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The Relationship between Alterations in Skeletal Muscle Amino Acid Transport and NA+/K+-Pump Activity in Endotoxic Rats

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THE RELATIONSHIP BETWEEN ALTERATIONS IN SKELETAL MUSCLE AMINO ACID TRANSPORT AND NA^+/K^+-PUMP ACTIVITY IN ENDOTOXIC RATS

by

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VITA

Michael Donald Karlstad, son of Mr. and Mrs. Donald M. Karlstad, was born on July 9, 1957 in Norfolk, Virginia. Michael attended elementary and high school in Romeoville, Illinois. He graduated from Romeoville High School in June of 1975.

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Michael has accepted a post-doctoral fellowship under the direction of Dr. Bruce R. Bistrian and Dr. George L. Blackburn in the Department of Medicine at Harvard University in Boston, Massachusetts.

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CHAPTER I

INTRODUCTION

Recent hospital reports from the United States (82,83) have documented a high incidence of Gram-negative septic shock and a high mortality in these patients. McCabe et al (83) have projected that there might be approximately 300,000 cases of sepsis and 100,000 septic deaths each year. Bacterial endotoxin is a constituent of the outer cell wall of Gram-negative bacteria and has been implicated in the pathogenesis of septic shock (96,129). Intravenous injections of endotoxin into animals result in a pattern of shock which is characterized by alterations of the cardiovascular (54) and reticuloendothelial systems (5,34) and at the cellular level by disturbances in the transport of nutrients (104) and electrolytes (12,103). These hemodynamic and cellular alterations are, for the most part, similar to that seen in human sepsis and septic shock.

The studies of Cuthbertson over half a century ago established that negative nitrogen balance and the progressive loss of skeletal muscle mass are characteristic of shock and trauma (28-31). Clowes et al (23) showed that fasted septic patients mobilize amino acids by degradation of muscle protein at rates which are three to five times higher than those observed in normal men and women in a postabsorptive state. The supply of amino acids from muscle and uptake by liver for

gluconeogenesis and protein synthesis have been shown to be essential to survival (20). However, the prolonged mobilization of amino acids from skeletal muscle protein could lead to loss of muscle mass and threaten survival (20,68). The mechanisms responsible for the degradation of skeletal muscle protein in the septic or traumatized patient are not well understood at present but may be due to alterations of the neuro-endocrine and immunological systems (7,32,35,125,126). The increase in net skeletal muscle proteolysis during sepsis is presumably due to an imbalance between skeletal muscle protein synthesis and degradation. There is little doubt that insulin regulates protein metabolism in skeletal muscle by stimulating protein synthesis and inhibiting protein degradation and by increasing the uptake of amino acids (39,46,61). It is possible that increased proteolysis during sepsis could be due to altered effects of insulin on protein synthesis/degradation and/or amino acid transport in skeletal muscle. The first objective of this dissertation was to assess amino acid transport in skeletal muscle and the regulation of this uptake process by insulin during endotoxic shock.

The transport of the neutral amino acids in skeletal muscle is carried out by several distinct transport systems which differ in their reactivities with substrates, ions and hormones (14,16,48,62, 109). System A has been shown to transport neutral amino acids such as alanine, proline, serine, glycine, and the non-metabolizable analog, alpha-aminoisobutyric acid (3,88,97). It has been shown that most if not all of the energy used to transport type A amino acids is supplied by the free energy of the Na^+-electrochemical gradient

(27,41,85). The Na^+ gradient hypothesis states that the transport of amino acids against concentration gradients does not require a direct coupling of metabolic energy to transport but is dependent on energy derived from the asymmetric distribution of sodium ions across the cell membrane (27). Thus, the driving force for the active transport of amino acids is dependent on both the activity of the Na^+/K^+-pump and membrane ion permeabilities to Na^+ and K^+. It follows, that a reduction of Na^+-electrochemical energy would adversely influence the transport characteristics of system A and decrease active amino acid transport.

Insulin-sensitive amino acid transport in skeletal muscle has been shown to be restricted to system A (3,88,97). Insulin causes membrane hyperpolarization (85,130) and stimulates amino acid transport in skeletal muscle (3,24,88). The increase in amino acid transport is due to an increase of Na^+-electrochemical energy available for the energization of system A amino acid transport (62,85). Numerous studies have indicated that there are significant alterations of Na^+/K^+ transport and membrane ion permeability in a variety of tissues during endotoxic shock and other forms of tissue injury (12,42,50,108, 117). Little is known about the effect of insulin on the transport of amino acids and ions in skeletal muscle during endotoxin shock. Therefore, the second objective of this dissertation was to assess Na^+/K^+ transport and the regulation of the transport process by insulin during endotoxin shock and relate the changes in amino acid transport to the alterations in active ion transport.

The specific objectives of this dissertation are as follows:

1. To determine if Na^+-dependent amino acid uptake and the regulation of the uptake process by insulin are altered in rat soleus muscle during endotoxin shock.
2. To determine if the kinetic behavior of system A amino acid transport is altered in rat soleus muscle during endotoxin shock.
3. To determine if intracellular and extracellular concentrations of Na^+ and K^+ are altered in rat soleus muscle during endotoxin shock.
4. To determine if the active fluxes of Na^+ and K^+ and the effect of insulin on ion fluxes are altered in rat soleus muscle during endotoxin shock.

CHAPTER II

SURVEY OF THE LITERATURE

Protein metabolism during trauma

The metabolic response to moderate or severe injury in man was first described by Cuthbertson over 50 years ago (29). He made the pioneering observation that negative nitrogen balance in a patient with a fractured femur is in part related to an increase in urinary nitrogen excretion and pointed to the possibility that muscle wasting might be responsible for the nitrogen loss (28,29). He wrote that, "there appears to be a general increase in catabolism, of protein in particular, to meet the enhanced metabolism of the repair process" (28). Others have confirmed this concept, and most evidence indicates that the primary source of catabolized protein is from skeletal muscle (21,23,30). To pursue his interest in protein metabolism Cuthbertson developed an experimental model of injury using the rat and investigated aspects of the metabolic response to major trauma which could not be easily studied in human patients (28,31). He found that fracture of the femur was followed by a definite loss of nitrogen, phosphorous, and creatine in the urine. These changes could not be fully explained by muscular atrophy of the injured limb (28,31). In fact, after weighing the limbs and muscles of injured animals, the loss of body substance could not be fully accounted for by the loss of muscle

mass from the site of injury or from the injured limb. There appeared to be a generalized increase in protein degradation in tissues distant from the injury as well as in the injured tissue. These were the first studies to establish that negative nitrogen balance and general muscle wasting are hallmarks of a localized trauma.

Numerous studies have shown that the rates of skeletal muscle protein synthesis and degradation are altered in a variety of catabolic conditions, including sepsis (21,23), injury (19), and surgical trauma (63), such that there is an increase in net protein breakdown and a progressive loss of body weight. The weight loss is generally related to the extent of injury or severity of infection and may approach 20 to 30% of body mass if adequate nutritional support is not initiated (68). In humans, severe episodes of visceral sepsis may result in nitrogen losses of over 200 grams, which is the equivalent of approximately 6000 grams of hydrated lean tissue. There are approximately 13,000 grams of protein in a well-nourished adult (68). It has been shown in patients with a major injury that the protein contribution to weight loss over a three-week period amounted to 10-13% of the total weight loss and approximately 50% of the total protein content (68). Although the increase in protein breakdown and accelerated release of amino acids from the peripheral tissues during the initial response to sepsis or trauma may be important for host defense, the loss of protein, if uncontrolled, may have lethal consequences.

Characteristics of cellular amino acid transport

The fact that septic or injured patients show losses of skeletal muscle protein suggests that there may also be profound changes in the regulation of amino acid metabolism in this tissue. Since skeletal muscle constitutes approximately 40% of the body mass, an alteration of muscle membrane transport processes responsible for the uptake and release of amino acids in muscle would be expected to have significant effects on the mobilization of amino acids in the body. Surprisingly, there are only a few studies of amino acid transport in skeletal muscle and as a consequence little is known about its role in the pathophysiology of septic, endotoxic or traumatic shock.

Neutral amino acid transport systems: In 1913 Van Slyke and Meyer discovered that amino acids are present at much higher concentrations in the muscle, liver, and other tissues than in plasma in the dog (118). They showed that liver could accumulate amino acids to levels much greater than those in plasma following an infusion of amino acids (118). These studies eventually led to the concept that uptake of amino acids in cells is an active process. The studies of Christensen and others (16,17,112) in Erlich ascites tumor cells have been largely responsible for our present concepts of amino acid transport. Christensen and his colleagues have shown that the acquisition of amino acids by cells is a reflection of the cellular requirement of amino acids of different chemical structures (14,112). They have shown that cells possess several amino acid transport systems, including systems A, L, and ASC for the transport of neutral amino acids. Numerous studies have supported the presence of distinct amino acid

transport systems for neutral amino acids in skeletal muscle and that many of these amino acids are transported by more than a single transport system (14,16,62,109).

Na-dependent amino acid transport systems: The amino acid transport systems in cells can be generally separated into two main categories, namely, Na^+-dependent and Na^+-independent transport (62). Neutral amino acids with short, polar or linear side chains, such as alanine, serine, proline, and the nonmetabolizable analog alpha-aminoisobutyric acid and its N-methylated derivative (N-methylaminoisobutyric acid) are preferred by the Na^+-dependent transport system A (14,62,109). The Na^+-dependency of system A has been explained by the fact that there is a greater affinity (lower K_m) between the transport protein and amino acid in the presence of high extracellular Na^+ concentration and a lower affinity (higher K_m) in the presence of low intracellular Na^+ concentration (48) Therefore, the influx of amino acid via system A is much greater than amino acid efflux due to the asymmetric distribution of Na^+ across the cell membrane. Amino acid transport by system A is often subject to trans-inhibition by intracellular substrates of this system. This phenomenon accounts for the decrease in amino acid transport seen after increasing the intracellular amino acid levels by prior incubation of the cells in an amino acid rich medium (15,109). Most evidence indicates that the major if not the only driving force for the active accumulation of amino acids by system A in mammalian cells is obtained from the electrochemical gradient for Na^+ (62,85). Another Na^+-dependent system, ASC, is most effective for linear amino acids with 3, 4 or 5 atoms in

the chain, and so named because it transports alanine, serine and cysteine (14,109). The system ASC is most easily differentiated from system A on the basis of its intolerance to N-methylated amino acids and its ability to be trans-stimulated by intracellular amino acids (15,109).

Na-independent amino acid transport system: The Na^+-independent system L transports amino acids with branches or rings on the side chain, such as leucine and phenylalanine, and is insensitive to the sodium concentration gradient across the cell membrane. The transport of amino acids by system L is characteristically increased by the presence of high intracellular levels of system L substrates, a phenomenon known as trans-stimulation (15,109).

Insulin regulation of system A amino acid transport: Hormonal regulation of amino acid transport in tissues such as heart, diaphragm, liver, fat cells, and soleus muscle is restricted to the Na^+-dependent route, system A (14,48,109). An increase in the magnitude of the Na^+-electrochemical gradient produces an increase in the transport activity of system A as a result of the dependence of this system on the movement of sodium ions. Insulin has been reported to hyperpolarize the membrane in skeletal muscle (85,130). Insulin may hyperpolarize the membrane by increasing the membrane permeability to potassium ion, by decreasing the membrane permeability to sodium ion, or by increasing the activity of electrogenic Na^+/K^+-ATPase (85,130). Insulin stimulated transport is generally accompanied by an increase in the V_{max} of uptake of amino acids by system A with no change in K_m of

uptake, consistent with an increase in the number of active system A transport carriers (78,88).

Muscle and plasma amino acid concentrations during shock

The intracellular amino acid pools and the transmembrane amino acid concentration gradients have been measured to show changes in the various transport systems responsible for the uptake and release of amino acids by cells. Vinnars et al (120) used a needle biopsy technique to sample human muscle from the quadriceps femoris before, and two to three days after major, uncomplicated abdominal surgery. Muscle tissue and plasma samples were analyzed in simultaneously obtained samples for essential and nonessential amino acids. There were marked changes in the concentrations of most amino acids in plasma and skeletal muscle in the surgical patients. Concentrations of the small neutral amino acids, glycine, serine, and alanine, which are preferentially transported by system A, tended to increase in muscle and slightly decrease in plasma as the result of uncomplicated surgical trauma. The concentration gradients for these amino acids across the cell membrane in muscle were markedly increased primarily due to an increase in muscle amino acid concentration. The amino acid which showed the largest change in muscle was a basic amino acid, glutamine. There was a 38% decrease in glutamine concentration in muscle and a 31% decrease in the concentration gradient for glutamine (120). There was no detectable change in plasma glutamine concentration in these patients. These results indicate that there are alterations of the intracellular amino acid pools in muscle and that these changes are not always reflected in the values found in plasma. Changes of the

intracellular amino acid pools could be due to disturbances of the membrane transport systems responsible for the uptake and release of amino acids by skeletal muscle.

Kapadia et al (63) demonstrated that intracellular glutamine concentration in skeletal muscle decrease rapidly in the dog following a standard surgical trauma, similar to the response observed in humans (6). These changes were associated with marked negative nitrogen balance and accelerated release of amino acids from the hindquarter of the dog six hours post-operation. Infusion of an amino acid mixture with or without glutamine, maintained the skeletal muscle intracellular amino acid pool and reduced the efflux of nitrogen from the hindquarter. These data suggest that skeletal muscle nitrogen balance may be related to the intracellular concentration of glutamine.

Askanazi et al (6) showed a marked depletion of nonessential amino acids in muscle occurred in both injury and sepsis due to a decrease (50%) in glutamine, which was equally marked in both states. However, the concentration of alanine and other system A amino acids were approximately twofold greater in septic muscle as compared to normal muscle. The concentration of alanine was decreased in septic plasma which is consistent with an increase in amino acid extraction by the liver for gluconeogenesis. These changes resulted in rather large increases in the concentration gradients of these amino acids in septic muscle. There were no significant changes in the concentration of branched-chain amino acids, leucine, isoleucine, and valine, in plasma during sepsis. However, there was significant intracellular accumulation of the branched-chain amino acids in muscle which caused

elevated amino acid concentration gradients across the cell membrane in sepsis.

In previous studies the calculation of the concentrations of the individual amino acids in muscle water was based on estimates of the intracellular and extracellular water content in muscle. In those studies, chloride ion was presumed to be passively distributed across the cell membrane according to the Nernst's equation and known control values of skeletal muscle membrane potential and plasma chloride concentration were employed to determine extracellular and intracellular water contents (8). The transmembrane potential was assumed to be -87.2 mv in both normal and septic/injured muscle. Studies have since shown skeletal muscle membrane potential and electrolyte distribution to be altered in various forms of injury (42,50,60,108,115,117). Thus previously reported values of intracellular amino acid concentrations in muscle are presumably not accurate. The investigators of these studies (6,63,120) are aware of these limitations and realized that the lack of measured values of membrane potential may lead to quantitative errors. The magnitude and directions of the transmembrane gradients calculated for amino acids in septic/injured muscle may vary considerably depending upon the magnitude of error of the estimates of the transmembrane potential. The uncertainty of values of intracellular amino acid concentrations could invalidate conclusions of the previous studies.

Endogenous mediators and protein metabolism

Current evidence suggests that many of the metabolic alterations observed in sepsis are reproducible in the experimental setting by the

administration of leukocytic pyrogen or interleukin-1 (IL-1), a heat-labile low molecular weight protein (15,000 daltons) synthesized from activated monocytic cells (7,32,121). These studies show that leukocytic pyrogen, in addition to producing fever, also stimulates protein catabolism in skeletal muscle and enhances acute-phase protein synthesis in liver (7,32,121). Endotoxin and a variety of other agents, such as Gram-negative bacteria, are capable of inducing the production of leukocytic pyrogen from human monocytes (32). It has been shown also that the pyrogenic properties of endotoxin and Gram-negative bacteria in humans and rabbits is likely due to the lipid A portion of the endotoxin molecule (32).

Wannemacher et al (121) reported that the injection of leukocyte endogenous mediator into rats markedly enhanced the rate of amino acid transport by system A and L and increased the synthesis of acute-phase proteins in the liver. Baracos et al (7) reported that incubation of rat soleus or extensor digitorium longus muscle with human leukocytic pyrogen caused a marked increase in net protein degradation, as indicated by increased release of tyrosine from the muscles. Net protein loss resulted from an increased rate of proteolysis, since leukocyte pyrogen did not alter protein synthesis, as measured by phenylalanine incorporation into protein (7). More importantly their studies demonstrated that the stimulation of protein degradation in muscle by leukocytic pyrogen was mediated by prostaglandin E_2 and could be prevented by pretreatment with cyclooxygenase inhibitors. Prostaglandin E_2 stimulated muscle proteolysis via stimulation of a lysosomal protease (7). There is evidence that a 4000 dalton peptide referred

to as proteolysis inducing factor (PIF) can induce muscle protein catabolism and hepatic protein synthesis and that this agent is the active circulating cleavage product of interleukin-1 (19,20). Clowes et al (20) have recently shown that the lack of appropriate increase in muscle release of amino acids and hepatic protein synthesis in response to elevated levels of PIF in the fasting patient leads to septic death.

Counter-regulatory hormones and protein metabolism

The counter-regulatory hormones cortisol, glucagon, and the catecholamines have been implicated as mediators of skeletal muscle protein catabolism (125,126). Bessey et al (9) showed that infusion of these hormones into healthy volunteers produce many of the metabolic disturbances that occur following injury, including an increased loss of nitrogen in the urine. Hulton et al (58) demonstrated that selective hormonal blockade of the counter-regulatory hormones by infusion of alpha- and beta-adrenergic antagonists or neurogenic blockade (epidural anesthesia) substantially reduced hindquarter amino acid nitrogen efflux and reduced urinary creatinine excretion in post-operative dogs. These results provide evidence that it is possible to diminish the increase in net skeletal muscle protein breakdown with hormonal blockade in the injured state.

The role of cation transport in skeletal muscle in shock-like states

The cation transport mechanism responsible for the active movements of Na^+ and K^+ across the cell membrane is due to a membrane-bound Na^+-K^+ ATPase (11). The sodium pump maintains the internal ionic environment. The failure of this pump would lead to a loss of

cellular functions linked to normal Na^+ and K^+ ion gradients (103). All membrane-located transport ATPases share the common characteristic of causing the hydrolysis of ATP to ADP and inorganic phosphate. The free energy released from the splitting of high energy phosphate bonds is used to actively transport ions across the cell membrane against their electrochemical gradient (105)

Na^+/K^+-ATPase is composed of two types of polypeptide chains of different molecular weights. The molecular weight of the entire enzyme complex is approximately 250,000 (111). The larger polypeptide has a molecular weight of 100,000 and phosphorylation by ATP and P_i occurs on this subunit (111), which also contains the binding sites for ATP (111) and ouabain (105). The smaller chain is a glycoprotein with a molecular weight of 50,000 (87). Thus the enzyme complex which is embedded within the cell membrane is thought to contain two 100,000 subunits and either one or two 50,000 subunits. The binding sites for ATP and ouabain occur on opposite sides of the membrane; phosphorylation occurs on the inside of the membrane (87) whereas the cardiac glycosides bind on the outside (105). The stoichometry of the Na-K ATPase is fairly well established from a variety of cell preparations at 3 Na^+ for 2 K^+ per mole of ATP (11,105,111). One of the most characteristic properties of the enzyme is its requirement for the presence of both Na^+ and K^+ (11,105,111,113).

