In recent years, recombinant DNA technology has been used to design insulin molecules that overcome the limitations of regular insulin in mealtime supplementation. However, safety issues have been raised with these alternatives, as the alteration of the three-dimensional structure may alter the interaction with the insulin and/or IGF-I receptors and therefore lead to the activation of alternate metabolic as well as mitogenic signaling pathways. It is therefore essential to carefully study acute and long-term effects in a preclinical state, as insulin therapy is meant to be a lifelong treatment. In this study, we determined in vivo the insulin receptor signaling characteristics activated by insulin glulisine (LysB3, GluB29) at the level of insulin receptor phosphorylation, insulin receptor substrate phosphorylation, and downstream signaling elements such as phosphatidylinositol (PI) 3-kinase, AKT, and mitogen-activated protein kinase. C57BL/6 mice were injected with insulin glulisine or regular insulin and Western blot analysis was performed for liver and muscle tissue. The extent and time course of insulin receptor phosphorylation and activation of downstream signaling elements after insulin glulisine treatment was similar to that of human regular insulin in vivo. Moreover, insulin signaling in hypothalamic tissue determined by PI 3-kinase activity was comparable. Therefore, insulin glulisine may be a useful tool for diabetes treatment. Diabetes 54:361–366, 2005

The introduction of new short- and long-acting insulin and the development of analogs with increased stability, less variability, and selective action will allow more individualized treatment strategies targeted to specific needs. Moreover, the use of these agents is expected to improve glycemic control in type 1 and type 2 diabetic patients (1,2). Recently, a number of insulin variants generated by genetic engineering were found to retard and stabilize absorption kinetics of insulin preparations. However, the altered amino acid sequence of human insulin carries the risk of altered interaction with the insulin and/or IGF-I receptors, which might lead to the activation of different pathways and potentially increase mitogenic activity (3).

Insulin and IGF-I are known to regulate a variety of growth-related and metabolic effects in target tissues. Although predominant activation of the IGF-I receptor correlates with increased mitogenic potency, stimulation of the insulin receptor is mainly associated with enhanced glucose transport, glycogen synthesis, and inhibition of hepatic gluconeogenesis as long as insulin receptor binding kinetics remain normal (3,4).

Insulin binds to its cell surface receptor and stimulates autophosphorylation of the β-subunit, followed by phosphorylation of substrates, including the insulin receptor substrate (IRS) protein family members. The IRS protein family consists of at least six members, IRS1–IRS6. Each IRS protein contains a highly conserved NH₂-terminal pleckstrin homology domain followed by a phosphotyrosine binding domain, which couples IRS proteins to the activated insulin or IGF-I receptor (5). After being phosphorylated by the receptor kinase, IRS proteins bind to effector proteins such as the regulatory subunit of the lipid kinase phosphatidylinositol (PI) 3-kinase. Its product, PI phosphate, activates a network of kinases such as AKT and atypical protein kinase C that are involved in processes like glucose transport, protein synthesis, and gene transcription (6).

With the recombinant human insulin analog insulin glulisine (LysB3, GluB29), the tendency to form dimers and hexamers is significantly reduced in subcutaneous adipose tissue due to genetic engineering, leading to a more rapid onset and shorter duration of action than human regular insulin. Recent in vitro data suggest that the novel insulin analog predominantly activates the IRS-2 signaling pathway, which raises a safety concern, as IRS-2 is known to be involved in cell proliferation and mitogenic signaling (7).

Here, we provide in vivo data on the impact of insulin glulisine on the insulin signaling cascade in peripheral tissues. C57BL/6 mice were injected with regular insulin or the analog insulin glulisine; insulin receptor phosphorylation and activation of downstream signaling elements were then analyzed in muscle, liver, and hypothalamic tissue. [³H]thymidine incorporation rates were also determined in C2C12 myoblasts. The resultant data suggest that activation of the insulin signaling cascade is not altered in...
mice stimulated with insulin glulisine compared with mice treated with regular human insulin, and that the mitogenic potential of insulin glulisine is comparable with that of regular human insulin.

