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4-HYDROXY-2-NONENAL LEVELS INCREASE IN THE PLASMA OF PATIENTS WITH ADULT RESPIRATORY DISTRESS SYNDROME AS LINOLEIC ACID APPEARS TO FALL

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Gas chromatography-mass spectrometry has been applied to the analysis of plasma linoleic acid and one of its oxidation products, 4-hydroxy-2-nonenal (HNE), in adult patients with the acute respiratory distress syndrome (ARDS). Peak areas of total ion chromatograms showed there to be negative correlations between loss of linoleic acid and formation of HNE (measured by selective ion monitoring) in 7 out 10 patients studied. When HNE was quantitated by selective ion monitoring, with reference to a pure standard of HNE and an internal standard of nonanoic acid, ARDS patients showed significantly increased levels of HNE (0.412 ± 0.023 nmol/ml) compared with normal healthy controls (0.205 ± 0.018 nmol/ml).

KEY WORDS: ARDS, 4-Hydroxy-2-nonenal, essential fatty acids, lipid peroxidation, oxidative stress, antioxidants, free radicals.

INTRODUCTION

Acute respiratory distress syndrome in adults (ARDS) is a form of lung injury brought about by a variety of clinical conditions not necessarily involving the lungs. Patients have poor survival prospects following the development of ARDS, with death occurring in some 50% of cases as a result of multi-organ failure or sepsis.[1]

Oxygen free radicals have been implicated in the progress of ARDS, and evidence for their deleterious participation has been indirectly measured as oxidative damage to plasma proteins[2] and lipids (reviewed in 3).

Activated neutrophils are known to accumulate in the lungs of patients with ARDS, and the reactive oxygen species (ROS) they produce such as O_2^- and H_2O_2 may in the presence of iron form the highly reactive hydroxyl radical ($\cdot OH$).[3] The $\cdot OH$ radical may also arise independently of iron catalysis via the reaction of superoxide (O_2^-) and nitric oxide ($N^{\cdot}O$) to form peroxynitrite ($ONOO^-$)[4] or possibly the reaction of superoxide with hypochlorous acid (HOCl).[5] High inspired O_2 concentrations (FiO_2) administered during ventilatory support may also result in increased ROS production.

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Previous analysis of plasma fatty acid changes in ARDS patients has shown decreased levels of the polyunsaturated fatty acid linoleic acid (18:2).[6] In addition, there appears to be a positive relationship between the decrease of total plasma linoleic acid and loss of plasma protein thiol groups[6] suggestive of free radical damage.

Previous studies in ARDS patients, and animal models of acute lung injury, have shown equivocal lipid peroxidation data in both plasma and bronchoalveolar lavage fluids.[7,8,9] Most studies have been based on non-specific measures of lipid peroxidation such as thiobarbituric acid reactivity (TBA) and diene conjugation.

Here we extend our previous studies[2,6] of oxidative damage in patients with established ARDS by examining the relationship between linoleic acid, the major polyunsaturated fatty acid of plasma, and the formation of a specific and sensitive marker of lipid peroxidation namely 4-hydroxy-2-nonenal. Using the technique of gas chromatography-mass spectrometry we show that ARDS patients have elevated plasma levels of 4-hydroxy-2-nonenel compared to normal healthy controls, and that falls in total plasma linoleic acid often parallel increases in 4-hydroxy-2-nonenal.

MATERIALS AND METHODS

Linoleic acid, fatty acid methyl ester standards (FAMES) and butylated hydroxytoluene (BHT) were obtained from the Sigma Chemical Company (Poole, Dorset). Nonanoic acid (98% pure) was obtained from the Aldrich Chemical Company (Gillingham, Dorset). 4-hydroxy-2-nonenal-diethylacetal (HNE-DEA) and 4-hydroxy-2-nonenal (HNE) were generous gifts from Professor H Esterbauer and Dr Kevin Cheeseman. All other chemicals were of the highest purity available from Fisons Instrumental Ltd (Loughborough, Leicester).

