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The hormonal and humoral regulators of myocardial protein metabolism : the in vivo measurement of the physiologic role of insulin and amino acids in cardiac protein kinetics

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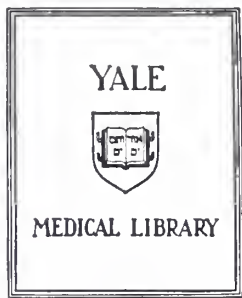*Yale University*

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IN VIVO ANABOLIC EFFECT OF INSULIN
AND AMINO ACIDS ON THE CANINE
HEART

DOUGLAS MIDDLETON DAHL

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Date

The Hormonal and Humoral Regulators of Myocardial Protein
Metabolism:

the *in vivo* measurement of the physiologic role of insulin and

amino acids in cardiac protein kinetics

Douglas Middleton Dahl

A thesis in partial fulfillment of the requirements for the degree

Doctor of Medicine

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Dedication

To my parents, Mary Middleton Dahl, M.D. and Douglas Seely Dahl, M.D., with enormous gratitude for their support, encouragement, and inspiring example.

Introduction

IN VIVO ANABOLIC EFFECT OF INSULIN AND AMINO ACIDS ON THE CANINE HEART. Douglas M. Dahl, Eugene J. Barrett, and Lawrence H. Young. Section of Cardiology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT. 1992.

Though it has been studied extensively *in vitro,* the physiologic role of insulin in regulating protein metabolism in the heart *in vivo* has not previously been determined. Using radioactively-labeled phenylalanine as a tracer molecule, we measured rates of protein synthesis and degradation in fasted, anesthetized dogs before and during a hyperinsulinemic (2mU/kg/min) euglycemic clamp, during a clamp and infusion of an amino acid solution, or during infusion with saline alone. We measured the uptake of radioactively-labeled phenylalanine from plasma and the release of unlabeled phenylalanine by cardiac muscle using arterial and coronary sinus catheterization during a continuous infusion of radioactive L-[ring-2,6-^{3}H] phenylalanine. After both a 16 hour (n=19) and a 36-hour fast (n=10), the heart was in net negative phenylalanine balance (-52±9 nmol/min, p<0.001; and -38±9, p<0.005 respectively), and those animals fasted 36 hours had a lower rate of protein degradation (81±13 vs. 121±12 nmol/min, p<0.05). Phenylalanine balance and rates of protein synthesis and degradation did not change in group I (n=10, fasted 36 hours) which received the hyperinsulinemic euglycemic clamp. In these animals, insulin produced a 30-40% decline in

plasma amino acid concentrations including branched chain (p<0.001) and essential amino acids (p<0.001). Group II (n=11, fasted overnight) received amino acid supplementation with the clamp. In these animals, myocardial phenylalanine balance changed from negative (-40±6 nmol/min) to neutral (6±7, p<0.05) because of an inhibition of heart protein degradation (118±12 vs. 60±7 nmol/min, p<0.001), with no change in rates of protein synthesis. Group III animals (n=8, fasted overnight) showed negative net phenylalanine balance which remained unchanged throughout the saline infusion. Hyperinsulinemia has a relative anabolic effect on the heart by inhibiting protein degradation. Hypoaminoacidemia blunts insulin's effect as an anabolic hormone.

We provide the first evidence in vivo that physiologic increments of plasma insulin have an important role in regulating myocardial protein turnover. Hyperinsulinemia appears to inhibit proteolysis of heart protein and has no effect of stimulating protein synthesis. The anti-proteolytic effect of insulin, however, is blunted by hypoaminoacidemia. The non-destructive method of measuring cardiac protein synthesis and degradation used in these large animal experiments offers the possibility of studying protein metabolism in humans and its response to a variety of interventions and disease states.

Background

Understanding the normal and pathologic growth of the heart is an important goal for physicians hoping to combat heart disease. When increased demands are placed on it, the heart adapts by growing in bulk. At first, the hypertrophied heart delivers greater power to meet the needs of the animal, but ultimately the enlarged heart fails as a pump. The process which initially improves the health of the organism eventually leaves it greatly weakened. This scenario is common in heart disease in humans. Disorders of the heart valves and hypertension are both common conditions in man which demand that the heart work harder to deliver blood. After a period which may last decades in which the enlargement of the heart restores hemodynamic equilibrium, the heart decompensates with often fatal consequences for the patient. This explains why the growth of the heart has been the focus of scientific study for over one hundred years (Zak, 1984).

The Importance of Understanding Myocardial Protein Kinetics

The heart is a muscular organ whose main function is to circulate blood throughout the body. As in other muscle tissues, the heart produces work through the coordinated contraction of millions of cells, each of which contains an elaborate contractile apparatus. The intracellular machinery for producing these carefully regulated contractions is composed largely of proteins.

Membrane channels, signal transduction proteins, and enzymes for cellular respiration support and regulate the function of the structural myofibrillar elements including actin and myosin. Taken together, protein comprises approximately 16% of the wet weight of muscle tissue.

Tissue protein undergoes continual turnover (Cahill, 1976). The cell degrades older proteins and replaces them with newly synthesized molecules. Because breakdown and synthesis occur simultaneously in each cell, the net flux of protein is considerably smaller than the individual rates for accumulation and degradation. Complex signals both within the cell and from outside it control the rates of these synthetic and degradative processes, so that depending upon these signals there may be net increase or decrease in total cellular protein. Thus the cell grows, remains at a constant mass, or atrophies in response to a large number of variations in nutritional supplies, hormonal signals, and work load.

Muscle serves two important functions for the organism. First, it has an obvious mechanical function in producing mobility of body and limbs. Second, as the body's largest reservoir of protein, it serves as a vital storehouse of nitrogen and carbon skeletons - in the form of amino acids - which are essential to support the normal growth and repair of other tissues. In times of nutritional deprivation or stress, the body also mobilizes these stores for use by the liver and kidneys as gluconeogenic substrates. In prolonged starvation, the body will sacrifice the strength of the muscle tissues in favor of providing metabolic

energy for the whole organism. In the long term - usually about 7 days in humans - the body adapts to nutritional deprivation so that muscle is relied upon less as a source of metabolic energy (Saudek, Vignati). The cost of this to the organism, however, is reduced functional capacity of the muscle tissue. Reduction of body protein by 8 to 17 percent in healthy people has been shown to compromise fitness. This amount of loss is commonly seen in critically ill hospital patients who often have accelerated catabolism and are unable to maintain adequate nutrition (reviewed in Wilmore, 1991).

It is well known that cardiac muscle hypertrophies and atrophies in response to functional demands placed on it. The transition seen in the heart at the time of birth nicely illustrates both phenomena. In the fetal heart, the right ventricle performs the bulk of the work of circulating blood. When the child breathes its first breaths, the right ventricle assumes a new role in providing volume at much reduced pressures to the newly low resistance pulmonary vascular bed. Concomitantly, the post-partum left ventricle must produce high pressures for the first time to meet the demands of the systemic circulation. The heart responds to this change in functional demands with rapid growth of the left ventricle and relative atrophy of the right ventricle. Experimental evidence has shown that this remodeling is associated with an accelerated rate of protein synthesis in the left ventricle which persists only until the size of the chamber is sufficiently large to accommodate its new workload efficiently (Robinson, 1990). In adults, pathologic hypertrophy of the left

ventricle is seen in people with stenotic or regurgitant valvular lesions and in systemic hypertension. Each of these conditions results in hypertrophy of the myocardium in response to increased work load to deliver the same volume of blood.

Controversy exists in the heart's role as a protein reservoir for the rest of the body. The heart's pumping function is so central to the survival of the organism that any functional compromise resulting from breakdown of its protein constituents could be very deleterious to the animal. The classical teaching was that "inanition has no harmful effect on the heart" (Vasquez, 1924). Several lines of evidence indicate that the heart responds differently than skeletal muscle to humoral signals which influence the rates of protein synthesis and breakdown, but the distinction is not consistent in all experiments (reviewed in Sugden, 1991). Review of studies of malnutrition in humans shows that myocardial skeletal muscle mass decline proportionally with resultant impairment in the heart's ability to generate cardiac output (Webb, 1986). The understanding of the regulation of heart growth has been impeded by the complexity of measuring the rates of protein synthesis and degradation in the heart in vivo. Diverse techniques have yielded sometimes conflicting evidence as discussed below.

Experimental Approaches to Quantifying Protein Synthesis

Investigators have used a variety of experimental methods to measure the rates of protein synthesis and degradation, and the

response of tissues to experimental interventions (reviewed in Waterlow 1978). Most methods employed to estimate the rate of protein synthesis, both in vitro and in vivo, involve measuring the incorporation of radioactively labeled amino acids into cellular protein during a specified time interval. The tracer molecules are administered as either a continuous infusion or with a flooding bolus (reviewed in Sugden, 1991). With this technique, the rates of synthesis in whole tissues or of individual proteins can be assayed. The expression of individual genes coding for specific proteins can also be measured through in vitro translation of mRNA isolated from the tissues under study (Dillman, 1983).

The accuracy of measurements of tissue protein synthesis which use labeled amino acid molecules rests on four assumptions. First, the specific activity of labeled amino acid in the measurable medium, e.g plasma, must be equivalent or have a known relationship to that of the immediate precursor pool within the tissue, the amino-acyl tRNA in the case of protein synthesis. Because proteins are continually turning over, the specific activity in the medium may be higher than that within the cytoplasm of the cell as that precursor pool is diluted by unlabeled amino acid from protein breakdown. This would potentially result in an underestimation of the protein synthetic rate using plasma specific activity. Second, any breakdown of proteins which contain labeled amino acid will also result in an underestimate of the synthetic rate. Third, the labeled amino acid must not undergo cellular loss of radioisotopic tracer which would alter the relationship between plasma and tRNA specific

activity. Fourth, the labeled amino acid itself must not behave differently, i.e. have lower affinity for cellular enzymes, than the native molecule.

The ideal amino acid tracer is one which rapidly equilibrates with the precursor pool and is not metabolized by the target tissue. Radioactively labeled phenylalanine, leucine, and tyrosine in culture medium each begin to be incorporated at linear rates into muscle protein (Morgan 1971, Jefferson 1977, Clark 1981). These amino acids are all transported with high activity by the L-system neutral amino acid transporter which transports branched chain and aromatic amino acids, but does not generate a transmembrane gradient of these molecules or maintain a large intracellular store (Banos 1973, Banos 1978, Morgan 1971, Cahill 1972, Waterlow 1978). Before incorporation into protein, amino acids are activated by acylation to tRNA. In tracer studies both in vitro and in vivo, comparison of the specific activity of total cellular amino acid with amino-acyl tRNA has demonstrated that the specific activity of the total cellular pool is lower (McKee 1978, Martin 1977, Everett 1981). This difference raises questions about the reliability of estimates which rely upon the assumption that exogenously introduced labeled amino acids freely equilibrate with all cellular amino acid pools. Fortunately, several studies have shown that extracellular specific activity closely reflects that of the cellular tRNA. In vivo studies administering a bolus of labeled leucine intravenously showed that plasma and leucyl-tRNA specific activities were nearly identical and that the equilibration was very rapid (<5 min.)

(Martin 1977, Everett 1981). Labeled phenylalanine at physiologic concentrations perfused into an isolated heart preparation showed that the specific activity of the medium and the phenylalanyl-tRNA were within 20% (McKee 1978). During continuous infusion in vivo, labeled phenylalanine has been demonstrated to equilibrate very rapidly between plasma and tissue tRNA precursor pools (Revkin, 1990). Thus in heart, measurements of labeled phenylalanine in plasma can be confidently used to estimate the specific activity of the immediate precursor pool to protein synthesis.

