Long-Lasting Designer Insulin with Glucose-Dependent Solubility Markedly Reduces Risk of Hypoglycemia

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Insulin analogs are key to blood glucose management for millions of people with diabetes. Nonetheless, the risk of hypoglycemia still exists because this insulin remains bioactive at normal or low blood glucose conditions. Here, the aim is to incorporate phenylboronic acids on insulin glargine to create a glucose-responsive designer insulin termed “PBA-F-glargine.” It is hypothesized that by inserting a glucose responsive moiety, this designer insulin increases the therapeutic window and reduces the risk of insulin-induced hypoglycemia. Chemical methods are used to incorporate phenylboronic acids into insulin glargine. Biochemical and cell-based assays are used to confirm that the designer insulin PBA-F-glargine preserves insulin bioactivity. In comparison to commercial glargine, in vitro experiments demonstrate that PBA-F-glargine has similar bioactivity and increased solubility that is glucose-dependent. In vivo experiments demonstrate that PBA-F-glargine has 88% bioactivity as compared to glargine at hyperglycemic levels, yet has only 30% bioactivity at euglycemic levels. This threefold difference in bioactivity demonstrates that PBA-F-glargine is responsive to glucose concentrations. In comparison to commercial glargine, PBA-F-glargine reduces iatrogenic hypoglycemia by 15-fold. In conclusion, PBA-F-glargine has a glucose-dependent in vivo bioactivity that markedly reduces the risk of hypoglycemia.

1. Introduction

Since the discovery of insulin nearly a century ago, many advancements in insulin design have allowed people with diabetes to improve their glycemic control; however, the risk of hypoglycemia is still a major barrier for tight glycemic control.[1,2] One problem is that commercially available insulin analogs are unable to modulate bioactivity in response to circulating glycemia and therefore have narrow therapeutic index. To address this challenge, the concept of a glucose-responsive insulin (GRI), or “smart” insulin, was proposed to mimic the glucose-stimulated insulin secretion in pancreatic beta cells.[3–5] To date, a number of studies of creating GRI have been developed using glucose-triggering signals from lectins,[6,7] glucose oxidases,[8,9] glucose transporters,[10] and phenylboronic acid (PBA).[11,12] The use of PBA to create glucose responsive properties is particularly useful since PBA is smaller in size compared to other sensing agents and is known to bind reversibly to cis-1,2 or cis-1,3 diols such as glucose thus creating a negative charge on the boronic acid (Figure 1A); a property then can be exploited to alter insulin absorption characteristics. Chemically modified insulin derivatives are therefore promising candidates for GRI designs.[13]

Insulin glargine (Lantus) is the most commonly used long-acting insulin for people with diabetes. The protracted mechanism of action for insulin glargine is due to the addition of two arginine residues in the B chain, which increases the isoelectric point (pI) of insulin to 6.7, thus lowering its solubility at physiological pH.[14,15] Once injected, insulin glargine precipitates at the injection site and is very slowly converted into hexamers, dimers, and monomers for absorption, thus providing a long-lasting and steady insulin entry into the bloodstream in vivo (Figure 1B). In the current set of experiments, we propose that with the addition of PBA to insulin glargine, high interstitial glucose levels will result in increased glucose binding and the resultant formation of negative charges to reduce the pI of the insulin, thus increasing insulin’s solubility and allow for more rapid entry into the bloodstream when glucose levels are high (Figure 1C). Conversely, when interstitial glucose levels are normal or low, there will be relatively less glucose available for binding to PBA, and in the absence of change in pI of insulin, insulin’s solubility will remain low and absorption into the bloodstream will remain slow. The goal of this insulin analog design is to combine the long-lasting benefits of insulin glargine with glucose-responsive properties that will enhance glycemic control, yet minimize the risk of iatrogenic hypoglycemia. Herein, we report our design and synthesis of “PBA-F-glargine” and its glucose-responsive properties. PBA-F-glargine has a similar in vitro bioactivity...
as insulin glargine. We further demonstrate that under varying glucose concentrations, PBA-F-glargine demonstrated a nearly threefold in vivo activity difference compared to insulin glargine and significantly reduced the extent of hypoglycemia. PBA-F-glargine insulin represents a new design in achieving glucose-mediated control of insulin based on protein solubility.

