# Differences in the Cellular Processing of $Asp^{B10}$ Human Insulin Compared With Human Insulin and $Lys^{B28}Pro^{B29}$ Human Insulin

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**Cellular metabolism studies were performed comparing human insulin with two rapid-acting analogs, $Lys^{B28}Pro^{B29}$ insulin (LysPro) and $Asp^{B10}$ insulin (B10-Asp). B10-Asp bound to isolated hepatocytes at 37°C to a greater extent than LysPro or native insulin, which were equivalent. The rate of degradation was similar for the three materials, resulting in a significant reduction in the degraded/bound ratio for the B10 analog. The processing of membrane-bound material was examined by incubating cells with hormone at 4°C, removing unbound insulin, and incubating the cells at 37°C. Again, binding was greater for B10-Asp versus LysPro or native insulin, with a reduction in the degraded/bound ratio. Hormone internalization and processing was examined by an acid wash of cells incubated with $^{125}I$(A14)-labeled hormone to remove surface-bound materials. The processing rate was slower for B10-Asp versus LysPro or native insulin. Cell extraction and examination on molecular-sieve chromatography confirmed that B10-Asp was processed at a slower rate than either LysPro or native insulin. Intact B10-Asp was found in the cell after 4 hours, whereas all native insulin and LysPro were degraded by 90 to 120 minutes. B10-Asp also caused a greater incorporation of thymidine into DNA in cultured cells than LysPro or native insulin, which were similar. These data show that the cellular processing of LysPro is essentially identical to that of native insulin. However, B10-Asp has markedly different properties and is processed much more slowly. The prolonged cell residence time of B10-Asp could contribute to its greater effects on cell growth and mitogenesis.**


INSULIN ANALOGS have great potential for diabetes therapy. With biosynthetic techniques, it is possible to produce targeted alterations in the insulin molecule for specific changes in its properties.[1] Ultimately, the goal is to produce insulin derivatives that can be used to mimic normal pancreatic function in clinical therapy.[2] In general, the pancreas provides basal insulin secretion for maintaining cellular function in the postabsorptive state and acute insulin release with nutrient intake. A major problem in the therapy for insulin-deficient patients has been obtaining the rapid increase in plasma insulin that normally occurs in the nondiabetic individual after a meal. To overcome this, an insulin analog that is absorbed rapidly from a subcutaneous injection site is needed.[3] $Lys^{B28}Pro^{B29}$ human insulin (LysPro) is such an analog; it has been shown to produce a more physiological plasma insulin profile and a smaller elevation in blood glucose than regular insulin when injected prior to a meal.[4-8]

Previously, another analog with aspartic acid substituted for histidine in position 10 of the insulin B chain ($Asp^{B10}$ human insulin [B10-Asp]) also was shown to be rapidly absorbed, and clinical trials were initiated.[9-11] However, animal studies showed that this analog had increased mitogenic activity, producing mammary tumors in rats.[12,13] Several possibilities for the mechanism of the augmented mitogenic activity of B10-Asp have been suggested, including increased binding to the insulin-like growth factor-I (IGF-I) receptor, increased insulin receptor binding, and prolonged stimulation of cells due to reduced degradation and clearance from the cell.[14] The present studies were performed to examine the degradation and cell processing of B10-Asp and LysPro compared with native insulin.

## MATERIALS AND METHODS

Human insulin, LysPro, and B10-Asp, all from Lilly Research Laboratories (Indianapolis, IN), were specifically labeled with $^{125}I$ at tyrosine A14 as previously described[15] and purified by high-performance liquid chromatography (HPLC).[16] The specific activity was 374 mCi/mg. Bovine serum albumin ([BSA] Fraction V, radioimmunoassay grade) was purchased from Sigma (St Louis, MO). Collagenase (Type 1) was obtained from Worthington Biochemical (Freehold, NJ). All other chemicals were at least of reagent grade. Male Sprague-Dawley rats were purchased from Sasco (Wilmington, DE) and treated according to the *Principles of Laboratory Animal Care* (National Institutes of Health Publication No. 85-23, revised 1985). Curve-fitting, statistical analysis, and graphing were performed using GraphPad Prism Version 2.01 (San Diego, CA).

