Reduced Action of Insulin Glargine on Protein and Lipid Metabolism: Possible Relationship to Cellular Hormone Metabolism

Janet Fawcett, Brian T. Tsui, Michael C. Kruer, and William C. Duckworth

Insulin analogues are used in the treatment of diabetes to mimic physiological insulin secretion. Glargine is used to provide basal insulin levels. Previous work has shown no differences in glucose uptake when glargine was compared to native insulin. The action of insulin on protein and lipid metabolism is studied infrequently, but these important actions should be considered with insulin analogues. In HepG2 cells, protein degradation was inhibited significantly less by glargine (15% over 3 hours) than by insulin (~20% over 3 hours) ($P < .05$). Lipid metabolism was investigated in 3T3-L1 cells. In these cells glucose oxidation to CO_2 was effected equally, but glargine was less potent than insulin at inhibiting epinephrine-stimulated lipolysis (EC_{50} = 1.4 *v* 0.35 nmol/L, $P < .001$) and at stimulating lipogenesis (EC_{50} = 1.27 *v* 8.06 nmol/L, $P < .01$). Since the action of insulin on protein and lipid metabolism has been suggested to be due to the metabolism of the hormone, we compared the cellular handling of ^{125}I[A14]-glargine to ^{125}I[A14]-insulin in HepG2 cells. While binding of glargine to the insulin receptor was identical to insulin, degradation of glargine was reduced compared to insulin (16.3% ± 0.3% *v* 21.6% ± 0.4% degraded/h, $P < .01$). Less degraded glargine than insulin was released from cells previously loaded with radiolabeled material (50.1% ± 2.4% *v* 58.3% ± 1.4%/2 h, $P < .02$). The amount of intact glargine released was concomitantly increased compared to insulin (44.8% ± 2.6% *v* 35.8% ± 1.4%/2 h, $P < .02$). These data provide further evidence for a relationship between insulin metabolism and insulin action on protein and lipid metabolism; however, the clinical relevance of these differences is hard to realize, since for the most part glargine, used as a basal insulin, is administered in addition to other shorter-acting insulin or analogues, and their effects will mask or reduce glargine effects on lipolysis and protein degradation. However, these studies do show that properties of insulin other than glucose metabolism and mitogenesis must be considered when studying insulin analogues.

THE TREATMENT of diabetes has been advanced by the introduction of various insulin analogues.[1,2] These analogues can be split into 2 basic groups as compared to regular human insulin: (1) shorter onset and shorter duration of action, which includes lispro and aspart; and (2) longer duration of action with no definitive peak of action such as glargine.[2] The aim has been to more closely mimic physiological insulin secretion[2] with the ultimate goal of normalizing blood glucose and reducing diabetic complications.[1-3] Insulin glargine is a long-acting insulin analogue that was designed to provide basal insulin requirements.[4] The B chain of the glargine molecule has 2 extra arginine residues added to the C-terminal end, and the asparagine residue in the A21 position substituted with a glycine (gly^{A21}, arg^{B31}, arg^{B32}-insulin). These alterations make glargine less soluble at physiological pH levels. Following injection of the acidic glargine preparation, the analogue precipitates in the subcutaneous tissue, which retards its absorption in the bloodstream and thereby increases its duration of action.[5]

The effect of glargine on glucose uptake has been studied previously and was found to be the same as the effect of insulin.[6,7] Glargine has the same mitogenic effects as insulin in all cell types tested[6-9] except one,[9,10] where an increase was seen with glargine compared to insulin in osteosarcoma cells. While increased binding of glargine to insulin-like growth factor-I (IGF-I) receptors has been reported,[6,9,10] this is most likely due to the cell type used.[11]

Insulin receptor binding has been shown to be similar for glargine and insulin whether to solubilized receptors[10] or receptors overexpressed in rat fibroblasts.[8] The phosphorylation of downstream signaling elements (IRS-1 and Akt) was also not significantly different between insulin and glargine.[6,8]

All previous work on the cellular effects of insulin glargine has focused on the effects on glucose metabolism.[6,7,10] However, insulin has a multitude of other metabolic activities, including effects on protein metabolism[12,13] and fat metabolism. One study[10] investigated lipogenesis in isolated mouse adipocytes and found glargine to be less effective than insulin. The effects of insulin on protein and fat metabolism are not necessarily due to simple receptor binding and activation of the tyrosine phosphorylation cascade, but may be related to insulin metabolism.[14]

In this study we compared the effect of glargine and native insulin on protein degradation, lipolysis, lipogenesis, and glucose oxidation in cultured cells. We also compared the cellular metabolism of the 2 analogues.

MATERIALS AND METHODS

Materials

HepG2 cells and 3T3-L1 fibroblasts were purchased from ATCC (Rockville, MD). Culture medium was purchased from Sigma (St Louis, MO). Fetal calf serum (FCS), donor calf serum (CS), and gentamicin were from Invitrogen (Rockville, MD). Dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma. ^{125}I-A14-iodoinsulin and recombinant human insulin were gifts from Eli Lilly (Indianapolis, IN). Recombinant human glargine was a gift of Aventis Pharmaceuticals (Bridgewater, NJ). ^{125}I-Na was purchased from NEN (Boston, MA). D-(U-^{14}C)-glucose was from Sigma. 6-^{3}H-thymidine and ^{3}H-leucine were purchased from Amersham Biosciences (Piscat-

From the Department of Medicine, Carl T. Hayden Veterans Affairs Medical Center, Phoenix, AZ; Molecular and Cellular Biology Program, Arizona State University, Tempe, AZ; and the Department of Medicine, University of Arizona, Tucson, AZ.

