

Membrane Transport in Human Skeletal Muscle

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1. Introduction

Membrane transport in human skeletal muscle can be studied with different techniques ranging from whole body exercise experiments to *in vitro* experiments with membranes obtained from human skeletal muscle. The membranes for these experiments are usually obtained with the biopsy technique.

2. Muscle biopsy

A muscle biopsy is a small sample of muscle. Theoretically this sample could be obtained by surgery. However, in muscle physiology the established method is the needle-biopsy method, usually called the Bergström technique (Bergström 1962). With this technique it is possible to obtain 10-100 mg wet weight of tissue or even more if suction is applied.

2.1 Use of muscle biopsies

In exercise physiology biopsies obtained before and after exercise have been used to obtain snapshots of the muscle content of ions and metabolites. For instance, the changes in muscle glycogen and glucose in association with muscle activity have been quantified. Changes in ion composition, for instance accumulation of lactic acid in muscle, the associated changes in pH, and changes in Na⁺ and K⁺ distribution have also been of interest. By repeated biopsies it has been possible to describe the recovery processes after exercise. Such measurements are usually combined with blood analysis of the same ions or metabolites (see exercise experiments below).

Muscle biopsies have also been used to obtain snapshots of the active genes. Measurements of transcriptional activity in human skeletal muscle have been difficult because of the large amount of muscle tissue needed to isolate nuclei. A new technique involving RT-PCR for performing nuclear "run-on" analysis made it possible to determine transcriptional activity in the small amount of tissue available from a needle biopsy. This opened the possibility to measure transcriptional activity before and in the recovery period in association with muscle activity (Pilegaard et al. 2000, Hildebrand & Neuffer, 2000).

2.2 Scope of the paper

The present review focuses on the use of biopsy material in studies of membrane transport in animals and especially in humans. The use of biopsy analysis and vesicles produced from

biopsies will be reviewed. It will be described that information obtained with this technique can be combined with information obtained from whole body experiments.

3. Membrane transport in general

3.1 Specific transport systems

Membrane transport involves all types of protein-mediated transport systems, including channels, carriers, exchangers and pumps.

3.1.1 Vesicle studies

In animal studies it is possible to use isolated muscle for transport studies. If a muscle is incubated with a radiolabeled compound it is possible to show uptake, and if specific inhibitors are known, it is possible to demonstrate that the uptake is mediated by specific membrane transport systems. However, a muscle consists of a high number of cells, it is therefore not possible to incubate all cells at the same time point, to control the gradient and to measure initial rate of uptake. It is therefore not possible based on intact muscle to determine the transport kinetic parameters K_m and V_{max} , the Michaelis- Menten parameters. In addition, this type of measurements can not be done in humans.

The use of vesicles made from human biopsies has solved these problems.

It has been known for long time that if tissue is homogenized, the membranes usually form small closed structures, called vesicles. The diameter of these vesicles is usually less than 0.5 μm . Such vesicles have been used for transport studies, but they are difficult to use because of the fast uptake/release of compounds due to the large surface/volume ration.

Sarcolemmal giant vesicles are produced with a different method (Burton & Hutter, 1990). Muscles are cut in pieces (but are not homogenized), the pieces are treated with collagenase and a high K^+ concentration (Juel 1991). The exact mechanism is not known. The effect of high potassium could be due to an osmotic gradient, but this is difficult to accept because the cells are not intact. Anyway, the outer membranes bud out and form large vesicles, which can be purified using a step density gradient and slow centrifugation. The purified vesicles are from 1-50 μm , with a median of 6 μm (Figure 1). This is the size of a red blood cell. Experiments with labeling of the extracellular part of the Na,K-ATPase with ouabain have shown no additional labeling if the vesicles were opened, which implies that the original outside is still facing outwards, this is usually called right side out. These vesicles are much better suited for transport studies because of the lower surface-volume ration. In the next sections this type of vesicles will be called sarcolemmal giant vesicles.