EXTRACTION AND DERIVATISATION OF PLASMA FATTY ACID METHYL ESTERS

The procedure used here has been described in detail elsewhere.[6] Briefly a modified Folch extraction was used; 0.75 ml of plasma was extracted for two (1 hour) periods firstly in (2:1) methanol-chloroform and then in (2:1:0.8) methanol-chloroform-water. The two supernatants produced after centrifuging were combined and further extracted in chloroform-water (1:1). The lower chloroform layer was collected and evaporated to dryness under a stream of oxygen-free nitrogen. The lipids within the residue were suspended in methanol-toluene 1:1 and 12.5 μl of concentrated sulfuric acid, and allowed to saponify and methylate for 15 hrs at 55°C under nitrogen. The FAMES produced were then extracted twice in 4:1 hexane-chloroform, containing saturated sodium chloride solution, washed in 0.33M sodium hydroxide, evaporated to dryness, and resuspended in dry acetone ready for gas chromatography-mass spectrometry (GC-MS). Samples were stored at -20°C for analysis.

Throughout the above procedures, samples were held at 4°C in oxygen free nitrogen. The antioxidant BHT was also added to prevent further peroxidation during processing.

FORMATION OF HNE FROM HNE-DEA

Hydroxy-2-nonenal was formed from the diethylacetal using the following method recommended by Professor Esterbauer (personal communication). Briefly, 0.5 ml of HNE-DEA 46 mg/ml in chloroform was evaporated to dryness under a stream of oxygen-free nitrogen at room temperature. To this was added 5.0 ml of 10^{-3} M hydrochloric acid, and the mixture shaken periodically for 1 hour until saponification was complete. The concentration of HNE was determined from uv spectra and found to be 20 mM. The HNE solution was extracted twice with 1 ml of chloroform[1] and the two chloroform extracts were combined and evaporated to dryness as before. The residue was subjected to the same derivatisation procedures as used for fatty acids described above. The resulting stock solution of HNE in 200 μl of acetone was stored at $-20°C$ under nitrogen for use. An internal standard solution of nonanoic acid methyl ester was also produced using the same derivatisation procedure.

PREPARATION OF HNE STANDARDS

From the stock solution five standards of 1.0, 0.75, 0.5, 0.2 and 0.1 nmol/ml HNE were prepared in acetone, each containing a nonanoic acid methyl ester internal standard equivalent to 0.01 μl/ml. Standards were stored at $-20°C$ under nitrogen prior to analysis.

LINOLEIC ACID PEROXIDATION AND ITS MEASUREMENT AS TBA-REACTIVITY AND BY GC-MS ANALYSIS

50 μl of pure linoleic acid, in clean glass tubes, were left open to the atmosphere at room temperature (25°C) for various times over a nine day period. Peroxidation was halted by adding 20 μl of BHT (1 mg/ml in methanol) and degassing for 1 min with a stream of oxygen-free nitrogen. Samples were capped and stored at $-20°C$. The stored samples were defrosted and suspended in 0.5 ml of methanol. 100 μl of each was then added to 0.5 ml of thiobarbituric aid (TBA) (1% w/v in 50 mM NaOH), and 0.5 ml of 25% v/v HCl, and heated at 100°C for 15 minutes. When cool, the chromogen was extracted into 3 ml of butan-1-ol, and measured at A532.

The remainder of each sample (in methanol) was combined with 0.5 ml of toluene and 12.5 μl of concentrated H_2SO_4, degassed, capped and treated at 55°C for 15 hours. When cool, 100 μl of internal standard was added (palmitic acid methyl ester 4 mg/ml in methanol). Further processing of samples for GC-MS fatty acid analysis was carried out as previously described.[6]

GC-MS OF TOTAL PLASMA FATTY ACID METHYL ESTERS AND HNE

2 μl of FAMES in dry acetone were injected into a Hewlett Packard 5890 series II gas chromatograph housing a WCOT fused silica 50 m C 0.25 mm ID CP SIL 88 capillary column (chrompack), using a splitless injection technique. The separation conditions used have been described in detail elsewhere.[6] A Fisons VG Trio 1000 mass spectrometer was used as a detector in the electron impact mode. FAMES were

detected as total ion chromatograms for masses between 50–650 amu. Peak assignments were based on retention times of known standards and characteristic fragmentation patterns.

