Evidence that Bacitracin Alters Intracellular Insulin Metabolism in Isolated Rat Hepatocytes

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SUMMARY

The effect of bacitracin on intracellular insulin degradation was investigated using an isolated rat hepatocyte preparation in which essentially all insulin degradation was due to cell-mediated processes. Bacitracin produced a concentration-dependent decrease in the degradation of insulin to products soluble in trichloroacetic acid, with a half-maximal effect at approximately 0.5 mM. These results were confirmed by analysis of extracted cell-bound radioactivity by Sephadex G-50 molecular sieve chromatography. Radioactive material eluting in the position of intact insulin from the G-50 column was further analyzed by reversed-phase, high-performance liquid chromatography. In addition to intact insulin, two peaks of radioactive material less hydrophobic than insulin were evident. Incubation of cells in the presence of 0.5 mM bacitracin significantly ($P < 0.05$) altered the distribution of radioactivity in these two peaks. These results indicate that bacitracin significantly affects hepatocyte insulin metabolism and suggest that the continued use of bacitracin in studies of hepatocyte-insulin interaction should be avoided.

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The demonstration by Terris and Steiner[1] that the initial step in insulin degradation by hepatocytes involves binding of the hormone to receptors has led to numerous studies concerning the membrane and intracellular events involved in insulin degradation. Current evidence suggests that subsequent to receptor binding, some of the bound insulin is internalized and is ultimately degraded to small peptides and amino acids within lysosomes.[2] Some of the bound insulin may also be degraded by membrane processes without internalization being required.[3] The majority of studies concerning the cellular fate of insulin have been conducted using isolated cells, and much of the uncertainty concerning insulin metabolism has resulted from the release of insulin-degrading activity into the extracellular compartment. Most isolated cell preparations have considerable insulin-degrading activity in the medium, presumably from broken or "leaky" cells damaged during isolation. A major factor contributing to the demonstration by Terris and Steiner[1] that cellular degradation is a receptor-mediated process was their ability to prepare hepatocytes that did not have extracellular degrading activity. Since the preparation of "non-leaky" isolated cells can be difficult, many investigators have attempted to circumvent this problem by including inhibitors of insulin degradation in the incubation medium. Bacitracin has been the agent most often used, since it is an effective inhibitor of isolated enzymes that degrade insulin.[4–6] The presumption behind its use, however, is that this inhibitor does not enter the cell and does not alter cellular insulin degradation.

In the present study, we have tested this hypothesis using an isolated hepatocyte preparation that has negligible extracellular insulin-degrading activity. With this cell preparation, bacitracin produced a dose-dependent inhibition of cell-mediated degradation and, furthermore, was found to alter the pattern of intracellular degradation products of insulin.

MATERIALS AND METHODS

Hepatocytes were isolated by a modification[7] of the method described by Terris and Steiner.[1] All isolation and incubation steps were performed using Krebs Improved Ringer II buffer containing 27.7 mM glucose, with the exception that the initial perfusate did not contain calcium. After isolation, cells were preincubated for 30 min in buffer containing 3% bovine albumin (BSA, Miles, fraction V, Pentex) before the start of an experiment, at which time they were harvested by centrifugation and resuspended in fresh BSA-containing buffer at a concentration of 10^6 cells/ml. Cell viability, as judged by trypan blue exclusion, was 94–97% at the start of an ex-

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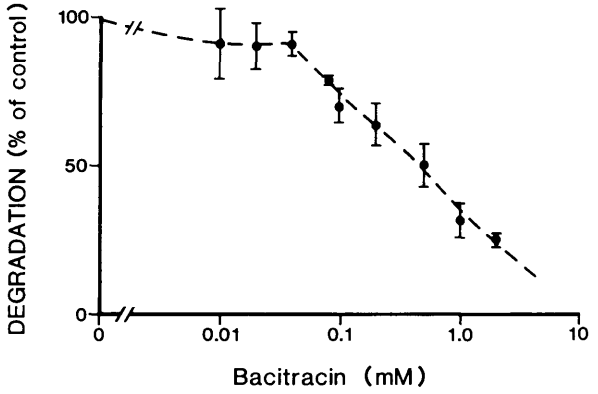