Hepatocytes were isolated by a modification[17] of the method of Terris and Steiner.[18] After isolation, the cells were incubated in Krebs improved Ringer II buffer with 5.5 mmol/L glucose and 3% BSA for 30 minutes at 37°C. The cells were then centrifuged and resuspended at a concentration of approximately $10^6$/mL in fresh buffer containing BSA. Radiolabeled insulin or one of the analogs were added ($\sim 5 \times 10^{-11}$ mol/L final) and the cells were incubated in one of three ways: (1) at 37°C with samples taken at the times indicated; (2) at 4°C for 30 minutes, washed and resuspended in fresh buffer at 37°C, and sampled; or (3) at 37°C for 15 minutes, washed with pH 5.0 buffer, resuspended in fresh pH 7.4 buffer at 37°C, and sampled. At the times indicated, the incubations were stopped by layering the cell suspension on silicone oil (density 1.02) over extraction mix and centrifuging for 1 minute in a microfuge to separate the medium from the cells. The extraction mix contained 7 mol/L urea, 3 mol/L acetic acid, 0.2% Triton X-100, and 100 μg/mL unlabeled insulin. The media were sampled, and the degradation of labeled insulin analogs was assayed by trichloroacetic acid (TCA) precipitation (10% final concentration). Media samples and cell pellets were analyzed by a gamma counter, and binding and degradation were calculated.

For examination of cellular degradation, the separated cell pellets were frozen in liquid nitrogen, an equal volume of extraction mix was added, and the sample was thawed and shaken overnight at 4°C. The

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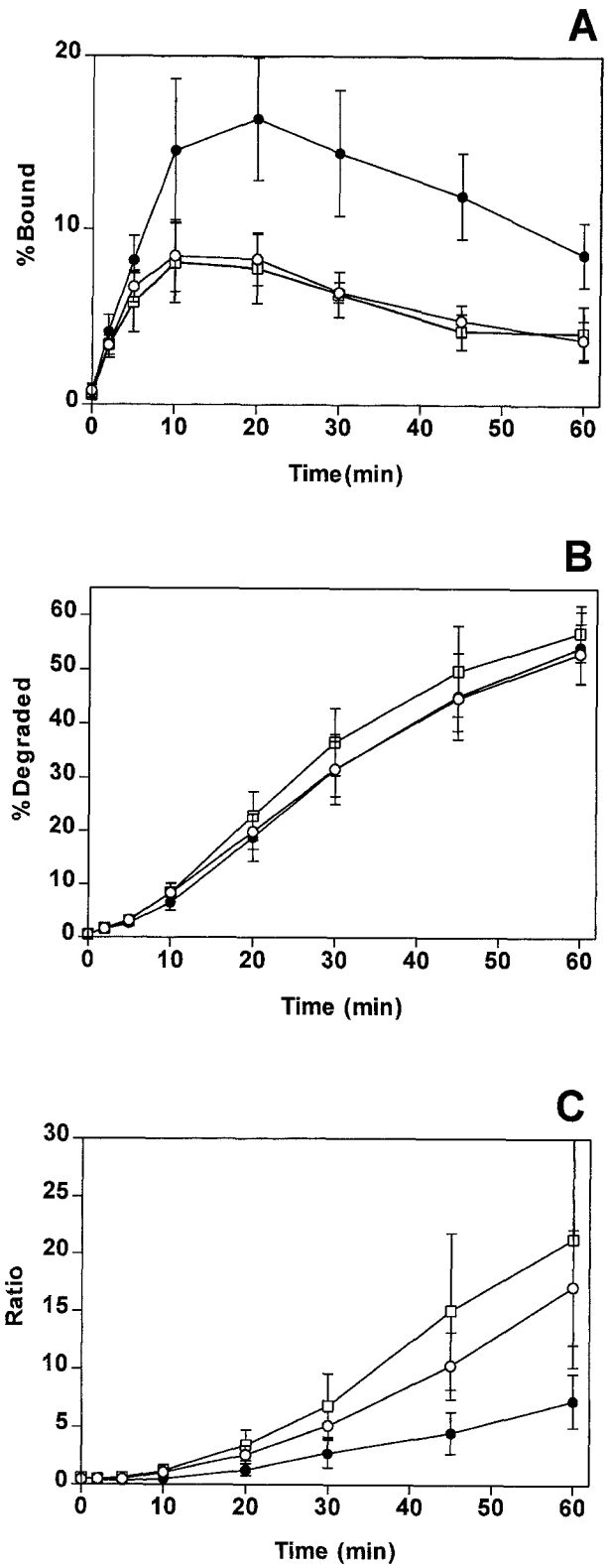



Fig 1. Binding and degradation of insulin analogs at 37°C. Isolated hepatocytes were incubated with radiolabeled insulin (○), B10-Asp (●), or LysPro (□) for the times indicated, and the amount of tracer associated with the cells and TCA solubility of label in the medium were determined. A, cell-associated radioactivity as a percent of total; B, TCA solubility of labels in the medium; C, ratio of the degradation to the amount of label bound at each time point.