HNE, although only present in plasma samples in low amounts, could nevertheless be detected using the sensitive technique of selective ion monitoring (SIM). Three characteristic masses were selected from the HNE standard total ion chromatogram (m/z 81, m/z 85, m/z 138) and monitored at the expected retention times. Identification was aided by the relative intensity of these ions.

QUANTITATION OF HNE IN PLASMA

Nonanoic acid was added to 0.75 ml of plasma, as an internal standard to give a final concentration of 0.1 μl/ml. Extraction and derivatisation procedures were carried out as previously described in this paper. 2 μl of sample was injected for GC-MS analysis and data acquired in the SIM mode. HNE peak area was measured from the three characteristic ions. Peak areas were corrected using selected ions (m/z 74, m/z 87, m/z 129) from the internal standard. Values were calculated from a standard curve r0.981, $p = 4.85 \times 10^{-4}$ over the range of 0.1 to 1.0 μg/ml HNE and expressed in nmol/ml of plasma.

Samples were analysed on each patient at approximately equal time points throughout their stay in intensive care. Each sample was analysed in triplicate to give a mean value $\pm$ SEM.

PATIENT POPULATION

Twelve patients with established ARDS were recruited into this study from a variety of different predisposing clinical backgrounds. The diagnostic criteria for ARDS in our Intensive Care Unit were as previously described.[2,6] Ethical Committee approval is not required in our Institution for studies on small volumes of blood taken for routine diagnostic purposes.

Blood samples were taken from the indwelling radial artery catheter at daily intervals where possible, and collected into lithium heparin blood bottles for immediate separation. Plasma was stored at $-20°C$ until the time of analysis and no longer than 4 weeks from collection of the first sample. Venous blood from twelve normal healthy controls (age range 19–36 mean 32 years) was separated and stored under the same conditions.

RESULTS

Using the GC-MS techniques described here it was possible to separate and quantitate 4-hydroxy-2-nonenal in plasma under the same analytical conditions used to separate and identify total plasma fatty acid methyl esters. When a pure standard of 4-hydroxy-2-nonenal was treated under these conditions we obtained the GC-MS total ion chromatogram (TIC) shown in Figure 1. The mass spectrum of the main peak assigned as 4-hydroxy-2-nonenal is also shown in Figure 2. The mass spectrum obtained for HNE suggests that it remains underivatised. The molecular ion (M^+) of m/z 156 (156.22) can be seen as well as the (MH^+) ion m/z 157 (Figure 1). No

