Counter-regulatory effects of procalcitonin and indoxyl sulphate on net albumin secretion by cultured rat hepatocytes

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Abstract

Background. Although hypoalbuminaemia is a significant predictor of mortality in haemodialysis (HD) patients, the pathophysiological mechanisms involved remain to be determined. Albumin is a negative acute-phase reactant and many proinflammatory substances are elevated in HD patients. We investigated factors that may affect liver albumin synthesis.

Methods. Hepatocytes were isolated from rat livers and were cultured with interleukin (IL)-4, IL-6, IL-12, tumor necrosis factor (TNF)-α, procalcitonin (PCT), a sensitive marker of infection, and indoxyl sulphate (IS), a uraemic toxin. Albumin levels in the supernatant were measured by enzyme-linked immunosorbent assay. Albumin mRNA expression was determined by reverse transcriptase polymerase chain reaction.

Results. IL-6 and TNF-α significantly decreased albumin levels in a dose-dependent manner (P < 0.01 and P < 0.05, respectively). In contrast, IL-4 and IL-12 did not modulate albumin production. PCT and IS significantly and dose-dependently increased albumin levels (both P < 0.01). PCT increased albumin mRNA expression in the hepatocytes (P = 0.05) and dose-dependently abrogated IL-6-induced suppression of albumin synthesis (P < 0.01). IS also blocked the IL-6-induced decrease in net albumin secretion (P < 0.01).

Conclusion. Our findings indicate that PCT and IS protect against suppression of hepatic albumin synthesis caused by proinflammatory cytokines, suggesting their potential role in preventing hypoalbuminaemia in HD patients.

Keywords: albumin; haemodialysis; IL-6; indoxyl sulphate; procalcitonin

Introduction

Albumin, the most abundant protein in the blood, is a strong predictor of mortality in haemodialysis (HD). During inflammation, there are decreases in plasma albumin concentrations, albumin synthesis and mRNA levels, while C-reactive protein (CRP) and α2-macroglobulin are increased [1]. Blood albumin is inversely correlated with serum CRP in dialysis subjects [2]. In a comprehensive study that used the elimination rate of [125I] human albumin, the estimated rate of albumin synthesis was reduced in dialysis patients having hypoalbuminaemia compared with those with normoalbuminaemia [3]. The normally occurring increases in albumin synthesis during feeding were also impaired in hypoalbuminaemic patients compared with normoalbuminaemic HD patients [4]. However, these findings were from comparisons between normoalbuminaemic and hypoalbuminaemic patients, and albumin metabolism was not directly compared between normal subjects and HD patients.

Recently, the absolute synthesis rate of albumin was found to be increased in normoalbuminaemic HD patients compared with control subjects. Giordano et al. [5] found a greater albumin and whole-body protein synthesis rate as well as an increased intravascular albumin pool in dialysis subjects despite the absence of overt inflammation. An increment in albumin synthesis in association with inflammation was also observed during HD treatment [6]. Kaysen et al. [7] reported that 74 HD patients had a significantly elevated albumin synthesis as well as an excess plasma volume expansion. Altogether, these observations suggest that hepatic albumin synthesis may be increased in uraemic subjects compared with the general population.

Circulating proinflammatory cytokines are elevated in HD patients. Many studies have demonstrated that proinflammatory cytokines can directly regulate the synthesis of acute-phase reactants in cultured hepatocytes. For example, interleukin (IL)-6 strongly suppresses
hepatic albumin synthesis, but stimulates the production of CRP [8,9]. Tumor necrosis factor (TNF)-z also blocks albumin synthesis by directly downregulating gene transcription [10]. IL-4, on the other hand, blocks hepatocyte production of acute-phase reactant proteins [11]. In addition, serum procalcitonin (PCT), a marker of infection [12], was shown to be elevated in non-infectious HD patients and correlated negatively with albumin levels [13]. Despite these data, little information is available on the exact nature of the substances that regulate hepatic albumin production.