Numerous investigations have shown significant alterations in cell membrane ion transport is a variety of tissues during shock and other forms of tissue injury (12,42,50,108,117). Sayeed (102) showed an inhibition of active Na^+/K^+ transport and an increase in membrane

permeability to Na^+ in liver slices from endotoxic rats. Haljamae and coworkers (50,51) measured Na^+ and K^+ in nanoliter samples of interstitial fluid obtained from skeletal muscle during hemorrhagic shock. They reported a decrease in muscle intracellular K^+ concentration and a much greater increase in interstitial K^+ concentration than that of plasma indicating that intracellular K^+ leaks out of muscle cells and accumulates in the interstitium. The K^+ in extracellular fluid aspirated directly from the interstitium was very high (8 to 12 mEq/liter). Studies of Illner and Shires (60) and Trunkey et al (115) have shown decreases in the resting membrane potential, cellular swelling with increases in intracellular Na^+ and Cl^- and reductions of intracellular K^+ and the extracellular volume in skeletal muscle during septic and hemorrhagic shock. Turinsky et al (117) have shown electrolyte disturbances in skeletal muscle underlying the burn wound. Burned muscles lose potassium and magnesium and gain sodium and calcium (117). Flear (37) demonstrated a decrease in the transmembrane Na^+ concentration gradient in skeletal muscle cells during endotoxin shock. Gibson et al (42) reported that a decrease in membrane potential on the order of 30 millivolts occurs in skeletal muscle cells during endotoxin shock in the dog. The reduction in membrane potential in skeletal muscle cells in dogs in endotoxin shock is comparable to changes reported by Trunkey et al (115) in septic shock. This change in membrane potential was associated with a decrease in intracellular K^+ and an increase in intracellular Na^+. These investigators postulated that the decrease in membrane potential was due to inhibition of the electrogenic Na^+/K^+ transport and not due to an increase in

permeability to Na^+ because the value of the measured resting membrane potential was not different than that which was predicted by the Goldman equation. These findings are at odds with those reported by Shires et al (108) who showed a disparity between the potential difference predicted by the Goldman equation and that measured in muscle during prolonged hemorrhagic shock. These results may be due to dissimilar effects of endotoxic and hemorrhagic shock on ion movements in muscle or to inaccurate estimates of Na/K membrane permeabilities used for calculation of the membrane potential.

CHAPTER III

ALPHA-AMINOISOBUTYRIC ACID TRANSPORT IN RAT SOLEUS MUSCLE DURING ENDOTOXIC SHOCK

INTRODUCTION

In a variety of pathological states including burn (89), sepsis (22,49), hemorrhagic (101) and endotoxic shock (56,94), there is a generalized resistance to the metabolic effects of insulin. Studies of carbohydrate metabolism during endotoxic shock have demonstrated a decrease in insulin-mediated glucose uptake by skeletal muscle (56,94). In addition to stimulating glucose uptake, insulin enhances the transport of amino acids (24,88,109) and ions (18,85) across the cell membrane in many tissues. Little is known about the effect of insulin on these transport processes in skeletal muscle during endotoxic shock.

The transport of neutral amino acids by System A in skeletal muscle is insulin sensitive and Na^+ dependent (69,85,88,92). Most if not all the energy required to actively transport amino acids by System A is derived from the transmembrane [Na^+] gradient and potential difference (27,85). Clausen and Kohn (18) demonstrated in rat soleus muscle a stimulation of radioactive Na^+ efflux by insulin. Insulin has also been shown to stimulate the Na^+-K^+-ATPase in purified plasma membranes from frog skeletal muscle (40). Zierler and Rogus (130) have recently demonstrated that hyperpolarization of rat skeletal muscle by insulin was an early response, occurring within one second, thus supporting the hypothesis that hyperpolarization may be a mechanism by which insulin produces its metabolic effects. Since insulin modulates the driving forces for the active accumulation of

type A amino acids, viz. [Na^+] gradient and potential difference, alterations in these forces during shock could attenuate amino acid transport by System A in the presence of insulin.

The purpose of this study was to determine if Na^+-dependent amino acid uptake and the regulation of this uptake process by insulin were altered in skeletal muscle during endotoxic shock in the rat.

METHODS

Male rats (140-160 g) purchased from the Holtzman Co. (Madison, WI) and were acclimated to the animal facilities for 2-3 days at 24°-26°C before use. A 12 hr light-dark cycle (7:00 A.M.-7:00 P.M., light) was maintained. Rats were fed (Wayne Rat Chow) everyday prior to the day of the experiment. Rats were fasted overnight (4:00 P.M.-8:00 A.M.) but allowed water ad libitum prior to experiments. Shock was induced in rats by intravenous (sublingual vein) injection of 1, 10, or 20 mg/kg Salmonella enteritidis endotoxin (Boivin, Difco Laboratories, Detroit, MI), suspended in 0.9% saline. Control animals were treated similarly but received an equal volume of 0.9% saline (0.5 ml/100 g). Rats were lightly anesthetized with ether prior to intravenous injections. Rats were decapitated five hours after the injection of endotoxin or saline and both soleus muscles were removed immediately and placed in chilled (8°-10°C) Krebs-Ringer bicarbonate buffer (KRB).

Analysis of blood and plasma metabolites

Mixed arteriovenous trunk blood was collected in heparinized tubes and transferred to heparin-lithium coated microfuge tubes for storage prior to the determination of glucose and lactate concentrations. Plasma glucose was determined using a model 23A Yellow Springs glucose analyzer (Yellow Springs, OH). For lactate determinations, one volume of whole blood was deproteinized with 2 volumes of 8% perchloric acid and centrifuged at a high speed for 10 minutes and the

resulting clear supernatant was added to a mixture containing lactic dehydrogenase (2.1 X 10^4 U/liter) and nicotine adenine dinucleotide (NAD) (3.1 X 10^4 mol/liter) (Calbiochem-Behring Corporation, La Jolla, CA). The formation of the reduced form of NAD was monitored in a Beckman spectrophotometer ACTA CIII, reading absorbance changes at 340 nanometers, to determine lactate concentration.

Determination of alpha-aminoisobutyric acid uptake

Intact soleus muscles were incubated in 25 ml Erlenmeyer flasks containing 2.2 ml KRB (pH 7.4), 5.6 mM glucose, 0.5 microcuries alpha-amino-(1-^{14}C)isobutyric acid (AIB) (4.0 micromolar) (Amersham, Arlington Heights, IL; specific activity 40-60 mCi/mmol) and 0, 1, 10 or 100 mU/ml crystalline bovine insulin (24.9 U/mg) (Sigma Chemical Company, St. Louis, MO). To determine AIB uptake in the absence of Na^+, Na^+ were substituted with choline. Gelatin (2 mg/ml) (Calf skin type IV, Sigma Chemical Company, St. Louis, MO) was added to flasks containing 1 mU/ml insulin to prevent adsorption of insulin to the glassware (3,85). Incubations were carried out under 95%O_2-5%CO_2 atmosphere at 37°C in a Dubnoff metabolic shaker incubator for 1, 2 or 3 hours. After incubation muscles were lightly blotted on Whatman (#42) filter paper. The wet weight was then determined and the tissue digested in 2 ml of NCS tissue solubilizer (Amersham). Radioactivity of dissolved muscles and media samples (0.1 ml) was determined in a liquid scintillation counter (Nuclear-Chicago) and radioactive counts were quench corrected using an external standard method. Tissue radioactivity was

converted into AIB contents (nmol) after dividing it by medium AIB specific activity (DPM/nmol AIB).

Determination of extracellular (inulin) space and total tissue water

Extracellular space was determined by measuring soleus muscle uptake of inulin-(^{14}C)carboxylic acid (0.2 microcuries) (Amersham, specific activity 17.1 mCi/mmol). The experiments to determine tissue inulin uptake were carried out separately but similarly to those of tissue AIB uptake. Total tissue water (ml/g) was calculated for each muscle from its wet weight/dry weight ratio. To determine their dry weights, muscles were incubated in a vacuum oven for 16 hours at 100°C.

Calculations

The extracellular (inulin) space was calculated as follows:

space (ml/g) = (inulin DPM/g muscle)/(inulin DPM/ml medium).

The intracellular fluid volume was calculated as the difference between the total tissue water and the extracellular space. The intracellular concentration of AIB was calculated by dividing the difference between the amount present in the total muscle and that in the extracellular space by the intracellular volume. The distribution ratio of AIB was calculated by dividing the intracellular concentration of AIB by the extracellular AIB concentration. Cellular AIB uptake was calculated as the amount present in the intracellular fluid divided by the dry weight of the muscle and was expressed in nmol/g dry tissue.

Statistical analysis

Data were analyzed using a two-way classification analysis of variance (ANOVA), a least significant difference (LSD) test, and Student's t-test modified for unpaired replicates. All data were expressed as mean $\pm$ S.E. A "P" value less than or equal to 0.05 was considered significant.

RESULTS

Blood lactate concentration in the endotoxic animals (20 mg/kg) significantly increased from a control value of 0.65 $\pm$ 0.06 mM (n = 44) to 2.09 $\pm$ 0.15 mM (n = 28) ($P < 0.001$). Plasma glucose concentration decreased from a control value of 89.8 $\pm$ 2.1 mg/dl (n = 55) to 61.1 $\pm$ 3.7 mg/dl (n = 41) in the endotoxic group ($P < 0.001$). Changes in these blood metabolites were accompanied by losses in the rat's ability to right themselves and by hemorrhagic bowel lesions.

The data in Table 1 show total tissue, extracellular and intracellular water in muscles during incubation at 37°C. Total tissue water was not altered during 1 to 3 hours of incubation in control or endotoxic muscles ($P > 0.05$). Extracellular water increased with the duration of incubation in both the control and endotoxic muscles ($P < 0.01$). The increases in extracellular water in the endotoxic group were equal to that seen in the controls. In both the control and endotoxic muscles, the increase in extracellular water with increasing incubation time could be due to permeation of inulin into cells. The concomitant decrease in the inulin-inaccessible space was taken to represent changes in tissues intracellular volume available for AIB distribution. The intracellular water decreased during the incubation in both the control and endotoxic muscles ($P < 0.01$) without accompanying changes in the total muscle water. However, there were no

TABLE 1. Effect of incubation time on water contents of control and endotoxic muscles

Group	n	Incubation period (hour)	Total tissue water (ml/g)	Extracell water (ml/g)	Intracell water (ml/g)
Control	12	1	0.80 ± 0.002	0.24 ± 0.01	0.56 ± 0.01
	12	2	0.80 ± 0.001	0.29 ± 0.01*	0.52 ± 0.01*
	24	3	0.81 ± 0.004	0.38 ± 0.01*	0.44 ± 0.01*
Endotoxin	16	1	0.81 ± 0.003	0.24 ± 0.01	0.56 ± 0.01
	9	2	0.81 ± 0.002	0.32 ± 0.01*	0.49 ± 0.01*
	12	3	0.81 ± 0.004	0.41 ± 0.01*	0.40 ± 0.01*

Values are means ± SE. n = number of muscles; * $p < 0.01$, compared to 1-hr incubation of own group. Water contents between groups were not significantly different ($p > 0.05$).

significant differences between the control and endotoxic muscle intracellular water contents ($P > 0.05$) at 1, 2, or 3 hours of incubation.

Addition of varying doses of insulin (1, 10, and 100 mU/ml) to the medium had no significant effect on the total tissue, extracellular or intracellular water in either group of animals for the 3 hour incubation period ($P > 0.05$) (data not shown). Also, there were no differences in the above 3 measurements between control and endotoxic muscles incubated without or with insulin at varying concentrations ($P > 0.05$).

The data presented in Figure 1 illustrates that the curve of basal cellular AIB uptake versus incubation time for control and endotoxic soleus muscles is nonlinear and approaches steady-state values with longer incubations. These findings are in agreement with previous studies that have shown that uptake of amino acids by System A is subject to trans-inhibition by the same amino acids as they are accumulated intracellularly causing a nonlinear uptake with increased incubation time (10,109). Basal cellular AIB uptake was significantly lower ($P < 0.01$) in the endotoxic muscles than in the control muscles at each corresponding time point. As shown in Figure 1, basal cellular AIB uptake by endotoxic soleus muscles was 25%, 28%, and 47% lower than controls at the 1, 2, and 3 hour incubation periods, respectively.

Figure 2 shows the effect of insulin (0-100 mU/ml) on cellular AIB uptake in control and endotoxic muscles. Although there was a significant stimulation of AIB uptake with insulin in both the control

Figure 1. Basal cellular AIB uptake by control and endotoxic (20 mg/kg) soleus muscles. Values are means ± SE for number of muscles given in parentheses. **Solid line**, control; **dashed line**, endotoxin. **Stars**, significant differences ($P < 0.01$) between control and endotoxic muscles at each time point.

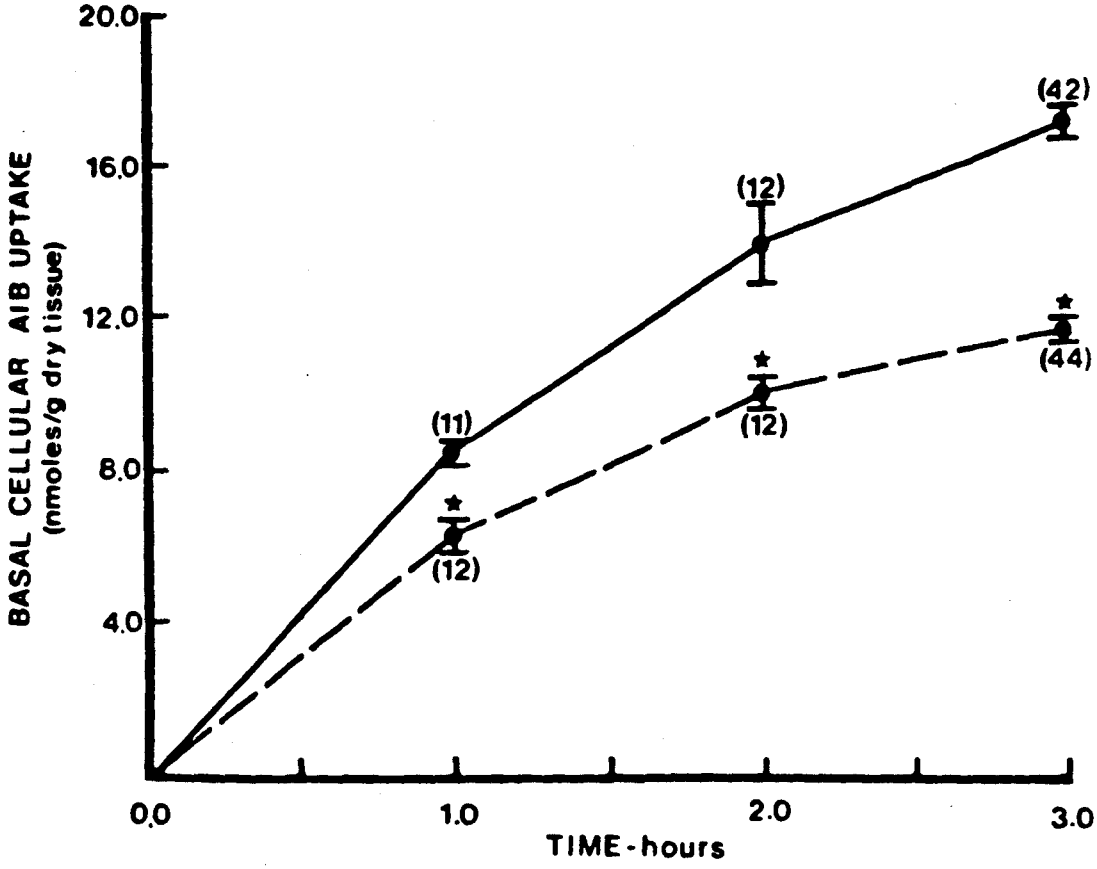
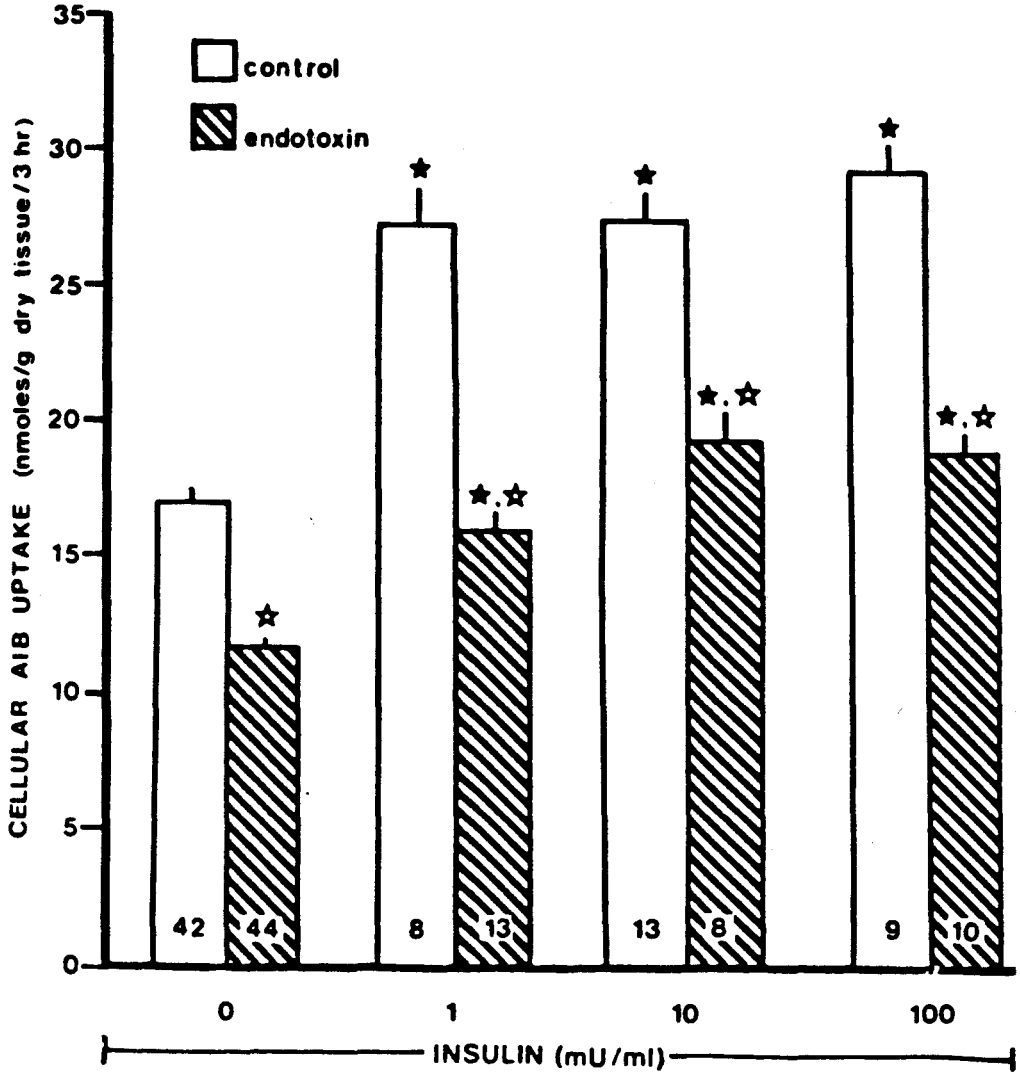

20.0
16.0
12.0
8.0
4.0
BASAL CELLULAR AIB UPTAKE
(nmoles/g dry tissue)
(42)
(12)
(11)
(12)
(44)
(12)
0.0
1.0
2.0
3.0
TIME - hours

Figure 2. Effect of varying concentrations of insulin on cellular AIB uptake by control and endotoxic (20 mg/kg) soleus muscles. Values are means ± SE for number of muscles given within bars. **Solid stars**, significant differences ($P < 0.005$) between insulin-stimulated AIB uptake and AIB uptake in the absence of insulin for each group. **Open stars**, significant differences ($P < 0.0001$) between control and endotoxic AIB uptake at each individual insulin concentration.


control
endotoxin
CELLULAR AIB UPTAKE (nmoles/g dry tissue/3 hr)
35
30
25
20
15
10
5
0
42
44
8
13
13
8
9
10
0
1
10
100
INSULIN (mU/ml)

and endotoxic muscles ($P < 0.01$, ANOVA), the insulin-stimulated AIB uptake in control muscles was significantly different (see Fig. 2 for statistics) from that in endotoxic muscles. All three insulin concentrations used, namely 1, 10 and 100 mU/ml produced comparable stimulatory effects in control or endotoxic muscles. Since these concentrations of insulin produce maximal effects (24,88), the differences in stimulated AIB uptake between control and endotoxic muscles incubated with any of these insulin concentrations represent endotoxin-induced changes only in the tissue's maximal response to insulin. Since we did not use subthreshold concentrations of insulin, our studies do not provide any insight into endotoxin-induced changes in submaximal insulin effects which would presumably vary in magnitude from changes in maximal insulin effects. Absolute differences between means of control and endotoxic muscle AIB uptake were: 5.4, 11.3, 8.2 and 10.2 nmoles/g dry tissue/3 hr at 0, 1, 10 and 100 mU/ml insulin concentrations, respectively. These values indicate that the endotoxin-related decrease in muscle AIB uptake was smaller in the absence of insulin than at insulin concentrations of 1, 10 and 100 mU/ml. This implies that the decrease in insulin response in endotoxic muscles was due to not only the decrease in basal AIB uptake but also a decrease in insulin sensitive AIB uptake. This observation was supported by the analysis of variance (2 x 2 factorial experiments: control X endotoxin vs basal X 1, 10 or 100 mU/ml insulin) showing significant interactions ($P < 0.01$) between endotoxin and insulin at concentrations of 1, 10 and 100 mU/ml.

