Development of a Long-Acting Insulin Analog Using Albumin Fusion Technology


The primary therapeutic goal for the treatment of diabetes is maintenance of a long-term near-normoglycemic condition and prevention of the onset or progression of the complications associated with the disease. Although several analogs of human insulin have been developed, the currently prescribed long-acting insulin analogs do not provide a stable basal glycemia for more than a few hours. Here, we report the development of Albulin, a long-acting insulin analog obtained by direct gene fusion of a single-chain human insulin to human serum albumin. Albulin showed an elimination $t_{1/2}$ of ~7 h in normoglycemic mice. In vitro pharmacodynamic profiles for Albulin characterized by receptor binding, inhibition of gluconeogenesis, induction of glucose uptake, and global regulation of gene expression in relevant cell types showed that Albulin produced similar activity profiles compared with that of recombinant human insulin. A single Albulin administration in vivo normalized blood glucose level in diabetic mice in a relatively peakless and sustained (24-h) fashion. A further reduction in glucose levels was achieved by administering a recombinant human insulin a few hours after Albulin injection in mice, indicating the potential for Albulin therapy in combination with available fast-acting insulin derivatives. In summary, Albulin displays characteristics of a potent long-acting insulin analog that can be evaluated for use as a novel insulin therapy for patients with insulin-dependent diabetes. Diabetes 54:251–258, 2005

RESEARCH DESIGN AND METHODS

Expression and purification of Albulin. A synthetic gene construct encoding a single-chain insulin containing the B- and A-chain of mature human insulin linked together by a dodecapeptide linker was obtained using four overlapping oligonucleotide primers and PCR amplification. The resulting PCR product was ligated in-frame between the signal peptide of HSA and the NH$_2$-terminus of mature HSA, contained within a pSAC35 vector for expression in yeast (pSAC35-Albulin). The same synthetic gene was also cloned in-frame between a myeloid progenitor inhibitory factor-1 signal peptide sequence and the NH$_2$-terminus of HSA into pC4, a mammalian proprietary expression vector (pC4Albulin). Chinese hamster ovary (CHO) cells were stably transfected with pC4Albulin with Lipofectamine 2000 (Invitrogen) using the manufacturer’s protocol. The Albulin-expressing single clones were selected based on HSA enzyme-linked immunosorbent assay (ELISA). Albulin resulting from expression in yeast or CHO cells was purified using conventional chromatographic methods. Studies reported in this article were performed using Albulin expressed in both yeast and CHO cells.

Receptor binding assays. Competition binding to the human insulin receptor was assayed in a 96-well plate using 0.6 × 10$^{-5}$ IM-9 B-cells/well in 10 µl complete medium (RPMI 10% fetal bovine serum) with 0.3 nmo/l $^{125}$I-labeled insulin (Amersham; specific activity, 2,000 Ci/mmol) in the absence or presence of various concentrations of unlabeled Albulin or insulin. Competition binding to the IGF-I receptor was assayed in rat L6 myoblasts (2 × 10$^{5}$/wells) using 0.3 nmo/l $^{125}$I-labeled IGF-I (Amersham; specific activity, 2,000 Ci/mmol). Binding reaction was performed at room temperature for 2 h on a shaker platform. The bound and unbound ligands were separated by centrifugation through 200 µl of 1.5 dibutylphthalate/1.0 bis (2-ethylhexyl)
phthalate mixture in a polyethylene microtube (Bio-Rad, Hercules, CA) for 20 s. The cell pellet in the bottom of the tube containing the bound radiolabeled ligand was cut off using a tube cutter and counted in a γ counter. Binding data were analyzed by Prism (GraphPad Software, San Diego, CA) to determine the concentration that results in 50% inhibition (IC50) values.

**Proliferation assays.** Proliferation assays were performed using rat B1C2 cardiomyocytes and mouse fibroblasts in vitro. After 12 h, 1 × 105 cells were plated in a 96-well plate and starved for 24 h in serum-free medium. Cells were treated with various concentrations of insulin or Albulin in 100 μl RPMI medium containing 0.5% fetal bovine serum for 24 h at 37°C. H-thymidine (5 μCi) was added to each well, and cells were incubated for another 6 h. Cells were harvested, and H-thymidine incorporation was measured on a scintillation counter.

