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The Effect of Insulin on Collagen Production in Isolated Chondrosarcoma Chondrocytes

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Summary: Insulin stimulated the incorporation of [^{3}H]-proline into collagen of freshly isolated chondrosarcoma chondrocytes. In addition, insulin enhanced incorporation of radiolabeled precursors into general protein, RNA, and proteoglycans. The stimulatory effects on collagen and non-collagen protein occurred within 2 h while the effects on RNA and proteoglycan were observed at 5 h and 8 h, respectively. All responses were obtained with physiological concentrations (1-2 nM) and were proportional to concentrations to 2 uM. These results demonstrate that insulin, in addition to exerting a general anabolic action on chondrocytes, also stimulates the incorporation of [^{3}H]-proline into a specific protein, i.e., collagen. The latter effect should provide a useful means to probe insulin's mechanism of action.

Introduction: Much recent work has centered on understanding the basic hormonal regulation of cartilage growth and development. However, technical difficulties associated with the tissue, as well as the limited amounts of cartilage available from laboratory animals, have impeded advances in this area. The Swarm rat chondrosarcoma is far superior to normal cartilage with respect to ease of manipulation and responsiveness to certain hormones both *in vivo* and *in vitro* [1-8]. Furthermore, the chondrosarcoma is similar to normal cartilage in that the tumor is pituitary-dependent and responds to insulin and growth hormone-dependent serum factors (i.e. somatomedins) [1-8]. In those studies the *in vitro* response to insulin was demonstrated using tumor minces [2-4] and cultured chondrocytes [6-8].

In the present report, we present evidence that several anabolic processes of isolated chondrosarcoma chondrocytes are stimulated by

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physiological concentrations of insulin. To our knowledge the stimulatory effect of insulin on the incorporation of [^{3}H]-proline into a specific protein, i.e. collagen, is an original observation. By analogy to other systems, e.g. progesterone/ovalbumin; estrogen/vitellogellin, the study of the relationship of insulin to collagen could prove to be extremely useful in studying the molecular mechanism of action of insulin.

Methods:

Preparation of Isolated Chondrosarcoma Chondrocytes - Tumors (generously provided by Dr. B. Caterson, Univ. of Alabama) were maintained in Sprague-Dawley strain rats (Zivic-Miller Labs, Allison Pk., PA) by serial subcutaneous implantation of minced pieces. 4-6 weeks later, tumors (40-100g) were excised and cleaned of associated fascia. Non-necrotic areas of the tumors were minced and the pieces rinsed thrice in 0.9% NaCl to remove blood cells. Pieces were placed in 0.2% collagenase (Type I or Type IA, Sigma) in medium [9] and shaken for 30 min. at 37°. Pieces were separated from released cells by passing the suspension through a fine mesh wire screen. Cells were pelleted, washed thrice with medium, counted in a hemacytometer chamber, and diluted to the appropriate cell concentration. In experiments in which [^{3}H] proline incorporation into collagen was measured, 0.1 mM ascorbic acid was present. Isolated cells were tested for viability using trypan blue exclusion. Approximately 80-90% of the cells excluded the dye both before and after the incubation.

Metabolic Studies - Various concentrations of insulin (bovine, Calbiochem) were added to 24-well plates (Costar) which contained 1-4 x 10^5 cells in a final volume of 1 ml. 2 uCi [2,3-^{3}H]-proline (20-40 Ci/mmol, New England Nuclear), or 1 uCi carrier-free [^{35}S]-Na_2SO_4 (New England Nuclear) or [5-^{3}H]-uridine (25 Ci/mmol, New England Nuclear), was added 2 h prior to stopping the reaction. The incubation was done at 37°. At the end of the incubation, the entire contents of the well were added to an equal volume of cold 10% trichloroacetic acid. Two drops of a 1% bovine serum albumin (Sigma) solution were added to aid pellet formation and the precipitate collected by centrifugation. Pellets were washed thrice with cold 5% trichloroacetic acid and, except for those samples containing [^{3}H]-proline (see below), solubilized with 50-100 ul of 88% formic acid at 60° prior to determination of radioactivity.

The [^{3}H]-collagen produced during incubation was determined as described by Peterkofsky and Diegelmann [10]. The purified collagenase used was graciously provided by Dr. R. Diegelmann and Nada Woods, Dept. of Surgery, Medical College of Virginia. Non-specific proteolytic activity in the enzyme preparation was negligible when tested against [^{3}H]-tryptophan-labeled proteins isolated from E. coli.

