Identification of Insulin-Like Growth Factor Binding Protein-2 as a Biochemical Surrogate Marker for the in Vivo Effects of Recombinant Human Insulin-Like Growth Factor-1 in Mice

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ABSTRACT

Recent studies indicate that a daily s.c. injection of 1 mg/kg of recombinant human insulin-like growth factor-1 (rhIGF-1) for 17 days is efficacious in enhancing the functional recovery of injured sciatic nerves in CD-1 mice. To identify and characterize surrogate marker(s) that are altered in association with the administration of an efficacious dose of rhIGF-1, dose-response curves (0.1, 1 and 10 mg/kg) and time course effects (0, 0.5, 3, 6 and 24 hr) were determined after acute (single) and chronic (once daily for 17 days) injections of rhIGF-1 in CD-1 mice. Plasma glucose levels decreased in a dose-dependent fashion after either acute or chronic injections of rhIGF-1 with maximal effects at 0.5 to 1 hr after administration of rhIGF-1. Among the three insulin-like growth factor binding proteins (IGFBPs) evaluated in the study, only IGFBP2 levels were consistently increased in a dose-dependent fashion with maximal effects 3 hr after the last of a series of injections of rhIGF-1. Furthermore, IGFBP2 levels increased at a dose of rhIGF-1 (1 mg/kg) that enhances the regeneration of injured sciatic nerves in mice. Chronic administration of insulin at doses that cause comparable decreases in plasma glucose to that of rhIGF-1 did not alter IGFBP2 levels or enhance hindlimb function suggesting that the beneficial effects of rhIGF-1 occur via activation of the type-1 IGF receptor rather than the insulin receptor. Based on these criteria, IGFBP2 appears to be useful as a surrogate marker for determining the in vivo effects of rhIGF-1.

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ABBREVIATIONS: IGF-1, insulin-like growth factor-1; rhIGF-1, recombinant human insulin-like growth factor-1; IGFBP, insulin-like growth factor binding protein; TBS, Tris-buffered saline.
In Vivo Biochemical Effects of rhIGF-1

In addition to liver, which is the main source of IGF-1 in plasma, there are several local sources of IGF-1 (Schwander et al., 1983; Costefi-Peter et al., 1994) and consequently its actions are relatively widespread. There exists a highly complex family of IGFBPs that are thought to facilitate transport of IGF-1 and modulate the actions of IGF-1 on target cells. Although six different IGFBPs (1–6) have been identified (for review see Drop et al., 1992), in plasma three different IGFBPs which range between 24 and 55 kDa can be visualized reliably using a Western/ligand blot technique (Fazleabas and Donnelly, 1992). IGFBP3 appears as a doublet (48–55 kDa) due to different glycosylation states, IGFBP2 is a 34- to 36-kDa protein, and IGFBP4 has an M, of 24 kDa (Camacho-Hubner et al., 1989; Clemmons et al., 1989; Clemmons, 1993). In some studies, IGFBP1 (31 kDa) which is present in plasma at lower concentrations can be visualized by the Western/ligand blot technique. In addition to the IGFBPs, an ~150 kDa ternary complex exists consisting of IGFBP-3, IGF-1 and an acid labile subunit. IGF’s bioavailability is increased after proteolysis of this complex (Blat et al., 1994). The IGFBPs are thought to participate in several functions, including control of IGF transport in blood and out of the vascular compartment, localizing and modulating IGF’s to specific cell types and binding to receptors and regulating blood glucose levels (Lewitt and Baxter, 1991; Lewitt et al., 1991; Holly, 1991; for review see Clemmons, 1993).

Recently, rhIGF-1 has been under clinical evaluation for the treatment of amyotrophic lateral sclerosis. To facilitate selection of optimal dosage regimens and to assess the potential for tolerance it would be useful to identify a surrogate marker for rhIGF-1. By definition, a surrogate marker is a measure of some parameter associated with, but not a direct measure of, drug efficacy. It should be easily obtained from tissue or plasma, dose-responsive and, as a result, provide a basis for dosage selection in efficacy trials. The objectives of our study were to characterize biochemical effects of rhIGF-1 administration in an effort to identify a surrogate marker. As a secondary objective, we compared the effects of IGF-1 with those of insulin to determine the specificity of the regenerative effects of IGF-1.