Figure 3 shows the effect of various endotoxin doses on basal cellular AIB uptake in soleus muscle. Basal cellular AIB uptake was significantly lower ($P < 0.001$) in the endotoxic muscles than in the control muscles at each endotoxin dose. The distribution ratio of AIB was depressed to the same extent at 1, 10, and 20 mg/kg endotoxin as compared to the controls. These data indicate that basal cellular AIB transport is similarly affected over a wide range of endotoxin doses.

Table 2 shows the intracellular-to-extracellular AIB concentration ratio (distribution ratio) at various concentrations of insulin in control and endotoxic muscles. The ratio indicates the ability of the membrane transport system to accumulate AIB intracellularly against a concentration gradient. In both the control and endotoxic muscles (in the absence or presence of insulin), the distribution ratio was greater than one, indicating uphill AIB transport. However, uphill AIB transport was significantly depressed in the endotoxic muscles as compared to the controls in both the basal and insulin-stimulated state ($P < 0.01$). At insulin concentrations of 1, 10, and 100 mU/ml the uphill movement of AIB increased to a greater extent in the control muscles than in the endotoxic muscles.

In order to determine if the decreased AIB uptake by endotoxic muscle was due to an alteration in the Na^+-dependent portion of AIB uptake, Na^+ was removed from the incubation medium and replaced with another monovalent cation, choline (see Fig. 4). Previous investigators have shown that choline does not, by itself, affect total tissue water, extracellular space or membrane permeability to nonpermeant solutes (69,92). In the absence of Na^+, the basal uptake of AIB in

Figure 3. Effect of 0, 1, 10, and 20 mg/kg endotoxin on basal cellular AIB uptake by soleus muscles. Values are means ± SE for number of muscles given within bars. Stars, significant differences ($P < 0.001$) between control and endotoxic AIB uptake at each individual endotoxin dose.

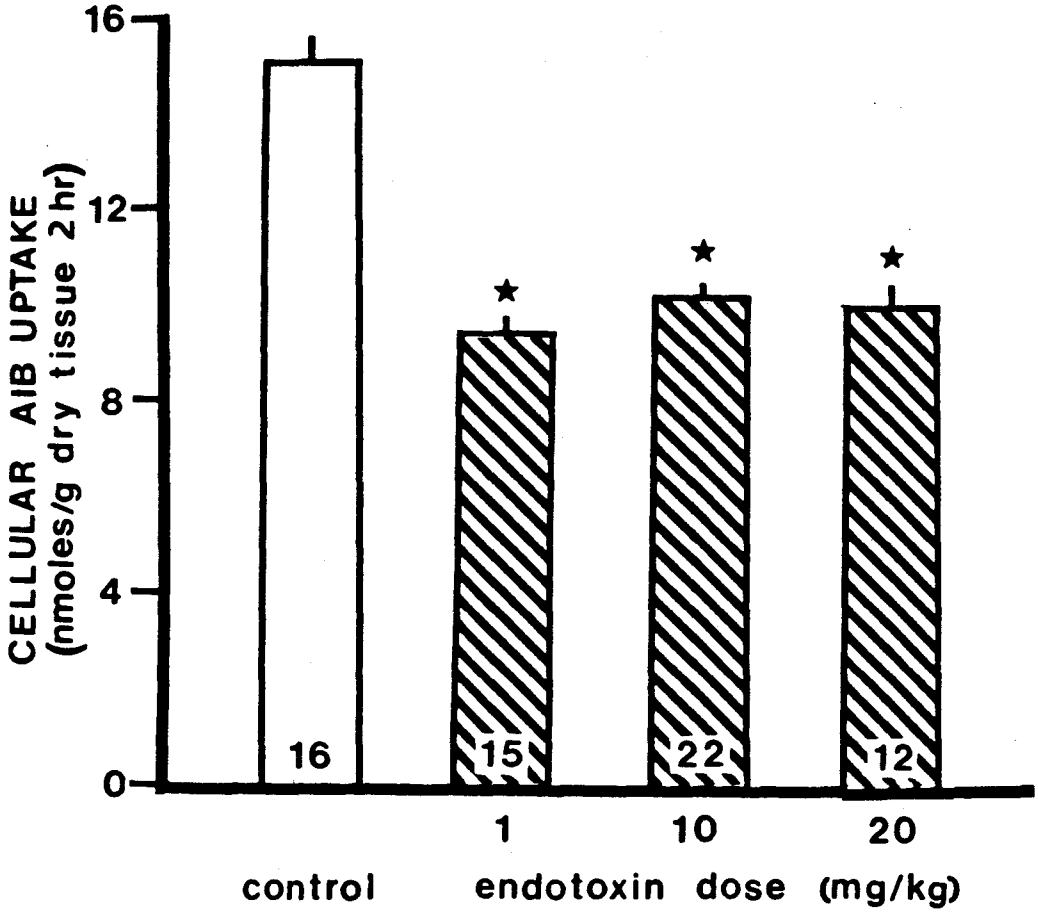
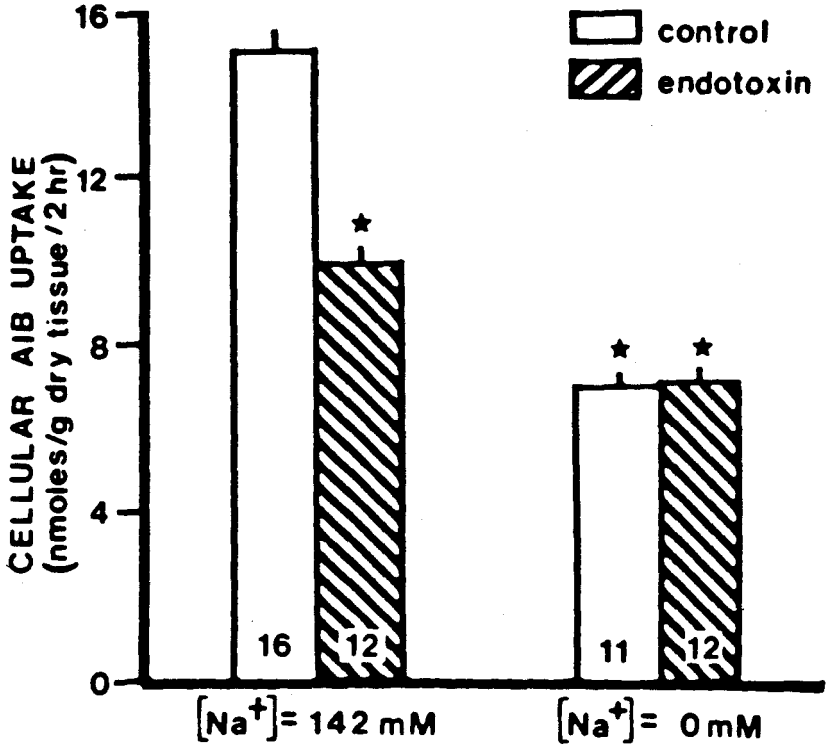

16
12
8
4
0
CELLULAR AIB UPTAKE
(nmoles/g dry tissue 2hr)
16
15
22
12
1
10
20
control
endotoxin dose (mg/kg)

Table 2. Effect of insulin on the intracellular-to-extracellular AIB concentration ratio (distribution ratio) in control and endotoxic muscles.

Group	Insulin (mU/ml) 0.0	1.0	10.0	100.0
Control	1.84 ± 0.05 (42)	2.86 ± 0.13[a] (8)	3.09 ± 0.11[a] (13)	3.24 ± 0.14[a] (9)
Endotoxin	1.36 ± 0.03[b] (44)	1.78 ± 0.08[ab] (13)	2.15 ± 0.12[ab] (8)	2.12 ± 0.06[ab] (10)

Values are means ± SE. Numbers in parentheses indicate numbers of muscles. [a] $p < 0.01$, compared to 0.0 mU/ml insulin within the control or endotoxic group. [b] $p < 0.01$, compared to corresponding controls.

Figure 4. Effect of Na^+ on cellular AIB uptake by control and endotoxic (20 mg/kg) soleus muscles. Values are means $\pm$ SE for number of muscles given within bars. Stars, values significantly different ($P < 0.001$) from control muscle AIB uptake in the presence of Na^+.


16
12
8
4
0
CELLULAR AIB UPTAKE
(nmoles/g dry tissue/2 hr)
control
endotoxin
16
12
11
12
[Na+]= 142 mM
[Na+]= 0 mM

controls was not significantly different from that in endotoxic muscles. The basal AIB uptake values were approximately one-half of that seen in the control muscles in the presence of Na^+. In the absence of Na^+, the distribution ratios were reduced to one in both groups of muscles. Furthermore, there was no significant effect ($P > 0.05$) of insulin (100 mU/ml) on AIB uptake in control or endotoxic muscles when Na^+ was absent from the medium.

DISCUSSION

This study has shown a decrease in basal and insulin-stimulated AIB uptake by the skeletal muscle of rats in endotoxic shock. In addition, the depression of basal cellular AIB uptake by endotoxic soleus muscles was the same over a twenty-fold range of endotoxin doses. We ascertained the onset of shock phenomenon in rats at 5-hr post-endotoxin by the occurrence of lactic acidemia, hypoglycemia, and hemorrhagic lesions in the bowel. Previous studies have documented changes in blood lactate (84) and glucose (94) along with hemodynamic alterations (hypotension, decreased cardiac output, and increased peripheral vascular resistance) after endotoxin administration in a variety of species (54).

The isolated rat soleus muscle, which we used for the assessment of amino acid transport has been shown to be a viable preparation, _in vitro_, by a number of investigators (13,18). The isolated muscles retain their excitability and 90% of their initial cellular potassium contents at the end of 6 hours of incubation at 35°C (70). The transport of oxygen and substrates to fibers within the muscle core and thus the viability of these fibers during the incubation most probably vary with the thickness of the muscle preparation. Previous investigators have indicated that facilitated sugar (70) and uphill amino acid transport (24) and active ion transport (18,71) by soleus muscle were readily measured when isolated rat soleus muscles were in the weight range of 30-40 mg. However, the present studies in soleus

muscles (55-65 mg) from 140 to 160 gram rats clearly demonstrated that muscles could accumulate AIB intracellularly against a concentration gradient and that the cellular amino acid uptake was sensitive to insulin.

AIB, a nonmetabolizable compound, has been shown to be a suitable substrate for investigating the transport of alanine, glycine and proline and other neutral amino acids by System A across the cell membrane in skeletal muscle (14,15,97,109). Riggs and McKirahan have provided evidence that, of the various neutral amino acid transport systems in rat diaphragm, System A is the only one that responds to insulin (97). Thus, a distinct feature of skeletal muscle AIB transport measurement is that it evaluates not only neutral amino acid transport by System A but also its response to insulin. The uptake of AIB into soleus muscle as studied here reflects its net influx into the muscle. Previous studies have shown that such net influx of AIB is a resultant of a carrier mediated uphill influx and a passive AIB efflux out of the muscle (24). The efflux process has been shown to be insensitive to insulin (24).

The reduction of the distribution ratio to one and a 54% decrease in control muscle AIB uptake in the absence of Na^+, as shown in this study, was comparable to the previous findings of Narahara and Holloszy who studied AIB uptake in frog sartorius muscle in Na^+-free media (88). Although a distribution ratio of one is consistent with a process of simple diffusion, this can also be indicative of a carrier mediated process without the capability of active transport. That the carrier continues to operate in the absence of active transport is

supported by experiments showing glycine competition of AIB transport in the absence of Na^+ (92). The fact that AIB uptake in Na^+-free media was the same in control and endotoxic muscles implies that the membrane carrier responsible for the facilitated transport of AIB into skeletal muscle was functional during endotoxic shock. The decrease in basal AIB uptake by endotoxic muscles was therefore due to an alteration in the Na^+-dependent portion of the uptake process. Na^+-dependent AIB uptake, calculated as the difference between AIB uptake in the presence and absence of Na^+, was decreased in shock to 36% of the control value. The decrease in the Na^+-dependent active AIB uptake could be due either to a diminution of $[Na^+]$ gradient or to an altered transmembrane electrical potential difference in skeletal muscle during shock. Previous studies have shown a direct dependency of active AIB transport on the $[Na^+]$ gradient and/or membrane potential (41,48,69,119). Since the $[Na^+]$ gradient and potential difference are maintained by the Na^+/K^+ pump, an alteration in this pump during shock could affect the active accumulation of AIB. Indeed, changes in membrane Na^+ transport during endotoxic shock has been implicated in cardiac muscle (91) and liver (102,103). Gibson et al (42) observed a reduction in transmembrane potential difference across skeletal muscle cell membranes in dogs during endotoxin shock. Furthermore, direct evidence of altered $[Na^+]$ gradient in skeletal muscle during endotoxic shock has been shown by Flear and coworkers (37,38) and others (42) who have reported increases in intracellular $[Na^+]$ and decreases in extracellular $[Na^+]$.

The decreased insulin effect on AIB uptake in endotoxic muscle was probably not due to just a decrease in basal AIB uptake. This interpretation was supported mainly by the analysis of variance of the data (see Results and Fig. 2). To explain the low magnitude of response of the AIB transport system to insulin in endotoxic muscles, one might postulate that the defect in the Na^+-dependent portion of AIB transport is also limiting insulin's ability to increase transport. Since insulin is known to increase the active accumulation of type A amino acids by enhancing the transmembrane [Na^+] gradient (18,85), the alteration in insulin's ability to increase AIB transport during endotoxic shock may be due to an inadequate insulin-enhancement of the [Na^+] gradient, i.e. an impairment in insulin activation of the Na^+-pump mechanism. Alternatively, the attenuated insulin response could be due to an impairment of the Na^+ pump during endotoxin shock.

The observed decrease in basal and insulin mediated AIB transport by skeletal muscle of animals in endotoxic shock would be expected to reduce the concentration of amino acids in cell fluid. This in turn could limit protein synthesis in skeletal muscle during endotoxic shock.

CHAPTER IV

EFFECT OF ENDOTOXIC SHOCK ON THE KINETICS OF SYSTEM A AMINO ACID TRANSPORT IN RAT SOLEUS MUSCLE

INTRODUCTION

Alterations in amino acid and protein metabolism associated with severe trauma or sepsis lead to a profound decrease in the mass of skeletal muscle protein (21,23,53,99,100,114). An increase in protein degradation in muscle presumably provides amino acids for energy in muscle and other tissues (23,99). Although the factors responsible for increased catabolism of muscle protein during trauma or sepsis are not fully understood, net skeletal muscle proteolysis could be due to an imbalance between protein synthesis and degradation.

Transport of amino acids into muscle has important effects on protein metabolism by regulating the composition of the intracellular pools of amino acids (15,55). We reported previously that active amino acid transport via system A is markedly reduced in rat soleus muscle during endotoxic shock (64). The stimulation of amino acid transport by insulin is also attenuated in endotoxic soleus muscle (64). Guidotti et al (48) suggested that of all the amino acid transport systems in cells the A system may be the most important because it is the only system which is subject to regulation by hormones and other stimuli (14,74,97,109). The depressed activity of system A amino acid transport in the presence or absence of insulin and the reduced ability of muscle cells to actively accumulate type A amino acids may limit the formation of new protein and thereby lead to net protein catabolism during endotoxic shock.

METHODS

Male rats (80-100 g) purchased from Holtzman Co. (Madison, WI) were allowed free access to water but fasted overnight prior to experiments. Endotoxic shock was produced in rats by intravenous (sublingual vein) injection of 20 mg/kg Salmonella enteritidis endotoxin (Boivin, Difco Laboratories, Detroit, MI), suspended in 0.9% saline. Controls were treated similarly but were given an equal volume of 0.9% saline (0.5 ml/100 g body wt.). Rats were lightly anesthetized with ether prior to injections. Five hours after injections, rats were killed by decapitation and both soleus muscles were removed with care to avoid any damage to the muscles and placed in chilled Krebs-Ringer bicarbonate buffer (KRB). Isolated soleus muscles were maintained at the resting length by attaching opposing tendons to an adjustable stainless steel holder.

Measurement of alpha-aminoisobutyric acid uptake

Isolated soleus muscles (40 mg) were incubated at 37°C for 20, 40 or 60 minutes in KRB (3 ml), pH 7.4, which was equilibrated with 95%O_2:5%CO_2 and contained 5.6 mM glucose, 119 mM NaCl, 4.7 mM KCl, 1.1 mM KH_2PO_4, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 24 mM $NaHCO_3$. The incubation medium also contained alpha-amino-(1-^{14}C)isobutyric acid (AIB) (0.23-1.4 microcuries/ml) (Amersham, Arlington Heights, IL; specific activity 40-60 mCi/mmol) with sufficient unlabeled AIB to yield AIB concentrations in the range of 0.1 to 20 mM. Soleus muscles were removed from the medium at the end of incubation and lightly blotted

on Whatman (#42) filter paper to remove adherent surface radioactivity. After weighing the muscles they were dissolved in 2 ml of TS-1 tissue solubilizer (Research Products International, Mount Prospect, IL). Scintillation fluor was added to vials containing dissolved muscles and media samples (0.1 ml). Radioactivity was counted in a Packard Tri-Carb 460C liquid scintillation counter and quenching of radioactive counts was corrected by an external standard technique.

To measure the transport of AIB into the intracellular compartment of skeletal muscle, it was necessary to determine the content of AIB in the extracellular compartment of the tissue. The experiments to determine tissue inulin uptake were carried out separately but similarly to those of tissue AIB uptake. The technique for measuring total tissue water and extracellular water was similar to that described previously (64). Extracellular water was determined by measuring the uptake of inulin[C^{14}]carboxylic acid (0.1 uCi/ml) by muscle after 20, 40 or 60 minutes of incubation. Total tissue water was calculated for each muscle from its wet weight to dry weight ratio. Dry weights were determined after drying muscles to a constant weight in a vacuum oven at 100°C. Intracellular water was calculated as the difference between total tissue water and extracellular water. Total AIB content (mol/g dry wt) was calculated by dividing tissue radioactivity (DPM/g dry wt) by the specific activity (DPM/mol) of AIB in the medium. Intracell AIB content was calculated as follows:

Intracell AIB Content = AIB Content - (Extracell Space X Medium [AIB])
(mol/g dry wt) (mol/g dry wt) (ml/g dry wt) (mol/ml)

Statistical analysis

Data were expressed as means ± SE. Results were analyzed using a one-way analysis of variance, least significance difference test, or Student's t-test modified for unpaired replicates. For the double reciprocal plots, the error values for the slope and the intercept were determined and used to calculate the SE for V_{max} and K_m. Since K_m = slope · V_{max}, the SE for V_{max} was determined first and compounded with the error of the slope to give SE for the K_m (59,119). Slopes were compared with analysis of covariance. A "P" value less than or equal to 0.05 was considered significant.

RESULTS

The data in Table 1 show total tissue, extracellular, and intracellular water in isolated soleus muscles. Total tissue water was not altered during 20, 40 or 60 minutes of incubation in control or endotoxic muscles ($P > 0.05$). Extracellular water increased with the duration of incubation in both the control and endotoxic muscles ($P < 0.05$). The increases in extracellular water in the endotoxic muscles were equal to those seen in the controls. Intracellular water decreased during the incubation in both the control and endotoxic muscles ($P < 0.05$) without accompanying changes in total muscle water. However, there were no significant differences between control and endotoxic muscle intracellular water contents at 20, 40 or 60 minutes of incubation ($P > 0.05$). We reported similar findings for water contents of soleus muscles incubated for periods up to 3 hours in vitro (64). The decrease in the inulin-inaccessible space in soleus muscle was taken to represent changes in the volume of the intracellular compartment available for AIB distribution.

