EARLY FATE OF SOMATOSTATIN IN THE CIRCULATION OF THE RAT AFTER INTRAVENOUS INJECTION

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SUMMARY

The products of somatostatin in the circulation have been investigated by high-pressure liquid chromatography. Plasma collected 1 min after intravenous injection of cyclic (oxidized) somatostatin showed a single ultraviolet absorbing peak. The total plasma content of this product was equivalent to 10–20% of the injected dose. Amino acid analysis showed that 80–90% of the material in the peak was [des-Ala\(\text{\textsubscript{1}}\)]-somatostatin and the remainder was unchanged peptide. [des-Ala\(\text{\textsubscript{1}}\)]-Somatostatin is rapidly formed in blood and plasma in vitro and according to other workers may be fully active. In contrast, 1 min after injection of linear (reduced) somatostatin, no products could be detected in the circulation. Incubations in vitro resulted in rapid conversion of the linear somatostatin to a product similar to the cyclic form. However, in vivo, very efficient clearance of linear somatostatin must occur even more rapidly than cyclization. In view of the very different clearance rates of the two forms of somatostatin, it is important to know whether endogenous somatostatin is released in the cyclic or the linear form. The absence of detectable concentrations of inactive peptide fragments in the circulation suggests that inactivation of somatostatin occurs in the tissues.

INTRODUCTION

It has been established by many workers that somatostatin is widely distributed throughout the body and has multiple inhibitory actions, among them inhibition of the secretion of growth hormone, insulin, glucagon and gastrin (Bloom, Mortimer, Thorner, Besser, Hall, Gomez-Pan, Roy, Russell, Coy, Kastin & Schally, 1974; Vale, Brazeau, Rivier, Brown, Boss, Rivier, Burgus, Ling & Guillemín, 1975). Somatostatin might, therefore, have future clinical applications in diabetes mellitus, possibly in combination with insulin (Gerich, 1976), or in the treatment of gastric acid hypersecretion (Lipmann & Borella, 1976). Although the short-lived effects of somatostatin are a limitation on its clinical usefulness, very little information is available as to its fate in vivo. Until it is understood how and where the molecule is taken up and degraded, no guidance can be offered as to which analogues should be synthesized in the search for longer acting and more potent substances.

In this study, synthetic linear and cyclic somatostatin (structures shown in Table 1) were injected intravenously into rats, and high-pressure liquid chromatography (HPLC) was used for the detection and identification of the peptides and their fragments in the blood.

MATERIALS AND METHODS

Materials

Disposable syringes used for extraction columns were from Gillette Surgical (Isleworth, Middlesex). Porous Teflon disks were cut from sheets obtained from Aerof Plastics...
Sphersorb ODS-silica (10 µm) was obtained from Phase Separations (Queensferry, Clwyd) and Porasil A (35–70 µm mesh) from Waters Associates (Northwich, Cheshire). Octadecyltrichlorosilane was obtained from the Aldrich Chemical Company (Gillingham, Dorset). The HPLC columns and accessories were obtained from Jobling Laboratory Division (Stone, Staffordshire). Male Wistar rats, from Charles River (U.K.) Ltd (Margate, Kent), were used throughout the experiments. Somatostatin was kindly supplied by Dr W. Rittel (Ciba-Geigy, Basle, Switzerland). Analar reagents were used whenever possible.

| Table 1. Structure of synthetic linear and cyclic somatostatins |
| (a) Cyclic (oxidized) form |
| Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys |
| (b) Linear (reduced) form |
| Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys |

**Methods**

**Preparation of linear somatostatin**

Cyclic somatostatin (5 mg, 3.05 µmol) was reduced with excess dithiothreitol (10 mg, 64.94 µmol) in 4 ml borax buffer (4% disodium tetraborate decahydrate, 3% boric acid), pH 8.1, for 40 min at room temperature. The solution of reduced peptide was acidified with 1% trifluoroacetic acid and extracted using the procedure described under 'Plasma extraction' to give an eluate which was diluted with 4 vol. water and freeze-dried. The recovery of peptide was 60–70% and measurement of the sulphydryl (—SH) content using Ellman's reagent (Ellman, 1959) showed 95% of it to be in the reduced form. The product gave a single peak on HPLC when monitored at 280 nm and with the Ellman reaction.

**Preparation of octadecasilyl Porasil (ODS–Porasil)**

Porasil Type A (200 ml) was washed in concentrated hydrochloric acid, water and methanol in turn. After gentle drying (40–50°C), and then several washes in a 50:50 mixture of chloroform and chloroform saturated with water, 20 ml octadecyltrichlorosilane in 200 ml dry chloroform were added. After 1 h, the supernatant fraction was decanted and the ODS–Porasil rinsed several times in dry chloroform and then methanol, and finally dried.