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Address reprint requests to Janet Fawcett, PhD, Endocrinology (CS/111E), Carl T. Hayden VA Medical Center, 650 E Indian School Rd, Phoenix, AZ 85012.

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away, NJ). Hyamine hydroxide was from Fisher (Pittsburgh, PA). All other chemicals were of at least reagent grade.

Cell Culture

HepG2 cells were grown in minimal essential medium Eagle (MEM) with 10% FCS and 10 μg/mL gentamicin in a 37°C humidified atmosphere of 5% CO_2/95% air. For all experiments, cells were plated at a density of 3.4×10^4 cells/cm^2 and grown to confluency in the above medium. For DNA synthesis experiments, cells were plated in 48-well plates and deprived of serum on day 4 after plating for 48 hours prior to the experiment.

3T3-L1 fibroblasts were plated onto either 6-well or 24-well cell culture plates at a concentration of 12,500 or 10,000 cells per well, respectively. Cells were maintained for 7 days to confluence with Dulbecco's modified Eagle's medium (DMEM) with 10% CS and 10 μg/mL gentamicin in a 37°C humidified atmosphere of 8% CO_2/92% air. Cells were then changed to differentiation medium, DMEM with 10% FCS, 10 μg/mL gentamicin, 0.25 μmol/L dexamethasone, 0.5 mmol/L IBMX, and 0.1 μmol/L insulin. After 4 days, when cells had differentiated into adipocytes, the medium was changed to insulin medium (DMEM with 10% FCS, 10 μg/mL gentamicin, and 0.1 μmol/L insulin). After 3 to 4 days, the adipocytes were maintained with adipocyte media (DMEM with 10% FCS and 10 μg/mL gentamicin) for an additional 7 to 14 days before experimentation.

Radioiodination of Glargine

Glargine was iodinated by the chloromine T method: briefly, 50 μL 0.5 mol/L sodium phosphate, pH 7.4, 2.7 mCi ^{125}I-Na, and 100 μL 0.5 mg/mL glargine in 0.01 mol/L HCl are mixed with chloramine T (50 μL 0.5 mg/mL) and incubated for 60 seconds at room temperature. The reaction was stopped with the addition of 50 μL 0.5 mg/mL sodium metabisulfite. Unreacted ^{125}I-Na was removed by passing the mixture through a desalting column (D-Salt Excellulose, Pierce, Rockford, IL) eluting with triethylammonium phosphate (TEAP) buffer (6.7 mL/L phosphoric acid [85%], 2.8 ml/L triethylamine, 7 g/L sodium perchlorate, pH 3.0). Fractions with greater than 95% trichloroacetic acid (TCA) precipitation were pooled and injected onto reverse-phase high-performance liquid chromatography (HPLC; C18, 10 μ Bondclone, Phenomenex, Torrance, CA) running at 5 mL/min. Fractions (0.2 mL) were collected. The HPLC elution buffers were as follows: buffer A—30% (vol/vol) acetonitrile in TEAP buffer; buffer B—42% (vol/vol) acetonitrile in TEAP buffer. The elution was as follows: 8 minutes isocratic 0% B, 3 minutes gradient to 20% B, 7 minutes isocratic at 20% B, 5 minutes gradient to 100% B, 5 minutes isocratic at 100% B, 1 minute gradient to 0% B, 5 minutes isocratic at 0% B. This separates the A14-monoiodinated glargine (at approximately 15 minutes retention time) from other radioactive peaks.

Binding Assay

Binding of ^{125}I-insulin or ^{125}I-glargine to HepG2 cells was performed at 4°C for 4 hours. This was determined to be maximal binding to HepG2 cells by incubating cells with ^{125}I-insulin or ^{125}I-glargine at 4°C for up to 8 hours (data not shown). Radioactivity (10,000 cpm) was added to the cells with either native insulin or unlabeled glargine (10^{-12} to 10^{-6} mol/L) in serum-free MEM containing 0.1% BSA. After incubation, unbound material was removed and the cells washed twice with PBS, pH 7.4. The cells were solubilized and counted in a gamma counter.

Degradation, Uptake, and Association of Radiolabeled Insulin or Glargine

HepG2 cells were incubated with radiolabeled insulin or glargine (10,000 cpm) in MEM containing 0.1% BSA for up to 2 hours at 37°C. At the end of the incubation, medium was removed and subjected to TCA precipitation (10% wt/vol final concentration). Cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, and acid washed (PBS, pH 5, for 15 minutes) to remove membrane-bound radioactivity. Any remaining radioactivity was considered to be intracellular.

Uptake and Degradation of Membrane-Bound Material

HepG2 cells in 6-well plates were incubated with 20,000 cpm/mL ^{125}I-insulin or ^{125}I-glargine in MEM with 0.1% BSA for 3 hours at 4°C to label cell surface receptors. The labeling media was then removed and the cells washed twice with ice-cold PBS, pH 7.4. Media (MEM + 0.1% BSA) at 37°C was then added to the cells and the cells incubated at 37°C for up to 2 hours. At the indicated times the cells were placed on ice, and the media removed and analyzed for degradation by precipitation in 10% (wt/vol, final concentration) TCA. The membrane-bound and intracellular material was separated as described earlier.

