Factors Controlling Protein Turnover in Heart Muscle

By Howard E. Morgan, D. Eugene Rannels, and Race L. Kao

ABSTRACT

Hearts perfused in vitro with buffer containing glucose and amino acids developed a block in peptide-chain initiation associated with a decline in the rate of protein synthesis, increased ribosomal subunits, and decreased polysomes. Provision of insulin or palmitate-albumin prevented or reversed development of the initiation block. Protein synthesis in anoxic hearts was inhibited by a restricted rate of peptide-chain elongation; ribosome profiles were more aggregated than those of control tissues. High-energy phosphate levels were reduced 50% by anoxia. Release of $^3$H-phenylalanine during perfusion reflected the rate of protein degradation. Net release of phenylalanine indicated that the rate of protein degradation exceeded that of synthesis in control hearts. Provision of insulin completely prevented or reversed this imbalance; palmitate was only partially effective. Since increased phenylalanine release was associated with increased numbers of autophagic vacuoles, cathepsin-D activity was estimated. "Available" activity increased from 20% to 33% of total during 3 hours of perfusion. Insulin prevented or reversed this increase.

Thus, a balance in protein turnover was maintained through concomitant effects of insulin to increase protein synthesis and to reduce protein degradation. These effects involved changes in ribosome cycle and lysosomal enzyme activities.

KEY WORDS

Langendorff perfusion insulin oxidizable substrates
ribosome cycle amino acid release cathepsin-D activity
anoxia phenylalanine

- Cardiac muscle cells appear to have a life that may be as long as that of the organ itself. However, individual components within the cell turn over with half-times in the range of 5 to 12 days. Growth or repair of heart muscle cells must involve more rapid rates of synthesis of cell constituents than of degradation. As seen in Figure 1, synthesis of the protein components of myocardial constituents appears to be affected by (1) availability of amino acids, adenosine triphosphate (ATP), and guanosine triphosphate (GTP) as substrates; (2) availability of transfer ribonucleic acid (tRNA), messenger ribonucleic acid (mRNA), ribosomes, and enzymes that catalyze peptide bond formation; (3) factors affecting rates of peptide-chain initiation, including fatty acids, insulin, and perhaps other hormones; and (4) factors controlling rates of peptide-chain elongation. Recent studies have suggested that ribosome-catalyzed reactions were the major restriction to protein synthesis in cardiac muscle cells. Mechanisms and regulation of protein degradation are poorly understood, but insulin appears to be involved in controlling the rate of degradation. Knowledge of the factors controlling protein synthesis and degrada-

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Pathway of protein turnover. Amino acids are supplied to the intracellular pool by either membrane transport or protein degradation. Intracellular amino acids are activated to form aminoacyl tRNAs by combination with transfer RNA (tRNA). Polymerization of activated amino acids into protein is catalyzed by a series of ribosome-catalyzed reactions that make up the ribosome cycle. These reactions include initiation of peptide chains on the ribosomes and elongation and termination of chains. Peptide-chain initiation involves binding of messenger RNA (mRNA) and the initiator tRNA, probably a methionyl-tRNA, to the small ribosomal subunit (40S) and is dependent upon initiation factors.1 Binding of the large subunit (60S) follows and requires guanosine triphosphate (GTP). Chain elongation involves progressive addition of activated amino acids determined by the code contained within mRNA and is dependent upon transfer factors.5 When the protein is complete, the peptide chain and ribosomal subunits are released into the cytoplasm. Protein degradation involves reactions catalyzed by proteases and releases free amino acids into the intracellular pool.

What Are the Factors Controlling Protein Synthesis In Vivo and In Vitro?

When hearts were removed from fed or fasted rats, about 15% of the ribosomal RNA was present as ribosomal subunits (Fig. 2). Presumably, the muscle have been asked. These questions are dealt with in the remainder of this paper.
remains was present as polysomes. These findings suggested that peptide-chain initiation was proceeding at a sufficiently rapid rate to make peptide-chain elongation the major rate-controlling reaction for protein synthesis. The rate of chain elongation was influenced by the availability of aminoacyl-tRNA, GTP, ribosomes, and mRNA and by factors that control steps in the process of chain elongation.