**Packing of columns for HPLC**

The ODS–silica high-pressure column consisted of a glass column (4 mm × 25 cm) dry-packed manually with 10 µm ODS–Sphersorb. Portions of silica (enough to fill a few cm of column at a time) were poured in and allowed to settle by tapping the column and then tamping down firmly using a rod with a close-fitting Teflon tip. A vacuum was applied to the lower end of the column each time the rod was withdrawn. This method gave columns showing high resolution.

**Animal experimentation procedure**

Rats, anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and then heparinized (0.2 ml of a 1% solution, w/v), were exsanguinated through the dorsal aorta 1 min after injection of 1 mg somatostatin (in 1 ml buffer) into a tail vein. The peptide was stored in 0.01 M-HCl, in saline (it was less stable in saline alone), and the pH was adjusted to approximately 7·0 with 0·1 M-disodium hydrogen orthophosphate immediately before injection.
Plasma extraction

Plasma obtained by the above procedure (4–6 ml) was passed through a small extraction column, consisting of 1 ml coarse ODS-silica (ODS-Porasil A 35–70 µm) packed between two porous Teflon disks in a 2 ml syringe barrel. The syringe plunger was used to apply the entire plasma sample to the column in 1 ml portions; the column was then washed with 1% aqueous trifluoroacetic acid (3 × 1 ml) and eluted with 2 ml 80% aqueous methanol : 1% trifluoroacetic acid (800 ml methanol and 10 ml trifluoroacetic acid made up to 1 litre with water). Extraction of a plasma sample took about 5 min. Under these conditions most (90% cyclic or 80% linear) of a dose of somatostatin added to whole blood was recovered from plasma, indicating very little uptake of peptide into the red blood cells. It was also possible to extract quite large volumes of whole blood; 90% of an added dose of cyclic somatostatin (50 µg) could be recovered from 4 ml blood.

High-pressure liquid chromatography

The eluate from the extraction column was diluted with 3 vol. 1% trifluoroacetic acid and pumped directly on to an ODS-silica high-pressure column. A linear solvent gradient was used for the separation and purification of samples. Two identical beakers containing equal volumes (50 ml) of starting solvent (aqueous 1% trifluoroacetic acid) and final solvent (80% acetonitrile : 1% trifluoroacetic acid) were connected by a glass siphon containing final solvent. Solvent from the first beaker was magnetically stirred and pumped into the column at a flow rate of 45·4 ml/h. A complete elution took about 1·5 h and during this time the column pressure ranged between 3·4 and 5·4 MPa. All solvents were kept warm on a hot-plate, to reduce the amount of dissolved air entering the system. Attention should be paid to ventilation. The column eluate was monitored for compounds containing tryptophan by u.v. absorption at 280 nm and also for free—SH groups using the Ellman reaction. During preparative experiments, only the u.v. monitor was used for detection. Otherwise, after the solvent had passed through a Uvicord it was continuously mixed with 4% borax : 1% NaOH buffer bringing the pH up to approximately 9·0, and then with Ellman's reagent (0·01% 5,5'-dithiobis(2-nitrobenzoic acid)) before entering a continuous flow colorimeter. At this pH any compounds containing —SH groups react, forming a yellow colour which is monitored at 420 nm. Using this system, very small (µg) amounts of linear somatostatin could be detected at both wavelengths. A high proportion (93%) of added somatostatin could be recovered from samples of plasma during the extraction and purification procedures. A number of background peaks were observed when control plasma extracts containing no somatostatin were monitored by u.v. Some of these peaks were found to be due to material eluting from the syringe, and Gillette syringes gave the lowest background (Fig. 1a). A control plasma extract was chromatographed to accompany each experiment.

Reduction of peptide products isolated from plasma

Fractions containing peptide obtained by HPLC were pooled, chilled (but not frozen) and dried in tubes in a vacuum desiccator. The residue was dissolved in 1 ml borax buffer, pH 8·1, and treated with excess dithiothreitol (twice the weight of peptide, estimated by measurement of peak area). One hour later the sample was diluted with 4 ml 1% trifluoroacetic acid, extracted as described under ‘Plasma extraction’ and chromatographed by HPLC.

