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THE MITOGENIC ACTIVITY OF INSULIN: An intrinsic property of the molecule

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SUMMARY

Higher than physiological concentrations of insulin stimulate the incorporation of [^{3}H]thymidine into DNA in a large variety of cells in culture. In order to exclude a contaminant of insulin preparations as responsible for this mitogenic action, porcine insulin was purified by reverse phase HPLC and assayed for mitogenic activity. HPLC- purified insulin had a mitogenic activity similar to that of impure insulin. Moreover, semisynthetic and synthetic insulins as well as HPLC-purified insulin derivatives such as monodesamido- and monoarginine insulin were also biologically active.

INTRODUCTION

Since the pioneering study of Gey and Thalhimmer in 1924 (1) it is known that the addition of insulin preparations to cells in culture enhances their growth. Although the principal role of insulin is the regulation of the distribution and utilization of metabolic fuels within the animal body, many studies have indicated that this peptide also stimulates the proliferation of a wide spectrum of cultured cells (2-11). Though several types of cells [human skin fibroblasts, chick embryo fibroblasts, fibroblast-like SWISS or BALB 3T3 cells, GH3, HeLa or neuroblastoma cells] and media under a variety of experimental conditions have been used in these investigations, a common result has been that only pharmacological doses of insulin preparations [usually 1 µg/ml] exhibited mitogenic activity and that these doses gave only small effects if compared to those obtained with other growth promoting agents such as epidermal or fibroblast growth factor (9, 12). Because higher than physiological concentrations of insulin are required the question has been raised whether the growth promoting

Abbreviations: DNA, desoxyribonucleic acid; RNA, ribonucleic acid; HPLC, High performance liquid chromatography; DMEM, Dulbecco-Vogt Modified Eagle's Medium; BSA, bovine serum albumin; RIA, radioimmunoassay; AUFS, Absorbance unit full scale; IGF, insulin-like growth factor, NSILA, non-suppressible insulin-like activity.

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effect of insulin preparations is due to a trace contaminant (11, 13) or is an intrinsic property of the insulin molecule.

It has been repeatedly emphasized since the studies by Harfenist and Craig (14) that commercial insulin preparations contain several biologically active contaminants such as glucagon (15) proinsulin (16) and pancreatic polypeptide (17), which in fact were first isolated and characterized from these preparations. It has been difficult, however, to obtain insulin preparations of very high purity with conventional chromatography because such techniques lack sufficient separation power. The insulin preparations of highest purity available so far are the so called mono - and single component insulins which are chromatographically and/or electrophoretically pure (18). Single component insulin has been shown to possess mitogenic activity in tissue culture studies (9).

With the use of high performance liquid chromatography (HPLC), a separation technique with much higher resolutive power and sensitivity than conventional chromatography it should be possible, to obtain insulin of better purity than presently available and to detect contaminants present in trace quantities. In this study we prepared HPLC-purified insulin and investigated whether the insulin molecule itself has an intrinsic mitogenic activity or a contaminant is responsible for this effect. We have also tested semisynthetic porcine and synthetic human insulin which are free of biological contaminants.

MATERIALS AND METHODS

Peptides: The following peptides were obtained as gifts: Bovine insulin lot. no. 615-D63-5, porcine insulin lot nos. 615-D63-10 and 615-1082B-108I (single component), monoarginine insulin lot. 615-D63-192-2, A-21-desamido-insulin lot. no. 615-1082B-75-3, zinc free sodium insulin lot. no. IDG-04-94-193 and porcine proinsulin lot. no. 615-D63-53 from Dr. Chance, Eli Lilly and Co., Indianapolis, IN U.S.A.; semisynthetic porcine insulin from Prof. H. Zahn, Aachen, Germany; human synthetic insulin from Drs. F. Märki and H.G. Scheibli, Ciba - Geigy, Basel, Switzerland.