**Glucose uptake assay in 3T3-L1 adipocytes.** Murine 3T3-L1 fibroblasts were grown and differentiated into adipocytes as previously described (16). A modification of a method (14) was used for the measurements of 2-deoxyglucose uptake. A 96-well plate containing 3T3-L1 adipocytes was washed twice with Dulbecco’s modified Eagle’s medium (DMEM) low glucose (Life Technologies). Insulin and Albulin were added for 30 min, followed by addition of 0.1 mmol/l 2-deoxy-[2,6-3H]glucose (0.5 mCi/mmol) for 10 min at 37°C.

**Glucose production assay.** A previously described protocol (15) was adapted for a 96-well format. 3T3-L1 cells were grown, and glucose in high-glucose DMEM containing 40 mmol/l sodium DL-lactate and 2 mmol/l sodium pyruvate were added to the wells (100 μl), and incubation was continued for 5 h at 37°C. The supernatant was then harvested, and the cells were lysed in 200 μl 1% (wt/vol) sodium dodecyl sulfate in PBS. Glucose concentrations were measured using the Amplex Red kit (#A22177) from Molecular Probes (Eugene, OR). Glucose concentrations were calculated using Prism software and adjusted for protein concentrations.

**Reporter assays.** The PEPCk (PEPCk-SEAP [secreted embryonic alkaline phosphatase]) reporter construct was based on the promoter-less pSEAPneo vector that contained the wild-type PEPCk promoter sequence from -600 to +69 fused to the SEAP gene. Stable H4IIE/PEPCk-SEAP reporter cells were treated with 0.5 mmol/l dexamethasone for 18 h to activate the PEPCk promoter before addition of Albulin or insulin. After a 48-h incubation period, conditioned media were removed, and SEAP activity was determined using the manufacturer’s recommended protocol (Tropix Phospha-Light System, Wellesley, MA). Glucose concentrations were calculated using Prism software and adjusted for protein concentrations.

**Statistical analysis.** Data from all in vivo studies using mice were analyzed by using a one-way repeated-measures ANOVA model with a subsequent pairwise treatment comparison. When the F test P value was <0.05, the treatment effect was considered significant.
yeast or in mammalian cells. Albulin consists of the B- and A-chain of human insulin (100% identity to native human insulin), linked together by a dodecapeptide linker and fused to the NH2-terminus of the native HSA (Fig. 1A). Albulin was collected from yeast supernatants at a concentration of 2–10 mg/l and purified to near 100% homogeneity. Western blotting using a monoclonal antibody raised against human proinsulin resulted in a strong signal with purified Albulin (Fig. 1C). NH2-terminal sequencing confirmed that the Albulin secreted from yeast or CHO cells was formed by the cleavage of the HSA signal peptide and started with the first eight amino acids of the human insulin B-chain (Phe-Val-Asn-Gln-His-Leu-Cys-Gly).

Receptor binding and proliferation studies. To characterize the in vitro binding properties of Albulin, competition binding assays were performed in various cell lines that express the insulin receptor (19). Competitive binding data demonstrated that Albulin bound to the insulin receptor in IM-9 cell lines with a binding affinity lower than that of insulin (Fig. 2A and Table 1). Similar results were

<table>
<thead>
<tr>
<th>Binding to insulin receptor</th>
<th>Insulin</th>
<th>Albulin</th>
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<tr>
<td>Estimated IC50 or EC50 (nmol/l)</td>
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<tr>
<td>Insulin- and Albulin-mediated proliferation in HSC2 cells</td>
<td>2.1</td>
<td>2.7</td>
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<td>Insulin/Albulin-mediated proliferation in L6 cells</td>
<td>1.40</td>
<td>0.45</td>
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<tr>
<td>Glucose uptake in 3T3-L1 adipocytes</td>
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<td>46.1</td>
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<tr>
<td>Glucose production in H4IIE hepatocytes</td>
<td>0.51</td>
<td>2.23</td>
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<tr>
<td>SEAP activity in PEPCK reporter assay</td>
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<tr>
<td>SEAP activity in SREBP reporter assay</td>
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<td>11.5</td>
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<td>SEAP activity in ME reporter assay</td>
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<td>SEAP activity in FAS reporter assay</td>
<td>39.7</td>
<td>27.1</td>
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IC50 and EC50 values derived from the in vitro pharmacodynamic studies shown in Figs. 2 and 3 are collected in this table to facilitate potency comparisons between the two agents.