Measurement of Protein Degradation

Protein degradation has been much more difficult to assess and no method has gained acceptance as a reliable measure (reviewed in Barrett 1989). Indirectly, the rate of protein breakdown is often inferred from the difference between the measured rate of protein synthesis and the change in mass of the tissue over a given interval. While each of the two measurements is reliable, their combination is problematic. Synthetic rates measured by isotope incorporation are accurate when the experimental time course is minutes to a few hours. After that, the error introduced by recycling of amino acids, and variations in the hormonal milieu becomes more significant. Changes in the mass of muscle protein are only accurately determined over the course of days during which time the rate of protein synthesis may have fluctuated significantly. Combining these two

measurements compounds the potential error in a value determined for protein degradation (Barrett, 1989).

The drug cyclohexamide is a powerful inhibitor of ribosomal translation of mRNA into protein. In an experimental system in which protein ·synthesis is blocked pharmacologically, amino acid release measured by dilution of tracer specific activity should provide a valid measure of protein breakdown. Myocardial protein degradation theoretically may be determined by measurement of the arteriovenous difference in amino acid concentration across the heart after administration of cyclohexamide. One drawback, however, is that cyclohexamide is a potent toxin and its full effect on tissues is unknown and may influence the process of protein degradation itself (Goldberg 1974). Another limitation is that it obviously cannot be used in human investigation.

The measurement of 3-methylhistidine release by the heart might theoretically also allow a direct assay of protein breakdown. Formed by methylation of histidine residues bound in actin (Asatoor 1967) and in some forms of myosin, 3-methylhistidine is not reutilized for protein synthesis once it has been released in the degradative process (Young, VR 1970). The arteriovenous difference in 3-methylhistidine across skeletal muscle beds is so small as to preclude anything but a semiquantitative measure of myofibrillar protein breakdown (Barrett 1989). With a blood flow rate per gram of tissue approximately twenty times that of skeletal muscle and a lower 3-methylhistidine content, cardiac muscle will produce a

significantly lower arteriovenous gradient and therefore be even more difficult to study precisely.

A Novel Approach to Quantifying Protein Turnover

One of the greatest limitations to understanding the normal physiology of myocardial growth is that direct tissue sampling is required for most methods described above. Whether in atrial tissue in vitro (Cohen, 1969), in the isolated, perfused heart (Chua, 1979; Smith, 1983), or in sacrificed experimental animals (Samarel, 1987; Preedy, 1989), the rates of protein synthesis and degradation and the effects of experimental interventions are determined by methods which require tissue analysis. To study the heart in vivo by these techniques would require an open-chest model. The extensive surgical preparation required for such studies would add a confounding factor in extrapolating results to the truly physiologic state. Furthermore, they would necessitate that large tissue samples be taken from the heart. This would have limited applicability in studying the effects before and after interventions within the same animal, and obviously could not be used in man.

A novel method for simultaneously directly assessing both protein synthesis and protein degradation in vivo using isotopically-labeled amino acids was developed in this laboratory (Barrett, 1987; Revkin, 1990; see methods). Because phenylalanine's only metabolic fate in muscle tissue is incorporation into protein (Williams, 1981) and it is a well-represented amino acid in heart muscle protein (Morgan, 1971), it

can serve as a useful estimator of heart protein kinetics. By measuring the flux of phenylalanine in and out of heart muscle, we can estimate the kinetics of total protein turnover. We measured the dilution of specific activity and the balance of radioactively labeled phenylalanine across the heart in vivo. This technique affords a nondestructive method of investigating the effects of a variety of experimental interventions and allowed us to examine the physiologic role of insulin and amino acids in the regulation of myocardial protein synthesis.

Factors Influencing Myocardial Protein Metabolism

A wide variety of investigations into the regulation of heart protein synthesis and degradation have been performed. Many have examined the effects of starvation on protein synthesis and degradation (Curfman, 1980; Crie, 1980; Preedy, 1984; Samarel, 1987). In animal models, the influence of anoxia and ischemia (Kao, 1976; Chua, 1979; Smith, 1983; Preedy, 1989), pressure overload (reviewed in Zak, 1984 and Barrett, 1989), and circulating concentrations of various amino acids (reviewed in Sugden, 1991) have been extensively examined because of their obvious clinical relevance. Though patterns have emerged, the results are inconsistent and often contradictory due to the myriad technical considerations outlined above.

Since its discovery in 1922 (Banting, 1922), insulin's role as a hormone signalling the plenitude of nutrients to tissues has been copiously studied. Many experiments investigating insulin's role in cardiac protein metabolism have again left a complex set of

findings. In the isolated perfused heart, insulin has repeatedly been shown to stimulate protein synthesis (Morgan, 1971; Rannels, 1975; Preedy, 1984). In diabetic animals in vivo, heart protein synthesis is diminished in comparison with normals. Insulin has been demonstrated to restore the synthetic rate toward that seen in control animals (Pain, 1974; Ashford, 1986), but showed no stimulatory effect in nondiabetic animals. In another study, glucose infusion in young, growing rats (with its resultant hyperinsulinemia) did stimulate heart protein synthesis (Garlick, 1988). The degradative rate of protein in the isolated heart is reduced by high levels of insulin (Rannels, 1975; Curfman, 1980). A similar effect has been demonstrated in diabetic rats in vivo (Ashford, 1986).

The Current Study

Throughout the day in the normal animal, periods of fasting alternate with periods of eating. Following a meal, insulin is released and circulates in plasma at high levels, signaling to diverse tissues throughout the body that nutrients are plentiful. Between meals, the concentration of insulin is markedly diminished, but always present in measurable amounts. Studies of insulin's influence on cardiac protein metabolism carried out in myocardial tissue in vitro or in isolated perfused hearts have yielded valuable data, but the results are difficult to extrapolate to the situation in vivo. Furthermore, as discussed above, degradative rates of cardiac protein have been particularly difficult to determine and therefore not addressed by many

studies which measure rates of protein synthesis. Recent data suggest that insulin's most prominent role in regulating protein metabolism is to inhibit proteolysis. This is true in human skeletal muscle in which insulin has no effect on skeletal muscle protein synthesis but does block proteolysis (Gelfand, 1987; Fryburg, 1990). Studies in the normal adult rat have shown that insulin does not have the effect of increasing heart protein synthesis (Ashford, 1986), thus it appeared particularly important that the influence of insulin on the heart's rate of protein degradation be more thoroughly addressed.

We sought to define insulin's effect on protein metabolism in a physiologic experimental system. Utilizing the nondestructive isotopic method for simultaneously measuring protein synthesis and degradation (Barrett, 1987; Revkin, 1990), we undertook the study of the effects of fasting and physiologic hyperinsulinemia, both with and without amino acid replacement, on the protein kinetics of the canine heart.

Experimental Methods

Animal preparation

Twenty-nine adult mongrel dogs, each weighing 20 to 25 kilograms, were fasted either overnight (18 hours) or for 36 hours before the beginning of the study. Anesthesia was induced with intravenous sodium pentobarbital (20 mg/kg) and maintained with either sodium pentobarbital or intravenous alpha chloralose urethane. The animals were intubated and ventilated with room air. An electrocardiogram was monitored continuously and arterial blood gas measurements were made at regular intervals throughout the experimental protocol. Arterial and coronary venous blood was sampled from catheters in the right femoral artery and coronary sinus. Arterial blood was sampled and blood pressure monitored through a 7 French arterial catheter. Arterial blood pressure was measured with a pressure transducer (P23XL, Spectramed, Inc., Oxnard, CA) and recorded by a multi-channel strip chart recorder (Gould Inc., Cleveland, OH). Under fluoroscopic guidance, two catheters were placed into the mid coronary sinus. One 6 French multipurpose catheter was placed for blood sampling. A second catheter, a 7 French Baim thermodilution flow catheter (Electrocatheter Co., Rahway, N.J.) was inserted into the mid coronary sinus to measure coronary venous flow rates by the thermodilution method (Ganz 1971, Pepine 1978). Position of the coronary sinus catheters was verified to be sufficiently distal in the coronary sinus to avoid admixture of coronary sinus and right atrial blood which occurs

with more proximal placement. The coronary sinus catheters were secured before the first sampling period and their positions reconfirmed by fluoroscopy at the conclusion of the experiment. The patency of all catheters was maintained with intermittent flushes of heparinized saline (Heparin 500 U/L in 0.9% saline).

Infusion Protocols

For 60 to 90 minutes prior to the initial blood sampling, each animal received a continuous infusion of L-[ring-2,6-^{3}H]-phenylalanine (0.75 mCi/min) (Amersham Corp., Arlington Heights, IL) via a hindlimb vein. After this tracer equilibration period, basal arterial and coronary sinus blood was sampled four times, and three coronary sinus flow measurements made during a 15-minute period. Following baseline sampling, a primed (4 mU/kg/min for 10 minutes) continuous (2 mU/kg/min) infusion of regular porcine insulin (Eli Lilly Corp., Indianapolis, IN) was begun. Blood glucose was maintained at baseline levels by adjusting the rate of intravenous infusion of 20% dextrose according to the hyperinsulinemic euglycemic clamp technique (DeFronzo 1979). We undertook three distinct experiments:

Group I - fasted 36 hours, received hyperinsulinemic euglycemic clamp alone for two hours.

Group II - fasted overnight, received hyperinsulinemic euglycemic clamp for two and a half hours and supplement with amino acid infusion.

Group III - fasted overnight, received two hour saline infusion alone.

Ten animals fasted for 36 hours underwent a two-hour euglycemic clamp. In the animals that received supplemental amino acids (n=11, overnight fasted), the clamp time was extended to two and a half hours to assure steady state plasma levels of the amino acids. The amino acids were given as a continuous infusion of a balanced solution (Travasol™, Clintec Nutrition Co., Deerfield, IL) to prevent the hypoaminoacidemia associated with insulin infusion (Schwartz, Fukagawa). The infusion of 1.2 mg/kg/min of Travasol™ delivered 6.2×10^{-2} mg/kg/min of unlabeled phenylalanine which maintained the plasma concentration of phenylalanine at approximately the baseline levels. An additional group of eight animals fasted overnight received infusions only of saline and labeled phenylalanine as above. During the last 15 minutes of each of these three infusion protocols, arterial and coronary blood samples were again drawn in quadruplicate and coronary sinus flow measurements by thermodilution were made in duplicate.

Analytic Measurements

The concentration of glucose in whole blood was measured using the glucose oxidase method (Yellow Springs Instruments, Yellow Springs, OH). Blood was collected in heparinized tubes and centrifuged for 10 minutes. The plasma was separated from each blood sample and diluted 1:1 with 6% sulphosalicylic acid to precipitate protein, centrifuged for 30 minutes, and aliquots of the acid extracts were taken to assay concentrations of neutral and acidic amino acids by an automated ion-exchange

chromatographic technique (Dionex D-500, Sunnyvale, CA) (Barrett 1987).

High performance liquid chromatography (HPLC) was used to measure phenylalanine concentrations more precisely. A second portion of supernatant (2ml) was passed over a Dowex 50W ion exchange column (Biorad Laboratories, Richmond, CA) which retains amino acids but not their oxo derivatives. Amino acids were eluted with 1M NH_4OH. The eluant was dried and reconstituted with 2% trichloroacetic acid. Next, 0.2 ml of this solution (equivalent to 0.5 ml of plasma) was applied to a reverse-phase HPLC column (Ultrasphere ODS [C-18, 5mm], Beckman Instruments, Inc., Fullerton, CA) and eluted isocratically with a mobile phase consisting of 16% methanol (vol/vol) containing a phosphoric acid buffer and heptane sulfonic acid as an ion pairing agent (Low UV-PIC B7, Waters Associates, Milford, Mass.). The column eluant was monitored for ultraviolet absorbance at 214 nm, and a sharply separated phenylalanine peak was eluted at 14-16 minutes. This fraction was collected in scintillation vials and counted for ^{3}H radioactivity by a liquid scintillation system (Packard Instruments, Downers Grove, Ill.). The mass of phenylalanine in each sample was calculated by comparing its peak area with that of prepared standards using a chromatography software package (Nelson Analytical, Cupertino, CA). Specific activity is calculated as the ^{3}H radioactivity divided by the mass of phenylalanine.

