Biochemical Pharmacology, Vol. 34, No. 19, pp. 3543–3546, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

IDENTIFICATION OF THE MAJOR BINDING PROTEIN OF SALMON CALCITONIN IN THE RAT

YOSHIHIRO SAITO, MASATO YASUHARA, KATSUHIKO OKUMURA and RYOHEI HORI
Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, 606, Japan

(*Received* 18 *December* 1984; *accepted* 13 *May* 1985)

Abstract—The major serum binding protein of salmon calcitonin (sCT) in the rat was identified. High-molecular-weight (HMW) forms of sCT, produced by the incubation of radioactive sCT in rat serum, were isolated by gel filtration and analysed by chromatofocusing. The major radioactive peak was eluted at the region of albumin in gel filtration, and this peak had a slightly higher pI than albumin on chromatofocusing.

Immunoreactivity of the radioactive peak fraction in chromatofocusing, examined by immunodiffusion, showed that the major protein in the fraction was albumin. Immunoreactivity of the radioactive HMW fraction isolated by gel filtration was studied by immunoprecipitation, indicating that a large portion of the fraction reacted with anti-rat albumin antiserum. These results suggested that the radioactive peak in chromatofocusing represents the complex of sCT and rat albumin.

Further, HMW forms of sCT were analysed by immunoelectrophoresis and autoradiography, and most of the radioactivity was found on the precipitation line of albumin.

These results demonstrate that the major binding protein of sCT in rat serum is albumin.

Though the immunochemical heterogeneity of endogenous calcitonin (CT) has been well-documented in plasma and tissue [1–14], details such as the biological activity of immunochemical forms other than the authentic one are not known.

On the other hand, there is little information on the forms of exogenous CT in blood plasma, which originates in a different species and reaches higher levels in the blood. So analysis of exogenous CT in blood and tissue should give useful information for therapy.

Previously we have shown that some of intravenously injected salmon CT (sCT) is converted into high-molecular-weight (HMW) forms *in vivo*, and that HMW forms similar to those observed *in vivo* can be produced by incubation of sCT with rat serum *in vitro*, and the major component of the HMW forms *in vitro* is thought to be the same one of those *in vivo* [15, 16].

In this study, HMW forms produced in rat serum were further analysed for their physicochemical and immunochemical properties to clarify the forms of exogenous sCT in blood.

MATERIALS AND METHODS

Preparation of radioactive tracers. Salmon calcitonin (Sandoz AG, Basel, Switzerland) and rat albumin (Cappel Laboratories, Inc., U.S.A.) were labelled with ^{125}I by the method of Hunter and Greenwood [17], and ^{125}I-labelled sCT was purified by gel filtration before use.

Preparation of HMW forms of sCT (HMWsCT). Radioactive sCT (12 mU/ml) was incubated in freshly isolated rat serum at 37° for 60 min to prepare HMWsCT with low radioactivity. In a separate experiment, HMWsCT with high radioactivity was prepared by incubation of radioactive sCT (2.8 U/ml) in rat serum.

Isolation of HMWsCT. The incubation mixture containing HMWsCT with low radioactivity (300 μl) was fractionated by gel filtration on a Sephadex G-200 column (1.6 × 75 cm; 0.03 M phosphate buffer with 0.2 M sodium chloride, pH 7.0, as eluant). The radioactive HMWsCT peak fractions were pooled, dialysed against distilled water, and lyophilized. For the separate experiment, HMWsCT with high radioactivity was isolated by gel filtration on a Sephadex G-50 column (0.9 × 20 cm, 0.1 M acetic acid as eluent) and lyophilized.

Chromatofocusing. The HMWsCT (about 30 mg) isolated by gel filtration on Sephadex G-200 was dissolved in Polybuffer 74-HCl, pH 4.0, diluted in distilled water 1:8, and put on a chromatofocusing column (1 × 20 cm) equilibrated with 0.025 M histidine–HCl, pH 6.2. The sample was eluted with diluted Polybuffer 74-HCl, pH 4.0 at a linear flow rate of 33 cm/hr.

