Increases in Free, Unbound Insulin-like Growth Factor I Enhance Insulin Responsiveness in Human Hepatoma G2 Cells in Culture*

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Insulin-like growth factor-binding protein (IGFBP)-1 binds to insulin-like growth factor (IGF)-I and -II with high affinity and has been shown to modulate IGF-I actions in vivo and in vitro. The synthesis of IGFBP-1 is suppressed by insulin, and administration of IGFBP-1 to rats results in impaired glucose metabolism. A synthetic peptide (bp1-01) has been shown to have a high affinity and specificity for human IGFBP-1 and to inhibit IGF-I binding. The current studies were undertaken to determine if, after incubation of bp1-01 with IGF-I/IGFBP-1 complexes, anabolic and insulin-like effects of IGF-I could be detected in human hepatoma (HepG2) cell cultures and to determine the receptor subtype(s) through which these effects were mediated. Incubation of HepG2 cells with bp1-01 (200 nM) increased IGF-I-stimulated protein synthesis by 44% and glycogen synthesis by 170% compared with stimulation by IGF-I alone. Incubation with bp1-01 also enhanced IGF-I-stimulated tyrosine phosphorylation of the IGF-I/insulin hybrid receptor and insulin receptor substrate 1. Exposure of the cells to bp1-01 alone enhanced glycogen synthesis and phosphorylation of IGF-I/insulin hybrid receptors. This was not a direct effect of bp1-01 because it did not bind to the receptor and did not activate tyrosine kinase activity in the presence of an anti-IGF-I receptor antibody. The addition of bp1-01 (200 nM) plus insulin to HepG2 cell culture medium resulted in increased tyrosine phosphorylation of the hybrid receptor, insulin receptor substrate 1, and the glycogen synthesis response compared with the effects of insulin alone. This enhancement of hybrid receptor phosphorylation and glycogen synthesis by bp1-01 peptide was diminished by preincubation with an inhibitory antibody for the α subunit of IGF-I receptor (αIR3). bp1-01 stimulated the hybrid receptor phosphorylation response to IGF-I, and this effect was inhibited by prior incubation of the cells with αIR3. In conclusion, bp1-01 competes with IGF-I for binding to IGFBP-1, which leads to release of free IGF-I from IGF-I/IGFBP-1 complexes. This released IGF-I stimulates biologic actions that are mediated predominantly through the IGF-I/insulin hybrid receptor.

Insulin-like growth factor (IGF)1-I is a potent mitogen that has anabolic actions and insulin-like actions in vivo and in vitro.

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1 The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; IGFI-R, insulin-like growth factor receptor; IRS-1, insulin receptor substrate 1; IR, insulin receptor; DMEM, Dulbecco’s modified Eagle’s medium; DMEM-H, DMEM with high glucose; DMEM-L, DMEM with low glucose.
including liver, spleen, skeletal muscle, and placenta (22). These hybrid receptors have been proposed to mediate IGF-I signaling in tissues where they represent the predominant receptor subtype (23). Recent studies have shown increases in hybrid receptor abundance in skeletal muscle of patients with type II diabetes (24), and gene targeting experiments that disrupt the normal function of these receptors in skeletal muscle lead to the development of insulin resistance (25). The major aims of this study were to determine whether this peptide could compete with IGF-I for binding to IGFBP-1 in HepG2 cell conditioned medium, to determine whether release of IGF-I from IGFBP-1 would result in alteration in insulin and/or IGF-I actions, and to compare the relative importance of each receptor subtype in mediating these effects.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human hepatoma (HepG2) cells were obtained from American Type Culture Collection (Manassas, VA). Tissue culture media, penicillin, and streptomycin were purchased from Invitrogen. [35S]Methionine was from ICN Biochemical Inc. (Costa mesa, CA), and [3H]D-glucose from Amersham Biosciences. Polyvinylidene difluoride transfer membranes (Immobilon-P) were obtained from Millipore. The anti-phosphotyrosine antibody (PY99), antibody for the β subunit of IGF-I receptor (β-IGF-R) (C-20), and anti-insulin receptor substrate-1 (IRS-1) antibody (A-19) were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA). The antibody for the α subunit of insulin receptor (αIR) (47-9 and S3-14) that has no cross-reactivity with IGF-R was from NeoMarker (Fremont, CA), and the antibody for the β subunit of insulin receptor (βIR) that has no cross-reactivity with IGF-R was from Upstate Biotechnologies (Lake Placid, NY). The antibody for the α subunit of IGFI-R (αIR3) was prepared and purified as described previously (26). Recombinant human IGF-I and IGFBP-1 binding peptide bp1-01 (CRAGPLQWLC6KFKY) were gifts from Genentech Inc. (South San Francisco, CA). [125I]-IGF-I (150 Ci/μg) was prepared as described previously (27).

