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INSULIN PROMOTED DECREASE IN THE PHOSPHORYLATION OF PROTEIN SYNTHESIS INITIATION FACTOR eIF-2

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Insulin stimulates cellular protein synthesis in calf chondrocytes in suspension culture. This enhanced synthetic activity is seen in association with a decrease in phosphorylation of the α subunit of protein synthesis initiation factor eIF-2. [^{32}P] associated with the α subunit is reduced approximately 50% by insulin treatment of chondrocytes incubated in [^{32}P] containing media. Identical or closely located amino acids in the eIF-2 α subunit are phosphorylated by the chondrocyte kinase(s) and the rabbit reticulocyte hemin regulated kinase as indicated by comparative peptide fragment analysis of [^{32}P] labeled α subunits.

Evidence for effectors of gene expression acting at the translational levels of protein synthesis in eukaryotes is well documented (for review see 1). The most clearly defined examples of translational control are the investigations showing the effect of hemin on the initiation of protein synthesis in reticulocytes and lysates derived from these cells.

Almost twenty years ago investigators first reported on the inhibition of protein synthesis they observed with reticulocytes incubated in hemin deficient media(2-4). This inhibtion was later shown to be at the level of initiation and to be associated with an ATP dependent phosphorylation of initiation factor eIF-2 by a hemin regulated kinase. More recent evidence indicates that phosphorylated eIF-2 can initiate only one round of protein synthesis as a result of its inability to react with a second factor required for dissociating it from the GDP generated during the initiation reaction(5-9). There are many who believe that control of protein synthesis via phosphorylation of eIF-2 may be involved in other cell types(10,11). We describe here the first report of an insulin induced stimulation of cellular protein synthesis associated with a decrease in the phosphorylation of the α subunit of eIF-2.

MATERIALS AND METHODS: Pure pork insulin was from Novo Industries; reticulocyte lysate, all radioactive material and scintillation cocktails were from New England Nuclear. Collagenase Type IV, pepstatin, leupeptin, N-ethyl maleimide and HEPES were from Sigma Chemical Co. Fetal calf serum and media

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were from Gibco. Traysylol was from FBS Pharmaceuticals, New York. S. aureus V8 protease was from Miles Laboratory, Elkhardt, Indiana.

Effect of Insulin on Protein Synthesis in Chondrocytes: Articular cartilage from radialcarpal joints of freshly slaughtered 9-14 day old calves was digested with clostridial collagenase as previously described(12). Cells were separated from non-digested material by passage through sterile cheesecloth, collected by centrifugation at 1000 x g for 5 min and the pellets washed twice in sterile saline. Chondrocyte pellets were resuspended in Ham's F12 supplemented with penicillin (150 u/ml), streptomycin (50 ug/ml), glutamine (4 mM), ascorbic acid (50 ug/ml) and 20 mM HEPES pH 7.3.

Freshly isolated cartilage cells in Ham's F12 medium were rested for 5 h at 2 x 10^6 cells/ml. One ml aliquots of cells were placed in tubes containing 1.0 ml of Ham's F12 medium. To each tube was added 50 uCi [^{3}H] proline (S.A. 23.7 Ci/mmole) and insulin to provide final insulin concentration from 0 to 160 nM. The cells were incubated without stirring for 2.5 h at 37° and harvested by centrifugation. The cell pellets were solubilized in 100 ul 0.1 N NaOH and incubated for 10 min at 37°. Samples were precipitated with TCA and processed for counting in a liquid scintillation counter as previously described(12).