Immunodiffusion. The samples obtained in chromatofocusing (the major radioactive peak and protein peak fractions), desalted and lyophilized, were analysed by immunodiffusion in an agar gel plate, which was prepared using 1.2% agar solution (0.05 M barbital buffer, pH 8.6) and was about 1.5 mm thickness. The distance between the adjacent margins of the two wells (each 2 mm in diameter) was 11 mm. After incubation at 28° for 20 hr, the plate was washed with saline, dried, and stained with Amidoblack 10B.

Immunoprecipitation. The HMWsCT with high radioactivity was analysed by immunoprecipitation using the double antibody technique. Radioactive HMWsCT (10 μg), a mixture of tracer amount of radioactive rat albumin and rat albumin (10 μg), and

a mixture of tracer amount of radioactive sCT and rat albumin (10 μg) were used. The antibodies were rabbit anti-rat albumin (first antibody) and goat anti-rat IgG (second antibody).

Immunoelectrophoresis and autoradiography. The HMWsCT (about 65 μg) with high radioactivity and rat serum (5 μl, as control) were electrophoresed in 1.2% agar equilibrated with 0.05 M barbital buffer, pH 8.6, at 8 mA/plate (2.6 × 7.6 cm) for 140 min and a trough was filled with 70 μl of antiserum. After incubation at 28° for 24 hr, the plate was washed extensively with saline, dried, and exposed to Sakura MARG film at −40° for 7 days. The film was developed with Konidole X and fixed with Konifix (a film, developing and fixing agents: Konishiroku Photo Ind. Co., Ltd., Tokyo, Japan). Finally the plate was stained with Amidoblack 10B.

RESULTS

Isolation of HMWsCT

The HMWsCT with low radioactivity was isolated by gel filtration (Fig. 1). A radioactive peak other than that for ^{125}I-sCT was seen at the region of albumin, which indicates the molecular weight of the major component of HMWsCT is close to 66,000. Radioactive peak fractions (hatched) were collected, dialysed and lyophilized.

Chromatofocusing of HMWsCT

Radioactive HMWsCT isolated in Fig. 1 was analysed by chromatofocusing (Fig. 2). There was a major radioactive peak eluted at pH 5.0 and a major protein peak at 4.8–4.9, which corresponds to the pI value of albumin.

Immunodiffusion

Major radioactive and protein peak fractions in Fig. 2 were analysed by immunodiffusion (Fig. 3).

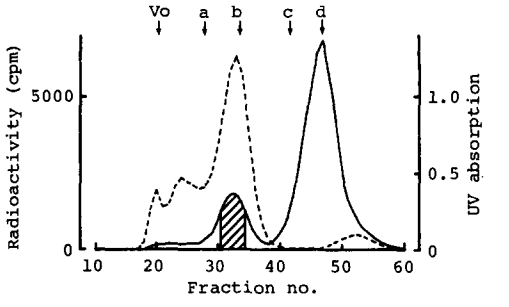

Fig. 1. Isolation of HMWsCT by gel filtration on Sephadex G-200. After incubation of ^{125}I-sCT (12 mU/ml) in rat serum at 37° for 60 min, the incubation mixture was fractionated by gel filtration. Radioactivity (——) and u.v. absorption at 280 nm (– – – –) were monitored. The arrows indicate the void volume (V_0) and the elution volume of the markers: (a) catalase (MW 232,000); (b) bovine serum albumin (MW 66,000); (c) chymotrypsinogen A (MW 25,000); and (d) ^{125}I-sCT (MW 3500).