When hearts were perfused in vitro with buffer containing normal plasma levels of amino acids and glucose, levels of polysomes decreased and levels of ribosomal subunits increased (Fig. 2). These findings, together with a fall in the rate of protein synthesis, indicated that a block in initiation of peptide chains had developed during perfusion of the heart. These changes provided an opportunity to identify substances that were involved in controlling peptide-chain initiation.

Since an adequate supply of amino acids and high-energy phosphates is required for optimal rates of protein synthesis, these substrates were measured in hearts that had developed a block in peptide-chain initiation. Hearts that were perfused with buffer containing normal plasma levels of amino acids and glucose maintained intracellular levels of most amino acids that were as high as those found in vivo. Glutamic acid, isoleucine, and leucine levels were somewhat lower, even though these amino acids were present in the perfusate. Addition of 5 times normal plasma levels of all amino acids to the perfusate increased protein synthesis about 20%, but did not result in a fall in ribosomal subunits to in vivo levels. These studies suggested that reduced intracellular levels of amino acids were not responsible for inhibition of protein synthesis at the initiation step. Similarly, the supply of high-energy phosphate in the form of ATP and GTP was well maintained during 1 hour of perfusion and did not appear to be responsible for the reduced rate of synthesis.

Can Insulin Overcome the Block in Peptide-Chain Initiation?

Insulin has been found to increase amino acid incorporation in heart and skeletal muscle. As seen in Figure 3, addition of insulin to perfusate that contained normal plasma levels of amino acids and glucose prevented loss of polysomes and accumulation of ribosomal subunits in the heart. The rate of phenylalanine incorporation was well maintained over three hours of perfusion in hearts exposed to the hormone. These findings indicated that insulin would prevent the development of a block in peptide-chain initiation during perfusion. In other experiments, the hormone has been found to reverse the block once it was allowed to develop. Addition of insulin after one hour of perfusion decreased
levels of ribosomal subunits and increased the rate of phenylalanine incorporation during the second hour.

Although insulin accelerated transport of some amino acids, the hormone did not appear to increase protein synthesis by virtue of raising intracellular amino acid levels. The hormone reduced intracellular levels of serine, threonine, phenylalanine, tyrosine, lysine, histidine, and arginine, but increased the level of alanine. Reduced intracellular levels of amino acids were interpreted in terms of a shift in the balance between rates of protein synthesis and degradation to favor the synthetic pathway. Increased alanine levels presumably resulted from a faster rate of glycolysis in the insulin-treated tissue. In other experiments, the hormone was found to have no effect on intracellular levels of adenosine triphosphate, but maintained higher levels of creatine phosphate during one hour of perfusion. These experiments suggested that the hormone effect on protein synthesis did not depend on increased availability of amino acids or ATP. The mechanism of the effect of insulin on chain initiation is unknown.

**Can the Block in Peptide-Chain Initiation Be Prevented by Other Oxidizable Substrates?**

Since fatty acids are the preferred substrate of cardiac muscle, the possibility that these substrates might protect the rate of protein synthesis was investigated by perfusing hearts with buffer containing glucose, normal plasma levels of all amino acids, 3% albumin, and palmitate. As seen in Figure 3, addition of fatty acid maintained the initial rate of phenylalanine incorporation. In addition, hearts perfused in the presence of palmitate contained increased levels of polysomes and lower levels of ribosomal subunits than controls, indicating that fatty acid had prevented the onset of the initiation block. Palmitate and other substrates, including acetate, acetoacetate, octanoate, and oleate, were found to reverse the block in chain initiation once it had developed.

These experiments indicated that either fatty acid or insulin was required for rates of chain initiation that were sufficiently rapid to maintain in vivo levels of ribosomal aggregation. In hearts perfused in the presence of insulin or fatty acid, peptide-chain elongation appeared to be the major restraint on protein synthesis, as it was in vivo.