2. Experimental Section

2.1. Peptide Synthesis

A chains were synthesized by a Biotage automated microwave peptide synthesizer (Initiator+ Alstra) using Fmoc solid phase synthesis. Peptide synthesis was carried out on 0.1 mmol scale with a standard HATU/DIEA protocol. For Fmoc deprotection, 20% piperidine in DMF was added and mixed for 5 min twice at 25 °C. For amino acid coupling, 0.2 M Fmoc-protected amino acid, 0.2 M HATU (coupling reagent) and 1.0 M DIEA (base) were prepared in DMF. In each cycle, 5 eq. amino acid, 5 eq. coupling reagent and 10 eq. base was added into the reaction vessel and mixed for 5 min at 75 °C (for cysteine and histidine, mix for 10 min at 50 °C; for arginine, mix for 15 min at 50 °C and couple twice). Upon completion of the peptide chain, resins were washed with DCM and dried using vacuum. Peptide was then cleaved by TFA, and further precipitated with cold ethyl ether, followed by HPLC purification and lyophilization. B chains were synthesized by a Prelude X peptide synthesizer without heating.

The synthesis protocol was the same as what is used for A chains except the coupling time. For amino acid coupling, the reaction was mixed for 30 min at 25 °C with nitrogen bubbling. Detailed PBA-F-glargine synthetic protocols are included in Supporting Information.

2.2. In Vitro Bioactivity Assay

To measure the bioactivity of human insulin, insulin glargine, and PBA-F-glargin, pAkt Ser473 levels were measured in a mouse fibroblast cell line, NIH 3T3, overexpressing human IR-B (a gift from A. Morrione, Thomas Jefferson University). The cells were authenticated by western blotting to assess their level of IR expression compared with that of parent 3T3 cells: The NIH 3T3 cells showed an approximately tenfold-higher level of expression than that of the parent. The NIH 3T3 cell line was cultured in DMEM (Thermo Fisher Scientific) with 10% FBS, 100 U mL⁻¹ penicillin–streptomycin (Thermo Fisher Scientific) and 2 µg mL⁻¹ puromycin (Thermo Fisher Scientific) and were shown to be free of mycoplasma contamination. For the assay, 40,000 cells per well were plated in 96-well plates with culture medium containing 1% FBS. 24 h later, 50 µL of insulin solution was pipetted into each well after the removal of the original medium. After a 30-min treatment, the insulin solution was removed, and a HTRF pAkt Ser473 kit (Cisbio, 64AKSPEH) was used to measure the intracellular level of pAkt Ser473. Briefly,

Figure 1. Proposed design of glucose-responsive PBA-F-glargine. A) Under high glucose conditions, the equilibrium shifts to negative-charged boronate complex upon glucose binding from the neutral boronic acid group. B,C) While insulin glargine has a slow and sustained release from subcutaneous depot, PBA-F-glargine was released in response to elevated glucose levels.
the cells were first treated with cell lysis buffer (50 µL per well) for 1 h under mild shaking. 16 µL of cell lysate was then added to 4 µL of detecting reagent in a white 384-well plate. After a 4-h incubation, the plate was read in a Synergy Neo plate reader (BioTek), and the data were processed according to the manufacturer’s protocol. The assays were repeated a total of four times (biological replicates). Mean EC50 values and their 95% confidence intervals were calculated (using Prism 8) after curve fitting with a nonlinear regression (one-site) analysis.

