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Note

Separation of somatomedins and somatomedin inhibitors by size exclusion high-performance liquid chromatography

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The somatomedins are a family of circulating peptides with structure and biological actions similar to those of insulin [1]. Growing cartilage can be stimulated in vitro by physiologic concentrations of somatomedins, and these factors are thought to mediate the growth-promoting actions of growth hormone [2]. The actions of somatomedins appear to be modulated by other circulating factors, the somatomedin inhibitors [3]. Although both somatomedins and somatomedin inhibitors can be detected in serum after fractionation by conventional gel permeation chromatography [4], these techniques provide poor separation and are not readily applicable to human or animal studies with large numbers of samples. In the present studies, we report the use of size exclusion high-performance liquid chromatography (HPLC) in the separation of somatomedins and inhibitors, and application to the examination of pathophysiology in animal models.

EXPERIMENTAL

Animals and serum

Male CD rats were obtained from Charles River Breeding Labs. and male Sprague—Dawley rats from King Animal Labs. Hypophysectomized male rats were used at 80—100 g. Normal rat serum was used as a source enriched in somatomedins, and was pooled from 90—150 g rats as previously described [4]. Sera from rats with severe diabetes were selected for enrichment in somatomedin inhibitors, and pooled as described [4]. Human serum was obtained from normal males and females, 20—55 years old. For studies of

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pathophysiology, serum was obtained from normal rats (120—160 g), fasted rats (weight loss 33 ± 1 g after three days), and unselected diabetic rats with moderate diabetes (mean serum glucose 441 mg/dl, two days after streptozotocin administration). All samples were kept at −20°C prior to study.

Bioassays

Somatomedin activity was determined as the ability of samples to stimulate sulfate uptake by hypophysectomized rat costal cartilage, as described previously [4]. This tissue responds to all somatomedins identified to date. Rat-to-rat variations require examinations in single animals in order to achieve acceptable precision in the bioassay, and samples from any column can only be compared after assay with cartilage from the same rat. Because of these inherent limitations in assay size (sixteen unknowns per rat), column eluates were usually divided into twelve to sixteen fractions. Stimulation by HPLC fractions was assessed at 0.4%, 2% or 5% (v/v) final concentration. Increases in sulfate uptake were expressed as percentage increase above buffer levels or as a percentage of stimulation provided by whole normal serum (1%, v/v) in the same assay. Somatomedin inhibitory activity was measured by the ability of samples to blunt cartilage stimulation by somatomedins, as described previously [4]. Cartilage incubations were supplemented with whole normal serum at 0.5% or 1% (v/v), and fractions added at 2% or 8%. In dose-response studies, potency was estimated by parallel-line analysis as described previously [5].

High-performance liquid chromatography

Toyo Soda TSK 2000 SW (10-μm modified silica) 100 × 7.5 mm and 600 × 7.5 mm size exclusion guard and separation columns, respectively, were eluted isocratically at 0.7 ml/min using a Beckman 112 pump. A Beckman 210 injector was used for sample application, and absorbance at 280 nm was monitored continuously with a Beckman 160 detector and a Kipp and Zonen BD40 recorder. Samples were eluted with 0.1 *M* ammonium formate, adjusted to pH 3.0 with concentrated formic acid. The eluate was collected in 1.40-min fractions (Pharmacia FRAC-100) beginning with elution of the void volume. Added $Na^{125}I$ appeared in fraction 13. Eluted fractions were lyophilized, relyophilized two to three times after addition of deionized water, reconstituted in 0.5 ml Krebs phosphosaline—amino acid buffer [3], and kept at −20°C prior to bioassay. All chemicals were of analytical-reagent grade. HPLC-grade water was prepared by passing deionized water through a Millipore Norganic cartridge and a Durapore filter, and buffers were filtered through a Durapore membrane (0.22 μm) and degassed before use.