Partial purification of eIF-2 from Insulin Treated and Control Chondrocytes Labeled with [^{32}P] Orthophosphate: Chondrocytes isolated by the procedure described above were suspended in 200 ml of phosphate free minimal essential medium at a cell concentration of 5 x 10^6/ml. 5 mCi [^{32}P] orthophosphate was added to the medium and incubation continued for 4 h. The cell suspension was then divided into two 100 ml bottles, one containing 9.6 ug of insulin, and both suspensions incubated for an additional 1 h. The cells were harvested by centrifugation at 1000 x g for 10 min and the cell pellets frozen at -80°.

eIF-2 was partially purified from [^{32}P] labeled insulin and control chondrocytes according to the method of Treadwell(13). Frozen cell pellets were each suspended in 5 ml of buffer containing 0.7% triton X-100, 500 mM KCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 50 mM NaF, 10 mM 2-mercaptoethanol, 1 uM Leupeptin, 1 uM Pepstatin, and 10 u/ml traysylol. The suspension was homogenized in an all glass motor driven homogenizer at 0° and centrifuged 7,000 x g 10 min. The supernatant was diluted to 25 ml with homogenization buffer without KCl and triton X-100 and applied to a CM-Sephadex (CM-50) column (bed vol - 5 ml) equilibrated with the same buffer containing 100 mM KCl. The column was washed with 40 ml of this buffer (flow rate 120 ml/h) and the fraction containing eIF-2 was eluted from the column with 6 ml of this buffer made 400 mM in KCl. The protein was precipitated by the addition of 2 volumes of acetone and cooled to -40°C. The precipitate was collected by centrifugation (15,000 x g 10 min), the supernatant decanted, and the pellet washed with cold acetone and lyophilized to dryness.

Phosphorylation of Reticulocyte eIF-2: One microgram of highly purified reticulocyte eIF-2 (90% pure) was incubated at 37° for 7 minutes with 0.5 ul of reticulocyte lysate (previously treated with N-ethylmaleimide to activate the hemin controlled kinase according to the procedure of Gross and Rabinovitz(14)) in a final volume of 50 ul containing 20 mM Tris Cl pH 7.0, 100 mM KCl, 2.5 mM Mg $(Ac)_2$, 0.1 mM ATP and 2 uCi gamma labeled [^{32}P] ATP (5,000 Ci/mMole).

Two dimensional Gel Electrophoresis: Samples were resolved by two dimensional gel electrophoresis according to the procedure of O'Farrell(15) except that the lysis buffer and focusing gels contained the following ampholines: 1.6% pH 3.5-10 and 0.4% pH 5-7. Acetone-precipitated protein in the partially purified eIF-2 preparations from control and insulin treated chondrocytes was dissolved in 9.2 M urea for protein determination and immediately diluted with an equal volume of lysing buffer. Identical amounts of protein, 70 ug as determined by the Bradford procedure(16), were applied to each isoelectric gel. On a separate isoelectric gel, 50 ug of the eIF-2 preparation from control chondrocytes together with 0.5 ug of reticulocyte eIF-2 (phosphorylated by incubation with [^{32}P] ATP) were co-electrophoresed. Two-dimensional gels were stained in

0.5% Coomassie blue, 50% methanol, 7% acetic acid and destained in 7% acetic acid. Gels were dried under vacuum and exposed to x-ray film for 18-48 hrs.

Protease Digestion of [^{32}P] Labeled α Subunit Isolated from Reticulocytes and Chondrocytes: Reticulocyte eIF-2 (0.5 ug) was labeled with [^{32}P] ATP as described above. 70 ug [^{32}P] chondrocyte eIF-2 was prepared from control cells as described. The α subunit of each preparation was resolved by two-dimensional gel electrophoresis and the [^{32}P] containing α subunit spots excised. Samples were digested with 40 ng S. aureus V8 protease and electrophoresed according to the procedure of Cleveland(17). Following electrophoresis gels were stained, dried and placed in contact with x-ray film for 72 h.

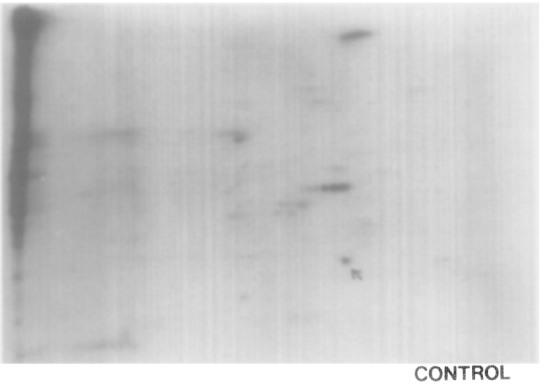
RESULTS

We obtained significant stimulation of [^{3}H] proline incorporation into calf articular chondrocyte protein with insulin concentrations as low as 0.16 nM, and nearly a two fold increase was observed at 16 nM. These results are in good agreement with those obtained by others using chondrocytes isolated from different species(18-20).