The radioactivity of all samples was determined using Bray's solution [11]. All counts were quench corrected.

Statistical Analysis - Significant statistical differences were detected using a one-way analysis of variance. These were assigned to specific groups using Dunnett's t-test [12].

Results: Continuous labeling studies showed the uptake of [^{3}H]-proline, [^{35}S]-sulfate and [^{3}H]-uridine by chondrocytes was linear and insulin

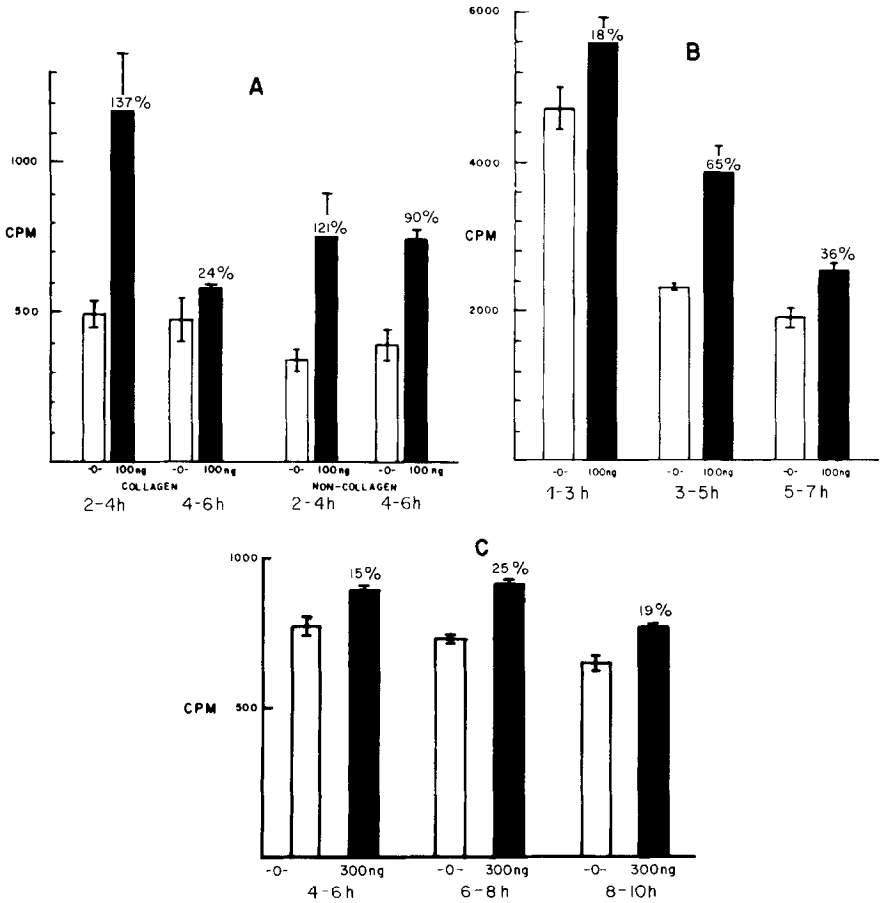

Figure 1. Time course of rates of incorporation of radiolabeled precursors into macromolecules during two hour labeling periods. 1-4 x 10^5 chondrocytes/ml were incubated in the presence (100 and 300 ng/ml) or absence of insulin at 37° with either 2 uCi [^{3}H]-proline (panel A) or 1 uCi [^{3}H]-uridine (panel B) or [^{35}S]-sulfate (panel C) for the indicated periods prior to stopping the incubation. Results are presented as CPM/well for [^{3}H]-uridine and [^{35}S]-sulfate experiments, and as CPM/40% aliquot for [^{3}H]-proline experiments as described in Methods. Values above solid bars indicate stimulation by insulin as % above control. Each value represents the mean ± SE of at least three determinations.

stimulated the uptake of the latter two radiolabels into macromolecules only marginally, i.e., 20%. As previous reports [2-4] had shown that labeling the tissue for a 2 h period prior to stopping the reaction could optimize the stimulation, similar experiments were performed with our system.