Methods

Animals. Male CD-1 mice (Charles River, Raleigh, NC) between 30 to 60 days of age were used in the studies. Before use, mice were housed with five mice per cage and maintained on a 12 hr light/dark cycle. All mice were allowed access to food and water ad libitum.

All studies were carried out under the guidelines of the Cephalon Institutional Animal Care and Use Committee. After either acute or chronic s.c. injections of rhIGF-1 (dosed at the same time of day), mice were anesthetized under CO2 and blood was obtained via intracardiac puncture. Blood samples were immediately introduced into Microtainer heparinized tubes (Becton Dickinson, Rutherford, NJ) and kept on ice for 10 min. Samples were spun at 20,000 × g in a table top Eppendorf centrifuge for 10 min. Plasma (supernatant) was aliquoted and frozen at -80°C until use.

Experimental design. For the acute rhIGF-1 injection study, 0.1, 1 or 10 mg/kg of rhIGF-1 were administered s.c. to mice that were killed at 0, 0.5, 1, 3, 6, 12 and 24 hr after the acute injection of rhIGF-1 (n = 5 at each time point/dose).

The time course of the chronic effects of rhIGF-1 treatment was evaluated by comparing the effects at 0, 0.5, 3, 6 and 24 hr after an acute challenge injection of 1 mg/kg of rhIGF-1 in two chronic treatment groups (n = 6 at each time point): 1) mice receiving injections with vehicle (0.1 M acetic acid) for 17 days and 2) mice receiving injections 1 mg/kg of rhIGF-1 for 17 days. The dose-response effects of chronic administration of rhIGF-1 was evaluated in mice injected daily with either 1) vehicle or 2) rhIGF-1 (0.1, 1 and 10 mg/kg) for 17 days (n = 6 at each dose). On the last day, both treatment groups were challenged with either 0.1, 1 or 10 mg/kg rhIGF-1. These mice were killed at 3 hr after the last challenge injection.

Insulin (from bovine pancreas) was obtained from Sigma Chemical Co. (St. Louis, MO) and was prepared in phosphate buffered saline and administered via s.c. route (1U/kg = 0.036 mg/kg; 10 U/kg = 0.36 mg/kg).

Sciatic crush. Mice were anesthetized on day 0 with a s.c. injection of ketamine (50 mg/kg), acepromazine (0.75 mg/kg) and xylazine (5 mg/kg). Both sciatic nerves were exposed near the hip and crushed for 10 sec using hemostats whose tips were covered with plastic tubing to produce a fairly uniform amount of damage. The incision was closed with autoclips and the mice allowed to recover from the anesthetic before being randomly assigned to different treatment groups.

Both sciatic nerves were crushed and vehicle, insulin (1 or 10 U/kg) or rhIGF-1 (1 mg/kg) was administered s.c. on the day of surgery and for the next 17 days. At various times thereafter, the mice were tested for return of function in both hind limbs by measuring the ability to grip an inverted wire screen.

Grip assay. Mice were placed individually on a wire screen mesh, which was turned over to assess the ability of the mice to correctly grip the screen with their hind paws. Each mouse was tested 10 times and the number of failed trials recorded. Each failed trial was scored as a “1” with a maximum score of “10” for the test. A correct response was when a mouse gripped the screen with its hind toes. If a mouse held onto the screen by hooking its ankles or legs through the screen, the trial was still recorded as a failure.

