Isolation and characterization of plasmin-generated bioactive fragments of IGFBP-3

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Booth, Barbara A., Mary Boes, Brian L. Dake, and Robert S. Bar. Isolation and characterization of plasmin-generated bioactive fragments of IGFBP-3. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E450–E454, 1999.—Insulin-like growth factor-binding protein-3 (IGFBP-3) was digested with plasmin, and the proteolytic fragments were isolated by HPLC and tested for bioactivity as measured by stimulation of glucose uptake in microvessel endothelial cells. Two of the pooled fractions of the digest stimulated glucose uptake. The major bioactive pool, at an estimated protein concentration <50 ng/ml, stimulated glucose uptake to 150% of control with greater stimulation and 220% of control at ~250 ng/ml. Two fragments were present in the bioactive fraction, the dominant one migrating at ~20,000 and the other at ~8,000. Both fragments bound $^{125}\text{I}$-labeled insulin-like growth factor and $^3\text{H}$-heparin. NH$_2$-terminal amino acid analysis of the bioactive peak yielded two sequences. One, representing the majority of the material, had an NH$_2$-terminal sequence identical to IGFBP-3; the second fragment began at amino acid 202 of IGFBP-3. In contrast to the bioactive fragments, intact IGFBP-3, at concentrations up to 130 µg/ml, had no bioactivity. These findings demonstrate that IGFBP-3 can be degraded into fragments that have potent bioactivities that are not present in the intact IGFBP-3 molecule.

endothelium; insulin-like growth factor-binding protein-3; bioactivity

THE INSULIN-LIKE GROWTH factors, IGF-I and IGF-II, are mitogenic proteins that are present in the circulation and in tissues bound to one of six high-affinity IGFBPs (IGFBPs; see Refs. 16, 17). IGFBP-3, the major carrier of circulating IGF, is highly susceptible to proteolysis both in vitro and in vivo. Several proteases have been shown to digest IGFBPs, including plasmin (3, 7–9, 21, 27), thrombin (7, 28), prostate specific antigen (12), other serine proteases (2, 29), cathepsin D (10, 11), matrix metalloproteases (15), and other metalloproteases (18, 24). Recent studies have demonstrated that proteolysis of IGFBP-3 and IGFBP-5 can enhance the effects of IGFs, presumably through liberation of IGF from the IGF-IGFBP complexes (5), as well as generate fragments of IGFBP-3 and IGFBP-5 with intrinsic bioactivity (22, 27). We previously demonstrated that limited exposure of IGFBP-3 to plasmin, thrombin, or serum reproducibly digested IGFBP-3 into defined fragments (7). In addition, synthetic, highly basic, heparin-binding peptides derived from the COOH-terminal third of IGFBP-3, IGFBP-5, and IGFBP-6 stimulated glucose uptake by microvessel endothelial cells (6).

In the present study, nonglycosylated recombinant human (rh) IGFBP-3 was exposed to plasmin, the resulting proteolytic fragments were separated by HPLC, and their ability to increase glucose uptake microvessel endothelial cells was tested. Whereas intact rhIGFBP-3 did not stimulate glucose uptake, at least one of the plasmin-generated fragments had potent bioactivity, stimulating glucose uptake even when present in nanogram amounts.

METHODS

Nonglycosylated, rhIGFBP-3 (420 µg; a gift from Celtrix, Santa Clara, CA) was incubated with 12 µg plasmip (American Diagnostica, Greenwich, CT) in the presence of 140 µg fatty acid-free BSA (Sigma, St. Louis, MO) for 20 min at 37°C in 0.05 M HEPES, 0.05 M NaCl, and 20 µl/Tween 80, pH 7.5. A BSA-plasmin control (no IGFBP-3) was similarly incubated. Immediately after the incubation, phenylmethlysulfonyl fluoride (PMSF; Sigma) was added to a concentration of 1 mM to inactivate the plasmin.

HPLC was performed on a semipreparative Vydac C$_{18}$ column (10 mm × 250 mm) flowing at 2.5 ml/min using a gradient from 0 to 80% acetonitrile in 0.06% trifluoroacetic acid, collecting fractions every 2 min. Because a large number of peaks were detected, eluted material was initially combined to give eight 10-min pooled fractions, i.e., 0–10 min, 10–20 min, etc., through 80 min. Samples from both the BSA/plasmin control and the IGFBP-3 digest were dried on a Speed-Vac (Savant, Farmingdale, NY) and stored at ~70°C.

Additionally, a BSA sample (140 µg) without plasmin and a plasmin sample (60 µg) without BSA were run on the HPLC column to confirm the elution peak of plasmin and the lack of digestion of BSA by plasmin.