**Tissue Culture**—HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium with high glucose (DMEM-H) (4 g/liter) supplemented with 10% FCS, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were grown in 5% CO2, 95% air at 37 °C and passaged using a split ratio of 1:4 after trypsinization.

**[125I]-IGF-I Binding Assay**—The capacity of IGFBP-1 in HepG2 cell conditioned medium to bind to [125I]-IGF-I and the effect of increasing concentrations of bp1-01 on binding were determined using the polyethylene glycol precipitation method as described previously (28). Duplicate tubes containing [125I]-IGF-I (25,000 cpm/tube) were incubated with 5 μl of serum-free DMEM that had been conditioned by HepG2 cells, 10 μl of HepG2 conditioned medium, 2 h at room temperature. Bound IGF-I and free IGF-I were separated by precipitation using 12.5% polyethylene glycol (M, 8,000). The pellets were washed in 6.25% polyethylene glycol, and bound [125I]-IGF-I was determined by gamma counting.

**[35S]Methionine Incorporation into HepG2 Cells**—HepG2 cells were grown to 80% confluence on 10-cm tissue culture dishes. The cells were rinsed three times with serum-free DMEM-H and incubated with the same medium for 30 h, and then various concentrations of bp1-01 (0–500 nM) were added directly, and the incubation was continued for 1.5 h. IGF-I (0 or 10 ng/ml) was added, and the incubation was continued for 10 min.

The cultures were washed once with ice-cold phosphate-buffered saline and solubilized in lysis buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 150 mM NaCl, 50 mM Hepes, pH 7.5, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 2 mM p-nitrophenylthiobenzoate, 1 μg/ml aprotinin, A, and 1 μg/ml leupeptin). The insoluble material was removed by centrifugation at 15,000 × g for 10 min, and the supernatant (900 μg of protein) was incubated with either anti-β-IGFI-R antibody, anti-IRS-1 antibody, or anti-β-IR antibody (1,300 dilution) overnight at 4 °C. The immune complexes were precipitated by incubation with protein A-Sepharose for 2 h at 4 °C followed by centrifugation at 7,000 × g for 1 min and washed with lysis buffer without phosphatase inhibitors four times. The proteins were resuspended in Laemmli sample buffer, separated by SDS-PAGE (7.5%), and transferred to a polyvinylidene difluoride membrane (0.45-μm pore size). The membrane was probed with a 1:1,000 dilution of anti-phosphotyrosine antibody (PY99) and visualized with enhanced chemiluminescence (Super-Signal CL-H substrate system; Pierce) and then exposed to Kodak X-AR film (Eastman Kodak Co.). The signal intensities were quantified by scanning densitometry and analyzed using NIH Image. In the experiments where the effect of insulin was to be determined, the cells that had been exposed to serum-free medium for 30 h were incubated with bp1-01 for 1.5 h and then exposed to insulin (0–1 μM) for 5 min. Further processing was the same as that described for IGF-I stimulation. Total protein content was measured using the biocinchoninic acid protein assay (Pierce).

**Separation of IGF-I/Insulin Hybrid Receptor and IGF-IIR or IR Homodimers**—To identify IGF-I/insulin hybrid receptor and IGF-IIR homodimers, HepG2 cells were solubilized in 1.0 ml of lysis buffer without a nonionic detergent. The insoluble material was removed by centrifugation, and supernatant was incubated with anti-αIR monoclonal antibody (1.0 μg) for 2 h at 4 °C and then incubated with anti-mouse IgG-agarose for 3 h at 4 °C. The immobilized anti-mouse IgG-agarose was sedimented by centrifugation. The supernatant (1.0 ml) was incubated with an anti-β-IGFI-R polyclonal antibody (1 μg) for 18 h at 4 °C. Immunoprecipitation and immunoblotting were conducted as described previously. To separate IGF-I/insulin hybrid receptors and the IR homodimer, the cells were lysed as described above, and then, after the removal of the insoluble material, the supernatant was incubated with an anti-IR monoclonal antibody (αIR3) (1.0 μg) for 18 h at 4 °C exposed to anti-mouse IgG-agarose for 3 h at 4 °C, and centrifuged. The supernatant was incubated with anti-βIR polyclonal antibody (1,300 dilution) for 18 h at 4 °C and then immunoprecipitated and immunoblotted as described previously.