IGF-1 immunoradiometric assay. After s.c. injections of rhIGF-1, the levels of IGF-1 attained in the plasma compartment were evaluated using the IGF-1 immunoradiometric assay kit from Diagnostic Scientific Laboratories Inc. (Webster, TX). The IGF-1 monoclonal antibody used in this assay detects human IGF-1 and does not cross-react with rat or mouse IGF-1. Hence the detection of IGF-1 in plasma reflects that of the rhIGF-1 injection only. Briefly, samples (100 μl, in duplicate) were acidified, to dissociate any complex bound to IGF-1, neutralized and assayed. Typically, 50 μl of the plasma sample and 200 μl of 125IIGF-1 were added to IGF-1 antibody coated plastic tubes and incubated at room temperature for 3 hr on an orbital shaker set at 140 r.p.m. Tubes were then overturned to drain off excess liquid and washed three times with 3 ml of distilled deionized water. Radioactivity in tubes were determined in a Wizzard gamma-counter (Wallac, Inc., Gaithersburg, MD). Pharmacokinetetic parameters were analyzed by non-compartmental pharmacokinetic analysis using a computer program written for PC-SAS (PC-SAS for Windows, Version 6.08, SAS institute, Cary, NC).

Plasma glucose determinations. Glucose levels were assessed in plasma samples of mice injected with rhIGF-1 or insulin using a glucose assay kit (Sigma Diagnostics, St. Louis, MO). Briefly, 10 μl of plasma samples (in duplicate) or glucose standards were incubated with 1 ml of glucose assay reagent for 10 min at room temperature. The reaction was terminated by the addition of 10 ml of 0.1 N hydrochloric acid. Samples were transferred to cuvettes and the plasma samples (in duplicate) were acidified, to dissociate any complex bound to IGF-1, neutralized and assayed. Typically, 50 μl of the plasma sample were added to 125IIGF-1 and not cross-react with rat or mouse IGF-1. Hence the detection of IGF-1 in plasma reflects that of the rhIGF-1 injection only. Briefly, samples (100 μl, in duplicate) were acidified, to dissociate any complex bound to IGF-1, neutralized and assayed. Typically, 50 μl of the plasma sample and 200 μl of 125IIGF-1 were added to IGF-1 antibody coated plastic tubes and incubated at room temperature for 3 hr on an orbital shaker set at 140 r.p.m. Tubes were then overturned to drain off excess liquid and washed three times with 3 ml of distilled deionized water. Radioactivity in tubes were determined in a Wizzard gamma-counter (Wallac, Inc., Gaithersburg, MD). Pharmacokinetetic parameters were analyzed by non-compartmental pharmacokinetic analysis using a computer program written for PC-SAS (PC-SAS for Windows, Version 6.08, SAS institute, Cary, NC).

Protein determination. Protein concentrations were determined in plasma after a dilution of 1:20 in distilled deionized water. Bovine plasma albumin (0.0625–2 mg/ml) was used to create a standard curve. Five μl of sample in triplicate was incubated with 300 μl of a 1:5 diluted stock solution of BioRad protein reagent (BioRad, Richmond, CA). Absorbance at 520 nm was detected in a spectrophotometer. Linear regression analysis was used to generate the standard curve and protein values were calculated from the linear regression equation.
Western/ligand blotting. IGFBPs were analyzed from plasma samples using a modified Western/ligand blot method (Hossenlopp et al., 1986; Fazleabas and Donnelly, 1992). Ten μl of 3x Laemmlli sample buffer (Laemmlli, 1970), [2% sodium dodecylsulfate, 125 mM Tris-HCl, 10% (w/v) glycerol] was added to each 20-μl sample buffer (Laemmlli, 1970), [2% sodium dodecylsulfate, 125 mM with 13tionship between increasing protein concentration (0.58–23.4 μg) of protein and ligand results in a corresponding increase in binding. Plasma samples containing 1% bovine serum albumin and 0.1% Tween-20 for 2 nights. Blots were washed three times (15 min for each wash) with TBS containing 0.1% Tween-20 followed by two washes in TBS. Blots were air-dried, placed in a Phosphor-Imager cassette and exposed for 1 day.