The samples were dissolved in 400 µl of 0.05 M HEPES, 0.15 mM NaCl, and 20 µl/Tween 80, pH 7.5, containing fresh PMSF (1 mM). 2-Deoxy-[$^{14}$C]glucose (Amersham, Arlington Heights, IL) uptake assays were performed on 25-µl aliquots of samples C1–8 and D1–8, using bovine microvessel endothelial cells, at passages 3–6, as described previously (6). In a later experiment, the 2-min fractions from the most bioactive area were individually tested for activity, and dose-response assays of the active fractions were performed. In a subsequent experiment, the HPLC fractions were collected by “peak” elution rather than by “time” to more effectively isolate the bioactive material.

Samples of the bioactive material derived from plasmin-treated rhIGFBP-3 were electrophoresed on a 16% SDS-polyacrylamide gel. Ligand blotting with a mixture of $^{125}$I-labeled IGF-I and $^{125}$I-IGF-II or with $^3$H-heparin was performed as previously described (7). Silver staining of the gel was done using a Bio-Rad Silver Stain kit (Bio-Rad, Richmond, CA) according to the instructions of the manufacturer.
RESULTS

HPLC of plasmin-digested rhIGFBP-3 yielded numerous peaks (Fig. 1). The HPLC profile shown in Fig. 1B was essentially identical in four separate experiments using plasmin to digest IGFBP-3. For comparison, the HPLC profile of the control BSA/plasmin sample without IGFBP-3 is illustrated in Fig. 1A. When these HPLC fractions were combined as 10-min pools (Fig. 1A: C1–C8; Fig. 1B: D1–D8), two pools, D7 and D8, significantly increased glucose uptake by microvessel endothelial cells (Table 1). D7 (25 µl) increased glucose uptake to 201%, whereas D8 (25 µl) increased uptake to 141% of control. Pools D1–D6 and C1–C8 had no bioactivity. When undigested rhIGFBP-3 was run on HPLC there was no bioactivity in these fractions, indicating that the effect on glucose uptake was not due to an impurity in the rhIGFBP-3 (data not shown).

D7 contained the trailing edge of a peak eluting at ~60 min, a small peak with a shoulder eluting at ~60–64 min, and the leading edge of a peak eluting just before 70 min. Although D8 was bioactive, D8 contained only peaks seen in the BSA-plasmin control, thus any additional bioactive material generated by the digest was present in very low concentration. On 125I-IGF ligand blot, D7 contained two faint bands at ~20,000 and ~8,000 (Fig. 2, lane A). These same bands also bound [3H]heparin, although the intensity of the bands was relatively low (Fig. 2, lane B). Silver staining of D7 revealed two faint bands at ~20,000 and ~5,300 (Fig. 2, lane C), corresponding to the same regions identified in 125I-IGF (Fig. 2, lane A) and [3H]heparin (Fig. 2, lane B) ligand blots. Based on the sensitivity of the silver stain, the bands at 20,000 and 8,000 were estimated to contain ~50 ng protein; the total protein in the D7 pool derived from the plasmin digest of 420 µg IGFBP-3 was estimated to be 1 µg (~500 ng/band). No 125I-IGF or [3H]heparin binding was observed with pool D8, and no silver-stained material was seen.

In a second experiment, the individual 2-min HPLC fractions corresponding to the bioactive (D7) area were collected, dried in a Speed-Vac, dissolved in 200 µl buffer, and analyzed in the glucose uptake assay. The two fractions containing the small peak with a shoulder

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean ± SE, %</th>
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<tr>
<td>Control</td>
<td>100 ± 7</td>
<td>P3</td>
<td>141 ± 14*</td>
</tr>
<tr>
<td>IGF-I</td>
<td>211 ± 8*</td>
<td>D1</td>
<td>103 ± 8</td>
</tr>
<tr>
<td>C2</td>
<td>115 ± 6</td>
<td>D2</td>
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<td>107 ± 7</td>
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<tr>
<td>C5</td>
<td>106 ± 3</td>
<td>D5</td>
<td>113 ± 9</td>
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<tr>
<td>C6</td>
<td>93 ± 3</td>
<td>D6</td>
<td>108 ± 5</td>
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<td>101 ± 1</td>
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<tr>
<td>C8</td>
<td>101 ± 4</td>
<td>D8</td>
<td>145 ± 5</td>
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Shown is percent of 2-deoxy[14C]glucose uptake relative to control ± SE. Insulin-like growth factor-I (IGF-I) was tested at 50 ng/ml; 18 amino acid P3 peptide was tested at 10 µg/ml; 25-µl aliquots of C1–C8 and D1–D8 were tested. C1–C8, control digest pools; D1–D8, IGFBP-3 digest pools. *P < 0.01 when compared with control by ANOVA and Dunnett's test.