**Statistical Analysis**—A paired Student’s t test was used to test for differences in the control and the test groups.

**RESULTS**

The Effect of bp1-01 Peptide on IGF-I/IGFBP-1 Binding—To determine whether bp1-01 peptide could compete with IGF-I for binding to the IGFBP-1 in HepG2 cell conditioned medium, [125I]-IGF-I binding was quantified in the presence of increasing concentrations of the bp1-01 peptide. Increasing concentrations of bp1-01 peptide inhibited IGF-I binding to IGFBP-1 in HepG2 conditioned medium, and half-maximal inhibition occurred with 140 nM, which is similar to the concentration reported by Lowman et al. (18) (e.g., 160 nM) using pure IGFBP-1 (Fig. 1).
IGFBP-1 Binding Peptide Increases IGF-I-stimulated
[^35S]^Methionine Incorporation into Protein by HepG2 Cells—
HepG2 cells were grown to 80% confluence in 24-well culture plates, and the ability of IGF-I to stimulate[^35S]^methionine incorporation into cellular protein in the presence of the bp1-01 peptide was determined. IGF-I stimulated the incorporation of[^35S]^methionine into HepG2 cells by 42 ± 2.1% above the basal level. Coincubation with bp1-01 (200 nM) increased this response to 83 ± 5.5% above the basal level (p < 0.05 compared with the effect of IGF-I alone) (Fig. 2). bp1-01 alone (200 nM) had no significant effect. Thus, the addition of bp1-01 peptide increased the IGF-I-stimulated protein synthesis presumably by inhibiting IGF-I binding to IGFBP-1, thus allowing more IGF-I to be available to interact with the IGF-I receptor.

IGFBP-1 Binding Peptide Increases IGF-I-stimulated
[^3H]Glucose Incorporation into Glycogen by HepG2 Cells—
IGF-I stimulated[^3H]glucose incorporation into HepG2 cells by 162 ± 18% above control (Fig. 3A). The addition of bp1-01 (200 nM) with IGF-I enhanced the cell glycogen synthesis response to each concentration of IGF-I that was tested. The maximal response was a 317 ± 18% increase (p < 0.01) (Fig. 3A). Because HepG2 cells secrete IGF-I into culture medium, the ability of increasing concentrations of bp1-01 to stimulate glycogen synthesis in the absence of exogenously added IGF-I was determined. HepG2 cells were grown to 80% confluence in 24-well culture plates, and the[^3H]glucose incorporation response to increasing concentrations of bp1-01 was measured, bp1-01 stimulated[^3H]glucose incorporation into HepG2 cells up to 71.8 ± 8.0% above the basal level without the addition of IGF-I or insulin (Fig. 3B). To exclude the possibility that this was a direct effect of the bp1-01 peptide, the identical peptide concentrations were tested in the presence of the anti-IGF-I-R antibody, aIR3 (an antibody that blocks IGF-I binding to IGF-I-R). This antibody completely blocked the glycogen synthesis response to bp1-01 (p < 0.01). The antibody that blocks binding of insulin to the insulin receptor (aIR) partially blocked the glycogen synthesis response (p < 0.05), but it was not as effective as aIR3. To determine that bp1-01 was not binding to the IGFII-R or IR, we quantified[^125I]bp1-01 binding and the effect of each anti-receptor antibody in competition for binding. Minimal binding (e.g., <0.1%) was detected, and it was not inhibited by either antibody (data not shown). These results are consist-
plates, and [3H]glucose incorporation into glycogen was determined as described under “Experimental Procedures” after stimulation with IGF-I in the presence (○) or absence (●) of bp1-01 (200 nM). B, HepG2 cells were grown to 80% confluence on 24-well tissue culture plates, and the effect of bp1-01 on glycogen synthesis in HepG2 cells in the absence of added IGF-I was determined (○). The results are expressed as the percentage increase over control cultures that were not exposed to bp1-01. Additional cultures were exposed to DMEM-L containing the same concentrations of bp1-01 in the presence of αIR (●) or αIR3 (▲) without IGF-I. Each value is the means ± S.E. of triplicate determinations.

