Communication

Insulin or a Closely Related Molecule Is Native to Escherichia coli*

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Evidence is presented for the presence in Escherichia coli of material very similar to insulin with regard to specific reactivity in the insulin radioimmunoassay and in the insulin bioassay.

Insulin which is found in large amounts in the β-cells of the pancreas in vertebrates has also been shown to be present in the most primitive of vertebrates and in complex invertebrates, which are evolutionarily about 500 million years old (see Refs. 1–8 of Ref. 1). We reported evidence for the existence of material extremely similar to insulin in three unicellular eukaryotes, viz. Tetrahymena pyriformis, a ciliated protozoon, and two fungi, Neurospora crassa and Aspergillus fumigatus (1). This finding suggested that the insulin molecule may have had its origin about 1 billion years ago.

In this study, we present evidence for the presence of similar material with both immunological and biological similarities to mammalian (or hagfish) insulin in a prokaryote, Escherichia coli K12, which suggests that insulin may have origins even earlier than previously thought, namely 2 billion years ago (2).

METHODS

Organisms—E. coli K12, purchased in powder form from the American Type Tissue Culture Collection (Rockville, MD), were maintained on nutrient broth slants (the nutrient broth had no detectable insulin by radioimmunoassay). Colonies were transferred to defined medium (Vogel’s Bonner cell medium) which contained citric acid, glucose, and salts, with no addition of serum or macromolecules. The cells were grown overnight in large volume fermenters at 37°C and harvested by centrifugation in a Sharples centrifuge. To exclude a possible contamination of the bacteria with insulin during the growth of the organisms, a minute aliquot of the starting E. coli was taken to another laboratory that processes only bacterial materials without vertebrate materials; bacteria grown and processed in this vertebrate- and insulin-free environment (Batch 4, Table I) gave similar results.

Extraction—The cell paste was weighed and homogenized using a Manton Gaulin homogenizer. Fifty liters of conditioned medium (Batch 3, Table I) were concentrated to 500 ml by evaporation. The homogenate or concentrated conditioned medium was then extracted and concentrated using a classic acid ethanol extraction procedure for extracting insulin from pancreas (1, 3). The reconstituted extract was applied to a column of Sephadex G-50 (fine) and eluted with 0.05 M (NH4)2CO3. Each effluent fraction was lyophilized and reconstituted in 1 ml of distilled water.

Radioimmunoassay—The insulin content of each fraction was determined by radioimmunoassay at 1:10 (final) dilution of the sample. Porcine insulin (purchased from Eli Lilly) and guinea pig anti-porcine insulin serum: (“first antibody”, Batch 619, purchased from the Department of Pharmacology, Indiana University, Indianapolis) were used in the radioimmunoassay. The insulin radioimmunoassay was performed by standard methods using 125I-labeled porcine insulin as the tracer and rabbit anti-guinea pig IgG as the “second antibody” (4, 5).

Exclusion of Nonspecificity in the Radioimmunoassay—To exclude the possibility that the gel-filtered extracts of E. coli contained substances that interfered in a nonspecific manner in the double antibody radioimmunoassay, we performed the following experiments. In the first experiment (I), the supernatants from the immunoassay (after removal of the immune precipitates) were reacted with trichloroacetic acid at a final concentration of 5%; 92% or more of the radioactivity was precipitated irrespective of whether extract had been present. In the second experiment (II), 125I-insulin was incubated for 3 days under conditions of the immunoassay in the presence and absence of extract and then filtered on Sephadex G-50; the profile of the radioactivity was the same irrespective of whether tracer had been exposed to extract. In Experiment III, 125I-insulin was incubated with or without extract under conditions of the assay for 3 days. Just before addition of the second antibody, an excess of anti-insulin antibody was added (under conditions such that the final concentration of guinea pig γ-globulin was effectively unlabeled); 90% or more of the radioactivity was precipitated irrespective of whether extract had been present. In Experiment IV, extract was incubated with a high concentration of 125I-insulin under conditions of the assay for 3 days. The mixture was then diluted (sufficiently so that its insulin content was negligible, i.e. true trace) and used in the radioimmunoassay. 125I-Insulin so exposed was indistinguishable from 125I-insulin incubated without extract. In Experiment V, extract was added to the immunoassay for human growth hormone, which utilizes guinea pig antibody to human growth hormone with the same carrier guinea pig γ-globulin and rabbit anti-guinea pig globulin used in the insulin assay; little or no effect of extract was observed.