In control experiments (Barrett, 1987), recovery of labeled phenylalanine from the Dowex column was greater than 95%.

Using the above HPLC method, the coefficient of variation of phenylalanine concentration was ±1.9%, ±1.4% for measurements of radioactivity, and ±2.2% for specific activity (Revkin, 1990). At the end of a three-hour infusion of labeled phenylalanine in the dog, more than 95% of the ^3H in plasma amino acids is present in phenylalanine and less than 1% is in its metabolic product tyrosine (Barrett, 1987).

Calculations

Rates of protein synthesis and degradation were determined by measurements of the kinetics of labeled phenylalanine in the heart. The derivation of the formulae used to estimate these rates begins with the equation which estimates protein degradation from dilution of plasma phenylalanine specific activity (SA):

Degradation = [Phe]$_{arterial}$ x {(SA$_{art}$/SA$_{cs}$) - 1} x flow   (1)

where [Phe] is the concentration of phenylalanine in arterial plasma, SA is the phenylalanine specific activity in arterial and coronary sinus plasma, and flow is the coronary sinus blood flow measured by thermodilution. This gives the rate of phenylalanine release from heart muscle protein in nmol/min.

Labeled amino acid is also extracted by myocardial tissue. The extraction ratio is given by the expression:

ER = (DPM$_{art}$ - DPM$_{cs}$) / DPM$_{art}$ (2)

where DPM is radioactive disintegrations per minute of labeled phenylalanine per milliliter of plasma.

Phenylalanine is neither synthesized nor metabolized by the heart. The difference between its rate of incorporation into protein and its rate of release by degradation of protein therefore defines its net balance across the heart:

phenylalanine balance = synthesis - degradation   (3)

where these values are expressed in nanomoles of phenylalanine per minute. The balance is determined directly by the measurements of the amino acid concentration and rate of flow across the heart:

$$\text{balance} = ([\text{Phe}]_{art} - [\text{Phe}]_{cs}) \times \text{flow}_{cs} \qquad (4)$$

By algebraically rearranging equations 4 and 1 and incorporating them into equation 3, a solution emerges by which protein synthesis can be determined directly:

$$\text{synthesis} = \{(\text{DPM}_{art} - \text{DPM}_{cs})/ \text{SA}_{cs}\}*\text{flow} \qquad (5)$$

The rates of incorporation and release of phenylalanine are converted to rates of tissue protein synthesis and degradation by correcting for the measured molar representation of phenylalanine in myocardial protein (290 nmol/mg).

*A Note on Authorship

*(In compliance with the recommendation of the Thesis Subcommittee of the Educational Policy and Curriculum Committee.)

The animal preparation and experimentation is a complex undertaking requiring the contribution of several workers. For the insulin and the insulin plus amino acids groups, Lawrence Young M.D., Eugene Barrett M.D. Ph.D., and I performed the bulk of the work. On several occasions I have performed each of the procedures described in "animal preparation" and "infusion protocols", including preparing the infusion solutions, performing the surgical cutdowns and placement of the arterial and venous catheters, and performing the euglycemic clamp. We were assisted by Jon Alderman, George Harnish, and Jill Seres. For the insulin alone group and approximately half of the insulin plus amino acids group, I performed all the separations and chemical analyses described in "analytic measurements" with the exceptions below. For all experiments, Ralph Jacob, Ph.D. ran the automated amino acid analyzer and Rosa Hendler M.D. measured insulin concentration by radioimmunoassay. Deborah Rauner processed the blood samples for half the insulin plus amino acids group and the entire saline infusion group.

Results

Metabolic effects of the euglycemic clamps.

Animals undergoing the euglycemic clamp should demonstrate evidence of the effects of insulin, including increased glucose disposal, while maintaining their plasma glucose concentration. In the animals fasted 36 hours, insulin infusion without amino acid replacement increased plasma insulin from 9±1 to 106±9 mU/ml (p<0.001). This hyperinsulinemia resulted in a whole body glucose utilization of 8.0±0.6 mg/kg/min and increased myocardial glucose uptake more than doubled (2.2-fold increase, p<0.005) (Table I). Whole blood glucose was maintained constant at basal levels (63±2 mg/dl) during insulin infusion without amino acid replacement.

Though the basal blood glucose concentrations in the animals fasted overnight that received the euglycemic clamp and amino acid infusion were significantly higher than in the 36-hour fasted animals (70±2 vs. 63±2, p<0.01), they had similarly low basal plasma insulin concentrations (9±1 mU/ml) and myocardial glucose disposal (2.6±0.8 and 3.1±0.6 respectively, p=not sig.). The overnight-fasted group receiving both insulin and amino acids responded with a whole body glucose disposal (8.7±0.8 mg/kg/min) and increased myocardial uptake (2.2-fold increase, p<0.001) not significantly different than the 36-hour fasted group at the

Table I: Arterial Glucose and Insulin, Whole Body and Heart Glucose Uptake

	Blood Glucose (mg/dl)	Plasma Insulin (μU/ml)	M (mg/kg/min)	Myocardial Glucose Uptake (mg/min)
Euglycemic Clamp (36-hour fast)				
Basal	63±2	9±1		3.1±0.6
Insulin	65±1	106±9*	8.0±0.6	6.8±0.9†
Euglycemic Clamp + Amino Acids (Overnight fast)				
Basal	70±2¥	9±1		2.6±0.8
Insulin	71±2§	106±14*	8.7±0.8	5.7±0.9*
Saline Infusion (Overnight fast)				
Basal	66±2	10±1		2.6±0.6
Saline	65±3	7±1		2.6±0.6

Data are mean values ± Standard Error of the Mean for the arterial concentrations of glucose and insulin and for myocardial glucose uptake during the basal period (Basal) and the last 15 minutes of the infusion protocols (Insulin or Saline).   M is the mean whole body glucose utilization rate during the euglycemic clamps (see Methods).   *$p < 0.001$ and †$p < 0.005$ compared to basal value determined by paired t test; ¥$p < 0.01$, §$p < 0.05$ compared to Euglycemic clamp by unpaired t test.

same circulating insulin concentration (106±14mU/ml) (Table I).

In the group of animals fasted overnight that received saline infusion alone, the baseline whole blood glucose, plasma insulin, and myocardial glucose utilization were similar to the overnight-fasted animals that received insulin and amino acid infusions. In the saline control group, these characteristics did not change during the course of the saline infusion (Table I).

Hemodynamics and Coronary Blood Flow

Heart rate, blood pressure, and coronary sinus blood flow were monitored throughout each experiment. Coronary sinus blood flow was similar in all three groups and did not change during the course of the experiments (Table II). At baseline, animals fasted 36 hours had slightly higher baseline heart rates and significantly greater mean arterial blood pressures than animals fasted overnight (p<0.02). All groups had consistent hemodynamic parameters except the insulin plus amino acid group which experienced a modest, but statistically significant 13% rise in arterial blood pressure during the course of the experiment (p<0.02 for systolic BP, p<0.05 for mean arterial pressure) (Table II).

Effects of Infusions on Amino Acid Concentrations

Concentrations of plasma amino acids were measured in quadruplicate plasma samples at baseline and again during the last 20 minutes of each infusion. Data are from an automated amino acid analyzer (see Methods). The mean values of individual

Table II: Hemodynamics and Coronary Blood Flow

	Heart Rate (min^{-1})	Systolic BP (mm Hg)	Mean BP (mm Hg)	Double Product (mmHg/min$\cdot$10^{-3})	Coronary Blood Flow (ml/min)
Euglycemic Clamp					
Basal	159±9	165±4	146±6	25.7±2.2	57±4
Insulin	154±11	167±3	149±5	25.3±2.2	58±6
Euglycemic Clamp + Amino Acids					
Basal	142±9	139±9	113±6	20.0±2.2	60±9
Insulin	139±7	157±5*	128±4†	22.1±1.7	63±9
Saline					
Basal	138±16	165±9	137±7	23.0±3.2	62±8
Saline	159±11	173±5	146±2	27.7±2.3	64±9

Data are mean ± Standard Error of the Mean of quadruplicate values measured prior to (Basal) and during the last 15 minutes of the infusion protocols (Insulin or Saline).   *p < 0.02 and †p < 0.05 compared to basal by paired t test.

amino acids from quadruplicate samples in each experimental group are shown in Table III. Figure 1 displays graphically the summed values for branched chain, essential, and total amino acids.

In the group of animals fasted 36 hours that received insulin alone, concentrations of nearly all amino acids measured - non-basic amino acids - decreased substantially (Table III). Infusion of insulin significantly reduced the concentrations of the essential amino acids threonine and valine ($p < 0.02$ for each), and methionine, isoleucine, leucine, tyrosine, and phenylalanine ($p < 0.001$ for each) (Table III). Overall, the measured essential amino acids decreased 37% during the euglycemic clamp. The total amino acid concentration fell 34% and the concentration of the branched-chain amino acids (leucine, isoleucine, and valine) fell 37% with insulin infusion (figure 1).

Because of the concern that the hypoaminoacidemia resulting from infusion of insulin alone may prevent the observation of an anabolic effect on myocardial protein turnover, we studied a second group in which a balanced amino acid solution was infused during the euglycemic clamp. Our goal was to maintain the plasma amino acid concentrations at levels observed prior to the beginning of the clamp. Figure 1 demonstrates that the overall concentrations of the branched-chain, essential, and total amino acids did not significantly change from baseline levels. Because of the concentrations of amino acids in our infusion mixture (Travesol™, see methods), individual amino acid concentrations varied though overall there was no significant

Table III: Arterial Amino Acid Concentrations (μM)

	Euglycemic Clamp		Clamp + A.A.		Saline	
	Basal	Insulin	Basal	Insulin	Basal	Insulin
Threonine	193±45	124±23[*]	139±14	123±9	154±13	146±14
Serine	94±8	66±8[†]	84±10	108±7	97±10	90±6
Asparagine	39±4[φ]	21±2[¥]	29±2	15±1[¥]	31±2	30±3
Glutamate	15±2	13±1	14±1	13±1	14±2	14±2
Glutamine	386±39	265±26[¥]	382±28	305±17[§]	445±34	427±44
Glycine	129±16	91±10[¶]	91±6	185±7[¥]	145±14	124±14
Alanine	375±72[φ]	280±36	209±16	382±14[¥]	210±24	169±15
Valine	160±14	123±16[*]	159±10	165±7	141±8	180±20[¶]
Cysteine	24±2	24±2	22±2	20±1	24±1	27±1
Methionine	30±2[θ]	15±2[¥]	26±3[θ]	33±2[*]	46±5	47±5
Isoleucine	34±4[θ]	10±1[¥]	39±4	59±2[¶]	54±7	86±14[*]
Leucine	90±11	41±8[¥]	101±9	70±3[¶]	96±8	136±18[§]
Tyrosine	30±4	17±2[¥]	25±2	13±1[¥]	31±2	36±4
Phenylalanine	44±3	32±2[¥]	49±4	74±4[¥]	50±2	59±3[¶]

Data represents mean values ± Standard Error of the Mean for arterial plasma amino acid concentrations during the basal period (Basal) and the last 15 minutes of either the euglycemic clamps (Insulin) or saline infusions (Saline). [*]p<0.02, [†]p<0.01, [¥]p<0.001, [§]p<0.05, [¶]p<0.005, compared to basal by paired t test. [φ]p<0.05 compared to 16 hour fasted animals and [θ]p<0.05 compared to saline infused animals (fasted 16 hours) by ANOVA. n=4 for threonine values available in each group.

