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EFFECT OF INSULIN-DEPENDENT CYTOPLASMIC REGULATOR ON MURINE MELANOMA KML CELL PROLIFERATION

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Insulin-dependent cytoplasmic regulator (IDR), the activity of which correlates with the insulin level in blood, was isolated from rat liver and fractionated on a Sephadex G-25 column. Radioautography established that IDR stimulates by 30% proliferation of murine melanoma KML cells in culture. This is indicated by ^{3}H-thymidine incorporation into the cell nucleus. It is proposed that insulin stimulates proliferation of the melanoma KML cell cultures through an intracellular intermediate, IDR.

Key words: insulin, insulin-dependent cytoplasmic regulator (IDR), gel filtration, cell culture proliferation.

We have previously reported that insulin-dependent cytoplasmic regulator (IDR), the activity of which correlates with the insulin concentration in blood, inhibits gluconeogenesis in kidney and liver sections [1], stimulates glycogen and protein synthesis, and possesses hypoglycemic activity [2]. We found that IDR, like insulin, decreases $[Ca^{2+}]_{in}$ in thymocytes owing to stimulation of Ca^{2+} transport into mitochondria and endoplasmic reticulum [3].

Insulin stimulates proliferation of several cell types in culture [4-8]. The biochemical mechanism of the effect of insulin on replication is unclear. It is proposed that prolonged stimulation of protein synthesis via phosphorylation of ribosomal serine of protein S6 leads to the synthesis of a peptide necessary to convert cells in phase G_1 of the mitotic cycle into phase S [6-9]. Multiplication of cells stalled in phase G_1 owing to removal of serum from the medium can be restored if insulin is added, i.e., insulin enhances the progress of retarded cells from phase G_1 to S-phase [7, 9]. Insulin-dependent replication occurs through the insulin tyrosine-kinase receptor and an unknown intracellular regulator. IDR apparently plays the role of the intercellular regulator.

Our goal was to modify the method of isolating a preparation containing IDR from rat liver. Considering that IDR can penetrate the plasma membrane [1-3], we studied the effect of IDR on proliferation of murine melanoma KML cells.

The literature procedure for isolating and purifying IDR is labor-intensive and includes four steps [10]. Two steps, chromatography on DEAE-cellulose and desalting on a Sephadex G-10 column, were eliminated by modifying the purification and isolation. The new conditions for gel filtration on Sephadex G-25 produced a fraction containing IDR as a simple and separate second peak (Fig. 1). The total activity of IDR relative to the previous version increases by 100 times because the IDR is separated better from ballast proteins under these conditions. The high content of IDR in this preparation enables its influence on metabolism not only under cellular and subcellular conditions but also in *in vivo* experiments to be studied.

Then we studied the effect of IDR on the proliferation of KML cell culture (murine melanoma). It was found that the IDR activity on proliferation of KML cells is dose-dependent. IDR at concentrations from 0.01 to 0.1 μg/mL stimulates proliferation from 7 to 30%, respectively, relative to the control as measured by ^{3}H-thymidine incorporation into the KML cell nucleus. Increasing the IDR concentration from 1.0 to 10.0 μg/mL induces the opposite effect. The proliferation of the cell culture decreases without question relative to the maximal effect (0.1 μg/mL) to the control level. This is consistent with a bell-shaped dependence of the IDR effect. We have previously reported such a bell-shaped concentrational dependence of IDR on cellular and subcellular levels [1, 2, 10]. Averages from five samples with standard deviations (M ± m) are given below.

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IDR concentration, μg/mL	^{3}H-Thymidine incorporation level, %	Cpm, counts/min
0	100	21900±100
0.01	107	23500±90
0.1	130	31020±120
1.0	115	25800±100
10.0	100	22070±80

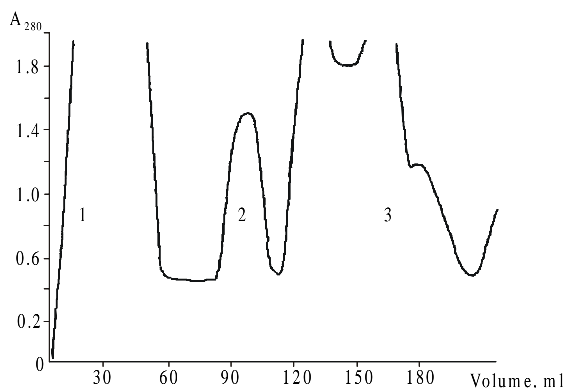

Fig. 1. Gel filtration of the thermostable fraction of rat-liver cytoplasm on a Sephadex G-25 column. IDR activity is found in peak 2.

