Fatty acids influence binding of cobalt to serum albumin in patients with fatty liver

G. Jayakumar Amirtharaj, Sathish Kumar Natarajan, Ashis Mukhopadhyya, Uday George Zachariah, Sudheer K. Hegde, George Kurian, K.A. Balasubramaniana, Anup Ramachandran

Department of Radiodiagnosis, Christian Medical College, Ida Scudder Road, Vellore-632004, India

Abstract

Human serum albumin binds ligands such as fatty acids and metals in circulation. Oxidative stress can modify albumin and affect ligand binding. This study examines the role of oxidative stress and fatty acids in modulating cobalt binding to albumin in patients with fatty liver. Elevated levels of malondialdehyde and protein carbonyls, indicative of oxidative stress were evident in serum of patients with fatty liver. A significant decrease in albumin–cobalt binding was also observed. Albumin isolated from patient serum also showed an increase in bound fatty acids. In vitro experiments indicated that while oxidant exposure or removal of fatty acids independently decreased cobalt binding to albumin, removal of fatty acids from the protein prior to oxidant exposure did not influence the oxidant effect on albumin–cobalt binding. These results suggest that oxidative stress and fatty acids on albumin can influence albumin–cobalt binding in patients with fatty liver by independent mechanisms.

1. Introduction

Human serum albumin (HSA) is synthesized in the liver and is the most abundant protein in blood. It has a single polypeptide chain of 585 amino acids (66,500 Da) with three domains — I, II and III [1]. Each domain consists of two sub domains A and B. The structure of albumin is stabilized by 17 disulphide bridges with a free thiol group at cysteine 34 which is prone to be oxidized [2]. Apart from maintaining colloid osmotic pressure, albumin also plays a role in transport of a number of ligands including hormones, hemin, bilirubin and fatty acids, as well as transition metals such as copper, cobalt, nickel, and cadmium [3,4]. Modifications on albumin have been demonstrated in numerous pathophysiological conditions such as acute coronary syndrome [5] and skeletal muscle ischemia [6]. These modifications can affect the protein’s ability to bind ligands, as seen in chronic renal and liver disease [7]. It has been demonstrated that the binding of albumin to cobalt is compromised in patients with myocardial infarction [8] and myocardial ischemia [4] and this forms the basis for the albumin–cobalt binding test (ACB) a marker for myocardial ischemia [4].

The binding of metals to albumin has been well studied, and four different metal binding sites are known: site A, site B, the N-terminal and cys-34 [9]. Among the metals, binding is best characterized for copper and nickel [3,10], which bind to the three N-terminal amino acid residues Asp-Ala-His. Cobalt is transported in circulation by albumin [3,4], similar to copper and nickel [3]. However, a recent study has demonstrated that cobalt also binds to sites A and B on the albumin molecule [4].

Another important ligand for albumin is fatty acids and HSA is the primary transporter for the delivery of fatty acids to tissues, having at least seven binding sites for this ligand [11]. Oxidative stress is one of the mechanisms by which modification of albumin can occur [7], and free radicals have been implicated in the etiology of liver disease, playing a role in progression from fatty liver to fibrosis and cirrhosis [12,13]. Since albumin is synthesized in the liver, and oxidative stress is known to be a feature of liver disease, we hypothesized that oxidative stress during fatty liver disease can cause modification on serum albumin, which may influence ligand binding properties of the protein. We tested this hypothesis by studying serum from patients with fatty liver and demonstrate that association of albumin with fatty acids is altered in an oxidative milieu and this can influence the cobalt binding capacity of the protein.

2. Methods

Tris (hydroxymethyl) aminomethane (Tris), purified human serum albumin (HSA), bromo cresol green, dithiothreitol (DTT), 2,4 dinitro phenyl hydrazine (DNPH), 1,1,3,3 tetrathoxy propane, catalase, xanthine and xanthine oxidase were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Cibachron Blue was from CIBA-GIEGY. All other chemicals and solvents used were of analytical grade.

2.1. Patients and control subjects

The study included 28 patients who were diagnosed with fatty liver by radiological findings, with high levels of alanine aminotransferase (ALT) and no history of alcohol consumption, diabetes mellitus or hyperlipoproteinaemia. Controls were 28 patients with irritable bowel syndrome and no liver involvement. The following blood investigations were done in all subjects — aspartate aminotransferase (AST), alanine aminotransferase...
(ALT), total bilirubin, alkaline phosphatase (ALP), albumin, total protein, total cholesterol, triacylglycerol, HbsAg and anti-HCV. The study was approved by the Institutional Research Committee and samples were obtained after informed consent.