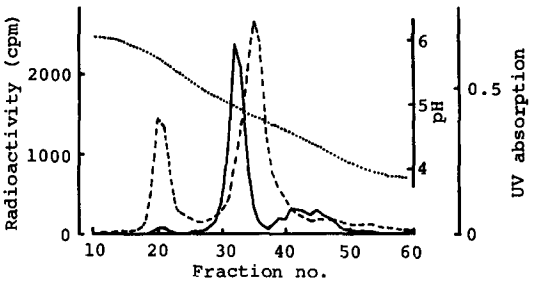

Fig. 2. Chromatofocusing of HMWsCT. The HMWsCT (30 mg) isolated in Fig. 1 was chromatofocused on a PBE 94 gel column, starting at pH 6.2, 0.025 M histidine–HCl; eluted with pH 4.0 Polybuffer 74–HCl at a linear flow rate of 33 cm/hr. Radioactivity (——), pH (· · · · ·), and u.v. absorption at 280 nm (– – – –) were monitored.

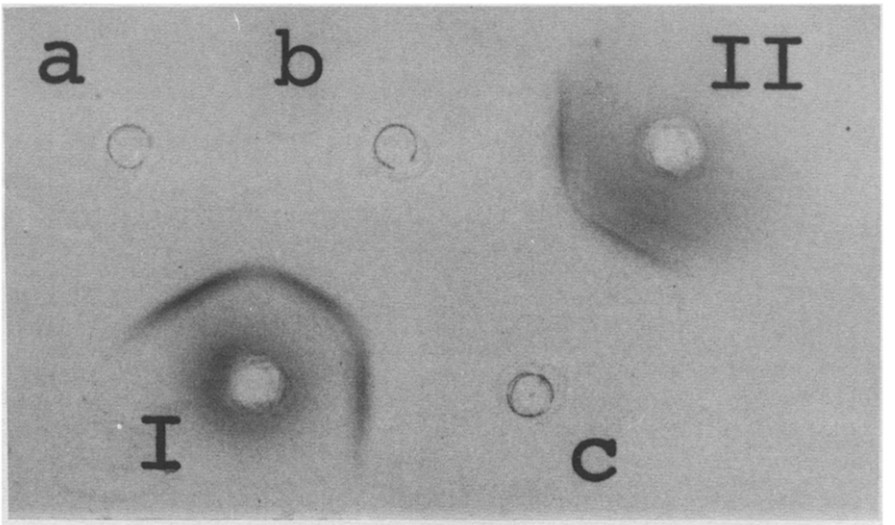

Fig. 3. Immunochemical properties of HMWsCT examined by immunodiffusion. Radioactive peak fraction (b, 10–20 μg of protein), protein peak fraction (c, 10–20 μg of protein), both isolated in Fig. 2 and rat albumin (a, 10–20 μg) were reacted with rabbit anti-rat serum antiserum (I, 5 μl) and rabbit anti-rat albumin antiserum (II, 5 μl) in agar gel plate at 28° for 20 hr.