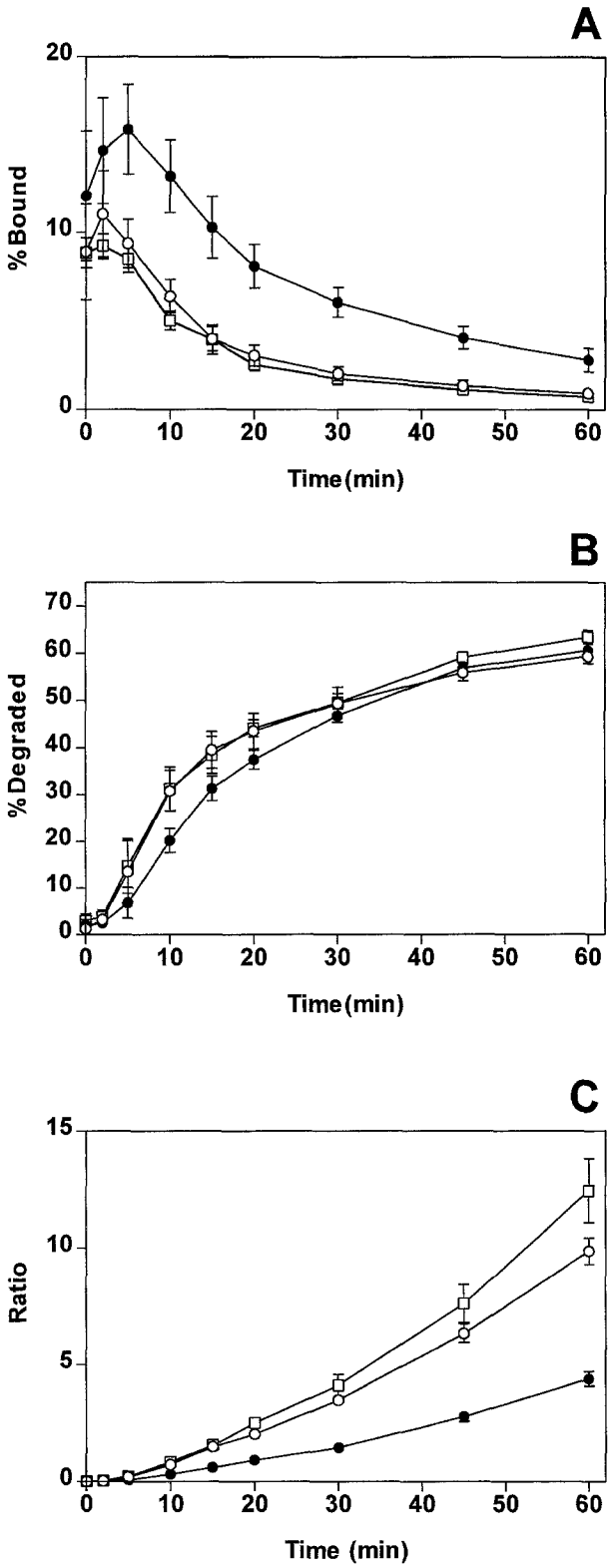



Fig 2. Binding and degradation of insulin analogs initially bound at 4°C. Isolated hepatocytes were incubated at 4°C with radiolabeled insulin (○), B10-Asp (●), or LysPro (□), and the subsequent processing of cell-associated material was monitored at 37°C. A, cell-associated radioactivity as a percent of total radioactivity in the 4°C incubation; B, TCA solubility of labels in the medium; C, ratio of the degradation to the amount of label bound at each time point. Mean values for time points 10 through 60 minutes are significantly different by ANOVA ($P < .01$).

sample was applied to a Sephadex G-50 (fine) column (0.9 × 50 cm) and eluted with 1 mol/L acetic acid, yielding a high–molecular weight (HMW) peak, an insulin-sized peak, and a low–molecular weight (LMW) peak.