If the extract were acting on the rabbit anti-guinea pig globulin (second antibody) to reduce its ability to bind to guinea pig γ-globulin or form precipitates, this effect would have been uncovered in Experiments III and V. If the extract were acting on the guinea pig γ-globulin to reduce its ability to interact with or be precipitated by second antibody or if the extract were acting on guinea pig γ-globulin to destroy the antigen binding sites (i.e. insulin or hormone binding sites), the effects would have been uncovered in Experiments III and V. If the extracts grossly altered the 125I-insulin (and thereby prevented it from interacting fully with first antibody), this would have been detected in Experiment III. Putative low molecular weight binding materials (that bind to 125I-insulin but do not change the migration of 125I-insulin on Sephadex G-50), while not detectable in Experiment II, were excluded by previous gel filtration and dialysis used to prepare the extracts. If the extract contained material that did not change the overall features of the 125I-insulin but simply destroyed its affinity (i.e. K = 0) for first antibody, this would have been picked up in Experiment III. The only remaining possibility is that the extract caused a reduction in (but not total loss of) the affinity of 125I-insulin for antibody. This possibility, while not entirely excluded by Experiment III, is partially excluded by Experiment IV. In summary, we have excluded with reasonable certainty the whole range of possible “nonspecific” effects in the assay.

Bioassay—Following gel filtration, the fractions corresponding to the peak of insulin immunoreactivity were pooled, lyophilized, and reconstituted in distilled water. The reconstituted pool was then tested for bioactivity and reprecipitated for immunoreactivity. The biological activity was measured as lipogenesis, i.e. incorporation of [3-14C]glucose into tolune-extractable lipids (6), by isolated fat cells.
prepared from epididymal fat pads of young Sprague-Dawley rats (7). To show that the bioactive molecules were reactive with anti-insulin antibody, duplicate aliquots were mixed with a 1:100 dilution of guinea pig anti-porcine insulin serum 619 or normal guinea pig serum before their addition to the bioassay. Normal guinea pig serum at this concentration had no effect on the bioassay.

RESULTS

When acid ethanol extracts of E. coli K12 were gel filtered on Sephadex G-50 (fine), a peak of insulin immunoreactivity was found in the region typical for insulin (Fig. 1, inset). On serial dilution over a 6-fold range, the gel-filtered material was very similar to the porc insulin standard (Fig. 1A). About 40 pg/g, wet weight, were recovered from the pooled peak fractions, measured both by radioimmunoassay and by bioassay. The bioactivity was largely neutralized in the presence of anti-insulin antibody (Fig. 1B, Table I). The conditioned medium in which the E. coli had been grown also contained insulin that was very similar and in approximately equal amounts to that isolated from the bacteria themselves (Table I, Batch 3), as previously demonstrated with *T. pyriformis* (1). It should be noted that the medium in which the bacteria were grown was composed only of citric acid, salts, and glucose with no serum or other macromolecules; when (*Tetrahymena*) medium was carried through the entire procedure and extracted, no insulin was detected (1).2

![Graph A](image1.png)

**Fig. 1. E. coli Sephadex G-50 extract.** *A* (inset), gel filtration. The extract was filtered on a column of Sephadex G-50 (fine; 1.5 × 60 cm; 1-ml fractions) and the immuno-reactive insulin measured at a 1:10 final dilution in the assay. The horizontal line, equivalent to 0.25 ng of insulin, indicates the sensitivity of the assay and the fractions that were tested. The arrow at the left indicates the void volume (214 ml-thyroglobulin) and the arrow at the right marks the salt peak (16 ml). Peak fractions from the gel filtration column were pooled, lyophilized, and reconstituted in distilled water, and then tested over a 6-fold dilution in the immunoassay (×). *B*, the reconstituted peak fractions were tested for biological activity by measuring the incorporation of [3H]glucose into toluene-extractable lipids in isolated rat adipocytes. The [3H]lipid produced is plotted as a fraction of the insulin concentration for the porcine insulin standard (dark solid line) or equivalent amount of *E. coli* extract (△) (determined by radioimmunoassay (RIA) against a pork insulin standard). Neutralization of this bioactivity was tested by measuring bioactivity of the sample assayed in the presence of anti-porcine insulin antiserum (△).

