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CREATINE KINASE ACTIVITY AND CARTILAGE ENERGY METABOLISM: CREATINE KINASE-CREATINE PHOSPHATE CIRCUIT ACTIVITY AND MACROMOLECULE SYNTHESIS

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Abstract

Earlier studies showed that there is significant creatine kinase activity in the epiphyseal growth cartilage, and there is stage-specific isoform expression. To further explore the role of this enzyme in cartilage development, we disrupted the creatine kinase - creatine phosphate (CK-CP) circuit using the creatine analogue, cyclocreatine. We found that while cyclocreatine decreased creatine kinase activity, the drug did not alter the energy status of the cell, nor did it cause a significant loss in adenine nucleotides. However, we noted that the glycolytic pathway was very sensitive to the drug. At high concentrations, cyclocreatine reduced glucose entry into the cell by 100%, and there was almost complete suppression of lactate generation. Surprisingly, there were minimal changes in creatine phosphate concentrations. Although cyclocreatine did not influence mitochondrial energy generation, the possibility existed that it could modulate other maturation-dependent activities of the cell. Indeed, we found that there was decreased expression of type II and X collagen and osteopontin. These results suggest that the circuit is primarily concerned with the transduction of energy rather than the generation or storage of high energy equivalents. In this role, the circuit would serve to facilitate nucleotide transport from sites of generation to sites of utilization.

Key Words: Chondrocytes, creatine kinase, creatine phosphate, energy metabolism, cyclocreatine, collagen gene expression.

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Introduction

The process of endochondral bone formation requires the presence of a secondary cartilage, the epiphyseal growth plate. During the short life span of the epiphyseal chondrocyte, there are extensive changes in morphology and gene expression. Alterations in chondrocyte phenotype are characterized by elevation in alkaline phosphatase activity (Fell and Robison, 1929; Stocum *et al*., 1979); increased expression of type X collagen mRNA (Schmid and Linsenmayer, 1985; Horton and Machado, 1988; Leboy *et al*., 1989); down regulation of type II collagen mRNA; increased osteopontin and osteonectin mRNA (Oshima *et al*., 1989); and a raised level of transforming growth factor-β (Thorp *et al*., 1992), c-*myc* (Farquharson *et al*., 1992), and 1,25-dihydroxycholecalciferol receptors (Suda *et al*., 1985). Accompanying these developmental changes, there are profound alterations in energy metabolism, a hallmark of chondrocyte maturation. For example, in the resting and proliferating zones of the cartilage, the cells exhibit significant oxidative activity, whereas in the hypertrophic zone, the terminally differentiated cells generate energy by processes that are linked to non-oxidative metabolism. Our previous investigations have shown that chondrocytes in the hypertrophic zone have an elevated NADH/nicotinamide adenine dinucleotide (NAD) ratio (Kakuta *et al*., 1986), a decreased adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio (Shapiro *et al*., 1982), a low energy charge ratio and utilize the creatine kinase - creatine phosphate (CK-CP) circuit (Funanage *et al*., 1992; Shapiro *et al*., 1992).

Although the coupling between energy generation and alterations in chondrocyte phenotype is poorly understood, there is mounting evidence to support the view that many genes are under stringent metabolic control (Robin *et al*., 1984; Ausserer *et al*., 1994; Semenza *et al*., 1994; Rajpurohit *et al*., 1996). Other studies point to the probability that there is tight coupling between the energy needs of the cell and its functional requirements (Bessman and Carpenter, 1985). In most tissues, the CK-CP circuit transfers energy-rich phosphoryl groups from intracellular energy generating sites to more distant energy utilizing

centers (Wallimann *et al*., 1992). The reaction is catalyzed reversibly by different isozymes of creatine kinase, especially BB, which have been shown to be present in the growth plate (Somjen *et al*., 1984; Shapiro *et al*., 1992). The transfer function of the CK-CP circuit can be summarized in the following equations:

$$\text{phosphocreatine} + MgADP^{-2} \longrightarrow \text{creatine} + MgATP^{-2}$$

$$MgATP^{-2} \longrightarrow MgADP^{-2} + Pi$$

The CK-CP circuit also functions as an energy buffer that maintains the ATP/ADP ratio of the cell, especially when there is elevated demand for energy ([ATP] low, [ADP] high). In tissues adapted to a low oxygen tension, or where there is a rapid change in metabolism, the circuit generates metabolic energy to meet cellular needs. From this viewpoint, the growth plate provides an excellent tissue in which to evaluate energy-coupled functional demands.

Our earlier studies have shown that there is significant creatine kinase activity in the epiphyseal growth cartilage and there is evidence of expression of specific isoforms during development. Thus, the MM isoform is expressed in the resting and proliferating cartilage, while in the hypertrophic zone, the MB and BB forms predominate (Shapiro *et al*., 1992). Inhibition of circuit activity impairs the growth and development of cartilage *in vivo*, as well as inhibiting the maturation of chondrocytes *in vitro* (Funange *et al*., 1992). Together, these observations indicate that creatine kinase activity is of critical importance in the normal development and function of the growth cartilage. In the present study, using maturing chondrocytes in culture, we examined the mechanism by which the CK-CP circuit influences chondrocyte function. Using the creatine analogue cyclocreatine, a compound which prevents ATP regeneration (Walker, 1979), we evaluated the importance of the CK-CP circuit in terms of its role in maintaining a reserve of high energy equivalents and as a "spatial energy buffer" facilitating "energy transport".