To determine whether an increase in protein and ligand results in a corresponding increase in binding, plasma samples containing 0.58, 2.93, 5.87, 11.7 and 23.4 μg protein were loaded on each SDS-PAGE gel (n = 6). After transfer into nitrocellulose membranes, each blot was probed with either 7.81, 15.625, 31.25, 62.5, 125 or 250 pM [125I]IGF-1 to determine whether an increase in protein and ligand results in a corresponding increase in binding. Blots were placed in cassettes and scanned using the Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). The densitometric values (volume) from the Phosphor Imager, i.e., 1-μl spots on the nitrocellulose membrane were first correlated with actual amounts of radioactivity (dpm) by counting aliquots directly in the gamma counter. The results demonstrated an excellent correlation (r² = 0.99) between the densitometric units and radioactivity detected using the gamma counter (dpm). Based on this quantitation, bands from each blot in densitometric units (volume) were converted into dpms and then into fmol of [125I]IGF-1. Further characterization of the Western/ligand blot method for IGFBPs revealed a linear relationship between increasing protein concentration (0.58–23.4 μg) and fmol bound for IGFBP3, IGFBP2 and IGFBP4. The optimal protein concentration and concentration of [125I]IGF-1 to visualize changes in binding to the IGFBPs after acute or chronic treatment found to be 5 to 10 μg and 31.25 to 62.5 pM, respectively.

Data analysis. Data were analyzed using a two-way analysis of variance. When significant effects of treatment were observed data were further analyzed by Duncan’s multiple range test. For the behavioral and biochemical studies, results are presented as mean ± S.E.M. To assess whether any treatment resulted in responses that were different from control values (sham/vehicle) on any given day, a Dunnett’s t test was carried out comparing all values to that of the control group. To determine whether any treatment was better than another treatment, the results of all groups were compared to one another using the Newman-Keuls test. Any comparisons with a P < .05 were considered to be significant.

Results

Determination of plasma rhIGF-1 levels after acute and chronic injections of rhIGF-1. The levels of rhIGF-1 in the plasma were determined in plasma samples after acute injections and were assayed using an IRMA assay. As shown in figure 1a, plasma concentrations of rhIGF-1 increased in a dose-dependent manner with the Cmax achieved 0.5 hr after administration. The levels of rhIGF-1 in plasma at 0.5 hr were 104.8 ± 5.8, 1197 ± 43.4 and 11580 ± 570 ng/ml after an acute injection of 0.1, 1 and 10 mg/kg of rhIGF-1, respectively.

After daily chronic injections with either vehicle or 1 mg/kg of rhIGF-1 for 17 days, mice were challenged with an acute injection of 1 mg/kg of rhIGF-1 and killed at various times after the challenge injection. Body weights monitored during chronic treatment indicated that no significant changes in the rate of body weight gain were observed between any of the groups during the course of the treatments (data not shown). As shown in figure 1b, peak plasma rhIGF-1 levels were attained at 0.5 hr after the challenge injection of rhIGF-1 in both treatment groups. The clearance of rhIGF-1 from plasma was similar in both treatment groups indicating that the t1/2 and clearance rates of rhIGF-1 in plasma are not
altered after chronic rhIGF-1 treatment (table 1). A slight decrease (P < .05) in rhIGF-1 levels was observed at 6 hr when compared to the acute challenge injection; however, it is questionable as to its biological significance.

The dose-response effects of chronic rhIGF-1 treatment were determined in mice killed 3 hr after the last injection, a time at which levels of IGFBPs were found to be maximally altered in initial studies. No significant differences in plasma levels of rhIGF-1 were observed between the chronic and acute challenge groups at either the higher (10 mg/kg) or lower dose (0.1 mg/kg) of rhIGF-1 (data not shown).

**Determination of plasma glucose levels after acute and chronic injections of rhIGF-1.** Because IGF-1 has been reported to result in hypoglycemia at high doses, plasma glucose levels were determined in samples from mice treated with an acute injection of rhIGF-1. Plasma glucose levels were significantly reduced between 0.5 and 1 hr, the time of peak plasma concentrations of rhIGF-1, after either the acute injection of 1 or 10 mg/kg of rhIGF-1 (fig. 2a). In order to determine the effects of chronic treatment with 1 mg/kg of rhIGF-1, the time course for the decrease in plasma glucose levels was determined in mice after the last of a series of repeated injections. As shown in figure 2b, neither potentiation nor tolerance to the peak effect of rhIGF-1 was observed after the acute challenge injection of 1 mg/kg in both treatment groups. However, although plasma glucose levels returned to baseline levels by 3 hr after the acute rhIGF-1 challenge injection in chronic vehicle-treated mice, they were still significantly lower in chronic rhIGF-1-treated mice. By 6 hr, plasma glucose levels in both groups returned to control levels. These results suggest that the decrease in plasma glucose levels is slightly prolonged after chronic treatment with rhIGF-1. This prolonged effect on plasma glucose at 3 hr after chronic treatment with rhIGF-1 was observed at all challenge doses of rhIGF-1 when compared to the chronic vehicle treated group (fig. 2c).