In this study we have investigated amino acid transport in skeletal muscle during endotoxic shock by using the non-metabolizable amino acid analog, alpha-aminoisobutyric acid (4,14). It has been shown that AIB is preferentially transported by system A in skeletal muscle which actively transports neutral amino acids with short polar or linear side chains, such as alanine, proline, serine and AIB (14,15,48,109). The initial rate of entry of AIB into skeletal muscle

TABLE 1. Effect of incubation time on water contents of control and endotoxic soleus muscles

Group	N	Incubation Time (min)	Total Tissue Water (ml/g)	Extracellular Water (ml/g)	Intracellular Water (ml/g)
Control	10	20	0.795 ± 0.002	0.228 ± 0.004	0.566 ± 0.004
	12	40	0.792 ± 0.002	0.254 ± 0.005*	0.538 ± 0.004*
	11	60	0.800 ± 0.003	0.282 ± 0.005*	0.519 ± 0.007*
Endotoxin	10	20	0.792 ± 0.002	0.222 ± 0.008	0.571 ± 0.008
	12	40	0.804 ± 0.003	0.252 ± 0.011*	0.552 ± 0.012*
	12	60	0.802 ± 0.002	0.280 ± 0.007*	0.523 ± 0.007*

Values are means ± S.E.; N, no. of muscles. Water contents between groups were not significantly different ($P > 0.05$). *$P < 0.05$, compared with 20-min incubation of own group.

cells of control and endotoxic soleus muscles was determined over a wide range of extracellular AIB concentrations (0.1 - 20 mM). The uptake of AIB was measured after 20, 40 or 60 minutes of muscle incubation. AIB entered the cells at a linear rate for 40 minutes but not for 60 minutes in all experiments. Therefore, the initial rates of entry for each concentration of AIB was calculated from slopes of regression lines fitted to AIB uptake measurements at 20 and 40 minutes and passing thru zero time. Correlation coefficients (r) calculated from slopes of regression lines were greater than 0.88 in all experiments ($P < 0.001$).

In Fig. 1 and 2 it can be seen that when control and endotoxic soleus muscles were incubated in media containing increasing concentrations of AIB the transport of AIB increased rapidly up to the extracellular AIB concentration of 1 mM and then more slowly and linearly at the higher extracellular AIB concentrations (9-20 mM) (Fig. 2). The transport rate did not reach a steady state level even at the highest AIB concentration (20 mM). As illustrated in the inset to Fig. 1, total AIB uptake in the endotoxic muscles was 32, 25, and 27% lower than control muscles at 0.1, 0.25, and 1.0 mM AIB ($P < 0.01$). However at 3.1 and 6.4 mM AIB, total AIB uptake in control muscles was not significantly different from the total uptake in endotoxic muscles ($P > 0.05$) (Fig. 1). Fig. 2 shows that at higher AIB concentrations (9.6, 14.8 and 20.0 mM) total AIB uptake in endotoxic muscles was 21, 22, and 28% greater than control muscles ($P < 0.05$).

Figure 1. Effect of concentrations of AIB (0.1 - 6.4 mM) on the rate of total AIB transport (v_t) in control and endotoxic soleus muscles. **Inset**, transport rates (v_t) for control and endotoxic muscles at AIB concentrations less than 1 mM. Values are means ± SE for 13 to 27 muscles. **Solid circle**, control; **open circle**, endotoxin. **Stars**, significant differences ($P < 0.05$) between control and endotoxic muscles at each concentration of AIB; **ns**, nonsignificant difference between control and endotoxic muscles ($P < 0.05$).

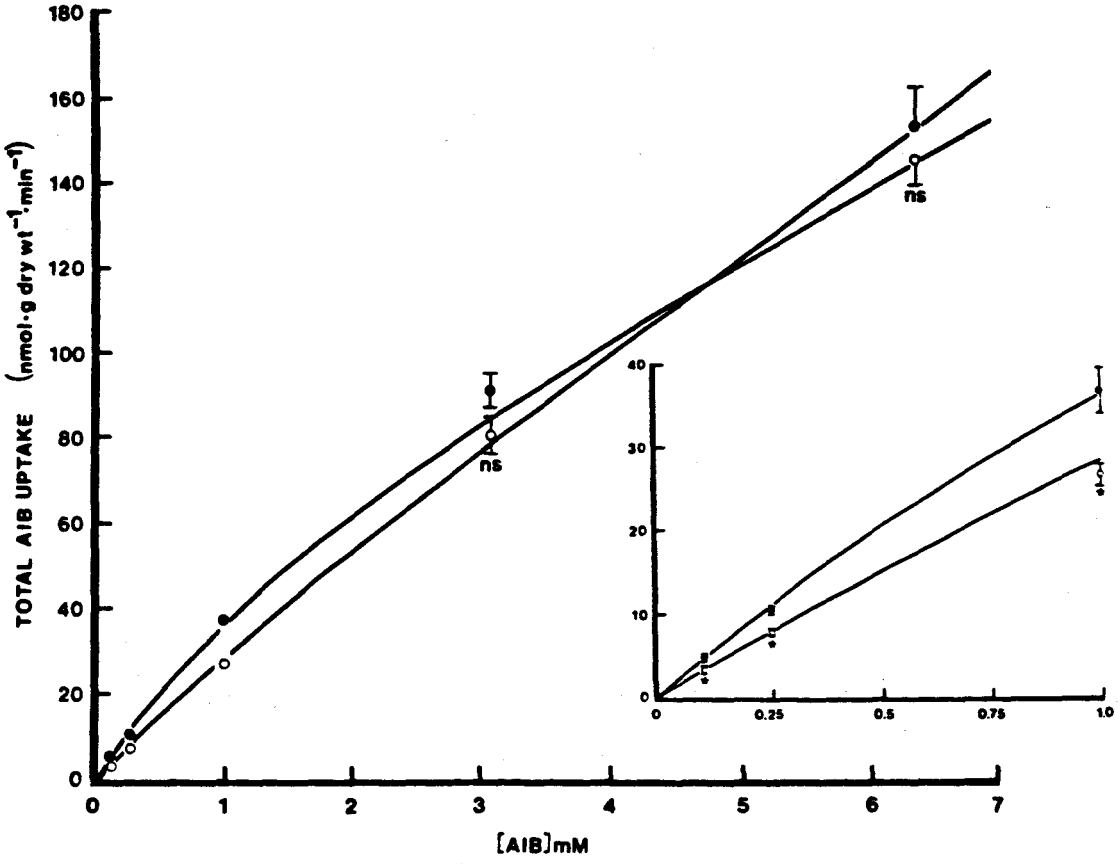
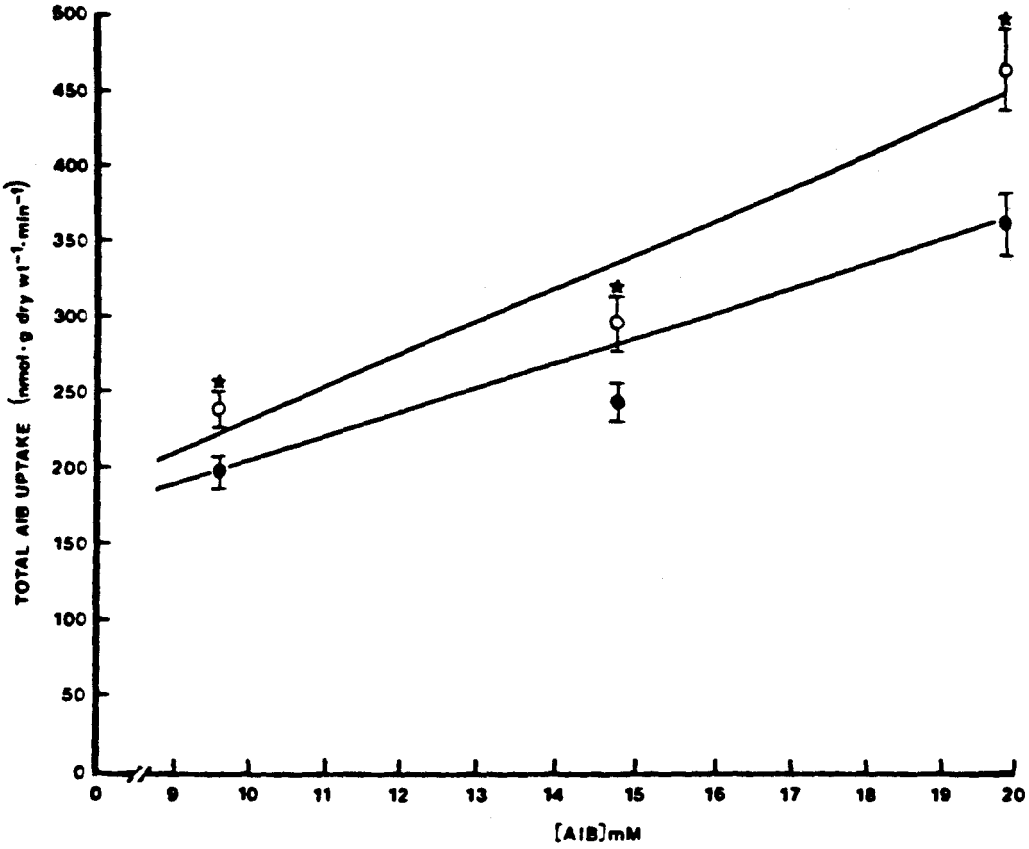

TOTAL AIB UPTAKE (nmol·g dry wt⁻¹·min⁻¹)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
[AIB]mM
ns
ns
40
30
20
10
0
0
0.25
0.5
0.75
1.0
*
*

Figure 2. Effect of concentrations of AIB (9.6 - 20.0 mM) on the rate of total AIB transport (v_t) in control and endotoxic soleus muscles. Values are means $\pm$ SE for 14 to 22 muscles. **Solid circle**, control; **open circle**, endotoxin. **Stars**, significant differences ($P < 0.05$) between control and endotoxic muscles at each concentration of AIB. See text for values of K_D.


TOTAL AIB UPTAKE (nmol · g dry wt⁻¹ · min⁻¹)
500
450
400
350
300
250
200
150
100
50
0
0
9
10
11
12
13
14
15
16
17
18
19
20
[AIB]mM

The fact that total AIB uptake was not saturated at AIB concentrations as high as 20 mM indicates that AIB uptake occurs by more than one process in skeletal muscle. These findings are similar to others (3,73,88,106,107) and have been interpreted to indicate that total AIB uptake in skeletal muscle is dependent on two parallel transport systems, one obeying Michaelis-Menton saturation-type kinetics and one failing to exhibit saturation and hence resembles a diffusion-like process. Total AIB uptake (v_t) can be separated into its component parts by the following equation:

$$v_t = v_c + v_d \quad (1)$$

where v_c and v_d respectively represent the carrier-mediated saturable and non-saturable components.

The non-saturable component of total AIB uptake can be represented by the following equation:

$$v_d = K_D(S) \quad (2)$$

where S is the concentration of AIB in the incubation medium and K_D is the diffusion constant. Measurements of total AIB uptake for control and endotoxic soleus muscles at the three highest concentrations of AIB were fitted to regression lines by least squares analysis and the slopes were taken to represent K_D (Fig. 2). The K_D for the non-saturable component in endotoxic muscle (0.36 hr^{-1}) was 38% greater than the controls (0.26 hr^{-1}) ($P < 0.01$).

The saturable component of AIB uptake can be represented by the Michaelis-Menton equation:

$$v_c = V_{max}/(K_m/S + 1) \quad (3)$$

where K_m is the Michaelis constant and V_{max} is the maximum rate of

entry. As shown in Fig. 3, saturable AIB uptake in muscles of endotoxic rats was decreased by 66% compared to control muscles. In close agreement with previous observations (14,20) the saturable component represented 67% and 13% of total AIB uptake in controls at 0.1 and 20 mM, respectively. However, the saturable component of total AIB uptake for endotoxic muscles was 33% and 7% of total AIB uptake at 0.1 and 20 mM, respectively.

The maximum rate of entry (V_{max}) and apparent K_m for saturable AIB uptake by control and endotoxic soleus muscles were determined from reciprocal plots of initial rates of entry, $1/v_c$, and extracellular AIB concentration, 1/S, according to the graphical method of Lineweaver-Burk (75) (Fig. 4). The method of least squares was used to fit regression lines to plots of $1/v_c$ against 1/S to determine the kinetic parameters, V_{max} and apparent K_m. The large decrease in the rate of entry of saturable AIB transport into endotoxic muscles was due to a 69% decrease in V_{max} ($P < 0.05$) (see below).

Group	number of muscles	V_{max} ($nmol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$)	K_m (mM)
Control	62	55.6 ± 3.3	1.68 ± 0.12
Endotoxin	67	18.3 ± 1.9	1.64 ± 0.19

The values of apparent K_m for saturable AIB transport were not different between control and endotoxic soleus muscles ($P > 0.05$).

Figure 3. Effect of low concentrations of AIB (0.1 - 3.1 mM) on the saturable component (v_c) of AIB transport in control and endotoxic soleus muscles. The saturable component of uptake was estimated for each AIB concentration by substracting the non-saturable component (v_d) from total AIB uptake (v_t) (see text, equations 1 to 3). Each point represents the slope as derived from linear regression analysis from these values (13 - 27 muscles) (as shown in Fig. 1 and 2). **Solid circle**, control; **open circle**, endotoxin.

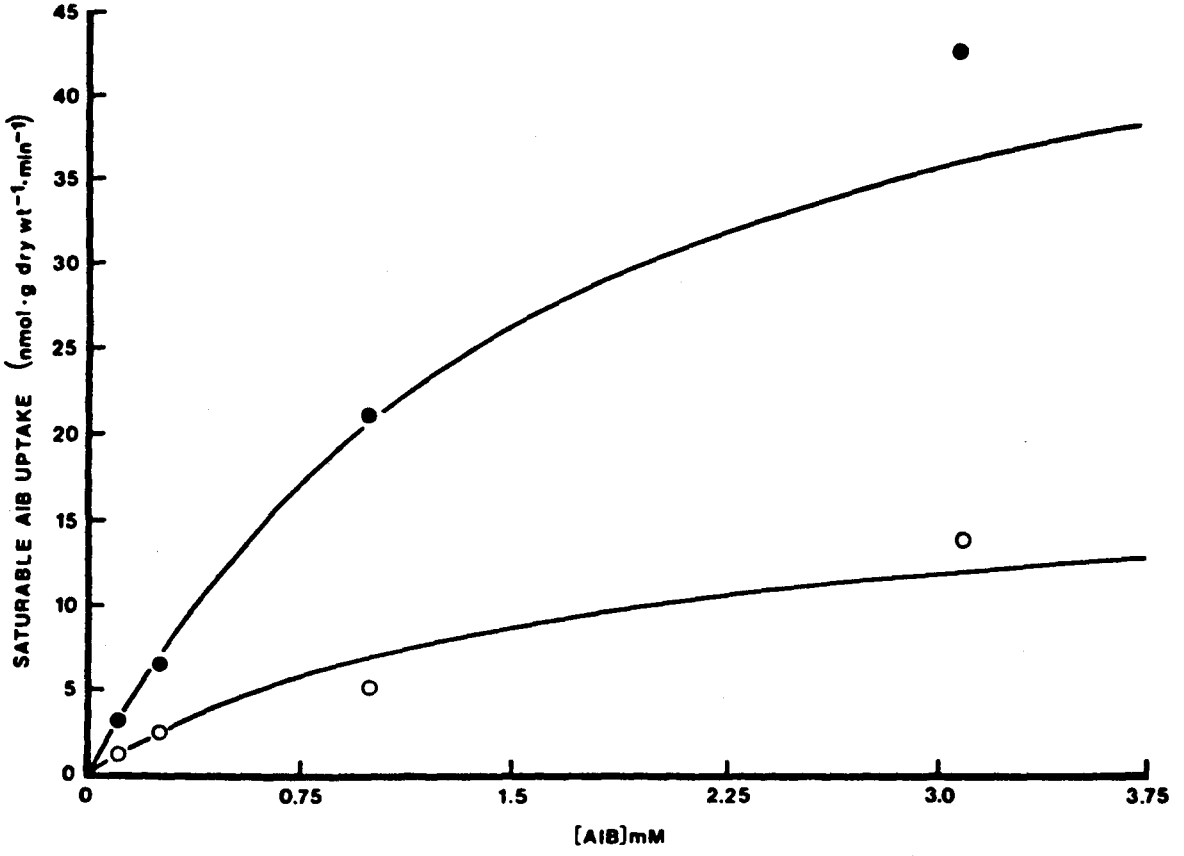
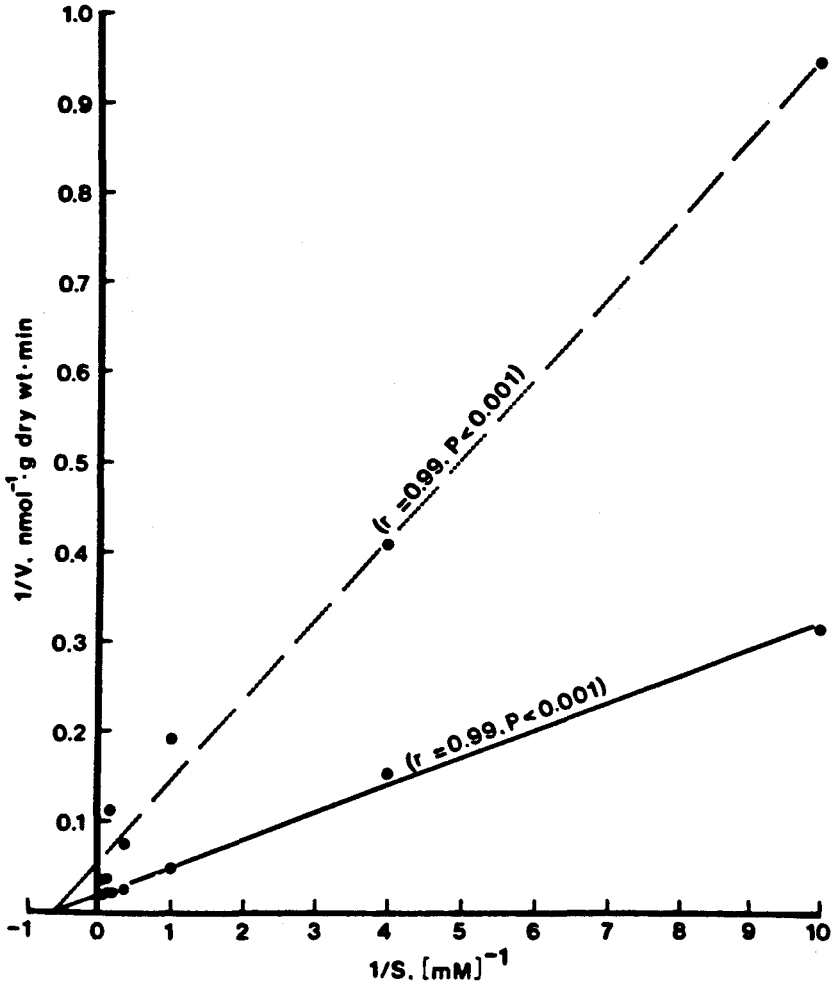

SATURABLE AIB UPTAKE (nmol·g dry wt⁻¹·min⁻¹)
45
40
35
30
25
20
15
10
5
0
0
0.75
1.5
2.25
3.0
3.75
[AIB]mM

Figure 4. Double reciprocal plot of initial rates of saturable AIB transport (v_c) versus AIB concentration for control and endotoxic soleus muscles. **Solid line**, control; **dashed line**, endotoxin. Correlation coefficients (r) and significance of individual regressior lines are shown (least squares analysis). Slopes are significantly different between control and endotoxic muscles ($P < 0.001$). See text for values of V_{max} and apparent K_m.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/V, nmol^{-1}·g dry wt·min
(r =0.99, P<0.001)
(r =0.99, P<0.001)
-1
0
1
2
3
4
5
6
7
8
9
10
1/S, [mM]$^{-1}$

DISCUSSION

In a previous study we established that manifestations of shock (lactic acidemia, hypoglycemia, and hemorrhagic lesions of the bowel) were evident in rats 5 hours after endotoxin (20 mg/kg) injection (64). It has been determined that the lethality of rats in this model is 32% at 5 hours post-endotoxin. Also, we showed that the capability for active amino acid transport by system A was substantially reduced in endotoxic rat soleus muscle (64). The purpose of this study was to determine if the reduction of AIB uptake in endotoxic muscle was due to changes in the kinetic behavior of system A amino acid transport. The rate of appearance of AIB in skeletal muscle cells was separated into a saturable, carrier-mediated process which fits Michaelis-Menton kinetic analysis and a passive diffusion process whose rate depends upon the diffusion constant (K_D) and the extracellular amino acid concentration (3,88,106,107). Previous studies showed that saturable AIB uptake was an energy dependent process with an apparent K_m for AIB of approximately 1-2 mM (3,73,88). The AIB diffusive process remains unsaturable even at AIB concentrations as high as 30 mM (73,88); saturation, however, may occur at higher AIB concentrations which would give a very high value of apparent K_m. The rate constant (K_D) for the non-saturable component and the apparent K_m for the saturable process obtained for control soleus muscles in this study are similar to those reported by Akedo and Christensen for rat diaphragm (3) and others for mouse soleus muscle (73).