Purification of peptides by high performance liquid chromatography: A Waters model 204 liquid chromatography system equipped with two M-6000A pumps, a 660 solvent programmer and a Schoeffel 770 variable UV detector was used. Separation was accomplished with a Supelcosil C18 column (15 X 0.4 cm, 5 µm particle size, Supelco, Bellafonte, PA). The mobile phase (flow rate 1.5ml/min) consisted of acetonitrile (glass distilled, Burdick's and Jackson, Muskegon, MI) in 0.25 M triethylammmonium phosphate pH 3.0 (19). Usually 500 µg of insulin preparation was subjected to HPLC. Peaks were identified by comparison with the retention times of insulin and known contaminants such as A-21-monodesamido- and B-31-monoarginine-insulin or proinsulin.

Desalting of peptides: For biological testing the purified insulins were desalted because of the possibility of buffer salt interference (20). For desalting the peptide solution was freed of organic solvent in vacuo and pumped through a C18 guard column (Brownlee Lab., Berkeley, CA) using the HPLC apparatus. The peptides which are retained on the column under these conditions were recovered after washing the column with 0.2 M acetic acid until the eluate was free of salt

by subsequent elution with 40% propanol in 0.2 M acetic acid. The propanol phase which contained the peptides was lyophilized.

Tissue cultures: Calf serum (control no. A981021) and Dulbecco-Vogt-modified Eagle's medium (DMEM) supplemented with 5.6 mM glucose/l (control no. 0782550) were purchased from Grand Island Biological Company, Grand Island, N.Y. Dexamethasone was from Merck, Sharp and Dohme, West Point, PA; bovine serum albumin (BSA, RIA grade lot no. 701841) from Calbiochem-Behring, San Diego, CA; glutamine from Nutritional Biochemicals Corporation, Cleveland, Ohio; fungizone from Squibb, Princeton, NJ; gentamycin from Schering, Kenilworth, N.J.; trypsin (1:250) from DIFCO Detroit, MN. Tritiated thymidine (specific activity 40-60 Ci/mol) was purchased from Amersham, Arlington Heights, IL. BALB 3T3 cells were a gift from Dr. Kenneth Brown, La Jolla, CA. Microtiter plates (96 wells) were obtained from Falcon, Oxnard, CA.

Assay of mitogenic activity: The incorporation of thymidine into BALB 3T3 cells was measured as described (21). Briefly, the cells were incubated in DMEM containing 5% calf serum, glutamine and antibiotics for 6-8 hours. Then the medium was changed to 0.2% serum containing 400 ng/ml dexamethasone. After 16-18 hours when the serum-starved cells became quiescent the peptides to be tested were added in DMEM containing 0.5% BSA. After 8-12 additional hours the cells were pulsed with 1 µCi [^{3}H]thymidine and 50 ng cold carrier in 10 µl DMEM for another 12-14 hours. The cells were harvested with cotton swabs and after trichloroacetic acid precipitation, the incorporated radioactivity was counted in a Tricarb liquid scintillation counter.

Statistical analysis: After analysis of variance differences between treatment groups were determined by the multiple tests of Dunnett and Duncan using the computer program EXBIOL (22).

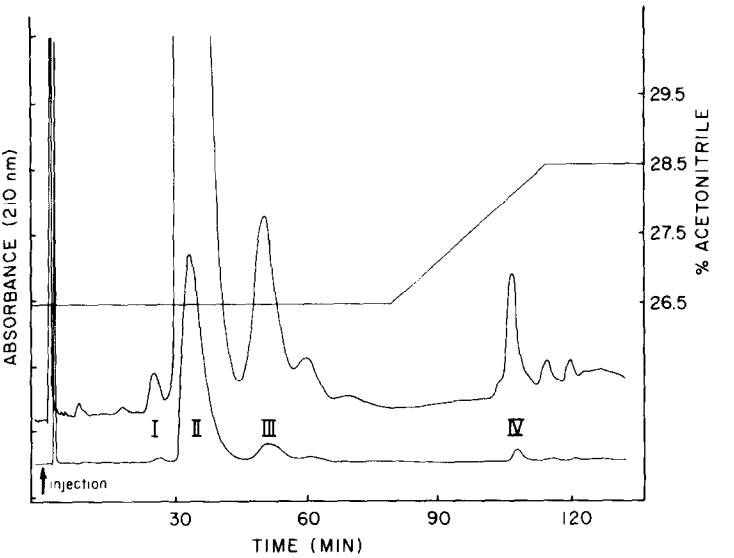

Figure 1: Reverse phase HPLC of 500 µg porcine single component insulin. Sensitivity: upper trace 0.02 AUFS, lower trace 0.2 AUFS (other conditions see Material and Methods) I: B-31 Monoarginine-Insulin; II: Insulin; III: A-21 Monodesamido-Insulin; IV: Proinsulin.