2.3. Circular Dichroism

All CD spectra were recorded on an AVIV Model 410 spectrophotometer (AVIV) in water in a 1 mm quartz cuvette (Starna) at 25 °C. Wavelength scans were performed at 1-nm resolution with 1-s averaging time. Data from double scans were averaged, blank subtracted, and normalized to mean residue ellipticity by the following equation: 

\[
\theta = 100 \times \theta/C \times l \times (n - 1)
\]

where \(C\) is concentration of protein in mM, \(l\) is path length in centimeters, and \(n\) is the number of residues in the protein. The concentrations of the protein samples used for CD experiments were 100 µM.

2.4. Solubility Determination

To an Eppendorf tube, 1 mg peptide was added and suspended in 100 µL PBS buffer (pH 5, 7, and 9) with various concentrations of glucose (0–400 mg dL\(^{-1}\)). The peptide was added in excess to make a saturated solution with incompletely dissolved peptides at the bottom. Samples were vortexed for 5 min and gently shaken for overnight. Then they were centrifuged at 12,000 rpm for 10 min. The concentrations of saturated peptide solutions were determined by Nanodrop based on absorbance at 280 nm and calculated extinction coefficient.

2.5. Animals

Male Sprague-Dawley rats (SASCO SD, Strain code: 400; Charles River Laboratories, Inc., Wilmington, MA) weighing 250–300 g were housed in polyacrylic cages and maintained under standard housing conditions (room temperature 22–24 °C with 12 h light/dark cycle) at the University of Utah. The animals had free access to food and water, and were acclimatized to handling for 1 week before experimental procedure. All procedures were performed in accordance with the United States National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Utah.

2.6. Glargine Sample Preparation

Commercial Lantus (100 U mL\(^{-1}\)) was purified by HPLC to obtain insulin glargine. Both lyophilized insulin glargine and PBA-F-glargine were dissolved in (3.63 mg mL\(^{-1}\)) diluent buffer, pH 4 containing similar constituents as of commercial Lantus diluent (30 µg zinc, 2.7 mg m-cresol, 20 mg glycerol 85%, 20 µg polysorbate 20).

2.7. Vascular Surgery

Rats were anesthetized with an intraperitoneal injection of ketamine/xylazine (75 mg kg\(^{-1}\) ketamine with 5 mg kg\(^{-1}\) xylazine) and an incision was made on the midline of ventral side of neck to implant vascular catheters under aseptic conditions. A microcatheter catheter (MRE 025, Braintree Scientific, Inc., Braintree, MA) was inserted into the right jugular vein and another catheter (MRE 033) was implanted into the left carotid artery. To maintain patency, all catheters were filled with a 40% polyvinylpyrrolidone (Sigma, MO) in heparin (1000 units mL\(^{-1}\); USP) and tunneled subcutaneously to place at the back of the neck. The animals were then allowed to recover in their home cages and returned to the animal facility.

2.8. Modified Euglycemic and Hyperglycemic Clamps

To evaluate the action of both PBA-F-glargine, and commercially available glargine insulin, modified euglycemic and hyperglycemic clamps were performed in nondiabetic and diabetic rats respectively. In these modified glucose clamps, the absorption/bioactivity characteristic of the insulin was investigated following the administration of a single subcutaneous injection of insulin (as opposed to an intravenous infusion as would occur in traditional glucose clamps). For euglycemic clamps in nondiabetic rats, one week after vascular surgery the nondiabetic control rats were fasted overnight and the arterial and venous catheters were exteriorized under isoflurane anesthesia and extended via connector for blood sampling and to attach to the infusion pump, respectively. After 90 min resting period, the basal glucose levels were measured from arterial blood samples obtained from awake, unrestrained rats using glucometer (Ascensia Contour BG monitors, Bayer HealthCare, IN). Following baseline blood glucose measurement, all rats were injected subcutaneously at time zero with either commercial glargine insulin (i.e., 0.5 mg kg\(^{-1}\)) or synthesized PBA-F-glargine insulin (0.5 mg kg\(^{-1}\)). Blood glucose was measured at 10-min interval throughout the clamp and a constant variable intravenous infusion of dextrose (50% w/v) was used to maintain euglycemia (90–110 mg dL\(^{-1}\)) for 4 h.