The decreased rates of AIB transport by the saturable component of system A in endotoxic soleus muscles was due to a decrease in V_{max} and not due to a change in apparent K_m. These results suggest that the reduction in the maximum rate of entry of AIB into skeletal muscle cells of endotoxic rats was likely due to a decrease in the number of system A transport carriers and/or a decrease in the activity of existing transporters in the membrane and not due to a change in membrane carrier affinity for AIB.

It has been demonstrated that the active transport of amino acids by system A requires the presence of extracellular Na^+ (62,64,88,92) and that most if not all of the energy used to transport type A amino acids is supplied by the free energy of the Na^+ electrochemical gradient (85). There is little doubt that the rate of Na^+-dependent amino acid transport and the steady-state level of amino acid accumulation are influenced by the electrochemical potential difference for Na^+ across the cell membrane (62,85,119). A decrease in the number and/or activity of existing transport sites in the cell membrane would, by itself, not be expected to decrease the steady-state level of type A amino acid accumulation. However, from thermodynamic considerations reduction of the Na^+-electrochemical gradient across the cell membrane should reduce the active accumulation of AIB by muscle cells (85,88,92). Although we have not measured the Na^+-electrochemical gradient in endotoxic muscle there is substantial evidence showing alterations of the Na^+ concentration gradient and membrane potential in skeletal muscle during endotoxic (37,42) and septic shock (38,60,115). We demonstrated in a previous study that active trans-

port of AIB and the near steady-state level of AIB accumulation were substantially decreased in endotoxic soleus muscles and that in the absence of extracellular Na^+ the transport of AIB was reduced to a passive uptake process which was not different between control and endotoxic muscles (64). These results suggested that the decrease in active AIB transport in endotoxic soleus muscles was due to an alteration of the Na^+-dependent portion of system A amino acid transport (64).

Geck and Heinz (41) have provided a kinetic analysis of several transport models which relates the effects of membrane potential on various transport parameters. They indicated that the magnitude of the membrane potential is an important factor in determining the K_m and V_{max} values of amino acid transport systems. It was demonstated that the membrane potential can be expected predominately to affect either the K_m or V_{max} of the transport system, depending on whether the loaded or unloaded carrier bears an electrical charge. Thus, it is possible than an attenuation of the membrane potential across the skeletal muscle cell membrane is responsible for decreasing V_{max} without affecting the K_m for AIB transport in endotoxic soleus muscles. It has been shown that inhibition of the Na/K-pump by strophanthin K in skeletal muscle reduces the Na^+ concentration gradient and membrane potential and decreases the active accumulation of AIB (85,92). Additionally, insulin has been shown to stimulate the active accumulation of AIB in skeletal muscle via hyperpolarizing the membrane and increasing the free energy of the Na^+ electrochemical gradient (85). The hyperpolarization of the membrane by insulin in

skeletal muscle increases V_{max} without any changes in K_m for AIB (73,78,80). Insulin might increase V_{max} by increasing the number and/or activity of transport carriers in the cell membrane (74,78). In skeletal muscle the effect of insulin on AIB transport is eliminated in the absence of extracellular Na^+ (64,88) and reduced in the presence of protein synthesis inhibitors (73,78). Therefore a decrease in the Na^+ electrochemical gradient in endotoxic soleus muscle could decrease V_{max} and not affect K_m which is in agreement with the kinetic analysis of this study. We have previously reported that the effect of insulin on AIB transport in soleus muscle was decreased during endotoxin shock (64). It is plausible that resistance of AIB transport to insulin was due to an inability of insulin to effectively increase the Na^+ electrochemical gradient and/or increase the number of active carriers.

The fact that the K_D was 38% greater in the endotoxic muscles as compared to the controls indicates that there is an increase in passive AIB diffusion in endotoxic soleus muscles. Diffusion of AIB into the cells of endotoxic soleus muscles accounted for a large proportion of total AIB uptake particularly at higher extracellular AIB concentrations. Moreover, the resultant diffusion of AIB represented a much greater proportion of total AIB accumulation in endotoxic muscles than was the case for AIB uptake by control muscles. Therefore at low extracellular AIB concentration, where the uptake of AIB occurs mainly by the saturable route, total AIB uptake by endotoxic muscles was significantly lower than controls due to a large decrease (69%) in saturable AIB transport. However, when the concentration of AIB in

the medium bathing the endotoxic muscles was increased above the concentration of AIB that would produce a half-maximal rate of entry, the saturable component makes up only a small proportion of total AIB uptake. Even though the saturable component was substantially decreased in the endotoxic muscles as compared to the controls this fact was not evident in measurements of total AIB uptake at concentrations of AIB beyond K_m. In fact at high extracellular concentrations of AIB the rate of AIB transport was actually greater in the endotoxic muscles as compared to the controls because K_D was substantially greater in the endotoxic muscles. Measurement of transport rates at high concentrations of AIB is an experimental technique which was devised by Akedo and Christensen (3) and used by others (73,78,88) to determine the diffusion constant (K_D) for the non-saturable component and was not intended to be representative of transport in the physiological range of amino acid concentrations. Thus, it should be kept in mind that the concentration of most neutral amino acids in plasma is less than 1 mM (23), which indicates that the uptake of neutral amino acids in vivo occurs primarly by a saturable mechanism, which is in the same concentration range where total AIB uptake by endotoxic soleus muscles was lower than controls.

An increase in the non-saturable component which presumably allows passive, reversible movement of amino acids into and out of cells may be a homeostatic mechanism during periods of stress which allows for the release of amino acids by skeletal muscle to be used, for example, by other tissues for fuel and by the liver for gluconeogenesis or other purposes (21,23). Indeed, Clowes et al (21,23) have

demonstrated that the release of amino acids from skeletal muscle and the uptake of amino acids by liver are accelerated during septic and post-traumatic states. However, prolonged mobilization of amino acids from intracellular pools may lead to loss of muscle mass and result in muscle weakness and threaten survival.

In conclusion, the results indicate that the mechanism of decreased amino acid transport in endotoxic soleus muscle is due to a decrease in the number of carriers and/or a decrease in the activity of existing transporters in the membrane and not due to a change in membrane carrier affinity for amino acid. Since the intracellular pools of amino acids might play an important role in the regulation of protein synthesis and since amino acid transport contributes to the intracellular pools, it is reasonable than an inhibition of amino acid entry into muscle might limit the formation of new protein and thereby contribute to protein catabolism during septic and endotoxic shock.

CHAPTER V

EFFECT OF ENDOTOXIC SHOCK ON SKELETAL MUSCLE INTRACELLULAR ELECTROLYTES AND SYSTEM A AMINO ACID TRANSPORT, *IN VIVO*

INTRODUCTION

Abnormalities of protein and amino acid metabolism in skeletal muscle during endotoxemia (64,65,110) and sepsis (20,23,99) may be due to alterations in the utilization of fuels at the cellular level. Accelerated release of amino acids from skeletal muscle and transfer to liver for gluconeogenesis and protein synthesis appear to be responses to endotoxin and sepsis that are essential to survival (20,99). Important as these responses may be, prolonged mobilization of amino acids from skeletal muscle leads to loss of muscle mass and threatens survival.

The capacity for active transport and accumulation of amino acids in skeletal muscle cells plays an important role in the dynamics of protein metabolism by regulating the composition of the intracellular amino acid pools (2,55). Intravenous infusion of balanced amino acid solutions to postoperative dogs diminishes the fall in skeletal muscle intracellular amino acid pools and reduces hindquarter amino acid efflux (63). These results provide evidence that skeletal muscle protein metabolism may be related to the composition of the intracellular amino acid pools. Insulin regulates protein metabolism in skeletal muscle by stimulating protein synthesis and inhibiting protein degradation and by increasing the uptake of amino acids through increased activity of Na^+-dependent system A amino acid transport (46,64,85). System A amino acid transport in skeletal muscle is dependent on the Na^+-electrochemical gradient to provide energy for

the active accumulation of amino acids (62,85). A decrease in that gradient will decrease the active accumulation of amino acids and presumably lead to a net release of amino acids from the intracellular amino acid pools.

We showed previously that system A amino acid transport in skeletal muscle and the stimulation of this process by insulin *in vitro* are decreased during endotoxic shock (64,65). Although isolated muscles incubated *in vitro* offer numerous advantages for controlled investigations of muscle metabolism, results from such studies may differ significantly from those measured *in vivo* due to the fact that tissues are removed from the effects of innervation, humoral factors, and other regulatory factors normally present *in vivo*. These factors are particularly important when investigating nutrient transport or substrate metabolism in tissues isolated from control and endotoxic animals because their effects on tissue metabolism and hormone sensitivity may not be evident under *in vitro* conditions.

The purpose of the present study was to assess skeletal muscle amino acid uptake, *in vivo*, during endotoxic shock. To assess relationship(s) between amino acid transport and ionic gradients, we have investigated *in vivo* changes in intracellular and extracellular Na^+, K^+ and Cl^- concentrations in muscle. The regulation of amino acid transport by insulin was assessed by measuring the endogenous insulin level and effects of injections of exogenous insulin into endotoxic rats.

METHODS

Determination of alpha-aminoisobutyric acid uptake

Male rats (Holtzman Company, Madison, WI) weighing 80-100 g were fasted overnight (16 hr) but allowed water ad libitum prior to experiments. The rats were anesthetized with ether for all injections and surgical procedures. To measure soleus muscle amino acid transport, alpha-amino(1-^{14}C)isobutyric acid (2 uCi/ml) (Amersham, Arlington Heights, IL; specific activity 40-60 mCi/mmol) and unlabeled AIB (Sigma Chemical Company, St. Louis, MO) was dissolved in 0.9% saline and given at doses of 1 mg/kg or 10 mg/kg by injection into the sublingual vein of rats (0.5 ml/100g). Shock was induced by the intravenous injection of 20 mg/kg Salmonella enteritidis endotoxin (Boivin, Difco Laboratories, Detroit, MI) suspended in 0.9% saline subsequent to the injection of AIB. Control animals were treated similarly but received an equal volume of 0.9% saline. AIB uptake in control and endotoxic soleus muscles was determined 1, 2, 3, 4 or 5 hours later and at 8 and 24 hours for the controls. Insulin was intravenously injected into control and endotoxic rats 5 hours after the administration of AIB. The effect of insulin on AIB uptake in soleus muscle was assessed 30 minutes after the injection of insulin. Pure crystalline bovin insulin (24.9 U/mg) was suspended in 0.9% saline and given in doses ranging from 50.0 mU/kg to 2500.0 mU/kg. A total of 5 doses of insulin were tested and compared with animals receiving 0.9% saline. Soleus muscles were removed and weighed and a

sample of arterial blood was drawn from the abdominal aorta and collected in heparin-lithium coated microfuge tubes for analysis of ^{14}C-labeled AIB. Plasma was obtained from each blood sample after centrifugation in a Beckman Microfuge 12 (Palo Alto, CA). Muscles and plasma samples (0.1 ml) were digested in scintillation vials containing 2.0 ml tissue solubilizer at 40°C for 16 hours. Scintillation fluor (13 ml) was added to vials containing dissolved muscles and plasma samples and the radioactivity was counted in a Tri-Carb 460C liquid scintillation counter. The quenching of radioactive counts was corrected using an external standard technique. Muscle (DPM/g) and plasma radioactivity (DPM/ml) were divided by the specific activity of AIB (DPM/mol) to determine muscle AIB content and plasma AIB concentration.

Determination of plasma glucose and insulin

Plasma from control and endotoxic rats was analyzed for glucose and insulin 1, 3 or 5 hours after the injection of 1 mg/kg AIB. Plasma glucose concentration was determined using a model 23A Yellow Springs glucose analyzer (Yellow Springs, OH). Plasma insulin was determined by radioimmunoassay, using a double antibody technique (Cambridge Medical Diagnostics, Inc., Billerica, MA).

Determination of muscle and plasma electrolytes

Electrolytes were determined in tissues of control and endotoxic rats 5 hours after the injection of 1 mg/kg AIB. Na^+ and K^+ were analyzed in plasma (0.1 ml) and nitric acid digestates of soleus muscles by means of atomic absorption spectroscopy (Perkin-Elmer,

model 603). Cl^- was analyzed in plasma and muscle by means of electrometric titration in a Buchler-Cotlove chloridometer (25,26). Plasma Cl^- was titrated in a solution containing 0.1 ml plasma, 4.0 ml 0.1 N HNO_3 in 10% glacial acetic acid and 4 drops of gelatin-indicator solution. Gelatin reagent contains the following dry, pulverized chemicals: Gelatin, Thymol and Thymol blue in the weight ratio of 60:1:1 (6.2 grams of this mixture is diluted in 1.0 liter hot water). The gelatin-indicator solution is used to ensure a smooth and reproducible amperometric current by preventing reduction of silver chloride and by facilitating a uniform deposition of reduced silver ions on the indicator cathode (25,26). For analysis of tissue chloride, a solution for titration was prepared by alkaline digestion of wet tissue, followed by protein precipitation, and perborate oxidation of sulfhydryl groups in the supernatant. To tubes containing wet tissue 3 ml 0.6 N NaOH was added and the tubes were covered with rinsed aluminum foil and heated in a boiling water bath for 30 minutes to effect complete dissolution of the tissue. For precipitation of protein and after cooling to room temperature, 3 ml of 4% $ZnSO_4.7H_2O$ in 0.4 N HNO_3 was added to each tube. Samples were shaken, set aside for 1 hour, centrifuged at 2500 r.p.m for 20 minutes, and a 2 ml aliquot of the clear supernatant was transfered to a titration vial. To each vial containing supernatant, 0.1 ml of fresh alkaline perborate solution (0.2 M $NaBO_3$ in 2.8 N NaOH) was added, and then mixed by gentle swirling. Vials were covered with Parafilm and left standing for 16 to 24 hours. Before titration, 3 drops of gelatin-

indicator solution and 0.5 ml of 1.3 N HNO_3 in 50% acetic acid was added to each vial (25,26).

Determination of extracellular (inulin) space and total tissue water

Inulin(^{14}C)carboxylic acid (0.1 uCi/kg) (Amersham, Arlington Heights, IL, specific activity 17.1 mCi/mmol) was injected intravenously (sublingual vein) into rats to determine the extracellular space of soleus muscle in vivo. Control and endotoxic rats were bilaterally nephrectomized to prevent the clearance of inulin from the plasma compartment by the kidneys (123,124). The kidneys were removed through an incision on either side of the rat after ligating the renal vessels. Care was taken during the procedure not to damage the adrenal glands or their blood supply. The incisions were closed with metal clips and the animals were allowed to recover for 30 minutes. The distribution of inulin in soleus muscle and plasma was determined 10, 20 or 30 minutes after the injection of inulin. The uptake of inulin by muscle and the plasma inulin concentration did not change after 10 minutes following its injection in controls and after 20 minutes following its injection in endotoxic rats. Because of these results, extracellular space was calculated in controls from the 10-30 minute values and in endotoxic muscles from the 20-30 minute values.

Calculations

Extracellular space (ml/g) was calculated as the ratio of muscle radioactivity (DPM/g) to plasma radioactivity (DPM/ml). The concentration of inulin in plasma was assumed to be equal to the concentration in muscle interstitial water. Tissue and plasma radioactivity

were determined as described previously. Total tissue water (ml/g) was calculated for each muscle from its wet weight to dry weight ratio. Muscles were dried in a vacuum oven at 100°C for 24 hours. Intracellular water (ml/g) was calculated as the difference between total tissue water and extracellular water. Extracellular AIB and electrolyte contents (mol/g) in muscle were calculated as the product of extracellular (inulin) space and AIB or electrolyte concentrations in plasma, respectively. Intracellular AIB and electrolyte contents were taken as the difference between total and extracellular AIB and electrolytes contents, respectively. Intracellular concentrations (mol/ml) of AIB and electrolytes were calculated by dividing the intracellular AIB and electrolytes contents by intracellular water. The distribution ratio of AIB was calculated by dividing the intracellular concentration of AIB by the extracellular AIB concentration.

Data analysis

Statistical analysis was performed using Student's t-test modified for unpaired replicates and one-way analysis of variance with means compared using least significant difference test. Results were expressed as means $\pm$ SE. $P \leq 0.05$ was considered significant.

RESULTS

The sequential changes in arterial plasma glucose and insulin concentrations for control and endotoxic rats are shown in Fig. 1 and 2. At one hour post-endotoxin, concentrations of glucose ($P < 0.05$) and insulin ($P < 0.001$) in the plasma of endotoxic rats were significantly greater than in the controls. Plasma glucose concentration in endotoxic rats was not different from controls at 3 hours ($P > 0.05$) but decreased to levels lower than controls at 5 hours post-endotoxin injection ($P < 0.05$). Plasma insulin concentration in endotoxic rats was approximately 2-fold greater than the controls at those time periods ($P < 0.001$). As glucose concentration increased in plasma there was a corresponding increase in insulin during the early phase of endotoxin shock (1-hr post-endotoxin). The decline of plasma glucose concentration during later stages of shock (3-5 hr post-endotoxin) was not accompanied by a corresponding decrease in plasma insulin concentration.

The intracellular concentrations of Na^+, Cl^-, K^+, and AIB were calculated using values of 0.216 ± 0.005 ml/g ($n = 18$) for the extracellular space and 0.774 ± 0.005 ml/g ($n = 18$) for the water content of control soleus muscles. In endotoxic soleus muscles, the intracellular concentrations were calculated using values of 0.168 ± 0.004 ml/g ($n = 36$) for the extracellular space and 0.771 ± 0.001 ml/g ($n = 36$) for the water content of the tissue. Whereas there were no differences in total muscle water ($P > 0.05$), the extracellular space

Figure 1. Plasma glucose concentration in control and endotoxic rats. Values are means ± SE for number of rats given within bars. Stars, values significantly different ($P < 0.001$) from control.