Materials and Methods

Experimental design

A chondrocyte culture system was used that mimics maturation events that are exhibited by cells in the growth plate. In earlier studies we reported that when cephalic sternal chondrocytes are treated with low doses of retinoic acid and ascorbic acid, the cells exhibit maturation-dependent changes in phenotype (Leboy *et al*., 1989, Iwamoto *et al*., 1993, Shapiro *et al*., 1994). Using this system, now we investigate the import of the CK-CP circuit on chondrocyte function. We treated the cells with selected doses of the creatine analogue, cyclocreatine. This agent serves as a substrate for the creatine kinase isozymes, and as it forms a stable non-metabolic phosphagen, the circuit can be selectively interrupted without altering other components of the cell's energy generating systems. A concentration range of 0.07-7.0 mM was chosen as studies by other workers have shown that cells are sensitive to this cyclocreatine dose without development of toxicity (Martin *et al*., 1994). Following treatment, we assessed how the drug modulated components of the CK-CP circuit, mitochondrial energy metabolism and glycolysis, and indicators of chondrocyte maturation. Finally, we examined the effect of the inhibitor on *de novo* protein synthesis, and the expression of type II and X collagen, and osteopontin.

Cell culture

Sternal chondrocytes from the cephalic portion of 14 day chick embryos were isolated according to the method of Iwamoto *et al* (1993). These cells were maintained in primary culture for 4-5 days. To minimize fibroblast contamination, secondary cultures were re-plated in 35 mm^2 12 well plates at a cell density of 0.28 x 10^6 cells per well in Dulbecco's modified high glucose Eagle's medium containing 10% Nuserum® (Becton Dickinson, Collabarative Biomedicals, Bedford, MA), 2 mM L-glutamine and 50 U/ml each of penicillin/streptomycin. To facilitate adhesion, cells were treated with 4 U/ml of hyaluronidase. Chondrocytes also received 25 μg/ml ascorbic acid for the initial 2 days; this value was increased to 50 μg/ml through the rest of the culture period. To activate the maturation process, the medium was supplemented with 35 nM all trans-retinoic acid dissolved in 95% ethanol. In experiments designed to measure mineral deposition, the medium contained 3 mM β-glycerophosphate. When confluent, chondrocyte cultures were treated with 0.07, 0.7 or 7.0 mM cyclocreatine (2-imino-1-imidazolidineacetic acid), which was obtained from Sigma Chemical Co. (St. Louis, MO). The medium was changed every day during the entire treatment period; cell morphology was evaluated by phase contrast microscopy.

Biochemical assays

Medium was collected throughout the cyclocreatine treatment period. At the end of each treatment period, the cell layer including the matrix was washed with cold phosphate buffered saline (PBS), and extracted in 0.1% triton X-100 in PBS and stored at -70°C until analyzed. Creatine kinase, was assayed by the method of Bergmeyer (1985). Alkaline phosphatase activity was estimated by monitoring the release of free p-nitrophenol at 410 nm according to the method of Leboy *et al* (1989). To measure creatine phosphate, cells were extracted in 6% ice cold perchloric acid and immediately frozen. Following neutralization, the samples were centrifuged at 14,000 x g and the supernatant used for analysis according to Bergmeyer (1985). DNA

measurements were made by the Hoechst 33258 dye binding method of Teixeira *et al.* (1995). Fluorescence was measured in a Photon Technology International spectrophotometer with the excitation and emission wavelengths set at 365 and 460 nm respectively. Protein levels were assayed by the Biorad (Cambridge, MA) BCA method according to the manufacturer's protocol. Medium glucose depletion (Schon, 1965) and lactate generation was determined by enzymatic analysis (Carroll *et al*., 1970). Calcium was measured in acid extracts of the cultures by the atomic absorption spectrophotometry.

Nucleotide analyses

The chondrocyte monolayer was washed with ice cold phosphate buffered saline, suspended in cold 2% perchloric acid and frozen at -70°C until analyzed. The pH of the extract was adjusted to 6.0 with 10 M KOH and centrifuged. The supernatant was filtered through a 10,000 MW cut off ultrafree MC filter (Millipore, Bedford, MA). The nucleotides present in the filtrate were separated and analyzed by high performance liquid chromatography (HPLC) using a C18 μ Bondapak reverse phase column (3.9 mm x 300 mm). Elution was achieved using a linear gradient generated by 0.1 M potassium phosphate buffer pH 6.0 and 0.1 M potassium phosphate buffer pH 6.0 with 10% methanol and detected at 254 nm (Stocchi *et al*., 1985). The energy charge was calculated from the formula:

$$\text{Energy charge ratio} = \frac{[\text{ATP} + (1/2)\,\text{ADP}]}{(\text{ATP} + \text{ADP} + \text{AMP})}$$