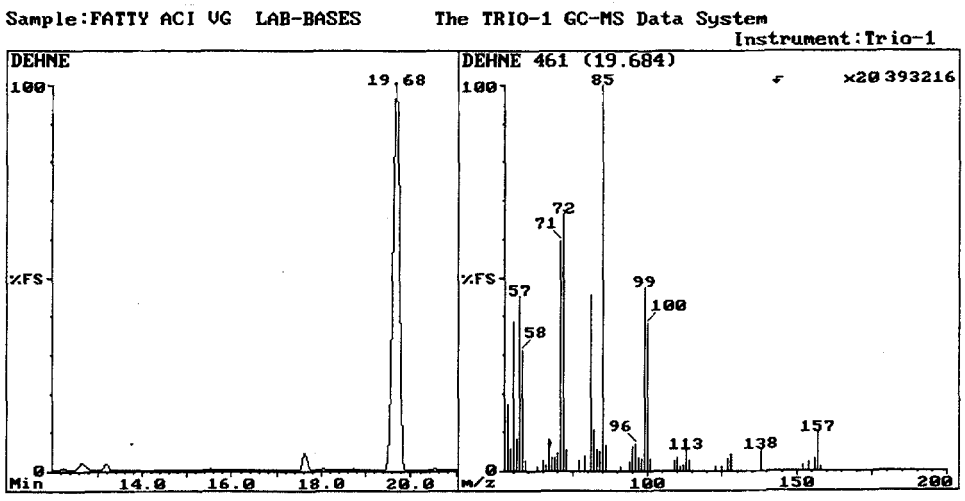

FIGURE 1 Figure 1 shows the total ion chromatogram for the HNE standard solution together with the mass spectrum obtained.

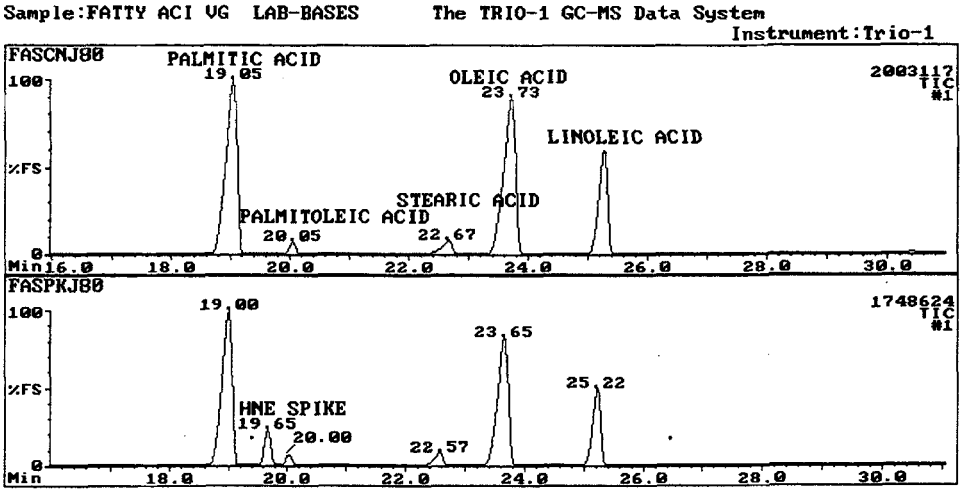

FIGURE 2 Figure 2 is representative of a total ion chromatogram of plasma total fatty acid methyl esters, from an ARDS patient, before and after spiking with HNE.

ions of higher mass which could be ascribed to a methyl derivative could be found. The m/z 138 ion corresponds to HNE $-H_2O$ ($M^+ - 18$) and the longer than expected retention time of HNE is because it is less volatile in this state. The internal standard and all other fatty acids could be identified from mass spectra as methyl esters. Total ion chromatograms of total plasma FAMES from an ARDS patient both before and after spiking the plasma sample with 4-hydroxy-2-nonenal are shown in Figure 2. The level of HNE in normal plasma is extremely low, and so the sensitive technique of selective ion monitoring (SIM) was applied. Three