For the hyperglycemic clamps, four days following vascular surgery, rats were intraperitoneally injected with streptozotocin (STZ; 65 mg kg\(^{-1}\)) to induce diabetes. Diabetic rats were used in these studies as they had the advantage of increased blood and interstitial glucose concentrations, ideal for investigating the effects of high glucose levels on insulin bioavailability. Three days after STZ injection and after an overnight fast, all diabetic rats were injected subcutaneously with either commercial glargine insulin (i.e., 0.5 mg kg\(^{-1}\)) or synthesized PBA-F-glargine insulin (0.5 mg kg\(^{-1}\)) at time zero and subjected to a similar clamp protocol except that the rats were clamped at hyperglycemic levels (≈400 mg dL\(^{-1}\)) for 4 h.
2.9. Insulin Tolerance Test with Glucose Challenge

Three days following STZ injection and after a 4–5 h fasting period, diabetic rats were subjected to an insulin tolerance test (ITT) with, or without, an additional glucose challenge. Blood glucose levels were measured from a tail nick every 15 min using glucometer. Following 30 min of baseline blood glucose measurement, rats were randomly injected with either commercial glargine insulin (1 mg kg\(^{-1}\)) or synthesized PBA-F-glargine insulin (1 mg kg\(^{-1}\)) subcutaneously at time zero and blood glucose levels were allowed to drop. For the ITT experiments that included a glucose challenge (to further examine the glucose-dependent action of insulin) rats were treated with intraperitoneal injection of bolus glucose (4 g kg\(^{-1}\), i.p.) at 150 min following the insulin injection.

2.10. Statistical Analysis

For all data presented in this manuscript, raw data was used without pre-processing except for bioactivity measurements where all data was normalized to the maximum signal. The results are represented as mean ± standard deviations (SD) for bioactivity and solubility evaluations (Figure 2); mean ± standard error of the mean (SEM) for in vivo experiments (Figures 4–6). Sample size for statistical analysis: \(n = 4\) for solubility measurements and \(n = 5–6\) for all in vivo experiments. Data were analyzed by student (unpaired) t-test. Repeated measures ANOVA (two-way) was performed to analyze the data for glucose clamps and ITT over the period of 4 h. Post-hoc analyses were performed by Tukey’s multiple comparison tests. A level of 5% probability was considered as statistically significant. All analysis was done by Prism.

3. Results

The chemical synthesis of PBA-F-glargine was achieved by using solid-phase peptide synthesis (Figure 2). We first synthesized the A and B chains separately. In order to avoid degradation of PBA under the harsh peptide coupling conditions, PBA was introduced at latest stage after the whole B chain was synthesized utilizing Lys(Dde) residues. Specifically, the Dde protecting group can be selectively deprotected using hydrazine solution. The ε-amino groups from lysines was then modified with the F-PBA group using amide coupling reaction. To form all
three disulfide bonds in a controlled manner, four different Cys protecting groups were used as described in a previous report by Liu et al.\cite{16} We first deprotected A6 Cys(S-tBu) using mercaptoethanol followed by activation with 2,2′-dithiobis(5-nitro pyridine) (DTNP). Next, A11 Cys(Mmt) was deprotected using 1% TFA to obtain the thiol. The A6-A11 intra-molecular disulfide bond was then formed through a disulfide substitution reaction. The A chain was then cleaved from the resin to give A7 Cys(Acm) and A20 free Cys (deprotection of Trt). Next, the A and B chain were then ligated through a similar disulfide substitution reaction. The last disulfide bond formation was formed using iodine to obtain PBA-F-glargine after HPLC purification (>98% purity). 2-fluorophenylboronic acid was used due to its similar pKatophysiological pH (Figure 1A). \cite{17}