L-[^{35}S]-methionine incorporation

Following 7 day treatment with cyclocreatine, cells were labelled with [^{35}S]-methionine (25 μCi/ml) in a low methionine medium for 1 hour. The medium was removed and the cells were washed thoroughly with cold PBS. The cell layer including the surrounding matrix was then extracted with 0.1% Triton-X 100. Equal amounts of protein (70 μg) was solubilized in Laemmli's (1970) sample buffer and resolved on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel under reducing conditions. The gel was stained with coomassie blue for protein visualization, and the autoradiogram was developed after over night exposure at -70°C. Radiolabelled methionine incorporation was measured in aliquots of protein from the cell extracts after precipitation with 5% ice-cold trichloroacetic acid. The pellet was washed twice with 5% cold trichloroacetic acid and once with cold ethanol in the presence of carrier bovine serum albumin (1.0 mg). The pellet was dried after the final wash, solubilized in 5 ml of scintillation fluid and counted. The [^{35}S]-methionine incorporation was expressed as cpm per μg protein.

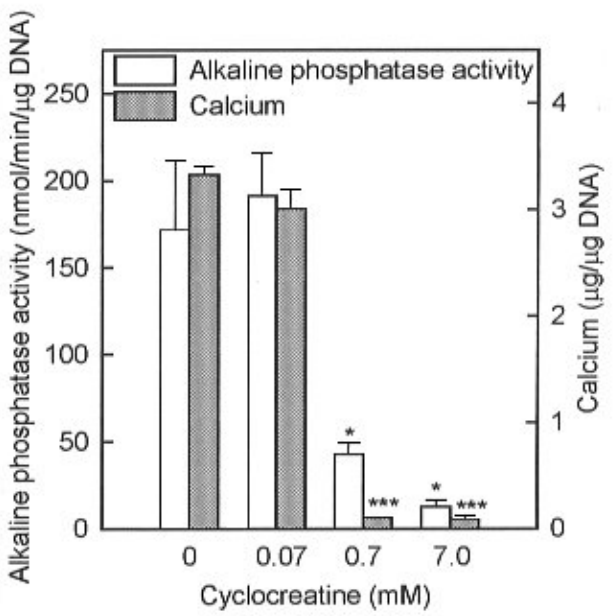

Figure 1. Effect of cyclocreatine on alkaline phosphatase activity and calcium accumulation by sternal chondrocytes. Chick embryonic cephalic sternal chondrocytes were maintained in culture until confluent and then treated with 35 nM all-trans retinoic acid and increasing doses of cyclocreatine for 7 days. Medium was supplemented daily with 3 mM β-glycerophosphate. The alkaline phosphatase activity and calcium content of the cultures were then determined and expressed per μg DNA. Values shown are mean ± standard error of mean (SEM); n = 3. Asterisks indicate a significant difference from untreated (0) control; *$p < 0.05$, ***$p < 0.001$.

Western blot analysis

After electrophoresis the proteins in the SDS-polyacrylamide gel were transblotted onto a nitrocellulose membrane. The proteins were blocked with low fat milk, and then exposed to a polyclonal rabbit antibody against chick type X collagen. Antibody binding was localized using peroxidase-conjugated goat anti-rabbit IgG and the color reagent 3,3'-diaminobenzidine was used as a substrate to visualize the protein.

Northern analysis

Total RNA was extracted from chondrocyte cultures treated with 0-7 mM cyclocreatine using TRIzol reagent (GibcoBRL, Grand Island, NY) according to the manufacturer's instructions. The RNA sample was denatured with formaldehyde and electrophoresed on a 1% agarose gel, each lane containing 15 μg total RNA. The RNA was then transferred to a maximum strength NYTRAN membrane (Scheicher and Schuell, Keene, NH) using a turboblotter.

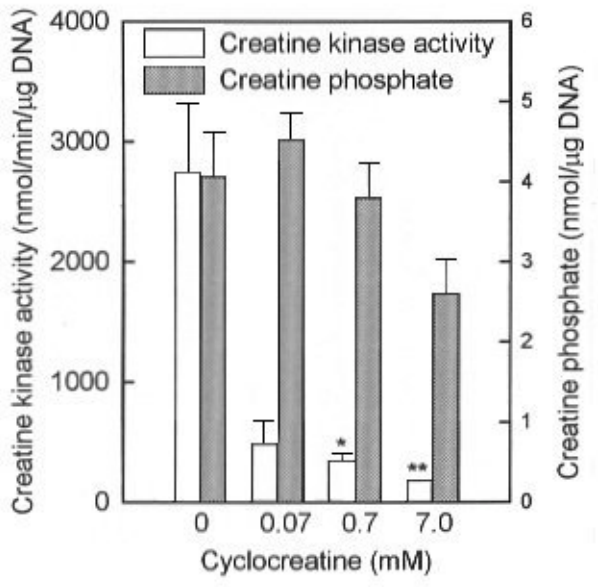

Figure 2. Creatine kinase activity and creatine phosphate levels in chondrocytes treated with cyclocreatine. Confluent 14 day sternal chondrocytes were treated with 35 nM retinoic acid and increasing doses of cyclocreatine for 7 days. Cells were extracted with 0.1% triton-X 100 or 6% perchloric acid. The creatine kinase activity or the creatine phosphate concentration was then determined. Values are mean ± SEM; n = 3. Asterisks indicate a significant difference from untreated (0) cells; *$p < 0.05$, **$p < 0.01$.