Figure 1A: Total Amino Acid Concentrations
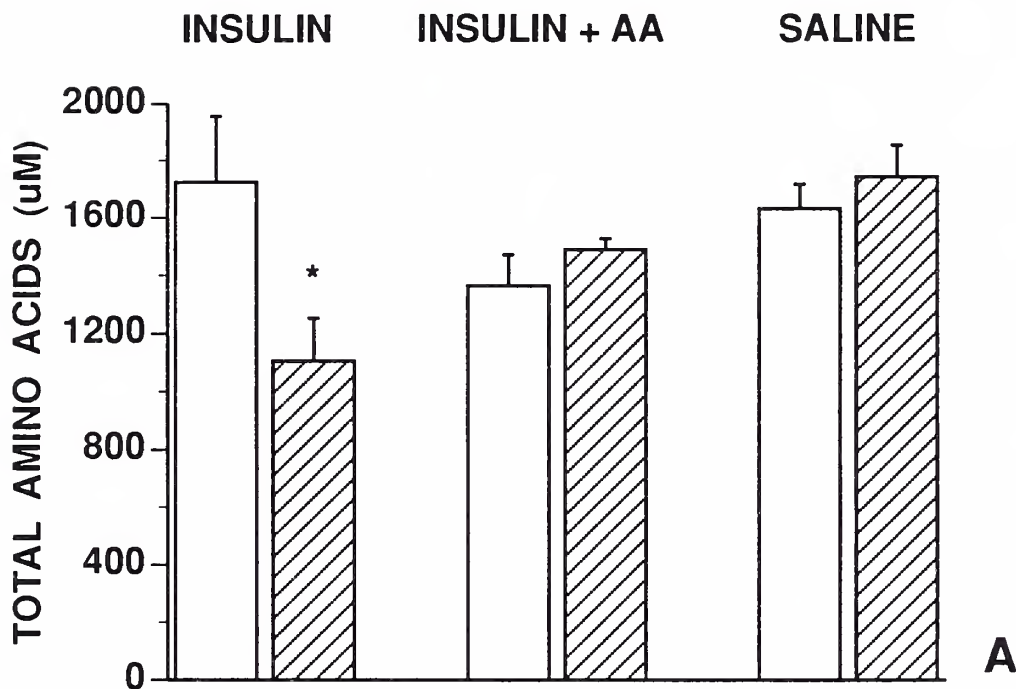
Arterial concentrations of total (non-basic) amino acids. Data are mean values ± standard error of the mean prior to experimental infusions (open bars) and during the last 15 minutes of the protocol (hatched bars). INSULIN represents the euglycemic clamp group, INSULIN + AA is the group which received the euglycemic clamp plus amino acids, and SALINE indicates the group which received saline infusion only. *p<0.005 compared with baseline state.

Figure 1B: Branched Chain Amino Acid Concentrations
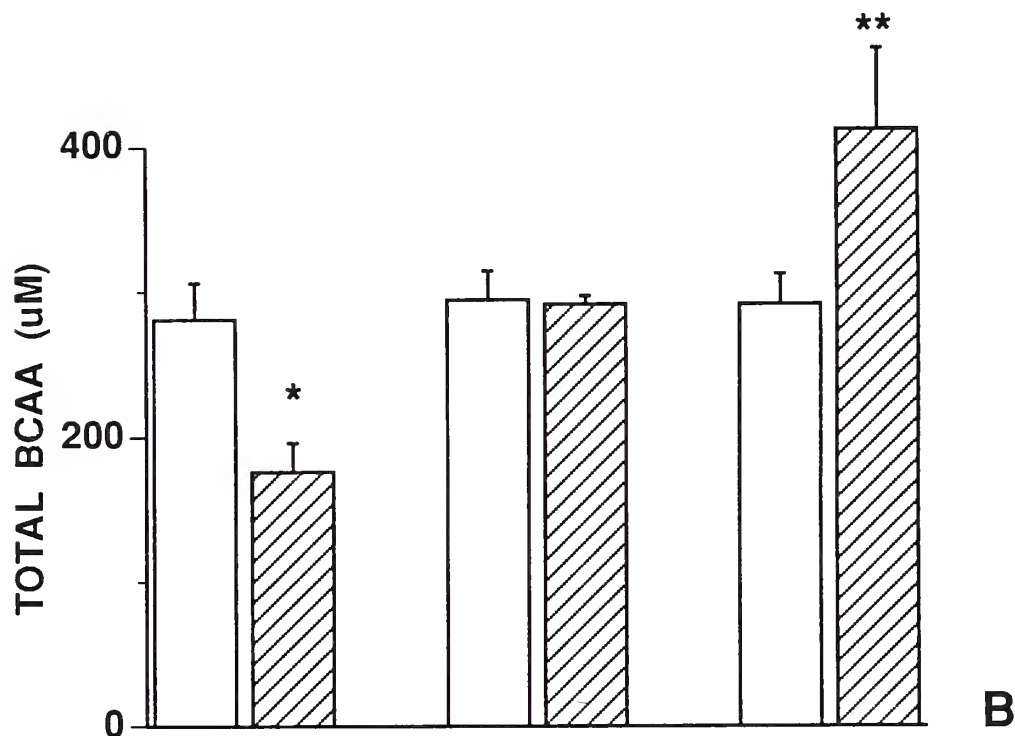
Arterial concentrations of total branched chain amino acids. Data are mean values ± standard error of the mean prior to experimental infusions (open bars) and during the last 15 minutes of the protocol (hatched bars). INSULIN represents the euglycemic clamp group, INSULIN + AA is the group which received the euglycemic clamp plus amino acids, and SALINE indicates the group which received saline infusion only. *p<0.005, **p<0.02 compared with baseline state.

Figure 1C: Essential Amino Acid Concentrations
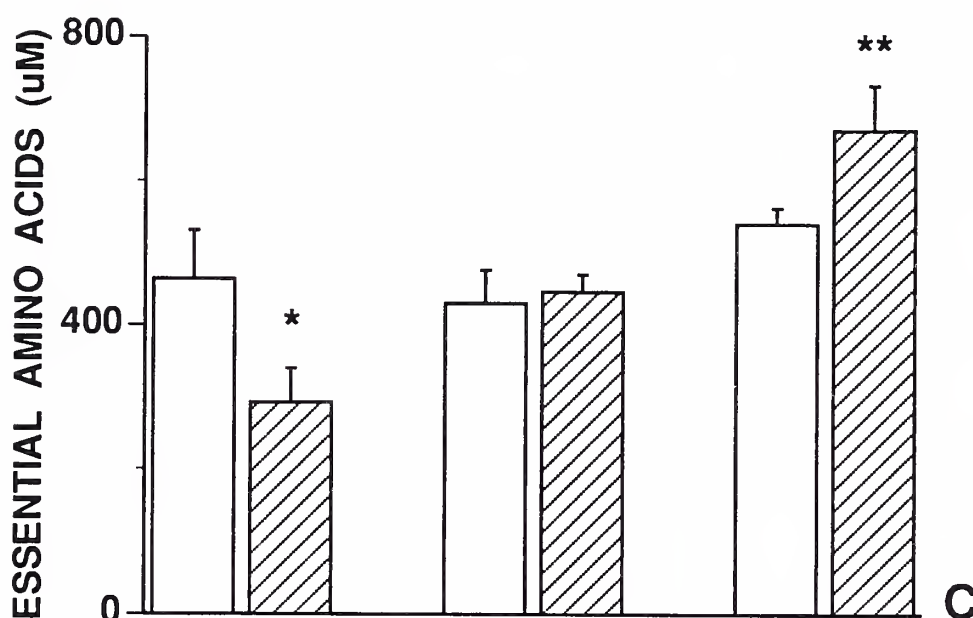
Arterial concentrations of measured essential amino acids.   Data are mean values ± standard error of the mean prior to experimental infusions (open bars) and during the last 15 minutes of the protocol (hatched bars).   INSULIN represents the euglycemic clamp group, INSULIN + AA is the group which received the euglycemic clamp plus amino acids, and SALINE indicates the group which received saline infusion only.   *p<0.005, **p<0.02 compared with baseline state.

change in the summed amino acid concentrations. The essential amino acids methionine, isoleucine, and phenylalanine showed a statistically significant increase in concentration. Of other essential amino acids, leucine decreased and valine and threonine were unchanged (Table III).

In the control group of animals receiving saline infusion alone that served to examine the metabolic effects of undergoing anesthesia and catheterization, the total amino acid concentration remained stable (figure 1). The concentrations of the essential and branched-chain amino acids, however, showed statistically significant increases (p<0.02 for each) over the two-hour infusion of saline (figure 1), as did the concentration of phenylalanine (Table III).

Rates of Synthesis and Degradation by Phe Kinetics

Insulin Infusion

After a fast of 36 hours, the concentration of phenylalanine was higher in the coronary sinus of 9 of 10 animals studied (mean difference 1.02 ± 0.22 nmol/ml, p<0.001). There was net negative balance of phenylalanine during the basal period (-38 ± 9 nmol/min, p<0.005 compared with the null hypothesis value of 0) which indicates that the rate of myocardial protein degradation exceeds the rate of synthesis. The rates of degradation and synthesis were estimated from the dilution of specific activity and extraction of labeled phenylalanine using equations 1 and 5 respectively (see methods). In the basal state, ^{3}H-phenylalanine

was significantly extracted from coronary arterial plasma (3 ± 1%, p<0.01) and ^{3}H-phenylalanine specific activity was significantly diluted (6 ± 1%, p<0.001). When incorporated into the above equations, the rate of myocardial protein degradation was 81 ± 13 nmol/min, while the rate of synthesis was 38 ± 17 nmol/min (figure 2). Following two hours of the euglycemic clamp, the phenylalanine balance and the rates of heart protein degradation and synthesis were not significantly different from the basal state (figure 2).

Insulin and Amino Acid Infusion

In overnight-fasted animals prior to insulin and amino acid infusion, we observed again a net negative phenylalanine balance (-40±6nmol/min, p<0.001 different from 0), with higher coronary sinus than arterial phenylalanine concentrations (mean difference 1.12±0.15 nmol/ml). There was significant basal extraction of ^{3}H-phenylalanine (5±1%, p<0.001) and dilution of ^{3}H-phenylalanine specific activity (7±1%, p<0.001) across the heart. This yielded rates of protein synthesis and degradation during the basal period of 79±8 nmol/min and 118±12 nmol/min respectively (figure 2). During the last 15 minutes of a two and one-half hour hyperinsulinemic euglycemic clamp with infusion of amino acids, the myocardial phenylalanine balance changed significantly (p<0.05) from negative (-40±6nmol/min) to slightly positive (6±15 nmol/min). The synthetic rate of protein did not significantly change, but there was inhibition of protein degradation which fell 50% from 118±12 nmol/min during the basal state to 60±7

nmol/min during the clamp (p<0.001).   Thus the net anabolic effect of insulin and amino acid infusion in these overnight-fasted dogs was due to inhibition of protein degradation, rather than stimulation of protein synthesis.

