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LINOLEIC ACID AND PROTEIN THIOL CHANGES SUGGESTIVE OF OXIDATIVE DAMAGE IN THE PLASMA OF PATIENTS WITH ADULT RESPIRATORY DISTRESS SYNDROME

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Patients with the acute lung injury syndrome ARDS are under oxidative stress from the disease and from treatment with high inspired oxygen concentrations. Oxidative stress can lead to molecular damage by a variety of reactive oxygen intermediates generated in the lung. In the present study we sequentially monitor changes in plasma total lipid linoleic acid fatty acid levels, using GC-MS, and express these as a function of changes in plasma protein thiol values. In nine out of eleven ARDS patients there was a relationship between loss of protein thiols and loss of total lipid linoleic acid. In three patients changes in protein thiols preceded changes in total lipid linoleic acid by several days. Parallel decreases in plasma total lipid fatty acid esters of linoleic acid and protein thiols are suggestive of oxidative stress leading to molecular damage.

1. INTRODUCTION

Adult respiratory distress syndrome (ARDS) is an acute form of lung injury characterised by refractory hypoxaemia secondary to non hydrostatic pulmonary oedema. Many clinical conditions, often not directly involving the lung, can precipitate ARDS. These include, sepsis, polytrauma, pancreatitis, severe burns, cardiopulmonary bypass, haemorrhagic shock, adverse drug reactions, aspiration of the lungs and oxygen toxicity (reviewed in 1).

Oxidative stress leading to molecular damage has been implicated in the disease process of ARDS, although evidence of lipid and protein damage based on plasma studies is sparse. Reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are known to arise in the lung from activated neutrophils (2). When suitable redox active iron complexes are available, O_2^- and H_2O_2 serve as precursors of the highly reactive and damaging hydroxyl radical ($\cdot OH$). Recent work suggests that $\cdot OH$ could also arise by a metal independent pathway whereby O_2^- reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$) which decomposes to yield $\cdot OH$ (3). Oxidative stress leading to oxidative damage is also an unfortunate consequence of the ventilatory support of ARDS patients with high inspired oxygen concentrations, essential to combat the refractory hypoxaemia associated with the disease.

In a previous study we sequentially monitored the plasma proteins of ARDS

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patients for evidence of oxidative damage by measuring changes in carbonyl and thiol levels, and found patterns of change in thiol levels that were different for survivors and non-survivors (4). Here, we extend this study to look at sequential changes in plasma total lipid fatty acid patterns, various non-specific oxidation products of lipids such as thiobarbituric acid reactive and autofluorescent materials, and their relationship to changes in plasma protein thiols.

2. MATERIALS AND METHODS

Fatty acid methyl esters (FAMES) and butylated hydroxytoluene (BHT), were obtained from the Sigma Chemical Company (Poole, Dorset). All other chemicals were of the highest grade, available from Fisons Instrumental Ltd (Loughborough, Leics).

2.1 Extraction and Derivatisation of Plasma Fatty Acids

To 0.75 ml of plasma was added 3.5 ml of methanol-chloroform mix (2:1) and 5 μl of BHT (100 μg/ml in methanol). The mixture was purged with oxygen-free nitrogen, capped and vigorously vortex mixed on six separate occasions for two minutes during a period of 1 hour. The tube contents were centrifuged at 8,500 rpm for 5 min, and the supernatant removed and stored under nitrogen at 4°C. To the remaining residue was added 4.5 ml of methanol-chloroform-water mix (2:1:0.8), and the samples treated as above. The supernatant from this centrifugation was combined with the first, and 5 ml of chloroform-water (1:1) added. The mixture was vigorously vortex mixed for 2 min, and centrifuged at 8,500 rpm for 10 min. The lower chloroform layer was carefully removed and evaporated to dryness at 55°C under a stream of nitrogen.

Fatty acids in the dry sample were derivatised by adding 0.5 ml of toluene (dry), 0.5 ml methanol (dry) and 12.5 μl of concentrated sulphuric acid. The samples were vortex mixed, gassed with nitrogen, capped and heated at 55°C for ten hours. To the resulting fatty acid methyl esters 0.25 ml of saturated NaCl solution was added and 1 ml of hexane-chloroform (4:1).