The stimulation produced by insulin during 2 h labeling periods for the various labeled precursors is depicted in Figure 1. Although insulin enhanced the incorporation of [^{3}H]-proline into collagen (67%)

and non-collagen protein (38%) within two hours in continuous labeling experiments, its effect at longer incubation periods was studied to determine maximum response. The maximal stimulation found with 18.5 nM (100 ng/ml) insulin occurred during the 2-4 h labeling period (Fig. 1A). During this period insulin increased the uptake of [^{3}H]-proline into collagen and non-collagen about equally, i.e., 137% and 121%, respectively. On the other hand, during the 4-6 h labeling period the stimulation of collagen declined to 24% while non-collagen protein remained relatively unaltered at 90%. Therefore, all further experiments with [^{3}H]-proline employed the 2-4 h labeling period. 18.5 nM insulin enhanced [^{3}H]-uridine incorporation maximally at 3-5 h (Fig. 1B). At this time period, the stimulation by insulin was 65% compared with 18% and 36% at the 1-3 h and 5-7 h labeling periods, respectively. Therefore, all subsequent experiments with [^{3}H]-uridine were done using a 3-5 h labeling period. Because the stimulation of sulfation was not detected until 8 hours during continuous labeling experiments, exposure periods with [^{35}S]-sulfate were performed at times after 4 hours (Fig. 1C). The stimulation of sulfation by 55.5 nM (300 ng/ml) insulin was maximal at the 6-8 h labeling period (25% above control). The labeling periods preceding and following this pulse time produced a 15% and 19% stimulation, respectively. Thus, all subsequent sulfation studies were done using a 6-8 h labeling period.

The effect of various concentrations of insulin on radiolabeled precursors into macromolecules is depicted in Fig. 2. Significant stimulation of the incorporation of [^{3}H]-proline into collagen (Fig. 2A) and non-collagen protein (Fig. 2B) occurred at concentrations as low as 1.8 nM. Half-maximal stimulation of both processes was approximately 2 and 3 nM, respectively. The maximal effect of 0.18 uM insulin on collagen and non-collagen protein was 196% and 159%, respectively. Insulin-enhanced uridine incorporation was concentration-dependent with 1.8 uM causing a 73% increase (Fig. 2C). As little as 1 nM insulin