Serum samples were acidified by addition of concentrated formic acid, in a ratio of 240:10, and incubated at either 37°C or room temperature for 1 h. Before application, samples were either Millipore-filtered (0.22 μm), or centrifuged (12 000 *g* for 5 min). Samples (200 μl, approximately 16 mg protein) were injected with a 250-μl Hamilton syringe. Each separation was followed by a 15—20 min rinse with eluting buffer, and the columns were kept in 20% methanol between studies.

RESULTS

Preliminary experiments (not shown) were conducted to determine suitable separation conditions. Porcine insulin and α-chymotrypsinogen-A were used as model proteins of size roughly comparable to that of the somatomedins and somatomedin inhibitors, respectively. Eluted peak separation and contour were not improved by 0.2 *M* buffer, or by decreasing flow-rate to 0.5 ml/min. Pre-acidification was necessary for separation of somatomedins from larger carrier proteins. Under these conditions, elution of protein standards [ovalbumin, molecular weight (MW) 45 000; α-chymotrypsinogen-A, MW 25 700; ribonuclease-A, MW 13 700; insulin, MW 5784; glucagon, MW 3485; and cyanocobalamin, MW 1355) was essentially linear when plotted as log MW versus K_{av} ($r = 0.99$).

Normal human serum was then examined, as shown in Fig. 1. Somatomedins were found in fractions 9 and 10, and somatomedin inhibitors in fractions 4—8. Adjacent fractions (not shown) had no significant stimulatory or inhibitory activity, respectively. Fractions with significant somatomedin activity had apparent MW 5050—16 000, and fractions with somatomedin inhibitory activity had apparent MW 16 000—340 000. Fractions 9 and 10 contained 0.4% of recovered 280-nm absorbing material, and fractions 4—8 contained 30.4% of 280-nm absorbing material.

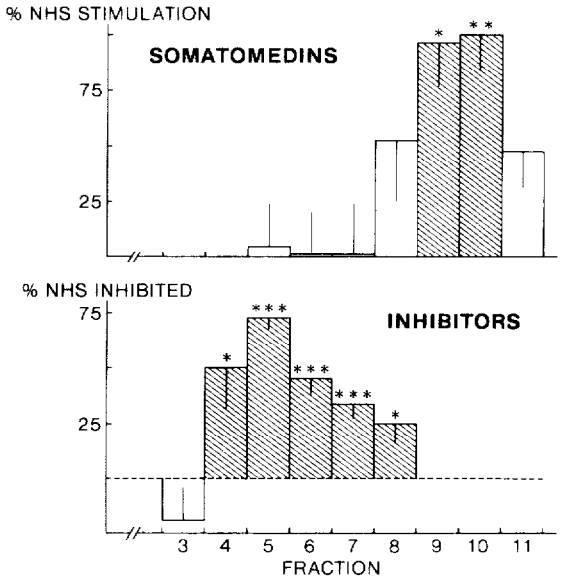

Fig. 1. Isocratic separation of somatomedins and somatomedin inhibitors in four and six samples of normal human serum (NHS), respectively, by HPLC. Whole serum was acidified, centrifuged, and 200 μl were applied to a TSK-2000 column eluted at 0.7 ml/min with 0.1 *M* ammonium formate, pH 3.0, as described in Experimental. Fraction collection began with the excluded volume, as determined with blue dextran. Somatomedin activity was expressed as stimulation of cartilage sulfate uptake relative to that produced by unfractionated NHS, and somatomedin inhibitor activity was expressed as percentage inhibition of cartilage stimulation produced by NHS. Hatching denotes fractions with statistically significant activity. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. Mean ± S.E.M.

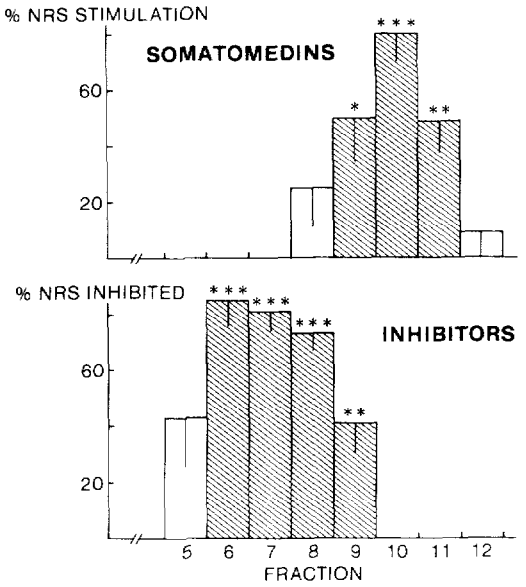

Fig. 2. Separation of somatomedins in normal rat serum (NRS) and somatomedin inhibitors in diabetic rat serum, six samples each, by HPLC. Procedures as in Fig. 1 and Experimental. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. Mean ± S.E.M.