FIGURE 1. Dose-response effect of bacitracin on insulin degradation by isolated rat hepatocytes. Isolated hepatocytes were incubated with ^{125}I-(A14)-iodoinsulin and the indicated concentration of bacitracin at 37°C for 30 min. Insulin binding and degradation were then determined as described in the text. Results shown are the mean ± SEM from three experiments.

periment and decreased by only 1–2% during 30 min of incubation. All additions to the cells (bacitracin or ^{125}I-(A14)-insulin) were dissolved or diluted in incubation buffer and aliquoted into the appropriate flasks immediately before the start of the experimental incubation period.

In all studies to be described, cells were continuously incubated with specifically labeled ^{125}I-(A14)-iodoinsulin[8] for the indicated period of time. Insulin binding and degradation was determined as described previously.[7] In preliminary experiments, the extent to which leakage of insulin-degrading activity into the incubation media might contribute to overall insulin degradation was evaluated. Based on an increase in the solubility of the ^{125}I-(A14)-iodoinsulin tracer material in 10% trichloroacetic acid (TCA), the rate of degradation of ^{125}I-labeled insulin in control cells was 33.6 ± 1.7%/30 min of incubation (N = 3). Degradation was almost totally cell mediated, since incubation buffer taken from control cells incubated for 30 min degraded only 1.95 ± 0.7% (N = 3) of the ^{125}I-iodoinsulin that was added during a subsequent 30-min incubation. Using the more sensitive method of HPLC analysis, degradation of ^{125}I-insulin by buffer taken from control cells was only 2.4% in a 30-min incubation. Thus, cellular release of insulin-degrading activity did not contribute significantly to overall insulin degradation in our system.

For the extraction of cell-bound insulin, aliquots (600 μl) of the cell suspension were taken and were overlaid on gradients in 1.5-ml microfuge tubes consisting of 750 μl of silicone oil (density = 1.02) on top of 100 μl of extraction mix. The extraction mix contained 7 M urea, 3 M acetic acid, 0.2% (vol/vol) Triton X-100, and 100 μg/ml unlabeled pork insulin as carrier. The tubes were carefully loaded into a Beckman Model B microfuge so as not to disturb the gradient and were spun for 1 min. Three phases resulted, consisting of cell-free buffer on top, silicone oil in the middle, and a lower phase of cell extract and pelleted cell debris. An aliquot of the cell-free buffer was removed and transferred to a tube held at the temperature of liquid nitrogen. The microfuge tube was then frozen in liquid nitrogen and severed just above the extraction mix-oil interface. The tube tips were collected and held at the temperature of liquid nitrogen until all samples had been processed. Additional extraction mix was added to both the buffer and tube tips in an amount equivalent to the total fluid volume of each. As a control for possible bacitracin effects that might occur during sample processing, bacitracin was added to one set of extracts from control cells at this point in the extraction process. In another control, ^{125}I-(A14)-iodoinsulin was added at this time to an extract from cells incubated in the absence of any added tracer. All samples were allowed to thaw and were shaken in a reciprocating shaker at 170 cycles/min at 6°C for 18 h. Samples were then centrifuged at 2000 × *g* for 10 min at 4°C to remove debris and an aliquot of the supernatant fluid was applied to a 0.9 × 55-cm column of Sephadex G-50 equilibrated with 1 M acetic acid. The column was eluted with 1 M acetic acid and 1-ml fractions were collected in BSA-coated polypropylene tubes. Based on the elution profile of radioactivity, fractions containing "insulin-sized" material were pooled and lyophilized. The lyophilized material from the Sephadex G-50 step was dissolved in 0.2 M ammonium phosphate, pH 4.0, and 250 μl was applied (50–100,000 cpm) to a reversed-phase, high-performance liquid chromatography (HPLC) column. Another aliquot was directly counted for the purpose of estimating recoveries, and the relative degradation of the sample was assessed by determination of its solubility in 10% TCA. Details of the HPLC elution conditions are presented elsewhere.[9] Briefly, the system consisted of a DuPont Zorbax C-8 column (4.6 mm × 25 cm; 6 μm particle diameter) maintained at 40°C. Samples were eluted with a buffer/acetonitrile system by a series of isocratic and gradient steps in which the acetonitrile concentration was increased from 23.75% (vol/vol) to 35% over a 70-min period. The buffer component of the elution solvents was 0.2 M ammonium phosphate, pH 4.0, for all steps. One-half-milliliter fractions were collected and were counted in a Tracor Analytic 1285 gamma counter at an efficiency of 85%. Recovery of radioactive materials from the HPLC column was typically 95–100% and the sample applied to the HPLC column was usually 2–3% TCA soluble.