Figure 2A: Cumulative Rates of Synthesis, Degradation, and Balance (Group I)
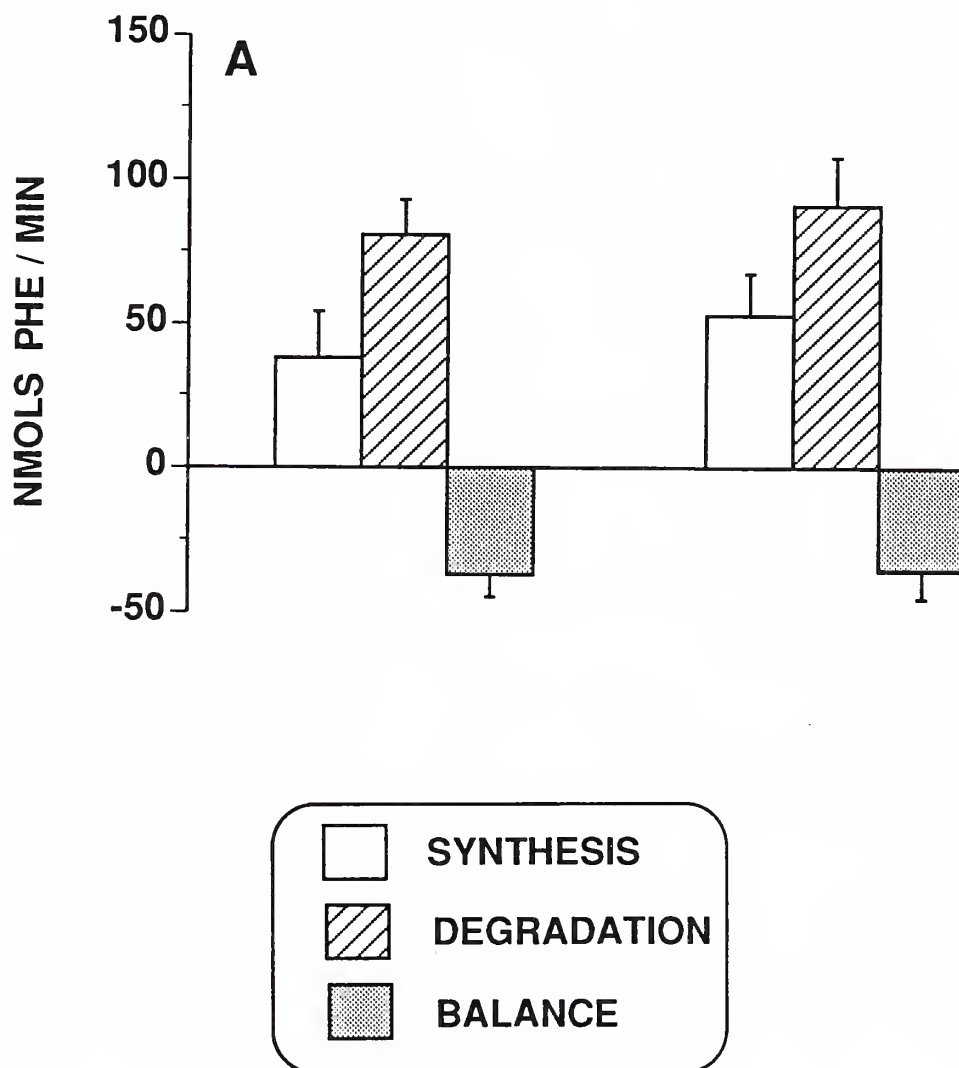
Rates of myocardial protein synthesis, degradation, and phenylalanine balance prior to experimental infusions (left group of bars) and during the last 15 minutes (right group of bars) of intervention.   Figure 1A is the euglycemic clamp group.   Data are mean values ± standard error of the mean.

Figure 2B: Cumulative Rates of Synthesis, Degradation, and Balance (Group II)
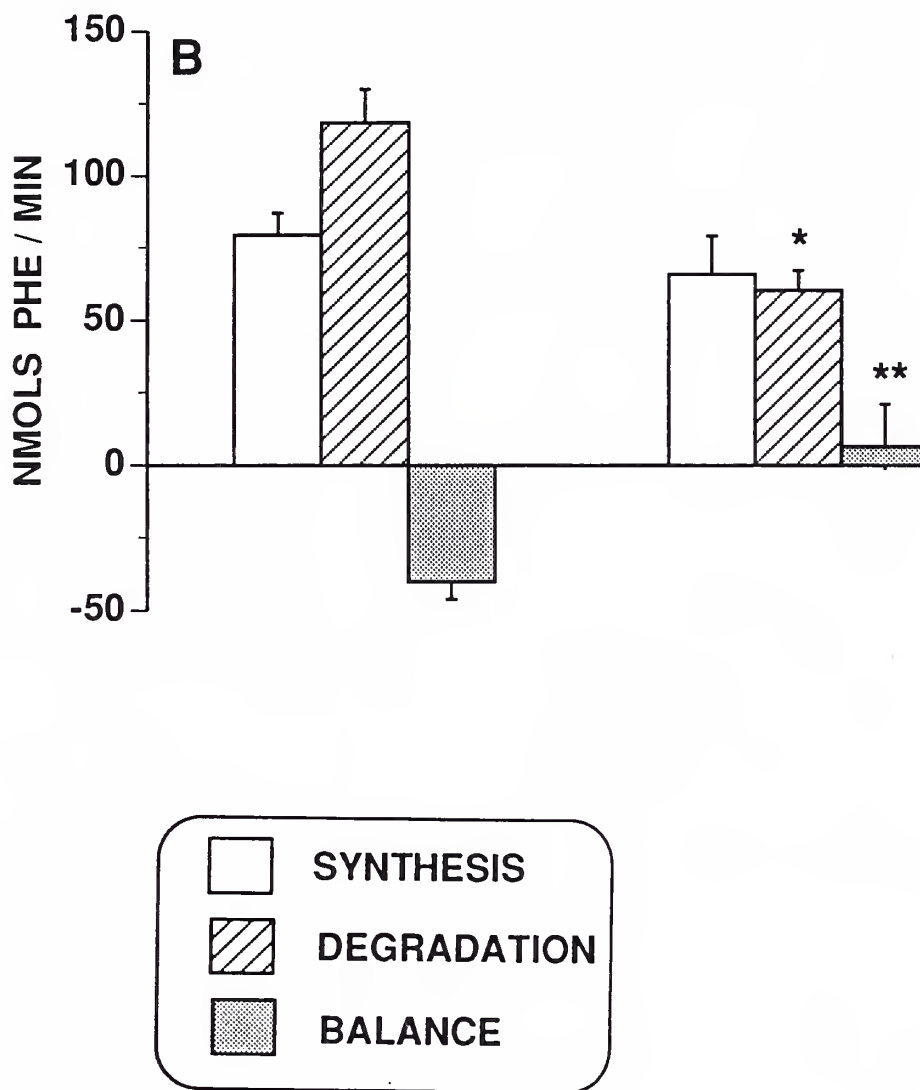
Rates of myocardial protein synthesis, degradation, and phenylalanine balance prior to experimental infusions (left group of bars) and during the last 15 minutes (right group of bars) of intervention. Figure 2B represents animals which received the euglycemic clamp with amino acid infusion. Data are mean values ± standard error of the mean. *p<0.05, **p<0.005 compared with pre-infusion values.

Figure 2C: Cumulative Rates of Synthesis, Degradation, and Balance (Group III)
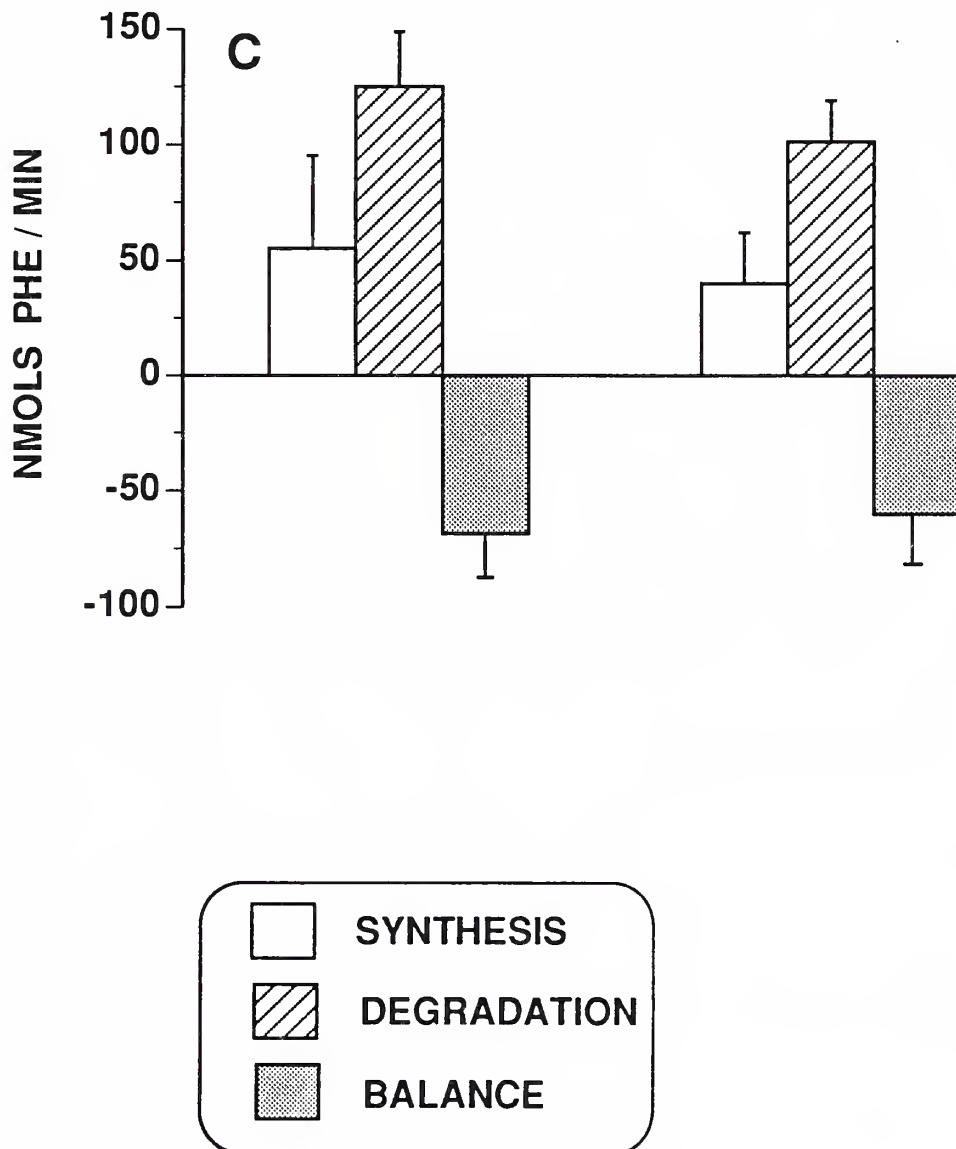
Rates of myocardial protein synthesis, degradation, and phenylalanine balance prior to experimental infusions (left group of bars) and during the last 15 minutes (right group of bars) of intervention. Figure 2C represents animals which received saline infusion alone. Data are mean values ± standard error of the mean.

Saline Infusion

Following an overnight fast, the animals in the saline control group also had a net negative arterial-coronary sinus phenylalanine concentration difference (-1.72±0.36 nmol/ml, p<0.005), and a negative phenylalanine balance (-68±19 nmol/min, p<0.01 compared to 0). Following two hours of saline infusion, phenylalanine balance remained negative (-60±17 nmol/min); a finding significantly different from the neutral balance achieved in animals receiving insulin and amino acid infusion (p<0.01). Myocardial protein synthetic rate did not change significantly during the experiment and was similar to the synthetic rates in the other experimental groups. The rate of heart protein degradation at the end of saline infusion (101±18 nmol/min) significantly exceeded that seen in overnight-fasted animals receiving insulin and amino acid infusion (60±7 nmol/min, p<0.05) (figure 2).

Discussion

Employing a novel method of measuring protein synthesis and degradation, we have provided the first evidence that physiologic hyperinsulinemia and circulating amino acids may have an important role in determining cardiac protein metabolism in vivo. Measurement of the balance of phenylalanine across the heart provides an overall assessment of net protein kinetics. Without the necessity of direct tissue biopsy for biochemical analysis, the isotope dilution method also permits the first analysis of the effects of experimental interventions within the same animal. Furthermore, with this technique one has the ability to measure simultaneously rates of synthesis and degradation and to monitor minute to minute the changes in heart protein metabolism with changing experimental conditions. While other investigators have determined rates of protein degradation by calculating the difference between rates of synthesis determined by isotope incorporation and change in heart mass over time (reviewed in Barrett, 1989), we rely on direct measurements of the dilution of tracer phenylalanine to provide an independent measure of degradation.