The main goal of the present study was to investigate direct effects of causative substances on albumin production in the liver. We first measured net albumin secretion and mRNA abundance in rat primary cultured hepatocytes in the presence of proinflammatory cytokines, PCT or growth factors. We also examined the effect of a uraemic toxin, indoxyl sulphate (IS), on the production of albumin in these hepatocytes. Finally, we examined whether PCT affects IL-6-induced suppression of albumin production.

Materials and methods

Materials

Male Sprague-Dawley rats, aged 5–6 weeks (150–200 g), were purchased from Japan SLC (Hamamatsu, Japan). Human recombinant TNF-α, IL-4, IL-6, IL-12, epidermal growth factor (EGF), aprotinin, penicillin–streptomycin solution, dextran and bovine serum albumin (BSA) were purchased from Sigma Chemical (St Louis, MO). Human PCT was obtained from Biomedical Laboratories Medicine (Brno, Czech Republic). Human recombinant hepatic growth factor (HGF) was a kind gift from Sumitomo Pharmaceuticals (Osaka, Japan). Human insulin-like growth factor (IGF)-1 was supplied by Fujisawa Pharmaceutical (Osaka), and IS was provided by Kureha Chemical Industry (Nagoya, Japan). Twenty-four collagen-coated wells were purchased from Iwaki Tecno Glass (Tokyo, Japan). Williams’ medium E and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Grand Island, NY). Rat serum albumin, polyclonal rabbit anti-rat albumin and peroxidase-conjugated polyclonal rabbit anti-rat albumin were from Nordic Immunology Laboratories (Tilbug, The Netherlands).

Primary culture of rat hepatocytes

Male Sprague-Dawley rats were housed in cages and provided with commercial pelleted diet and water ad libitum. Hepatocytes were isolated from the liver through collagenase-containing in situ perfusion via the portal vein, as described previously [14]. Briefly, rats were anaesthetized by pentobarbitone (50 mg/kg). After cannulation of the portal vein, the liver was perfused with a Ca2+-free perfusion solution that contained EGTA (ethyleneglycol-bis-N,N,N’,N’-tetraacetic acid), followed by a solution that contained Ca2+ and collagenase (0.5 mg/ml). After mincing in the first solution, the hepatic tissue was filtered through a nylon mesh to remove fibrous tissues and centrifuged at 800 r.p.m. three times each for 1 min. The cellular pellets were then re-suspended in culture medium. The viability of hepatocytes was tested by trypan blue exclusion. Hepatocytes containing >80% live cells were used for the study. After sowing subconfluent density of 1.2 × 105 cells/cm2 in culture plates, the cells were cultured in Williams’ medium E buffer containing 10% FBS, 10−9 M insulin, 10−9 M dexamethasone and 0.5% penicillin–streptomycin solution at 37°C under 95% air 5% CO2 gas mixture. At 4 h after plating, the culture medium was discarded and replaced with FBS-free Williams’ medium E containing 0.5 μg/ml aprotinin. IL-4, IL-6, IL-12, TNF-α, HGF, EGF, IGF-1, PCT (0–10 ng/ml) and IS (0–100 μg/ml) were then separately added. BSA and dextran (0–8 g/dl) were also added at the same time to examine the effect of colloid oncotic pressure. In separate experiments, different doses of PCT and IS in the presence of IL-6 (10 ng/ml) were added. After 20 h, the wells were washed twice with PBS, and the cells were cultured for an additional 6 h under the same conditions. The supernatants were then collected and kept frozen at −20°C until measurement of albumin concentration.

