Cleavage of Thyroxine-Binding Globulin During Cardiopulmonary Bypass

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Thyroxine-binding globulin (TBG) is a noninhibitory member of the serine protease inhibitor (serpin) superfamily. A characteristic serpin cleavage product of TBG has been demonstrated in sera of septic patients. We find that a similar cleavage product appears in serum during the rapid decline of immunoassayable TBG and thyroxine (T_4) that is associated with the inflammatory response to cardiopulmonary bypass (CPB). In vitro cleavage of TBG by the serine protease, neutrophil elastase induces a conformational change that has previously been shown to weaken affinity for T_4. In vitro protease cleavage also decreases immunoassayable TBG, probably because the conformational change decreases the availability of the TBG epitopes to the measuring antibody. Thus, the rapid decrease in immunoassayable TBG concentration previously attributed to accelerated clearance is caused in part by the proteolytic cleavage per se. The evidence for proteolysis of TBG concurrent with the decrease in serum T_4 during CPB is consistent with the proposed release of T_4 from TBG to cells showing serine protease activity.

A RAPID AND SELECTIVE decrease in immunoassayable thyroxine-binding globulin (TBG) occurs during the brief intense inflammatory response associated with the use of the pump oxygenator in cardiopulmonary bypass (CPB).[1-4] Because the rate at which the TBG concentration decreases is much faster than can be accounted for by the half-life of TBG, these results support the hypothesis that the decreased TBG concentrations of inflammatory nonthyroid illness result from increased consumption rather than decreased synthesis of TBG.[2] We have proposed that this is caused by serine protease cleavage of TBG at inflammatory sites.[2] TBG is a member of the serine protease inhibitor (serpin) superfamily but is not a functional protease inhibitor.[5] Like other serpins, TBG offers a "bait" to serine proteases such as neutrophil elastase (NE). This results in the loss of a 4- to 5-kd carboxyterminal fragment of the 54-kd protein and a major change from the s (stressed) to the r (relaxed) conformation.[6] In the case of cortisol-binding globulin (CBG), a serpin closely related to TBG, the characteristic serpin cleavage and conformational change have been shown to decrease the protein's affinity for cortisol.[6,7] This suggests that CBG releases cortisol to inflammatory sites rich in NE. Although NE cleavage causes a similar conformational change in TBG, this change was not found to affect thyroxine (T_4) binding to TBG.[6] However, subsequent studies have described weakened T_4[8-10] or triiodothyronine[11] binding by TBG after proteolytic cleavage by exposure to NE or activated polymorphonuclear leukocytes in vitro. Thus, serine protease cleavage of TBG is a potential mechanism for T_4 release at sites where serine protease activity is expressed.[6,7] Whether such cleavage of TBG occurs in vivo has been difficult to determine. However, by preliminary removal of albumin, which can interfere with Western blotting,[12] we have been able to show a characteristic 49- to 50-kd serpin cleavage product of TBG in the sera of septic patients.[10] To test the hypothesis that such cleavage causes the rapid decrease in immunoassayable TBG and T_4 concentrations during the inflammatory response, we sought to identify the 49- to 50-kd TBG cleavage product in sera from CPB patients previously described[2] and to determine whether cleavage of TBG can account for the observed decrease in immunoassayable TBG.

SUBJECTS AND METHODS

Subjects

Sera that had been collected from 3 of the patients in a study of the effects of CPB surgery on thyroid hormone–binding proteins and thyroid hormone concentration[2] were examined for the presence of cleaved TBG. Eleven aliquots of sera obtained from 3 CPB patients preoperatively and 4, 12, 24, and 72 hours after incision had been stored frozen at −20°C. Twelve sera from normal subjects that had been similarly stored for approximately the same period were used as controls. The studies in which these sera had been obtained were approved by the institutional review board, and informed consent was obtained from each subject.

Laboratory Methods

TBG determination. TBG concentrations were measured by the GammaDab [^{125}I]TBG radioimmunoassay (RIA) kit (Incstar, Stillwater, MN). This is a sandwich method in which immobilized TBG antibody captures serum TBG, which is then detected by its ability to bind ^{125}I T_4.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting of sera. The sera were examined for evidence of TBG cleavage using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting for TBG. To reveal the endogenous TBG, it was necessary to remove albumin. This was done by a modification of the method of Rengarajan et al.[12] Affi-Gel Blue Beads (Bio-Rad Laboratories, Hercules, CA) were washed with 20 mmol/L potassium-phosphate buffer, pH 7.1. Twenty milliliters of serum diluted with 230 mL of potassium phosphate buffer was added to the beads, and the mixture was shaken gently for 3 hours at room temperature and then centrifuged. An equal volume of Tris-glycine SDS sample buffer containing 5% mercaptoethanol was added to an aliquot of each supernatant and incubated at 100°C for 5 minutes. Aliquots of 25 mL of the incubated samples were subject to SDS-PAGE (4% stacking gel, 12% separating gel) and electrotransferred to nitrocellulose membranes (Immobilon-p; Millipore Corp, Bedford, MA) blocked with 2% nonfat dry milk. The membranes were incubated overnight with goat antiserum against human TBG (Lot/ch-B:94680-01; Incstar) diluted 1:500 in 2% nonfat dry milk. They were then