Each of the analogs was tested for susceptibility to degradation by insulin-degrading enzyme. Rat skeletal muscle insulin-degrading enzyme was obtained as previously described[19] to the ammonium sulfate precipitation step. The only insulin-degrading activity in this preparation is due to insulin-degrading enzyme. Degradation of radiolabeled insulin or analog was assayed by HPLC as previously described.[20] Loss of radiolabel from the peak of intact peptide was determined at the times shown, and expressed as the amount of intact analog remaining.

The relative mitogenicity of the analogs was assessed using $^{3}$H-thymidine incorporation. Rat H4-II-E hepatoma cells (American Type Culture Collection, Rockville, MD) were grown to a subconfluent state in minimum essential medium Eagle (MEM) with 10% fetal bovine serum at 37°C and 5% $CO_2$. The cells were serum-deprived overnight, and then changed to serum-free MEM with 0.1% BSA and the indicated concentrations of insulin analogs and incubated for 20 hours. $^{3}$H-thymidine (0.1 μCi/mL) was added and the cells were incubated another 4 hours. The cells were then treated with 10% TCA to precipitate DNA. The precipitate was solubilized in 0.1% sodium dodecyl sulfate and 0.1 mol/L NaOH and analyzed in a beta counter.

## RESULTS

Freshly isolated hepatocytes were incubated at 37°C with LysPro, B10-Asp, and native insulin, all labeled on Tyr$^{A14}$. B10-Asp bound to cells to a greater extent than either LysPro or native insulin but the total degradation was similar, resulting in a markedly reduced degraded/bound ratio for the B10 analog. The rate of degradation is therefore much slower for cell-associated B10-Asp versus LysPro or native insulin, which are equivalent (Fig 1).

At 37°C, cell-associated insulin includes both membrane-bound and internalized insulin and degradation products. To examine more clearly the receptor-bound material and subsequent cellular processing, isolated hepatocytes were incubated at 4°C with the three A14-labeled materials and then washed and resuspended at 37°C. The results are very similar to the experiments shown in Fig 1, with greater binding of the B10 analog, similar degradation rates for native insulin and LysPro, and a reduced degraded/bound ratio for B10-Asp (Fig 2).

The next series of experiments was directed at comparing the processing of internalized material. Cells were incubated at 37°C to achieve maximal binding of the labeled insulins and then acid-washed to remove surface-bound material. The cells were resuspended in fresh buffer without insulin, and the release of intact and degraded hormone was measured. Expressed as either relative (using 0 time as 100%) or absolute, the B10 analog was released more slowly than either native insulin or LysPro, which were equivalent. The amount of released material that was degraded was similar for all three insulins (Fig 3).

Since several studies have suggested that alterations in the intracellular processing of insulin may affect the intermediate and long-term effects of the hormone,[17,21] we examined the intracellular degradation products of the analogs and insulin.