RESULTS

The insulin of highest purity so far tested in culture for mitogenic activity has been porcine single component insulin (9). Reverse phase HPLC of such a preparation indicated the presence of at least ten compounds (Figure 1). The main peaks had identical retention times as B-31-monoarginine-, A-21-monodesamido-insulin, insulin and proinsulin standards. B-31-monoarginine- and A-21-monodesamido-insulin differ from insulin by the additional presence of an arginine residue or the absence of an amino group respectively. Similar chromato grams were obtained with other insulin preparations such as porcine and bovine insulin preparations (data not shown).

The mitogenic activity of impure porcine insulin of HPLC-purified insulin and monodesamido insulin, as well as zinc free and semisynthetic porcine insulin is shown in figures 2 and 3. Zinc free insulin was tested because zinc is known to be an important cofactor for RNA- and DNA-biosynthesis and hence for cell replication (23) and most insulin preparations contain zinc as a constituent. Semisynthetic insulin (prepared by reoxidizing purified A- and B-chains obtained from insulin) was tested because it is unlikely that a biological contaminant of impure insulin would still be present after the steps involved in preparing this insulin. For comparison human synthetic insulin was also tested (figure 3). All these insulin preparations stimulated the incorporation of thymidine in a dose

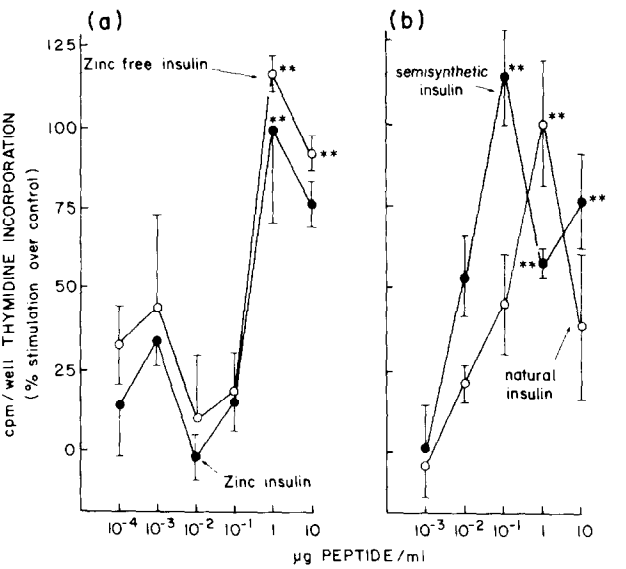

Figure 2: Dose response curves of the stimulation of DNA-synthesis in BALB-3T3 cells by (a) zinc containing and zinc free porcine insulin preparations and by (b) porcine natural insulin and semisynthetic porcine insulin [n = 3 or 6 (controls), values are mean ± SEM, ** $p < 0.01$, exp. 22762 and 23262].

