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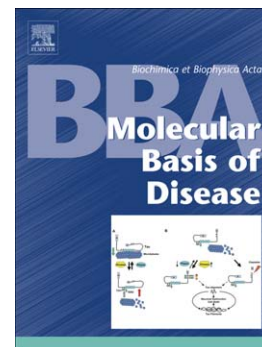
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Oxidative Damage of Albumin in Advanced Liver Disease

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Abstract

Albumin has a number of biological functions and the serum albumin level is related to prognosis in advanced liver disease. Oxidative stress is believed to play an important role in the pathogenesis of liver failure. The aim of the present study was to characterize oxidative modification of albumin in patients with various degrees of liver failure and to investigate implications for its binding function. Patients with liver cirrhosis (n=10), acute-on-chronic liver failure (n=8) and healthy controls (n=15) were included in the study. Three fractions of albumin were separated by HPLC according to the redox state of cysteine-34 and detected by fluorescence as well as UV absorption. Carbonyl groups were measured as a marker of oxidative modification in plasma proteins and, by western blotting, on albumin. Progressive oxidative modification of albumin was found with increasing severity of liver failure indicated by an increased content of carbonyl groups and oxidation of cysteine-34. Fluorescence properties of albumin were altered by oxidation and, in patients with acute-on-chronic liver failure, by high plasma levels of bilirubin. This alteration of albumin fluorescence by bilirubin provides evidence for a preferred binding of bilirubin to the fully reduced form of albumin.

Introduction

Albumin, a 66.5 kDa protein, is quantitatively the most important plasma protein [1]. It is the main determinant of plasma oncotic pressure and exhibits many other biological functions, such as transport of endogenous and exogenous compounds, modulation of capillary permeability, neutrophil adhesion and activation, hemostasis and free radical scavenging (for review see [2]). The serum albumin level is an important prognostic factor in advanced liver disease [3]. Its main therapeutical uses include maintenance of intravascular volume, prevention of circulatory dysfunction and hepatorenal syndrome and, more recently in extracorporeal liver support, removal of potentially toxic substances accumulating in liver failure (for review see [4]).

Albumin is the major extracellular source of reduced sulfhydryl groups, which are potent scavengers of reactive oxygen and nitrogen species [5]. Depending on the redox state, there are three major species of albumin: mercaptalbumin (HMA) with a free thiol group on cysteine-34, nonmercaptalbumin1 (HNA1) with cysteine, homocysteine or glutathione bound by a disulfide bond and nonmercaptalbumin2 (HNA2) with cysteine oxidized to sulfenic or sulfonic acid [6, 7]. HMA and HNA1 are in a dynamic exchange with small thiol-containing compounds and disulfides in the blood [8]. The sulfenic acid state was described as a reactive intermediate in the formation of HNA1 [9, 10]. Protein sulfenic and sulfonic acids have long been considered to be irreversibly oxidized but recently reversible oxidation/reduction of these species in redox regulation has been described [11, 12].

Oxidative stress is believed to play a major role in the pathogenesis of acute-on-chronic liver failure (ACLF) [13]. Sogami et al. described decreased relative amounts of HMA in patients with various liver diseases as compared to healthy controls [14]. This was

confirmed by Watanabe et al., who also reported an increase of oxidized albumin with severity of liver disease [15]. Oxidized albumin shows altered binding capacities for several substances used to assess albumin function: Decreased bilirubin binding was reported for in vitro oxidized albumin [16] and decreased binding of dansylsarcosine was found in plasma of patients with endstage liver disease [17, 18]. We recently reviewed the relation of oxidative modification of albumin to its binding properties [19]. However, the pathophysiological impact and the clinical implications of albumin modification in liver disease have not yet been studied in detail and no data exist on the redox state of albumin in ACLF, a condition with liver failure as well as secondary end-organ failure associated with poor prognosis.

The aim of the present study was to investigate oxidative modification of albumin and its relation to physiological properties of albumin in patients with advanced liver disease.