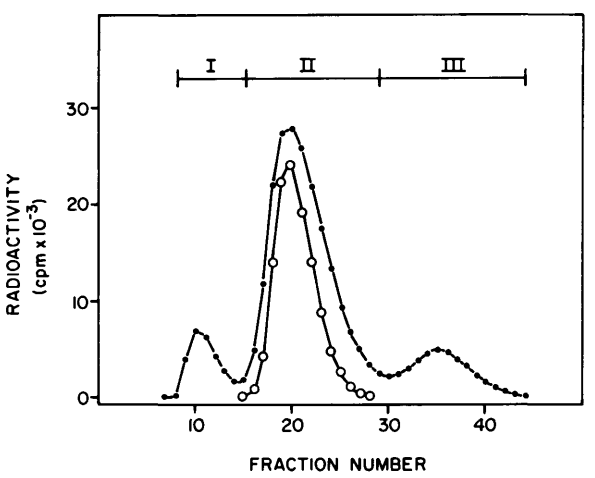

FIGURE 2. Sephadex G-50 elution profile of cell-bound radioactivity extracted from isolated hepatocytes. Cells were incubated with ^{125}I-iodoinsulin either alone or in the presence of 0.5 mM bacitracin for 30 min. The cell-bound radioactivity was extracted and subjected to Sephadex G-50 molecular sieve chromatography as described in the text. A representative elution profile of radioactivity from control cells (●---●) is shown, as is the elution profile of intact ^{125}I-(A14)-iodoinsulin (○---○).

TABLE 1
Distribution of extracted cell-bound or media radioactivity by Sephadex G-50 molecular sieve chromatography (% of total radioactivity)

	Cell extract			Media extract		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
Control cells	12.7 ± 1.6	76.6 ± 3.1	10.6 ± 1.5	5.1 ± 0.4	76.8 ± 4.8	18.1 ± 4.4
Bacitracin added after incubation*	9.0 ± 1.7†	78.7 ± 3.2	12.3 ± 1.6	5.0 ± 0.5	75.2 ± 5.7	19.8 ± 5.7
Cells incubated with bacitracin	10.8 ± 1.3	83.0 ± 2.1†	5.9 ± 0.7†	4.3 ± 0.5	85.1 ± 3.4†	10.7 ± 3.0†

Isolated hepatocytes were incubated for 30 min at 37°C with ^{125}I-(A14)-iodoinsulin in either the absence or presence of bacitracin (0.05 mM). Cells and incubation media were extracted and applied to a Sephadex G-50 column as described in the text. Peak 1, peak 2, and peak 3 refer to the radioactivity contained in fractions 9–14, 15–29, and 30–42, respectively, as shown in Figure 2. Results shown are the mean ± SEM from three separate experiments.
*Refers to control cells incubated with ^{125}I-labeled insulin alone, to which bacitracin (0.5 mM) was added to both cells and media at the initial step of extraction.
†Significantly different from control values ($P < 0.05$) as determined by ANOVA and Student-Newman-Kuels multiple range test.