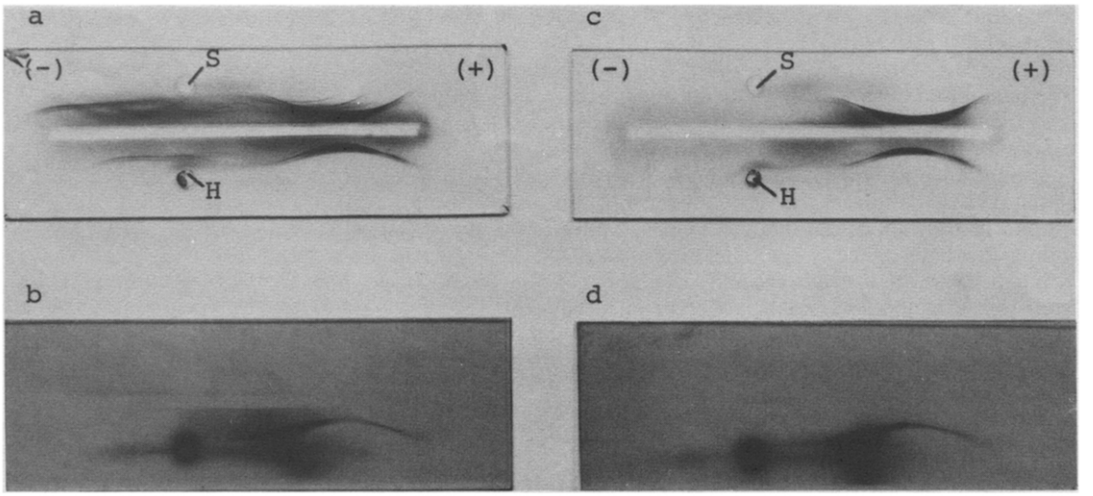

Fig. 4. Immunoelectrophoresis and autoradiography of HMWsCT. Immunoelectrophoresis of HMWsCT (H) and rat serum (S) was performed using rabbit anti-rat serum antiserum (a) or rabbit anti-rat albumin antiserum (c): 65 μg of HMWsCT, 5 μl of rat serum, and 70 μl of antisera were used. Autoradiography was done with the same plates: (b) for (a), and (d) for (c).

Precipitation lines for rat albumin, the major protein peak fraction, and the major radioactive peak fraction, reacting with anti-rat serum, were fused, and those for the major protein peak and radioactive peak fractions, reacting with anti-rat albumin, were also fused.

Immunoprecipitation

Immunoreactivity of HMWsCT with high radioactivity was estimated by immunoprecipitation, and was compared to that of radioactive rat albumin or radioactive sCT. The HMWsCT was precipitated by 71% of the total radioactivity, which is more than two-thirds of the value for rat albumin (97%); sCT was precipitated by only 2%.

Immunoelectrophoresis and autoradiography

Using anti-rat serum antiserum, several kinds of serum protein were observed after staining (Fig. 4a), but radioactivity was present on the precipitation line of albumin (Fig. 4b). When anti-rat albumin antiserum was used, the precipitated protein was albumin only (Fig. 4c), and the distribution of radioactivity was similar to that in Fig. 4b (Fig. 4d).

DISCUSSION

That some protein binding of pig CT occurs in pig or human plasma was suggested by Leggate *et al.* [13], who measured CT by bioassay, but the binding protein has not been determined. Habener *et al.* [18] showed that incubation of ovine or salmon CT labelled with ^{125}I in bovine serum resulted in protein-bound radioactivity; they did not examine the properties of the product or the mechanism of its formation. Our previous work showed that some of the sCT injected intravenously or incubated in rat serum was bound by disulfide bridges to a serum protein with a molecular weight close to that of albumin [15, 16]. Thus we know that some part of exogenous CT is bound to serum protein. Here it was found that the major serum binding protein of sCT in the rat is albumin by using gel filtration, chromatofocusing and immunochemical analysis of HMWsCT.

The difference observed on chromatofocusing in the elution position of the major peaks of radioactivity and u.v. absorption (Fig. 2) suggests that radioactive HMWsCT represents either a complex of sCT and a minor serum protein with a pI value close to that of albumin, or a complex of sCT and serum albumin with a pI that has become slightly higher than that of albumin because of conjugation with sCT, which has a high pI (pI = 10.4). Figure 3 shows that the major protein in the major radioactive and in the major protein peak fractions was albumin, supporting the latter possibility. Immunoprecipitation study showed that HMWsCT reacted for the most part with anti-rat albumin antiserum, which also means that HMWsCT is chiefly composed of sCT conjugated with serum albumin. That the value of precipitated radioactivity for HMWsCT was slightly lower than that for rat albumin may reflect (1) altered conformation of rat albumin upon conjugation with sCT, (2) the release of radioactive sCT from HMWsCT during incubation in the presence of bovine serum albumin, or (3) contamination with a conjugate of sCT and serum protein other than albumin. The gap between the radioactive and the protein peaks (Fig. 2) was also observed on chromatofocusing of sCT-rat albumin conjugate (data not shown), which was prepared by the incubation of sCT in rat albumin solution, suggesting that HMWsCT with a slightly higher pI than that of albumin is the conjugate of sCT and albumin. Finally, immunoelectrophoresis and autoradiography (Fig. 4) clearly demonstrated that the major serum binding protein of sCT was albumin. As shown in Figs 4b and 4d, radioactivity was chiefly on the left half of the precipitation line of albumin, which