**Determination of plasma IGFBP levels after chronic injections of rhIGF-1.** In initial experiments, commercially available mouse plasma was used to analyze the three IGFBPs by Western/ligand blots. Incubation of a second nitrocellulose blot with a 1000-fold excess of unlabeled rhIGF-1 prevented specific binding of [125I]IGF-1 to the IGFBPs (data not shown). Addition of high concentrations (100 ng/ml) of rhIGF-1 to mouse plasma samples did not alter the levels of the IGFBPs suggesting that endogenous amounts of IGF-1 are not carried along with IGFBPs during gel electrophoresis (data not shown).

After Western/ligand blot analysis, three major bands can be visualized as shown in lane 1 (fig. 3a). The 48- to 55-kDa doublet reflects IGFBP3 and the 34- to 36-kDa singlet reflects IGFBP2. The estimated M_r sizes of each of the forms was determined by comparison with defined M_r size standards and are consistent with previous published reports.

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C_{max} (mg/ml)</th>
<th>T_{max} (hr)</th>
<th>t_{1/2} (hr)</th>
<th>CL/F (ml/min/kg)</th>
<th>V/F (liter/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>1047.3</td>
<td>0.5</td>
<td>5.1</td>
<td>2.54</td>
<td>1.11</td>
</tr>
<tr>
<td>Chronic</td>
<td>944.1</td>
<td>0.5</td>
<td>4.6</td>
<td>2.60</td>
<td>1.05</td>
</tr>
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(Camacho-Hubner et al., 1991; Clemmons, 1993). The identity of the two IGFBPs was further confirmed by comparison of the bands with those of purified rhIGFBP2 and rhIGFBP3 (lanes 2 and 3, fig. 3a). The 24-kDa band has been previously reported to be IGFBP4 (Camacho-Hubner et al., 1991; Clemmons et al., 1993). Due to the unavailability of purified recombinant IGFBP4, direct identity comparisons could not be made, hence we can only assume that this band is IGFBP4. In some of the experiments, a faint band at ~ 31 kDa (presumably IGFBP1) was observed. Densitometric analyses using the Phosphor Imager revealed that the value of this band were near background levels and could not be measured reliably. Therefore, further characterization and quantitation of only IGFBP2, IGFBP3 and 24-kDa protein (IGFBP4) were carried out.

Figure 3b shows a representative Western blot of the chronic time course effects of rhIGF-1. Three hours after an acute challenge injection of 1 mg/kg of rhIGF-1, IGFBP2 levels in plasma maximally increased in mice chronically injected with either vehicle or 1 mg/kg rhIGF-1 (fig. 4a). This effect was confirmed by Western immunoblot technique using a polyclonal antibody to IGFBP2 (obtained from UBI, data not shown). At 6 hr after the challenge injection of rhIGF-1, mice chronically receiving injections with rhIGF-1 had significantly elevated levels (~ 2-fold) of plasma IGFBP2 when compared to mice chronically injected with vehicle. Levels of IGFBP2 in plasma at 24 hr after the rhIGF-1 injection (defined as baseline) were not significantly different from IGFBP2 levels in naive (noninjected) mice indicating that the IGFBP2 does not accumulate in plasma during the series of repeated injections. As shown in figure 4b, the increase in IGFBP2 in response to an acute challenge injection of rhIGF-1 is dose dependent. The low dose of rhIGF-1 (0.1 mg/kg) did not alter IGFBP2 levels; however, at the 1-mg/kg dose of rhIGF-1, only mice chronically treated with rhIGF-1 showed an significant increase (~ 2.7-fold) in IGFBP2 levels. Although the acute challenge injection of 1 mg/kg of rhIGF-1 did not increase IGFBP2 in vehicle-treated mice, it is likely that 1 mg/kg rhIGF-1 is a threshold dose because in the previous experiment, an acute injection of 1 mg/kg rhIGF-1 caused an increase in IGFBP2 levels (fig. 4a). At 10 mg/kg of rhIGF-1, IGFBP2 levels were significantly elevated in both treatment groups (fig. 4b). Furthermore, at this dose, IGFBP2 levels were greater in the chronic rhIGF-1 group compared to the acute rhIGF-1 (chronic vehicle, acute rhIGF-1 challenge injection) group.