RESULTS

Plasma extraction and HPLC

The techniques described for plasma extraction and high-resolution chromatography are based on the powerful adsorption of peptides from aqueous solutions on to hydrophobic octadecasilyl silica. The peptides can subsequently be eluted with an aqueous
solvent mixture containing a relatively hydrophobic component, such as methanol or acetonitrile, and a volatile electrolyte (1% trifluoracetic acid) to minimize ion-exchange effects. High-pressure ODS-silica columns have been reported to give good separations of peptides (Burgus & Rivier, 1976; Gruber, Stein, Brink, Radhakrishnan & Udenfriend, 1976; Krummen & Frei, 1977). A peptide mixture can be separated on a high-pressure ODS-silica column with a linear solvent gradient (Bennett, Hudson, McMartin & Purdon, 1977) and the elution position of any particular peptide then depends largely on the content of hydrophobic residues. Elution time was found to be extremely sensitive to solvent composition: a decrease of a few per cent in the concentration of organic solvent increased the elution time severalfold. Therefore, a diluted extract could be directly pumped on to a high-resolution ODS-silica column since dilution with several volumes of water lowered the concentration of organic solvent sufficiently to allow any peptides present to adsorb strongly to the ODS-silica. Somatostatin extracted and purified by this method was recovered intact, as shown by amino acid analyses of column fractions. The high-pressure column of 10 µm particle size ODS-silica gave better resolution than untreated silica, with quantitative recoveries.

Animal experiments

One minute after the i.v. administration of 1 mg cyclic somatostatin to rats, a single peak, corresponding in position to somatostatin, could be detected in addition to the usual u.v. absorbing products found in control plasma extracts (Fig. 1c). The plasma concentration of peptides in this peak was estimated by comparison of the peak area with those obtained using known quantities of somatostatin. Using a published estimate of blood volume
Fate of somatostatin in the circulation

for the rat (Hobbs, 1967), the total quantity in the circulation was calculated and found to account for 10–20% of the injected dose. Testing with Ellman’s reagent showed that this peak contained no reduced (linear) peptide. After reduction with dithiothreitol (see Methods) the product chromatographed as a single peak, detected by u.v. absorption and Ellman’s reaction, corresponding in position to reduced somatostatin, indicating an absence of peptide-bond cleavage between residues 3 and 14. This was confirmed by amino acid analysis. However, only 0·1–0·2 of a residue of alanine was found to be present and since alanine is the NH₂-terminal amino acid this indicates that 80–90% of the peptide in the peak must be in the form of [des-Ala¹]-somatostatin, the remainder being intact peptide (Table 2).

Table 2. Amino acid composition of product isolated from plasma collected 1 min after i.v. injection of 1 mg somatostatin into male rats

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theoretical</th>
<th>Control extract</th>
<th>Plasma extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1</td>
<td>1·04</td>
<td>1·14</td>
</tr>
<tr>
<td>Thr</td>
<td>3</td>
<td>2·95</td>
<td>3·10</td>
</tr>
<tr>
<td>Ser</td>
<td>1</td>
<td>1·00</td>
<td>1·04</td>
</tr>
<tr>
<td>Gly</td>
<td>1</td>
<td>1·01</td>
<td>0·10</td>
</tr>
<tr>
<td>Ala</td>
<td>1</td>
<td>1·36</td>
<td>1·18</td>
</tr>
<tr>
<td>Cys†</td>
<td>2</td>
<td>3·00</td>
<td>3·00</td>
</tr>
<tr>
<td>Phe</td>
<td>2</td>
<td>2·01</td>
<td>1·95</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>0·38</td>
<td>0·44</td>
</tr>
</tbody>
</table>

* Combined estimate due to lack of separation.
† Partially destroyed during acid hydrolysis.

Plasma and a control (pure somatostatin in water) were both extracted, purified by high-pressure liquid chromatography (see Methods), dried (see Methods) and analysed after acid hydrolysis.

After this observation, a number of tests were carried out in vitro. The results (Table 3) showed an extremely rapid conversion to the des-Ala¹ form, particularly in whole blood; the reaction was slowed down, but not totally inhibited, by EDTA.

Table 3. Formation of[des-Ala¹]-somatostatin during 2 min incubations of cyclic somatostatin with plasma and whole blood, with and without EDTA, at 37°C

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Plasma</th>
<th>Whole blood</th>
<th>Plasma + EDTA</th>
<th>Whole blood + EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>[des-Ala¹]-Somatostatin (%)</td>
<td>63</td>
<td>95</td>
<td>27</td>
<td>61</td>
</tr>
</tbody>
</table>

Cyclic somatostatin (200 μg) was incubated in 6 ml plasma or 12 ml whole blood, extracted and then purified by high-pressure liquid chromatography. The experiment was repeated with the addition of 110 μmol EDTA. The alanine content relative to phenylalanine was used to determine the percentage of [des-Ala¹]-somatostatin present.

One minute after an i.v. injection of 1 mg linear somatostatin, no peptides containing —SH groups could be detected in plasma, compared with a control plasma extract (Fig. 2a and b). In addition, no u.v. absorbing products were present indicating that less than 2% of the injected somatostatin remained in the circulation at this time. A number of experiments were carried out in vitro in which linear somatostatin was incubated in plasma at 37°C, and portions of plasma were removed, extracted and chromatographed at various time intervals. As shown in Fig. 3, over a period of 3 min the u.v. absorbance of the somatostatin peak remained constant. However, the amount of —SH-containing peptide rapidly decreased, falling to less than 1% by 3 min. It was concluded that the linear peptides had
been oxidized to the closed-ring form which eluted from the column in the same place as somatostatin. A control sample incubated in Krebs–Ringer bicarbonate buffer containing calcium ions remained fully reduced.