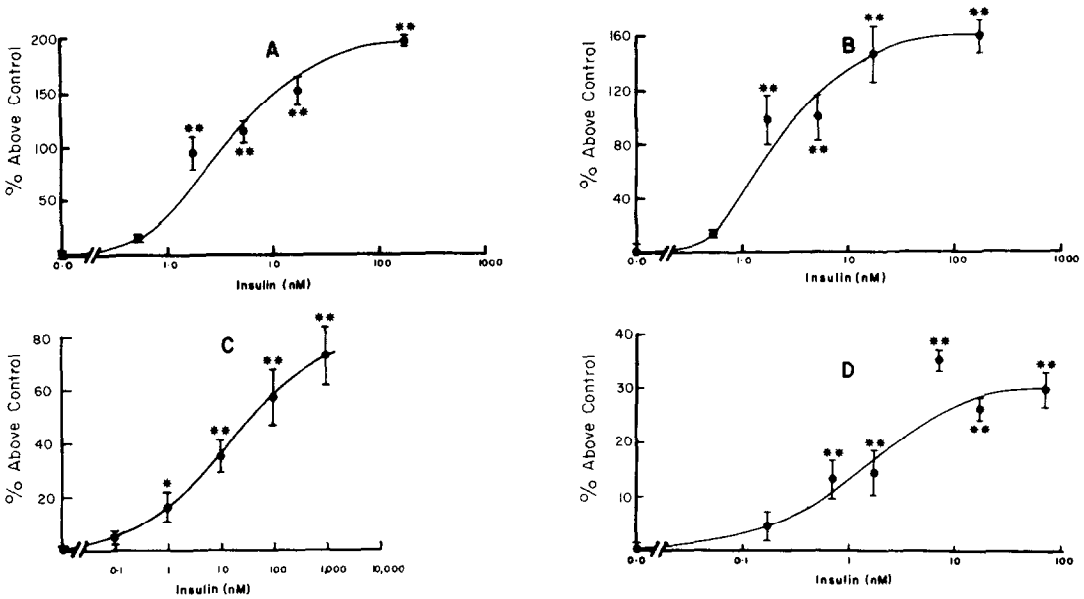

Figure 2. Stimulation of the incorporation of radiolabeled precursors into macromolecules by various concentrations of insulin. 1-4 x 10^5 chondrocytes/ml were incubated at 37° with or without insulin in the presence of either 2 uCi [^{3}H]-proline (panel A, collagen; panel B, non-collagen protein) or 1 uCi [^{35}S]-sulfate (panel C) or [^{3}H]-uridine (panel D) for the appropriate labeling periods (see text). Each graph is from a representative experiment in which each point represents the mean of triplicate determinations. Control values for the incorporation of [^{3}H]-uridine into RNA was 2328 ± 38 CPM/well, of [^{35}S]-sulfate into proteoglycans was 1502 ± 16 CPM/well, and of [^{3}H]-proline into collagen and non-collagen protein was 760 ± 37 CPM/40% aliquot and 1156 ± 67 CPM/40% aliquot, respectively. (*, $p < 0.05$; **, $p < 0.01$)

caused a significant response with half-maximum stimulation being about 10 nM. Sulfation was increased in a concentration-dependent fashion (Fig. 2D) with the minimum effective concentration being 0.74 nM. The maximum stimulation, i.e., 38%, was obtained with 7.4 nM insulin, and half-maximal stimulation was attained at 2.1 nM.

Discussion: Microscopic examination of hematoxylin- and eosin-stained chondrocyte preparations revealed that >90% of the cells were chondrocytes. Of the remaining cell types, erythrocytes comprised the majority. Contamination by the latter did not significantly contribute to the metabolic responses measured. Thus, erythrocytes incorporated negligible amounts of radiolabeled precursors into collagen and non-collagen protein, proteoglycans and RNA (data not shown).

The results presented clearly demonstrate that freshly isolated Swarm rat chondrosarcoma chondrocytes incorporate radiolabeled precursors into collagen and non-collagen proteins, proteoglycans and RNA and that these processes are stimulated by insulin in vitro. The rapid response and sensitivity of the cells to insulin are comparable to those obtained by McCumbee and Lebovitz using tumor pieces [2-4]. It appears, therefore, that the brief collagenase treatment we employed had little or no effect on the metabolic activity of isolated chondrocytes.

The rates of incorporation of both [^{3}H]-proline and [^{35}S]-sulfate into macromolecules during the 2 h exposure periods for the control chondrocytes remained relatively constant (Fig. 1). In contrast, the uptake of [^{3}H]-uridine by the control was initially rapid (1-3 h), declined (3-5 h), and remained relatively constant thereafter (5-7 h); thus suggesting, among other possibilities, that some factor(s) may become rate limiting. The ability of insulin to increase the amount of label incorporated into macromolecules in the first two instances appears to be due to net synthesis. Because proline is actively transported into the cell, increases in the radioactivity in both collagen and non-collagen protein may be due to an increase in the specific activity of the prolyl-tRNA pool. However, since the cells are incubated in the presence of 70 uM proline for a 2 h period prior to adding label, the effect of insulin on the specific activity of the prolyl-tRNA pool probably is small. The increase in sulfation reflects an increase in proteoglycan synthesis as sulfate freely diffuses into the cell and is not compartmentalized [9]. Stimulation of the incorporation of [^{3}H]-uridine into RNA is in part due to insulin's ability to prevent the rapid decline of the rate of uptake (Fig. 1B) and may reflect an increase in the specific activity of the UTP pool. This latter possibility will require further investigation.

The chondrosarcoma chondrocytes respond to a range of insulin concentration that is physiologically meaningful. The half-maximum

stimulation of sulfation and [^{3}H]-proline incorporation into collagen and non-collagen protein was between 2 to 4 nM. Serum insulin levels in fetal rats range from 1 nM (basal) to 3-4 nM during a period of hyperinsulinemia prior to birth [13]. Thus, it appears that the chondrocytes are responsive to insulin concentration ranges found in fetal animals.

Collagen, by virtue of the fact that it comprises approximately 40% of total tissue protein [14], is one of the major proteins synthesized by chondrosarcoma tissue. In our experiments, [^{3}H]-collagen represented 15-20% of the total radioactive protein. In this regard, the freshly isolated cell system appears to be admirably suited for metabolic as well as hormone mechanism of action studies. Others have demonstrated the stimulation of proteoglycans by insulin and multiplication-stimulating activity in cultured chondrosarcoma chondrocytes [6-7]. The synthesis and hormonal regulation of structural matrix components, that is, collagen and proteoglycans, could provide new insights into cartilage growth and development.

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