![FIG. 1. Structure and purification of Albulin. A: Domain structure of the single-chain insulin fused to the NH2-terminus of recombinant HSA to create Albulin. B and C: Analysis of Albulin purified from yeast supernatants. A: A Comassie blue staining of 2.5 µg Albulin after SDS-PAGE analysis in nonreducing (NR) and reducing (R) conditions is shown (B). Purified Albulin was resolved by SDS-PAGE and immunobotted with mAb against human serum insulin (C).](image)

![FIG. 2. In vitro properties of Albulin. A: Binding to human insulin receptor. IM-9 cells were incubated with 0.1 nmol/l 125I-insulin in the absence or presence of various concentrations of insulin or Albulin. B: Binding to the IGF-I receptor. L6 cells were incubated with 0.1 nmol/l 125I-IGF-I in the absence or presence of various concentrations of insulin or Albulin. C and D: Insulin- and Albulin-induced proliferation in H9C2 (C) and L6 (D) cells. E: Insulin and Albulin effects on glucose uptake in 3T3-L1 adipocytes. F: Insulin and Albulin effects on glucose production in H4IIE cells.](image)
obtained using CHO cells overexpressing the human insulin receptor, rat H4IIE hepatoma cells, and human HepG2 hepatoma cells (data not shown).

Insulin can also bind to the IGF-I receptor with a lower affinity than to the insulin receptor (20). To determine the binding affinity of Albulin to the IGF-I receptor, a competition binding assay was performed using 125I-labeled IGF-I in L6 cells. As shown in Fig. 2B, Albulin completely displaced 125I-IGF-I, and the binding affinity was slightly lower (threefold) than that of insulin (Table 1).

High affinity for IGF-I receptors of some insulin analogs have been correlated with growth-promoting activity (21). Moreover, certain insulin analogs with higher affinity toward the insulin receptor can produce higher mitogenic responses in vitro (22). To compare Albulin’s mitogenic effects to that of insulin, proliferation assays were performed with insulin and Albulin using two commonly used myoblast cells: H9C2 (Fig. 2C) and L6 (23) (Fig. 2D). Albulin induced proliferation of cells in a dose-dependent fashion showing similar (H9C2) to threefold-higher (L6) potency than that of insulin (Table 1).

**In vitro activity of Albulin: glucose uptake and production.** Insulin activities include stimulation of peripheral glucose disposal and inhibition of hepatic glucose production (24). The ability of Albulin to mediate these two key biological functions was assayed in vitro, and comparison was made with the activity of recombinant human insulin. First, the effect of insulin and Albulin on glucose uptake in 3T3-L1 adipocytes was investigated. Pretreatment of the cells with insulin or Albulin resulted in a dose-dependent increase in 2-deoxyglucose uptake with very similar characteristics (Fig. 2E). Maximal glucose uptake and EC50 values (Table 1) were not significantly different between the two proteins. Similar results were obtained using cultured human adipose cells (data not shown). We then examined whether Albulin could regulate the net glucose production in H4IIE hepatoma cells (Fig. 2F). Pretreatment of the cells with Albulin led to a dose-dependent inhibition of the amount of glucose released by H4IIE cells in the incubation medium. Albulin was as potent as recombinant human insulin at inhibiting gluconeogenesis in this system (Table 1).

**In vitro activity of Albulin: regulation of gene expression.** In addition to the direct activation of signaling pathways in target tissues, insulin regulates tissue functions by controlling the transcription of numerous genes involved in growth and metabolism (25). We have used two separate strategies to compare the ability of Albulin and insulin to regulate gene transcription.

The first approach was to evaluate reporter assays using promoter regions of four human genes (e.g., *PEPCK, SREBP, malic enzyme,* and *FAS*) that are known to be regulated by insulin (Fig. 3). PEPCK is a rate-limiting enzyme in hepatic gluconeogenesis, and the *PEPCK* gene is regulated by insulin (26). Our data showed that treatment of the *PEPCK*-SEAP reporter with either Albulin or insulin strongly inhibited the secretion of SEAP in a dose-dependent manner. The expression profiles observed in the cDNA array analysis were confirmed for a subset of the 32 genes with distinctive regulation patterns (*DEPP, ID3, ADM,* and *CRABP2*) through qPCR (Fig. 5).

**In vivo control of glycemia.** The ability of Albulin to reduce hyperglycemia was tested in STZ-induced diabetic mice. A single subcutaneous injection of Albulin in freely fed diabetic mice produced a gradual decline in blood glucose level, with the lowest levels recorded at 6 h postinjection (Fig. 6A). The reduction in blood glucose and insulin can induce binding of SREBPs to their cognate sequence with approximately similar efficiency (Table 1). We also tested the effect of Albulin and insulin on the transcription of two other key enzymes involved in hepatic fatty acid metabolism: malic enzyme and FAS (28). Treatment of FAS and *malic enzyme* reporters with Albulin or insulin strongly stimulated the secretion of SEAP in a dose-dependent and comparable manner.