2.2. Oxidative stress parameters

Serum was used for the assessment of oxidative stress parameters. Protein carbonylation was determined spectrophotometrically based on the reaction of 2,4-dinitrophenyhydrazine with protein carbonyls [14] and the results were expressed as nmol carbonyl/ml of serum. Malondialdehyde, the end product of lipid peroxidation was measured by the thiobarbituric acid method using 1,1,3,3 tetramethoxy propane as standard [15].

2.3. Assessment of cobalt binding to albumin

The serum samples were assessed for cobalt binding as described with slight modifications [4]. Briefly, serum samples were incubated with cobalt chloride 0.232 mM (final concentration) and made up to 100 µl with phosphate buffer saline (PBS pH 7.4) in wells of a microtiter plate. This was incubated for 10 min to allow the cobalt to bind to the albumin. At the end of the incubation, the unbound cobalt was measured by reducing cobalt chloride with 1.6 mM DTT (final concentration), adding 200 µl of saline and reading the absorbance at 490 nm. Absorbance readings were also measured in parallel wells, where only cobalt chloride and DTT were present, without serum. The amount of bound cobalt was calculated from subtracting the value for unbound cobalt from that of the wells which had only cobalt and DTT without serum.

2.4. Separation of albumin from serum

For separation of albumin from serum, 100 µl serum was incubated with 250 µl of blue sepharose beads (Cibacron Blue) in Tris HCl buffer (0.05 M pH 8) for 30 min, following which the beads were separated by centrifugation at 4000 rpm for 10 min. These beads were then washed with Tris HCl buffer, following which the bound albumin was eluted with 100 µl of Tris HCl (0.05 M)/NaSCN (0.2 M).

2.5. In vitro oxidant exposure

For examining the direct effect of oxidative stress on cobalt binding, normal serum (2.5 g/dL) as well as purified human serum albumin (2.5 g/dL) was treated with either hydrogen peroxide (200 µM) and copper sulphate (CuSO4) (200 µM), or xanthine (1 mM) and xanthine oxidase (100 µu) for 30 min at 37 °C. Following treatment, the cobalt binding capacity was estimated as mentioned above. To confirm roles, if any, of hydrogen peroxide or superoxide in these changes, experiments were also carried out in the presence and absence of catalase (1000 U) or superoxide dismutase (SOD) (1000 U) respectively. To evaluate the role of hydroxyl radicals in the process, experiments were done with and without thiorrea (50 mM) and mannitol (50 mM), which have been used to scavenge hydroxyl radicals [16].

2.6. Lipid analysis from albumin

Total lipids were extracted from albumin as described [17]. The lower organic phase was concentrated using nitrogen, resuspended in a small volume of chloroform/methanol (2:1) and used for lipid analysis. Neutral lipids were separated by thin layer chromatography (TLC) on silica gel G plates using the solvent system of hexane/diethyl ether/acetatic acid (80:20:1, by vol). Spots corresponding to standard fatty acids run in parallel were identified by iodine exposure and eluted. The eluted fatty acids were methylated with methanol and concentrated HCl for 4 h at 70 °C, following which the fatty acid methyl esters were extracted with 1 ml of heptane. Individual fatty acid content was then quantitated using gas chromatography as described [18].

2.7. Removal of fatty acid from purified HSA

Albumin (5.0 g/dL) was mixed with activated charcoal (2.5 g) and the pH of the solution was lowered to 3.0 by the addition of 0.2 N HCl. The solution was then placed in an ice bath and mixed for 1 h, following which the charcoal was removed by centrifugation at 3000 rpm and the solution pH adjusted back to 7.0 by addition of 0.2 N NaOH. Control experiments were carried out to confirm that changes in pH did not influence the cobalt binding assay.

2.8. Estimation of albumin

Albumin content was measured using the bromocresol green (BCG) method [19].

2.9. Statistical analysis

Results are expressed as mean±S.E.M. Statistical calculations were performed using SPSS software for windows (version 9.0). Mann–Whitney and Student’s t test were performed for statistical analysis. A P value of less than 0.05 was taken to indicate statistical significance.