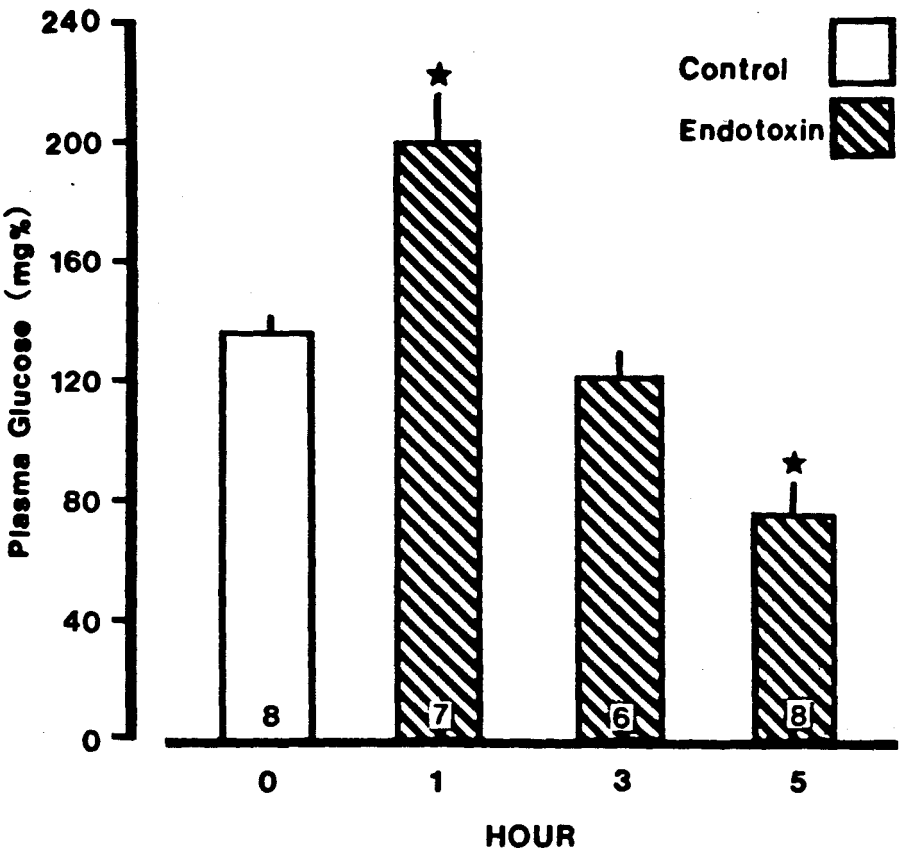
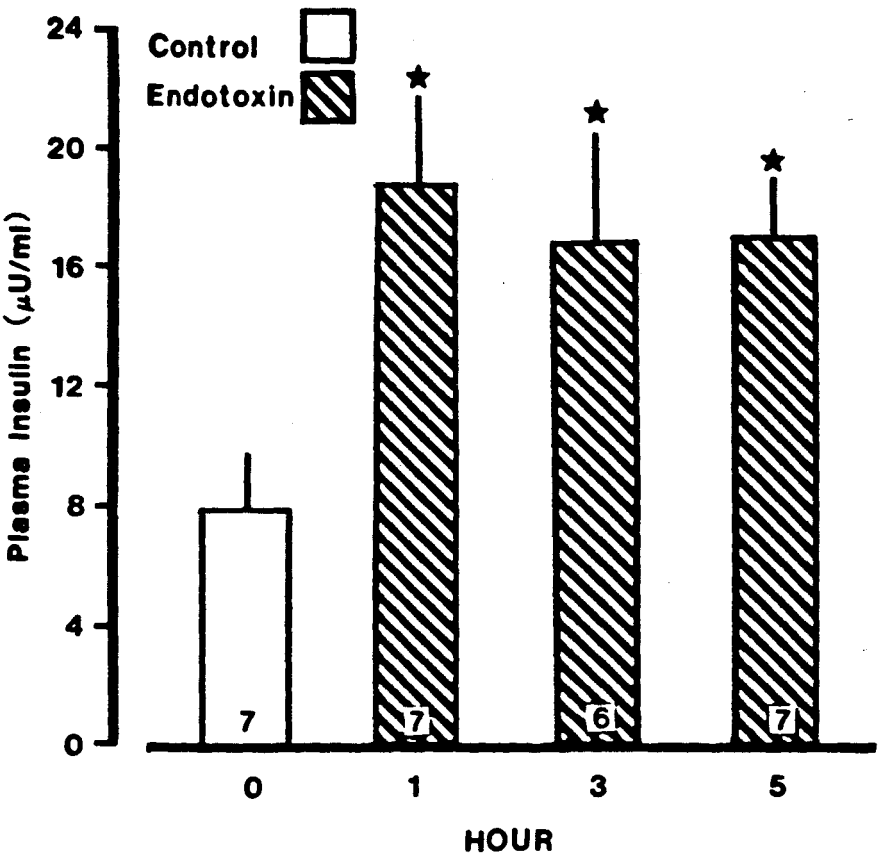

240
200
160
120
80
40
0
Plasma Glucose (mg%)
Control
Endotoxin
8
7
6
8
0
1
3
5
HOUR

Figure 2. Plasma insulin concentration in control and endotoxic rats. Values are means ± SE for number of rats given within bars. Stars, values significantly different ($P < 0.05$) from control.


Control
Endotoxin
Plasma Insulin (μU/ml)
24
20
16
12
8
4
0
7
7
6
7
0
1
3
5
HOUR

in control muscles was significantly greater from that measured in endotoxic muscles ($P < 0.001$). The intracellular space in control muscles (0.558 $\pm$ 0.008 ml/g; n = 18) was significantly different from the endotoxic muscles (0.603 $\pm$ 0.004 ml/g; n = 36) ($P < 0.001$). The increase in intracellular water in endotoxic soleus muscles could be due to a shift of fluid from the extracellular space as there was no change in total tissue water. In endotoxic rats, total tissue and extracellular water in muscle was measured at 1, 3, and 5 hours post-endotoxin injection. The total water and intracellular and extracellular spaces determined 1-hr after endotoxin injection were not significantly different from the values at 3 and 5-hr ($P > 0.05$) and were therefore averaged and considered to representative of water contents for all endotoxic groups. The size of the extracellular space reported for control soleus muscles in this study are in close agreement with those reported by Turinsky (116) and Hom et al (57) who, respectively, used sulfate and sucrose as extracellular markers. Flear (38) and others (42) have shown cellular swelling and marked decreases in the extracellular fluid volume in muscle cells during endotoxin shock. Since previous studies have shown that the size of the extracellular space is not affected by insulin, the extracellular and intracellular spaces reported above were used for calculation of intracellular and extracellular solute concentrations in rats injected with insulin (18,64).

Table 1 shows the effect of endotoxin shock on the intracellular and extracellular concentrations of Na^+, Cl^-, and K^+ in soleus muscle. A small decrease in plasma Na^+ ($P < 0.005$) and increase plasma K^+

TABLE 1. Effect of endotoxic shock on electrolyte concentrations in soleus muscle

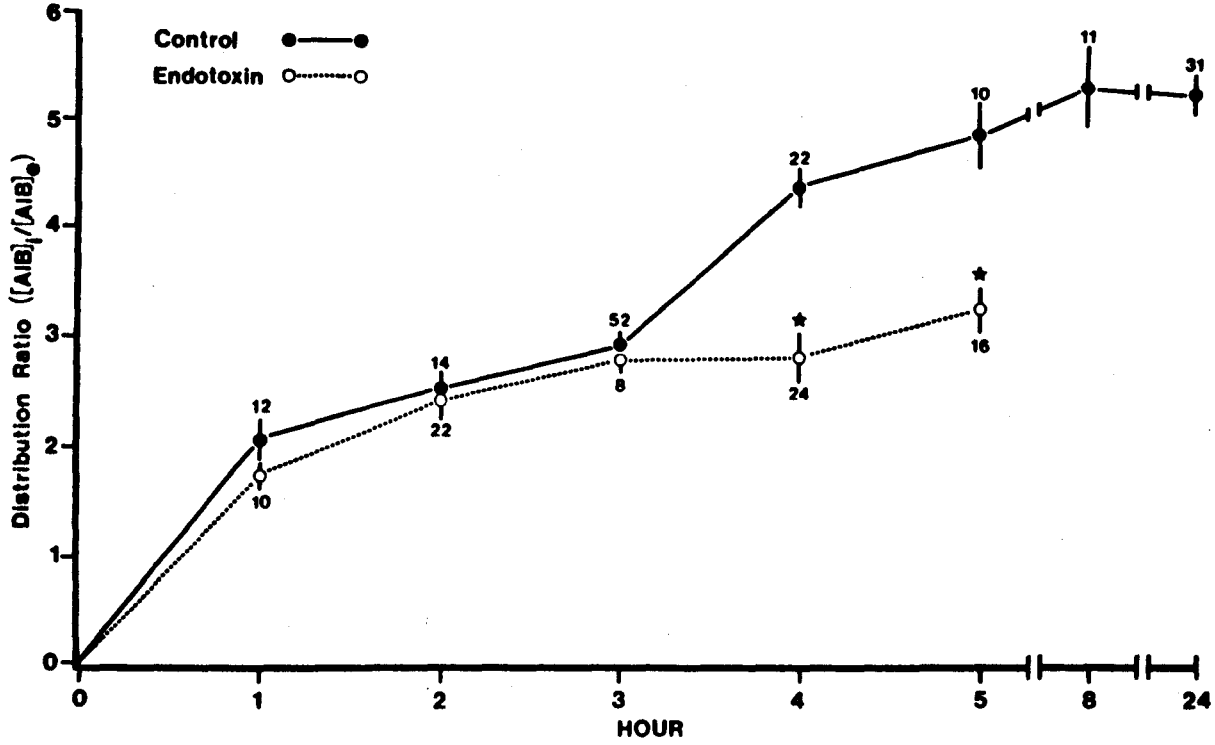
	Extracellular Electrolytes[1] (mmol/L)			Intracellular Electrolytes (mmol/L cell water)		
Group	[Na]	[K]	[Cl]	[Na]	[K]	[Cl]
Control	134 ± 0.9	4.5 ± 0.3	101 ± 1.0	9.3 ± 1.1	164 ± 1.5	4.1 ± 0.6
(n)	(6)	(6)	(16)	(12)	(12)	(16)
Endotoxin	119 ± 1.3*	5.4 ± 0.3**	102 ± 1.0	15.9 ± 0.9*	145 ± 1.7*	14.6 ± 0.9*
(n)	(11)	(11)	(9)	(21)	(21)	(10)

[1]Concentrations in plasma.
Values are means ± SE; n, no. of muscles.
* $P < 0.0005$
** $P < 0.05$

($P < 0.05$) was observed in endotoxic rats as compared to controls. There was no significant difference in plasma Cl^- concentration between control and endotoxic rats ($P > 0.05$). Changes in the intracellular concentrations of electrolytes in the endotoxic muscles included a 70% increase in Na^+, a 11% decrease in K^+, and a 257% increase in Cl^- as compared to control muscles ($P < 0.005$). Since chloride has been shown to be distributed passively across the skeletal muscle cell membrane (122), the chloride equilibrium potential can be quantitatively described by the Nernst equation. The calculated chloride equilibrium potential was significantly lower in endotoxic muscles (-52.7 ± 1.6 mv; $n = 18$) as compared to the control muscles (-89.4 ± 3.5 mv; $n = 16$) ($P < 0.0001$). Alterations in muscle and plasma electrolyte concentrations and resting membrane potential during endotoxic shock could be due either to an increase in the Na^+/K^+ permeability ratio and/or to a decrease in the activity of the Na^+/K^+-pump in the endotoxic soleus muscles.

Figure 3 shows the intracellular-to-extracellular AIB concentration ratio (distribution ratio) in control and endotoxic soleus muscles at various times after i.v. injection of 1 mg/kg AIB. Active AIB transport is indicated by the fact that AIB distribution ratios are greater than one in both control and endotoxic soleus muscles. The maximum distribution ratio of AIB in control muscles was reached by 4 hours and longer exposure to AIB (up to 24 hr) did not significantly increase the distribution ratio ($P > 0.05$). The maximum steady state level of AIB distribution in the endotoxic soleus muscles was evident by 3 hours and was not significantly different at 4 and 5 hours

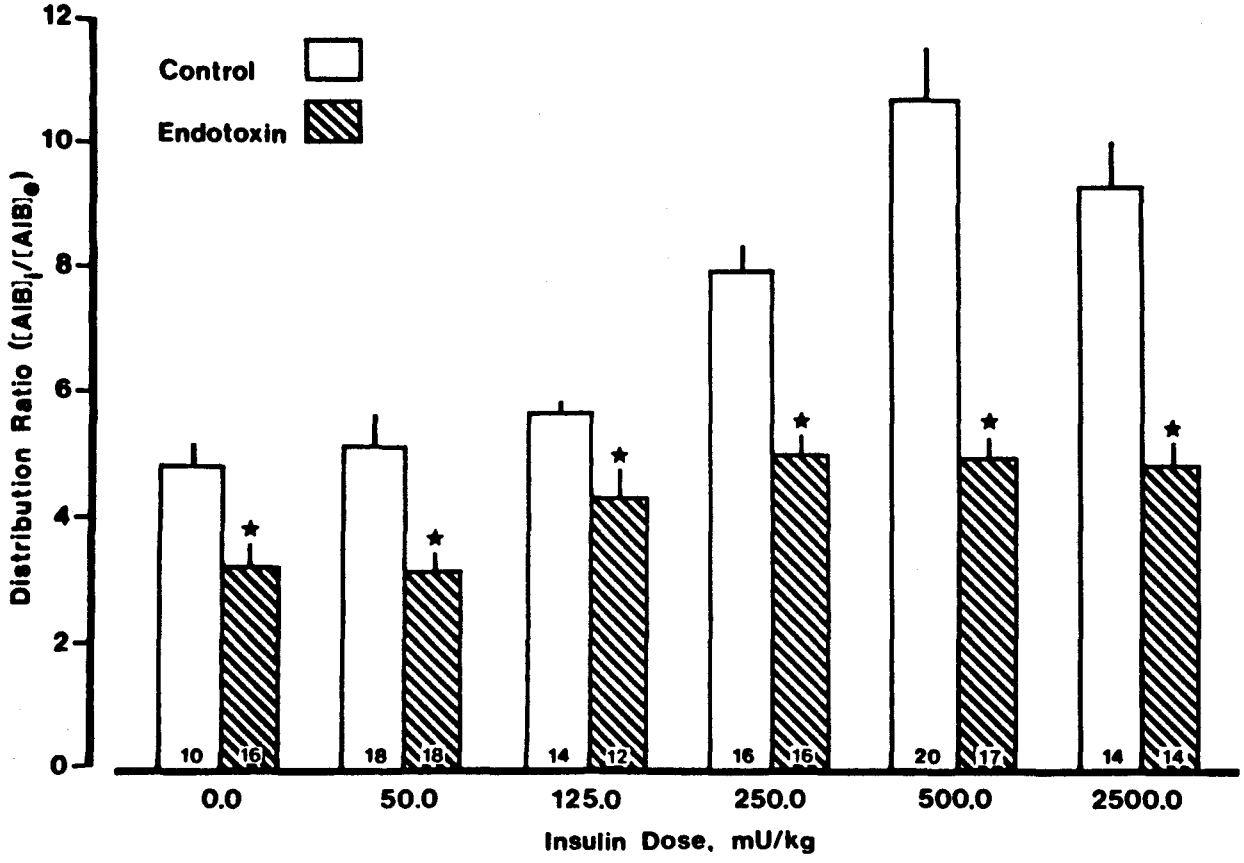
Figure 3. AIB distribution ratios in control and endotoxic soleus muscles at various times after intravenous injection of AIB. Values are means ± SE for number of muscles given at each time point. Stars, significant differences ($P < 0.0001$) between control and endotoxic muscles at each time point.


Control
Endotoxin
Distribution Ratio ((AIB)i/(AIB)e)
HOUR
0
1
2
3
4
5
8
24
6
12
10
14
22
52
8
22
24
10
16
11
31

($P > 0.05$). It can be seen that steady state accumulation capacity of endotoxic soleus muscles for AIB at 4 and 5 hours is significantly lower than controls ($P < 0.0001$). The steady state AIB distribution ratio has been shown to be an accurate index of AIB transport capability in skeletal muscle because it is independent of plasma AIB concentration or changes in AIB delivery to muscle (44,98). Changing the concentration of AIB in plasma 10-fold (by altering the dose) increased the content of AIB in muscle but did not produce significant changes in the distribution ratio. In control rats the distribution ratio of AIB in soleus muscles 4 hours after i.v. injection of 1 mg/kg or 10 mg/kg AIB was 4.4 ± 0.2 ($n = 22$) and 4.2 ± 0.2 ($n = 14$), respectively ($P > 0.05$). These results indicate AIB accumulation capacity in endotoxic soleus muscle is lower than control muscles due to changes in the AIB transport system *per se* and not due to differences in muscle blood flow or plasma AIB concentration.

Since insulin is known to stimulate amino acid transport in muscle (62,64,85,88) and the fact that steady-state AIB distribution ratios in endotoxic soleus muscles were lower than controls in the presence of elevated plasma insulin levels (Fig. 2), experiments were performed to determine if insulin's effect on amino acid transport in muscle is attenuated during endotoxic shock. The effect of insulin on the steady-state distribution ratio of AIB (5 hrs) in soleus muscle was assessed 30 minutes after an intravenous injection of insulin (0-2500 mU/kg) to control and endotoxic rats (Fig. 4). The lowest dose of insulin (50 mU/kg) did not increase the distribution ratio of AIB in control and endotoxic soleus muscles ($P > 0.05$). AIB distribution

Figure 4. AIB distribution ratios in control and endotoxic soleus muscles after the intravenous injection of insulin. Values are means ± SE for number of muscles given within bars. Stars, values significantly different ($P < 0.0001$) between control and endotoxic AIB distribution ratios at each individual insulin dose.


Control
Endotoxin
Distribution Ratio ([AIB]i/[AIB]e)
0
2
4
6
8
10
12
10
16
18
18
14
12
16
16
20
17
14
14
0.0
50.0
125.0
250.0
500.0
2500.0
Insulin Dose, mU/kg

ratios were significantly greater in controls than in endotoxic muscles at each insulin dose ($P < 0.0001$). The absolute difference over basal AIB distribution ratios in endotoxic muscles were smaller than controls at insulin doses of 250, 500 and 2500 mU/kg. The maximum distribution ratio of AIB achieved in the endotoxic soleus muscles with maximal insulin stimultation was substantially lower from that observed in controls. These results indicate that the decrease in maximal insulin response in endotoxic rats was due to not only the decrease in the steady state AIB distribution ratio but also to a decrease in insulin-sensitive AIB transport.

DISCUSSION

Our results demonstrate that active AIB transport and insulin's ability to stimulate this transport system are substantially reduced in rat soleus muscle, *in vivo*, during endotoxic shock. The changes in muscle intracellular electrolytes *in vivo* during shock indicate an alteration of the transmembrane movement of ions. The onset of endotoxic shock was characterized by hyperinsulinemia and blood glucose alterations which included an early hyperglycemia progressing to hypoglycemia by 5 hours of shock. The latter findings are similar to those reported by other investigators (128). In a recent study we found similar changes in plasma glucose concentration along with lactic acidemia and hemorrhagic lesions of the bowel in endotoxic rats (64). The lethality of rats 5 hours after intravenous injection of endotoxin was approximately 32% (65).

This study has shown that the *in vivo* steady state distribution ratio of AIB in endotoxic muscles was substantially lower than in control muscles. Previous studies have indicated that varying the plasma concentration of AIB 100-fold does not affect the steady state AIB distribution ratio in soleus muscle even though there are changes in the cellular content of AIB (44,98). In this study, AIB distribution ratios in control and endotoxic muscles were compared at time periods which permitted muscles to maximally accumulate AIB. Thus, the decrease in the AIB distribution ratio in endotoxic soleus muscle was not a function of plasma AIB concentration or AIB delivery to the

muscle but rather was due to an intrinsic alteration of system A amino acid transport. This does not mean that changes in blood flow or supply of nutrients to muscle do not participate in the endotoxin related decrease in system A amino acid transport.

The present study has indicated that the intracellular concentrations of Na^+ and Cl^- in endotoxic muscles were approximately 2-fold greater than in control muscles and the intracellular concentration of K^+ in endotoxic muscles was considerably lower than the controls. These alterations in electrolyte concentrations in endotoxic soleus muscles could be due to an increase in the ratio of membrane permeability to Na^+ relative to K^+ and/or a decrease in the activity of the Na^+/K^+-pump. This would mean a decrease in Na^+-electrochemical energy for the support of secondary active transport processes. Since the active transport of AIB by system A requires the presence of extracellular Na^+ and that most if not all of the energy used to transport type A amino acid is supplied by the free energy of the Na^+ electrochemical gradient (85), a decrease in the energy of this gradient is likely to reduce the active accumulation of AIB in skeletal muscle cells. Numerous investigations have shown alterations in membrane Na^+/K^+ transport in a variety of tissues during shock and other forms of tissue injury (42,50,60,102,103,108,115). Approximation of the membrane potential for control and endotoxic muscles, by the chloride equilibrium potential value, also support the inference of a decreased Na^+-electrochemical gradient. Taken together, the lower values of transmembrane potential and of Na^+ and K^+ gradients in endotoxic

muscles, as compared with controls, do indicate a substantial loss of Na^+-electrochemical energy.

We have previously shown *in vitro* that the Na^+-dependent component of system A amino acid transport and stimulation of the uptake process by insulin are attenuated in endotoxic rat soleus muscles (64). The mechanism of amino acid transport alterations was shown to be due to a decrease in the maximum velocity (Vmax) of transport and not due to a change in the apparent affinity (Km) for AIB (65). This finding indicates that there is a decrease in the number and/or activity of existing transport carriers in the membrane for AIB (65). Geck and Heinz (41) provided a kinetic analysis of various transport models which indicate that the energy of the membrane potential can affect the maximum velocity of transport. Since the magnitude of the electrochemical potential gradient for Na^+ across the cell membrane determines the extent of AIB accumulation in skeletal muscle, the decrease in active AIB transport measured *in vitro* and *in vivo* are consistent with a decrease in the availability of energy of the Na^+-electrochemical gradient in muscle.