The other approach to determine Albulin’s ability to regulate gene transcription was based on cDNA microarray profiling using insulin-responsive cells after Albulin or insulin treatment. A wide-ranging comparison of the effects of Albulin and insulin on global gene expression in human primary adipocytes was performed through cDNA array analysis. Of the 6,476 genes represented on the array, 32 were consistently up- or downregulated at least twofold in at least three of the treatment groups (either Albulin or insulin) compared with the respective control. These 32 genes were organized using unsupervised hierarchical clustering (29) (Fig. 4). Changes in gene expression after treatment with insulin and Albulin were very similar, and no genes were identified that were regulated exclusively by either ligand. The striking similarity of responses to Albulin and insulin is illustrated in the dendrogram arrangement of treatments at 6 h, where responses to the two ligands were found to be more similar than the effects of treating with the same molecule at different concentrations (1× and 10×). The expression profiles observed in the cDNA array analysis were confirmed for a subset of four genes with distinctive regulation patterns (*DEPP, ID3, ADM,* and *CRABP2*) through qPCR (Fig. 5).
levels induced by Albulin was relatively slow (peakless), and glycemia remained significantly ($P < 0.05$) lower than control values in vehicle-treated mice for up to 24 h. There was no significant difference between HSA- and vehicle-treated control mice. When compared with HSA control mice, the highest dose of Albulin (10 mg/kg) showed a significant ($P < 0.01$) reduction in glycemia at 24 h, and all four Albulin doses (1–10 mg/kg) lowered ($P < 0.05$) glucose level up to 12 h postinjection.

To evaluate whether currently available short-acting insulin analogs retain the ability to induce hypoglycemia in the presence of Albulin, diabetic mice treated with Albulin (2.5 mg/kg s.c.) were reinjected with Humulin R (5.0 units/kg s.c.). Albulin alone was able to maintain a reduced glucose level for almost 24 h (Fig. 6B). As expected, Humulin R alone induced a sharp and fast hypoglycemia (peak ~1 h), after which glucose levels returned to high basal levels within 3 h. When Humulin R was reinjected to mice treated with Albulin, an additional reduction ($P < 0.01$) in glucose levels was observed at 1 h after Humulin injection.

In the next experiment, a glucose challenge was given to fasted diabetic mice to mimic prandial events (Fig. 6C). Predictably, Humulin R alone produced significant hypoglycemia when administered just before the glucose challenge in mice. Whereas Albulin alone (Albulin-vehicle) was able to reduce glucose levels for the initial 3 h, it did not significantly enhance glucose clearance after the glucose challenge compared with controls. When mice were injected with Humulin R 3 h after Albulin administration and then immediately challenged with glucose, the combination therapy was able to produce a significant ($P < 0.05$) reduction in glucose compared with mice treated with Albulin alone.

**In vivo pharmacokinetics of Albulin.** Plasma concentrations after intravenous and subcutaneous dosing of Albulin are presented in Fig. 7A. The results of the pharmacokinetic analyses are reported in Table 2. A 1.0 mg/kg dose of Albulin was detectable for the 72-h duration of this study after both intravenous and subcutaneous administration. Albulin appears to be largely confined to the plasma after intravenous injection with a volume of
distribution of 48 ml/kg. Clearance was 4.9 ml·h⁻¹·kg⁻¹ after intravenous injection. The apparent volume of distribution was 132 ml/kg after subcutaneous injection, and apparent clearance was 13.5 ml·h⁻¹·kg⁻¹. The increased apparent volume of distribution and apparent clearance values reflect the 36% subcutaneous bioavailability of Albulin relative to intravenous injection. The half-life of Albulin is ~7 h when given intravenously or subcutaneously. The absorption t₁/₂ was 4 h after subcutaneous injection. Research has shown that the interspecies pharmacokinetic behavior of a wide range of therapeutic proteins can be predicted using the equation \( Y = a W^b \), where \( Y \) is the parameter of interest, \( a \) is the allometric coefficient, \( W \) is the body weight (in kilograms), and \( b \) is the allometric exponent (30). If the 4.9 ml·h⁻¹·kg⁻¹ clearance of Albulin observed in a 23-g mouse is scaled (allometric exponent of 0.75) to predict clearance in a 70-kg human, a clearance value of 0.66 ml·h⁻¹·kg⁻¹ is obtained. The predicted elimination half-life of Albulin in a 70-kg human would be ~50 h based on scaling of the mouse data (Fig. 7B).