In response to the challenge injection of 1 mg/kg of rhIGF-1, an increase in IGFBP3 levels was not observed in either treatment group (fig. 4a). After chronic treatment a small but significant decrease (24%) in IGFBP3 levels was observed after an injection of 0.1 mg/kg of rhIGF-1 when compared to noninjected or vehicle injected controls (fig. 4b). At the 1-mg/kg dose of rhIGF-1, IGFBP3 levels were similar to that of controls. After the 10-mg/kg challenge injection of rhIGF-1 in chronic rhIGF-1-treated mice, a significant increase (1.2-fold) was observed in plasma IGFBP3. Analyses of the 24-kDa band (IGFBP4) levels revealed that changes in the levels of this protein were not observed at any of the doses of rhIGF-1 in either of the groups (fig. 4, a and b).

**Comparison of the effects of insulin with rhIGF-1 after sciatic nerve crush.** To examine whether a peptide similar to rhIGF-1 that also has the ability to decrease
plasma glucose could be efficacious in 1) enhancing regeneration of the sciatic nerve and 2) increasing IGFBP2 levels, we determined the effect of insulin on grip and IGFBPs after sciatic nerve injury.

In initial experiments the time course and dose-response effects of insulin on plasma glucose levels were determined (data not shown). From these studies, we determined that 1 and 10 U/kg of insulin caused a similar decrease in plasma glucose to that of 1 and 10 mg/kg of rhIGF-1. To compare the effects of multiple injections of insulin and rhIGF-1 on behavioral parameters associated with sciatic nerve function and IGFBP2 levels, CD-1 mice with bilateral crushes of the sciatic nerves were injected daily for 17 days with vehicle for insulin (phosphate-buffered saline), insulin (1, 10 U/kg), vehicle for rhIGF-1 or 1 mg/kg of rhIGF-1. Mice were tested for their ability to grip an inverted screen in 10 trials. As shown in figure 5a, the rate of recovery of grip ability was significantly enhanced after daily injections with rhIGF-1 but not after insulin. The effects of multiple injections of insulin and rhIGF-1 on plasma glucose at 0.5 hr (significant decrease in
Both agents significantly decreased plasma glucose 0.5 hr after the injection. The effects of 1 U/kg insulin on plasma glucose was not significantly different from that after 1 mg/kg of rhIGF-1.

We have demonstrated in above experiments that IGFBP2 appears to be a potential surrogate marker for the effects of rhIGF-1. To assess the effects of repeated injections of insulin on IGFBP2 and compare the effects to that of rhIGF-1, IGFBP2 levels were determined in plasma 3 hr (maximal effects on IGFBP2) after the last injection. As shown in figure 5c, a significant increase in IGFBP2 was observed after 1 mg/kg of rhIGF-1.
rhIGF-1. An increase in IGFBP3 but not IGFBP4 (24-kDa protein) levels were also observed (data not shown). Conversely, no significant alterations in the levels of any of the IGFBPs were observed after repeated administration of 1 or 10 U/kg insulin (fig. 5c).