RESULTS

Figure 1 shows the effect of increasing concentrations of bacitracin on ^{125}I-iodoinsulin degradation by isolated hepatocytes. A concentration-dependent effect on degradation was observed, with significant inhibition at a concentration as low as 0.04 mM. A concentration-dependent effect on ^{125}I-iodoinsulin binding (cell-associated radioactivity) was also observed, with 0.2 mM or higher producing a 50% increase in binding (data not shown). Since this hepatocyte preparation had negligible extracellular degrading activity (see above), these results implied an effect of bacitracin on cell-mediated insulin degradation.

We, therefore, examined possible bacitracin effects on intracellular insulin degradation using HPLC. Cells were incubated for 30 min at 37°C in the absence and presence of bacitracin (0.5 mM). Samples were extracted and subjected to molecular sieve chromatography as described under MATERIALS AND METHODS. As previously described,[10] radioactivity eluted from the Sephadex columns in three peaks, a high-molecular-weight component in the void volume, a peak with the elution volume of insulin, and a peak of low-molecular-weight materials eluting in the salt peak (Figure 2). The distribution of radioactivity in the three peaks is shown in Table 1. Essentially all of the radioactivity (99%) of the insulin not exposed to cells eluted in the insulin peak, demonstrating the quality of the tracer.[8] Cells incubated with ^{125}I-iodoinsulin in the presence of bacitracin had a significant decrease in low-molecular-weight products and an increase in insulin-sized material both in the medium and in the material extracted from the cells as compared with control cells ($P < 0.01$), again consistent with an effect of bacitracin on cell-mediated degradation. Addition of bacitracin to extracts from control cells resulted in a slight, but statistically significant, decrease in the amount of radioactivity eluting as peak 1, the high-molecular-weight peak. The reason for this effect is not known.

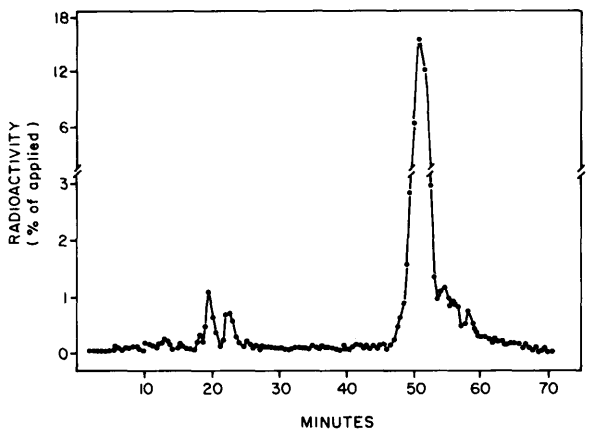

FIGURE 3. High-performance liquid chromatographic analysis of insulin-sized cell-bound radioactivity extracted from control hepatocytes. Sephadex G-50 column fractions containing "insulin-sized" material (fractions 15–29 in Figure 2) were pooled, lyophilized, and then dissolved in 0.5 ml of HPLC solvent buffer. One-half of the sample was applied via a 250-μl sample injector loop onto a C-8 reversed-phase column. Results shown are from a representative sample obtained from a control cell incubation. For reference, unlabeled pork insulin and intact ^{125}I-(A14)-iodoinsulin elute at 33 and 51 min, respectively.

The radioactivity in each "insulin" peak (peak 2 of Figure 2) was pooled, lyophilized, and analyzed by HPLC as described above. Figure 3 shows the elution pattern of insulin-sized material extracted from control cells. Most of the radioactivity eluted at 51 min in the position of unaltered ^{125}I-(A14)-iodoinsulin, but two additional peaks, eluting at 19 and 23 min, are evident. The distribution of radioactivity in these two product peaks was significantly altered ($P < 0.01$) by incubation of cells with bacitracin (Table 2). A significant decrease in the amount of product eluting at 19 min (peak