**RESEARCH DESIGN AND METHODS**

We obtained 10-week-old male C57BL/6 mice from Charles River and allowed them to acclimatize for 2 weeks. They were maintained on a normal light/dark cycle and kept on a regular diet. Glucose levels were sampled from mouse tail bleeds using a Glucometer Elite (Bayer, Elkhart, IN). All procedures were approved by the local Animal Care and Use Committee.

**In vivo insulin stimulation and Western blot analysis.** For in vivo stimulation, a bolus of regular human insulin or insulin glulisine (1 IU/kg body wt) was injected intraperitoneally. For short-term stimulation, 2 IU of insulin were injected into the inferior vena cava. Control animals received a comparable amount of diluent. Tissues (liver, muscle, and hypothalamus) were removed at the indicated time points and homogenized at 4°C, as previously described (8). Homogenates were allowed to solubilize for 30 min on ice and were clarified by centrifugation at 12,000 g for 20 min. To detect insulin-stimulated tyrosine phosphorylation, supernatants containing 0.5 mg of total protein were immunoprecipitated with antibodies directed against the COOH-terminal of the insulin receptor (KKNGRILTLPRSNPS), IRS-1 (QHLRLSSSSGLRY), and IRS-2 (QSQPQPGDNSWRTI). Visualization of immunocomplexes after gel electrophoresis and Western blotting with the antiphosphotyrosine antibody 4G10 was performed with the nonradioactive enhanced chemiluminescence system. Blots were subsequently stripped and reprobed to reveal expression of total protein. To assess activation of downstream signaling elements, tissue lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with anti-phospho-AKT and anti-phospho–mitogen-activated protein (MAP) kinase antibodies (Cell Signaling, Beverly, MA). The insulin used in this study was a gift from Aventis Pharmaceuticals.

**Assay of PI 3-kinase activity.** Tissue lysates were immunopurified with anti-PY20 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies and immunocomplexes were absorbed to protein A-sepharose for 12 h. Immunoprecipitates were washed three times and pellets were directly incubated with 0.1 mg/ml L-(32)P-PI (Sigma) and 50 μmol/l [γ32P]-ATP (Perkin Elmer) at room temperature for 10 min. After 150 μl of 1 mol/l HCl was added, lipids were extracted twice with 450 μl chloroform/methanol (1:1 by vol). Products were separated by thin-layer chromatography, as previously described (9). 32P-labeled phospholipids were detected by autoradiography.

**[3H]thymidine incorporation into C2C12 myoblasts.** To measure [3H]thymidine incorporation, C2C12 cells were grown in Dulbecco's modified Eagle's medium (4.5 g/l glucose and 10% FCS) in six-well culture plates, and subsequently starved for 24 h in serum-free medium. After cells were stimulated with insulin for 16 h, [3H]thymidine (0.5 μCi/ml) was added for 4 h. The dishes were then rinsed twice with ice-cold PBS and once with 10% trichloroacetic acid. After 20 min, dishes were washed once with ice-cold 10% trichloroacetic acid. Cells were then lysed with 500 μl of 0.2 N NaOH/1% SDS and neutralized with 0.5 ml of 0.2 N HCl. Radioactivity was determined by liquid scintillation counting.
Statistical analyses were done with a two-sided unpaired Student's t test. Data are expressed as means ± SE.

RESULTS

Autophosphorylation of the insulin receptor after stimulation with insulin glulisine in vivo. Evidence that activation of the insulin/IGF-I signaling pathway is a pivotal element in cell proliferation and induction of mitogenic activity has been provided by in vitro and in vivo studies. To determine whether rapid insulin receptor activation by insulin glulisine differs in terms of the kinetic and/or extent, 12-week-old C57BL/6 male mice were stimulated with a bolus insulin injection into the inferior vena cava. Tissue was dissected after 10 min, and Western blot analysis was performed for liver and muscle tissue. Insulin receptor phosphorylation was markedly enhanced in both muscle and liver 10 min after injection (Fig. 1A), but was comparable with that in animals treated with regular insulin or insulin glulisine, suggesting an equal insulin receptor autophosphorylation kinetic. Looking at early downstream signaling elements, an equal increase in tyrosine phosphorylation of IRS-1 and -2 was observed (Fig. 1B and C).