Because the addition of bp1-01 enhanced the cellular response to insulin as well as IGF-I, we determined its effect on insulin-stimulated IR and IRS-1 phosphorylation. Insulin (1.0 nM) stimulated tyrosine phosphorylation of both IR (Fig. 7A) and IRS-1 (Fig. 7B) compared with insulin alone. In contrast, bp1-01 alone had no effect. When the experiment was repeated three times, scanning densitometry showed that the intensities of the phosphorylated IR or IRS-1 bands from the cultures that received bp1-01 followed by stimulation with 1 nM insulin were increased by 65 ± 5.9% and 120 ± 18%, respectively, above the level that was detected with insulin stimulation alone.

Because bp1-01 enhanced the effect of insulin but had no effect when added alone, these results strongly suggest that peptide inhibitor increased the cellular responsiveness to insulin by increasing the interactions between free IGF-I and either IGF-I-R or the IGF-I/insulin hybrid receptor.

To determine which receptor(s) was mediating the effects of bp1-01, phosphorylation of IGF-I/insulin hybrid receptor and the insulin receptor were analyzed after insulin or bp1-01 exposure. Stimulation of HepG2 cells with 200 nM bp1-01 peptide alone showed enhanced phosphorylation of a band corresponding to the IGF-I/insulin hybrid receptor plus the insulin receptor (Fig. 8A, a, lane 3, 1). In contrast, IGF-I-R phosphorylation was not increased (Fig. 8A, a, lane 4, 2). Insulin alone also stimulated phosphorylation of this band (Fig. 8A, a, lane 5, 1). The combination of bp1-01 plus insulin stimulated an addi-

FIG. 4. Effects of bp1-01 on [3H]glucose incorporation into HepG2 cells after stimulation with insulin. HepG2 cells were seeded at a density of 6 × 10^4 cells/cm^2 in 24-well tissue culture plates and grown for 24 h. The medium was replaced with serum-free DMEM-L, and the incubation was continued for 24 h. Then 0 or 200 nM bp1-01, 1 nM insulin, and 0.05 μCi/well [3H]glucose were added directly, and the incubation was continued for 2 h. The incorporation of [3H]glucose into glycogen was measured as described under “Experimental Procedures.” In some experiments, αIR and/or αIR3 were added for 1 h before the addition of bp1-01, insulin, and [3H]glucose. The results are expressed as the percentage of increase over control cultures that were incubated with DMEM-L without insulin or bp1-01. Each value is the mean ± S.E. of triplicate determinations.
IGFBP-1-associated IGF-I Enhances Insulin Responsiveness

Fig. 5. Effects of bp1-01 on IGF-I-stimulated phosphorylation of IGF-I-R, IRS-1, and IR. HepG2 cells were grown to 80% confluence on 10-cm tissue culture dishes. The cultures were incubated with serum-free DMEM-H for 30 h. The indicated concentrations of bp1-01 were added directly and incubated for 1.5 h, and then 0 or 10 ng/ml IGF-I was added for 10 min. The cells were solubilized in lysis buffer and immunoprecipitated with either anti-β-IGF-I-R (A), IRS-1 (B), or β-IR (C) antibody. The proteins were analyzed by 7.5% SDS-PAGE followed by immunoblotting using anti-phosphotyrosine antibody (PY99). Scanning densitometry values (expressed as arbitrary scanning units) for the bands shown in the top panel of A were: lane 1, 2,705; lane 2, 1,689; lane 3, 6,884; lane 4, 12,636; and lane 5, 11,929. Scanning densitometry values for the bottom panel of A were: lane 1, 7,612; lane 2, 7,782; lane 3, 8,950; lane 4, 10,576; and lane 5, 10,859. For the top panel of B, scanning densitometry values were: lane 1, 0; lane 2, 3,624; lane 3, 0; and lane 4, 7,607. For the bottom panel of B, scanning densitometry values were: lane 1, 11,391; lane 2, 10,394; lane 3, 8,612; and lane 4, 10,215. For the top panel of C, the values were: lane 1, 0; lane 2, 3,461; lane 3, 0; and lane 4, 8,296. For the bottom panel of C, they were: lane 1, 12,287; lane 2, 13,335; lane 3, 13,071; and lane 4, 10,625. All values represent the mean of three separate experiments.