Release of Intracellular Material

HepG2 cells in 6-well plates were incubated with 100,000 cpm/mL ^{125}I-insulin or ^{125}I-glargine in MEM with 0.1% BSA for 30 minutes at 37°C. Cells were then rinsed with PBS, pH 7.4, and acid washed (PBS, pH 5) to remove cell surface material. The cells, which now contained only internalized analogue, were then incubated in MEM + 0.1% BSA for up to 2 hours, with frequent sampling of the media to determine release of intact (TCA-precipitable) and degraded (TCA-soluble) radioactivity. The volume removed (100 μL) was replaced with fresh media. At the end of the incubation, the cells were washed and solubilized to determine the radioactivity remaining. The amount of insulin or glargine in the cells at the beginning of the experiment was calculated as the sum of the released material and the material remaining at the end of the incubation.

^{3}H-Thymidine Incorporation

The mitogenic potency of insulin and glargine was determined by incorporation of ^{3}H-thymidine into cells. This was performed as previously described[15] in 48-hour serum-deprived HepG2 cells.

Protein Degradation

This was performed as previously described.[16,17] Cells in 24-well plates were incubated overnight with ^{3}H-leucine to label intracellular proteins. The labeling medium consisted of leucine-free MEM with 10% FCS, 10 μg/mL gentamicin, and 0.5 μCi/mL ^{3}H-leucine. The cells were then washed twice with incubation/wash medium (MEM + 0.1% BSA + 2 mmol/L leucine + 20 mmol/L N-[Tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid [TES], pH 7.5) and incubated for up to 3 hours at 37°C with or without insulin or glargine. The incubation medium contains 2 mmol/L unlabeled leucine to prevent reincorporation of any released ^{3}H-leucine into new proteins. At the end of the incubation an equal volume of 6 mol/L acetic acid containing 2% Triton X-100 was added to the cells on ice. The solubilized cells/medium mix was analyzed by precipitation in 10% TCA, and the percent degraded was taken as the percent TCA-soluble radioactivity. Dose response to insulin and glargine was determined by incubating the cells with 10^{-12} to 10^{-6} mol/L analogue for 3 hours.

Lipogenesis

Hormone-stimulated storage of glucose into lipid was assayed using 3T3-L1 cells plated in 6-well dishes. The adipocyte media was aspirated and the cells were rinsed with PBS, pH 7.4. Krebs-Ringer-HEPES (KRH) buffer containing 2% BSA, 0.55 mmol/L glucose, 0.2 μCi/mL D-(U-^{14}C)-glucose, and various concentrations (10^{-6} to 10^{-12} mol/L) of insulin or glargine. The cells were incubated in a humidified atmosphere at 37° C at 8% CO_2/92% air for 1 hour. To stop further

incorporation, the plates were placed on ice for 1 hour. The cells were scraped off the bottom of the wells and collected with the incubation media. The cell/medium mix was extracted with chloroform. One milliliter of the chloroform extract was placed in a scintillation vial and counted in a liquid scintillation counter.

Lipolysis

Lipolysis was measured by both glycerol and nonesterified fatty acid (NEFA) release. For these experiments, 3T3-L1 adipocytes in 24-well plates were used. The adipocyte media was aspirated and the cells were rinsed with PBS, pH 7.4. Lipolysis was stimulated using 0.1 μmol/L epinephrine in KRH buffer, pH 7.4, with 0.1% BSA. Insulin or glargine (10^{-6} mol/L to 10^{-12} mol/L) was incubated with epinephrine for 2 hours at 37°C. After incubation, the media was immediately removed and stored at −20°C. Glycerol release was measured using methods previously described.[18] NEFA release was measured using a commercially available spectrophotometric kit (Waco Chemicals, Richmond, VA).

Glucose Oxidation

Hormone-stimulated oxidation of glucose to CO_2 was assayed using 3T3-L1 cells plated in 6-well dishes. The adipocyte media was aspirated and the cells rinsed with PBS, pH 7.4. KRH with 2% BSA and 0.55 mmol/L glucose (1 mL) was added per well. The cells were scraped off the wells and pipetted up and down to evenly distribute the cells. One milliliter of the cell suspension was then added to a 20-mL vial containing 1 mL KRH with 2% BSA, 0.55 mmol/L glucose, 0.4 μCi/mL D-(U-^{14}C)-glucose and various concentrations (2×10^{-6} M to 2×10^{-12} mol/L) of insulin or glargine (final concentration of hormone, 10^{-12} to 10^{-6} mol/L). The cells were then gassed with a stream of 5% CO_2/95% O_2 for 5 seconds before being sealed with an airtight rubber gasket attached to a plastic well (Kontes Glass, Vineland, NJ). The well contained a 2 cm × 1 cm piece of Whatman Filter Paper (Clifton, NJ) soaked with 0.3 mL of hyamine hydroxide. The vials were then incubated in a shaking 37° C water bath for 1 hour. To ensure complete release of CO_2, 0.3 mL 10N H_2SO_4 was added to the cells through the gasket using a syringe. After an additional 30 minutes of shaking water bath incubation, the wells were clipped from the gasket into scintillation vials. To acidify the sample to reduce chemiluminescence, 0.5 mL of 1 mol/L HCl was added to the scintillation vial before the addition of scintillation cocktail.

Statistical Analysis

Differences between insulin and glargine were analyzed by Student's *t* test and $P < .05$ was taken as significant.