Thus, the results suggest that IDR, like insulin, stimulates the growth and regulation of cells. IDR may be located on the plasmalemma or penetrate into the cell and stimulate cells if they are resting in the G_0 stage after dividing, increasing the pool of dividing cells, because the signal from IDR enters the cell through the plasma membrane [1-3, 10] by an as yet unknown mechanism.

The proliferative activity of insulin is connected, on one hand, to the activation of cAMP-dependent proteinkinase *A*, tyrosine-dependent proteinkinase, and mitogen-dependent proteinkinase [4-9, 11]. On the other hand, a new hypothesis is being promoted [12] according to which insulin receptors on the nuclear membrane [13] interact directly with insulin or with an internalized insulin—receptor—tyrosinekinase complex or certain peptides that apparently have an affinity for the insulin receptor on the nuclear membrane [13]. This hypothesis proposes that the insulin receptor on the nuclear membrane can interact with DNA and regulate gene expression [13]. The activity of nucleosidephosphatase that is localized in the nuclear membrane and stimulates the release of mRNA into the cytoplasm increases as a result of the interaction of insulin with the membrane of isolated nuclei [14]. It should be noted that the results upon which the hypothesis of the direct nuclear interaction of protein—peptide hormones is based are somewhat indirect and were obtained from isolated nuclei in *in vitro* experiments.

This hypothesis does not stand up to criticism about insulin because there is not yet direct proof that insulin enters the cell, interacts directly with intracellular membranes, and participates in intracellular metabolism in *in situ* and *in vivo* experiments.

Our results could agree with this hypothesis only if insulin not in the free state or the insulin—receptor complex of the plasma membrane but IDR interacts with the nuclear receptor that has affinity for insulin and several insulin-like growth factors (epithelin, nerves, thrombocytes, etc.) and possibly with another specific or nonspecific IDR receptor in the nuclear membrane. Therefore, the following pathway for the effect of insulin on cell proliferation can be proposed: interaction of insulin with receptor—tyrosinekinase in the plasmalemma → formation of IDR (as yet unknown from what) → interaction of IDR with receptor in the cell nucleus → signal transfer to DNA → transcription and translation to mRNA → protein synthesis. Apparently the effect of insulin on cell proliferation is mediated by an intracellular agent, IDR.

EXPERIMENTAL

IDR Activity. IDR activity in fractions was determined by a modification of mitochondrial respiration rate in a suspension of rat thymocytes [15] compared with the effects of insulin in physiological concentrations (1-10 μE/mL).

IDR Isolation. A preparation with high IDR activity was prepared by homogenizing rat liver in 0.3 M saccharose with 10 mM Tris-buffer. The liver/medium ratio was 1:1. The homogenate was centrifuged at 30,000 g for 20 min. The supernatant was heated at 97°C for 7 min. The denatured proteins and membrane fragments were settled by centrifugation after heating. The transparent supernatant containing the IDR activity was concentrated in a rotary evaporator to 2-3 mL and placed on a Sephadex G-25 column (25×600 mm). The optical activity at 280 nm was measured in the resulting fractions.

IDR activity was detected in a separate fraction of peak 2, which eluted immediately after passage of the void volume (Fig. 1). The eluate was 0.05 M NaCl.

Proliferation activity was studied as follows. Murine melanoma KML cells [16] were cultured in vials to 80,000 cells/mL in 3 mL of RPMI 1640 nutrient medium with 10% calf embryo serum, 200 mM L-glutamine, and antibiotics. IDR in various doses and 0.5 μCi (1 mCi) ^{3}H-thymidine per vial were simultaneously added. The vials were cultured for 24 h at 37°C in a CO_2 incubator. The culture medium was poured off. The cells were transfered to a filter, washed three times with phosphate buffer at pH 6.8, three times with 5% trichloroacetic acid for 10 min, then three times with distilled water for 10 min, and dried. The filters were counted in scintillant (toluene—POPOP-PPO) in a β-counter [17].

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