Materials and Methods

Patients

Eighteen patients with liver cirrhosis admitted to the Department of Internal Medicine, Medical University of Graz, between March 2003 and May 2005 were enrolled in this study. The study population comprised 8 consecutive patients with ACLF being evaluated for extracorporeal liver support as well as 10 patients with liver cirrhosis (LC) who were candidates for liver transplantation. ACLF was defined as acute deterioration of liver function over a period of 2-4 weeks, associated with a precipitating event, with jaundice and either hepatic encephalopathy or hepatorenal syndrome, and with a high SOFA (Sepsis-related Organ Failure Assessment) or APACHE II (Acute Physiology and Chronic Health Evaluation II) score [20]. The study protocol was approved by the Ethics Committee of the Medical University of Graz and informed consent was obtained in accordance with the Declaration of Helsinki. Blood samples were immediately centrifuged at 4°C and plasma aliquots were stored at -70°C until batch analysis. Bilirubin, albumin, creatinine, prothrombin time (international normalized ratio, INR), and C-reactive protein (CRP) were routinely assessed. To estimate severity of liver disease, Child-Pugh score and the model for end-stage liver disease (MELD) were calculated [3, 21]. Plasma samples from 15 age and sex-matched healthy blood donors were used as controls.

Albumin analysis

Albumin was fractionated by high performance liquid chromatography to give three peaks according to cysteine-34 in the free sulfhydryl form (HMA), as a mixed disulfide (HNA1) or in a higher oxidation state (HNA2) as previously described [22]. Plasma samples were diluted 1:100 with 0.1 M sodium phosphate, 0.3 M sodium chloride, pH 6.87, filtered

through a Whatman 0.45 μm nylon filter (Bartelt Labor- & Datentechnik, Graz, Austria). Thereafter, 20 μL were injected into the HPLC system. Separation was performed using a Shodex Asahipak ES-502N 7C anion exchange column (7.5 x 100 mm, Bartelt Labor- & Datentechnik, Graz, Austria) with 50 mM sodium acetate, 400 mM sodium sulfate, pH 4.85 as mobile phase. For elution a gradient of 0 to 6 % ethanol and a flow rate of 1 ml/min applied by a FLUX Rheos 4000 gradient pump (Spectronex, Vienna, Austria) were used. The column was kept at 35°C. Detection was carried out by fluorescence at 280/340 nm and UV absorption at 280 nm in series with a Jasco 821FP fluorescence detector (Spectronex, Vienna, Austria) and a Waters 2487 UV/VIS detector (Waters, Vienna, Austria), respectively. Quantification was based on peak heights determined by EZ Chrom Elite chromatography software (VWR, Vienna, Austria). The fluorescence/UV absorption ratio was obtained as the ratio of the peak heights from the fluorescence and UV chromatograms. A commercial 20% albumin solution obtained from Behring GmbH (Vienna, Austria) was used as a control.

Quantitative carbonyl protein determination

Quantification of carbonyl groups using an ELISA assay was carried out as described [23, 24] with slight modifications. In brief, oxidized human serum albumin was used as standard. Oxidized albumin standards were prepared from a commercial 20% albumin solution (Behring, Vienna, Austria) as described in [23] using iron ions/ascorbic acid as oxidizing agents after removing the stabilizer by intense dialysis. Standards and samples were diluted to give a final protein concentration of 4 mg/ml. For protein derivatization, 45 μL dinitrophenylhydrazine solution (10 mM dinitrophenylhydrazine in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5) were mixed with 15 μL sample and incubated for 45 minutes, vortexing every 15 minutes. Afterwards, 5 μL of each solution were added to 995 μL PBS (phosphate buffered saline, 10 mM, pH 7.4).

Duplicate 200 μ L aliquots were added to wells of a Nunc Immuno Plate Maxisorb. Plates were incubated over night at 4°C and then washed 4 times with 300 μ L PBS containing 0.05 % Tween20 using the ELx405 microplate washer (Bio-Tek, Vermont, USA). Then, 200 μ L PBS containing 0.2% I-Block and anti-dinitrophenylhydrazine-horseradish peroxidase antibody (1:1000) were added to each well and incubated at 37°C for one hour. After an additional washing step, 200 μ L super signal ELISA maximum sensitivity substrate (Pierce, Rockford, USA) were added to each well. Light emission was measured on a chemiluminescence reader (Lumistar, BMG, Germany).