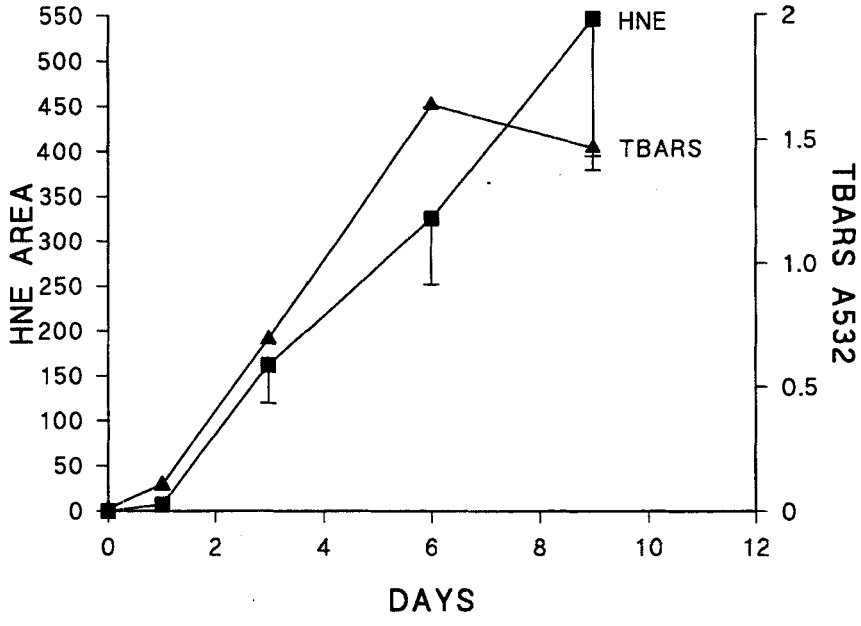

FIGURE 3 Figure 3 showed the time-dependent increase in HNE (peak area) formed from linoleic acid oxidised in air, together with the formation of thiobarbituric acid-reactive substances (TBARS) measured at the same time points. Means of 3 separate analyses and standard deviations are shown.

characteristic ions were selected from the mass spectrum of the total ion chromatogram of HNE (see Figure 1), and data acquired at the expected retention times. Chromatograms obtained by selective ion monitoring of total plasma fatty acid patterns also showed the expected HNE peak. The mass spectra showed the selected ions at the correct relative intensities, which aided identification.

Peroxidation of bulk linoleic acid in air over a nine day period showed increasing formation of HNE (selective ion monitoring) and this accompanied an increasing formation of thiobarbituric acid-reactive substances (Figure 3).

Percentage changes in total plasma linoleic acid values (as methyl esters) of ARDS patients were determined from total ion chromatograms, using palmitic acid methyl ester as an internal reference standard. 4-Hydroxy-2-nonenal was measured in the same samples from peak areas using SIM, the molecular ion of palmitic acid methyl ester (m/z 270) was used as an internal standard. ARDS patients showed, in many cases, an inverse correlation between loss of total plasma linoleic acid and the formation of HNE (Table 1, figures 4, 5). Quantitation of HNE present in plasma of ARDS patients was performed using selective ion monitoring, a standard curve of HNE and an internal standard of nonanoic acid methyl ester. This technique showed there to be significantly increased amounts of HNE (0.412 ± 0.023 nmol/ml $p < 0.0001$) in ARDS plasma compared to normal healthy control plasma (0.205 ± 0.018 nmol/ml). These normal values were in close agreement with previously published result.[14,18] In addition, non-surviving ARDS patients had higher levels (0.447 ± 0.027 nmol/ml) compared to surviving ARDS patients (0.365 ± 0.041 nmol/ml) (Table 1). Our results may be an underestimate of the true value since

TABLE 1
Relationships between plasma total linoleic acid and 4-hydroxy-2-nonenal (HNE) in ARDS patients

Sampling of ARDS plasma in AICU Patient Code	HNE nmol/ml Early	Intermediate	Late	Outcome	Relationship between peak areas of HNE and linoleic acid Correlation (r)	Significance (p)	Number of samples
A	0.482 ± 0.029	0.169 ± 0.009	0.157 ± 0.004	S	−0.794	0.010	9
B				S	−0.740	0.095	7
C	0.792 ± 0.028	0.823 ± 0.017	0.174 ± 0.002	S	−0.872	0.004	8
D				S	−0.757	0.018	9
E				S	−0.867	0.011	6
F	0.157 ± 0.007	0.260 ± 0.013	0.293 ± 0.011	S			
J	0.438 ± 0.034	0.681 ± 0.027	0.137 ± 0.037	N/S	−0.902	0.001	9
K				N/S	−0.260	0.530	9
L	0.205 ± 0.006	0.130 ± 0.007	0.137 ± 0.002	N/S	−0.170	0.680	8
M	0.416 ± 0.022	0.360 ± 0.054	0.557 ± 0.020	N/S			
N	0.603 ± 0.043	0.616 ± 0.086	0.545 ± 0.084	N/S	−0.065	0.860	9
O				N/S	−0.623	0.072	9