Figure 1 shows the results obtained with two dimensional gel electrophoresis of [^{32}P] labeled chondrocyte protein present in a 0.4 M KCl eluent from a CM-Sephadex column. Although this figure represents the results obtained from one experiment, the same protocol was repeated eight times with virtually identical results. The α subunit of chondrocyte eIF-2, indicated by an arrow in the figure, was identified by its co-migration on 2-d gel electrophoresis with the α subunit of highly purified reticulocyte eIF-2 obtained from two sources (see Acknowledgements). The lower panel shows the results obtained with lysates from insulin treated cells and the upper panel the control. The figure shows that the [^{32}P] containing material co-migrating with the α subunit is more intensely labeled in the control lysate.

This difference is approximately 2 fold as demonstrated by the results in Table I. Autoradiograms from six experiments were scanned using a densitometer (Schoeffel Instruments). To normalize the data one protein, for which the degree of phosphorylation did not change significantly in response to insulin, was used as an internal standard. The relative density of the eIF-2 α subunit was calculated from the ratio of the heights (above adjacent background) of peaks corresponding to the α subunit and the internal standard.

The results of the S. aureus V8 protease digestion of reticulocyte and chondrocyte [^{32}P] labeled eIF-2 α subunit are shown in Figure 2. The digestion products of the α subunit from both sources yield virtually identi-



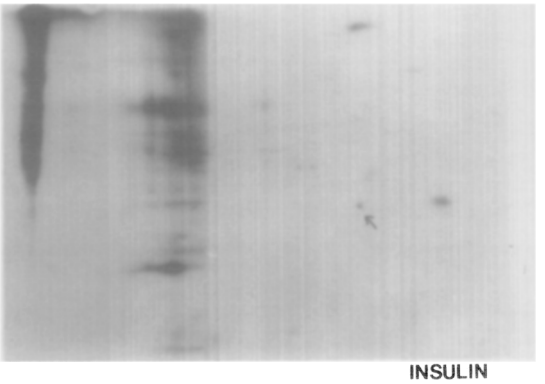


Figure 1: Two-dimensional gel electrophoresis of phosphorylated chondrocyte eIF-2. Chondrocytes were labeled with [^{32}P] orthophosphate in the presence and absence of insulin. Cell homogenates were prepared for chromatography on CM-Sephedex as described in the experimental section. Equal amounts of protein from the 400 mM KCl column eluent were applied to the first dimension gels. The arrow indicates the chondrocyte eIF-2 α subunit. The isoelectricfocusing is from left to right with the acid region to the right. Upper panel represents an autoradiograph of results obtained with control lysates, the lower panel with lysates prepared from insulin treated chondrocytes.

Table I

Experiment	Relative Density of eIF-2 α spot		Ratio
	Control	Insulin	(Control/Insulin)
1	2.0	0.94	2.12
2	2.28	0.96	2.37
3	0.61	0.32	1.90
4	1.62	0.60	2.70
5	2.28	1.52	1.50
6	3.80	1.17	3.25

Radioactivity associated with eIF-2 α subunit from insulin-treated and control chondrocytes. Autoradiograms of two dimensional gels of partially purified eIF-2 prepared from insulin treated and control chondrocytes were scanned using a densitometer and the relative densities calculated as described in the experimental section. Significance of difference as determined by Student's t-test using paired control and insulin values: $p < 0.02$.