The fact that steady state AIB distribution ratios are decreased in endotoxic soleus muscles in spite of high plasma insulin concentration suggests that the effect of insulin on amino acid transport in soleus muscle may be attenuated during endotoxic shock. Soleus muscles from endotoxic rats showed consistently lower insulin-stimulated AIB accumulation at each insulin dose than control muscles. Although exogenous insulin administration to rats stimulated active AIB transport in the endotoxic muscles, insulin was unable to increase the

distribution ratio of AIB in endotoxic muscles to levels seen in control muscles even in the basal state (saline injected). Insulin increased the distribution ratio of AIB approximately 2-fold in control muscles. The attenuation of the insulin mediated increase in the distribution ratio of AIB in endotoxic muscles may be related to an inability of insulin to increase sufficiently the Na^+ electrochemical potential gradient in soleus muscle. Insulin has been shown to stimulate the Na^+-K^+-ATPase in purified plasma membranes from frog skeletal muscle (40). Zierler and Rogus (130) have reported that in the rat caudofemoralis muscle insulin produces an immediate membrane hyperpolarization by about 9 mv. Flatman and Clausen (36) have shown an *in vivo* hyperpolarizing action of insulin by recording membrane potentials in an exposed soleus muscle of the anesthetized rat. Intravenous injection of 2000 mU/kg insulin to a rat produced a 15 mv hyperpolarization in the soleus muscle (36).

The results of this study indicate that the lowered ability of system A to actively accumulate AIB and the relative lack of insulin sensitivity in endotoxic skeletal muscle cells may be related to a decrease in Na^+-electrochemical energy. These changes could presumably lead to a loss of neutral amino acids from muscle's intracellular amino acid pools and thus provide a plausible explanation for increased amino acid efflux from skeletal muscle during endotoxemia (110) and sepsis (20,99).

CHAPTER VI

EFFECT OF INSULIN ON THE NA^+/K^+-PUMP IN SOLEUS MUSCLE DURING ENDOTOXIC SHOCK IN THE RAT

INTRODUCTION

Electrogenic Na^+/K^+ transport is responsible for the maintenance of normal transmembrane Na^+ and K^+ gradients and the regulation of cell volume in skeletal muscle cells (43,103). A change in the membrane potential and intracellular electrolyte concentrations can affect the transport of amino acids, activation of glycolytic enzymes, and peptide bond formation for protein biosynthesis (79,85,93). A metabolic role of Na^+/K^+ transport in skeletal muscle is also evident from the observation that insulin can hyperpolarize the cell membrane via potentiation of active Na^+/K^+ transport or redistribution of ions due to changes in membrane ion permeability (18,85,130). The membrane hyperpolarization increases the free energy of the Na^+-electrochemical gradient which is, in part, used for activation of amino acid transport in skeletal muscle cells.

Septic and endotoxic shock produces decreased transmembrane potentials and altered electrolyte concentration gradients in skeletal muscle (38,42,60,115). It is likely that these changes are due to decreased Na^+/K^+ transport activity or altered membrane permeability to these ions. There may also be a lack of ion regulation by insulin in muscle. Little is known about the effect of insulin on ion transport during shock. In previous studies, we have shown that soleus muscles from rats in endotoxic shock exhibit decreased transmembrane electrolyte gradients and decreased basal and insulin-sensitive amino acid uptake by transport system A (64,65, see Chapter 5). These

alterations may be related to alterations of cell membrane Na^+/K^+ transport in muscle during shock.

In this study, we have evaluated basal and insulin-stimulated Na^+/K^+ transport *in vitro* by assessing ^{22}Na efflux and ^{86}Rb uptake in control and endotoxic soleus muscles.

METHODS

Male Sprague-Dawley rats weighing 80 to 100 grams were allowed free access to water but fasted overnight prior to experiments. Endotoxic shock was produced in rats by intravenous (sublingual vein) injection of 20 mg/kg Salmonella enteritidis endotoxin (Lipopolysaccharide B, Difco Co., Detroit, MI), suspended in 0.9% saline. Control rats were treated similarly but were given an equal volume of 0.9% saline. Rats were lightly anesthetized with ether prior to intravenous injections. Animals were decapitated 5 hours after the injection of endotoxin or saline and both soleus muscles (40 mg) were removed with care to avoid any damage to the muscles. Isolated soleus muscles were maintained at the resting length during loading and washout procedures by attaching opposing tendons to an adjustable stainless steel holder.

For measurement of Na^+ fluxes, soleus muscles were incubated for 90 minutes in Krebs-Ringer-bicarbonate (KRB) buffer (pH 7.4) containing 5.6 mM glucose, 1 mg/ml bovine serum albumin (fraction V) and a trace quantity of ^{22}Na (7 uCi/ml) (Amersham, Arlington Heights, IL). A 90 minute incubation period was chosen for these studies because the uptake of ^{22}Na into the cellular compartment reaches a constant level within this time (18). Incubations and washes were carried out under 95%O_2-5%CO_2 atmosphere at 37°C in a Dubnoff metabolic shaker incubator. The efflux of ^{22}Na was determined by washing the muscles in a series of flasks containing 5 ml isotope-free KRB for 70 minutes at 5

minute intervals. The effect of a sodium pump inhibitor on the efflux of ^{22}Na from control and endotoxic soleus muscles was assessed by incubating muscles with 1 mM ouabain (Strophanthin-G) and ^{22}Na (7 uCi/ml) for 90 minutes and by washing muscles in isotope-free KRB media (5 ml) containing ouabain (1 mM) in a series of flasks for 70 minutes at 5 minute intervals. The radioactivity released into the medium during each interval was counted in a Nuclear-Chicago gamma counter. To estimate muscle radioactivity at various times during the washout, radioactivity remaining in the muscle at the end of the washouts was determined and added in a retrograde manner to that released during each washout period. A plot of percentage of initial muscle radioactivity as a function of washout time yielded the ^{22}Na efflux curve.

The efflux of ^{22}Na can be divided into two parts, one rapid, the other slow, representing the efflux of isotope from the extracellular and from the cellular compartment (86,90). Cellular ^{22}Na efflux curves were fitted to a monoexponential equation:

$$(t_i) = A \cdot e^{-\lambda t}$$

where (t_i) is the percentage of initial counts remaining at time t; A is the initial counts associated with the cellular compartment; and λ is the rate coefficient (min^{-1}) of ^{22}Na efflux from the cellular compartment. The curves were fitted by an exponential (least squares) regression method and the correlation coefficients (r) calculated from the slopes of regression lines were greater than 0.98 and statistically significant for each muscle ($P < 0.01$).

Active ^{86}Rb influx was determined by measuring the cellular uptake of ^{86}Rb by control and endotoxic soleus muscles in the presence or absence of insulin (100 mU/ml). The rubidium tracer, ^{86}Rb, was used to study K influx instead of ^{42}K, because its half-life is more suitable for these prolonged experiments (18.7 days for ^{86}Rb versus 12.4 hours for ^{42}K). ^{86}Rb is a K analog that has a similar affinity for the Na/K transport system (76). Tissues were equilibrated in isotope-free KRB media for 10 minutes and then incubated for 10, 20 or 30 minutes in KRB media containing 0.45 uCi/ml ^{86}Rb (Amersham, Arlington Heights, IL). Muscles were removed from the medium at the end of the incubation and blotted on Whatman (#42) filter paper to remove adherent surface radioactivity. The wet weight was determined and the tissue was digested in tissue solbulizer and total tissue and incubation medium radioactivity was counted with a Tri-Carb 460C liquid scintillation counter (Packard). Intracellular ^{86}Rb was determined by correcting for the amount of ^{86}Rb trapped in the extracellular space which was assumed to be equivalent to that of the medium. We previously measured total tissue, extracellular (inulin) space and intracellular water contents in control and endotoxic soleus muscles (65). The cellular content of ^{86}Rb in control and endotoxic muscles was calculated using a value of 0.25 ± 0.005 ml/g wet weight ($n = 12$) for the extracellular space (65). Because insulin does not affect total tissue, extracellular or intracellular water contents in control or endotoxic soleus muscle this same value was used for insulin experiments (18,64).

Statistical analysis

Results were expressed as means ± SE. The kinetic parameters derived from ^{22}Na efflux curves were compared using Student's t-test modified for unpaired replicates. ^{86}Rb uptake by muscles was compared using the unpaired Student's t-test. A paired t-test was used to analyze the effect of insulin on ^{86}uptake in control or endotoxic muscles. A "P" value less than or equal to 0.05 was considered significant.

RESULTS

Figure 1 shows the time course of ^{22}Na release from control and endotoxic soleus muscles which were loaded and washed in the absence or continued presence of ouabain (1 mM). Under these conditions, unidirectional ^{22}Na efflux shows a rapid initial decrease followed by a much slower and approximately exponential fall. The initial loss of ^{22}Na presumably represents a loss of isotope from the extracellular compartment (86,90). Under all conditions tested, the washout of isotope from the extracellular compartment was nearly complete within 25 minutes. The slower loss of ^{22}Na represents efflux from the cellular compartment (86,90). The extrapolation of the linear portion of the efflux curves to the zero time intercept gives the percentage of total ^{22}Na radioactivity in the muscles that is found in the cellular compartment.

Table 1 and Figure 1 shows that the percentage of total muscle ^{22}Na radioactivity found in the cellular compartment of endotoxic muscles is 40% greater than the control muscles ($P < 0.02$). No apparent differences were detected for the rate coefficient and $t_{1/2}$ of the efflux process between control and endotoxic muscles in the absence of ouabain ($P > 0.05$). However, the rate of ^{22}Na efflux was significantly greater in the untreated endotoxic muscles as compared to the controls ($P < 0.001$). The control and endotoxic muscles that had been exposed to ouabain during both loading and washout yielded lower rate coefficients and higher $t_{1/2}$ values and differed significantly from

Figure 1. Effect of ouabain on ^{22}Na efflux in control and endotoxic soleus muscles. Solid circle, control; Open circle, endotoxin; Solid line, basal; Dashed line, ouabain. (see Table 1 for statistics).

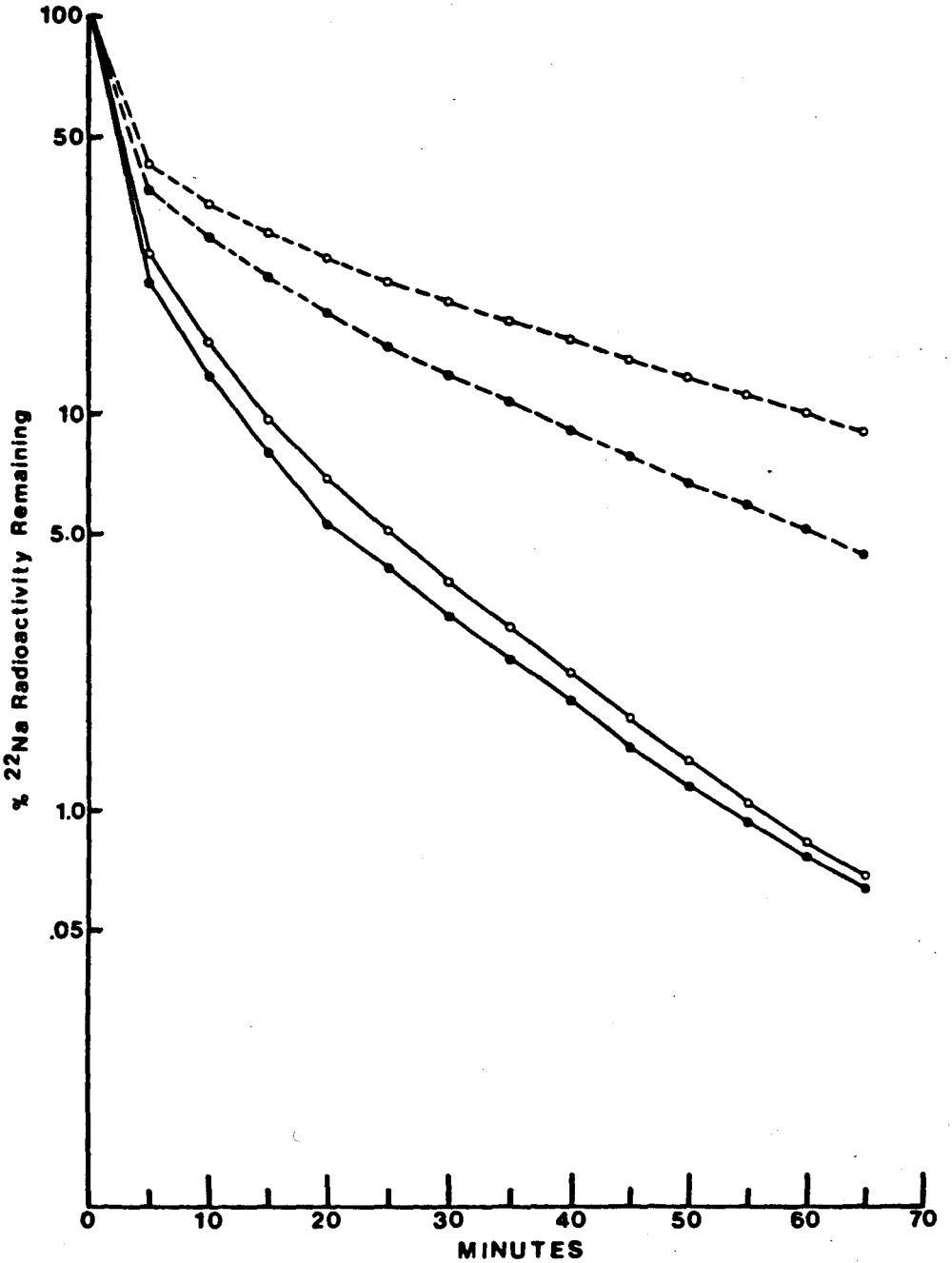

100
50
10
5.0
1.0
.05
% 22Na Radioactivity Remaining
0
10
20
30
40
50
60
70
MINUTES

Table 1. ^{22}Na efflux from control and endotoxic muscles in the presence or absence of ouabain

	n	Cell ^{22}Na Space (%)	Rate Coefficient (min^{-1})	$T_{1/2}$ (Min)
Control	6	12.0 ± 0.9	0.047 ± 0.002	15.0 ± 0.5
Endotoxin	6	16.8 ± 1.4[a]	0.050 ± 0.002	14.0 ± 0.6
Control + Ouabain (1 mM)	6	30.4 ± 2.1[b]	0.030 ± 0.001[b]	23.2 ± 0.9[b]
Endotoxin + Ouabain (1 mM)	6	36.1 ± 1.4[b]	0.022 ± 0.001[b]	32.6 ± 1.7[b]

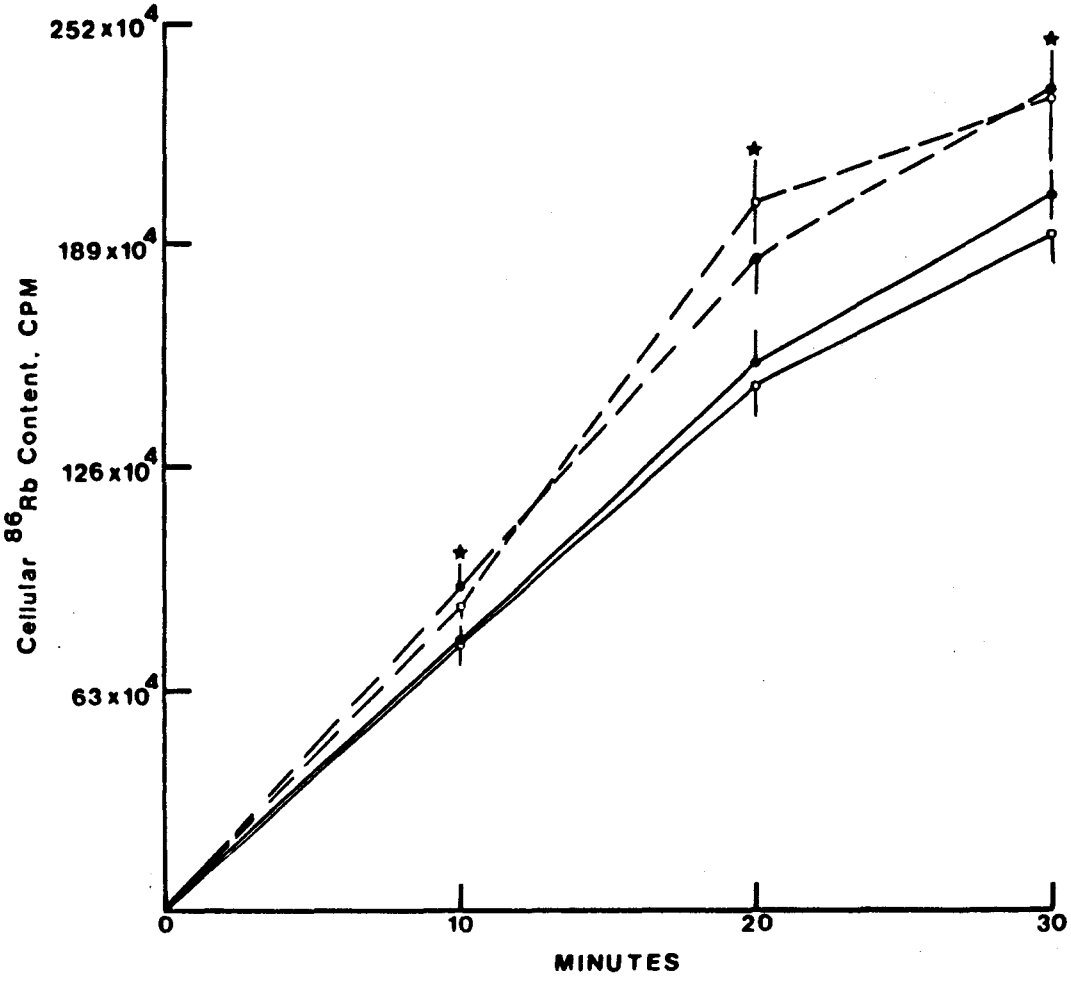
Values are means ± SE; n, no. of muscles. [a] $P < 0.02$, compared with control muscles in the absence of ouabain. [b] $P < 0.001$, compared with corresponding muscles in the absence of ouabain.

muscles in the absence of ouabain ($P < 0.001$). Ouabain significantly increased cellular ^{22}Na radioactive content in control and endotoxic muscles approximately 2-3 fold ($P < 0.0001$). The changes in ^{22}Na efflux curves in the presence of ouabain are consistent with inhibition of active cellular extrusion of isotope.

To compare the rate coefficient and the rate of ouabain-sensitive ^{22}Na efflux in control and endotoxic muscles, we substracted the mean values for the rate coefficient in the presence and absence of ouabain and multiplied the difference with the mean intracellular ^{22}Na pool measured in the absence of ouabain (Table 1). The rate of ouabain-sensitive ^{22}Na efflux was expressed as the percent of radioactive change per minute. The rate coefficient for ouabain-sensitive ^{22}Na efflux in endotoxic muscles (0.028 min^{-1}) was 1.7-fold greater than in control muscles (0.017 min^{-1}). As a result, the half-life for ouabain-sensitive ^{22}Na efflux in endotoxic muscles (24 min) was reduced by 40% as compared to the controls (40 min). These findings indicate that the intrinsic activity of the Na-pump, which is independent of the intracellular Na pool (67), is markedly increased with endotoxic shock. Rates of ouabain-sensitive ^{22}Na efflux was more than doubled for endotoxic muscles (0.470 %/min) as compared to control muscles (0.204 %/min). This increase in due to a combination of increases in both the rate coefficient and cellular ^{22}Na pool.

Figure 2 shows that ^{86}Rb uptake by control and endotoxic muscles in the presence or absence of insulin is approximately linear for incubation periods of 10-20 minutes. ^{86}Rb uptake into the cellular compartment in the absence of insulin at 10, 20 and 30 minutes was not

Figure 2. Effect of insulin of ^{86}Rb uptake in control and endotoxic soleus muscles. Values are means $\pm$ SE for 6 muscles at each time point. **Solid circle**, control; **Open circle**, endotoxin; **Solid line**, basal; **Dashed line**, insulin. **Stars**, significant differences ($P < 0.05$) between basal and insulin-stimulated ^{86}Rb uptake in control and endotoxic soleus muscles at each time point.