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Table 1. TBG: TBG/Albumin Ratios

	Time (h)				
Patient	0	4	12	24	72
1	2.50: 0.65	0.73: 0.32	0.63: 0.23	2.10: 0.65	2.00: 0.61
2	4.25: 0.94	2.25: 0.68	1.20: 0.41	1.20: 0.43	
3	2.05: 0.66	0.68: 0.34	0.98: 0.34	0.98: 0.39	1.60: 0.55

NOTE. TBG measured in mg/dL; albumin measured in g/dL.

washed twice with 2% nonfat dry milk and twice with Tris-buffered saline TBS; 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) and incubated with affinity-purified alkaline phosphatase–conjugated rabbit anti-goat immunoglobulin (Ig) G (lot 66H4824; Sigma, St Louis, MO) diluted 1:5,000 in TBS for 2 hours, washed twice with TBS and twice with distilled water. The membranes were then incubated with alkaline phosphatase substrate solution containing BCIP/NBT (Sigma) in 10 mL of distilled water until the intensity of the bands became adequate.

Elastase cleavage of TBG and heat inactivation. To determine the effect of NE cleavage on immunoassayable TBG, purified TBG (Sigma) and normal sera with approximately the same TBG content were incubated with NE (Sigma) at a TBG:elastase ratio of 100:1 by weight at 37°C for 1 hour. The reaction was stopped by adding phenylmethylsulfonyl fluoride (PMSF) 0.02 mg (Sigma. In duplicate samples, heat inactivation at 60°C for 1 hour was used to determine if the antibodies used here for RIA and radial immunodiffusion (RID) of TBG were sensitive to changes of TBG conformation.[13,14] TBG concentration was determined by immunoassay (GammaDab [^{125}I]TBG RIA kit; Incstar) and RID (Bind A RID; The Binding Site Ltd, Birmingham, England).

RESULTS

TBG and TBG–Albumin Ratio

As shown previously for the group of CPB patients as a whole,[2] the TBG concentrations in the sera of the 3 CPB patients studied here decreased markedly within 4 hours after the start of surgery, when the patients had been on cardiopulmonary bypass for approximately 2 hours (Table 1). To correct for dilution and shifts in protein distribution space during the bypass, TBG concentrations (mg/dL) are also shown as ratios to albumin (g/dL). The average preoperative TBG concentration of the CPB patients was 2.93 mg/dL, and that of the normal subjects was 2.58 ± 0.65 mg/dL.

Serpin Cleavage Product of TBG in CPB Sera

Figures 1 and 2 show that normal subjects have a single TBG band at 54 kd, which is the molecular weight of the TBG marker. A second band, about 4 to 5 kd smaller, appears in the CPB sera, Figs 3, 4, and 5. This slightly lower-molecular-weight band, which was previously identified in sepsis patients,[10] is consistent with the expected serine protease cleavage product of TBG.[6] In patient 3 (Fig 5), the 49- to 50-kd band is already present in the preoperative serum but becomes more intense intraoperatively. None of the 12 control sera showed the 49- to 50-kd band.

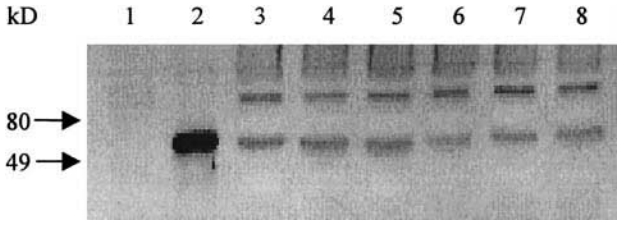

Fig 1. SDS-PAGE and immunoblotting of TBG from 12 control sera. Lane 1, molecular weight standards (Bio-Rad Lab, Hercules, CA); lane 2, purified human TBG; lanes 3 through 8, control sera. Intact serum TBG is seen at 54 kd, the same molecular weight as the TBG marker.

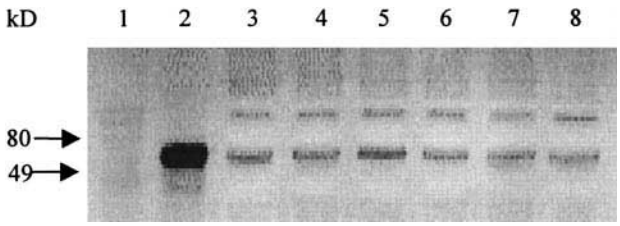

Fig 2. SDS-PAGE and immunoblotting of TBG from 12 control sera. Lane 1, molecular weight standards (Bio-Rad Lab, Hercules, CA); lane 2, purified human TBG; lanes 3 through 8, control sera. Intact serum TBG is seen at 54 kd, the same molecular weight as the TBG marker.