**What Factors Restrict Protein Synthesis by Limiting the Rate of Peptide-Chain Elongation?**

Rapid rates of chain elongation depend upon availability of optimal levels of aminoacyl-tRNA and GTP, activity of the transferase enzymes, and the total number of ribosomes. A 50% reduction in the tissue levels of high-energy phosphates in anaerobic muscle was associated with an inhibition of protein synthesis (Fig. 4). In association with the inhibition of phenylalanine incorporation, lower levels of ribosomal subunits were found in anaerobic muscle, suggesting that chain elongation was inhibited to a greater extent than chain initiation.

The block in chain elongation was more clearly exposed when insulin was present to facilitate
chain initiation. Hormone-treated tissue that was perfused under either aerobic or anaerobic conditions contained low levels of ribosomal subunits, indicating that chain elongation was the major restriction to protein synthesis. Although the hormone was able to restore in vivo levels of ribosomal aggregation in anaerobic hearts, phenylalanine incorporation was unaffected.

In other experiments, the block in chain elongation was not found to be associated with a fall in intracellular levels of free amino acids. Further studies will be required to determine whether factors in addition to the fall in GTP levels were involved in the inhibition of chain elongation.

**What Factors Affect Net Protein Turnover by Regulating the Rate of Protein Degradation?**

Levels of protein within cardiac muscle may vary as a result of changes in the rates of either protein synthesis or degradation. Rates of degradation have been estimated by measuring release of phenylalanine from the heart. Since this amino acid is not synthesized or degraded by heart muscle, changes in perfusate levels reflected the balance between protein synthesis and degradation. If these rates were equal, net release of phenylalanine would not occur.

An approximation of the rate of degradation could be made by measuring the rate of dilution of the specific radioactivity of the free-phenylalanine pool. This method underestimates the degradation rate by the extent to which exchange of phenylalanine across the cell membrane prevents equilibration of the specific activities of the intracellular and extracellular pools of the amino acid. As seen in Figure 5, hearts that were perfused with buffer containing glucose and normal plasma levels of amino acids had a more rapid rate of degradation than synthesis of protein as evidenced by net release of phenylalanine. Since these hearts were removed from rats in which the hearts were still increasing in size, net phenylalanine release indicated either that the rate of degradation had increased or that the rate of synthesis had decreased during the first hour of perfusion in vitro. Net release of phenylalanine was even more rapid during the third hour of perfusion.

When insulin was added to the perfusate, net release of phenylalanine was inhibited during either the first or third hour of perfusion. This indicated that the hormone prevented the imbalance between...

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The pathway of protein degradation is poorly defined, but appears to involve proteolytic enzymes that are contained within lysosomes. Hearts that were perfused for three hours in the absence of insulin contained large membrane-limited vacuoles that were prevalent in the perinuclear region and in the rows of mitochondria. Addition of insulin prevented the development of vacuoles. These structures are believed to represent autophagic vacuoles. They contained membranous elements which in some instances were recognizable as mitochondrial fragments. Similar vacuoles have been described in liver cells. These observations suggested that changes in the permeability of lysosomal membranes could provide a basis for varying rates of protein degradation in control and insulin-treated hearts.

Cathepsin-D activity was assayed in heart homogenates as an index of the availability of lysosomal enzymes. "Available" activity was taken as that assayable in the absence of Triton, and total activity as that found in the presence of detergent. It should be noted that homogenization of the tissue can be expected to disrupt lysosomes, so that the "available" activity is likely to be higher than in intact hearts. Total activity was unchanged by perfusion either in the presence or absence of insulin (Fig. 6). In unperfused hearts, approximately 20% of total activity was "available"; this percentage increased to about 33% after 3 or more hours of perfusion with glucose-containing buffer. When insulin was included in the perfusate, activity of the "available" enzyme remained near that found in unperfused tissue. When insulin was added after 3 hours of perfusion in its absence, activity of the "available" enzyme fell to that in unperfused hearts.

These findings, together with the development of autophagic vacuoles, suggested that variations in the rate of protein degradation correlated with changes in the structure of lysosomes and the availability of their enzymes.