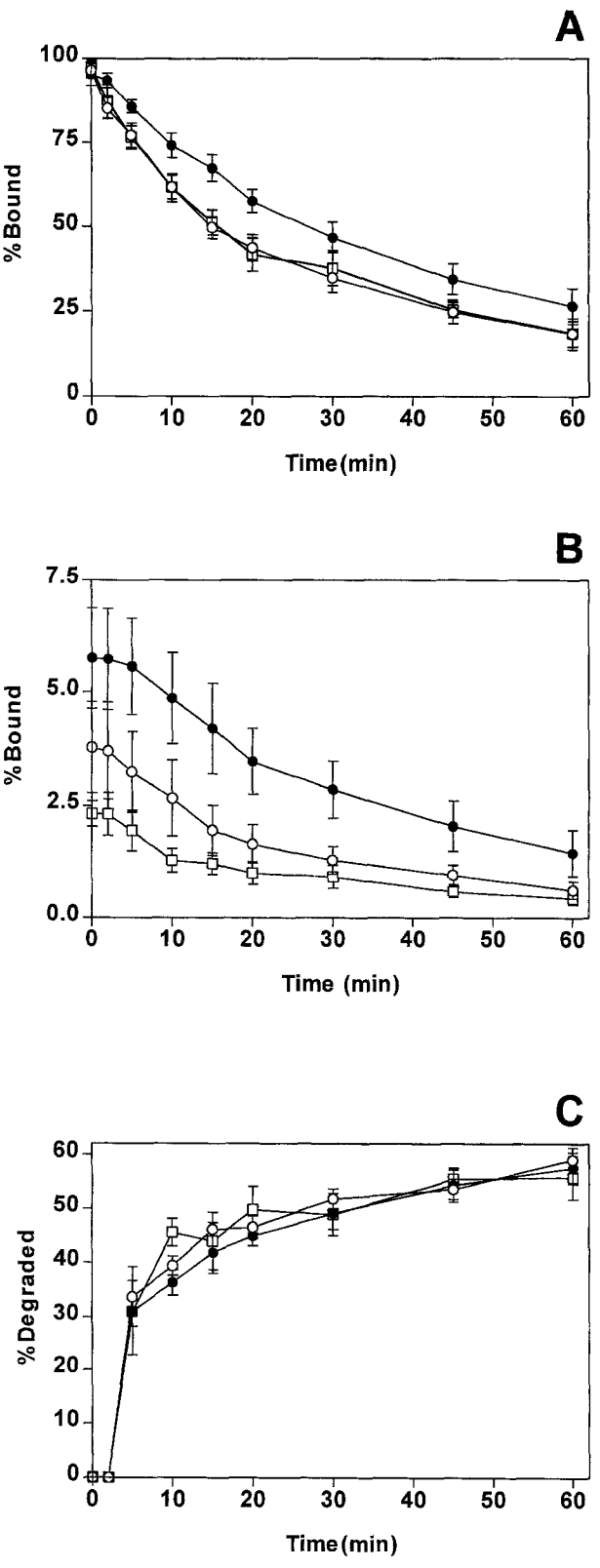
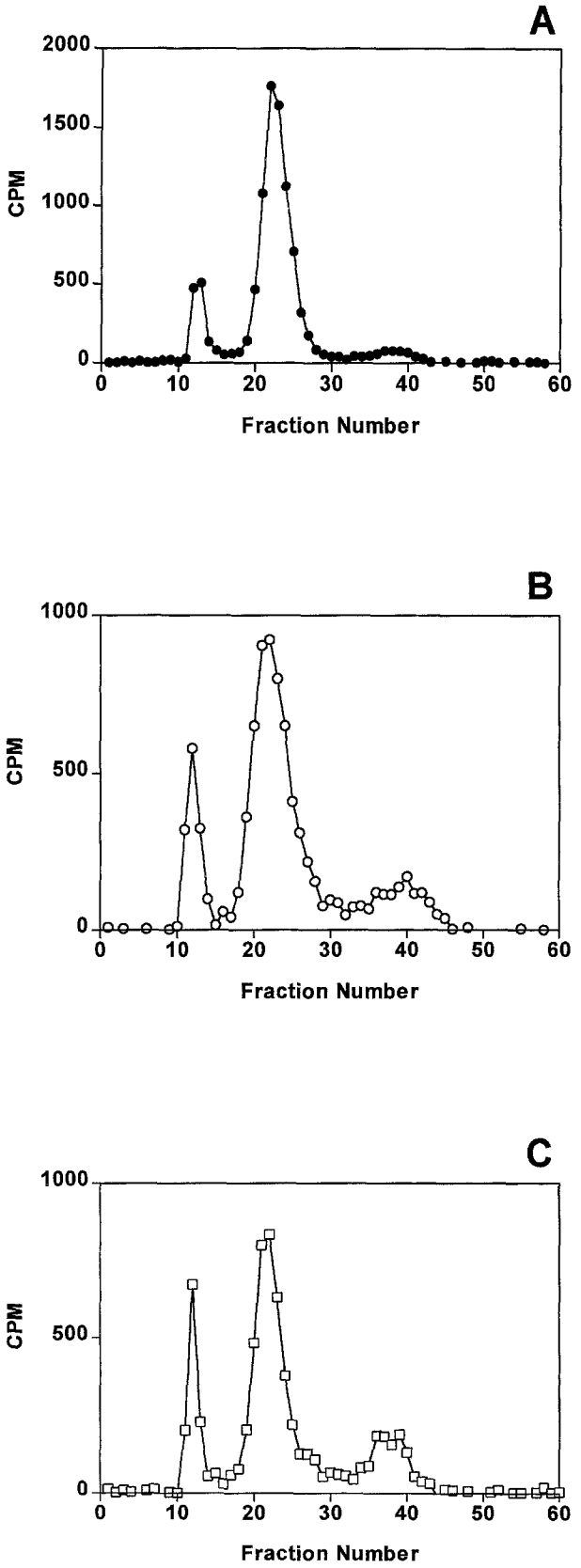