To determine whether PBA incorporation had an effect on secondary structure of insulin, we evaluated the insulins using near-UV circular dichroism (CD). All insulin molecules were observed to have CD spectra consistent primarily with \( \alpha \)-helical secondary structure (Figure 3A). To measure in vitro bioactivity, a cell-based insulin receptor activation assay was performed using pAkt level as indication of bioactivity (Figure 3B). Both insulin glargine and PBA-F-glargine have a similar EC\(_{50}\) (12 nm) for signal activation. Human native insulin has a twofold higher bioactivity than insulin glargine, which is consistent with literature reports.\cite{18} We next measure the solubility profile of both glargine molecules. First, both insulin glargine and PBA-F-glargine have high solubilities at pH 5 and 9 with a much lower solubility at pH 7, which is consistent with the biochemical design of insulin glargine (Figure 3C). Noted, at pH 7, the solubility of PBA-F-glargine is lower than one fourth of that of insulin glargine (0.06 vs 0.28 mg mL\(^{-1}\)) most likely due to the hydrophobic nature of PBAs. Next, we measure the solubility profile at pH 7 with various glucose concentrations (Figure 3D). While insulin glargine has the same solubility from 0 to 400 mg mL\(^{-1}\) glucose, PBA-F-glargine has a \( \approx \)2.5-fold increased solubility over the same range. This result supports our hypothesis that PBA-F-glargine has an increased solubility at high glucose conditions due to the negative charge from the boronate complex and the hydrophilic sugar attachment; thus demonstrating the biochemical basis of the glucose responsiveness.

To establish the in vivo glucose responsiveness of PBA-F-glargine, we performed euglycemic and hyperglycemic clamp studies to compare and contrast the in vivo biological activities of both PBA-F-glargine and commercially available insulin glargine. After a subcutaneous injection of insulin (0.5 mg kg\(^{-1}\)) blood glucose levels were well-matched (by experimental design) for both insulin glargine and PBA-F-glargine-treated rats during both the euglycemic and hyperglycemic clamp protocols (Figure 4A). During the hyperglycemic clamp \( (\approx 400 \text{ mg dL}^{-1} \text{ glucose}) \), the glucose infusion rate needed to maintain hyperglycemia for the PBA-F-glargine-treated rats was 88% that of the insulin glargine-treated rats (Figure 4B). However, during the euglycemic clamp \( (\approx 100 \text{ mg dL}^{-1} \text{ glucose}) \), the rate of exogenous glucose infusion in the PBA-F-glargine-treated rats was markedly reduced (to about 30% that of the insulin glargine-treated rats) (Figure 4B) and this difference was highly significant.
Figure 4. Glucose clamp studies. A) Blood glucose levels (mg dL$^{-1}$) during euglycemic ($\approx$100 mg dL$^{-1}$) and hyperglycemic ($\approx$400 mg dL$^{-1}$) clamps performed in nondiabetic control and STZ-diabetic rats, respectively. In both euglycemic and hyperglycemic clamps, rats were injected subcutaneously with either insulin glargine (0.5 mg kg$^{-1}$) or PBA-F-glargine (0.5 mg kg$^{-1}$) at time zero. Data are expressed as mean ± SEM (n = 5–6 per group). Statistics were calculated by repeated measures ANOVA (two-way) followed by post hoc test with Tukey’s comparisons. B) Average glucose infusion rates (mg kg$^{-1}$ min$^{-1}$) are shown for the last hour of euglycemic ($\approx$100 mg dL$^{-1}$) and hyperglycemic ($\approx$400 mg dL) clamps. Data are expressed as mean ± SEM (n = 5–6 per group). *p < 0.05, **p < 0.01 versus glargine; Student $t$ (unpaired) test.