**What Roles Do Levels of Insulin, Fatty Acids, Amino Acids, and High-Energy Phosphates Play in Controlling Growth and Repair of Heart?**

When in vivo levels of all these factors were present, protein synthesis appeared to proceed at an optimal rate. In this situation, peptide-chain elongation limited the rate of protein synthesis. Restriction at this step depended on the activity of enzymes that catalyzed peptide-bond formation...
Accelerated synthesis of RNA is well known in cardiac hypertrophy. Hypertrophy of skeletal muscle involves both increased rates of protein synthesis and decreased protein degradation. Decreased destruction of myocardial proteins may contribute to growth of the hypertrophying heart. However, the contribution of protein degradation to hypertrophy and atrophy of the heart has not been adequately defined.

Damage to the anoxic or ischemic heart appeared to involve inhibition of protein synthesis and accelerated destruction of myocardial constituents. As noted above, insulin was effective in stimulating peptide-chain initiation in anoxic myocardium, but could not overcome the restraint on peptide-chain elongation that accompanied a deficiency of high-energy phosphates. Since both insulin and fatty acids restrained protein degradation, addition of these factors to anoxic or ischemic hearts offers the possibility of restraining destruction of myocardial components. This possibility has not been explored.

The roles of insulin and fatty acid in growth and repair of the heart involved stimulation of protein synthesis and inhibition of protein breakdown. The effect on the synthetic pathway was at the level of peptide-chain initiation. Restraint of protein degradation by insulin was associated with appearance of fewer autophagic vacuoles within the heart and lower activity of lysosomal enzymes. Understanding of the control of protein turnover by these and other factors offers the possibility of identifying the critical changes that are responsible for growth and repair of the myocardium.

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Discussion

Dr. E. I. Chazov, Moscow, U.S.S.R.: I am interested, first of all, in the percentage of disaggregation of polysomes with your methodology. Did you study this question?

Dr. Morgan: In unperfused hearts about 15% of the ribosomal RNA appears to be in the form of ribosomal subunits. After an hour or so of perfusion, this goes up to about 30% and then appears to remain essentially constant, so that polysomes will begin at about 85% and go down to something in the range of 70%.

Dr. Chazov: You have taken these percentages of polysomal disaggregation into consideration in your evaluation?

Dr. Morgan: We haven't tried to relate the rate of peptide-bond formation per polysome. It would appear, in fact, that the rate of protein synthesis might fall more than the number of polysomes, but I don't think our analysis has been good enough to settle that point.

Dr. Chazov: We have now begun to study these same questions in our laboratory, and we're interested in the following problem: Did you study the regulation of the process of synthesis at the level of peptide-chain termination? If so, how does insulin influence this process?

Dr. Morgan: The method we've used would not distinguish between a factor that affected elongation of a peptide chain as contrasted with termination. This analysis would include both of these steps as one phenomenon. I don't have any data on the control of peptide-chain termination. In terms of the effect of insulin on this overall process, there is no effect of insulin on the rate of protein synthesis in hearts in which peptide-chain initiation is accelerated by addition of fatty acids. This would argue against an effect of insulin on either elongation or termination of chains.

Dr. E. Morkin, Boston, Massachusetts: It has been suggested that the rate of protein synthesis increases very quickly, perhaps within an hour, following an increase in pressure load in isolated perfused hearts. Does this happen in your preparation? If so, is the increase in total synthesis the result of increased initiation of chains or more rapid translation on the ribosome?

Dr. Morgan: We haven't investigated the effects of pressure development as yet. The work to which you refer was carried out by Dr. Hjalmarsen, using the isolated working rat heart preparation. In that situation it appeared that work increased both the initiation and elongation of chains, but in those
studies the variety of the factors that could be involved in this effect was not studied. Additional work will be needed to identify these factors.

**Dr. S. E. Severin, Moscow, U.S.S.R.:** It is most important to discuss the mechanism of the effect of insulin. Of the three possible mechanisms of insulin's effect, which appears most likely: the first, permeability of the membrane, the delivery of amino acids into the cell; the second, the direct influence of insulin upon the initial stages of synthesis; or the third, the endurance of the formation of greater quantities of energy-rich compounds necessary for the process of biosynthesis?