**Fig 3. Binding and degradation of insulin analogs cell-associated after acid-washing. Isolated hepatocytes were incubated at 37°C for 15 minutes with radiolabeled native insulin (○), B10-Asp (●), or LysPro (□) and then acid-washed. The subsequent processing of cell-associated material monitored at 37°C is shown. A, cell-associated radioactivity as a percent of total radioactivity in each sample; B, cell-associated radioactivity as a percent of total radioactivity in the initial incubation before acid-washing. Mean values for time points 10 through 30 minutes are significantly different by ANOVA ($P < .05$). C, TCA solubility of labels in the medium.**




Isolated hepatocytes were incubated with $^{125}$I(A14)-labeled hormones and extracted and gel-filtered on Sephadex G-50. With this approach, labeled material elutes from Sephadex G-50 in three peaks, a HMW peak, an insulin-sized peak, and a LMW peak of degradation products (Fig 4). A comparison over time of changes in the proportion of these peaks is shown in Fig 5. The cell-extracted insulin-sized peak decreases with incubation time and the LMW and HMW peaks increase, reflecting cell processing and degradation of the labeled materials. By 90 to 120 minutes, essentially no intact insulin can be extracted from cells incubated with LysPro or native insulin, whereas almost 50% of cell-associated B10-Asp elutes from the Sephadex G-50 column in the position of intact insulin. Reflecting the slowed cellular processing of B10-Asp, the LMW and HMW products are consistently lower from this analog versus LysPro or native insulin.

Since insulin-degrading enzyme is primarily responsible for initiating insulin breakdown in the cell, we examined the relative susceptibility of each analog to cleavage by this enzyme. Figure 6 shows the amount of intact insulin or analog remaining over time when incubated with a partially purified preparation of insulin-degrading enzyme. Insulin and LysPro were degraded at the same rate ($t_{1/2} \approx 9$ minutes), but B10-Asp was lost much more slowly ($t_{1/2} \approx 30$ minutes).

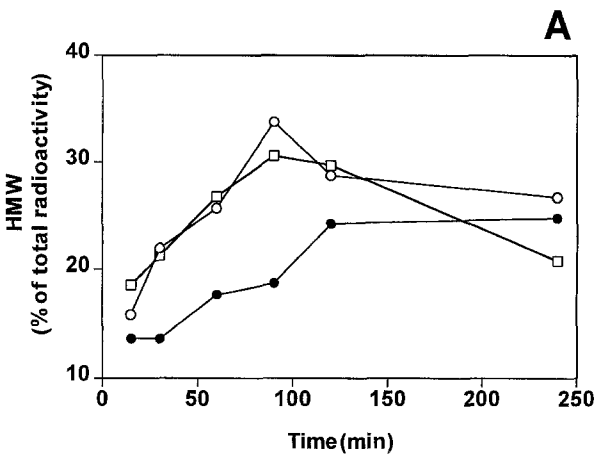
Evidence for the increased mitogenicity of B10-Asp is shown in Fig 7. The median effective concentration ($EC_{50}$) for insulin, LysPro, and B10-Asp was $4.8 \times 10^{-9}$, $1.8 \times 10^{-9}$, and $6.4 \times 10^{-10}$ mol/L, respectively. Thymidine incorporation was statistically significantly higher in cells treated with B10-Asp compared with either LysPro or native insulin.