To test an extended and more physiological response, including regulation of downstream insulin receptor signaling elements, we further injected mice intraperitoneally with regular insulin or insulin glulisine (1 IU/kg body wt). After the injection, there was no difference in the blood glucose–lowering effect between the two insulins. Blood glucose levels dropped significantly from 110 ± 15 to 60 ± 10 mg/dl with regular insulin and to 74 ± 16 mg/dl with insulin glulisine by 20 min (control versus regular insulin or insulin glulisine: P < 0.001) and down to 53 ± 20 mg/dl with regular insulin and 63 ± 7 mg/dl with insulin glulisine by 30 min (control versus regular insulin or insulin glulisine: P < 0.001). There was no significant difference in the blood glucose–lowering effect between the two insulins at each time point (P > 0.1). Moreover, significant changes in blood glucose levels could not be detected 10 min after injection (P = 0.2). Therefore, later time points were analyzed in the further experiments to test downstream insulin receptor signaling pathways.

Looking at insulin receptor phosphorylation, we observed identical results in mice stimulated intraperitoneally with regular insulin or insulin glulisine in muscle (Fig. 2A) and liver (Fig. 2B) tissue. These data suggest that the
amino acid exchange of insulin glulisine does not alter insulin receptor activation. Moreover, tyrosine phosphorylation of the insulin receptor was already upregulated after 10 min in muscle and liver (data not shown), suggesting that the phosphorylation kinetics observed in this study were not dependent on the administration route.

Effect of insulin on tyrosine phosphorylation of IRSs in muscle and liver tissue. After insulin stimulation, IRS-1 is readily tyrosine phosphorylated by the activated insulin receptor. To determine whether activation of IRS-1 is altered after stimulation with insulin glulisine, the tyrosine phosphorylation state of the IRS-1 protein was evaluated in mice treated with insulin. In contrast to data obtained from myoblasts in vitro (7), IRS-1 tyrosine phosphorylation in muscle (Fig. 3A) and liver (Fig. 3B) was comparable for the two groups throughout the 30-min experiment.

Recent studies have revealed that insulin glulisine predominantly activates the IRS-2-dependent pathway (7,10). Because enhanced IRS-2 signaling has been proposed to be involved in mitogenic signal transduction, the phosphorylation state of IRS-2 was determined in vivo in lysates of muscle and liver after insulin stimulation. Mice were stimulated with insulin and tissue was harvested after 20 and 30 min, respectively. Phosphorylation of IRS-2 in muscle (Fig. 4A) and liver (Fig. 4B) was detected by Western blot analysis using an anti-phosphotyrosine antibody. Concurrent immunoblots revealed a comparable increase in IRS-2 phosphorylation for both insulins throughout the experiment.

Activation of PI 3-kinase in muscle and liver tissue after insulin stimulation. To test IGF-I-like activities, PI 3-kinase activation was determined in mouse liver and muscle after mice were stimulated with insulin glulisine and compared with activation with regular insulin. Tissues were removed 5 and 10 min after insulin injection, and a PI 3-kinase assay was performed. The data suggest that the activation levels in muscle (Fig. 5A) and liver (Fig. 5B) from mice treated with insulin glulisine are equal to those stimulated with regular insulin.

Effect of insulin on the phosphorylation of AKT and MAP kinase in vivo. Looking at downstream signaling elements, the serine/threonine kinase AKT has emerged as a focal point for many signal transduction pathways regulating GLUT4 translocation, glycogen synthesis, lipogenesis, and gene expression. Growth factor-mediated activation of PI 3-kinase leads to the generation of PtdIns-3,4,5-P3, which recruits inactive protein kinase B from the cytosol to the plasma membrane. Ser473 is then phosphory-
ylated, indicating activation (6). Treatment of mice with insulin led to an activation of AKT in muscle (Fig. 6A) and liver (Fig. 6B). Quantification of kinetics by scanning densitometry normalized to the total amount of AKT protein are given below immunoblots. Scanning data obtained from five independent experiments are expressed as fold increase over basal (untreated) phosphorylation ± SE.