RESULTS

Effect on Protein Degradation

An important action of insulin rarely considered in metabolic studies is that of inhibition of protein degradation. HepG2 cells labeled overnight with ^{3}H-leucine were incubated for up to 4 hours with 10^{-7} mol/L insulin or glargine. Both insulin and glargine had to be present on the cells at least 3 hours to have an inhibitory effect on protein degradation (data not shown). A dose response of HepG2 cell protein degradation to insulin and glargine is shown in Fig 1. Although there was no significant difference in the potency of insulin and glargine (EC_{50} = 0.15 *v* 0.17 nmol/L, respectively, P = not significant [NS]), glargine was less effective at inhibiting protein degradation than was insulin (protein degradation reduced to 84.4% ± 1.1 *v* 79.4% ± 1.4% of no additions, respectively, $P < .05$).

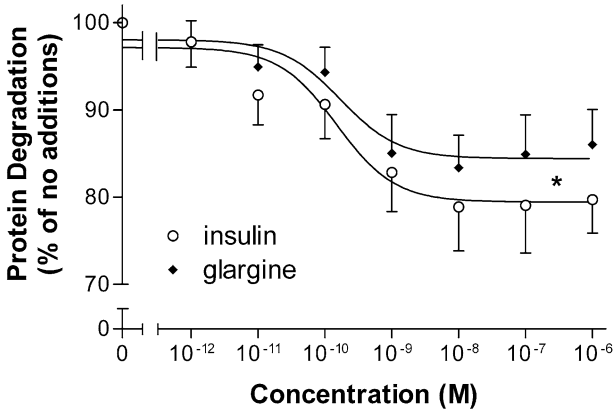

Fig 1. Effect of insulin and glargine on protein degradation in HepG2 cells. Cells were labeled with [^{3}H]-leucine (1 μCi/mL) overnight, washed twice with medium containing 2 mmol/L unlabeled leucine, and incubated for 3 hours at 37°C in the same medium with or without insulin or glargine (10^{-12} to 10^{-6} mol/L). The reaction was stopped with the addition of an equal volume of 6 mol/L acetic acid containing 2% Triton X-100. Protein degradation was determined by precipitation of the cell/medium mix in 10% TCA. Percent TCA soluble was taken as degraded proteins. In the absence of insulin or glargine, the cells degraded 14.1% ± 0.5% and 12.9% ± 0.8%/3 h, respectively (P = NS). Data are means ± SEM of 6 experiments performed in triplicate. *$P < .05$

Lipolysis and Glucose Metabolism

To investigate the effects of glargine on fat metabolism we used 3T3-L1 adipocytes. Both insulin and glargine were antilipolytic (Fig 2), reducing both NEFA and glycerol release. Glargine was significantly less potent than insulin (EC_{50} = 1.4 *v* 0.35 nmol/L, respectively, $P < .001$) for NEFA release (Fig 2A). Similarly, glycerol release was significantly effected (EC_{50} = 5.7 *v* 0.66 nmol/L, glargine *v* insulin, respectively, $P < .001$) (Fig 2B). Investigation of glucose incorporation into lipid (lipogenesis) also revealed that glargine was less potent than insulin (EC_{50} = 8.1 *v* 1.3 nmol/L, glargine *v* insulin, respectively, $P < .01$) (Fig 3). While glargine was less antilipolytic and lipogenic than insulin, there was no significant difference between the 2 analogues on glucose oxidation to CO_2 (EC_{50} = 11.8 *v* 10.6 nmol/L, glargine *v* insulin, respectively, P = NS) in 3T3-L1 adipocytes.

Binding

Figure 4A shows that unlabeled insulin displaced ^{125}I-insulin from HepG2 cells with an EC_{50} of 1.4 nmol/L. Glargine was equipotent (EC_{50} = 2.1 nmol/L). Insulin and glargine also displaced ^{125}I-glargine in a similar manner (EC_{50} = 0.79 and 0.82 nmol/L, respectively) (Fig 4B).

Degradation and Cellular Interactions

HepG2 cells were incubated for up to 2 hours with ^{125}I-insulin or ^{125}I-glargine. Degradation was measured as described in the Methods by precipitation in 10% TCA. HepG2 cells degraded 21.6% ± 0.4%/h ^{125}I-insulin and 16.3% ± 0.3%/h ^{125}I-glargine ($P < .01$) (Fig 5A). Figure 5 also shows the uptake (B) and association (C) of insulin and glargine by