Effect of Elastase and Heat Treatment on Immunoassayable TBG

Figure 6 shows the effects of NE and heating on immunoassayable TBG. In the absence of serum, in vitro exposure of TBG to NE, which has been previously shown to cause TBG cleavage,[8-11] resulted in a 39% decrease in immunoassayable TBG concentration by RIA. In serum, the same concentration of NE caused only a 7% decrease in immunoassayable TBG, presumably because of serine protease inhibitors that neutralize NE. Heating at 60°C for 1 hour resulted in similar decreases of immunoassayable TBG in the absence (81%) and presence (89%) of serum. With RID, qualitatively similar results were obtained, although absolute values differed (Table 2).

DISCUSSION

The appearance of the 49- to 50-kd TBG cleavage product in serum confirms that characteristic serine protease cleavage of TBG occurs in CPB. This is probably because of the associated inflammatory response because similar cleavage is produced by NE[6,7] and by activated polymorphonuclear leukocytes.[10] The relatively low initial TBG concentration in patient 3 (Table 1)

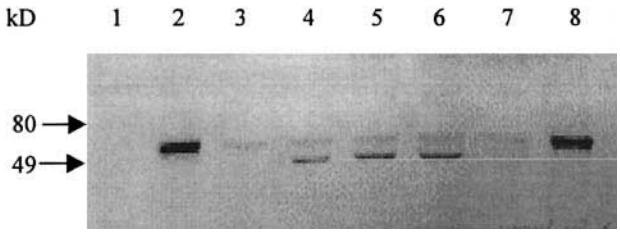

Fig 3. SDS-PAGE and immunoblotting of TBG from sera of patient 1. Lane 1, molecular weight standards; lanes 2 and 8, purified human TBG; lane 3, serum obtained preoperatively; lanes 4 through 7, sera obtained 4, 12, 24, and 72 hours after incision.

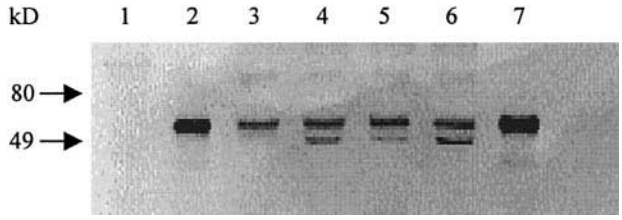

Fig 4. SDS-PAGE and immunoblotting of TBG from sera of patient 2. Lane 1, molecular weight standards; lanes 2 and 7, purified human TBG; lane 3, serum obtained at preoperative period; lanes 4 through 6, sera obtained 4, 12, and 24 hours after incision.

and the presence of the 49- to 50-kd TBG before surgery (Fig 5) suggest that proteolytic cleavage of TBG, perhaps caused by inflammation, may have occurred before surgery. However, chart review did not show a prior inflammatory condition other than the coronary artery disease.

The question arises whether protease cleavage of TBG can account for the decrease in the ratio of immunoassayable TBG to Alb that was previously described as "selective consumption of thyroxine binding globulin."[2] This might be expected on the basis of the conformational change associated with cleavage.[6] Heating to 60°C, which decreases TBG affinity for T_4,[13] has been described as also decreasing its affinity for TBG antibody.[14] These effects are presumably caused by conformational change. In this study, we show that exposure to NE, like heat inactivation, decreases immunoassayable TBG by both the RIA and RID methods, which demonstrated a rapid decrease in TBG concentration during CPB in our previous report.[2] Because the RIA method depends on both antibody and T_4 binding of TBG, an apparent decrease in TBG concentration can reflect not only a decreased TBG but also decreased TBG affinity for T_4 or the assay antibody. For the RID, an apparent decrease in concentration can be caused by decreased TBG affinity for antibody as well as a decrease in TBG concentration. Thus, it appears that the apparent rapid decrease in immunoassayable TBG during the inflammatory response associated with CPB may be explained, at least in part, by the conformational changes induced by characteristic serpin cleavage of TBG. It remains to be determined whether there is an accelerated clearance of the 49- to 50-kd cleavage product. Our findings support the hypothesis that the previously demonstrated rapid release of T_4 during the acute inflammatory reaction associated with CPB is caused by serine protease cleavage of TBG.