**Discussion**

Several lines of evidence suggest that IGF-1 plays a key role in nerve regeneration and sprouting (Henderson et al., 1983; Near et al., 1992; Gehrmann et al., 1994; Caroni et al., 1994; Contreras et al., 1995). Furthermore, recent studies suggest that systemic administration of rhIGF-1 for 17 days enhances the rate of functional recovery of injured sciatic nerve of adult mice (Contreras et al., 1995). The results of the latter study suggest that a bell-shaped dose-response curve exists for rhIGF-1, and although 1 mg/kg of rhIGF-1 was the most efficacious dose for enhancing functional recovery in injured sciatic nerves of mice, 10 mg/kg of rhIGF-1 was modestly effective in recovery of hindlimb function. Our purpose was to identify a biochemical surrogate marker in plasma that is altered at a dose that causes a therapeutic

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**Fig. 5.** Comparison of the chronic in vivo effects of insulin and rhIGF-1 after bilateral crushes of the sciatic nerve. a) Effect of insulin and rhIGF-1 on recovery of grip. Each mouse was tested five times for the ability to grip an inverted screen. Results are presented as the mean ± S.E.M. *Value is significantly different from the sham/vehicle group (P < .05, Dunnett’s t test). b) Effect of insulin and rhIGF-1 on plasma glucose. Plasma glucose was measured in mice injected daily with vehicle, 1 and 10 U/kg insulin or vehicle and 1 mg/kg of rhIGF-1 for 17 days. All groups were challenged with an acute injection of rhIGF-1 and blood was taken before death at 0.5 hr after the challenge injection. Bars represent the mean ± S.E.M. of five mice. Values differing from appropriate vehicle control: *P < .05. c) Effect of insulin and rhIGF-1 on plasma IGFBP2 levels. Plasma IGFBP2 was measured by Western/ligand blot method in mice injected daily with vehicle, 1 and 10 U/kg insulin or vehicle and 1 mg/kg of rhIGF-1 for 17 days. All groups were challenged with an acute injection of rhIGF-1 and blood was taken before death at 3 hr after the challenge injection. Bars represent the mean ± S.E.M. of five mice. Values differing between sham and crush mice: #P < .05. Values differing from sham-vehicle: *P < .05.
effect and does not result in biochemical tolerance after chronic treatment. Based on the results of our study, changes in IGFBP2 fit the criteria for a surrogate marker for chronic rhIGF-1 treatment.

In response to either acute or chronic administration of rhIGF-1, plasma glucose levels decrease to similar levels with a maximal effect observed at 0.5 hr after injection. This suggests that neither potentiation nor tolerance to the peak hypoglycemic effects of rhIGF-1 were observed. A decrease in plasma glucose in response to IGF-1 administration is consistent with previous reports which show that IGF-1 has insulin-like activity on blood glucose levels (Zapf et al., 1986; Snyder and Clemons, 1990; for review see Froesch et al., 1985). The decrease in plasma glucose levels was found to recover to control levels more slowly after chronic rhIGF-1 treatment when compared to an acute rhIGF-1 injection. Although the mechanism of action and physiological role of this effect is unclear, it is possible that this effect could be due to altered IGFBP levels. For example, IGFBP1 has been shown to regulate blood glucose levels by counteracting the insulin-like activity of IGF-1 in vitro (Drop et al., 1979; Ritvos et al., 1988) and in vivo (Lewitt et al., 1991). In our assay we cannot reliably measure IGFBP1; however, it is tempting to speculate that changes in IGFBP1 levels or function after chronic treatment with rhIGF-1 could retard the return of plasma glucose to normal levels.

Another possibility for observing this prolonged decrease in plasma glucose would be if the levels of rhIGF-1 accumulate in plasma after repeated rhIGF-1 injections, i.e., there is an alteration in the pharmacokinetic parameters of rhIGF-1 after chronic treatment. However, this does not appear to be the case since changes in the clearance rate or \( t_{1/2} \) of rhIGF-1 after chronic treatment were not observed in our studies. Because both acute and chronic injections of rhIGF-1 alter plasma glucose levels at a dose that is efficacious at enhancing the functional recovery of injured sciatic nerves (1 mg/kg), it fits the criteria for a surrogate marker. However, it is important to note that circadian rhythms, nutrition and stress can also affect plasma glucose levels and, therefore, plasma glucose cannot be used as a surrogate marker.

In response to 1 mg/kg of acute or repeated rhIGF-1 injections, plasma IGFBP2 levels showed a dramatic increase (208%) with peak effects attained at 3 hr after the challenge injection. This effect is consistent with a previous study showing that in human subjects, a daily injection of IGF-1 showing that in human subjects, a daily injection of IGF-1 for injection. This effect is consistent with a previous study (208%) with peak effects attained at 3 hr after the challenge.