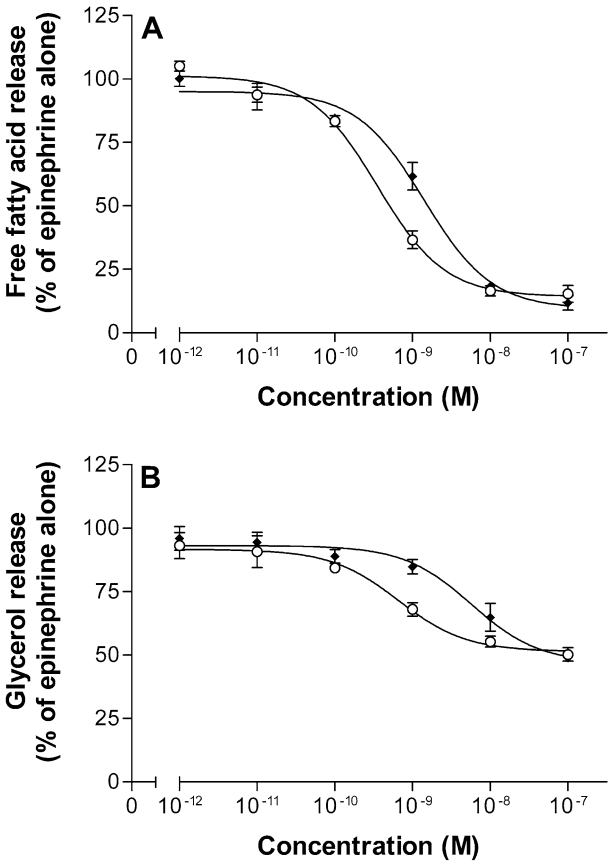

Fig 2. Antilipolytic effect of insulin and glargine on epinephrine stimulated lipolysis in 3T3-L1 cells. Cells were incubated with 0.1 μmol/L epinephrine and 10^{-12} to 10^{-7} mol/L insulin (○) or glargine (◆) for 2 hours at 37°C. Medium from the cells was assayed for free fatty acids (A) and glycerol (B). The data are presented as % of epinephrine alone and are mean ± SEM of 4 experiments performed in triplicate.

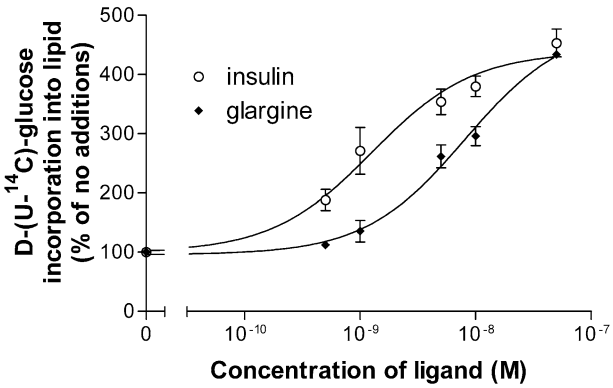

Fig 3. Glucose incorporation into lipid in 3T3-L1 adipocytes. Cells were incubated with insulin or glargine for 1 hour in the presence of D-(U-^{14}C)-glucose. Lipid was extracted by chloroform and counted in a beta counter. Data are mean ± SEM of 3 experiments performed in triplicate.

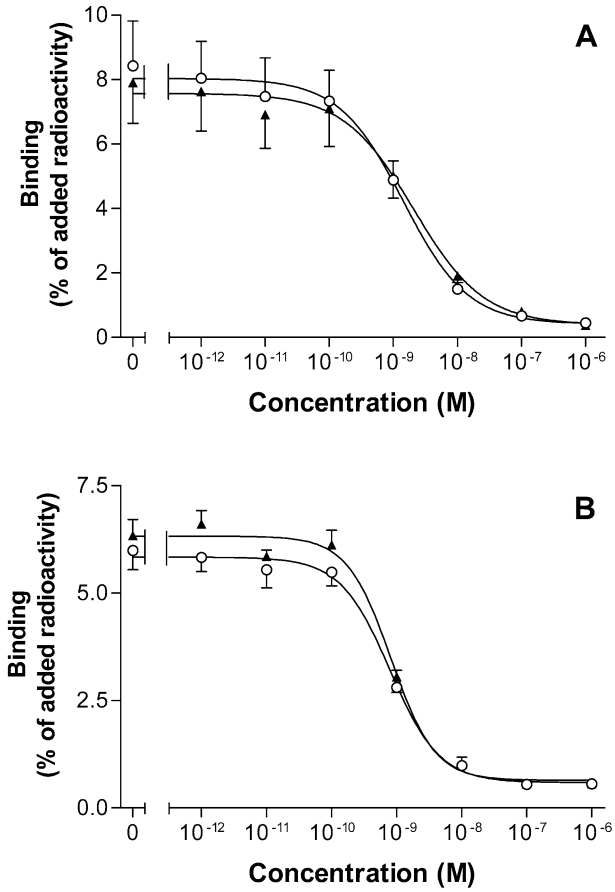

Fig 4. Binding of ^{125}I-iodo[A14]-insulin (A) and ^{125}I-iodo[A14]-insulin glargine (B) to HepG2 cells: competition with native insulin (○) and insulin glargine (◆). HepG2 cells were incubated at 4°C for 4 hours with 10,000 cpm/mL radioactivity with or without native insulin or insulin glargine (10^{-12} to 10^{-6} mol/L). Solubilized cells were counted in a gamma counter to determine binding. Data are means ± SEM of 3 experiments performed in triplicate.

HepG2 cells. Less glargine was internalized compared to insulin (Fig 5B). At early time points there was also less glargine on the membrane, but this difference was lost at time points greater that 45 minutes.