The mixture was vigorously vortex mixed and centrifuged to separate the phases. The top layer (hexane) was retained and stored at 4°C under nitrogen, and the same procedure as above repeated on the remaining lower phase. The two hexane extracts were combined and 1 ml of 0.33 M NaOH added. Tube contents were vigorously vortex mixed and centrifuged at 8,500 rpm for 8 min. The upper phase was carefully removed and evaporated to dryness at 55°C under nitrogen. The dry fatty acid methyl esters were resuspended in 200 μl of acetone (dry) for analysis by GC-MS.

2.2 Total Plasma Thiobarbituric Acid Reactivity (TBAR)

To new clean glass tubes were added 200 μl of plasma, 0.5 ml of thiobarbituric acid (TBA) 1% in 50 mM NaOH, and 0.5 ml of 25% v/v HCl. The tubes were capped and heated at 100°C for 10 min. When cool, 1.5 ml of butan-1-ol was added, and the tube contents vigorously vortex mixed for 2 min, before centrifugation at 9,000 rpm for 8 min. The upper butanol layer was carefully removed, and the fluorescence measured at 554 nm with excitation at 532 nm. Relative fluorescence intensity units (RFIU) were ascribed to the samples based on a standard of rhodamine B (3×10^{-6}M) set to 100 units under the same wavelength settings.

2.3 *Total Lipid Autofluorescence*

200 μl of plasma, 0.3 ml of NaCl 0.15 M, 0.5 ml methanol and 1.0 ml chloroform were mixed in new clean glass tubes, and vigorously vortex mixed for 2 min. Phases were separated by centrifugation at 9,000 rpm for 5 min. The upper phase together with the protein pellet were carefully removed. 0.1 ml of methanol was added to 'clear' the lower chloroform phase for spectrofluorimetry. Autofluorescence was measured against appropriate blanks at 430 nm with excitation at 360 nm (5). RFI units were calculated relative to a standard of tetraphenylbutadiene (10^{-7}M) set to 100 units at the same wavelengths.

2.4 *Gas Chromatography-Mass Spectrometry of Fatty Acid Methyl Esters*

2 μl of the acetone solution of FAMES were injected into a Hewlett Packard 5890 series II gas chromatograph housing a WCOT fused silica 50 × 0.25 mm ID, CP sil 88 capillary column. A splitless injection technique was used with a purge time of 5.5 mins and temperature of 150°C. The column head pressure was 10 psi. The heating profile was run at 65°C for 3 mins, a ramp of 15°C/min to 200°C, held for 1 min, followed by a ramp of 8°C/min to 220°C and held for 10 mins.

Detection was by positive ion electron impact mass spectrometry using a Fisons VG Trio 1000 mass spectrometer. Total ion chromatograms were obtained for masses between 50–650 amu after a solvent delay of 16 mins. Peak assignments were based on retention times of known FAME standards, molecular ions, characteristic ion fragmentation patterns and the NBS library. The percentage of linoleic acid present was calculated using palmitic acid as the non-oxidisable 100% reference value.

2.5 *Plasma Proteins and Thiol Groups*

Plasma thiols are predominantly associated with plasma proteins, and these were measured using the Ellman (6) technique. Total plasma proteins were determined using a Sigma Kit assay based on the Lowry procedure. Plasma thiol values were expressed as nmol/mg of protein present.

3. RESULTS

Eleven patients with clinically diagnosed ARDS (see Table 1) were recruited into the study from a variety of different clinical backgrounds. Blood samples were taken

TABLE 1

	Clinical Problems	Sex/Age	Days in ITU	1st sample Time after development of ARDS	Outcome
A	Abdominal sepsis	F/37	13	24 hrs	Survived
B	Aspiration pneumonia	F/23	20	48 hrs	Survived
C	Multiple trauma	M/18	30	24 hrs	Survived
D	Fat embolisation	F/16	12	48 hrs	Survived
E	Aspiration/fit	F/20	17	48 hrs	Survived
F	Multiple trauma	M/9	26	10 days	Non survivor
G	Multiple trauma	F/16	32	3 days	Non survivor
H	Anaphylaxis/sepsis	F/22	17		Non survivor
I	Lung surgery	M/68	19		Non survivor
J	Pre-eclampsia	F/22	19	48 hrs	Non survivor
K	Ectopic pregnancy	F/19	16		Non survivor

from indwelling radial artery catheters during their stay in intensive care at the time intervals shown in appropriate Figures. Total protein, thiol and TBAR measurements were carried out within the shortest possible time on freshly separated plasma, whereas total lipid fatty acid analysis and autofluorescence were performed on plasma stored at −20°, for no longer than three weeks. All patients, except 'J', were fed by total parenteral nutrition (TPN), and as part of this some were given a lipid feed 'Intralipid' at various times during management. Careful note was made of these times which are shown in Table 2 and Figures 1 and 2.