**Dr. Morgan:** We have attempted to evaluate the role of an insulin effect on amino acid transport by measuring both the intracellular levels of free amino acids in the heart and the rate of entry of labeled amino acids from the perfusate. Insulin reduces the intracellular level of most amino acids. This would be consistent with the fact that the hormone stimulates protein synthesis and inhibits protein degradation. These effects would lead to depletion of the intracellular pool.

The effects of insulin on the entry of amino acid into the heart can be demonstrated using a non-metabolized amino acid, AIB, and a minority of the natural amino acids. There is no effect of insulin on the intracellular level of either ATP or GTP. The creatine phosphate levels, however, are higher in hearts perfused in the presence of insulin. I am not able to evaluate the physiological significance of these findings to the stimulation of protein synthesis. We do not know what the mechanism of the effect of insulin on initiation of peptide chains might be. It does not appear from studies in other laboratories that insulin reduces the cyclic-AMP level in heart muscle as it does in adipose tissue and a number of other cells; but in terms of a positive statement, I have no information.

**Dr. W. F. H. M. Mommaerts, Los Angeles, California:** Would this methodology also be suitable for investigating the mechanism of an effect of thyroxine upon specific aspects of protein synthesis? In our laboratory it was found some years ago that after hypophysectomy the myosin of cardiac muscle changes its properties in relatively few days and, as an ATP-splitting enzyme, remains only about half as active. This is a very interesting precedent of affecting gene expression at the functional level. Perhaps this methodology is suitable for explaining the mechanism by which it occurs.

**Dr. Morgan:** We have attempted to look at the rate of synthesis of light and heavy chains of myosin in hearts of hypophysectomized rats and those treated with thyroxine. These experiments suggest that the light and heavy chains are synthesized at about the same rate and that these rates are not largely affected by the presence of thyroxine. In principle, this methodology could be used for studies of this type and offers the possibility of avoiding many of the difficulties that arise in vivo with problems of precursor pools, specific activities, and other difficulties that Dr. Rabinowitz, I am sure, will describe to us in greater detail.

**Dr. A. M. Chernukh, Moscow, U.S.S.R.:** Would some pathological conditions characterized by disturbances in permeability where one can see products of protein degradation change the situation?

**Dr. Morgan:** In the last year or two, Doctors Neely, Rovetto, and I have devised a model of myocardial ischemia using the isolated perfused rat heart. In this model ischemia has a large effect upon the integrity of the myocardium. There appears to be very rapid destruction of the membranous elements of the cell as well as of the myofibrils and the intercalated disc. In relation to one of the questions that Professor Chazov raised earlier, the contact between the heart muscle cells is rapidly lost in ischemia. These changes could contribute to the genesis of cardiac arrhythmias. Whether changes in the intercalated disc can be prevented by insulin or other factors is the topic of current studies.

**Dr. Chazov:** Do you have any data regarding the interrelationship of the influence of insulin and other hormones on the rate of synthesis of catecholamines?

**Dr. Morgan:** The only experiments we have carried out in regard to catecholamines have been in association with Dr. Hjalmarson. He had found that catecholamines would inhibit the rate of protein synthesis. We looked into the effect of dibutyryl cyclic AMP on rates of synthesis. This compound, when added to the perfusate, reduces the rate of peptide-chain elongation. It also leads to a reduction in the ATP level of the heart. Whether the reduction of energy levels or other factors, such as phosphorylation of ribosomes, accounts for the inhibition is unknown.

**Dr. Chazov:** As I understand, catecholamines and insulin are, so to speak, opposite factors insofar as their activities are concerned. Can one influence the other to regulate the speed of synthesis?

**Dr. Morgan:** In the experiments we carried out, we attempted to see whether insulin would overcome the inhibitory effects of dibutyryl cyclic AMP. In these experiments the hormone stimulated peptide-chain initiation, but it did not over
come the inhibitory effects of the cyclic nucleotide on chain elongation.