Measurement of albumin concentration

The concentration of albumin present in the medium during the final 6 h was measured by enzyme-linked immunosorbent assay (ELISA). In brief, 0.5 μg/100 μl polyclonal rabbit anti-rat albumin dissolved in 0.05 M sodium carbonate (pH 9.6) (coating buffer) was plated in each well of a 96-well plate for 1 h at room temperature. After double washing with 50 mM Tris and 0.14 M NaCl containing 0.05% (v/v) Tween 20 (pH 8.0), the response was blocked with 200 μl of 50 mM Tris, 0.15 M NaCl containing 1% BSA (pH 8.0) for 30 min. After two washes, 1:50 diluted samples of standard rat serum albumin dissolved in 50 mM Tris and 0.15 M NaCl containing 1% BSA and 0.05% Tween 20 (pH 8.0) were added to each well and incubated at room temperature for 1 h. After double washing, the wells were incubated with 0.02 μg/100 μl peroxidase-conjugated polyclonal rabbit anti-rat albumin dissolved in sample/conjugated diluents for 1 h at room temperature. After washing, 100 μl of equal volumes of 3,3’,5,5’-tetratramethyl benzidine peroxidase substrate and peroxidase solution B (H2O2 at a concentration of 0.02% in citric acid buffer) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added, and the samples were kept at room temperature for 10–15 min. The reaction was terminated by adding 100 μl of 1 M H2SO4. Absorbance was measured at 450 nm with a microplate reader (EL340, Biotek Instruments, Winooski, VT). This ELISA system did not cross-react with bovine or rat serum albumin (data not shown). Cellular protein concentration was determined by the Bradford method [15]. The rate of net albumin secretion was determined by dividing albumin content in the supernatant by cellular total protein in each well (ng/μg protein). The presented data represent the average values of 2–11 rats. Each measurement was performed in duplicate. All data were expressed as percentage of control, because the rate of albumin production was different in each rat.

RNA extraction and semi-quantitative analysis of albumin mRNA by RT–PCR

Total RNA was isolated from the primary culture of rat hepatocyte by ISOGEN, acid guanidine thiocyanate-phenol-chloroform solution (Nippon Gene, Toyama, Japan). The
quality of RNA products was assessed from spectrophotometric measurements at 260 and 280 OD and quantitated by the absorbance at 260 nm. Before each reaction, 1 μg of total RNA from rat hepatocytes was reverse-transcribed by incubating at 65°C for 15 min and placing the sample on ice for 5 min, then at 25°C for 10 min, and finally at 42°C for 1 h, followed by inactivation of enzyme at 99°C for 5 min in a 20 μl reaction mixture using a 1st Strand cDNA Synthesis Kit for reverse transcriptase polymerase chain reaction (RT–PCR) (Roche Molecular Biochemicals, Indianapolis, IN).

The albumin primers were synthesized by referring to the sequence of the albumin cDNA, the 5′ primer (5′-TTCGCCA AGTACATGGTGTGAG-3′) and the 3′ primer (5′-GGTCTT TCTACAAGAGGCTG-3′), which yielded a 373 bp PCR product. The primers used for GAPDH were 5′-AATGC ATCCTGCACCAACCA-3′ and 5′-GATGCCATATTCAT TGTCATA-3′, which yielded a 515 bp PCR product. An intron was not included between the two primers. PCR was performed on a PTC-0150 MiniCycler (MJ Research, Waltham, MA) using 1.5 μl of the above aliquots in a total mixture volume of 25 μl of AmpliTaq Gold (Applied Biosystems, Branchburg, NJ). The PCR protocol for albumin consisted of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and extension at 72°C for 1 min, and this was repeated 16 times. The PCR technique for GAPDH consisted of denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min and extension at 72°C for 2 min, and this was repeated 19 times. We selected this protocol because the PCR product increased linearly from 13 to 17 cycles for albumin, and from 18 to 20 cycles for GAPDH. An initial heating at 94°C for 9 min and a final extension at 72°C for 10 min were performed for all PCR products.

From each PCR production, the amplified products were run on 2.0% agarose gel (1.3% Nusieve GTG agarose; 1.3% agarose (Life Technologies, Carlsbad, CA) by Mupid- BioWhittaker Molecular Applications, Rockland, ME) and run on 2.0% agarose gel (1.3% Nusieve GTG agarose; 1.0% agarose (Life Technologies, Carlsbad, CA) by Mupid-BioWhittaker Molecular Applications, Rockland, ME). The semi-quantitative analysis of this RT–PCR product was conducted by factoring GAPDH PCR products from three different samples.