Though the isotope dilution method overcomes many of the limitations inherent in previously employed techniques to study cardiac protein synthesis, several theoretical constraints pertain in interpreting results from the current study. We assume, though do not prove, that the tritium-labeled phenylalanine employed as a tracer molecule is handled identically to the native amino acid.

Furthermore, we assume that the size and specific activity of the intracellular free phenylalanine pool is not changing during the measurement period. There is no evidence that an isotope effect exists for tritium-labeled phenylalanine or that phenylalanine is concentrated in a cellular store, so with the constant plasma levels maintained in our protocol it is unlikely that a significant gradient exists. The major uncertainty in the current method is the assumption that venous phenylalanine specific activity we measure closely approximates the specific activity of phenlylalanyl-tRNA in cardiac muscle cells. As protein is degraded inside cells, unlabeled phenylalanine is released to mix with the labeled molecule, lowering specific activity relative to that in arterial blood. Though in previous investigation in this laboratory in rat hearts phenylalanine has been shown to equilibrate rapidly among the tRNA, free cellular, and venous plasma pools (Revkin, 1990), we cannot exclude a significant species difference at work in the dogs. It is technically impractical, unfortunately, to quantify the tRNA specific activity in large animals because of the enormous quantity of radioactive tracer molecule it would require for valid measurements.

To study accurately the kinetics of protein turnover in the heart, it was necessary to refine the isotope dilution technique as originally described (Barrett, 1987). Because of the heart's significantly greater oxygen requirement, the rate of blood flow per gram of tissue is 30-fold higher in cardiac than in skeletal muscle (Liang, 1982). Assuming comparable rates of protein turnover, the much greater flow means that a much lower

fractional extraction of amino acid is necessary to provide for protein synthesis and the dilution of specific activity from protein degradation is also substantially less. This explains the significantly narrower arteriovenous differences in phenylalanine radioactivity and specific activity seen across the heart than across skeletal muscle in previous studies from this laboratory (Revkin, 1990; Barrett, 1987; Gelfand, 1987). With the introduction of phenylalanine analysis by HPLC in the current experiment, the new precision in our measurements of amino acid balance (1.8% coefficient of variation vs. 5-7% with previous method) allowed us to investigate cardiac protein metabolism having minimized the systematic error in this measurement. In the experimental group which received amino acid supplementation, total phenylalanine increased 50% from baseline. This resulted in even smaller fractional dilution of specific activity, but the precision of this refined technique permitted us to obtain reproducible measurements even in this experimental group.

We studied the effects of physiologic interventions on three groups of fasted dogs. We measured the rates of protein synthesis and degradation in animals fasted 36 hours before and after a two-hour hyperinsulinemic euglycemic clamp. A second group, fasted overnight, received the insulin clamp and an infusion of an amino acid solution for two and one-half hours. We studied a third group, also fasted overnight, before and after a two-hour infusion of saline. Though in our experiments, each animal is an independent study which serves as its own control, the saline

group served as another control to disclose any unforeseen metabolic effects of the anesthesia and immobilization inherent in our experimental design.

We report five important findings from the study of these three experimental groups. First, after a brief overnight or 36-hour fast, the heart is in a net catabolic state as indicated by negative protein balance. Second, the degradative rates in 36-hour fasted animals are significantly lower than those fasted overnight. Third, insulin has no discernable effect on the rate of protein synthesis in these fasted animals in combination with glucose alone or glucose and amino acids. Fourth, physiologic concentrations of insulin inhibit this net catabolism by stopping proteolysis. Finally, the anti-proteolytic effect of insulin is inhibited by the systemic hypoaminoacidemia induced by insulin infusion.

Although contrary to the intuitive impression that cardiac muscle would be preserved during fasting, our finding that the canine heart is in a net catabolic state even after a brief fast is consistent with older studies in small animals and parallels more recent data in large animals and man. In rats, starvation results in a net loss of protein in both the whole body and heart (Preedy, 1984), though there is some evidence that the heart undergoes a slower net degradation than other muscles (reviewed in Sugden, 1991). We did not measure skeletal muscle kinetics in this study, but a previous investigation using the same isotope dilution method in dogs has demonstrated that it is also in a net catabolic state after an overnight fast (Barrett, 1987). Separate studies

performed by this laboratory have also demonstrated that both cardiac and skeletal muscle are in negative phenylalanine balance in the post-absorptive state in man (Young, 1991; Gelfand, 1987).

We found that the rate of myocardial protein degradation was lower in animals fasted 36 hours than in those fasted overnight (81±13 vs 121±12 nmol/min, p<0.05). Previous studies have demonstrated that in both the rat (Preedy, 1984) and rabbit (Samarel, 1987), fasting inhibits protein synthesis. By assessing protein degradation indirectly by taking the difference between changes in heart mass and measured rates of protein synthesis, Samarel reported fasting had no significant effect on the rate of degradation. Our study which estimates protein degradation directly and independently suggests that cardiac protein loss may decline as the fasting period increases.

In the present investigations, the rates of protein synthesis were unchanged by hyperinsulinemia in any of the experimental groups. This contrasts with earlier findings both in vitro and in vivo. In isolated, perfused hearts, where addition of insulin to perfusate stimulates protein synthesis (Rannels, 1975; Flaim, 1983, Preedy, 1984). Application of these findings to humans or large animals is complicated, however, by the artificial and highly catabolic nature of the isolated, perfused rat heart and the generally superphysiologic concentrations of insulin used in these experiments. In young, growing rats with streptozotocin-induced diabetes, insulin increases the rate of protein synthesis (Pain, 1974; Ashford, 1986). Interpretation of these observations is difficult in the diabetic animal with its complex array of

compensatory endocrinologic mechanisms operating (reviewed in Sugden, 1991). Furthermore, insulin served only to restore the rate of protein synthesis to that seen in fasted controls; it did not stimulate synthesis to that seen in normal rats fed *ad libitum*. Recent data from this lab in older rats demonstrates that insulin alone does not increase cardiac protein synthesis (McNulty, 1989). These findings do not preclude the possibility that there is some physiologic significance to the low, but measurable levels of circulating insulin in the basal state in this study or in any normal post-absorptive animal. Insulin at these low levels may serve as a permissive hormone, priming cells to receive stimulatory signals from other circulating molecules.

Our finding that insulin infusion, when coupled with maintenance of basal concentrations of amino acids, inhibits proteolysis is consistent with previous studies in the rat heart and other investigations of the metabolic influence of insulin. Because of the greater methodologic difficulties inherent in quantifying protein degradative rates (see introduction), few careful studies have addressed this issue in the heart. In isolated, perfused hearts, insulin at high concentrations has been shown to decrease protein breakdown (Rannels, 1975; Curfman, 1980). Ribosomal protein degradation was shown to be particularly sensitive to inhibition by insulin in vivo in diabetic rats (Ashford, 1986). Again, the pertinence to humans or large animals of findings in these complicated systems is difficult to assess. Our results are quite consistent with recent studies in human forearm skeletal muscle (Gelfand, 1987) and in whole body in man

(Fukagawa, 1985; Tessari, 1986). Numerous discrete mechanisms of protein breakdown have been described (reviewed in Kettelhut, 1988). Insulin appears to block protein degradation by inhibiting autophagy by lysosomes (Rannels, 1975).

That there was no effect of insulin in the group fasted 36 hours and not repleted with amino acids may be explained by insulin resistance or by hypoaminoacidemia. Though the animals had been fasted for 36 hours, there is no evidence that they had become resistant to insulin. Myocardial glucose utilization increased dramatically in response to insulin and whole body glucose disposal was similar to the groups fasted only overnight (Table I). Furthermore, studies in human forearm skeletal muscle demonstrate that even after a 60-hour fast when muscle is markedly resistant to insulin-mediated glucose disposal, it remains sensitive to insulin's anti-proteolytic effects (Fryburg, 1990).

The generalized hypoaminoacidemia resulting from insulin infusion in the 36-hour fasted animals probably explains the lack of effect on proteolysis. In man, insulin also decreases plasma concentrations of amino acids (Fukagawa, 1986). The complementary role of insulin and amino acids as circulating regulators has been demonstrated in studies of whole body protein metabolism in man (Fukagawa, 1989). Volunteers undergoing the hyperinsulinemic euglycemic clamp without amino acid supplementation had little change in whole body proteolysis. When amino acid levels were maintained near basal levels, however, insulin's anti-proteolytic effects were manifest

(Flakoll, 1989). Another study has implicated the branched-chain amino acids (which drop dramatically during insulin infusion) as responsible for determining the organisms sensitivity to insulin's regulation of proteolysis (Frexes-Steed, 1990).

We attempted to maintain plasma amino acids near basal levels during insulin infusion by administering a balanced amino acid solution. Although changes in individual amino acids did occur (alanine and glycine increased and leucine fell significantly from basal levels), we were successful overall in maintaining the concentrations of essential, branched chain, and total measured amino acids (Table III and figure 2). In studies of forearm skeletal muscle in vivo, it is possible to infuse insulin locally and study the effects of insulin without changing systemic amino acid levels (Gelfand, 1987), but this is technically impractical in studying cardiac metabolism. Glutamine and leucine have been implicated as regulators of muscle protein synthesis, but have their stimulatory effect at much higher concentrations than those achieved in our study (reviewed in Sugden, 1991). Though in our design we cannot formally exclude the possibility that one or more of the amino acids alone is responsible for the effect observed, our results are compatible with those observed in similar experiments in whole body protein metabolism in which plasma leucine concentrations are maintained at basal levels (Flakoll, 1989). Furthermore, in our overnight-fasted groups, glutamine and leucine levels changed significantly from basal, but did not consistently correlate with changes in measured synthetic or degradative rates (Table III and figure 1). The statistically

significant rise in phenylalanine concentration with infusion of amino acids .

The only significant change in hemodynamic parameters observed was a 10% increase in blood pressure during the course of the two and one-half hour infusion of insulin and amino acids (Table II). This increase may be explicable by the amino acid infusion or by variability in our delivery of anesthesia during this longer experiment. Insulin in superphysiologic doses may activate the sympathetic nervous system independent of hypoglycemia; in the physiologic doses administered in our study, however, this phenomenon generally does not occur (Liang, 1982). Though it is possible that this increase in demand on the heart contributed to the observed decrease in proteolysis, several factors argue against this being significant. First, other parameters which tend to parallel myocardial work, e.g. the calculated cardiac double product or coronary blood flow, were unchanged throughout the experiment. Second, studies of the influence of increased demand on myocardial protein kinetics, which measure the effects of doubling systolic pressures, show that this change results primarily in increased protein synthesis (Gordon,1986), and has no effect on the rate of protein degradation (Smith, 1983). In our study we observed a considerably more modest change in systemic resistance.

In summary, we have observed that insulin has a significant influence on protein kinetics in the canine heart. In many large animals, nutrients are consumed in large quantities several times per day. The consumption of a big meal may be associated with a

significant secretion of insulin which lasts for two to three hours. Between meals, insulin levels fall and bodily stores of carbohydrates and amino acids must provide the chemical substrates for diverse biochemical processes. In an experimental system which closely approximates this true physiology of large animals, we have demonstrated that insulin inhibits the catabolism of heart protein found after a brief period of fasting. This finding may be relevant in many situations in which patients undergo prolonged periods of inadequate nutrition or their hearts suffer some injury; both scenarios which are found in hospitals every day. Furthermore, this may help explain why people with hypertension and high circulating levels of insulin (because they are resistant to the effects of insulin on glucose disposal) are often found to have remarkably enlarged hearts. With an understanding of the physiology of these processes, it may be possible to devise ways to improve the care of these patients.