The profile of somatomedins and somatomedin inhibitors in rat serum was comparable to that of human serum, as shown in Fig. 2. Somatomedins (in normal rat serum) were found in fractions 9—11, and somatomedin inhibitors (in diabetic rat serum) were found in fractions 6—9, slightly retarded as compared to inhibitors in human serum. Of total eluted somatomedins from normal rat serum 28% were found in fraction 9, versus 13% of total eluted somatomedin inhibitors from diabetic rat serum. Somatomedins had apparent MW 2600—16 000, (fractions (9—11) and somatomedin inhibitors apparent MW 9000—98 200. Fractions 9—11 contained 0.3% of recovered 280-nm absorbing material, and fractions 6—9 had 3.7%.

Such HPLC separation permitted rat models of pathophysiology to be tested for alterations in circulating, biologically active somatomedins and somatomedin inhibitors, as shown in Fig. 3. Somatomedin activity was measured in pooled fractions 9—11, and somatomedin inhibitor activity in pooled fractions 6—8 (apparent MW 16 000—98 200). Somatomedins were undetectable in samples from hypophysectomized or diabetic rats (cartilage sulfate uptake at buffer levels), and marginally higher in fasted rats (somatomedin potency 6% compared to normal rats). In contrast, somatomedin inhibitors were present in all groups of animals. Diabetic rats exhibited twenty-fold increases in inhibitor potency compared to normal animals, fasted rats exhibited three-fold increases, and no change was seen in hypophysectomized rats. Inhibitory dose-response lines had slopes which did not differ significantly, indicating qualitative similarity of the factors being measured. Lesser quantities of somatomedin inhibitors were found in pooled fractions 4 and 5 (apparent MW 98 200—340 000), with no differences among animal groups.

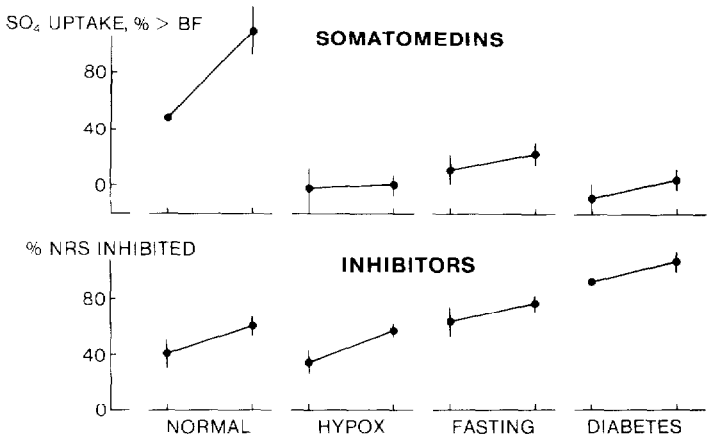

Fig. 3. Somatomedins and somatomedin inhibitors in normal, hypophysectomized (hypox), fasted, and diabetic rats, determined after preacidified serum was subjected to size exclusion HPLC (pH 3.0). Lines connect points denoting bioassay responses to two concentrations of somatomedins (upper panel) and somatomedin inhibitors (lower panel); in each case, the lower concentration is on the left. Somatomedin activity (pooled fractions 9—11) was measured by stimulation of cartilage SO_4 uptake [above buffer level (BF)] at 0.4% and 2% sample, and somatomedin inhibitor activity (pooled fractions 6—8) was measured by the ability of samples (at 2% and 8%) to decrease stimulation by 1% added normal rat serum (NRS). Mean ± S.E.M. for four samples each.