**Dr. J. Gergely, Boston, Massachusetts:** It is well established that polypeptide chains of different sizes are synthesized on polysomes of correspondingly different sizes. Of course, this is of particular interest in muscle, since the heavy chain of myosin is much longer than practically any other polypeptide. I wonder if you have looked at a differential change in distribution of polysomes that might reflect differential changes in the rates of the synthesis and degradation of various proteins?

**Dr. Morgan:** We have measured the rate of the synthesis of myosin as a characteristic muscle protein. This would associate the effects of a hormone such as insulin with the cardiac muscle cells as contrasted to effects on connective tissue or other elements within the heart. The effects of insulin and fatty acid can be observed on the rate of myosin synthesis. On the other hand, quantitative recovery of polysomes from a muscle is difficult. About 75% of the polysomes are lost in the pellet of myofibrils in homogenates of heart muscle. We have not attempted to do a detailed analysis of size distributions of polysomes. An advance in the technology of recovery of these particles will be required before this will be successful.

**Dr. A. M. Vikhert, Moscow, U.S.S.R.:** How does the force of contraction of the isolated heart influence the synthesis of protein?

**Dr. Morgan:** I don’t have any information on that topic.

**Dr. L. F. Nikolaeva, Moscow, U.S.S.R.:** I would consider it very interesting to investigate the effect of hypoxia on the rate of protein synthesis, and also to examine the effect of experimental myocardial infarction. I understand that the latter will be quite difficult, as we know that the process of myocardial infarction leads to quite different conditions in different parts of the heart. Do you have some results concerning this process in hypoxia?

**Dr. Morgan:** Yes. We’ve attempted to see whether insulin would improve the rate of protein synthesis in hearts exposed to buffers containing a wide range of oxygen tensions. In a completely inoxic heart, insulin is unable to increase the rate of protein synthesis. If the buffer is gassed with a mixture containing 10% oxygen, a portion of the inhibitory effect of hypoxia is reversed by insulin. This effect is not large, however, and Dr. Rannels and I are more interested in the effects of the hormone on protein degradation, hoping that the effects may be larger than they appear to be in maintaining the rate of synthesis.

**Dr. Robert B. Jennings, Chicago, Illinois:** I would like to turn the discussion to your results at three hours of perfusion with glucose alone. I think the results of your and Dr. Rabinowitz’s studies have both been very striking, particularly in showing the high turnover rate of myocardial components. I assume that three hours of perfusion with glucose alone and the resultant occurrence of autophagic vacuoles are associated with either increased degradation or decreased synthesis.

The lysosomal study suggests that there is greater activity of at least one proteolytic enzyme. Is insulin’s effect due to providing an increased glycolytic flux—that is, increased energy for synthesis? How do you explain how the increased lysosomal enzyme activity reverts to control activity after administration of insulin? What is the significance of this observation?

**Dr. Morgan:** In a heart perfused for three hours with glucose as substrate, the rate of protein degradation is faster than the rate of protein synthesis. This is due to both an accelerated rate of degradation and an inhibition of synthesis. The effects that we observed on cathepsin-D activity were also seen on the activity of β-acetyl glucosaminidase, another lysosomal enzyme. When insulin is added after three hours of perfusion in its absence, the “available” activity of this enzyme goes down as well. We have examined the ultrastructure of these hearts. It would appear that the number of autophagic vacuoles begins to go down again after 90 minutes of exposure to the hormone. I have no idea as to the biochemical connection between the addition of insulin and the stability of the lysosomes contained in the tissue.

**Dr. Steven E. Mayer, La Jolla, California:** The discussion about insulin and catecholamines brings to mind some observations that have been made recently which indicate that cholinergic stimulation of liver cells and of leukocytes decreases the stability of lysosomal membranes, whereas beta-adrenergic stimulation increases their stability. It might be worthwhile to look at the effects of tonic adrenergic and cholinergic stimulation of the heart on protein degradation.

**Dr. Morgan:** Yes, I think it would be very interesting. We have looked at the effect of dibutyryl cyclic AMP on the release of phenylalanine from the heart. The cyclic nucleotide reduced the rate of phenylalanine release, suggesting that it might inhibit degradation, but these are preliminary experiments. We haven’t investigated this in detail.
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