Statistical analysis

Data are expressed as means±SD. Differences between groups were examined using a Wilcoxon rank sum test. Effects of dose-dependency were examined by Jonckheere’s test for trend analysis. Statistical significance was set at P < 0.05.

Results

Effect of colloid oncotic pressure on albumin production

To confirm the ability of our system to regulate hepatic albumin synthesis, we first examined the direct effects of colloid oncotic pressure on albumin production. We altered dextran and BSA levels, since increases in oncotic pressure is known to strongly suppress albumin mRNA and protein levels in several types of cultured hepatocytes [16]. High levels of dextran induced a significant and dose-dependent suppression of albumin secretion (P < 0.01, n = 3). A significant reduction in albumin production was first visible at 2 g/dl dextran (Table 1). BSA also inhibited albumin production in a dose-dependent manner (P < 0.01, n = 4). This effect became significant at 2 g/dl, and at 8 g/dl BSA albumin production was completely inhibited (Table 1).

Effect of proinflammatory cytokines on albumin synthesis

We next examined the positive effect of growth factors for albumin secretion in our system. HGF significantly and dose-dependently increased net albumin secretion in rat hepatocytes (P < 0.01; Figure 1, right). At 1 ng/ml, HGF increased albumin levels in the media to 115 ± 9% (P < 0.01, n = 5) and further increased it to 141 ± 8% at 10 ng/ml (P < 0.01, n = 6). EGF also significantly and dose-dependently increased albumin concentration (P < 0.01). There were significant increases in albumin secretion at 1 ng/ml (108 ± 14%, P < 0.05, n = 5), 5 ng/ml (116 ± 15%, P < 0.01, n = 5) and 10 ng/ml (133 ± 7% of the control values, P < 0.01, n = 5). Although net albumin secretion tended to increase following the addition of 10 ng/ml IGF-1 (n = 4), this increase was not statistically significant (Figure 1). These data indicated that we obtained a functional system of primary hepatocyte culture.

Effect of proinflammatory cytokines on albumin production

IL-6 significantly and dose-dependently decreased net albumin secretion (P < 0.01, Figure 1). Albumin concentrations in the supernatant correlated negatively and significantly with IL-6 concentrations (1 ng/ml, 76 ± 7% from 100% control levels; 5 ng/ml, 69 ± 7%; 10 ng/ml, 63 ± 7%; P < 0.01, n = 8). TNF-α also significantly and dose-dependently reduced albumin synthesis (P < 0.01); it produced maximal inhibition (84 ± 5% of control levels) at 10 ng/ml (P < 0.05, n = 4) (Figure 1). In contrast, both IL-4 (0.5–10 ng/ml, n = 4) and IL-12 (0.5–10 ng/ml, n = 5) did not affect albumin production in rat hepatocytes (Table 2).
Effects of PCT and IS on albumin production

PCT significantly and dose-dependently stimulated albumin secretion ($P<0.01$). There was a significant increase in albumin production at 1 ng/ml, and this reached 117±7% of basal levels at 10 ng/ml ($P<0.01$, $n=6$) (Figure 2). IS also significantly and dose-dependently enhanced albumin production ($P<0.01$). Significant increases were found at 10 mg/ml (116±7% from 100% control, $P<0.01$, $n=11$), 25 mg/ml (119±9%, $P<0.01$, $n=11$), 50 mg/ml (122±12%, $P<0.01$, $n=11$) and 100 mg/ml (132±18%, $P<0.01$, $n=10$) (Figure 3). In addition, IS dose-dependently stimulated albumin secretion in the presence of 4 g/dl albumin in the supernatant ($P<0.01$). A significant increase in albumin secretion was found at 50 µg/ml (122±11% from 100% baseline, $P<0.05$, $n=4$) and 100 µg/ml (154±19%, $P<0.05$, $n=4$) (Figure 4).