Figure 5. Insulin tolerance test (ITT). A) Blood glucose levels (mg dL$^{-1}$) during ITTs (ITTs) performed in STZ-diabetic rats. After obtaining baseline blood glucose readings, rats were injected at time zero with either insulin glargine (1 mg kg$^{-1}$) or PBA-F-glargine (1 mg kg$^{-1}$) subcutaneously. Data are expressed as mean ± SEM (n = 5–6 per group). Statistics were calculated by repeated measures ANOVA (two-way) followed by post hoc test with Tukey’s comparisons. B) Time (min) during which the blood glucose levels remained below 70 mg dL$^{-1}$ during the ITT in rats injected with either insulin glargine (1 mg kg$^{-1}$) or PBA-F-glargine (1 mg kg$^{-1}$). Data are expressed as mean ± SEM (n = 5–6 per group). **p < 0.01 versus glargine; Student $t$ (unpaired) test.

(p < 0.01). This result indicates that PBA-F-glargine has similar in vivo bioactivity as insulin glargine under hyperglycemic conditions, but has greatly reduced activity under euglycemic conditions. This 2.9-fold difference in relative bioactivity demonstrates the glucose responsiveness of PBA-F-glargine in vivo.

Given its reduced bioactivity under lower glucose conditions, we hypothesize that PBA-F-glargine may be less likely to cause hypoglycemia. To evaluate the potential for insulin-induced hypoglycemia, high dose (1 mg kg$^{-1}$) ITTs were performed in streptozotocin (STZ)-induced diabetic rats. In the absence of glycemic clamp conditions, the subcutaneous administration of both insulin glargine and PBA-F-glargine (1 mg kg$^{-1}$) lowered blood glucose levels (Figure 5A). The nadir blood glucose levels reached in insulin glargine-treated rats was 40 mg dL$^{-1}$. An equal dose of PBA-F-glargine resulted in a more gradual lowering of blood glucose and a nadir blood glucose level of 102 mg dL$^{-1}$. To quantify the hypoglycemic potency of these insulins, the duration of time during which the blood glucose remained hypoglycemic (<70 mg dL$^{-1}$) was quantified. PBA-F-glargine-treated rats remained hypoglycemic for a significantly shorter duration as compared to commercial glargine-treated rats (Figure 5B). This 15-fold less hypoglycemic potency demonstrates that PBA-F-glargine portends a reduced risk of causing hypoglycemia as compared to insulin glargine.

To further investigate whether changes in blood glucose level alters the in vivo bioactivity of the two insulin analogs, we again evaluated bioactivity and hypoglycemic potency following rescue glucose administration. In this experiment, an intraperitoneal
injection of bolus glucose (4 g kg\(^{-1}\)) was administered to both insulin glargine and PBA-F-glargine insulin-treated rats 150 min after subcutaneous insulin injection. The glucose bolus resulted in a spike in blood glucose levels in both experimental groups; however, blood glucose returned to hypoglycemic levels in rats treated with insulin glargine, whereas blood glucose returned to normoglycemic levels in rats treated with PBA-F-glargine (Figure 6A). Evidence of ongoing insulin absorption/action was noted by the maintenance of normal or low glucose levels for greater than 5 h in both groups of diabetic animals. This persistent insulin action is particularly noteworthy in the setting of a (likely) counterregulatory response to hypoglycemia. Again, the duration of time during which the blood glucose remained hypoglycemic (<70 mg dL\(^{-1}\)) following the glucose bolus was significantly shorter in the PBA-F-glargine-treated rats as compared to commercial glargine-treated rats (Figure 6B).

**4. Discussion**

This study was undertaken with a goal to increase the therapeutic index of insulin. The innovation was based on the use of phenylboronic acids to convert insulin glargine into PBA-F-glargine allowing for glucose-dependent solubility at physiological pH. Unlike the steady release of commercially available insulin glargine into the bloodstream from the subcutaneous depot; the in vivo bioactivity profiles (Figure 5B) would suggest that PBA-F-glargine exhibits relatively high absorption under high glucose conditions and markedly less absorption under lower glucose conditions. Interestingly, a lesser rate of insulin absorption from the subcutaneous depot (under euglycemic conditions) would give PBA-F-glargine the theoretical advantage of prolonging the duration of insulin action. Such a therapeutic advantage could be investigated in follow up experiments.