3. Results

Patients with fatty liver on ultrasound, who were recruited for the study, had a significantly higher BMI when compared to controls (Table 1). Serum levels of alanine transaminase were also significantly higher in patients when compared to controls, as were serum triglyceride levels. No difference in levels of gamma-glutamyltransferase was

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Fatty liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>19/9</td>
<td>20/8</td>
</tr>
<tr>
<td>Age (year)</td>
<td>41.17±9.55</td>
<td>41.32±8.75</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.03±3.0</td>
<td>25.20±4.0</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>165.25±42.6</td>
<td>185.84±43.8</td>
</tr>
<tr>
<td>TAG (mg/dL)</td>
<td>144.9±73.1</td>
<td>232.08±130.65</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>32.38±11.39</td>
<td>45.33±20.66</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>33.65±19.76</td>
<td>77.55±57.56</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>90.07±26.65</td>
<td>111.59±35.48</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>0.66±0.34</td>
<td>0.74±0.41</td>
</tr>
</tbody>
</table>

Fig. 1. Protein carbonyl (A) and malondialdehyde (B) levels in serum from controls (□) and patients with fatty liver (■). Experiments were carried out as described in the Methods section. The data is expressed as mean±SEM, n=28. (*P<0.05).
seen between controls and patients (data not shown). Interestingly, there was no significant difference in levels of serum albumin between patients and controls.

Initial experiments were then carried out to evaluate oxidative stress in serum from patients with fatty liver and the results are shown in Fig. 1. Total protein oxidation assessed by protein carbonyls was significantly increased (P<0.05) in serum of patients with fatty liver (Fig. 1A). This was accompanied by a significant increase in malondialdehyde (P<0.05) (Fig. 1B) compared to control subjects.

Since albumin is the predominant protein in serum and has been shown to be susceptible to oxidative modification, it is possible that the increase in protein carbonyl content could reflect modification on the protein. To determine if oxidative stress in serum had any functional significance on albumin, we investigated the cobalt binding capacity of serum albumin in patients with fatty liver. As seen in Fig. 2, a slight but significant decrease in cobalt binding was seen in serum from patients with fatty liver, when compared to controls.

To determine whether oxidative stress could have an effect on albumin–cobalt binding, in vitro studies were then carried out using normal serum as well as purified human serum albumin. Exposure to hydrogen peroxide and copper sulfate resulted in a decrease in cobalt binding capacity of albumin on pure HSA (Fig. 3A). Hydrogen peroxide alone had no effect, while copper sulfate alone showed a slight decrease which was not statistically significant. A similar result was obtained in serum as well (Fig. 3B) where the decrease was prevented by the presence of catalase (Fig. 3B), confirming the role of hydrogen peroxide in the process.

Experiments were also conducted using other physiologically relevant oxidant generating systems such as the xanthine/xanthine oxidase system, which generates superoxide radicals. Surprisingly, cobalt binding to albumin was not altered in the presence of xanthine/xanthine oxidase (Fig. 4A). This suggests that the superoxide anion per se does not influence albumin–cobalt binding. However, the superoxide anion can be converted to hydrogen peroxide by superoxide dismutase. To confirm the results with direct hydrogen peroxide exposure, experiments were repeated with the xanthine/xanthine oxidase system in the presence of superoxide dismutase, with and without copper sulfate. Corroborating the results from Fig. 3, incubation with xanthine/xanthine oxidase+superoxide dismutase, which would produce hydrogen peroxide alone, had no effect on albumin–cobalt binding (Fig. 4A). However, inclusion of copper sulfate resulted in a significant decrease in albumin–cobalt binding, an effect which was reversed by addition of catalase (Fig. 4A). All these data suggest that only hydroxyl radicals, produced by the Fenton reaction when hydrogen peroxide and copper are present, could result in decreasing cobalt binding to albumin. To confirm this, experiments were repeated in the presence of thiourea and mannitol, which can scavenge hydroxyl radicals [16]. As seen in Fig. 4B, the presence of thiourea and mannitol was able to significantly reverse the effect of hydrogen peroxide and copper on albumin–cobalt binding, confirming the role of the hydroxyl radical in the process.