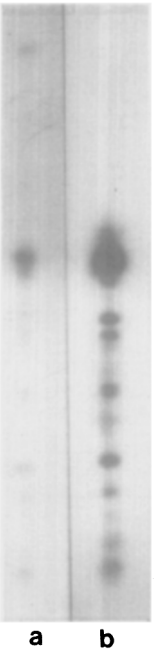


Figure 2: SDS gel electrophoresis of phosphorylated chondrocyte eIF-2 α and reticulocyte eIF-2 α after incubation with V8 protease. Phosphorylated reticulocyte eIF-2 was prepared as described in the experimental section. Phosphorylated chondrocyte eIF-2 was prepared from cells incubated with [^{32}P] orthophosphate; the α subunits were resolved by 2-dimensional gel electrophoresis. The [^{32}P] containing subunit areas of the gels were excised and applied to an SDS polyacrylamide gel and electrophoresed after the addition of buffer and V8 protease as described in the experimental section. Lane A of the autoradiograph represents digestion products of phosphorylated reticulocyte α subunit and lane B chondrocyte α subunit.

cal radioactive products as determined by SDS gel electrophoresis, confirming the identity of the indicated [^{32}P] containing spot as chondrocyte eIF-2 α.

eIF-2 was enriched 400-500 fold by CM-Sephadex chromatography of the lysates prior to electrophoretic analysis; the appropriateness of the procedure for calf chondrocyte eIF-2 had been established in preliminary experiments not reported here. It should be emphasized that great care was taken during the preparation of samples to minimize variation in protocol so a precise comparison could be made. Within an experiment, protein content and radioactivity in the acetone precipitated 0.4 M CM sephadex eluent derived from control and insulin treated chondrocytes were virtually identical. Throughout the purification procedure, samples were maintained at 0-4° in buffer containing 50 mM NaF 1 mM EDTA (conditions inhibiting protein phosphatase activity(21)).

DISCUSSION

We previously showed that phosphorylation of ribosomal protein S6 is associated with the insulin-promoted stimulation of protein synthesis in chondro-

cytes(22). Further analysis of the translational machinery reveals a decrease in the amount of radioactivity associated with the α subunit of eIF-2 in chondrocytes incubated with insulin. This result suggests that the insulin stimulation of protein synthesis may be due at least in part to this observed difference.

eIF-2 phosphorylated on its α subunit has been shown to be inactive as an initiator of protein synthesis in reticulocyte lysates. Recent data suggest that the inhibition is the result of the phosphorylated factor's inability to react with (or be released from) another protein synthesis factor variously known as GEF, RF, eIF-2B and anti-inhibitor(5-9). This second factor normally combines with eIF-2-GDP releasing the GDP generated during the initiation process. The eIF-2-bound RF is displaced by GTP to reform an eIF-2-GTP complex which can initiate another round of protein synthesis. Thus a decrease in the phosphorylation of eIF-2 α should stimulate the activity of this factor and the rate limiting initiation step of protein synthesis.

Evidence for the identification of the phosphorylated component as the α subunit of eIF-2 is the following: (a) eIF-2 activity co-purifies with this component during CM-Sephadex chromatography, (b) phosphorylated reticulocyte eIF-2 α subunit co-migrates on 2-dimensional gel electrophoresis with this component and (c) [^{32}P] containing S. aureus V8 protease digestion products of reticulocyte and chondrocyte eIF-2 α have identical mobility on gel electrophoresis. The apparent identity of the phosphorylated digestion products of chondrocyte and reticulocyte eIF-2 α is of interest; it suggests that identical or closely located amino acids in the reticulocyte and chondrocyte α subunits are targeted for phosphorylation by the reticulocyte hemin regulated kinase and the chondrocyte eIF-2 α kinase.

It has been demonstrated that even under conditions of active protein synthesis some eIF-2 α is phosphorylated in reticulocyte lysates. The results we obtained are in agreement in that chondrocytes contain phosphorylated α even in the absence of known inhibitors of protein synthesis and that the anabolic hormone, insulin, decreases this phosphorylation. It is not known whether this observed decrease is an insulin mediated increase in phosphatase activity or a decrease in α kinase activity.

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