Fig. 6. Effects of bp1-01 and/or IGF-I stimulation on the phosphorylation of IGF-I/insulin hybrid receptors or IGF-I receptor homodimers. HepG2 cells were grown to 80% confluence as described under “Experimental Procedures.” The media were replaced with serum-free DMEM-H, and the incubation was continued for 30 h. Then 0 or 200 nM bp1-01 was added directly, and the incubation was continued for 1.5 h. The cells were stimulated with IGF-I (10 ng/ml) for 10 min and then solubilized in lysis buffer without ionic detergent and immunoprecipitated with αIR3 (lanes 1, 3, and 5) and the supernatants were immunoprecipitated with anti-β-IR (lanes 2, 4, 6, and 8), and both sets of precipitates were analyzed by immunoblotting using an anti-phosphotyrosine antibody (top panel) or anti-β-IR (bottom panel). Scanning densitometry values for the bands shown in the top panel were (from the left): 1,069, 4,884, 6,118, 10,729, 105, 234, 291, and 201. Bottom panel values were 6,528, 6,975, 7,269, 6,018, 8,348, 11,206, 7,811, and 7,029.

Fig. 7. Effects of bp1-01 on insulin-stimulated phosphorylation of IR and IRS-1. HepG2 cells were grown to 80% confluence as described under “Experimental Procedures.” The media were replaced with serum-free DMEM-H, and the incubation was continued for 30 h. Then 0 or 200 nM bp1-01 was added directly, and the incubation was continued for 1.5 h. Subsequently, the cells were stimulated with 1 nM insulin for 5 min. The cells were solubilized in lysis buffer and immunoprecipitated with either anti-β-IR (A) or IRS-1 (B) antibody. The proteins were analyzed by 7.5% SDS-PAGE with Western immunoblotting using anti-phosphotyrosine antibody (PY99). Scanning densitometry values for the bands shown in the top panel of A were: lane 1, 0; lane 2, 0; lane 3, 4,605; lane 4, 7,130; lane 5, 40,254; and lane 6, 45,377. Values for the bottom panel of A were: lane 1, 9,324; lane 2, 9,285; lane 3, 9,555; lane 4, 10,342; lane 5, 8,634; and lane 6, 8,930. Values for the top panel of B were: lane 1, 0; lane 2, 22,206; lane 3, 0; and lane 4, 45,928. Values for the bottom panel of B were: lane 1, 16,891; lane 2, 14,385; lane 3, 20,085; and lane 4, 15,189. All values represent the mean of three separate experiments.

Fig. 8. Effects of bp1-01 on insulin-stimulated binding to insulin receptors. This result supports the conclusion that the effect of bp1-01 is due solely to hybrid receptor stimulation by IGF-I that is released from IGFBP-1. This result further supports the conclusion that the effect noted with the bp1-01 peptide in Fig. 5C was due to the hybrid receptor phosphorylation. To further confirm this finding, the experiments were repeated, and several time points were examined. When bp1-01 (400 nM) alone was incubated with HepG2 cells, only the IGF-I/insulin hybrid receptor was phosphorylated, and phosphorylation of this receptor was completely inhibited by preincubation with αIR3 (Fig. 8B, a and b). To confirm that hybrid receptor phosphorylation resulted in downstream signaling, phosphorylation of IRS-1 was analyzed. Insulin stimulated pretreated with αIR3. Because αIR3 does not block ligand binding to insulin receptors, this result supports the conclusion that the effect of bp1-01 is due solely to hybrid receptor stimulation by IGF-I that is released from IGFBP-1. This result further supports the conclusion that the effect noted with the bp1-01 peptide in Fig. 5C was due to the hybrid receptor phosphorylation. To further confirm this finding, the experiments were repeated, and several time points were examined. When bp1-01 (400 nM) alone was incubated with HepG2 cells, only the IGF-I/insulin hybrid receptor was phosphorylated, and phosphorylation of this receptor was completely inhibited by preincubation with αIR3 (Fig. 8B, a and b). To confirm that hybrid receptor phosphorylation resulted in downstream signaling, phosphorylation of IRS-1 was analyzed. Insulin stimulated
Six forms of IGFBPs have high affinities for IGF-I, and the affinity of each protein for IGF-I is greater than that of the IGF-I receptor. IGFBP-1 has been shown to inhibit cellular responses to IGF-I by preventing IGF-I binding to its receptor; therefore, dissociation of IGF-I from the IGF-I-IGFBP-1 complex represents a potential mechanism for increasing free IGF-I and receptor activation. Recently, Loddi et al. (31) demonstrated that displacement of IGFBPs from several forms of IGFBPs, using a compound that has high affinity for IGFBPs and no affinity for IGF-I receptors, increased free IGF-I in cerebrospinal fluid, and this resulted in increased IGF-I actions. However, that compound is not specific for a single form of IGFBP. Because HepG2 cells synthesize and secrete IGFBP-1, -2 and -4, in the current study we utilized bp1-01, a molecular mimic that binds specifically to IGFBP-1 and not to other forms of IGFBPs, such as IGFBP-3 (18).