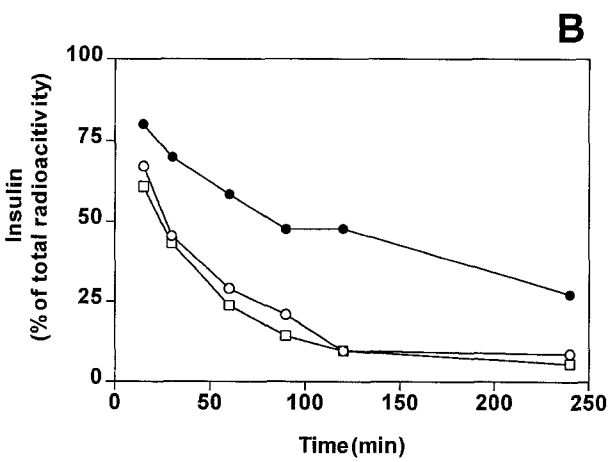
## DISCUSSION

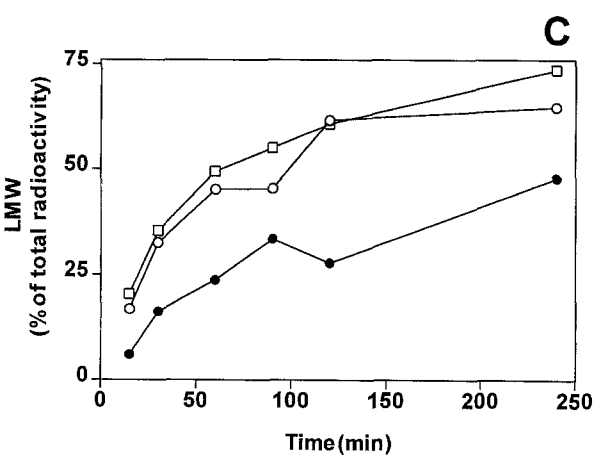
As information has accumulated, the complexity of cellular insulin processing and insulin signaling has become more apparent.[22] The actions of insulin are diverse, with effects on acute metabolic events such as glucose transport and metabolism, intermediate-term changes in cellular metabolism including lipid and protein turnover, and long-term events such as cell growth and mitogenesis. Not surprisingly, the signal transduction pathways for the various actions of insulin are not identical. Similarly, multiple cellular processing pathways exist. Many data suggest that cellular processing is involved in some actions of insulin.

For clinical management of diabetes, the acute metabolic effects of insulin have been the major focus, with blood glucose control the primary endpoint. However, it has become more apparent that this is insufficient. Not all of the actions of insulin are accurately reflected by changes in glucose levels; for adequate assessment of clinical care, other properties must be considered. This necessity extends to the agents used for treatment and was emphasized by clinical trials with B10-Asp.

**Fig 4. Sephadex G-50 (fine) profile of insulin analogs. Isolated hepatocytes were incubated with radiolabeled analogs for 15 minutes at 37°C, and the cells were separated from the media. Cell-extracted radiolabel was assayed on G-50 columns to assess degradation. A, B, and C, results for B10-Asp, native insulin, and LysPro, respectively. The first peak (~fraction 12) is HMW material. The second peak (~fraction 22) is insulin-sized material. The last broad peak (~fraction 36-43) is LMW degradation products.**



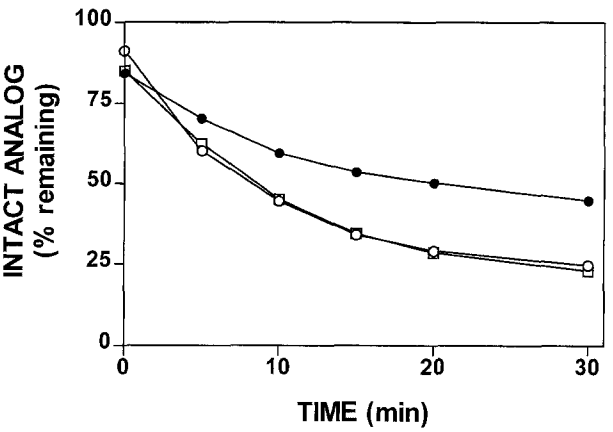




**Fig 6. Time course of insulin analog degradation by insulin-degrading enzyme. The amount of intact radiolabeled analog remaining (as a percent of the starting cpm) is shown versus time of incubation for each of 3 insulin analogs: native insulin (○), B10-Asp (●), and LysPro (□).**

This insulin analog has desirable properties from the standpoint of glucose control but undesirable effects on mitogenesis.[12,13]

LysPro also has highly desirable effects on glucose control in clinical use[23] and is comparable to native insulin with respect to its effects on the growth of normal mammary epithelial cells in culture[24]; furthermore, its safety has been demonstrated by a complex battery of toxicity studies.[25,26] Several possible explanations for the divergent properties as compared with B10-Asp have been suggested. One possibility is differential binding to the IGF-I receptor. In relative terms, the IGF-I receptor mediates cell growth to a greater extent than the insulin receptor. Insulin analogs with increased affinity for the IGF-I receptor would therefore have increased growth and mitogenic effects. B10-Asp has a greater affinity for the IGF-I receptor than native insulin, whereas LysPro has only a 1.6-fold increased affinity.[24,27] IGF-I receptor binding is thus one potential mechanism for the increased mitogenic effects of B10-Asp. The affinity of LysPro for the IGF-I receptor is only 0.2% of the affinity of IGF-I. However, the B10-Asp analog has only 0.3% of the affinity of IGF-I for its receptor, but 62% of its mitogenic activity.[28] Thus, the insulin/IGF-I receptor interaction is likely irrelevant.[14] Other mechanisms require examination.