HNE values shown are the mean ± SEM of three separate analyses. S = survivors, N/S non-survivors. This study was initially concerned with the total ion chromatogram for linoleic acid as % changes, and its relationship to change in peak area of HNE (measured as a selective ion chromatogram). In both cases palmitic acid was used as an internal standard. Where HNE values are given, these were obtained by selective ion monitoring with reference to pure HNE standard curve and an internal standard of nonanoic acid. Where no values are given suitable samples were not available for analysis.

AICU = Adult Intensive Care Unit. S = survivor, N/S = non-survivor of ARDS.

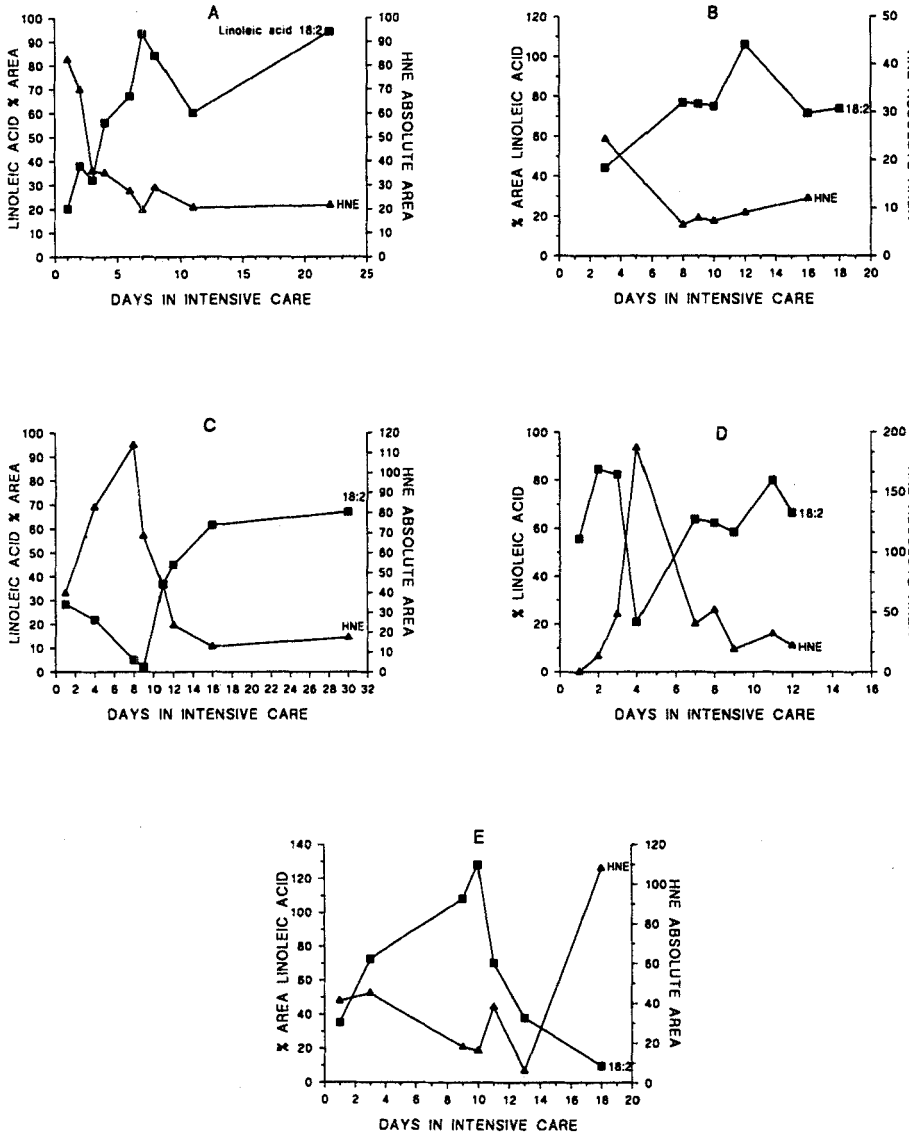

FIGURE 4

others have shown that deuterated HNE is not completely recovered from plasma.[19] It is likely that some HNE remains bound to protein thiol groups, but since percentage loss of HNE remains constant[19] differences found are valid.