Co-administration of PCT and 10 ng/ml IL-6 caused attenuation of IL-6-induced suppression of net albumin secretion. Albumin concentrations in the presence of 100 ng/ml and 1 µg/ml PCT were 72±9% and 87±3% of basal levels, respectively, and these suppressions were significantly greater than with IL-6 alone (55±5%, $P<0.01$) (Figure 5, left). IS (100 µg/ml) also significantly attenuated IL-6 (10 ng/ml)-induced decreases in albumin secretion (84±9% vs 55±5% from 100% basal values, $P<0.01$) (Figure 5, right).

Expression of albumin mRNA

Exogenous IL-6 (10 ng/ml) decreased the albumin/GAPDH mRNA ratio (85% from 100% basal level, $P=0.05$), and HGF (10 ng/ml) increased albumin mRNA abundance (147% of basal level, $P=0.05$) in rat primary cultured hepatocytes. PCT (1 µg/ml) enhanced the albumin/GAPDH mRNA ratio (140% of the basal level, $P=0.03$). IS (100 µg/ml) also...
Recent studies have demonstrated that several proinflammatory cytokines exert a regulatory role on albumin production and degradation [8–10]. Castell et al. [8] demonstrated that IL-6 caused a 50% suppression of albumin transcription in cultured human hepatocytes. In addition, Ramadori et al. [9] found comparable inhibitory effects of TNF-α and IL-6 on albumin synthesis in a human hepatoma cell line. Based on this background, we designed the present study to compare the effects of human TNF-α, IL-4, IL-6 and IL-12 on in vitro hepatic albumin production using primary cultures of hepatocytes. We found that IL-6 caused a greater alteration of net albumin secretion than TNF-α. IL-6 also decreased albumin mRNA abundance to 85% from basal levels. In contrast, IL-4 and IL-12 did not influence albumin production. These findings indicate that IL-6 but not TNF-α may play an important role during the suppression of hepatic albumin production in inflammatory diseases.

Blood PCT, a 116-amino acid polypeptide, was reported to be acutely elevated by infection and by pancreatitis [12]. Blood PCT, which has a half-life of 1 day [17], reached a peak level at 6 h after infusion of TNF-α. PCT is present in abundance in human liver [18] and is secreted into the blood during sepsis [19]. Recent studies demonstrated that PCT inhibited lipopolysaccharide-induced TNF-α production from human whole blood cells [20], indicating that PCT may partly act through inhibition of monocyte activation. These observations suggest that PCT is produced mainly in the liver and that it may interact with proinflammatory cytokines. In stable HD patients, blood PCT was slightly but significantly elevated to 0.68 ng/ml (normal <0.5 ng/ml), and it was positively correlated with CRP, but negatively with albumin and prealbumin levels [13]. Unlike CRP, PCT does not appear to act as a proinflammatory marker, since raised PCT did not affect 2-year mortality in HD subjects [21]. In our study, we found that, at PCT doses >10 ng/ml, which are similar to levels found in infected HD patients [22], hepatic net albumin secretion was stimulated. PCT also directly enhanced albumin mRNA abundance by 140% from basal levels and abrogated IL-6-induced suppression of albumin secretion. These findings suggest that PCT may be a factor that increases albumin production and counteracts proinflammatory actions in the liver.

Uraemia is known to influence the nutritional status of dialysis patients. In experimental uraemic animals, hepatic albumin synthesis was initially reported to be reduced due to enhanced degradation of albumin mRNA [23]. However, recent clinical studies have demonstrated that albumin synthesis was increased rather than decreased in HD patients [5–7]. During a single HD session, a significant increase in albumin synthesis was found that corresponded to elevated blood levels of IL-6 [6]. Thus, hepatocytes may operate compensatory mechanisms to increase albumin synthesis during uraemia. However, it is not known whether uraemic substances directly modify albumin production in the liver.

Recently, we found that blood IS was elevated to ~100 μg/ml (normal <1.9 μg/ml) in HD subjects, and this was positively correlated with time on HD and
blood carbonyl stress markers but not with inflammatory markers such as CRP and soluble TNF-α receptor p80 [24]. IS is known to directly reduce whole-body oxygen consumption in rats [25]. In addition, IS dose-dependently inhibited binding of tryptophan to albumin, thereby increasing the free fraction of tryptophan, which leads to a rise in brain serotonin synthesis that regulates food intake [26]. Daily HD also improves nutritional status that was associated with a reduction in blood IS [27]. IS directly induces oxidative stress in human renal proximal tubular cells [28]. IS also suppresses lymphocyte blast formation and IL-2 production in vitro [29]. Because these findings strongly suggest that IS possesses metabolic and immunologic properties, we used IS to examine the impact of uraemic toxins on albumin metabolism.

