Fig.2. Reverse-phase HPLC analysis of free fatty acids with DC: (A) duodenal juice, 0.5 ml extracted as in fig.1 and resuspended in 100 μl methanol; 20 μl was injected; (B) bile, 0.5 ml, processed as for duodenal juice; (C) phospholipids from 0.5 ml bile, hydrolysed with pancreatic extract, extracted as above, resuspended in 100 μl methanol; 20 μl was injected; detector, 234 nm.

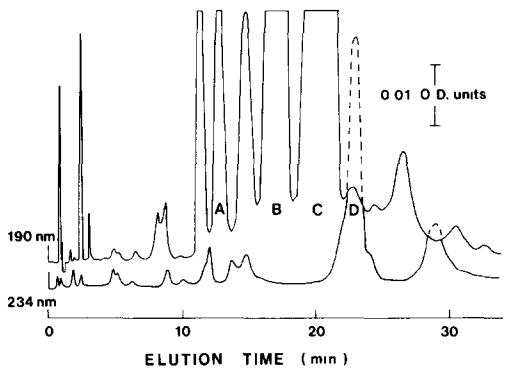

Fig.3. Reverse-phase HPLC analysis of hydrolysed serum free fatty acids. Serum (0.5 ml) was hydrolysed with pancreatic extract, extracted with chloroform/methanol, dried under nitrogen and resuspended in 100 μl methanol; 20 μl was injected; (A) linolenic acid; (B) arachidonic acid; (C) linoleic acid; (D) diene-conjugated fatty acid.

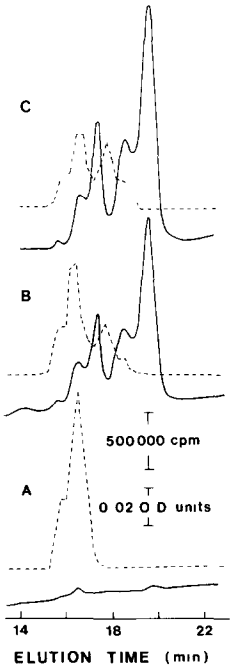

Fig.4. Gradient elution HPLC of UV-irradiated [1-^{14}C]linoleic acid and albumin; (A) before UV irradiation; (B) 60 min UV irradiation; (C) 120 min UV irradiation; A_{234}; (——) cpm.

These derivatives were also more polar than the diene-conjugated fatty acid in biological fluids.

UV irradiation of linoleic acid [procedure (iv)] did not produce DC despite a 40% depletion in the parent fatty acid after 2 h irradiation. By contrast, fatty acids irradiated in the presence of protein [procedure (v)] did produce diene-conjugated fatty acid derivatives. On the basis of HPLC mobility these derivatives were slightly less polar than their parent fatty acid. They were not hydroperoxides or hydroxyl fatty acids. One of the diene-conjugated derivatives produced by irradiating linoleic acid in the presence of albumin was chromatographically indistinguishable from the diene-conjugated fatty acid present in biological fluids. The same product in lower yield was obtained by the irradiation of linoleic acid with human gamma globulin. Irradiation with amino acids or glutathione [procedure (vi)] did not produce DC.

Irradiation of [1-^{14}C]linoleic acid with albumin analysed by gradient elution HPLC generates 4 diene-conjugated derivatives as minor products (fig.4).

4. DISCUSSION

Initial studies with HPLC have deepened rather than dispelled the uncertainty surrounding the origin and nature of diene-conjugated material in biological fluids. On exposure to free radical activity polyunsaturated lipids yield a number of diene-conjugated products which can be mapped out as a series of peaks. Whatever the starting lipid or means of generating free-radical activity, none of these peaks co-chromatograph with the peak (or peaks) that account for most or all the measurable DC in extracts from serum, bile or duodenal juice. The reason for this now appears to be clear. To produce in vitro the diene-conjugated products formed in vivo a fatty acid must be exposed to free radical activity in the presence of protein. Linoleic acid yields four diene-conjugated and two non-diene-conjugated products which have a chromatographic elution time close to that of the parent compound and which contain no oxygen outside the carboxyl group. The possible reaction sequences can be summarised as follows, the diene-conjugated products being *italicised:*

(1)	Linoleic acid (C18:2 9cis, 12cis)	C18:2˙ + H˙	Free-radical initiation
(2)	C18:2˙	*C18:2˙*	Double bond rearrangement leading to DC
(3)	Protein absent: *C18:2˙* + O_2	*C18:2-00˙*	Classical peroxidation
(4)	Protein present:		
	(a) C18:2˙ + Prot	C18:2 (9cis, 12trans) + Prot˙	Stabilised non-DC isomerisation
	(b) C18:2˙ + Prot	C18:2 (9trans, 12cis) + Prot˙	
(5)	(a) *C18:2˙* + Prot	*C18:2 (9cis, 11cis)* + Prot˙	
	(b) *C18:2˙* + Prot	*C18:2 (9cis, 11trans)* + Prot˙	Stabilised DC isomerisation
	(c) *C18:2˙* + Prot	*C18:2 (10cis, 12cis)* + Prot˙	
	(d) *C18:2˙* + Prot	*C18:2 (10trans, 12cis)* + Prot˙	

In our experimental systems the bulk of linoleic acid followed pathways 4a and 4b; some followed pathways 5a–5d; the remainder followed pathway 3. Other mechanisms are theoretically possible but would require more than one free-radical event per molecule. Albumin could be replaced by other proteins (e.g., human γ-globulin) but not by amino acid residues or by glutathione (either in the presence or absence of glutathione peroxidase). The changes in the protein component of our experimental system have not yet been studied in detail but may be similar to those in [11]. The lipid–protein interaction results in free radical isomerisation of C18:2 fatty acid without oxygen addition and not in classic lipid peroxidation. It is these isomers which account for DC in biological fluids.

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