Fig. 1. HPLC profiles of BSA/plasmin control (A) and 20-min plasmin digest of 420 µg insulin-like growth factor-binding protein (IGFBP)-3 (B). C1–C8 (A) and D1–D8 (B) are fractions pooled over 10 min of collection; areas 1 and 2 (B) represent the bioactive fractions detected in subsequent HPLC runs.

Fig. 2. 125I-labeled insulin-like growth factor (IGF) ligand blot (lane A), [3H]heparin blot (lane B), and silver stain (lane C) of the bioactive D7 pool; 40 µl of material was loaded in each lane of the SDS-PAGE gel. 125I-IGF ligand blot was exposed for 8 h, and [3H]heparin ligand blot was exposed for 1–4 days. M, relative molecular weight.
seen in the D7 pool (Fig. 1, fractions 1 and 2) were both active (Fig. 3). Fraction 1, eluting at 60–62 min and containing the leading edge of the peak, stimulated glucose uptake to 151% of control when endothelial cells were exposed to 2 µl of fraction 1, 217% of control with 10 µl of fraction 1, and 234% with 25 µl (Fig. 3). For comparison, a maximal dose (50 ng/ml) of IGF-I stimulated glucose uptake to 273% of control in this experiment. Fraction 2, eluting at 62–64 min and containing the trailing edge of the peak and the shoulder, was less active, stimulating glucose uptake to 182% of control when cells were incubated with 25 µl of fraction 2. Protein estimates, based on relative peak area for fraction 1, indicated that 2 µl contained ~14 ng protein. Because the glucose uptake assay was performed in a volume of 0.3 ml, this material was active at <50 ng/ml. All other HPLC fractions from the D7 region of the digest and all of the corresponding fractions from the control run were inactive in the glucose uptake assay.

In a third experiment, fraction 1 was subjected to NH₂-terminal amino acid analysis. Two NH₂-terminal sequences were obtained. The dominant protein, representing ~90% of the total protein in this fraction, had an NH₂-terminal sequence of GASSAGLGPVVRXEP, corresponding to the NH₂-terminus of IGFBP-3. The second protein, representing <10% of the material, appeared to have an NH₂-terminal sequence of VLSLP, corresponding to the sequence beginning at amino acid 202 of IGFBP-3. A fragment beginning at this location and extending to the COOH-terminus of IGFBP-3 would have an estimated molecular weight of ~8,000. However, this NH₂-terminal sequence follows an asparagine rather than a lysine or an arginine as would be expected from the specificity of plasmin. Due to the small amount of material generated by the conditions used in the plasmin digestion, and the limited amount of rhIGFBP-3 available for further experimentation, we were unable to further separate these peptides by HPLC or other methods.

The effect of the bioactive peak on glucose uptake was tested in the presence of a half-maximal stimulatory dose of IGF-I. IGF-I (2 ng/ml) stimulated glucose uptake to 164±6% of control while a submaximal stimulatory dose of the bioactive peak stimulated uptake to 138±5% of control. When endothelial cells were exposed to the same concentrations of IGF-I and the bioactive material combined, their effects were additive, increasing glucose uptake to 192±8% of control (Fig. 4).

**DISCUSSION**

In the present study, limited digestion of IGFBP-3 with plasmin, a proteolytic enzyme potentially generated at the endothelial surface from plasminogen, yielded a reproducible pattern on HPLC with two bioactive areas. A mixture of two fragments was isolated from the major area of bioactivity, which stimulated glucose uptake to a level approaching the maximal stimulatory effect of IGF-I, the most potent known stimulus of glucose uptake in the microvessel endothelial cells. The dominant protein in the bioactive peak had an NH₂-terminal sequence identical to IGFBP-3.

Previously, synthetic peptides of 18 and 34 amino acids corresponding to amino acids 215–232 (P3) and 199–232 (P334) of IGFBP-3 and amino acids 201–218 (P5) of IGFBP-5 were shown to stimulate glucose uptake by 40–80% in microvessel endothelial cells when the peptides were present at 10 µg/ml (6). The bioactive fragment(s) isolated from the plasmin digest in the present study demonstrated greater bioactivity than P3 or P334 when present at a substantially lower concentration, i.e., <50 ng/ml vs. 10 µg/ml for P3. This suggested that the added structure of the plasmin-derived bioactive fragment(s) of IGFBP-3 either substantially increased the bioactivity of the P3 moiety or components of the bioactive fragment(s) other than P3...
account for its potent bioactivity; the latter possibility was suggested by NH$_2$-terminal sequence analysis of the bioactive material. Results of this study thus indicate that IGFBP-3, known for its sensitivity to several proteases, may play a further role on proteolysis, a concept recently suggested by Mohseni-Zadeh and Binoux (22) for IGFBP-3 and by Abrass et al. (1) for IGFBP-5.