Carbonyl protein detection by Western blot

Protein derivatization was carried out as described for ELISA. Excess guanidine hydrochloride was eliminated by Ultrafree Centrifugal Filter (Millipore, Billerica, USA). Subsequently, protein content was determined by BCA protein assay (Pierce, Rockford, USA) and 10 μ g were separated electrophoretically on a 5-12.5 % polyacrylamide gel. Two gels were run simultaneously using the Mini-Protean 3 system (BioRad, Hercules, USA). One gel was stained with PhastGel Blue R (Pharmacia, Uppsala, Sweden) and the other one was transferred on a Immuno Blot PVDF membrane. The electrophoretical protein transfer was carried out using the Trans-Blot SD semi-dry electrophoretic transfer system (BioRad, Hercules, USA). Afterwards, unspecific binding sites were blocked for 3 hours in 40ml PBS containing 0.2 % casein. Thereafter, the membrane was incubated for one hour with 40 ml PBS containing 0.2 % casein and anti-dinitrophenylhydrazine-horseradish peroxidase antibody (1:4000). Three washing steps followed, each 10 minutes, with 40 ml PBS containing 0.05 % Tween20. Afterwards, the membrane was developed with super signal western maximum sensitivity substrate (Pierce, Rockford, USA) and the PVDF membrane was scanned using the AlphaImager Imaging system (Alpha Innotech Corporation, San Leandro, USA).

Statistics

Results are given as mean \pm SD unless indicated otherwise. Groups were compared with Student's t-test. Pearson's correlation coefficient was used to assess relationships between variables.

Results

The characteristics of the study population are shown in Table 1. The severity of the disease is indicated by the MELD (model for end-stage liver disease) and the Child-Pugh score. As expected, the plasma levels of albumin were significantly decreased in patients with severe liver disease while the bilirubin levels were increased. The content of carbonyl groups in plasma proteins was slightly increased in LC ($0.22 \pm 0.08 \mu\text{mol/g}$ protein, $p < 0.05$) but markedly increased in ACLF ($0.68 \pm 0.29 \mu\text{mol/g}$ protein, $p < 0.05$) compared to healthy controls ($0.18 \pm 0.04 \mu\text{mol/g}$ protein). Using western blot to investigate the appearance of carbonyl groups in albumin, we could show that albumin is a target of oxidation in plasma and that albumin of patients with ACLF has a severely increased content of carbonyl groups. (Fig. 1). On densitometric analysis ($n=6$ per group), the band density was significantly increased in ACLF vs. LC and controls ($p < 0.05$) while the slight increase in LC patients vs. controls was not significant (Fig. 1).

We routinely determine the distribution of albumin to HMA, HNA1 and HNA2 by HPLC with fluorescence detection. However, using samples from ACLF patients we found that the results obtained by fluorescence detection differ considerably from those obtained by UV detection (Fig. 2c). In a commercial albumin solution UV and fluorescence detection gave the same results (Fig. 2d). This is also true for samples from healthy subjects (Fig. 2a). The distribution of albumin species in patients and controls obtained by HPLC with UV detection are presented in table 2. While HMA was significantly decreased, both oxidized species were significantly increased in LC and ACLF patients.

We further investigated the relation of the fluorescence intensity to UV absorbance in the chromatograms of the three albumin species derived from controls, LC and ACLF patients (Fig. 3). In control samples HMA and HNA1 gave the same ratio of fluorescence/UV absorbance. In HNA2, however, the ratio dropped to 67 % of the value

for HMA ($p < 0.001$), indicating decreased fluorescence intensity in relation to UV absorbance. In LC samples HNA1 had a slightly higher ($p < 0.001$) and HNA2 a lower ($p < 0.05$) signal ratio compared to HMA. In ACLF samples these differences were even more pronounced. Comparing the same albumin species of different patient groups we found that HNA2 shows the same low signal ratio in both patient groups and controls. For HNA1, values in ACLF samples were decreased by 26% ($p < 0.001$) while LC samples were not different from controls. For HMA, LC samples showed a signal ratio which was about 10 % lower than that of controls ($p < 0.001$) and ACLF samples had a fluorescence intensity /UV absorbance ratio of only 36 % compared to control samples ($p < 0.001$).

The ratio of fluorescence intensity/UV absorbance decreased with increasing bilirubin concentrations (Fig.4). When extrapolating the fluorescence intensity/UV absorbance ratio to a bilirubin level of 0, HMA and HNA1 gave similar numbers (268 and 278, respectively), but the value for HNA2 was distinctly lower (196). The correlation coefficients for the ratio of fluorescence intensity/UV absorbance vs. plasma bilirubin were 0.97 ($p < 0.0001$) in HMA, 0.91 ($p < 0.0001$) in HNA1 and 0.41 ($p < 0.05$) in HNA2.