STATISTICAL ANALYSIS

Data are expressed in the text and tables as mean ± SEM. Linear regression analysis

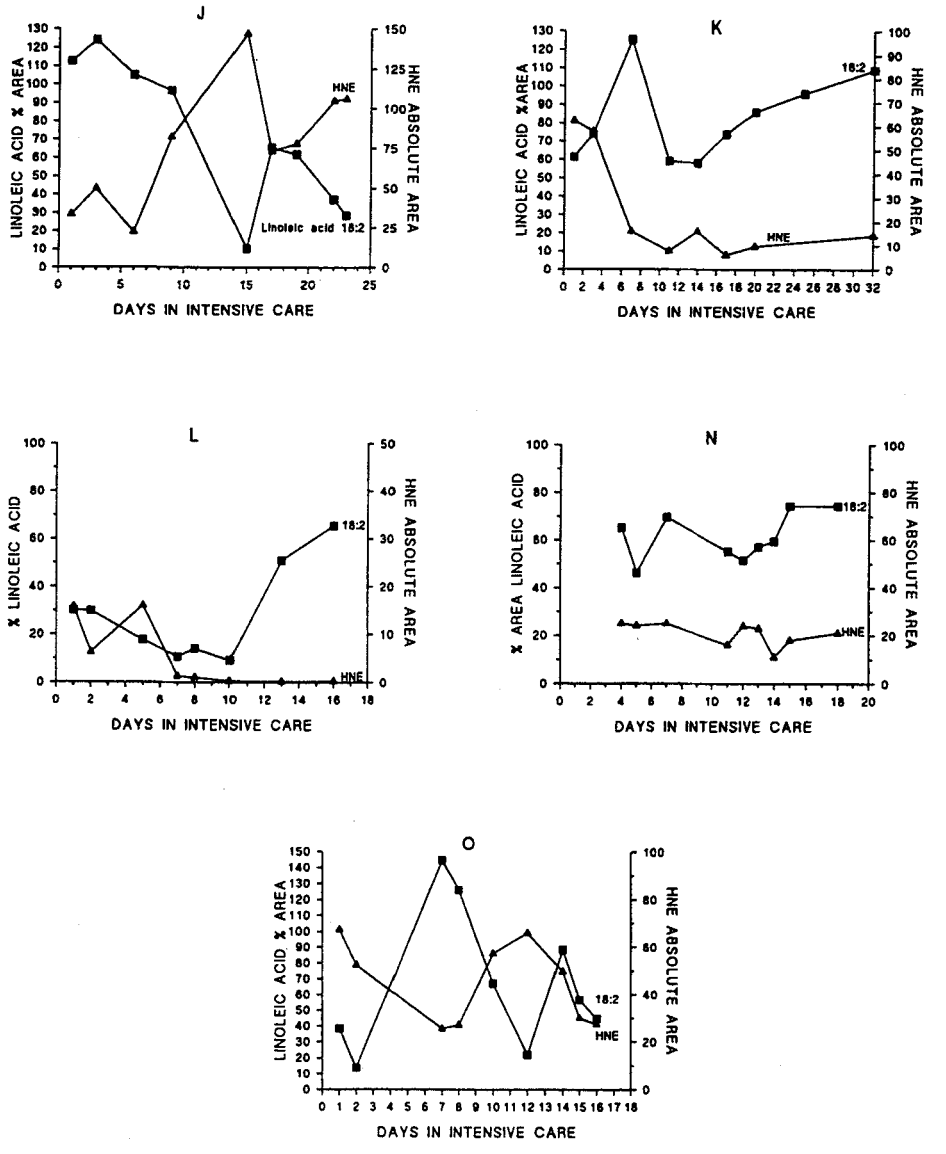

FIGURE 4/5 Figures 4 and 5 display the relationship between changes in plasma linoleic acid and HNE of ARDS patients over a period of time. Figure 4 shows survivors, and figure 5 non-survivors of the disease.

was used to assess the correlation between variables. The unpaired Student's test was used to analyse data with equal variances and the Welch alternative *t* test used when variances were significantly different. 'P' values less than or equal to 0.05 were considered statistically significant.