[3H]thymidine incorporation into C2C12 myoblasts. Looking at mitogenic activity, [3H]thymidine incorporation rates in C2C12 myoblasts stimulated with increasing concentrations of regular insulin or insulin glulisine were determined. Insulin glulisine stimulated [3H]thymidine incorporation to the same extent as regular insulin, suggesting a comparable mitogenic potential (Fig. 7C).

Activation of PI 3-kinase in hypothalamic tissue after insulin stimulation. Recent data suggest that insulin signaling in the brain is important in maintaining body weight regulation, as abnormalities in hypothalamic signaling lead to hyperphagia and weight gain (11). We therefore assayed the activation of the insulin signaling cascade in the hypothalamus of mice treated with an acute intravenous dose of regular insulin or insulin glulisine. The results obtained indicate that the regulation of the early insulin signaling cascade was equivalent between the two groups, suggesting intact insulin signaling in the central nervous system (Fig. 8).

DISCUSSION

Modifications of the amino acid sequence of the insulin molecule have resulted in prandial analogs with pharmacokinetic characteristics that more closely mimic the physiological insulin response to a meal after injection (12,13). Moreover, basal day-long glycemic control can be reached with new long-acting analogs that show distinguished absorption kinetics (14).

So far, >300 insulin analogs have been produced, but most of them have been removed from use due to safety concerns after careful in vitro and in vivo examination (15). Given that insulin treatment is most often required over a patient’s entire life, it is mandatory to extensively determine potential side effects in a preclinical state, as alteration of the three-dimensional structure affects insulin receptor activation and may lead to altered metabolic
as well as mitogenic signaling (3). Insulin glulisine is a new rapid-acting insulin intended for mealtime insulin delivery via subcutaneous injection or external infusion pump. The pharmacodynamics of insulin glulisine are similar to those of insulin lispro and insulin aspart in that insulin glulisine display a more rapid onset and shorter duration of action than regular human insulin due to the lack of hexamere formation.

We earlier tested insulin receptor binding characteristics and receptor auto- and dephosphorylation kinetics of insulin glulisine in rat-1 fibroblasts overexpressing the human insulin receptor and found no alterations compared with regular human insulin (16). However, additional in vitro data accumulated over the past year suggest that insulin glulisine differs slightly in terms of downstream activation of the insulin signaling cascade. In rat cardiomyocytes, a prominent phosphorylation of IRS-2 has been described, with only marginal IRS-1 phosphorylation (7). Comparable results were obtained for human skeletal muscle cells incubated with an extensive amount of insulin glulisine (7). Moreover, an inhibition of cytokine and fatty acid–induced apoptosis was described in a rat insulinoma cell line through activation of IRS-2 and was favored as an antiapoptotic activity of insulin glulisine, thereby opening a new field for optimized insulin treatment (10). However, these data also raise a safety concern, as IRS-2 is involved in cell proliferation and mitogenic signaling. On the other hand, in vitro studies in murine and human cell lines have never revealed changes in cell proliferation due to the incubation with insulin glulisine, even when high concentrations of insulin have been used (17).

Here we report for the first time on in vivo data for insulin glulisine from C57BL/6 mice that were treated with physiological dosages of either regular insulin or insulin glulisine. To overcome the effect of subcutaneous absorption kinetics and focus on direct insulin receptor signaling characteristics, insulin administration in this study occurred by injection in the inferior vena cava or intraperitoneally.

In our in vivo experiments, insulin glulisine activated the insulin receptor as well as insulin receptor substrates to the same extent as regular human insulin. Moreover, activation of PI 3-kinase and downstream signaling elements was equal in liver, muscle, and hypothalamic tissue, suggesting comparable in vivo activation of the insulin signaling cascade. These results are in contrast to the in vitro data obtained from myoblasts, cardiomyocytes, human skeletal muscle cells, and an insulinoma cell line (7,10), most likely due to the extensive amount of insulin and the nature of the cell lines used in those studies.

The current in vivo results support the use of the novel insulin analog insulin glulisine as a rapid-acting insulin analog with unaltered insulin receptor signaling characteristics.

ACKNOWLEDGMENTS

We are grateful to Katrin Brodbeck and Roman Werner for their excellent technical assistance.

REFERENCES