Hybridization was performed at 45°C in 5x Denhart's solution, 6x SSPE, 2% SDS, 100 µg/ml of sonicated salmon sperm DNA and 50% formamide. The membranes were washed with 1x SSC and 0.25x SSC twice consecutively with each buffer and exposed to Kodak film at -70°C for 1-3 days. The plasmids used were the chick type II collagen cDNA pCs2 (Young *et al*., 1984) type X collagen pDLr10 (Leboy *et al*., 1986), osteopontin cDNA MMPP2 (Moore *et al*., 1991) and a 556 bp cDNA fragment from chick β-actin cDNA plasmid (Engel *et al*., 1982).

Results

Figure 1 shows the alkaline phosphatase activity of confluent embryonic chick sternal chondrocytes treated with 35 nM retinoic acid in the presence of different doses of cyclocreatine. In the absence of cyclocreatine a significant increase in alkaline phosphatase activity is seen by 7 days, the phosphatase activity has increased 20 fold. Confirming our earlier studies, a parallel increase in calcium accumulation by the cultured chondrocytes is also seen (Shapiro *et al*., 1994). Cyclocreatine significantly inhibits the retinoic acid-dependent rise in alkaline phosphatase activity in a dose-dependant manner. Thus, the phosphatase activity is decreased by 75% when the medium contains 0.7 mM cyclocreatine; activity is inhibited by 93% at 7.0 mM. Addition of creatine or creatine phosphate to the medium had a minimal effect on the alkaline phosphatase activity (data not shown). In the presence of cyclocreatine at concentrations of 0.7 and 7.0 mM, there is a failure to accumulate calcium. Microscopic evaluation of the culture throughout the treatment period indicates that the cells maintain their polygonal shape and there was no evidence of cell death.

As expected, after 7 day treatment with cyclocreatine, the creatine kinase activity is decreased in a dose-dependent manner (Fig. 2). Activity is inhibited over 80% at the lowest dose of cyclocreatine (0.07 mM). The cyclocreatine-dependent decrease in creatine kinase activity, however is not accompanied by a fall in creatine phosphate levels. Figure 2 shows that up to 0.7 mM cyclocreatine, the concentration of creatine phosphate is held constant. Indeed, when the concentration is raised to 7.0 mM, while the mean creatine phosphate levels fall, the decrease is not significantly different from the values seen in control (untreated) cells.

To assess the role of the CK-CP circuit in maturing chondrocytes, we treated cells with the inhibitor and measured selected parameters of chondrocyte function. As a first indicator of energy metabolism, we measured glucose depletion and lactate generation by cells treated with cyclocreatine. Figure 3A shows that the rate of glucose consumption increases linearly for the first 4 days in culture. From day 4 to 7, the rate is maximal at 5.5 µM glucose consumed/ml medium/24 hours. In the presence of cyclocreatine, there is a progressive decrease in both the initial and maximal uptake rates. Indeed, at the highest cyclocreatine concentration, the rate of glucose uptake fell progressively through the culture period to a low of 1.5 µM glucose consumed/ml medium/24 hours. Changes in glucose depletion are matched by concomitant changes in the rate of lactate generation. Figure 3B indicates that untreated cells exhibit an initial 4 day linear increase in lactate generation and then the rate becomes maximal at 18 µM lactate/ml medium/24 hours. In line with the glucose values, the presence of cyclocreatine causes a decrease in initial and maximal lactate synthesis rates. Again, at the highest inhibitor levels, the rate of lactate generation is low throughout the culture period, and at 7 days, the lactate concentration is 4 fold lower than the control (no inhibitor) levels.

To study the interaction of the CK-CP circuit with mitochondrial energy status of the chondrocytes, we inhibited creatine phosphate generation with the highest concentration of the inhibitor (7.0 mM) for 7 days and