DISCUSSION

The regulation of circulating growth-related factors cannot yet be probed fully by radioligand assay techniques. Although two somatomedins have been isolated and can now be measured by radioimmunoassay [6], recent studies indicate that additional species with somatomedin-like activity are present in the circulation [7, 8]. The somatomedin inhibitors are even less well characterized [9], and at present can be measured only by bioassay. Thus, biological assay systems must be used to quantitate the "total" physiologic contribution of the various somatomedins, and to measure circulating somatomedin inhibitors as well. Such assays require the use of buffer components which either can be removed easily or do not interfere with the biological response, and also require prior separation of somatomedins from somatomedin inhibitors in individual samples.

Since recovery of somatomedins from ion-exchange separations has often been poor, somatomedins and somatomedin inhibitors have largely been separated on the basis of size. Previous reports from our laboratory and other workers [4, 10] have indicated that in samples of serum examined at neutral pH, the somatomedin inhibitors are smaller than the somatomedins (bound to carrier proteins), but at acidic pH the somatomedin inhibitors are larger than non-carrier bound somatomedins. Such differences in size have been exploited in studies of somatomedins and somatomedin inhibitors in samples subjected to conventional gel chromatography [11, 12]. However, these procedures are slow and separation may be relatively poor, with 20—40% of somatomedin activity eluting in the region where 30—60% of somatomedin inhibitory activi-

ty is found [13]. The HPLC approaches employed in the present studies resulted in improved separation, and the rapidity of the procedures facilitated initial physiological examinations. Additional improvements in separation might be obtained by decreasing sample size and the use of buffers of higher ionic strength.

Using animal models, circulating somatomedins and somatomedin inhibitors were measured to examine the applicability of the HPLC separations. Decreases in somatomedins were found in fasted and diabetic as well as hypophysectomized rats. Somatomedin inhibitors were markedly elevated in diabetic animals and modestly elevated with fasting, but unchanged by hypophysectomy. These observations are consistent with the hypothesis of regulation of net circulating somatomedin activity by nutrition and insulin as well as growth hormone [9], and suggest that nutrition and insulin affect both somatomedins and somatomedin inhibitors, whereas growth hormone affects only the somatomedins. In combination, these findings indicate that size exclusion HPLC can be combined successfully with bioassay determinations in the assessment of normal and abnormal physiology.

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REFERENCES

1 L.S. Phillips and R. Vassilopoulou-Sellin, N. Engl. J. Med., 302 (1980) 371.
2 W.D. Salmon, Jr. and W.H. Daughaday, J. Lab. Clin. Med., 49 (1957) 825.
3 L.S. Phillips, V.R. Bajaj, A.C. Fusco and C.K. Matheson, Diabetes, 32 (1983) 1117.
4 L.S. Phillips, D.C. Belosky, H.S. Young and L.A. Reichard, Endocrinology, 104 (1979) 1519.
5 L.S. Phillips and A.C. Herington, Comput. Biomed. Res., 8 (1975) 362.
6 J. Zapf, Ch. Schmid and E.R. Froesch, Clin. Endocrinol. Metab., 13 (1984) 3.
7 P.N. Cockerill, H.J. Cornell and A.C. Herington, J. Endocrinol., 85 (1980) 267.
8 W.F.P. Blum, M.B. Ranke and J.R. Bierich, Excerpta Med. Int. Congr. Ser., 652 (1984) 313.
9 L.S. Phillips and T.G. Unterman, Clin. Endocrinol. Metab., 13 (1984) 145.
10 W.D. Salmon, Jr., L.A. Holladay and V.J. Burkhalter, Endocrinology, 112 (1983) 360.
11 M. Binoux, C. Lassarre and D. Seurin, Acta Endocrinol. (Copenhagen), 93 (1980) 83.
12 V.R. Bajaj, A.C. Fusco and L.S. Phillips, Clin. Res., 30 (1982) 720A.
13 L.A. Stivaletta and S. Goldstein, unpublished results.