Of greater potential relevance are the differences in insulin effects on glucose metabolism versus cell growth. The signaling mechanisms for insulin effects on glucose transport and metabolism are clearly different from those for cell growth. The signal for glucose effects is produced within seconds after insulin exposure to cells. The signal for growth effects requires hours of insulin-cell interaction. Obviously, alterations in the insulin molecule that extend its cellular association would potentially increase its mitogenic effect. Our current studies show an

**Fig 5. Time course of cell-associated degradation of insulin analogs determined by size-exclusion chromatography. The relative amount (as percent of total radiolabel) of the 3 peaks in Fig 4 is followed over time for each of 3 insulin analogs: native insulin (○), B10-Asp$^{B10}$ (●), and LysPro (□). A, increase of HMW material; B, decrease of insulin-sized material; C, increase of LMW degradation products.**

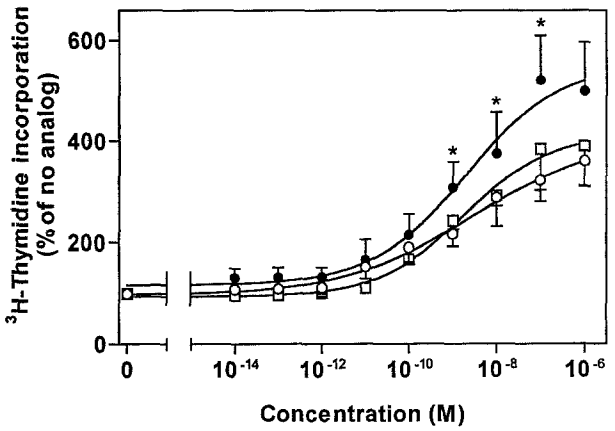



**Fig 7. Dose-response curve for $^3$H-thymidine incorporation of insulin analogs. $^3$H-thymidine incorporation is shown as a percent of the value without hormone added for each of 3 insulin analogs: native insulin (○), B10-Asp (●), and LysPro (□). *B10-Asp significantly different *v* native insulin ($P < .05$).**

increased mitogenic effect (Fig 7) in cells with undetectable levels of IGF-I receptor,[29] indicating that the insulin receptor is a mediator.

B10-Asp has properties that increase its residence time within the cell, as shown in the present and previous studies.[12,14] Receptor binding is increased and dissociation is decreased. Cellular degradation as reflected by the ratio of binding to degradation is decreased.[27] This results in a prolonged cellular residence of receptor-bound and intact hormone, and thus cell retention of biologically active insulin.

In contrast to B10-Asp, the cellular binding and metabolism of LysPro is essentially indistinguishable from native insulin. The overall cellular handling reflected by incubation at 37°C of insulin and LysPro is identical. The metabolism of membrane-bound (4°C incubation) and internalized (acid-washed cells) hormone is also identical. Degradation rates as measured by TCA precipitation and Sephadex G-50 chromatography are identical, whereas cell-associated B10-Asp remains more intact.

Our results (Fig 6) with enzymatic cleavage of the analogs suggest the mechanism for the increased cellular residence of B10-Asp. Degradation by insulin-degrading enzyme of insulin and LysPro proceeded at essentially identical rates. However, B10-Asp was degraded much more slowly. Since insulin-degrading enzyme is the primary mechanism for the initiation of degradation of insulin in the cell, this slower rate of degradation indicates that intact B10-Asp should exist in the cell for longer periods, as shown. Thus, an altered metabolism of insulin analogs can have significant effects on insulin action.

Intracellular insulin metabolism results in the production of insulin-sized degradation products. While the mechanisms are not well understood, alterations in the production and content of these products result in alterations of some but not all actions of insulin. As a generalization, alterations in the intracellular processing of insulin have little effect on glucose metabolism but may have significant effects on protein and fat metabolism and cell growth.

The results of the present study support the possibility that the augmented effect of B10-Asp on mitogenesis may be related to an increased cellular residence time. This increased cell residence is due to an increase in binding and internalization without a concomitant increase in degradation. LysPro does not share the degradation-resistant properties and does not demonstrate the enhanced mitogenic activity. This firmly supports the clinical value of the LysPro analog, and more importantly, it provides an additional approach to understanding the diverse effects of insulin.

## ACKNOWLEDGMENT

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