**DISCUSSION**

We report here the characterization of a potent long-acting insulin analog, Albulin, engineered using albumin fusion technology. Others have obtained biologically active single-chain insulins through replacement of proinsulin C-peptide with peptide linkers of various sizes, resulting in a covalent and permanent attachment of the A- and B-chain of insulin (31,32). To determine the influence of single-chain engineering and HSA fusion on the binding characteristics and biological activity of human insulin, Albulin was tested in a range of in vitro assays.

Albulin displayed a lower affinity for the human insulin receptor, as well as a slightly lower affinity for the IGF-I receptor, when compared with human recombinant insulin. Analogs of insulin that result from changes in the native amino acid sequence can display growth-promoting activities higher than those of recombinant human insulin, a characteristic usually attributed to higher affinity for the IGF-I receptor (20,21). The long-acting insulin analog glargine, despite a six- to eightfold increase in IGF-I receptor binding affinity and mitogenic potency (21), has yet to be associated with any mitogenic-related safety issues. Albulin displays a lower affinity for the IGF-I receptor and no more than a threefold increase in growth-promoting activity when compared with recombinant human insulin; it is therefore unlikely to trigger abnormal mitogenic responses in humans.

The two key functions of insulin (i.e., inhibition of gluconeogenesis in the liver and stimulation of glucose uptake in peripheral tissues) were tested in vitro using
Albulin in hepatic and adipose cell lines, respectively. In both systems, Albulin behaved like insulin, with little or no loss of activity. When the regulatory activities of Albulin and insulin on gene transcription were compared using transcription reporter assays, as well as a global gene expression pattern analysis, very few differences were observed between the two proteins.

Pharmacokinetic studies performed in mice can provide information relating to the molecule's pharmacokinetics properties in humans, especially when the data are compared with studies using analogs for which clinical data are available. The elimination half-life of subcutaneously administered Albulin in mice was found to be ~7 h. In comparison, the reported elimination half-life of subcutaneously administered recombinant human insulin in mice is ~10 min (33). Based on scaling of the mouse data (30), the predicted elimination half-life of Albulin in a 70-kg human would be ~50 h.

Finally, in in vivo studies, a single subcutaneous injection of Albulin (1–10 mg/kg) was potent enough to induce a dose-dependent progressive reduction of blood glucose levels in severely hyperglycemic STZ-induced diabetic mice. Albulin was able to maintain a near-normoglycemic state for long periods of time while enabling short-acting Humulin R to induce an additional decrease in basal glucose levels. The combination of Albulin and Humulin R was also efficacious in the context of a mealtime increase in glucose levels. These data indicate that Albulin has the

**TABLE 2**

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<tr>
<th>Pharmacokinetic analyses of Albulin (1.0 mg/kg) in male C57BL/6 mice following intravenous and subcutaneous administration</th>
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<tbody>
<tr>
<td><strong>Intravenous</strong></td>
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<tr>
<td>AUC_{0-\infty} (h · ng · ml^{-1})</td>
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<td>t_{1/2abs} (h)</td>
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<td>t_{1/2elim} (h)</td>
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<tr>
<td>V or V/F (ml/kg)</td>
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<td>Bioavailability (%)</td>
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Data are estimates ± SE. *Calculated as l/absorption rate + l/elimination rate. AUC_{0-\infty}, area under the plasma concentration curve extrapolated to infinity; t_{1/2abs} absorption half-life; t_{1/2elim} elimination half-life; CL, total clearance; CL/F, apparent clearance after extravascular administration; t_{max}, time to maximal plasma concentration; C_{max}, maximal plasma concentration; MRT, mean residence time; V, volume of distribution; V/F, apparent volume of distribution after extravascular administration; NA, not applicable.
promise to be used in a dual-therapy setting with short-acting insulin analogs.

In conclusion, we have engineered a long-acting insulin analog, Albulin, using albumin-fusion technology. Albulin displays improved in vivo pharmacokinetic and pharmacodynamic profiles while retaining the in vitro potency of recombinant human insulin. Albulin has shown to have the potential as a therapeutic option for the maintenance of long-term, near-normoglycemia for the treatment of diabetes.

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REFERENCES


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