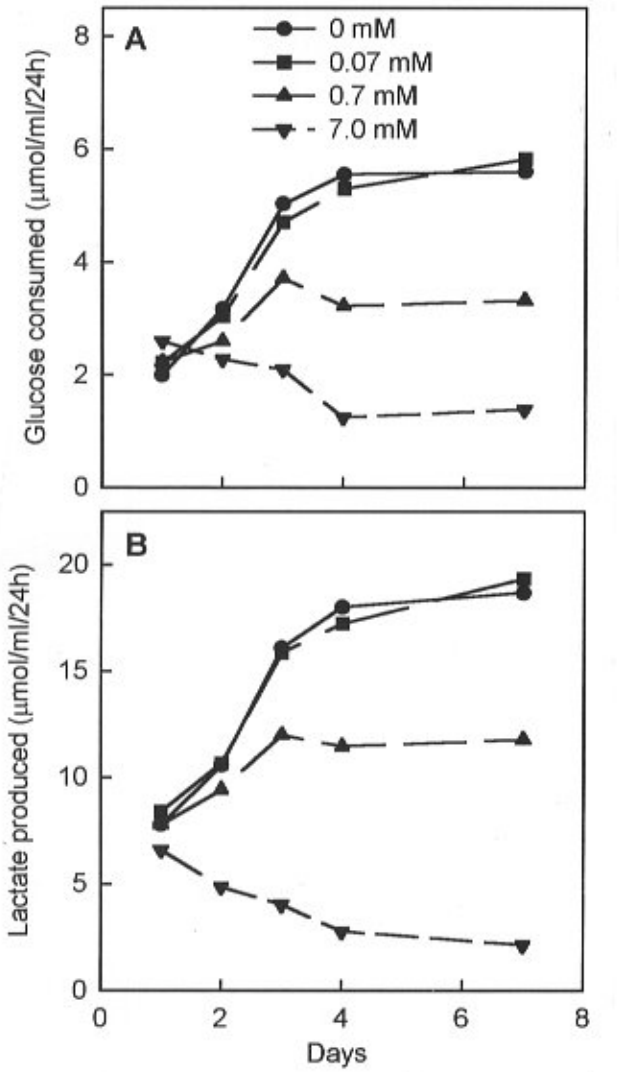

Figure 3. Glucose consumption and lactate generation by chondrocytes treated with cyclocreatine. Confluent embryonic chondrocytes were treated with 35 nM retinoic acid and increasing doses of cyclocreatine for 7 days. Medium was collected for analysis every 24 hours throughout the entire duration of the experiment; (**A**) glucose, (**B**) lactate. Each point is an average of 3 sets of determinations.

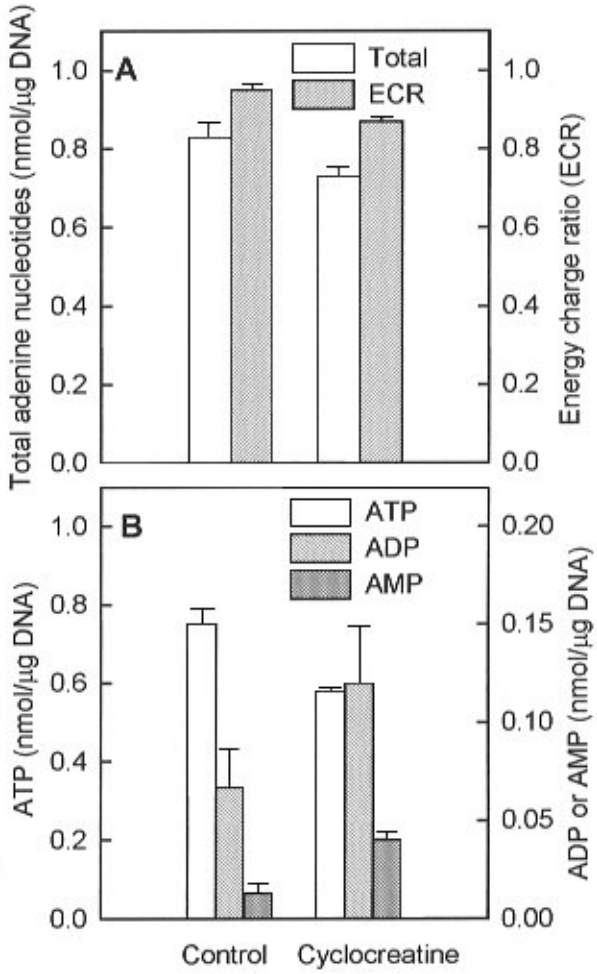

Figure 4. Adenine nucleotide values of chondrocytes treated with cyclocreatine. Chondrocytes were treated with 35 nM retinoic acid and 7.0 mM cyclocreatine for 7 days. The cells were washed with phosphate-buffered saline and extracted with 2.0% ice cold perchloric acid for analysis by HPLC. (**A**). Total adenine nucleotides and energy charge ratio (ECR); (**B**) ATP, ADP and AMP levels. Values are mean ± SEM; n = 3.

determined individual and total cellular adenine nucleotides by HPLC. We noted that in the presence of the inhibitor, there is little change in the total nucleotide level (Fig. 4A). In terms of individual nucleotides, there is a 23% decrease in ATP levels, and a two and four fold elevation in ADP and AMP levels, respectively (Fig. 4B). However, when expressed as total nucleotides or as the energy charge ratio, the values are virtually unaltered (Fig. 4A).