| Batch | Wet weight | Pooled fractions (RIA) | Pooled fractions (Bioassay) | Antibody neutralization % decrease |
|-------|------------|------------------------|-----------------------------|----------------------------------|
| 1. Cells | 52 | 4.0 | 3.5 | 2.5 | 90 |
| 2. Cells | 195 | 9.3 | 5.0 | 5.8 | 85 |
| 3. Cells | 54 | 5.0 | 3.0 | 3.0 | 90 |
| Conditioned medium (50 liters) | 3.2 | 2.2 | 2.0 | 60 |
| 4. Cells | 300 | 2.5 | |

In addition to the typical insulin peak, there is a distinct peak of immunoreactive material (Fig. 1B, Table I) that elutes slightly later than insulin. The nature of this material is unknown and we have not characterized it further. We have observed it previously in variable proportions in extracts of other unicellular organisms as well as extracts of extrapancreatic tissues of mammals. To our knowledge, this position does not correspond to a known insulin derivative that reacts in this radioimmunoassay, but could represent a modification of insulin, produced by the cell during the extraction procedure.
Insulin or a Closely Related Molecule Native to E. coli 6535

**FIG. 2. Specificity of the insulin bioassay.** To demonstrate the specificity of the effects in the bioassay, we studied two authentic insulins (guinea pig insulin, gift of C. C. Yip, and pork insulin), two extracts (E. coli and *Tetrahymena*), an insulin-like growth factor (multiplication stimulating activity (M.S.A.) purified from conditioned media of buffalo rat hepatoma, gift of M. Rechler), and two unrelated materials that produce insulin-like biological effects (vitamin B12 and spermine). Note that the amount of bioactivity in the *E. coli* and *Tetrahymena* extracts is predicted by their content of immunoreactive insulin, measured against a pork insulin standard. Furthermore, addition to the bioassay of antireceptor antibody (monovalent F(ab')2' derived by papain digestion of autoantibodies directed against the insulin receptor from patients with Type B extreme insulin resistance) blocked the bioactivity of the two extracts (95% neutralization), the two insulins (90-95%), and multiplication stimulating activity (75-100%) but had no effect on the bioactivity of vitamin B12 and spermine, which are thought to stimulate the rat adipocytes at steps beyond the insulin binding site of the receptor. Anti-porcine insulin antibody blocked the effect of porcine insulin (100% neutralization) and of the two extracts (95%) but not that of the other materials (which are known to lack reactivity with antipork insulin antibodies). Finally, as expected, rabbit anti-guinea pig insulin antibody neutralized only guinea pig insulin (75% neutralization). The data for the *E. coli* extract are the same as those in Fig. 1 and for the *Tetrahymena* extract as in Ref. 1.

and that most of the bioactivity was exercised by a molecular species recognized by anti-porcine insulin antibodies but not by antibodies against guinea pig insulin. The concentration of insulin in *E. coli* (40 pg/g, wet weight) assumes that *E. coli* insulin is as reactive as pork insulin in our assay and that we recover 100% of the insulin present in the cell. While insulin at this level is equivalent to 0.1 (or more) of the concentration of insulin in the plasma of fasting adults, it represents only 1 insulin molecule/100 *E. coli* cells because these bacteria are so small. However, if we assume that *E. coli* insulin is only as reactive in our assays as is the insulin of a primitive vertebrate (hagfish), the values for *E. coli* should be multiplied by 40-fold. If recovery of insulin from the *E. coli* is 3%, as we have shown for *Tetrahymena*, then values for *E. coli* need to be multiplied again by 30. If concentrations of the hormone (picograms/g) fluctuate (as we have recently found for another peptide in *Tetrahymena* during growth), then values may need to be multiplied again by 5-10-fold. Overall, the molar concentration of insulin in *E. coli* may be up to 1,000-10,000-fold higher than reported here.