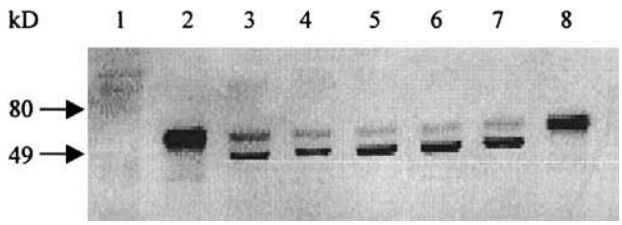

Fig 5. SDS-PAGE and immunoblotting of TBG from sera of patient 3. Lane 1, molecular weight standards; lanes 2 and 8, purified human TBG; lane 3, serum obtained preoperatively; lanes 4 through 7, sera obtained 4, 12, 24, and 72 hours after incision.

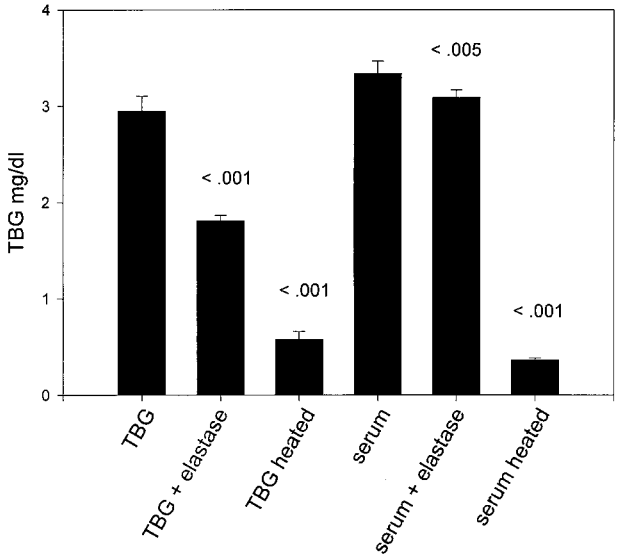

Fig 6. Effects of elastase and heat treatment on immunoassayable TBG. TBG, 0.75 μg, was incubated with NE, 0.0075 μg, at 37°C for 1 hour. Heat treatment was at 60°C for 1 hour. Results shown are the mean and SD of 6 determinations. The effect of elastase was greatly inhibited in the presence of serum.

Both the weakened T_4 binding of the 49- to 50-kd TBG cleavage product as well as variable effects of cleavage on the affinity of various measuring antibodies may contribute to apparent discrepancies in the relationship between ratios of free to bound T_4 ratios and immunoassayable TBG.[2,15] Specifically, a TBG assay sensitive to the 49- to 50-kd TBG cleavage product would show an increase in the free-to-bound T_4 ratio disproportionate to the decrease in TBG concentration, as has been reported by Chopra.[15]

In conjunction with evidence that elastase cleavage of TBG weakens thyroid hormone binding and releases T_4 from TBG in vitro,[8-10] the present observation of the rapid appearance of a TBG cleavage product during the inflammatory response associated with CPB supports the conclusion that inflammatory proteases release T_4 from TBG in vivo. This is similar to the proposed function of CBG proteolysis in cortisol release at sites of inflammation.[6,7] Because of circulating serine protease inhibitors, cleavage of TBG and release of T_4 is unlikely to occur in the general circulation. The recent demonstration of saturable high-affinity cellular TBG binding sites on cells[16] provides a potential locus for TBG cleavage and release of T_4. An analogous mechanism appears to release insulin-like growth factor I (IGF-I) to cellular sites by proteolysis of IGF-binding

Table 2. TBG by RID

	mg/dL (mean ± SD, n = 6)	% of Control
Purified TBG	4.0 ± 0.55	100%
Purified TBG + elastase	2.4 ± 0.33	60%
Purified TBG heated	1.4 ± 0.17	34%
Serum	1.9 ± 0.65	100%
Serum + elastase	2.1 ± 0.32	100%
Serum heated	<0.34	0%

protein 3.[17] Release of T_4 from TBG by local serine protease activity is consistent with the previously described accumulation of tracer activity in a pneumonic process after injection of ^{125}I T_4.[18] It is interesting to consider possible functions of T_4 release during inflammation. Tissue destruction during inflammation is associated with remodeling, and the release of T_4 may be involved in this process. The deiodinative metabolism of T_4 by polymorphonuclear cells at an infected site also could produce bactericidal concentrations of iodine.[19,20] Robbins[21] estimates that the release of approximately one half of the plasma bound T_4 into a small inflammatory site could produce bactericidal levels of iodine by leukocyte deiodination. The ability of proteases to release T_4 from TBG may not be limited to the inflammatory process. We suspect that TBG cleavage by cellular proteases is an ongoing process, even in the absence of overt inflammation, but does not accumulate sufficient cleavage products to be routinely demonstrable. Thus, local access to TBG-bound thyroid hormone may be determined in part by cellular exhibition of TBG-binding sites and proteolytic activity.

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