In concert with the energetic measurements, we also evaluated how cyclocreatine altered critical functions such as cellular proliferation and protein synthesis. Exposure of the chondrocyte culture to 0.07-7.0 mM cyclocreatine for 7 days did not cause any alterations in DNA levels, indicating that the drug did not influence cell proliferation (Fig. 5). Since there is also little change in lactate dehydrogenase activity throughout the treatment period, the results suggest that there is minimum drug toxicity (not shown). However, at doses of 0.7 mM and 7.0 mM, the total protein content is decreased by 18 and 42% respectively. We also examined [^{35}S]-methionine incorporation into chondrocyte proteins, and found that inhibition of creatine kinase activity has a dramatic effect on the rate of protein synthesis. Thus, the rate of incorporation of [^{35}S]-methionine into newly

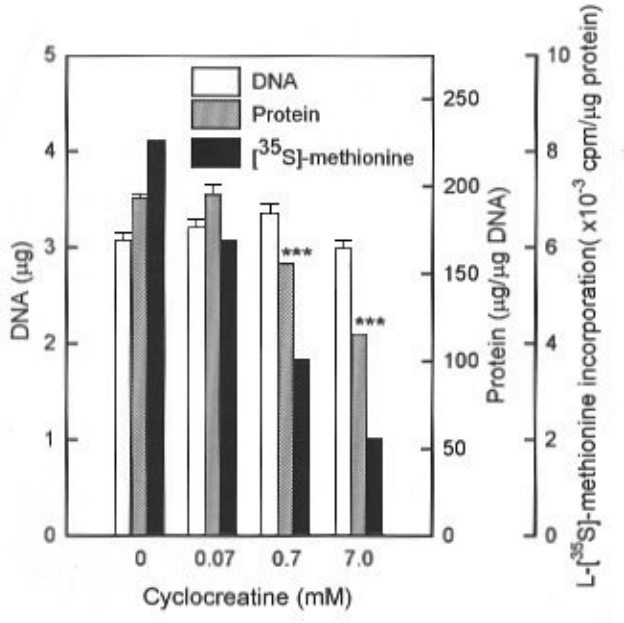

Figure 5. DNA, total protein and L-[^{35}S]-methionine incorporation in chondrocytes treated with cyclocreatine. Confluent chondrocytes were treated with 35 nM retinoic acid and increasing concentrations of cyclocreatine for 7 days. On the final day of the experiment, medium was supplemented with [^{35}S]-methionine for one hour. The cell layer was extracted with 5% trichloroacetic acid and the radioactivity of the precipitated protein determined. Values are mean ± SEM; n = 3. Values for the radiolabelled methionine incorporation are average of two experiments. Asterisks indicate a significant difference from untreated (0) cells ; ***$p < 0.001$.

synthesized proteins fell by 19% at 0.07 mM and by 80% at 7.0 mM cyclocreatine.

To ascertain if inhibition of the circuit resulted in a decrease in specific proteins, we resolved the radiolabelled proteins extracted from the cyclocreatine-treated chondrocytes on a 10% SDS-polyacrylamide gel under reducing conditions. Figure 6 shows that with increasing concentration of cyclocreatine, there is a proportional decrease in [^{35}S]-methionine incorporation; there is no obvious change in any particular low or high molecular weight component. However, Western blot analysis indicated that when the cyclocreatine concentration is between 0.7-7.0 mM, the expression of type X collagen is almost totally inhibited.

To verify whether the effect on type X collagen synthesis was specific, or whether the inhibitor affected other extracellular matrix components, we examined the expression of type II and X collagen and osteopontin by Northern analysis. Figure 7 shows that cyclocreatine inhibits the expression of type II and X collagen and osteopontin in a dose-dependant manner.

Discussion

The role of the CK-CP circuit in cartilage function has received limited study and its role in endochondral bone growth is unknown. While there are grounds for linking circuit activity to a unique aspect of the chondrocyte maturation process, a specific function has yet to be delineated. Nevertheless, in line with what is known of circuit function in other tissues, it is plausible to consider that the circuit could serve to conserve energy and provide a mechanism to shuttle energy equivalents to intracellular compartments that are sites of high energy use. The results of the present study provide strong evidence that CK-CP shuttle is required for functions linked to chondrocyte maturation and energy transport, and not *a priori* to energy conservation. We showed that in the presence of cyclocreatine, a drug that alters the efficiency of the circuit, there was inhibition of markers associated with chondrocyte maturation (low alkaline phosphatase activity, decreased calcium accumulation, low level expression of type II and X and osteopontin messages and decreased protein synthesis) with no change in cell morphology or number. Direct measurement of creatine phosphate levels indicated that the actual concentration of the phosphagen was low when compared with adenine nucleotides, little influenced by changes in creatine kinase activity and relatively unresponsive to alterations in the glycolytic rate. Thus, from an energetic perspective, we conclude that the shuttle functions in chondrocytes as a transducer of energy, rather than serving as an energy store or buffer. As such, the circuit responds to immediate energy demands, and interference with the circuit, influences events linked to energy use, not storage.