Cellular Processing

If cells were allowed to bind ^{125}I-insulin or ^{125}I-glargine on the cell surface (ie, at 4°C), and then washed free of unbound material, moving the cells to 37°C caused the cells to internalize radiolabeled material. Hormone disappeared from the membrane (Fig 6A) and appeared inside the cell (Fig 6B). Eventually, degraded material appeared in the medium (Fig 6C). On average the cells bound 7.5% ± 1.2% of added ^{125}I-insulin and 5.9% ± 1.4% of added ^{125}I-glargine (P = NS). After 2 minutes, 40% of the bound radioactivity was still residing on the membrane (Fig 6A), and 43% of insulin and 35% of glargine were internalized (Fig 6B). A small amount of material was already degraded (Fig 6C) and some intact material was released into the medium (12% insulin, 20% glargine) (Fig 6D). The rate of disappearance from the membrane was identical in

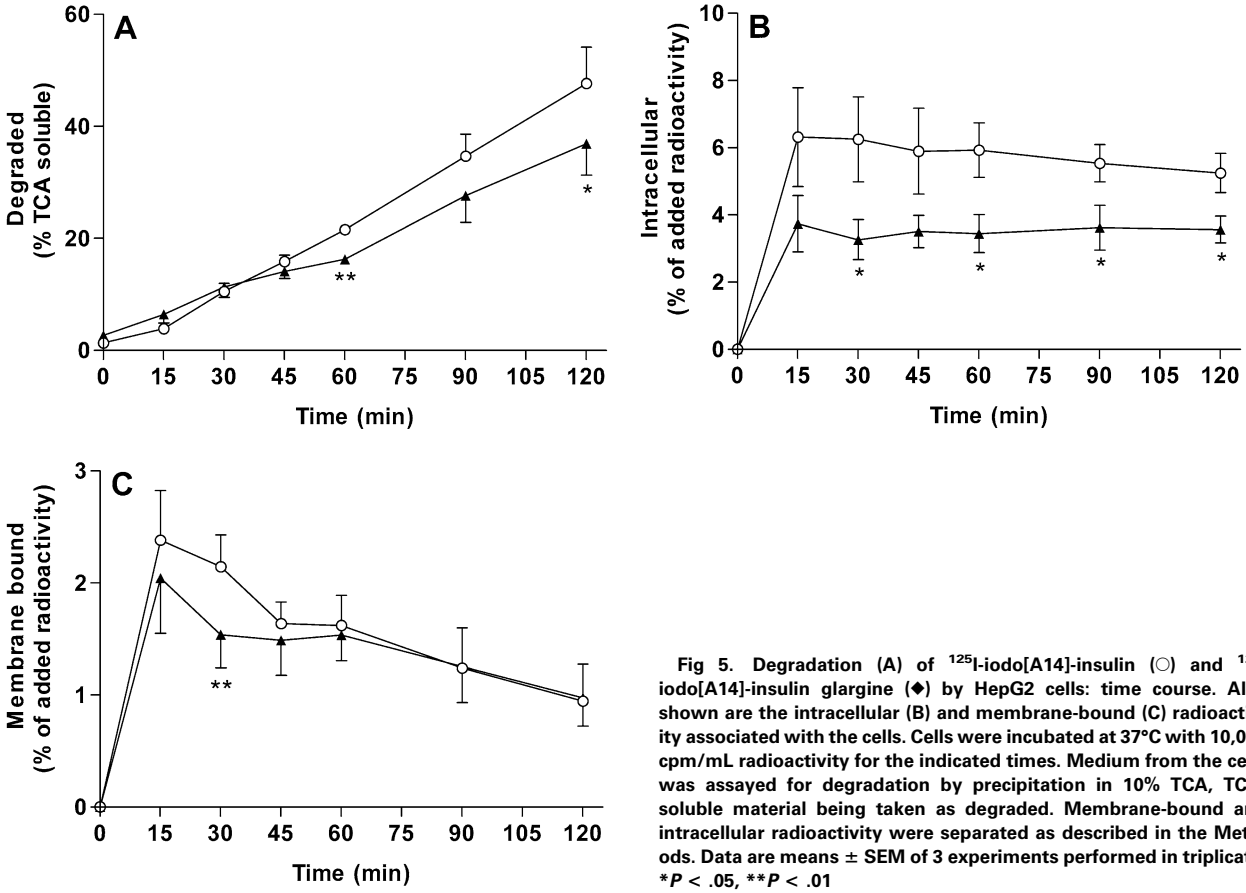

Fig 5. Degradation (A) of ^{125}I-iodo[A14]-insulin (○) and ^{125}I iodo[A14]-insulin glargine (◆) by HepG2 cells: time course. Also shown are the intracellular (B) and membrane-bound (C) radioactivity associated with the cells. Cells were incubated at 37°C with 10,000 cpm/mL radioactivity for the indicated times. Medium from the cells was assayed for degradation by precipitation in 10% TCA, TCA-soluble material being taken as degraded. Membrane-bound and intracellular radioactivity were separated as described in the Methods. Data are means ± SEM of 3 experiments performed in triplicate. *$P < .05$, **$P < .01$

the 2 analogues (Fig 6A), but more insulin than glargine went into the cell (Fig 6B), while more glargine than insulin was released into the medium without being internalized (Fig 6D).

The release of degraded material per se can be analyzed by loading cells with radiolabelled hormone for 30 minutes, acid washing to remove cell surface–bound material, and incubating at 37°C with fresh medium to look at released material (Fig 7). After 2 hours, 58.3% ± 1.4% of insulin initially internalized was degraded and released. Significantly less glargine was released degraded (50.1% ± 2.4% over 2 hours, $P < .02$). The amount of intact glargine released was concomitantly elevated compared to insulin (44.8% ± 2.6% *v* 35.8% ± 1.4% over 2 hours, respectively, $P < .02$).

DISCUSSION

Insulin analogues are useful tools in the treatment of diabetes.[1,2] Different analogues have been designed to have a shorter duration of action (eg, Lys^{B28},Pro^{B29}-insulin (lispro), Asp^{B28}-insulin [insulin aspart]) or a longer, flatter action profile (glargine, Novosol, Novo Nordisk, Basal, Denmark).[5] When used in combination, these analogues produce a serum insulin profile that more closely resembles that seen in nondiabetics.