In the previous studies, bp1-01 was shown to release sufficient IGF-I from the IGF-IGFBP-1 complexes to activate IGF-I receptor phosphorylation in MCF-7 cells (18). The current study demonstrates conclusively that the IGFBP-1 binding peptide (bp1-01) binds to IGFBP-1 and competes with IGF-I for binding. The results also show that bp1-01 does not bind to the IGF-I receptor. Because αIR3, the anti-IGF-I receptor blocking antibody, inhibited the effects of bp1-01 on glycogen synthesis, and it inhibited IGF-I/insulin hybrid receptor activation by bp1-01, we conclude that activation of this receptor in HepG2 cells is due to release of bound IGF-I from IGFBP-1. Although bp1-01 binding to IGFBP-1 could directly modulate IGFBP-1 binding to HepG2 cell surfaces, a direct action of IGFBP-1 would not be inhibited by αIR3. Therefore, we conclude that the effects demonstrated with bp1-01 are due to inhibition of IGF-I binding to IGFBP-1, leading to increased association of free IGF-I with the hybrid receptors.

IGF-I has been shown to stimulate glycogen synthesis through IGF-I-R and IR, and it can stimulate tyrosine phosphorylation of both receptors (19). Although insulin has been reported to stimulate glycogen synthesis through both IR and IGF-I-R in HepG2 cells, it has not been shown to stimulate tyrosine phosphorylation of IGF-I-R (32). Activation of either receptor in HepG2 cells has been shown to evoke quantitatively similar biologic responses (19). Therefore, we used HepG2 cells as an in vitro model to investigate the effects of increasing free IGF-I concentrations on both the insulin-like and anabolic actions of IGF-I. When added alone, bp1-01 stimulated glycogen phosphorylation, and this effect was enhanced by bp1-01. The response to bp1-01 was inhibited by preincubation with αIR3 (Fig. 8C). These results are consistent with the conclusion that bp1-01 dissociates the endogenous IGF-I from the IGF-I-IGFBP-1 complex and that the released IGF-I is mediating a significant part of these effects through an interaction with IGF-I/insulin hybrid receptor.