TABLE 2
Distribution of Sephadex G-50 peak 2 radioactivity on HPLC

	Percent of total radioactivity		Ratio peaks A/B
	Peak A	Peak B	
Control cells	3.02 ± 0.21	3.30 ± 0.33	0.92
Bacitracin added after incubation	3.66 ± 0.07	3.42 ± 0.11	1.07
Cells incubated with bacitracin	1.76 ± 0.39*	2.94 ± 0.37	0.60*

Fractions comprising the "insulin-sized" material eluting from a Sephadex G-50 column (see Table 1 and Figure 2) were pooled and subsequently applied to a C-8 reversed-phase HPLC column. Results shown are the mean ± SEM from three separate experiments. Peak A and peak B refer to the material eluting from the HPLC column at 19 and 23 min, respectively (see Figure 3).
*Significantly different from control cells ($P < 0.05$).

TABLE 3
Relative distribution of insulin degradation products eluting from Sephadex G-50 (total cpm)

	Sephadex G-50 pool		
	Front	Middle	Back
HPLC peak			
Peak A	1431	4750	2550
Peak B	1834	3830	1757
Ratio (peaks A/B)	0.78	1.24	1.45

Control cells were incubated with ^{125}I-labeled insulin for 30 min at 37°C. A cell extract was prepared and applied to a Sephadex G-50 column as described in the text. The peak of "insulin-sized" material (peak 2, Figure 2) was divided into thirds, designated as front (fractions 15–18, Figure 2), middle (fractions 19–22, Figure 2), and back (fractions 23–29, Figure 2). Each pool was analyzed by HPLC and the radioactivity eluting as peak A (19 min) or peak B (23 min) in each pool (front, middle, or back) is shown.

A) was observed in cells incubated with bacitracin. No alteration was detected in the HPLC pattern of insulin-sized products from the media (data not shown), further suggesting that bacitracin alters intracellular pathways of insulin metabolism.

Misbin and Almira[11] previously reported finding an insulin degradation product that eluted slightly ahead of intact insulin by molecular sieve chromatography. To evaluate the relative sizes of the two products that we observed, experiments were performed in which the insulin-sized material eluting from the G-50 column was divided and pooled approximately in thirds, corresponding to fractions 15–18, 19–22, and 23–29 in Figure 2. As shown in Table 3, the relative distribution of the two products between the three subfractions differed. HPLC peak B eluted from the G-50 column slightly ahead of peak A. However, when these two peaks from the HPLC were pooled and rechromatographed on Sephadex G-50, a single, nearly symmetrical peak was obtained (Figure 4), indicating the differences in size of these materials to be relatively minor. Similar observations have previously been made with insulin degradation intermediates from cultured fibroblasts.[12] A subsequent HPLC analysis of the insulin peak shown in Figure 4 again yielded two peaks of radioactivity eluting at 19 and 23 min (data not shown), indicating that the inability to resolve these two products on the second G-50 column was not due to their deterioration after the initial HPLC separation.

DISCUSSION

Results of the present study indicate that bacitracin has significant effects on cellular insulin degradation. The hepatocyte preparation used in the present study has essentially no insulin-degrading activity in the medium; thus insulin degradation is primarily due to the interaction of insulin with the cell. Bacitracin produced a dose-dependent inhibition of insulin degradation by these cells, suggesting that this inhibitor does in fact alter cellular insulin degradation.

By Sephadex G-50 chromatography, the cells incubated with bacitracin had lesser amounts of labeled material eluting in the salt peak, indicating reduced degradation to low-molecular-weight products. The labeled material eluting in the same area as intact insulin was further examined by HPLC. Several studies have shown that not all of the insulin-sized material eluting from Sephadex G-50 after exposure of the hormone to either purified insulin-degrading enzymes or to cells is intact insulin.[12–15] Thus, assessment of insulin degradation based on its elution profile from a molecular sieve column or its solubility in trichloroacetic acid tends to underestimate the amount of non-intact hormone. By HPLC analysis of the "insulin" peak, we were able to demonstrate two products that eluted differently from ^{125}I-(A14)-iodoinsulin. Stentz et al.[12] recently reported a similar result using HPLC separation of labeled material extracted from fibroblasts. Of interest was our finding that the relative proportions of the two products was different in cells incubated with bacitracin as compared with cells incubated without bacitracin, further supporting the suggestion that bacitracin has effects on cellular processing and degradation of insulin. Although bacitracin has been shown to inhibit the activity of isolated insulin-degrading enzymes,[4–6] we currently have no evidence that the bacitracin effect on the pattern of insulin degradation products is a direct result of this inhibition.