Correlation analysis revealed that the percentages of HMA, HNA1 and HNA2 as well as the carbonyl content were significantly related to bilirubin ($p < 0.05$). In addition, HNA2 and carbonyl content correlated with MELD, Child-Pugh score and prothrombin time (international normalized ratio). For creatinine no significant correlation was found.

Discussion

Structure and function of albumin are impaired in advanced liver disease by different mechanisms: Plasma levels are decreased due to reduced synthesis, albumin is oxidatively modified, and binding sites are occupied by high levels of bilirubin. We herein demonstrate that, in advanced liver disease, severe oxidative damage of albumin is associated with decreased binding of bilirubin.

Carbonyl groups in proteins are a well established marker of oxidative stress [25, 26]. The carbonyl content of plasma proteins was significantly increased in our ACLF patients. Which of the plasma proteins is the most sensitive to oxidative modification has so far been a matter of debate [27, 28]. However, we could show by western blot analyses that albumin is a target of carbonylation. The extent of oxidative modification depends on the severity of the disease. In patients with LC, albumin fractions were significantly shifted from HMA to HNA1 and HNA2, while the increase of carbonyl groups was about 20 % and not significant. In patients with ACLF the percentage of HNA1 was further increased, the percentage of HNA2 was increased up to fourfold compared to controls and carbonyl groups were about three times the control value.

As binding and transport of different compounds is an important function of albumin, oxidative damage of albumin is of high clinical relevance. However, binding data of albumin from patients with severe liver disease are scarce and have to be interpreted with caution mainly for two reasons: (i) isolation of albumin for binding experiments is laborious and may produce artifacts and (ii) binding studies with plasma from patients with severe liver dysfunction are hampered by the presence of high levels of other ligands such as bilirubin that make it impossible to distinguish between altered binding due to differences in albumin structure and effects due to the presence of these ligands. Increases

of bilirubin levels may be hundredfold in ACLF patients. In healthy subjects plasma contains about one mole bilirubin per 100 moles albumin. In contrast, in ACLF patients the molar ratio of bilirubin to albumin ranges around 1:1 as estimated from their mean plasma levels observed in our study.

We found differences in the relation of fluorescence intensity to UV absorption in HMA, HNA1 and HNA2. The reason for impaired fluorescence may be two-fold: First, oxidative damage of albumin was shown to reduce its fluorescence intensity [29]. Second, reduced fluorescence intensity may be due to quenching by various other compounds accumulating in plasma of patients with liver failure, including bilirubin [26]. In ACLF patients both factors are present: (i) albumin is severely oxidized as monitored by the percentage of HNA2 and carbonyl groups and (ii) the levels of bilirubin are extremely high. The fluorescence/UV absorbance ratio of the highly oxidized HNA2 is essentially the same in both patient groups and controls (Fig. 3) and hence independent from the bilirubin content of the plasma. Therefore the lower ratio of HNA2 compared to HMA cannot be due to a quenching effect of bilirubin but is most likely achieved by oxidative damage. HNA1 and HMA show similar ratios in controls and LC patients, but in ACLF patients the ratio is decreased for HNA1 and even more for HMA. HMA is a reduced form of albumin with respect to cysteine-34 and it may be expected that also the other domains of the molecule are less oxidatively damaged compared to HNA. Since ACLF patients showed the highest plasma concentrations of bilirubin, the severely decreased fluorescence of HMA from ACLF patients can be attributed to the high concentrations of the fluorescence quencher bilirubin rather than to oxidative damage. In ACLF patients HMA has the lowest fluorescence/UV absorption ratio compared to HNA1 and HNA2, indicating that fluorescence quenching by bilirubin mainly takes place in HMA. Thus we

conclude that oxidative damage impairs bilirubin binding of albumin and HMA has a higher affinity for bilirubin compared to HNA1 and HNA2.

This interpretation is supported by the correlation of the fluorescence/UV absorbance ratio to the bilirubin concentration (Fig. 4). Increasing bilirubin suppresses fluorescence only slightly in HNA1 and HNA2. The steeper slope for HMA indicates a preferred binding of bilirubin to the reduced form of albumin. In contrast, HNA2 shows a lower fluorescence intensity even in the extrapolated absence of bilirubin and the dependence of the ratio on bilirubin is much weaker. These data suggest an increasing bilirubin binding affinity in the order $\text{HNA2} < \text{HNA1} < \text{HMA}$. The decreased percentages of HMA as well as the lower total albumin concentrations in plasma of patients provide the pathophysiological basis for impaired albumin function in advanced liver disease.