Albumin in circulation also binds to fatty acids and patients with fatty liver have been shown to have increased serum levels of fatty acids [20]. To determine if the fatty acid content on albumin could influence the protein’s binding to cobalt, blue sepharose was used to extract serum albumin from controls and fatty liver patients. The blue sepharose extraction resulted in isolation of serum albumin from both controls as well as fatty liver patients (data not shown). Fatty acid analysis from this albumin indicated that levels of oleic, myristic and lauric acid were increased in patients with fatty liver when compared to controls (Fig. 5). These were the only fatty acids which were consistently detected as being associated with albumin from patients with fatty liver and controls.
But can this increase in fatty acids influence cobalt binding to albumin? To examine this, in vitro experiments were carried out where fatty acids were removed from purified human serum albumin and the cobalt binding capacity of the samples analyzed. Fatty acid removal from albumin was confirmed by TLC (data not shown). Interestingly, as seen in Fig. 6, cobalt binding to albumin decreased in samples where fatty acid had been removed, suggesting that fatty acid binding could influence cobalt binding to albumin.

The results so far suggest that oxidative stress is present in serum from patients with fatty liver, accompanied by a decrease in cobalt binding to albumin. In vitro exposure to free radicals or removal of fatty acids also decreases cobalt binding on albumin, suggesting that patients with fatty liver may have decreased fatty acids bound on albumin. Interestingly, however; analysis of albumin from patients with fatty liver showed an increase in fatty acids bound to albumin. This indicates that fatty acids may have a protective effect on maintaining albumin–cobalt binding. However, would removal of fatty acids aggravate the effect of oxidative stress on albumin–cobalt binding? This was tested in vitro where purified human serum albumin was subjected to oxidative stress subsequent to removal of fatty acids from the protein. As seen in Fig. 6, removal of fatty acids did not exacerbate the decrease in albumin–cobalt binding induced by oxidative stress, suggesting that oxidative stress and fatty acids influence albumin–cobalt binding by separate mechanisms.

4. Discussion

Albumin, the predominant protein in circulation is mainly synthesized in the liver and binds to a number of ligands; serving as transport vehicle for numerous endogenous compounds including bilirubin, hemin, heavy metals and fatty acids [21]. The protein is known to be affected in conditions such as exposure to cigarette smoke [22,23], diabetes and renal diseases [24,25]. Structural alterations on human serum albumin have been described in renal disease such as uremia and chronic liver disease such as liver cirrhosis [7]. One of the causes of modification on albumin is oxidative stress [26] and oxidized albumin has been detected in the blood of patients suffering from diabetes [24] and kidney disease [25]. Oxidative stress has also been demonstrated in conditions such as non alcoholic fatty liver disease (NAFLD) [27] and non alcoholic steatohepatitis (NASH) [28,29] conditions which can develop from fatty liver. It has been suggested that albumin, being synthesized in the liver could be exposed to free radicals, resulting in conformational changes which may affect its binding property [7]. Our initial studies focused on oxidative markers in serum from patients with fatty liver, and the increase in malondialdehyde and protein carbonyl indicates the presence of lipid peroxidation and protein oxidation, suggestive of oxidative stress. But does this result in functional alterations on serum albumin?

Altered cobalt binding to albumin has been reported in myocardial infarction (MI) [8] myocardial ischemia [4] and acute coronary syndrome [5] and this has been used as a diagnostic test to determine susceptibility to undergo MI [4]. However, it has also been shown that reactive oxygen species such as superoxide and hydroxyl radicals modify albumin during myocardial disease [26], suggesting a role for free radicals in inducing change in cobalt binding. Our data indicates that oxidative stress in serum of patients with fatty liver is accompanied by a slight but significant decrease in cobalt binding to albumin. But is oxidative stress and change in cobalt binding linked, or are they two unrelated observations? ROS generation in vitro has been shown to produce structural changes in human albumin as well as synthetic N-terminus tetra- and octa-peptides from the molecule, with loss of cobalt binding capacity [30]. Our in vitro experiments produced similar results, where exposure to hydrogen peroxide in the presence of copper resulted in a decrease in albumin–cobalt binding. The reversal of this effect with catalase confirms the role of $\text{H}_2\text{O}_2$ in mediating the effect. More specifically, this effect seems to be mediated by the hydroxyl radical, since superoxide generated by the xanthine/xanthine oxidase system had no effect and thiourea and mannitol were able to prevent the decrease in albumin–cobalt binding. Thiourea and mannitol have been used as...
scavengers for the hydroxyl radical [16] and albumin and other proteins have been shown to be selectively susceptible to hydroxyl radicals in comparison to the superoxide and hydroperoxyl radicals [31,32]. This data is also in accordance with previous studies where simultaneous presence of H₂O₂ and copper was required to alter cobalt binding of albumin [26]. The experiments also suggest that copper does not interfere with cobalt binding, probably because they bind at different sites on the protein [26].