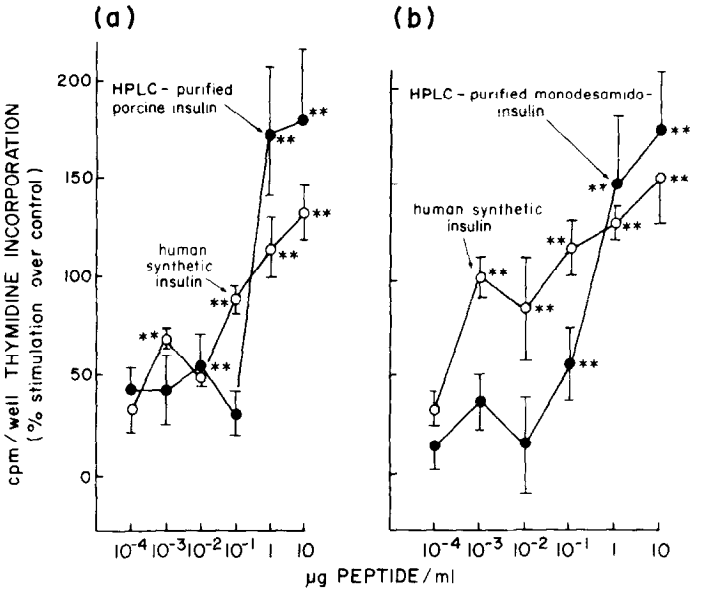

Figure 3: Dose response curves of stimulation of DNA-Synthesis in BALB 3T3 cells by (a) HPLC-purified porcine insulin and human synthetic insulin and by (b) HPLC-purified porcine monodesamido-insulin and human synthetic insulin [n = 4 or 9 (controls), values are mean ± SEM, ** $p < 0.01$, exp 23793 and 23794]. Note that the curves for human synthetic insulin in panels (c) and (b) are somewhat different. The data were obtained in two different experiments using cells at different passages.

dependent manner. With concentrations ranging from 100 pg/ml to 10 µg/ml a sigmoidal dose response curve was obtained. Whereas the maximal stimulation of thymidine incorporation by impure insulin was reached at concentrations of 1 to 10 µg/ml, purified and synthetic preparations were active at lower levels. Maximal thymidine incorporation was usually not more than a doubling or tripling of the control levels which is consistent with results reported by other investigators (2-11).

DISCUSSION

The hypothesis that the mitogenic activity of insulin preparations in tissue culture is due to a trace contaminant has been raised since the discovery of this activity. Various approaches such as the testing of insulin preparations purified by conventional chromatography (9), competitive binding studies with structurally related peptides (24) or the determination of the degradation rate of insulin in tissue culture (8), have been used in order to evaluate whether the activity is an intrinsic property of the insulin molecule. None of these experimental approaches has yielded conclusive results. We therefore decided to

approach the problem again by purifying insulin by means of high performance liquid chromatography and by testing synthetic insulin. Reverse phase HPLC is known to possess much higher resolutive power for the separation of peptides than other chromatographic techniques. This has been demonstrated for many closely related peptides (for a review see 25) and even small proteins such as neurophysins (26) or hemoglobin variants (27). With this technique is it is not only possible to completely separate known contaminants from insulin (monodesamido- and monoarginine insulin or proinsulin) but also a number of other unknown substances. We realize, however, that even with the best separation method the presence of a single peak is no proof of homogeneity. Yet in view of the high resolutive power of reverse phase HPLC for peptides it is unlikely that a contaminant causing the mitogenic activity is still co-chromatographing with insulin. Further evidence in support of the hypothesis that the mitogenic activity is intrinsic to insulin is the finding that both desamido- and monoarginine insulin (data for the latter not shown) even after purification by HPLC, show the same qualitative and quantitative activity as HPLC-purified insulin. A final and very strong indication of the intrinsic mitogenic activity of insulin preparations is the observation that semisynthetic porcine and synthetic human insulins indeed possess mitogenic activity. The synthetic human insulin used has been shown to be biologically equivalent to natural insulin in bioassays (28).

If the mitogenic activity of insulin is due to an intrinsic property of the molecule which only occurs at higher concentrations then this observation needs further explanation. Receptors for insulin have been described in 3T3 cells and other fibroblasts in tissue culture (29). In physiological concentrations insulin binds to these receptors and evokes classical metabolic effects such as the stimulation of glycolysis (30). At supraphysiological levels insulin may bind to the receptors of other structurally related peptides. Insulin-like growth factors (IGF, formerly called NSILA) are closely related to proinsulin and insulin (31). Evidence for a crossreactivity of IGF-receptors and insulin and vice versa has been presented (24). Moreover, the simultaneous addition of insulin and IGF only creates additive effects of these peptides (32), which we also have found in BALB 3T3 cells (data not shown).

A likely explanation for the mitogenic activity of insulin is its capability to crossreact with receptors for structurally related peptides (insulin-like growth factors) which are involved in the control of cell division.

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