DISCUSSION

Adult patients with the acute lung injury syndrome ARDS are under severe oxidative stress from the disease process, and from treatment with high concentrations of inspired oxygen. Oxidative stress leads to molecular damage, and the purpose of our recent studies to assess molecular damage in plasma[2,6,10] has been to identify ARDS patients at greatest risk. Previous studies[8] including our own[6] have failed to provide convincing evidence of oxidative damage to plasma lipids in ARDS patients based on non-specific assay procedures such as the TBA test, although others have reported significant increases.[7] We have, however, observed significant decreases in total plasma linoleic acid levels[6] during disease progression, and these changes often closely follow evidence of oxidative protein damage.

Damage to the plasma proteins of ARDS patients is seen as a loss of thiol groups and an increase in carbonyl residues,[2] with significantly different patterns between survivors and non-survivors. Linoleic acid is the major polyunsaturated fatty acid of human plasma, and quantitatively therefore the most susceptible to oxidative damage. Esterbauer and his co-workers have shown that polyunsaturated fatty acids yield 4-hydroxy-2-nonenal (HNE) as a peroxidation product[11] especially when iron is present.[12] Mechanistic studies have confirmed the essentiality of n-6 fatty acids and iron catalysis for the formation of HNE.[13] in agreement with previous studies the peroxidation of pure linoleic acid in air at room temperature yielded a time-dependent formation of thiobarbituric acid-reactive material, and a concomitant formation of HNE.[12] The latter served as a control for GC-MS monitoring. In an attempt to better understand our previous findings of a close relationship between a falling plasma linoleic acid and falling protein thiol values in ARDS patients, we have monitored the appearance of HNE using the specific and sensitive technique of gas chromatography-mass spectrometry with selective ion monitoring. Patients with established ARDS showed significantly higher levels of HNE in their plasma compared with normal healthy controls ($p < 0.0001$). Non-survivors of the disease appeared to have higher levels than survivors although the difference was not quite statistically significant ($p = 0.0853$). In 7 out of 10 of the ARDS patients studied there was an inverse correlation between loss of linoleic acid and the formation of HNE.

4-Hydroxy-2-nonenal is considered to be a major toxic aldehydic product of lipid peroxidation (reviewed in 14, 15) directly involved in some of the adverse effects of oxidative stress leading to molecular damage. Concentrations of HNE in the millimolar range have been observed to be acutely toxic to cells, depleting glutathione and protein thiols, disturbing calcium homeostasis, and inhibiting DNA, RNA and protein synthesis, respiration and glycolysis (reviewed in 14, 15). At lower concentrations approaching the physiological range ($< 0.1\ \mu M$) HNE has been shown to be chemotactic and to modulate adenylate cyclase, guanylate cyclase and phospholipase C activities (reviewed in 14, 16). The ability of HNE to decrease protein thiols[16] may provide a link in our ARDS patients between loss of protein thiols, loss of linoicic acid, and a rise in HNE. It should be noted, however, that HNE is only one minor component of a large number of aldehydes produced during the peroxidation of polyunsaturated fatty acids, many of which will react with protein sulphydryl groups. Although not free radicals, aldehydes, as products of free radical damage to lipids and proteins, have considerable chemical reactivity, acting as messenger and signal molecules with the potential to communicate tissue damage

to other sites of the body.[17] Molecules such as these may contribute to multiorgan damage characteristic of disease progression in ARDS patients.

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