Patients with ARDS show increased levels of protein damage when compared with age-matched intensive care control patients and normal healthy subjects (4). Plasma protein thiol values, mainly associated with albumin, show the most dramatic changes during intensive care management. As a group ARDS patients have lower thiol levels (4.56 ± 0.50 nmol/mg protein) than controls (6.55 ± 0.52 nmol/mg protein). However, when ARDS patients are grouped as survivors and non-survivors significantly ($p = 0.001$) higher plasma protein thiol levels are found in survivors (6.24 ± 0.09 nmol/mg protein) compared with non-survivors (3.56 ± 0.16 nmol/mg protein) (4). These values are summarised in Table 2 together with those of normal healthy controls (approximately age-matched).

When changes in total lipid plasma fatty acids, and their oxidation products, were matched with thiol changes, the following results were obtained (summarised in

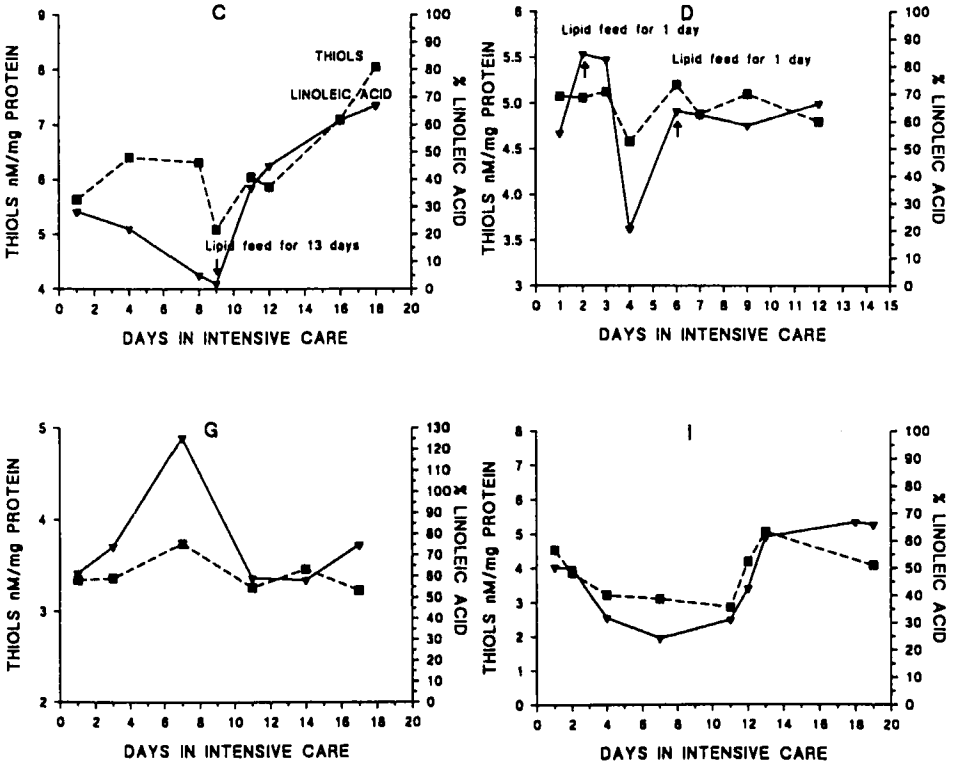

FIGURE 1 Shows changes in plasma protein thiols (■) and linoleic acid (▽) levels in patients C, D, G and 1. These patients showed a high correlation between the two parameters (see Table 2).