We used cyclocreatine to probe the import of the circuit in relationship to chondrocyte function during maturation. Cyclocreatine is a creatine analogue that serves as a substrate for creatine kinase forming a phosphorylated compound; as the phosphocyclocreatine is stable, it inhibits regeneration of ATP through the CK-CP circuit (LoPresti and Cohn, 1989). That cyclocreatine disrupted the chondrocyte circuit was supported by the observation that when cells were treated with the analogue there was decreased creatine kinase activity. However, the changes in creatine kinase activity were not accompanied by a parallel fall in the concentration of creatine phosphate. One explanation for the maintenance of creatine phosphate levels is that cyclocreatine may change both the rate of synthesis of creatine phosphate as well as its breakdown. As a result, the steady-state phosphagen levels would not be expected to change. It is also possible that at sites of energy

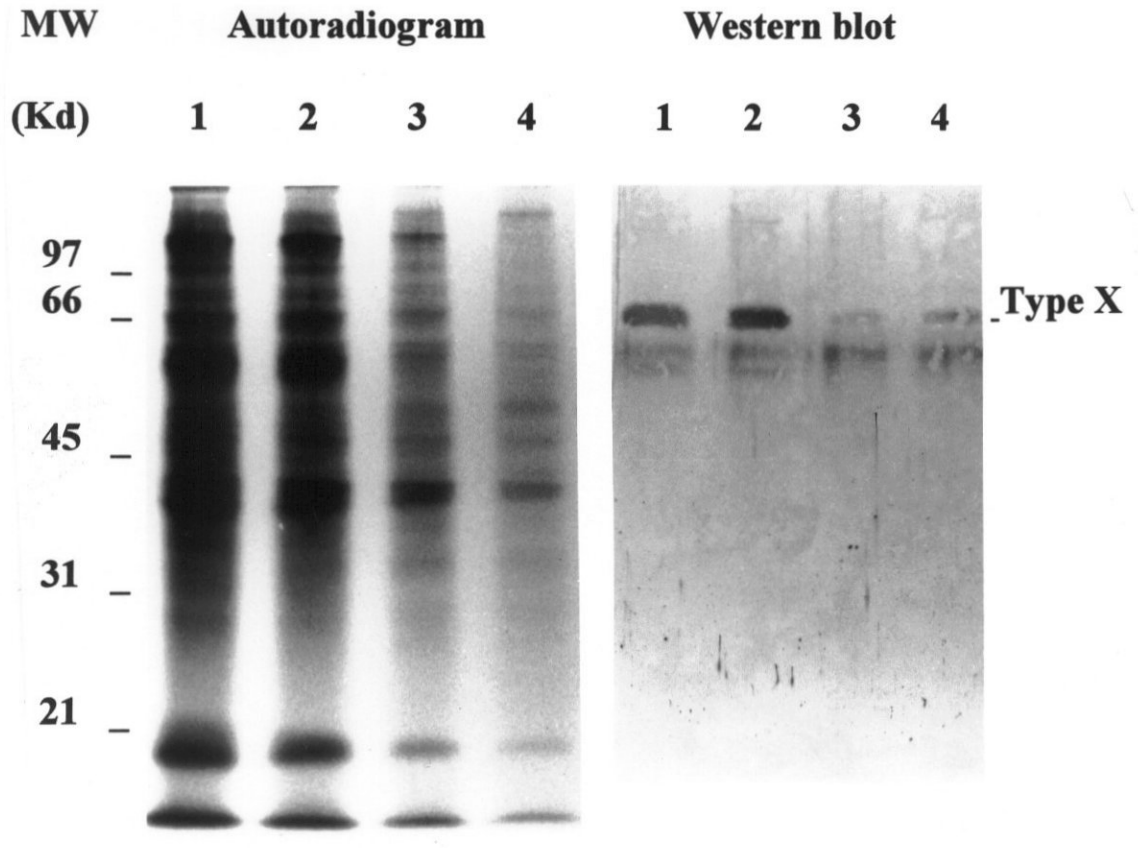

Figure 6. Autoradiogram and western blot of L-[^{35}S]-methionine labelled proteins. Chondrocytes were treated with retinoic acid (35 nM) and increasing doses of cyclocreatine for 7 days. Protein was extracted with Laemmli's buffer and equal amounts of protein (70 μg) were resolved on 10% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred to nitrocellulose and probed with an antibody to type X collagen. Lane 1, control; lane 2, 0.07 mM; lane 3, 0.7 mM; lane 4, 7.0 mM cyclocreatine respectively.

utilization, the isozyme responsible for regeneration of ATP from creatine phosphate is selectively inhibited by cyclocreatine.

In terms of the energy economy of the cell, we were surprised to find that cyclocreatine did not alter mitochondrial function. Experimentally, we noted that the drug was ineffective in disrupting the energy status of the cell, as evidenced by the unaltered energy charge ratio, nor did it cause significant loss in adenine nucleotides. From this perspective, our results indicated that creatine kinase activity is unlikely to be coupled with mitochondrial function. This point of view is consistent with results of earlier studies of chondrocyte energy metabolism. Thus, we have shown that chondrocytes, especially those that have been treated with agents that promote hypertrophy, generate much of their metabolic energy through glycolysis and there is minimal reliance on mitochondrial oxidative phosphorylation (Shapiro *et al*., 1994). Indeed, even when treated with uncoupling agents, chondrocytes remain viable and still maintain their phenotype (unpublished results). In terms of the circuit itself, in an earlier study we demonstrated that there was stage specific expression of the MM and BB isozymes (Shapiro *et al*., 1992), but there was no evidence of mitochondrial isozymes. For this reason, in this communication, we suggest that creatine kinase activity is extra-mitochondrial and limited to the delivery of creatine phosphate/ATP to energy consuming processes in the cell.