Insulin-induced hypoglycemia is the most serious acute complication of insulin therapy. Although the introduction of fast- and long-acting insulin analogs have led to reduced risk of hypoglycemia as compared to native insulins, these insulin analogs still have narrow therapeutic window, since once injected, insulin absorption into the bloodstream is continuous and independent of ambient glucose concentrations\(^{[19]}\) (Figure 1B). Consistent with this notion of persistent and unregulated insulin action, we noted that pharmacological dose of commercially available insulin glargine caused prolonged hypoglycemia (Figure 5A). Even after a rescue bolus of glucose, hypoglycemia recurred in insulin-glargine-treated rats indicating prolonged insulin action (Figure 6A). PBA-F-glargine also demonstrated prolonged insulin action but variable potency. The glycemic profile of PBA-F-glargine appeared to demonstrate a waning effect of insulin action/absorption as glucose level approached euglycemia; but following the glucose bolus and resulting hyperglycemia, the rapid return of glucose levels to euglycemia (i.e., 180–270 min) is indicative of enhanced insulin activity/absorption under hyperglycemic conditions. These glucose responsive properties of PBA-F-glargine are consistent with the in vitro and with the clamp experiments.

In summary, an equimolar dose of glucose responsive PBA-F-glargine demonstrated less hypoglycemia potency, as noted by, a) a lowering of blood glucose level to only a nadir of 102 mg dL\(^{-1}\), and b) either a 15-fold reduced duration of hypoglycemia (Figure 5B) or complete elimination of hypoglycemia (Figure 6B). Several factors may have contributed to this beneficial hypoglycemia-sparing effect of PBA-F-glargine. a) Independent of relative bioactivity measurements ascertained during the steady-state portion of the glycemic clamp experiments, the more gradual decline in blood glucose levels during the ITT with PBA-F-glargine (as compared to insulin glargine) is consistent with slower absorption following subcutaneous injection (Figure 5A). This decreased rate of absorption of PBA-F-glargine at a physiological pH is consistent with our in vitro findings (Figure 3C) and likely represents the hydrophobic nature of PBAs. b) A 12% lower bioactivity for PBA-F-glargine under hyperglycemic conditions (Figure 4B) may have made a minor contribution to this observed effect. c) As blood glucose approached euglycemia, it is
proposed that the markedly reduced solubility of PBA-F-glargine at euglycemia (Figures 3D and 5B) prevented the development of hypoglycemia. Of particular note, it is proposed that this slower onset and lower in vivo activity of PBA-F-glargine is not due to a reduction in intrinsic insulin-stimulatory activity because PBA-F-glargine and commercially available insulin glargine demonstrated identical bioactivity in activating insulin receptor signaling in vitro (Figure 3B). Thus, PBA-F-glargine demonstrated an overall decrease in solubility, as noted in vitro (Figure 3C) and also noted by slower onset of action noted in vivo (Figure 5A); yet PBA-F-glargine insulin did demonstrate an increased relative solubility under high glucose conditions in vitro (Figure 3D) and in vivo (Figure 4B) thus demonstrating glucose responsive properties. Overall, these novel findings indicate that it is possible to manipulate the bioactivity profile of insulin by altering the overall solubility of glargine in a glucose responsive fashion.

In conclusion, we provided evidence for the synthesis of a glucose responsive PBA-F-glargine. We further demonstrated its superior therapeutic property in preventing hypoglycemia as compared to insulin glargine. To our best knowledge, this is the first demonstration of using protein solubility to achieve glucose responsiveness. This unique mechanism can also be coupled with other reported designs to create even more optimal GRI.[100] We propose that the rapid clinical development of insulin derivatives with glucose responsive properties will help people with insulin-treated diabetes achieve glycemia goals while reducing the risk for iatrogenic hypoglycemia.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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