**DISCUSSION**

Since its introduction in 1960 (4), the radioimmunoassay for insulin has been extensively characterized and its specificity shown to be restricted to insulin. In order to react in this radioimmunoassay, a substance must contain the A and B chains of the insulin molecule joined by disulfide bridges (9, 10). However, amino acid homology is also very important. The insulin-like growth factors (I and II) and guinea pig insulin have the three characteristic disulfide bridges, have A and B chains that have 50-65% amino acid homology with those of pork insulin, and produce insulin-like bioeffects through the insulin receptor, yet are virtually nonreactive in this radioimmunoassay (11, 12). Likewise, fish insulin with 70% homology to pork insulin reacts with reduced affinity in this radioimmunoassay (9). Since the material in our *E. coli* extracts reacts specifically in the radioimmunoassay (experiments to rule out nonspecific effects are described under "Methods"), we conclude that the material probably has amino acid homology similar to mammalian insulins, at least in the immunoreactive region of the molecule.

Since the immunoreactivity in our extracts was recovered on gel filtration in the same place as insulin, we suggest that the material in our extracts of *E. coli* has a size-shape similarity to insulin and is unlikely to be proinsulin. The reactivity of this material in the bioassay and neutralization of this reactivity by the specific receptor blocking antibody suggests that in addition to having that region of the molecule which reacts in the radioimmunoassay, it has the additional regions which are needed for bioactivity, i.e. the region necessary for binding to the insulin receptor (i.e. affinity), and the region necessary for activation of the metabolic pathway (i.e. intrinsic activity). That the bioactivity was neutralized by anti-insulin antibody indicates to us that the bioactive and immunoreactive regions are present on the same molecule. In addition, the neutralization excluded the possibility that the effect was that of an insulin-like growth factor, somatomedin, or other material that can produce insulin-like bioeffects but which lacks the sites that interact with anti-insulin antibody. That the bioactivity/immunoactivity ratio of the material in *E. coli* is close to unity suggests that it is functionally (and structurally) very closely related to the common mammalian insulins such as pork, beef, human, rat, and mouse. Note that chicken insulin, turkey insulin, and fish insulins have bio/immunoactivity substantially greater than one, whereas proinsulin and other insulin precursors typically have bio/immunoactivity substantially less than one, and the insulin-like growth factors and other somatomedins (as well as guinea pig insulin) all have no reactivity in the immun assay, despite their insulin bioactivity which is exercised through the insulin receptor.

The most primitive vertebrate insulin (hagfish insulin) that has been characterized has a bio/immunoactivity ratio of close to unity; both its bioactivity and its reactivity in the immunnoassay for pork insulin are markedly reduced (13). Thus, we cannot distinguish whether the insulin in our extracts of unicellular prokaryotes and eukaryotes is closer to hagfish insulin or to a mammalian insulin, because both have bioimmunoactivity ratios of unity. This possibility of poor cross-reactivity may in addition explain our seemingly low levels of insulin in unicellular organisms. In addition, since the bacteria in our culture are not homogeneous, we are unable to ascertain whether each individual cell is making insulin.

In addition to demonstrating insulin in unicellular organisms, we have also shown the presence of similar amounts of insulin in the conditioned medium in which the cells were grown. This suggests that the cells are capable of both synthesis and release of insulin, although we have not excluded the possibility that the insulin is released by cell lysis rather than by a biologically meaningful secretory process.

The present finding of material that is very similar to insulin in prokaryotes extends the finding of human chorionic gonadotropic-like material in other bacteria (14), as well as our findings of insulin, adrenocorticotropic hormone, and somato-
Insulin or a Closely Related Molecule Native to E. coli

statin-like material in Tetrahymena. This confirms our earlier suggestion that many extrapancreatic (both neural and non-neural) tissues of mammals are capable of making insulin (15).

Although we have not demonstrated a function for these peptides as messenger signals in the unicellular organisms, there are a number of reasons that strongly suggest that these peptides do in fact function at this level: (a) the very strong conservation of the biologically important regions of the molecule; (b) the known effects of vertebrate hormones (e.g. opiates, catecholamines) in protozoa, that is inhibited by specific receptor blocking agents; the in vivo and in vitro effects of mammalian insulin on E. coli (18); and (c) the existence of humoral signalling mechanisms for cell-cell communication by slime mold, yeast, and Myxobacteria (19-21).

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