252 x 10^4
189 x 10^4
126 x 10^4
63 x 10^4
Cellular 86Rb Content, CPM
0
10
20
30
MINUTES

significantly different in the endotoxic muscles as compared to the control muscles ($P > 0.05$). These data indicate that active ^{86}Rb influx is not altered during endotoxic shock. Insulin (100 mU/ml) significantly increased ^{86}Rb influx into the cellular compartment of control and endotoxic muscles at each time point ($P < 0.05$). The rate of ^{86}Rb influx into the cellular compartment of control and endotoxic muscles was determined by linear regression analysis of the 10-20 minute uptake values in the absence (-I) or presence of 100 mu/ml insulin (+I) (see below).

		^{86}Rb Uptake (CPM x 10^{-3}/g wet wt·min^{-1})	
Group	(N)	-I	+I
Control	12	77.7 ± 2.9	92.4 ± 3.7*
Endotoxin	12	76.1 ± 3.9	93.8 ± 4.1*

(N), indicates the number of muscles; *, values significantly different ($P < 0.001$) from ^{86}Rb uptake in the absence of insulin. ^{86}Rb uptake was not significantly different between control and endotoxic muscles in the absence or presence of insulin ($P > 0.05$). These findings indicate that the basal rate and the insulin-stimulated rate of active ^{86}Rb influx in muscle are not altered with endotoxic shock.

DISCUSSION

Numerous studies have implicated gross alterations of Na^+/K^+ transport in skeletal muscle by measuring tissue ion and fluid contents and potential difference across the cell membrane during shock (42,50,51,108,115, see Chapter 5). These studies, however, have not established whether the changes in cellular electrolytes and membrane potential in skeletal muscle are due to alterations of membrane permeability to ions or active electrogenic $Na^+/K+$ transport. The increased rate coefficient for the ouabain-sensitive component of ^{22}Na efflux and no change in the rate of ^{86}Rb uptake in endotoxic muscles clearly indicates that the activity of the Na/K-pump is not attenuated with endotoxic shock. Furthermore, insulin-sensitive ^{86}Rb uptake was not different between control and endotoxic soleus muscles. Taken together, these results suggest that altered transmembrane Na/K concentration gradients and membrane depolarization in endotoxic muscles is not due to decreased Na/K-pump activity.

In this study we estimated cellular Na in control and endotoxic soleus muscles by extrapolation of the cellular component of ^{22}Na efflux curves to the time zero intercept. This method has been shown to provide an accurate estimate of cellular Na content (86). Clausen and Kohn (18) and others (81) have shown that approximately 12% of total Na content in rat soleus muscle is found in the cellular compartment. We calculated a similar value for cellular ^{22}Na content in control muscles and showed a 40% increase in cellular ^{22}Na content in

endotoxic muscles. The increase in cellular Na in the presence of ouabain in both the control and endotoxic muscles is consistent with an inhibition of active Na transport. The difference between the mean values for cellular Na content in the absence of ouabain for control and endotoxic soleus muscles was not appreciably different than that calculated in the presence of ouabain. This finding indicates that ouabain inhibited active ^{22}Na efflux to a similar extent in control and endotoxic soleus muscles.

Previous studies have shown that ^{22}Na efflux from the cellular compartment in muscle includes both active and passive movements (18,52,67). It was demonstrated that about one-third of the cellular ^{22}Na efflux was due to passive ^{22}Na movement and about two-thirds was due to active, ouabain-sensitive efflux (18). These findings compare favorably with active and passive ^{22}Na efflux rates calculated for control muscles in this study. In contrast, the proportion of active to passive cellular ^{22}Na efflux rates were not maintained in endotoxic muscles. For example, it was shown that whereas ouabain inhibited the rate of cellular ^{22}Na efflux to about 64% in controls, there was greater inhibition of ^{22}Na efflux by ouabain, to about 44%, in muscles from endotoxic rats. These data show an increase of ouabain-sensitive Na transport in endotoxic shock. This increase is apparently due not only to a shift in the kinetic properties of the Na/K-pump *per se*, as evidenced by an increased rate coefficient for the ouabain-sensitive component, but also to increased intracellular Na content in endotoxic muscle. It has been shown that with an increase in the intracellular Na concentration in muscle, the increase in Na efflux is linearly

related to the cube of intracellular Na concentration (52,67). Thus, it seems reasonable that the rate of total cellular ^{22}Na efflux was greater in endotoxic muscles because both the intracellular Na content and intrinsic Na/K-pump activity were increased.

Intracellular Na concentration in muscle is maintained at a steady-state level by balancing passive and active Na effluxes across the cell membrane with passive Na influx (43,103). Most studies indicate that with endotoxic (38,42) or septic shock (60,115) there is an increase in intracellular Na concentration, a decrease in intracellular K concentration, and membrane depolarization in skeletal muscle cells. The results of this study would indicate that electrolyte disturbances in muscle with shock are not due to decreased Na/K transport but may be due to increased cell membrane permeability to Na relative to K.

^{86}Rb, a functional analog of K, has been shown to be a suitable substrate for investigating the transport of K by Na/K-ATPase across the cell membrane in skeletal muscle (76,81). Fehlmann and Freychet (33) and others (95) have reported that insulin increases ouabain-sensitive uptake of ^{86}Rb by freshly isolated hepatocytes and adipocytes, respectively. It has been shown that the uptake of ^{86}Rb is primarily an active process in soleus muscle (18). Therefore, we have assumed that ^{86}Rb enters muscle cells via the Na/K-pump. Insulin has been shown to stimulate ouabain-sensitive Na and K transport by rat soleus muscle (18). Thus, a distinct feature of skeletal muscle ^{86}Rb uptake is that it evaluates not only the activity of the Na/K-pump but also its response to insulin.

Our finding that active ^{86}Rb influx in muscle was not affected with endotoxic shock was surprising since ouabain-sensitive ^{22}Na efflux in endotoxic muscle suggested an elevated rate of Na/K transport. We can only speculate as to how the transport of ^{22}Na and ^{86}Rb in endotoxic muscle were affected in a paradoxical manner. One possible explanation would be that the coupling ratio between the transport of Na and K was not maintained with endotoxic shock. Since the rate coefficient for ouabain-sensitive ^{22}Na efflux was elevated approximately 1.7-fold in endotoxic muscles with no change in the rate of ^{86}Rb uptake, it is plausible that the coupling ratio for Na and K transport increased from a control value of 3:2 to 5:2. This would suggest an increase in the electrogenicity of the Na/K-pump in muscle during endotoxic shock. However, the increase in electrogenic Na pumping is contrary to skeletal muscle depolarization shown in septic (60,115) and endotoxic shock (42) and in our previous studies in which membrane potential was approximated from measured chloride equilibrium potential (see Chapter 5). This disparity may be due to a greater membrane depolarization effect of altered membrane permeability (P) to Na relative to K in endotoxic muscle than Na/K-pump hyperpolarization via electrogenic pumping.

Streptozotocin- and alloxan-induced diabetes in rats are characterized by low plasma insulin levels and increased intracellular Na content and membrane depolarization in skeletal muscle (47,86). These studies indicate that normal plasma insulin levels may play a role in the regulation of the Na/K-pump and , more importantly, maintenance of the resting membrane potential *in vivo* (47,85,86). Since insulin

equally stimulates the Na/K-pump in control and endotoxic muscles, the alterations in intracellular Na concentration and membrane potential in shock muscle was probably not due to a change in insulin stimulation of the pump *per se*. The increase in intracellular Na concentration and decrease in membrane potential could alternatively be due to an insulin-independent increase in P_{Na}/P_K with shock. The concept that decrease membrane potential and increased intracellular Na concentration may be independent of circulating insulin levels during shock, is supported by our findings of electrolyte and membrane potential alterations in spite of hyperinsulinemia (see Chapter 5).

CHAPTER VII

SUMMARY AND CONCLUSIONS

In a variety of pathological states, including sepsis, traumatic injury, and endotoxic shock, there is a marked negative nitrogen balance and a severe loss of skeletal muscle protein (20,21,125). A number of studies have indicated that incorporation of amino acids into protein is regulated by the composition of the intracellular amino acid pools (15,55). The active transport of many of the neutral amino acids across the skeletal muscle cell membrane is due to transport system A (14,16,97). The active transport of amino acids by this transport system is increased by insulin and is dependent on the free energy of the transmembrane Na^+-electrochemical gradient (48,62,85). Amino acid and electrolye transport may be adversely affected in skeletal muscle because the free energy of this gradient is decreased with endotoxic shock. This dissertation examined the transport of neutral amino acids by system A in order to determine if this transport process is affected in skeletal muscle during endotoxic shock. The relationships between amino acid and electrolyte transport and the action of insulin on these transport processes in endotoxic skeletal muscle were also investigated.

The first study was designed to determine if Na^+-dependent amino acid transport and the regulation of this transport process by insulin

were altered in skeletal muscle during endotoxic shock in the rat. Amino acid transport was measured in vitro using alpha-aminoisobutyric acid (AIB), a non-metabolizable substrate of the Na^+-dependent, amino acid concentrating, transport system (A system) (14,16). This study showed a decrease in basal and insulin-stimulated amino acid transport by soleus muscle of rats in endotoxic shock. In addition, the depression of basal amino acid transport by endotoxic muscles was similarly affected over a twenty-fold range of endotoxin doses.

The effect of Na^+ on basal amino acid transport in control and endotoxic muscles was investigated to determine whether the decreased amino acid uptake was due to an alteration of the Na^+-dependent portion of transport system A. Na^+-dependent AIB uptake, calculated as the difference between AIB uptake in the presence and absence of Na^+, was decreased in the endotoxic muscles to 36% of the control value. Na^+-independent AIB uptake was the same in control and endotoxic muscles. There was no significant effect of insulin on AIB uptake in either group of muscles when Na^+ was absent from the medium. These findings suggest that the decrease in both basal and insulin-stimulated AIB transport was due to the decrease in Na^+-dependent AIB transport by skeletal muscle during endotoxic shock. Since previous studies have shown a direct dependence of active AIB transport on the energy of the Na^+-electrochemical gradient, it was postulated that the decrease in Na^+-dependent AIB transport was due to a dimunition of the transmembrane Na^+ concentration gradient or potential difference in skeletal muscle during endotoxic shock. The altered ability of

insulin to increase AIB transport in endotoxic soleus muscle may be due to inadequate enhancement of Na^+-electrochemical energy.

The second study evaluated the kinetic behavior of system A amino acid transport in order to elucidate the mechanism of amino acid transport disturbances in soleus muscle during endotoxic shock. A multicomponent analysis was used to separate initial rates of AIB transport into two parallel transport processes, a saturable carrier-mediated component and a non-saturable passive diffusive process. The maximum rate of saturable AIB transport (V_{max}) in the endotoxic muscles was 69% lower than control muscles. The apparent K_m for saturable AIB transport was not different between control and endotoxic muscles. The diffusion constant for the non-saturable process in the endotoxic muscles was 38% greater than control muscles. It was concluded that alterations of system A amino acid transport in endotoxic soleus muscles was due to an increase in passive AIB diffusion and a decrease in the number and/or activity of existing carriers and not to a change in carrier affinity for AIB. These results elucidate the mechanism for the decreased amino acid transport by soleus muscle in endotoxic shock.

In the third study soleus muscle intracellular electrolytes and AIB uptake and its regulation by insulin were investigated during endotoxic shock, in vivo. It was shown that the maximum level of active AIB transport by endotoxic muscles was 34% lower than control muscles at a time when plasma insulin concentration in endotoxic rats was two-fold greater than control rats. The effect of exogenous insulin injections on AIB transport was significantly lower in

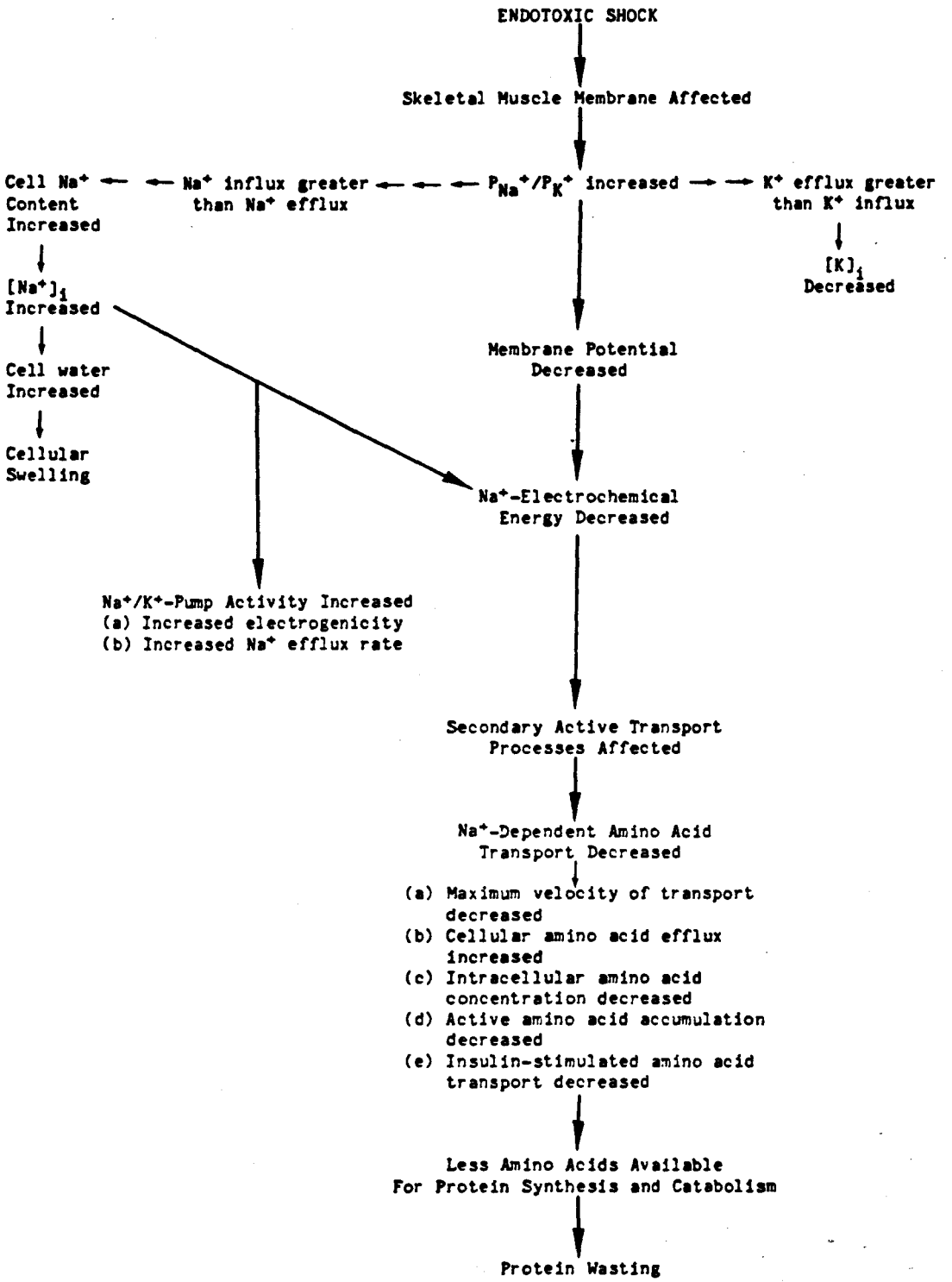
endotoxic muscles than in control muscles. The intracellular electrolyte concentrations in endotoxic soleus muscles were substantially altered when compared to control muscles. The finding that the transmembrane Na^+ concentration gradient and chloride equilibrium potential were markedly reduced in endotoxic soleus muscles suggests decreased Na^+-electrochemical energy available for active AIB transport in endotoxic soleus muscles. The endotoxic-related changes in soleus muscle intracellular electrolytes indicate a gross alteration of the transmembrane movement of ions. These changes could be due to either an increase in the Na/K permeability ratio and/or a decrease in the activity of the Na/K-pump in the endotoxic soleus muscles. The attenuation of the insulin-stimulated increase in active AIB transport in endotoxic muscles may also be related to an inability of insulin to increase sufficiently the Na^+-electrochemical potential gradient and thus its energy in soleus muscles.

To evaluate basal and insulin-stimulated Na/K transport, ^{22}Na efflux and ^{86}Rb uptake were measured in control and endotoxic soleus muscles in the fourth study. The results showed that the rate of active ^{86}Rb uptake was the same in control and endotoxic soleus muscles. The ability of insulin to stimulate the Na/K-pump was not altered with endotoxic shock. These findings indicate that Na/K-pump activity in muscle is not depressed during endotoxic shock. Yet this study shows that cellular Na^+ content in endotoxic soleus muscle was 40% greater than in control muscles. This endotoxin-related increase in cellular Na^+ content in muscle is corroborated by the observed increase in intracellular Na^+ concentration measured in freshly

excised unincubated soleus muscle (see Chapter 5). The increase in the rate of ^{22}Na efflux in endotoxic soleus muscles, compared to controls, may be attributed to the increase of intracellular Na^+ content and the increase in the rate coefficient for ^{22}Na efflux in endotoxic muscle. The data also suggest that the coupling ratio for Na^+ and K^+ transport may increase from a control value of 3:2 to 5:2 in endotoxic soleus muscle. This would mean an increase in the electrogenicity of active Na/K transport. However, the increased, rather than decreased, intracellular Na^+ levels suggest that although Na^+ efflux may be enhanced with the increased electrogenic Na/K-pump activity, the active movement of Na^+ and K^+ was apparently not sufficient to prevent changes in muscle Na^+ and K^+ content because of a much greater increase in leakage of Na^+ into and K^+ out of muscle cells. Overall, the results of this study indicate that decreased transmembrane Na^+/K^+ concentration gradients and membrane depolarization in endotoxic muscle is not due to decreased Na/K-pump activity but may be due to increased cell membrane permeability to Na^+ relative to K^+.

In overview, a sequence of events leading to cellular alterations produced with endotoxic shock can be proposed from the results of this dissertation. The scheme of events provided in Fig. 1 is based on data from the experiments in this dissertation. Changes in relative membrane permeabilities (P) to Na^+ and K^+ could be responsible for membrane depolarization and thus alterations in amino acid transport during endotoxic shock. Morever, the transmembrane Na/K concentration gradients would tend to decrease if the passive fluxes of Na^+ and K^+

Figure 1. Sequence of events leading to cellular alterations produced with endotoxic shock.


ENDOTOXIC SHOCK
Skeletal Muscle Membrane Affected
Cell Na+ Content Increased
Na+ influx greater than Na+ efflux
P_{Na^+}/P_{K^+} increased
K+ efflux greater than K+ influx
$[K]_i$ Decreased
$[Na^+]_i$ Increased
Cell water Increased
Cellular Swelling
Membrane Potential Decreased
Na+-Electrochemical Energy Decreased
Na+/K+-Pump Activity Increased
(a) Increased electrogenicity
(b) Increased Na+ efflux rate
Secondary Active Transport Processes Affected
Na+-Dependent Amino Acid Transport Decreased
(a) Maximum velocity of transport decreased
(b) Cellular amino acid efflux increased
(c) Intracellular amino acid concentration decreased
(d) Active amino acid accumulation decreased
(e) Insulin-stimulated amino acid transport decreased
Less Amino Acids Available For Protein Synthesis and Catabolism
Protein Wasting

were not balanced by active transport driven in the opposite direction by the Na/K-pump. Fig. 1 shows the decrease in transmembrane Na/K concentration gradients and membrane potential with endotoxic shock. The increase of intracellular Na^+ concentration would increase Na/K-pump activity and lead to increased rate of Na^+ efflux. Apparently, the increase in electrogenic Na/K-pumping was not sufficient to prevent changes in the intracellular concentrations of Na^+ and K^+ or membrane potential with shock. Water enters cells with Na^+ and Cl^- and contributes to cellular swelling. The decreased transmembrane potential and Na^+ concentration gradient reduces the free energy of the Na^+-electrochemical gradient. The loss of Na^+-electrochemical energy would decrease both the rate of Na-dependent amino acid transport and the active accumulation of amino acids in skeletal muscle cells. An attenuation of insulin's effect on amino acid transport may be due to an inability of insulin to sufficiently increase the energy of the Na^+-electrochemical gradient for active AIB transport. In addition, cellular amino acid efflux would increase and the concentration of intracellular amino acids would tend to decrease. The decrease in the level of amino acids in cell fluid would presumably limit the availability of amino acids for energy production and protein synthesis. This could contribute to increased skeletal muscle protein catabolism and accelerated loss of skeletal muscle protein with endotoxic shock.

CHAPTER VIII

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BIBLIOGRAPHY

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

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