TABLE 2

ARDS Patients () No samples	Linoleic acid as % total fatty acids mean ± SEM	Nutrition	TBARS mean ± SEM (RFIU)	Autofluorescence mean ± SEM (RFIU)	Thiols nmol/mg protein	Linoleic acid/thiol correlation 'r' values	Thiols μM
A (9)	60.6 ± 9.96	TPN(lipid)	66.1 ± 3.13	17.1 ± 1.37	6.07 ± 0.32	•	345.0 ± 24.90
B (7)	74.4 ± 6.80	TPN(lipid)	51.0 ± 2.45	-	6.26 ± 0.24	−0.17	350.7 ± 57.70
C (8)	33.5 ± 6.80	TPN(lipid)	-	32.6 ± 3.46	6.40 ± 0.24	+0.75	377.3 ± 30.00
D (9)	63.8 ± 6.40	TPN(lipid)	50.0 ± 1.90	41.7 ± 11.30	4.83 ± 0.12	+0.69	345.3 ± 7.10
E (6)	75.2 ± 13.40	TPN(lipid)	47.7 ± 2.85	16.2 ± 4.87	5.90 ± 0.49	−0.29	417.9 ± 19.80
F (9)	71.3 ± 13.40	TPN	43.3 ± 1.16	21.0 ± 4.87	3.39 ± 0.17	+0.38	309.1 ± 12.70
G (9)	82.8 ± 7.90	TPN	78.7 ± 3.20	36.8 ± 2.97	3.57 ± 0.13	+0.81	252.7 ± 12.26
H (8)	28.4 ± 7.20	TPN	33.5 ± 1.32	-	3.10 ± 0.15	•	273.6 ± 21.40
I (9)	47.3 ± 3.33	TPN	36.5 ± 1.56	21.2 ± 2.40	3.68 ± 0.24	+0.82	266.5 ± 14.60
J (9)	61.4 ± 3.33	Enteral	56.0 ± 2.70	4.04 ± 0.29		+0.30	265.5 ± 11.20
K (9)	66.8 ± 15.00	TPN(lipid)	26.6 ± 1.40	17.2 ± 1.01	5.48 ± 0.19	•	318.3 ± 8.45
ARDS mean ± SEM	60.5 ± 5.20		48.9 ± 5.00	25.5 ± 3.60	4.80 ± 38.0		320.2 ± 15.80
Healthy controls (6)	97.5 ± 12.50		36.6 ± 5.00	20.3 ± 5.26	6.90 ± 0.57		542.0 ± 42.30
Intralipid Solution (4)	225.0 ± 35.00						

• Delayed
TPN = Total parenteral nutrition
RFIU = Relative fluorescence intensity units

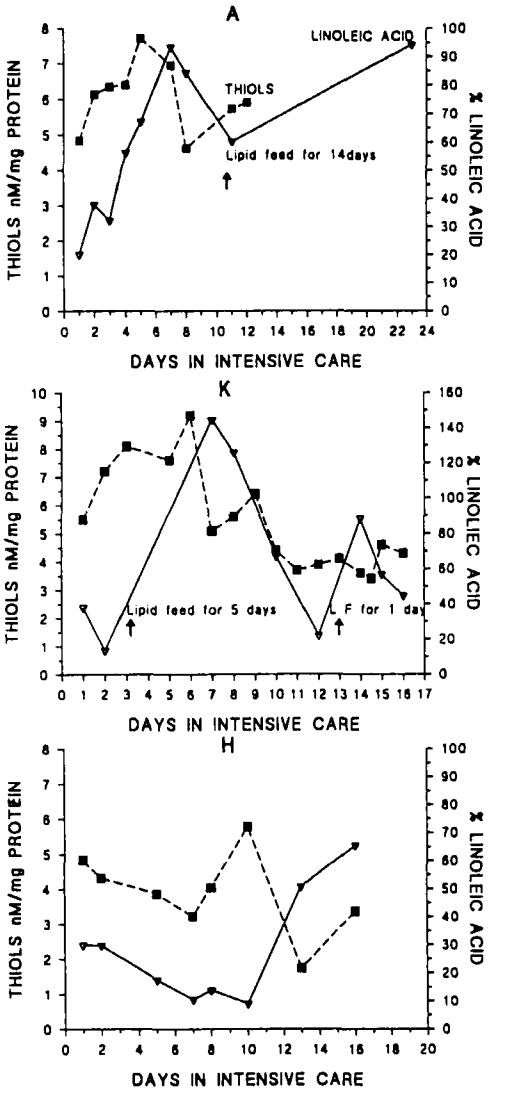

FIGURE 2 Shows changes in plasma thiols (■) and linoleic acid (▽) levels in patients A, K and H. These patients showed thiol changes 1 to 2 days in advance of those seen for linoleic acid.