The clinical relevance of albumin dysfunction in advanced liver disease is supported by studies showing reduction in severity of hepatic encephalopathy and improved survival of cirrhotic patients with spontaneous bacterial peritonitis and hepatorenal syndrome treated with albumin infusion [30-32]. Infusion of albumin [33] as well as albumin dialysis using the Molecular Adsorbents Recirculating System (MARS) [18, 34-37] have been shown to improve the circulatory dysfunction as evidenced by an increase in mean arterial pressure and systemic vascular resistance. This improvement in systemic hemodynamics might be due to a reduction in vasodilation following removal of nitric oxide and its metabolites [38] which results in deactivation of the neurohumoral systems and a decrease in plasma levels of renin, aldosterone, norepinephrine and vasopressin [33, 39, 40].

In summary, our findings indicate that albumin is oxidatively modified in patients with advanced liver disease depending on its severity. While oxidation is relatively mild in liver cirrhosis, severe modifications occur in ACLF. As bilirubin is preferentially bound by the fully reduced form of albumin, impaired binding of bilirubin and other ligands is likely to occur in liver cirrhosis and more so in ACLF.

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List of abbreviations

ACLF, acute-on-chronic liver failure; HMA, human mercaptalbumin; HNA1, human nonmercaptalbumin1; HNA2, human nonmercaptalbumin2; PBS, phosphate buffered saline; LC, liver cirrhosis.

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Figure Legends

Fig. 1. Representative Western blots of carbonyl groups in albumin (a). Plasma proteins were separated in duplicate gels and stained as described in the Methods section. A, protein stain; B, carbonyl stain. Blotted albumin was visualised after derivatization with dinitrophenylhydrazine. The integrated density of the carbonyl-stained albumin bands of 6 patients of each group were determined and the means \pm SD are given (b). LC, liver cirrhosis; ACLF, acute-on-chronic liver failure.

Fig. 2. Representative chromatograms. Albumin in plasma from a control subject (a), from a LC patient (b), from an ACLF patient (c) and from a commercial albumin solution (d) were separated by HPLC. Peaks indicated as 1, 2 and 3 represent HMA, HNA1 and HNA2, respectively. F and UV indicate fluorescence and ultraviolet detection, respectively.

Fig. 3. Ratio of fluorescence intensity/UV absorbance in albumin fractions after HPLC separation. Mean values \pm standard deviations are shown. Grey bars, controls; black bars liver cirrhosis, white bars, acute-on-chronic liver failure.

Fig. 4. Fluorescence of albumin species depends on the plasma bilirubin concentration. The ratio of fluorescence intensity/UV absorbance on HPLC is plotted against plasma bilirubin concentration. (●), HMA; (Δ), HNA1; (○), HNA2.

Table 1: Patient characteristics

	Age (y)	INR	Albumin (g/L)	Bilirubin (mg/L)	Creatinine mg/L	CRP (mg/L)	MELD ^a	Child- Pugh score
ACLF (n=8)	58±4	2.17±0.73 ^c	29.3±3.4 ^b	283±91 ^{b,c}	23±18 ^b	70±42 ^{b,c}	33±11 ^c	12±1 ^c
LC (n=10)	57±6	1.3 ±0.11	32.4±6.9 ^b	23±16 ^b	12±7 ^b	13±18 ^b	13±4	9±2
CONTROL (n=15)	55±5	n.a.	47.6±2.4	6±3	8±1	2±2	n.a.	n.a.

INR, international normalized ratio; CRP, C-reactive protein. n.a., not available.