is consistent with the pI of radioactive HMWsCT being slightly higher than that of albumin (Figs 2 and 5).

We have already shown that protein-bound sCT linked by disulfide bridges can release sCT in the presence of thiols [16], and here we identified the major binding protein. These results suggest that sCT bound to albumin may be a depot form of this hormone in the blood, and should help in understanding the heterogeneity and the biological significance of various forms, especially of the transporting forms in blood plasma of exogenous sCT that is now used therapeutically.

REFERENCES

1. P. H. Tobler, F. A. Tschopp, M. A. Dambacher, W. Born and J. A. Fischer, *J. clin. Endocr. Metab.* **57**, 749 (1983).
2. S. Morimoto, Y. Okada, T. Onishi, S. Takai, A. Miyauchi, S. Lee, Y. Nishioka and Y. Kumahara, *Endocrinol. Jpn.* **28**, 583 (1981).
3. W. C. Dermody, M. A. Rosen, R. Ananthaswamy, W. M. McCormick and A. G. Levy, *J. clin. Endocr. Metab.* **52**, 1090 (1981).
4. D. Goltzman and A. S. Tischler, *J. clin. Invest.* **61**, 449 (1978).
5. B. A. Roos, J. G. Parthemore, J. C. Lee and L. J. Deftos, *Calcified Tissue Res.* **225**, 298 (1977).
6. R. H. Snider, O. L. Silva, C. F. Moore and K. L. Becker, *Clin. chim. Acta* **76**, 1 (1977).
7. O. L. Silva, R. H. Snider, K. L. Becker and C. F. Moore, *J. Endocr.* **73**, 183 (1977).
8. L. J. Deftos, B. A. Roos, D. Bronzert and J. G. Parthemore, *J. clin. Endocr. Metab.* **40**, 409 (1975).
9. F. R. Singer and J. F. Habener, *Biochem. biophys. Res. Commun.* **61**, 710 (1974).
10. S. B. Baylin, K. C. Wieman, J. A. O'Neil and B. A. Roos, *J. clin. Endocr. Metab.* **53**, 489 (1981).
11. J. A. O'Neil, R. S. Birnbaum, A. Jacobson and B. A. Roos, *Endocrinology* **108**, 1098 (1981).
12. P. H. Tobler, M. A. Dambacher, W. Born, P. U. Heitz, R. Maier and J. A. Fischer, *Cancer Res.* **43**, 3793 (1983).
13. J. Leggate, A. D. Care and S. C. Frazer, *J. Endocr.* **43**, 73 (1969).
14. S. I. Girgis, C. J. Hillyard, I. MacIntyre and M. Szelke, in I. MacIntyre and M. Szelke (Eds.) *Molecular Endocrinology*, p.175. Elsevier/North Holland Biomedical Press, Amsterdam (1977).
15. R. Hori, Y. Saito, M. Yasuhara and K. Okumura, *J. Pharmacobio-Dyn.* **7**, 910 (1984).
16. K. Okumura, Y. Saito, M. Yasuhara and R. Hori, *J. Pharmacobio-Dyn.* **7**, 917 (1984).
17. W. M. Hunter and F. C. Greenwood, *Nature, Lond.* **194**, 495 (1962).
18. J. F. Habener, F. R. Singer, L. J. Deftos and J. T. Potts, Jr., *Endocrinology* **90**, 952 (1972).