Fig. 2. Application of high-pressure liquid chromatography (HPLC) to investigation of plasma after administration of linear somatostatin. Extraction of plasma and HPLC were carried out as described in Methods. (a) Control plasma with 20 µg linear somatostatin added; (b) plasma collected 1 min after i.v. injection of 1 mg linear somatostatin.

Fig. 3. Behaviour of linear somatostatin in plasma in vitro. Linear somatostatin (100 µg) was incubated with 2 ml portions of plasma at 37 °C and samples were taken at 10, 30, 90 and 180 s for extraction and high-pressure liquid chromatography (see Methods). The portion of the chromatogram containing the single peak corresponding to linear and cyclic somatostatin is shown at each time; the upper trace (absorbance at 280 nm) measures cyclic and linear peptide and the lower trace (Ellman’s (1959) reaction for —SH groups) measures the linear peptide only.
Incubation of cyclic somatostatin in plasma showed no evidence of reduction to the linear form; the oxidation in vitro thus appears to be irreversible and catalysed by some plasma constituent, such as trace metal ions or even an enzyme.

**DISCUSSION**

The detection methods used in this study necessitated the administration of large doses of peptide, but rapid clearance from the circulation was nevertheless observed for both forms of somatostatin (cyclic and linear); this is in keeping with widespread observations of only a brief duration of action after i.v. injection. The single product of cyclic somatostatin in the circulation, namely [des-Ala¹]-somatostatin, formed by rapid cleavage in vivo and in vitro probably retains full biological activity, since structure–activity studies showed that removal of the first two NH₂-terminal amino acids does not alter the potency in certain systems (Vale et al. 1975). However, until somatostatin and [des-Ala¹]-somatostatin have been widely tested for possible dissociation in their inhibitory effects on different target tissues, the biological significance of the conversion to [des-Ala¹]-somatostatin remains an open question. In addition, it is possible that under the conditions of most bioassays performed in vivo, responses to administered somatostatin may in fact be caused by [des-Ala¹]-somatostatin. By the same criterion, potencies assessed for linear somatostatin in many biological test systems may actually refer to a cyclized molecule, subsequent to our observations of rapid oxidation in plasma in vitro. Low biological activities of linear analogues unable to form the intramolecular disulphide bond have been reported (Vale et al. 1975; Garsky, Clark & Grant, 1976) suggesting that the cyclic structure is necessary for biological activity.

It has been demonstrated that somatostatin and somatostatin-like peptides are present in appreciable quantities in the extrahypothalamic brain, stomach and pancreas (Arimura, Sato, Dupont, Nishi & Schally, 1975; Dubois, 1975; Hökfelt, Hellerström, Efendic, Johansson, Luft & Arimura, 1975). Thus it is quite possible that in some instances somatostatin could be acting as a local hormone, and as such it would have to be rapidly inactivated upon release to prevent it from being returned to the blood and affecting other target tissues.

The clearance of linear somatostatin (more than 98% in 1 min) is surprisingly efficient, even compared with that of cyclic somatostatin. Therefore, the question of whether somatostatin is released from its storage cells in the reduced or oxidized form is of considerable interest. Although somatostatin has been isolated in the cyclized form, cyclization could have occurred during isolation and purification. Most cells contain glutathione with the redox potential lying strongly in favour of the reduced state and it is therefore quite possible that inside the cell, somatostatin is in the linear (reduced) form. If this were so, upon its release any peptide reaching its target receptors might undergo ring closure whilst the remainder would be rapidly removed from the circulation. In this way the requirement for rapid inactivation of a locally acting hormone could be satisfied.

The experimental results have shown that clearance of the linear form must occur before appreciable cyclization can take place, since the cyclic form, once produced, will leave the circulation more slowly. The disappearance of the linear form seems to be too rapid to be accounted for in terms of peptide actually leaving the capillary beds. Perhaps the presence of free —SH groups leads to direct binding to the capillary walls.

In conclusion, investigation of products in the blood has shown that linear and cyclic somatostatin are rapidly cleared from the circulation. Only one fragment was detected and this was [des-Ala¹]-somatostatin, which is probably fully biologically active. It appears that inactivation of somatostatin must occur after it has entered the tissues and a full understanding of the behaviour of this peptide will require further investigation of its fate once it has left the circulation.
The authors would like to thank Mr Bruce Evans for carrying out the amino acid analyses.

REFERENCES