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Group I - Arterial DPM (basal)

time from start of clamp	-15	-10	-5	0	Mean	Coefficient of variation
Dog #1	4702	4370	4692	4582	4587	0.03
Dog #13		3616	3438	3800	3618	0.04
Dog #3	6180	5742	5732	5278	5733	0.06
Dog #5	4160	4226	3768	4254	4102	0.05
Dog #6	8014	8822	8666	8774	8569	0.04
Dog #7	5170	5390	5334	5444	5335	0.02
Dog #9	3324	3452	3144	2904	3206	0.06
Dog #10	4280	4308	4276	4272	4284	0.00
Dog #11	3596	3668	3620	3748	3658	0.02
Dog #12	4216	4150	4478	4706	4388	0.05
Mean	4849	4774	4715	4776	4748	0.04
SEM	486	506	511	503	502	

Group I - Coronary Sinus DPM (basal)

time from start of clamp	-15	-10	-5	0	Mean	Coefficient of variation	Basal DPM	Extraction Fraction
Dog #1	4232	4396	4440	4434	4376	0.02	211	0.05
Dog #13	3370	3536	3626	3528	3515	0.03	103	0.03
Dog #3	5466	5234	5172	4810	5171	0.05	563	0.1
Dog #5	3918	4208	3944	4052	4031	0.03	72	0.02
Dog #6	8512	8542	8464	8570	8522	0.00	47	0.01
Dog #7	5064	5110	5200	5412	5197	0.03	138	0.03
Dog #9	3428	3404	3252	3376	3365	0.02	-159	-0.05
Dog #10	3956	4240	4180	4264	4160	0.03	124	0.03
Dog #11	3426	3566	3520	3708	3555	0.03	103	0.03
Dog #12	3890	4136	4326	4488	4210	0.05	178	0.04
Mean	4526	4637	4612	4664	4610	0.03	138	0.029
SEM	494	476	474	475	478		57	0.01
							t= 2.43	2.33

Appendix 1: Degradations per Minute (DPM)

Group I - Arterial DPM (post-insulin)

time from start of clamp	105	110	115	120	Mean	Coefficient of variation
Dog #1	4732	5326	5376	5654	5272	0.06
Dog #13	2616	2696	2980		2764	0.06
Dog #3	5832	5946	5890	5778	5862	0.01
Dog #5	5056	5368	4964	5012	5100	0.03
Dog #6	7410	6830	7218	6954	7103	0.03
Dog #7	4990	4932	5506	5504	5233	0.05
Dog #9	3116	2928	3168	3260	3118	0.04
Dog #10		3696	3676	3888	3753	0.03
Dog #11	4360	4462	4412	4542	4444	0.02
Dog #12	4050	4776	4718		4515	0.07
Mean	4685	4696	4791	5074	4716	0.04
SEM	475	411	412	413	428	

Group I - Coronary Sinus DPM (post-insulin)

time from start of clamp	105	110	115	120	Mean	Coefficient of variation	Net Extraction DPM	Net Extraction Fraction
Dog #1	4514	4780	4830	4732	4714	0.03	558	0.11
Dog #13	2704	2794	2636	2806	2735	0.03	29	0.01
Dog #3		5796	5456	5810	5687	0.03	174	0.03
Dog #5	4670	4302	4520	4704	4549	0.03	551	0.11
Dog #6	7160	6748	7198	7198	7076	0.03	27	0.00
Dog #7	4748	4780	5324	5068	4980	0.05	253	0.05
Dog #9	2948	2872	2908	3080	2952	0.03	166	0.05
Dog #10	3620	3612	3832	3692	3689	0.02	64	0.02
Dog #11	4318	4388	4418	4452	4394	0.01	50	0.01
Dog #12	4232	4358	4260	4422	4318	0.02	197	0.04
Mean	4324	4443	4538	4596	4509	0.03	207	0.04
SEM	431	384	415	406	402		63	0.01
						t=	3.29	3.62

Appendix 1: Degradations per Minute (DPM)

Group II - Arterial DPM (basal)

time from start of clamp	-15	-10	-5	0	Mean	Coefficient of variation
Dog #15			3072	3174	3123	0.02
Dog #17	4876	4762	4730	4954	4831	0.02
Dog #18	3694	3460	3316	3268	3435	0.05
Dog #19	2908	2818	2470	2876	2768	0.06
Dog #20	2648	2532	2710	2608	2625	0.02
Dog #21	3016	3040	3034	2976	3017	0.01
Dog #30	3086	2814	2886		2929	0.04
Dog #23	4126	3910		3692	3909	0.05
Dog #24	3648	3632	3798	3714	3698	0.02
Dog #25	2092	2070	2130	2088	2095	0.01
Dog #31		2408	2408	2428	2415	0.00
Mean	3344	3145	3055	3178	3168	0.03
SEM	279	255	240	257	232	

Group II - Coronary Sinus DPM (basal)

time from start of clamp	-15	-10	-5	0	Mean	Coefficient of variation	Basal DPM	Extraction Fraction
Dog #15	3016	3030	2984	2910	2985	0.02	138	0.04
Dog #17	4528	4510	4552	4624	4554	0.01	277	0.06
Dog #18	3284	3210	3116	3272	3221	0.02	214	0.06
Dog #19	2702	2512	2700	2774	2672	0.04	96	0.03
Dog #20	2394	2204	2536	2638	2443	0.07	182	0.07
Dog #21	2958	3088	2946	2898	2973	0.02	44	0.01
Dog #30	2972	2694	2734		2800	0.04	129	0.04
Dog #23	3758	3784	3738	3790	3768	0.01	142	0.04
Dog #24	3460	3458	3602	3566	3522	0.02	177	0.05
Dog #25	1926	1932	1936	1994	1947	0.01	148	0.07
Dog #31		2380	2406	2302	2363	0.02	52	0.02
Mean	3100	2982	3023	3077	3022	0.03	145	0.04
SEM	229	227	217	243	219		20	
					t =		7.09	8.27

Appendix 1: Degradations per Minute (DPM)

Group II - Arterial DPM (post-insulin)

time from start of clamp	135	140	145	150	Mean	Coefficient of variation
Dog #15	2962	2972	2812	2852	2900	0.02
Dog #17	4660	4598	4508	4624	4598	0.01
Dog #18	4470	4298	4374	4468	4403	0.02
Dog #19	3566	3554	3624	3812	3639	0.03
Dog #20	4372	4488	4372	4596	4457	0.02
Dog #21		3480		3596	3538	0.02
Dog #30	2718	2748	2786	2822	2769	0.01
Dog #23	3968	4016		3992	3992	0.00
Dog #24		4774	4696	4716	4729	0.01
Dog #25	2638	2628	2582	2630	2620	0.01
Dog #31	2024	1910	1998	1944	1969	0.02
Mean	3486	3588	3528	3641	3601	0.02
SEM	296	269	315	274	267	

Group II - Coronary Sinus DPM (post-insulin)

time from start of clamp	135	140	145	150	Mean	Coefficient of variation	Net DPM	Extraction Fraction
Dog #15	2804	2910	2252	2868	2709	0.10	191	0.07
Dog #17	4384	4626	4442	4588	4510	0.02	88	0.02
Dog #18	4358	4262	4132	4330	4271	0.02	132	0.03
Dog #19	3472	3568	3622	3682	3586	0.02	53	0.01
Dog #20	4244	4334	4438	4534	4388	0.02	70	0.02
Dog #21	3398	3462	3484	3504	3462	0.01	76	0.02
Dog #30		2762	2804	2718	2761	0.01	7	0.00
Dog #23			3784	3914	3849	0.02	143	0.04
Dog #24		4546	4552	4504	4534	0.00	195	0.04
Dog #25	2596	2562	2502	2586	2562	0.01	58	0.02
Dog #31	2050	2018	1956	1858	1971	0.04	- 2	0.00
Mean	3413	3505	3452	3553	3509	0.02	92	0.02
SEM	291	276	269	268	257		19	
							$t=$ 4.82	4.53

Appendix 1: Degradations per Minute (DPM)

Group III - Arterial DPM (basal)

time from start of saline	-15	-10	-5	0	Mean	Coefficient of variation
Sal #1	7824	7884	7716	7614	7760	0.01
Sal #2	4576	4932	5276	5374	5040	0.06
Sal #4	4504	4392	4488	4282	4417	0.02
Sal #5	5974	5670	6160	6048	5963	0.03
Sal #6		4008	4776	4022	4269	0.08
Sal #7		4642	5210	5560	5137	0.07
Sal #8	5370	5464	5066	5374	5319	0.03
Sal #9	5972	6243	6565	6937	6429	0.06
Mean	5703	5404	5657	5651	5542	0.05
SEM	455	409	357	402	380	

Group III - Coronary Sinus DPM (basal)

time from start of saline	-15	-10	-5	0	Mean	Coefficient of variation	Extraction DPM
Sal #1	6550	7630	7900	7660	7435	0.07	325
Sal #2	4538	4772	4916	5128	4839	0.04	201
Sal #4		4392	4594	4586	4524	0.02	-108
Sal #5	5718	5606	5892	5920	5784	0.02	179
Sal #6	4306	4522	4768	4774	4593	0.04	-324
Sal #7	4654	4584	4620	5016	4719	0.04	419
Sal #8	4882	5071	4930	5120	5001	0.02	318
Sal #9	5565	5747	5817	6080	5802	0.03	627
Mean	5173	5291	5430	5536	4270	0.04	205
SEM	281	353	370	332	325		

Appendix 1: Degradations per Minute (DPM)

Group III - Arterial DPM (post saline)

time from
start of saline

	105	110	115	120	Mean	Coefficient of variation
Sal #1	9002		9736	8326	9021	0.06
Sal #2	6058	6082	6422	6292	6214	0.02
Sal #4	5084	4908	4768	4528	4822	0.04
Sal #5	6078	6252	5782	6248	6090	0.03
Sal #6	5294	5412	5238	5840	5446	0.04
Sal #7	5690	5598	5700	5622	5653	0.01
Sal #8	6588	5580	6170	5734	6018	0.07
Sal #9	7171	8126	7718	7352	7592	0.05
Mean	6371	5994	6442	6243	6357	0.04
SEM	416	363	527	382	442	

Group III - Coronary Sinus DPM (post saline)

time from
start of saline

	105	110	115	120	Mean	Coefficient of variation	Extraction DPM
Sal #1	8370	9350	8308	9098	8782	0.05	240
Sal #2	5870	5900	6174	6464	6102	0.04	112
Sal #4	4916	4374	4884	5020	4799	0.05	24
Sal #5	6198	6152	5902	6190	6111	0.02	-21
Sal #6	5062	5446	5268	4770	5137	0.05	310
Sal #7	5534	5496	5584	5546	5540	0.01	113
Sal #8	5922	5755	5910	6159	5937	0.02	82
Sal #9	6574	6612	6690	6962	6710	0.02	882
Mean	6056	6136	6090	6276	6139	0.03	218
SEM	359	480	348	448	405		

Appendix 1: Degradations per Minute (DPM)

Group I - Arterial [Phe] (nmol/ml)(basal)

time from start of clamp	-15	-10	-5	0	Mean	Coefficient of variation
Dog #1	56.80	53.80	55.80	56.40	55.70	0.02
Dog #13		52.72	52.85	53.83	53.13	0.01
Dog #3	31.30	30.00	29.50	28.20	29.75	0.04
Dog #5	30.50	31.60	30.40	29.10	30.40	0.03
Dog #6	57.40	62.60	58.60	56.90	58.88	0.04
Dog #7	39.59	40.28	39.64	41.10	40.15	0.02
Dog #9	50.96	53.16	47.10		50.41	0.05
Dog #10	47.39	47.10	49.02	50.09	48.40	0.03
Dog #11	47.89	48.34	48.48	48.55	48.32	0.01
Dog #12	39.31	40.72	40.80	42.18	40.75	0.02
Mean	44.57	46.03	45.22	45.15	45.59	0.03
SEM	3.33	3.27	3.15	3.62	3.18	