^a MELD score was obtained using the MELD calculator at the Mayo Clinic website (<http://www.mayoclinic.org/gi-rst/mayomodel5.html>);

^b p<0.05 vs. controls, ^c p<0.05 vs. LC

Table 2: Percentages of albumin species in patients and controls obtained by HPLC with UV detection.

	fHMA %	fHNA1 %	fHNA2 %
ACLF	42.7±10.0 ^a	41.4±7.4 ^a	15.4±3.4 ^a
LC	53.3±5.3 ^a	39.6±6.1 ^a	7.5±2.4 ^a
CONTROL	65.1±4.2	30.8±4.0	4.1±1.1

a, p<0.05 compared to controls

fig 1

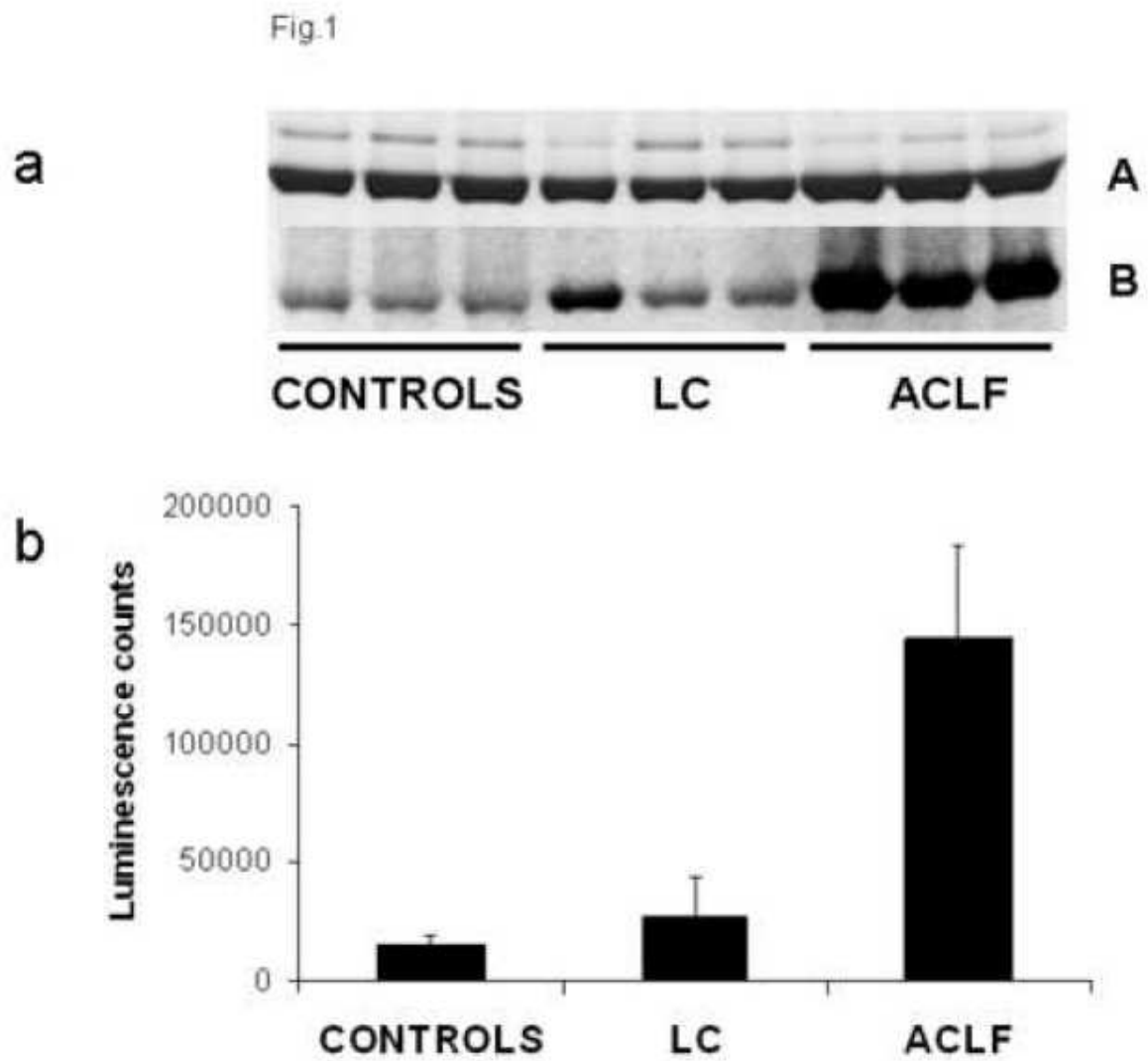


fig 2

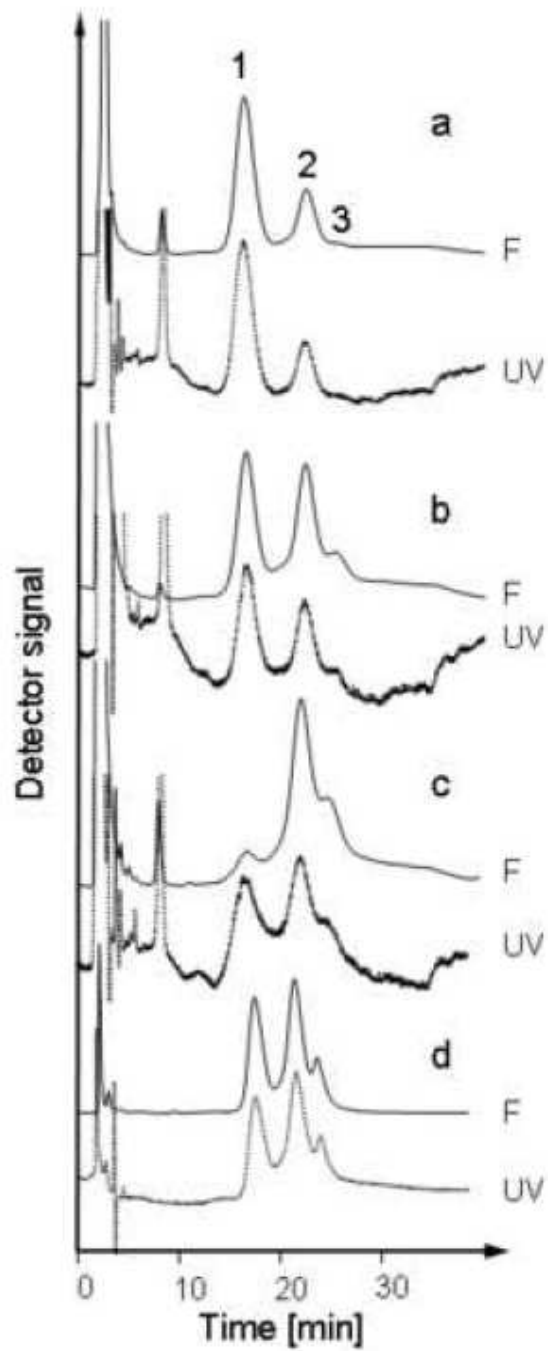


fig 3

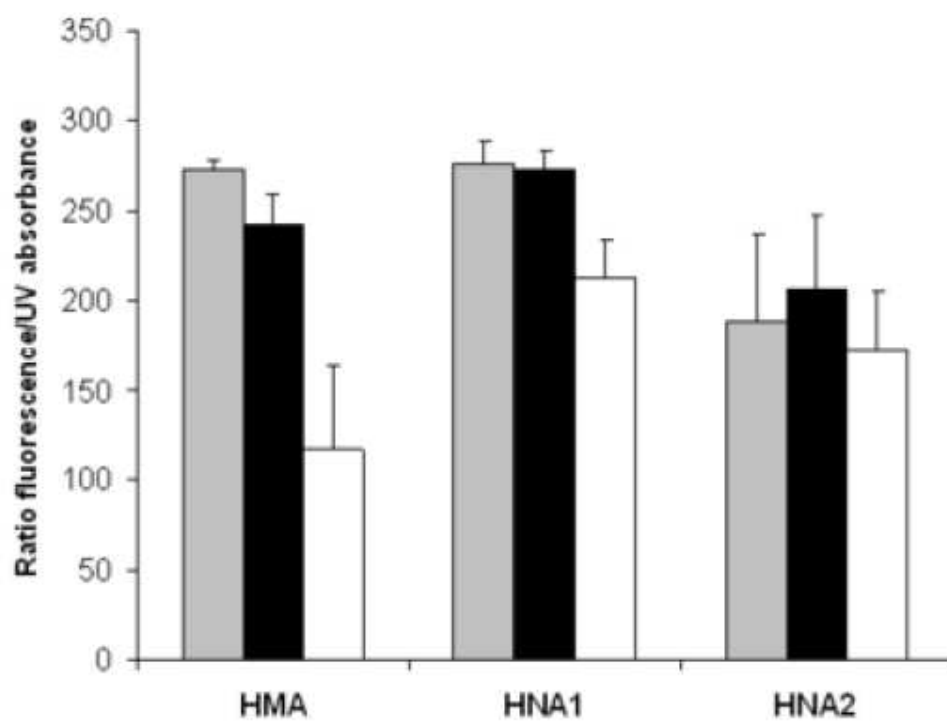


fig 4