**DISCUSSION**

FIG. 8. Effect of bp1-01 and/or insulin stimulation on the phosphorylation of IGF-I/insulin hybrid receptors, insulin receptor homodimers, and IRS-1. A, HepG2 cells were grown to 80% confluency as described under “Experimental Procedures.” The media were replaced with serum-free DMEM-H, and the cells were incubated for 30 h. At that time, some cultures (b) received 10 nM αIR3, and some cultures did not (a). The incubation was continued for 1.5 h, and then some cultures were stimulated with 1 nM insulin for 5 min. The cells were solubilized in lysis buffer without ionic detergent and immunoprecipitated with anti-αIR (1). The supernatants were immunoprecipitated with anti-α-IGFI-R (2), and both sets of precipitates were analyzed by immunoblotting using an anti-phosphotyrosine antibody. a, scanning densitometry values for the bands shown in top panel were from the left: 0, 0, 2,005, 0, 5,844, 94, 7,829, and 113; for the middle panel, they were 7,480, 7,952, 8,003, 8,674, 8,483, 8,128, 7,779, and 7,532; and for the bottom panel, they were 2,929, 0, 5,309, 0, 7,090, 0, 5,465, and 0. b, scanning densitometry values for the bands shown in top panel were from the left: 0, 0, 0, 0, 9,888, 0, 10,062, and 0; for the middle panel, they were 8,552, 6,859, 9,314, 6,364, 7,470, 6,889, 6,612, and 6,087; and for the bottom panel, they were 9,618, 0, 9,063, 0, 12,018. For the bottom panel of a, the values were 9,156, 0, 9,075, 0, 8,660, 0, 8,893, and 0. The values for A and B represent the means of three separate experiments. C, all cultures were serum-starved for 30 h, and then some cultures were exposed to αIR3 for 30 min, followed by the addition of bp1-01 for 30 min. Insulin (1.0 nM) was added for 5 min, and then the cells were solubilized in lysis buffer and immunoprecipitated with anti-IRS-1 antibody. Scanning densitometry values for the bands shown in the top panel were from the left: 0, 0, 5,447, 3,608, 3,622, 0, 3,234, and 0. Scanning densitometry values for the bottom panel were 9,708, 12,395, 12,030, 13,341, 13,352, 15,798, 13,556, and 14,659. The values for C represent the mean of two separate experiments.
IGF-I to the hybrid receptor that is activated by transphosphorylation after binding of IR (19, 32). Previous studies have reported that enhancement of insulin-stimulated glycogen synthesis is competitive with the binding of IGF-I to hybrid receptors or that insulin activation of IR is contributing to the glycogen synthesis response obtained after the addition of insulin plus bp1-01. Our results show that addition of bp1-01 to conditioned medium of HepG2 cells did not increase tyrosine phosphorylation of IR, and it did not enhance the effect of insulin on hybrid receptor phosphorylation preincubation with aIR3. This finding further supports the conclusion that bp1-01 is stimulating glucose synthesis primarily though the activation of hybrid receptors by IGF-I that has been released from IGFBP-1 and that the inhibitory effect of aIR may partially inhibit IGF-I/hybrid receptor activation. This inhibition of hybrid receptor activation could result in blocking the activation of downstream signaling components in the insulin signaling pathway because signal transfer through the IR β subunit kinase of the hybrid receptor that is activated by transphosphorylation after binding of IGF-I to the hybrid receptor α subunit results in activation of downstream signaling components that are also activated by IR (19, 32). Previous studies have reported that 40–50% of IGFBP-1 complexes form hybrid receptors in HepG2 cells (35). bp1-01 enhancement of insulin-stimulated glycogen synthesis is completely inhibited by aIR3, which further suggests that binding IGF-I to the α subunit of the hybrid receptor is required. Furthermore, bp1-01 enhanced the increase in glycogen synthesis that was stimulated with insulin, which strongly suggests that this response is due to increased free IGF-I because the response to bp1-01 under these conditions was inhibited by aIR3. Therefore, taken together, the results support the conclusion that the predominant mechanism by which bp1-01 functions is inhibition of IGF-I binding to IGFBP-1, thus resulting in enhancement of the activation of hybrid receptors.

Although bp1-01 alone stimulated glycogen synthesis, when added alone, it had no effect on protein synthesis (Fig. 2). We conclude that this difference is due to the difference in sensitivity of these two processes to stimulation by IGF-I. When the results of Figs. 2 and 3 are compared, the lowest concentration of IGF-I caused a significant increase in glycogen synthesis (1% ± 7%; n < 0.01), whereas it had no effect on protein synthesis (18% ± 4%; n, nonsignificant). Because the concentration of IGF-I that is released from IGFBP-1 by the addition of bp1-01 is low (e.g., probably <10 ng/ml), differential sensitivity of these two responses to IGF-I stimulation probably explains the difference in these responses to bp1-01.

Numerous studies have shown decreased IGF-I and increased IGFBP-1 levels in patients with catabolic states such as poorly controlled diabetes (7, 36), hypothyroidism (37), and starvation (38) and in mothers with intrauterine growth-restricted fetuses (39). In these states, insulin resistance that is characterized by hyperinsulinemia is frequently observed. In patients with insulin resistance, IGF-I administration has been shown to reduce the elevated glucose and insulin levels and to restore insulin sensitivity (13, 14). On the other hand, patients with other types of insulin-resistance such as Cushing disease (40), acromegaly (41), polycystic ovarian syndrome (42), and premature adrenarche (43) have low plasma IGFBP-1 concentrations, suggesting that it plays no role in the development of their insulin resistance. In most patients with poorly controlled type I or type II diabetes, IGFBP-1 concentrations are increased, presumably due to impaired insulin action in the liver. Our studies demonstrate that the dissociation of the IGF-I/IGFBP-1 complex could lead to increased free IGF-I that is available to bind to IGF-I receptor and IGF-I/insulin hybrid receptor, and as a result of increased IGF-I/insulin hybrid receptor interaction, there could be a subsequent increase in insulin sensitivity. These results suggest that this may be an important mechanism for enhancing insulin sensitivity in cells that possess abundant IGF-I/insulin hybrid receptors.

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