Table 2): Non-specific products of lipid peroxidation, such as total TBARS and autofluorescent products, showed no correlations with plasma thiol or total lipid fatty acid changes in any of the ARDS patients (data not shown). When expressed as mean values neither group were significantly different from normal controls.

The mean values for total lipid linoleic acid esters in each patient series varied greatly with no clear pattern emerging for ARDS survivors or non-survivors (Table 2). Levels of plasma linoleic acid esters appeared to be lower in ARDS patients than in normal controls in spite of the former group receiving lipid feeds containing high levels of linoleic acid (Table 2). However, this difference was not statistically

significant. When sequential changes in linoleic acid are plotted for each patient against thiol values a strong correlation is seen in most cases (Figures 1 and Table 2). Sometimes, changes in thiol levels tended to precede changes in linoleic acid by one to two days (Figure 2). When this occurs no 'r' value is shown (Table 2). A relationship between thiols and linoleic acid levels was also seen in patients who did not receive lipid feeds with their TPN (Table 2). As total lipid linoleic acid levels decreased there was a strong tendency for oleic and palmitoleic acids to increase in the plasma (data not shown). Arachidonic acid, a minor polyunsaturated fatty acid component of plasma lipids, also decreased in parallel with linoleic acid.

4. DISCUSSION

Patients with ARDS are under oxidative stress from the disease process and from treatment with high inspired oxygen concentrations. As a consequence, molecular damage occurs to a variety of biological molecules including proteins, lipids and DNA. At present it is not clear whether such damage is salvageable by intervention with protective agents, or whether it significantly affects patient survival rates.

Evidence of oxidative damage has most clearly been demonstrated in bronchoalveolar lavage fluid taken from the lungs of ARDS patients, where decreased levels of reduced glutathione (7), increased lipid hydroperoxides (8), and oxidised alpha-1-antiproteinase (9) were found. In plasma, however, the picture is less clear, with depleted ascorbate (8,11) but contradictory reports concerning plasma vitamin E (8,10,11,12), and lipid peroxide levels (8,10), the latter sometimes detected as thiobarbituric acid reactive substances (10).

When studying sequential changes in plasma biochemical values in ARDS patients we note large day to day variations, suggesting that the analysis of single time point samples may sometimes be misleading when compiling statistical data on the syndrome. When ARDS patients are studied on a daily basis during their stay in intensive care, they show evidence of increased plasma protein damage with raised levels of protein carbonyls and decreased levels of protein thiols (4). When these patients are grouped as survivors and non-survivors of the disease, we see higher thiol levels that increase as lung injury resolves in survivors, but low and often falling thiol levels in the non-survivors (4).

Here, we extended these protein studies to look for changes in total lipid linoleic acid esters, the major oxidisable polyunsaturated fatty acid of human plasma. Non-specific tests for evidence of lipid peroxidation products in plasma, such as measurement of total TBARS and autofluorescent material characteristic of Schiff bases (5), were carried out in parallel with fatty acid analysis. The non-specific assays showed slightly increased values but these were not significantly different from normal healthy controls in contrast to previously reported TBAR values (10). Plasma total lipid linoleic acid levels in ARDS patients, however, underwent large daily changes, often increasing after administration of a lipid feed. Based on plasma fatty acid profiles previous studies have suggested that ARDS patients show lipid patterns characteristic of essential fatty acid deficiency (10).

Linoleic acid is an essential fatty acid used in the biosynthesis of prostanoids, via arachidonic acid, and therefore loss from plasma cannot necessarily, or at all, be attributed to increased free radical oxidations. In order to attempt to assess the contribution to loss from oxidative stress we plotted changes in total lipid linoleic acid against changes in plasma protein thiol values. This data showed that in nine out of

eleven ARDS patients there was a relationship between the loss of plasma protein thiols and total lipid linoleic acid. In three of the patients changes in protein thiols preceded changes in linoleic acid by several days, supporting the proposal that plasma proteins are more sensitive markers of oxidative damage than plasma lipids (13).

We suggest that decreases in plasma total lipid linoleic acid levels, monitored in parallel with a loss of protein thiol groups, are suggestive of oxidant stress leading to molecular damage.

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