Group I - Coronary Sinus [Phe] (nmol/ml)(basal)

time from start of clamp	-15	-10	-5	0	Mean	Coefficient of variation	Net Extraction
Dog #1	53.20	58.60	56.30	59.00	56.78	0.04	-1.08
Dog #13	51.50	53.33	54.52	51.93	52.82	0.02	0.31
Dog #3	31.60	30.90	29.90	28.20	30.15	0.04	-0.40
Dog #5	32.90	34.10	31.00	29.90	31.98	0.05	-1.58
Dog #6	63.40	60.90	59.70	58.40	60.60	0.03	-1.73
Dog #7	40.73	41.25	40.76	41.83	41.14	0.01	-0.99
Dog #9	53.03	52.35	49.84	51.16	51.60	0.02	-1.19
Dog #10	50.42	50.28	49.96	51.27	50.48	0.01	-2.08
Dog #11	48.37	49.63	49.15	49.85	49.25	0.01	-0.94
Dog #12	39.15	41.52	41.69	43.00	41.34	0.03	-0.59
Mean	46.43	47.29	46.28	46.45	46.61	0.03	-1.02
SEM	3.18	3.17	3.23	3.38	3.21	0.22	t= -4.67

Appendix 2: Phenylalanine Concentrations (nmol/ml)

Group I - Arterial [Phe] (nmol/ml)(post-insulin)

time from start of clamp	105	110	115	120	Mean	Coefficient of variation
Dog #1	39.90	41.00	39.70	41.00	40.40	0.01
Dog #13	26.50	27.80	29.64		27.98	0.05
Dog #3	26.70	27.30	27.10	27.80	27.23	0.01
Dog #5	20.90	21.60	19.90	20.30	20.68	0.03
Dog #6	39.00	37.50	37.40	36.60	37.63	0.02
Dog #7	27.15	27.24	27.24	28.37	27.50	0.02
Dog #9	38.71	39.84	39.79	40.09	39.61	0.01
Dog #10	41.07	39.82	38.80	38.54	39.56	0.03
Dog #11	37.11	37.95	37.44	38.11	37.65	0.01
Dog #12	26.91	31.21	29.45		29.19	0.06
Mean	32.40	33.13	32.65	33.85	32.74	0.03
SEM	2.34	2.18	2.18	2.63	2.21	

Group I - Coronary Sinus [Phe] (nmol/ml)(post-insulin)

time from start of clamp	105	110	115	120	Mean	Coefficient of variation	Net Extraction
Dog #1	42.80	43.70	42.60	42.60	42.93	0.01	-2.52
Dog #13	30.44	31.74	27.53	30.91	30.16	0.05	-2.18
Dog #3		27.10	26.70	27.90	27.23	0.02	-0.01
Dog #5	21.80	19.10	20.40	21.10	20.60	0.05	0.07
Dog #6	40.60	38.90	38.80	40.30	39.65	0.02	-2.03
Dog #7	27.75	27.40	28.35	28.82	28.08	0.02	-0.58
Dog #9	38.73	38.92	38.72	40.23	39.15	0.02	0.46
Dog #10	40.62	40.16	40.56	40.53	40.47	0.00	-0.91
Dog #11	39.65	39.15	38.94	40.39	39.53	0.01	-1.88
Dog #12	32.64	30.28	29.94	30.95	30.95	0.03	-1.76
Mean	35.00	33.65	33.25	34.37	33.87	0.02	-1.13
SEM	2.39	2.44	2.38	2.32	2.34		0.34
						t=	-3.34

Appendix 2: Phenylalanine Concentrations (nmol/ml)

Group II - Arterial [Phe] (basal)

time from start of clamp	- 15	- 10	- 5	0	Mean	Coefficient of variation
Dog #15			54.86	57.52	56.19	0.02
Dog #17	53.52	53.49	53.42	54.34	53.69	0.01
Dog #18	44.10	41.84	43.11	42.15	42.80	0.02
Dog #19	38.68	38.13	35.88	38.30	37.75	0.03
Dog #20	30.20	28.68	29.34	26.70	28.73	0.04
Dog #21	56.93		51.47	50.83	53.08	0.05
Dog #30	45.69	43.97	43.80	44.48	44.49	0.02
Dog #23	71.14	71.84		67.96	70.31	0.02
Dog #24	57.11	57.34	59.12	56.49	57.52	0.02
Dog #25	37.41	37.13	37.37	36.51	37.11	0.01
Dog #31		57.44	54.83	53.98	55.42	0.03
Mean	48.31	47.76	46.32	48.11	48.82	0.02
SEM	3.95	4.18	2.97	3.37	3.40	

Group II - Coronary Sinus [Phe] (basal)

time from start of clamp	- 15	- 10	- 5	0	Mean	Coefficient of variation	Basal Extraction
Dog #15	58.47	56.92	55.81	55.37	56.64	0.02	-0.45
Dog #17	54.42	53.99	55.01	54.97	54.60	0.01	-0.90
Dog #18	44.30	42.51	45.46	45.19	44.37	0.02	-1.57
Dog #19	39.09	38.12	39.36	38.18	38.69	0.04	-0.94
Dog #20	29.99	30.44	30.49	30.42	30.34	0.07	-1.61
Dog #21	58.82	54.56	52.29	51.01	54.17	0.02	-1.09
Dog #30	46.78	45.66	45.67	46.06	46.04	0.04	-1.56
Dog #23	72.36	73.35	71.22	71.47	72.10	0.01	-1.79
Dog #24	57.90	57.44	60.22	58.29	58.46	0.02	-0.95
Dog #25	38.02	38.03	38.30	38.90	38.31	0.01	-1.21
Dog #31		56.43	55.51	55.11	55.68	0.02	-0.27
Mean	50.02	49.77	49.94	49.54	49.95	0.03	-1.12
SEM	3.79	3.48	3.30	3.27	3.37		0.14
						t =	-8.02

Appendix 2:   Phenylalanine Concentrations (nmol/ml)

Group II - Arterial [Phe] (post-insulin + AA's)

time from start of clamp	135	140	145	150	Mean	Coefficient of variation
Dog #15	75.52	76.55	74.16	65.74	72.99	0.06
Dog #17	86.52	85.05	84.35	85.91	85.46	0.01
Dog #18	81.00	79.26	80.49	81.82	80.64	0.01
Dog #19	60.06	60.46	60.24	60.08	60.21	0.00
Dog #20	69.84	71.13	68.44	72.02	70.36	0.02
Dog #21		66.73		65.19	65.96	0.01
Dog #30	65.03	64.80	63.62	65.08	64.63	0.01
Dog #23	86.50	87.86		88.13	87.50	0.01
Dog #24		96.84	95.27	96.79	96.30	0.01
Dog #25	52.32	53.63	54.10	55.11	53.79	0.02
Dog #31	61.64	58.75	61.14	59.77	60.33	0.02
Mean	70.94	72.82	71.31	72.33	72.56	0.02
SEM	2.76	2.86	2.87	2.75	7.97	

Group II - Coronary Sinus [Phe] (post-insulin + AA's)

time from start of clamp	135	140	145	150	Mean	Coefficient of variation	Net Extraction
Dog #15	76.64	77.31	69.55	68.74	73.06	0.05	-0.07
Dog #17	84.94	86.67	84.33	86.63	85.64	0.01	-0.19
Dog #18	81.78	76.17	77.20	83.01	79.54	0.04	1.10
Dog #19	59.82	60.38	60.65	61.04	60.47	0.01	-0.26
Dog #20	68.85	71.76	72.67	72.63	71.48	0.02	-1.12
Dog #21	67.10	66.97	65.48	65.11	66.17	0.01	-0.20
Dog #30	64.92	65.02	65.22	65.61	65.19	0.00	-0.56
Dog #23			84.83	85.37	85.10	0.00	2.40
Dog #24		94.48	94.42	94.36	94.42	0.00	1.88
Dog #25	54.50	54.61	53.61	55.34	54.52	0.01	-0.72
Dog #31	62.39	61.34	60.74	59.69	61.04	0.02	-0.71
Mean	68.99	71.47	71.70	72.50	72.42	0.02	0.14
SEM	3.21	3.73	3.56	3.70	3.59		0.33
						t =	0.43

Appendix 2: Phenylalanine Concentrations (nmol/ml)

Group III - Arterial [Phe] (basal)

time from

start of saline	-15	-10	-5	0	Mean	Coefficient of variation
Sal #1	46.44	44.37	42.90	40.93	43.66	0.05
Sal #2	44.93	45.72	45.92	46.74	45.83	0.01
Sal #4	46.41	46.64	45.76	44.74	45.89	0.02
Sal #5	55.55	53.37	57.29	55.15	55.34	0.03
Sal #6	52.90	47.85	54.10	51.05	51.48	0.05
Sal #7	60.90	60.78	62.87	61.30	61.46	0.01
Sal #8	45.88	46.61	45.14	48.50	46.53	0.03
Sal #9	43.88	43.95	44.47	46.43	44.68	0.02
Mean	49.61	48.66	49.81	49.36	49.36	0.03
SEM	2.17	2.01	2.59	2.27	2.21	

Group III - Coronary Sinus [Phe] (basal)

time from

start of saline	-15	-10	-5	0	Mean	Coefficient of variation	Extraction
Sal #1	42.18	47.80	46.91	46.56	45.86	0.05	-2.20
Sal #2	46.21	48.27	47.36	46.84	47.17	0.02	-1.34
Sal #4		48.42	48.51	48.57	48.50	0.00	-2.61
Sal #5	55.35	55.29	56.68	57.54	56.22	0.02	-0.88
Sal #6	54.32	55.60	55.34	55.43	55.17	0.01	-3.70
Sal #7	62.22	60.10	60.11	64.20	61.66	0.03	-0.19
Sal #8	46.12	47.40	47.83	49.62	47.74	0.03	-1.21
Sal #9	45.70	46.24	46.14	47.04	46.28	0.01	-1.60
Mean	50.30	51.14	51.11	51.98	51.08	0.02	-1.72
SEM	2.69	1.80	1.91	2.27	2.06		

Appendix 2: Phenylalanine Concentrations (nmol/ml)

Group III - Arterial [Phe] (post saline)

time from start of saline	105	110	115	120	Mean	Coefficient of variation
Sal #1	40.36	40.45	41.81	36.49	39.78	0.05
Sal #2	48.39	48.96	51.58	52.07	50.25	0.03
Sal #4	46.30	46.36	44.62	42.14	44.86	0.04
Sal #5	57.67	58.64	54.83	61.54	58.17	0.04
Sal #6	60.52	63.15	61.15	67.35	63.04	0.04
Sal #7	65.27	65.32	65.40	67.55	65.89	0.02
Sal #8	56.83	57.06	55.78	58.52	57.05	0.02
Sal #9	46.68	48.19	47.70	46.86	47.36	0.01
Mean	52.75	53.52	52.86	54.07	53.30	0.03
SEM	3.01	3.11	2.86	4.10	3.25	

Group III - Coronary Sinus [Phe] (post saline)

time from start of saline	105	110	115	120	Mean	Coefficient of variation	Extraction
Sal #1	41.95	44.86	38.97	42.40	42.05	0.05	-2.27
Sal #2	50.13	49.46	52.90	53.50	51.50	0.03	-1.25
Sal #4	47.48	43.35	46.52	48.60	46.49	0.04	-1.63
Sal #5	59.30	61.79	59.84	62.61	60.89	0.02	-2.72
Sal #6	63.13	64.77	63.89	62.60	63.60	0.01	-0.56
Sal #7	67.34	67.13	70.96	67.14	68.14	0.02	-2.26
Sal #8	58.82	57.51	60.36	59.61	59.08	0.02	-2.03
Sal #9	47.83	48.75	49.44	49.08	48.78	0.01	-1.42
Mean	54.50	54.70	55.36	55.69	55.06	0.02	-1.77
SEM	3.14	3.28	3.66	3.04	3.24		

Appendix 2: Phenylalanine Concentrations (nmol/ml)

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