We had previously (4) demonstrated the presence of a "bioactive IGFBP" in a multiplication stimulating activity (MSA) affinity-purified preparation derived from medium collected from cultured pulmonary artery endothelial cells. This bioactive "IGFBP" increased glucose uptake to 200–300% of control values (4). Despite the finding that the bioactive material(s) was purified based on its affinity for IGF (MSA), recombinant IGFBP-1 through IGFBP-6, as well as IGFBP-4 purified by HPLC from the endothelial cell-conditioned media, did not stimulate glucose uptake. Considering our current data and that of others (1, 2, 8, 22, 27), it is possible that the bioactive component(s) in this endothelial preparation was a bioactive proteolytic fragment(s) of IGFBP-3. The bioactive fragment(s) described in this report, as well as the material in the conditioned media (4), each had affinity for IGF and stimulated glucose uptake in microvessel endothelial cells to >200% of control. In fact, after HPLC of concentrated conditioned media from pulmonary artery endothelial cells, bioactive components stimulating glucose uptake in endothelial cells were found in the same HPLC region as the bioactive material from the plasmin-digested rhIGFBP-3 (data not shown).

The generation of bioactive fragment(s) from IGFBPs may be a process seen in vivo. IGFBP-3 binds to the surface of endothelial cells; plasmin and thrombin, as well as the IGFBP-3 proteases contained in serum, can be present in increased concentrations at the endothelial surface, such as at wound and thrombotic sites, as well as during the third trimester of pregnancy. The concentration of IGFBP-3 and IGFBP-5 proteases at a given endothelial surface could potentially mimic the conditions studied in this in vitro report. Should bioactive IGF BP-3 fragments be formed in this environment, they could potentially be released into the circulation, affect regional endothelial function, or be transported to subendothelial tissues supplied by the involved vasculature.

The potential roles of IGFBP-3 and IGFBP-5 continue to expand. Both binding proteins associate with IGF and the acid-labile subunit to form circulating 150,000 complexes that likely contribute to the regulation of the endocrine functions of the IGFs (26). They modify the effects of IGFs, being capable of inhibiting or potentiating IGF actions. IGFBP-3 and IGFBP-5 also manifest bioactivities independent of IGF, with IGFBP-3 inhibiting growth of human breast cancer cells (23) and inhibiting IGF-binding factor-4 proteolysis (14), whereas IGFBP-5 stimulates the migration of rat mesangial cells (1) and increases thymidine incorporation in DNA by rat osteoblasts (25). Finally, NH$_2$-terminal and COOH-terminal fragments of IGFBP-3 and IGFBP-5 have recently been described that either mimic the properties of the intact binding protein or elicit effects that are not observed with the intact binding protein. Thus a 16,000 NH$_2$-terminal fragment of IGFBP-3 has been reported to both bind IGF but inhibited the mitogenic actions of IGF, insulin, and basic fibroblast growth factor (BFGF) on mouse embryo fibroblasts (22); although intact IGFBP-3 also had an inhibitory effect on IGF-stimulated growth, it did not influence insulin or BFGF-stimulated growth. A similar NH$_2$-terminal fragment of IGFBP-3 showed weak IGF binding but significant insulin binding and inhibited insulin receptor autophosphorylation (27). Eighteen and 34 amino acid COOH-terminal portions of IGFBP-3 and IGFBP-5 have been shown to stimulate glucose uptake by microvessel endothelial cells (6), stimulate mesangial cell migration in the absence of IGF (1), and inhibit proteolysis of IGFBP-4 (14). The COOH-terminal region of IGFBP-3 and IGFBP-5 also appears to be important for cell attachment of the binding protein, nuclear localization, and interactions with proteases such as plasmin/plasminogen (1, 8, 13, 14).

These diverse functions of IGFBP-3 and IGFBP-5 clearly extend the complexity of the IGF-IGFBP system. At the same time, they also provide several new and specific mechanisms whereby IGFBP-3 and IGFBP-5, two IGF Bs with both overlapping and distinct properties, can impact the multiple biological systems that are likely to be affected by IGFBP-3, IGFBP-5, and their metabolites.

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