Insulin glargine is a human insulin analogue that precipitates at the pH of tissues at the injection site and as such is slowly absorbed into the circulation.[5] It has no pronounced activity peak and is used as a basal insulin in patients with diabetes.[4,19]

Insulin has effects not only on glucose uptake and metabolism, but also on protein[12,13] and fat metabolism. These are intermediate actions of insulin requiring receptor exposure time in the range of minutes to hours. These effects have been tied to the cellular processing of insulin[17,20-24] by insulin-degrading enzyme.[14,25] The present study supports the association of insulin processing to both the inhibition of protein degradation and insulin action on lipid metabolism. The action of glargine to inhibit protein degradation is reduced compared to insulin as is its antilipolytic and lipogenic actions and cellular metabolism. Glargine's surface binding to these cells is identical to that of insulin, and previous work has shown that the phosphorylation of the both the insulin receptor and second messengers (IRS-1 and Akt) are identical.[6,8,26] Glucose uptake is unchanged,[6,7] as is glucose oxidation (this study), both actions associated with the insulin receptor phosphorylation cascades.[27]

The altered activity of glargine compared to insulin may also be due to preferential binding to either type A or type B insulin receptor. Type A receptors have a higher binding affinity for insulin, higher affinity for the IGF, and a higher internalization rate than type B.[28,29] Since glargine has a higher affinity for the IGF-1 receptor than native insulin,[6,7,9,10] it may be postulated that glargine may preferentially bind to the type A receptors. This difference is probably not large enough to generate the altered actions seen. Type B receptors are the predominant

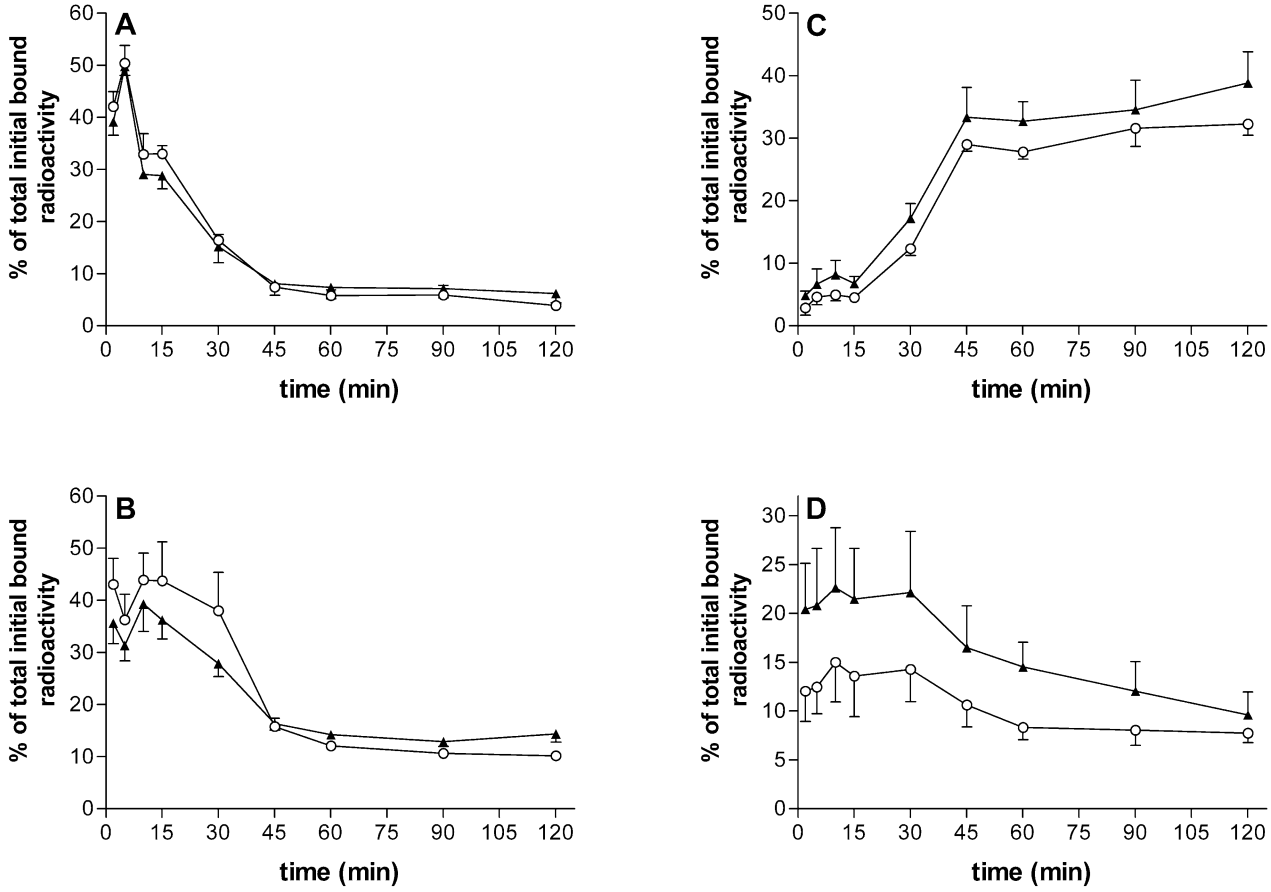

Fig 6. Uptake of ^{125}I-insulin (○) and ^{125}I-glargine (◆) in cells previously labeled on the cell surface. Following incubation of cells with 20,000 cpm for 3 hours at 4°C to label the cell surface, cells were washed twice with PBS, pH 7.4, and placed in fresh medium without radioactivity. At time points to 2 hours, the medium was removed and subjected to TCA precipitation (C—TCA-soluble; D—TCA-precipitable). The cells were rinsed and subjected to acid wash (membrane-bound material, A). Radioactivity remaining in the cells was taken to be intracellular (B). Data are means ± SEM of 3 experiments performed in triplicate.