**Fig. 5.** Effects of procalcitonin and indoxyl sulphate on IL-6-induced suppression of albumin production in primary rat hepatocyte cultures. Procalcitonin (100 ng/ml or 1 μg/ml) partially but significantly blocked IL-6 (10 ng/ml)-induced inhibition of albumin synthesis in hepatocytes (left panel). Similarly, indoxyl sulphate (100 μg/ml) significantly abrogated IL-6 (10 ng/ml)-induced suppression of albumin synthesis (right panel). Values are the means of 2–8 rats. Each experiment had its own control, which was set as 100%. Each point represents means ± SD for all experiments. *P < 0.01 vs IL-6 alone.

**Fig. 6.** Albumin mRNA levels in primary rat hepatocyte cultures. Hepatocyte growth factor (10 ng/ml) increased albumin mRNA abundance (147% of basal level, P = 0.05), but IL-6 (10 ng/ml) suppressed abundance (85% of basal level, P = 0.05). Procalcitonin (1 μg/ml) increased the albumin mRNA/GAPDH ratio to 140% of basal levels (P = 0.03). Similarly, indoxyl sulphate (100 μg/ml) increased albumin mRNA expression to 136% of basal levels (P = 0.03). Each value was the mean of three different rats. Each experiment had its own control, which was set as 100%. Each point represents means ± SD for all experiments. *P < 0.01 vs IL-6 alone.
We found that IS levels, which were similar to levels found in pre-dialysis serum, increased albumin secretion and mRNA abundance in hepatocytes. Since IS has been closely correlated with dietary protein intake [24], dietary protein-derived indole may mainly stimulate albumin production in the liver, thereby leading to elevations in blood albumin in HD subjects. In addition, since IS prevented the IL-6-induced suppression of albumin production, IS may operate defensively against cytokine-induced suppression of album synthesis. Further studies are needed explore whether PCT and IS can change albumin expression by modulating nuclear transcription factors such as hepatocyte nuclear factor 1.

There were some limitations in the present study. First, we did not evaluate albumin kinetics, such as intracellular uptake or degradation. However, albumin degradation was unlikely during our 6-h incubation, since we found that albumin in the medium produced a single band by western blot using a polyclonal antibody (data not shown). In addition, co-incubation with actinomycin, an inhibitor of protein synthesis, substantially reduced albumin levels in medium, indicating a major role for albumin synthesis. Furthermore, we had preliminary confirmation that albumin was linearly and constantly secreted into the supernatant during the 24-48 h of incubation (data not shown), indicating a minor role for albumin reuptake. Secondly, we did not evaluate direct synthetic rates of isotopically labelled amino acids into albumin. Thirdly, we only examined net albumin secretion in normal hepatocytes, and it is unclear whether our results would be similar to results with hepatocytes from uraemic animals. Finally, the impact of IL-6 on albumin mRNA may have been weak because of the lower concentration of dexamethasone (10−9 M), since negative effects of IL-6 on albumin mRNA are dependent upon the presence of >10−8 M dexamethasone [30].

In conclusion, we demonstrated that PCT increased hepatic albumin mRNA and net albumin secretion from primary cultured rat hepatocytes. In addition, PCT and IS partly attenuated the inhibitory effect of IL-6 on albumin secretion. These findings suggest that PCT and IS may work protectively against IL-6-induced suppression of hepatic albumin synthesis during inflammation in dialysis patients.

Acknowledgement. We thank Kureha Chemical Industry Co. Ltd (Tokyo, Japan) for kindly providing IS.

Conflict of interest statement. None declared.

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Received for publication: 5.12.02
Accepted in revised form: 10.10.03