We examined the relationship between glycolysis and the CK-CP circuit by treating chondrocytes with cyclocreatine and then determining glucose uptake and lactate generation. In contrast to mitochondrial function, we noted that the glycolytic pathway was very sensitive to the drug. At 0.7 mM, cyclocreatine reduced glucose entry into the cell by 100%, while at the highest drug concentration (7 mM), glucose uptake was below basal levels. Perusal of Figures 3A and 3B indicates that the changes in glucose and lactate levels occur in concert with each other. However, since the magnitude of change in

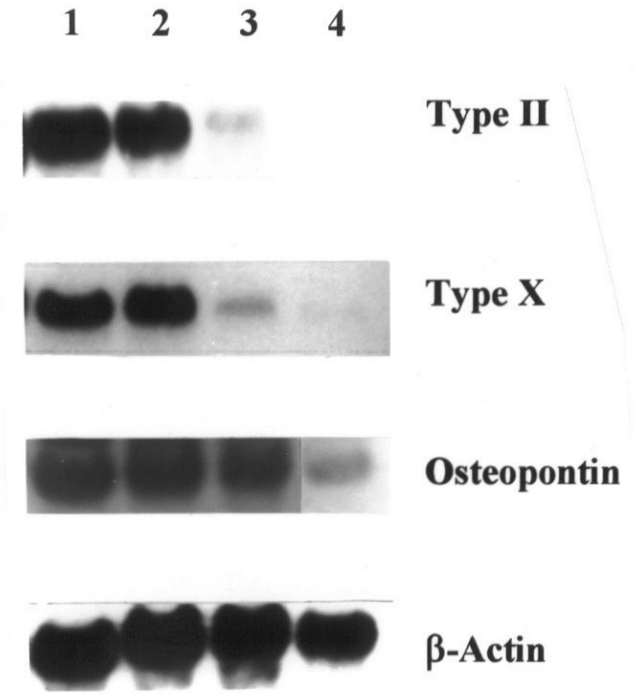

Figure 7. Northern analysis of Type II and X collagens, osteopontin and β-actin expression in chondrocytes treated with increasing doses of cyclocreatine for 7 days. 15 μg of total RNA was loaded in each lane and electrophoresed on a 1% agarose gel. The RNA was transblotted and hybridized with the respective probes and exposed to Kodak film at -70°C for 1-3 days. Lane 1, control; lane 2, 0.07 mM; lane 3, 0.7 mM; lane 4, 7.0 mM cyclocreatine respectively.

lactate levels is greater than that of glucose, it is likely that the drug inhibited utilization of substrates other than glucose. Indeed, if it is assumed that each mole of glucose can be converted to two moles of lactate (Rajpurohit *et al*., 1996), then other substrates are being degraded by the cells and used as a source of metabolic fuel.

While cyclocreatine did not influence mitochondrial energy status, as measured by the adenylate energy charge ratio, the possibility existed that it could modulate other maturation-dependant functions of the cell. Indeed we observed that a number of cellular activities (type X collagen protein biosynthesis, type II and X collagen and osteopontin gene expression; alkaline phosphatase activity and calcium accumulation) were compromised by cyclocreatine. These observations confirm an earlier report that noted that following inhibition of creatine kinase there was inhibition of protein and lipid synthesis (Carpenter *et al*., 1983). Accordingly, it is likely that the CK-CP circuit is required for chondrocyte maturation. In terms of mechanisms, since the cyclocreatine does not change the energy status of the cell, any attenuation in chondrocyte function, probably reflects a change in the availability of high energy compounds at sites of macromolecule synthesis. From this point of view, the possibility exists that in chondrocytes, the primary function of the circuit is to transduce energy rather than generate or store high energy equivalents.

Acknowledgments

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Discussion with Reviewers

Reviewer V: While the authors have evaluated the importance of the CK-CP shuttle in terms of chondrocyte energy metabolism and biosynthetic reactions they have not delineated a role for the system in the maturation process. Based on their own work or work emanating from other laboratories, would the authors like to speculate on a function of this interesting enzyme system?

Authors: First, we need to comment that findings from the study clearly show that contrary to most other tissues, the CP-CK shuttle does not contribute in a significant way to the energetic status of chondrocytes during the maturation process. Moreover, chondrocytes appear to be unique in that they do not have a mitochondrial energy generating system that utilizes the CP-CK circuit. However, if the circuit is blocked, although the energy charge is still maintained, there is marked inhibition in protein synthesis and a reduction in the expression of a number of key extracellular matrix proteins. How CP or CK can influence gene expression or the translational process is unknown. However, a very exciting paper indicates that creatine phosphate may serve as a cofactor in splicing reactions involved in RNA processing (Hirose and Manley, 1997). Indeed, other phosphagens that were used in this study were found to be far less efficient than CP. Thus, the possibility exists that the CP-CK shuttle regulates gene expression at an earlier stage than initially presumed. We now propose to examine the role of CP in chondrocyte pre-mRNA synthesis.

Additional Reference

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