Previously studies have shown that plasma levels of IGFBP2 are not regulated by glucose infusion, insulin or circadian rhythms (Clemons et al., 1991). We have also demonstrated that the response of IGFBP2 is specific for rhIGF-1 because chronic administration of insulin, failed to significantly alter IGFBP2 levels. Furthermore, we have shown that low doses of insulin, which decreases plasma glucose comparable to that after rhIGF-1 does not enhance hindlimb function. These experiments suggest that the effects of rhIGF-1 on IGFBP2 levels and regeneration of hindlimb function are modulated via the type-1 IGF receptor and not via the insulin receptor. Because both acute and chronic injections of rhIGF-1 alter plasma IGFBP2 levels at a dose that is efficacious at enhancing regeneration of the sciatic nerve function, and do not result in biochemical tolerance, IGFBP2 appears to be useful as surrogate marker for the chronic effects of rhIGF-1.

Because both acute and chronic injections of rhIGF-1 alter plasma IGFBP2 levels at a dose that is efficacious at enhancing or the rate of degradation of IGFBP2. A recent study showed that after sciatic nerve axotomy in neonates, IGFBP2 mRNA increases with a concomitant increase in IGF-1 (Gehrmann et al., 1994). Whatever the case may be, it is clear that biochemical tolerance to the increase in IGFBP2 was not observed after chronic rhIGF-1 treatment.

Chronic administration of 10 mg/kg of rhIGF-1 led to greater increases in IGFBP2 levels in plasma. Although efficacy has been observed at this dose of rhIGF-1, it appears to be less effective than 1 mg/kg of rhIGF-1 at promoting recovery of hindlimb function (Contreras et al., 1995). However, after administration of 10 mg/kg rhIGF-1, we find that plasma glucose levels decrease to about 50% of control levels. Therefore, it is possible that complications such as decreases in plasma glucose at high doses could negate the positive effect of neuronal regeneration.

In Vivo Biochemical Effects of rhIGF-1

In Vivo Biochemical Effects of rhIGF-1

Previous studies indicate that changes in IGFBP3 are more variable and can be influenced by the particular conditions of treatment. For example, twice daily s.c. injections of 0.04 mg/kg of IGF-1 for 7 days in growth hormone receptor-deficient patients did not alter IGFBP3 (Gargosky et al., 1993). However, low doses of IGF-1 (0.1–0.125 mg/kg) in fasted human subjects for 7 days slightly decreased IGFBP3 levels (Baxter et al., 1993). These studies have not used high enough doses of IGF-1 to observe any increases in IGFBP3. In contrast to injections, infusion studies in humans show that IGFBP3 increases in response to IGF-1 (Clemons et al., 1989; Quin et al., 1994). Our results suggest that the effects of rhIGF-1 on IGFBP3 are dose-dependent. Although low doses of rhIGF-1 (0.1 mg/kg) decrease IGFBP3 levels, a dose of 10 mg/kg of rhIGF-1 was found to modestly increase IGFBP3 levels. However, because IGFBP3 did not increase in association with a functionally efficacious dose of rhIGF-1, IGFBP3 is not a useful surrogate marker for the chronic in vivo effects of rhIGF-1.

IGFBP4 (24-kDa protein) did not change in response to any of the chronic rhIGF-1 treatments. Consistent with previous reports, this binding protein is not altered after rhIGF-1 administration (Clemons et al., 1989) and therefore does not fit the criteria for a surrogate marker for the effects of rhIGF-1.

In conclusion, we have identified significant increases in the levels of IGFBP2 after repeated injections of an efficacious dose (1 mg/kg) of rhIGF-1. Furthermore, we have determined that this repeated administration did not result in tolerance to IGFBP2 increases. Taken together, we suggest that IGFBP2 would be useful as a biochemical marker for determining the chronic in vivo effects of rhIGF-1. The observation that rhIGF-1, but not insulin, increases both IGFBP2 levels as well as recovery of hindlimb function is consistent with the hypothesis that the effects of rhIGF-1 are mediated via the type-1 IGF but not the insulin receptor.

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