receptors on insulin-sensitive tissues (liver, muscle, fat)[30] and are more sensitive to insulin.[31,32] HepG2 cells have been shown to express type A receptors exclusively[30] or to a level of approximately 45% to 50%.[33] However, the cells used in the latter study were deprived of serum for 4 days, while the cells in the former study were not. Cells in the protein degradation and hormone metabolism studies here were not deprived of serum, suggesting that the HepG2 cells used here have a population of approximately 100% type A insulin receptors. The cell types used in this study have been shown to express different levels of the 2 insulin receptor isoforms (HepG2 cells express mainly type A,[30] while 3T3-L1 adipocytes express mainly type B isoforms[32]) but while the B isoform may signal more effectively than the A isoform towards the tyrosine phosphorylation cascade proteins and subsequent effects on glucose uptake and metabolism, the actions of insulin on protein and lipid metabolism have been shown to not be associated with this pathway. Insulin's effect on protein and lipid metabolism requires cellular metabolism of the hormone.[17,20-24] The relationship between different receptor isoforms and cellular hormone metabolism is not understood and requires further study.

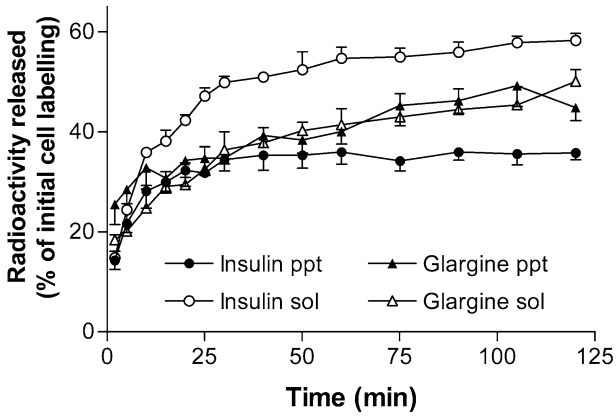

Fig 7. Release of intracellular insulin or glargine from cells preloaded with ^{125}I-insulin or ^{125}I-glargine. Cells were incubated with radioactivity for 30 minutes at 37°C, acid washed, and incubated for 2 hours in fresh, radioactivity-free medium. At the times indicated 100-μL aliquots were taken and analyzed by TCA precipitation. Fresh medium (100 μL) was added back to the cells. TCA-soluble (sol) was taken as degraded and TCA-precipitable (ppt) was taken as intact material. Data are means ± SEM of 5 experiments performed in triplicate.

Previous work with other insulin analogues, in various cell types and cell free systems, has shown a correlation between hormonal metabolism and their action on protein metabolism,[22,25] while binding per se of the analogues to cell surface receptors did not correlate to the relative inhibition of protein degradation.[17] Binding and degradation assays were not performed in 3T3-L1 cells in the current study. 3T3-L1 cells do bind[34] and degrade (unpublished observation) insulin in a manner similar to other insulin-responsive cell types.

It is also postulated that increased binding to IGF-1 receptors increases the mitogenic activity[35] of an insulin analogues (eg, Asp^{B10}-insulin).[36,37] However, the mitogenic activity of glargine is not increased compared to insulin[6-9,26] (data not shown) except in studies using osteosarcoma cells.[9,10] This is probably due to the different characteristics of the osteosarcoma cell line.[11]

As a basal insulin, glargine is used in combination with short-action insulin forms, either analogues or exogenous. This basal bolus approach is extremely effective in glucose control. The availability of other insulin forms makes is unlikely that the small differences between glargine and native insulin on fat and protein metabolism will have significant clinical effects. This conclusion needs to be tested in whole animal or clinical studies, especially since some of the rapidly absorbed insulin analogues also have minor differences from native insulin in their effects on nonglucose pathways. The important conclusion is that attention must be given to nonglucose effects of insulin in the evaluation of insulin analogues.

In fact, the properties of glargine could have clinical benefits. The reduced degradation could provide a longer action and increase its efficacy as a basal insulin.

In a study[38] with patients with type 2 diabetes previously treated with regular insulin, switching to glargine as a basal insulin produced less weight gain than the insulin-treated group. The authors suggested that this might be due to the lower incidence of hypoglycemic episodes seen in the glargine-treated group compared to the insulin-treated group. The patients would treat hypoglycemic episodes by increasing caloric intake. However, an alternative explanation could be the fact that glargine is less antilipolytic and less lipogenic than insulin, thereby promoting fat breakdown and/or attenuating fat deposition.

These studies support the concept that evaluation of insulin analogues should consider more than glucose metabolism and mitogenesis. Effects on protein and fat turnover are also important. Long-term effects on nonglucose pathways of insulin analogues must be evaluated. The findings in this study and previous studies with other analogues are supporting of the use of these materials in clinical practice, but also remind us that long-term follow-up is required.

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