The data so far indicates that oxidative stress in patients with fatty liver results in modification of albumin, such that the protein's binding to cobalt is altered. But what is the mechanism? Binding of transition metals such as copper and nickel is proposed to occur at the N-terminus of the albumin molecule at the amino acid sequence Asp-Ala-His-Lys [3]. Earlier studies examining albumin–cobalt binding in the context of cardiovascular disease have used peptides with the N-terminal sequence and shown that it also binds cobalt and loss of the sequence results in decreased cobalt binding [30]. This was suggested as the mechanism for decreased cobalt binding in myocardial infarction [4]. However, cobalt may bind to additional sites on albumin [33] and a recent study has demonstrated that the principle cobalt binding site on HSA are sites A and B in the interior, with the N-terminal site being of lesser importance [9]. A recent study by Bar-Or et al. using plasma from controls and patients with myocardial ischemia indicates that binding to N-terminal may not be the only mechanism for albumin–cobalt binding [34]. This indicates that alternate mechanisms for the decrease in cobalt binding exist, involving other ligands binding in the interior of the molecule.

HSA is the primary transporter for the delivery of fatty acids (FA) to tissues, the FA:HSA ratio being between 0.1:1 and 2:1 under physiological conditions and rising to 6:1 or greater in peripheral vasculature during fasting or exercise [35,36]. The binding of FA to HSA can also increase in pathological conditions such as diabetes, liver disease and cardiovascular disease [37]. At least seven binding sites for fatty acids have been identified in albumin [11], saturated fatty acids binding with increasing affinity as their chain length increases [38]. As mentioned earlier, four different metal binding sites have been identified in albumin [9] and it has been reported that fatty acids and cadmium compete with each other to bind to site A on albumin [21]. According to Mothes et al. cobalt binds to sites A and B on the albumin molecule [9]. But can fatty acids on albumin modulate the protein's cobalt binding activity? Our data shows that this seems to be the case, since removal of fatty acids from HSA results in decreased cobalt binding to the protein.

But what is the relationship between oxidative stress, fatty acids and cobalt binding on albumin in the context of fatty liver disease? Based on the data on oxidative stress and cobalt binding from patients, it seemed that free radicals decrease albumin–cobalt binding, which agrees with the in vitro oxidative exposure experiments. The role of fatty acids in the context is a bit more complex. The in vitro experiments indicate that fatty acid removal compromised cobalt binding; but patients with fatty liver (who had decreased cobalt binding to albumin) showed significant increases in oleic, myristic and lauric acids bound to albumin. The increase in fatty acids on albumin is probably expected, since patients with fatty liver have increased levels of free fatty acids [20], where the rate of FFA uptake is unregulated and therefore directly proportional to plasma FFA concentrations [39]. Though palmitic acid and stearic acid are present in serum, it has been shown that between bovine and human serum albumin, palmite binds more tightly to bovine whereas laurate and oleate bind more tightly to human serum albumin [40,41]. This could be the reason why we were unable to detect palmitate on the albumin isolated from controls or patients. The data then suggests that fatty acids have a protective effect in maintaining albumin–cobalt binding, though the in vitro experiments suggest that mechanisms for this protection are independent of oxidative modification. The protection by increased albumin-fatty acid binding may also explain the relatively small decrease in albumin–cobalt binding in patients with fatty liver. Since conditions such as cardiovascular disease are also associated with increase in serum free fatty acids [42] and oxidative stress [43], it is possible that this mechanism may hold true in cases of myocardial infarction as well.

In conclusion, our studies demonstrate that oxidative stress in serum of patients with fatty liver is associated with a decrease in cobalt binding capacity of albumin. This is accompanied by an increase in fatty acid binding on albumin in these patients, which probably attenuates the effect of free radicals on cobalt binding to the protein.

Acknowledgements
The Wellcome Trust Research Laboratory is supported by the Wellcome Trust, London. Funding for this study was from the Christian Medical College Fund Research Fund.

References


[40] A.A. Spector, Fatty acid binding to plasma albumin, J. Lipid Res. 16 (1975) 165–179.