By subfractionating the peak of insulin-sized material eluting from the G-50 column and analyzing each pool by HPLC, it was evident that peak A was eluted from G-50 slightly after peak B. Although the possibility of differential adsorption of the fragments to the gel cannot be excluded, the likely explanation for these results is that peak A is smaller than peak B. Whether a precursor-product relationship exists between these two degradation products has yet to be determined in our system, although Stentz et al.[12] provided evidence consistent with such a relationship in their fibroblast system. A number of other investigators have also reported insulin degradation products of a size similar to that of intact insulin.[13–15] Due to methodologic differences, it is not possible to determine at present if peaks A or B in our system correspond to the degradation products reported by others. Neither peak A nor B appears to be isolated insulin A-chain,

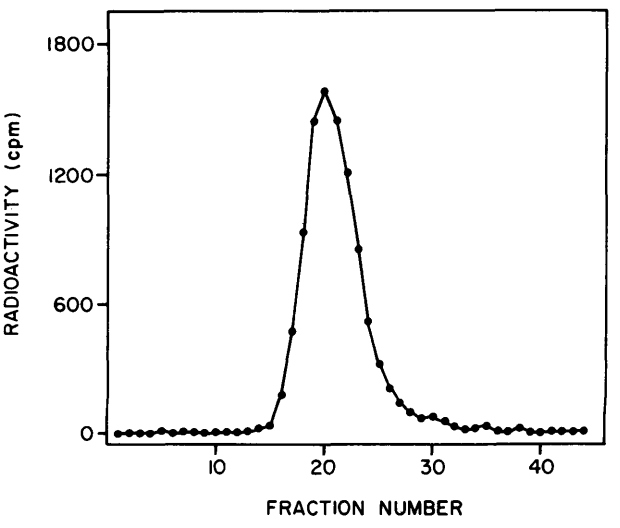

FIGURE 4. Sephadex G-50 elution profile of post-HPLC insulin degradation products. Peak tubes from the products eluting from HPLC at 19 or 23 min (see Figure 3) were individually pooled and lyophilized. Each sample was redissolved in 1 ml of 1 M acetic acid and the two samples then combined and applied to a column of Sephadex G-50. As described in the text, the column was eluted with 1 M acetic acid and the radioactivity contained in each 1-ml fraction was determined by gamma scintillation spectrometry.

however, since reduced A-chain elutes considerably after intact insulin in our system (F. G. Hamel, unpublished observations).

Bacitracin has been reported to have multiple effects on cells, including an alteration in binding of α_2-macroglobulin,[16,17] direct effects on lipolysis in isolated adipocytes,[18] and oxidation of pyruvate in hepatocytes.[19] In addition, several studies have suggested that bacitracin may have effects on cellular insulin degradation. Using primary cultures of hepatocytes, which had no insulin-degrading activity in the media, Duckworth et al.[10] found a significant effect of bacitracin on insulin degradation, as did Juul and Jones.[20] Recently, Bonser et al.[21] reported that bacitracin inhibited ^{125}I-insulin internalization and degradation by hepatocytes and recommended that the use of bacitracin in receptor-ligand studies be discontinued. We would support this conclusion and emphasize that the effects of bacitracin on cells may be multiple. Not only does it alter internalization but it also affects intracellular insulin metabolism, as reflected by significant alterations in the relative proportions of intracellular degradation products.

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