It must be noted that this type of vesicles can only be produced from fresh muscle material; vesicles can not be produced from frozen material.

Other types of vesicles have been called giant vesicles; in the present paper the name sarcolemmal giant vesicles is restricted to vesicles made with collagenase and K^+ treatment of muscle.

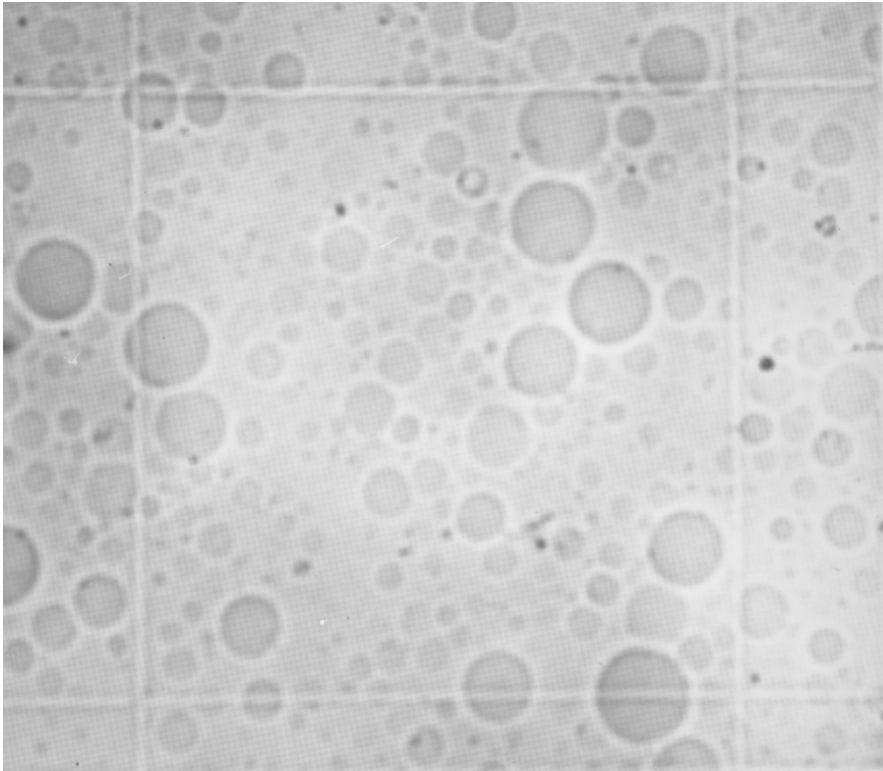


Fig. 1. Vesicles viewed in the light microscope. These vesicles were produced from human biopsy material. Grid: 200 μ m.

3.1.2 Lactate transport studies

Lactate transport was first described in red blood cells. Later it was shown with isolated muscle that the lactate uptake in incubated muscle can be inhibited by unspecific transport inhibitors (SH-group binding or cinnamate). But this method could not be used to obtain the Michaelis-Menten parameters. The first study of lactate transport with sarcolemmal giant vesicles was carried out with vesicles produced from rat muscle. With this technique it was possible to demonstrate saturation, the effects of inhibitors, trans-acceleration, and to determine the Michaelis-Menten parameters in different experimental situation: zero-trans efflux and equilibrium exchange (Figure 2, right).

The Michaelis-Menten parameters for lactate/ H^+ co-transport in human skeletal muscle were obtained with vesicles made from needle biopsies (Juel et al. 1994). This is one of the first membrane transport studies in humans. The use of vesicles made this possible. Lactate transport (quantified as tracer fluxes) was determined with different lactate concentrations in the vesicles and outside the vesicles. This setup and the use of inhibitors to quantify simple diffusion made it possible to calculate the Michaelis-Menten parameters K_m and V_{max} both for zero-trans experiments and equilibrium exchange experiments (Figure 2).

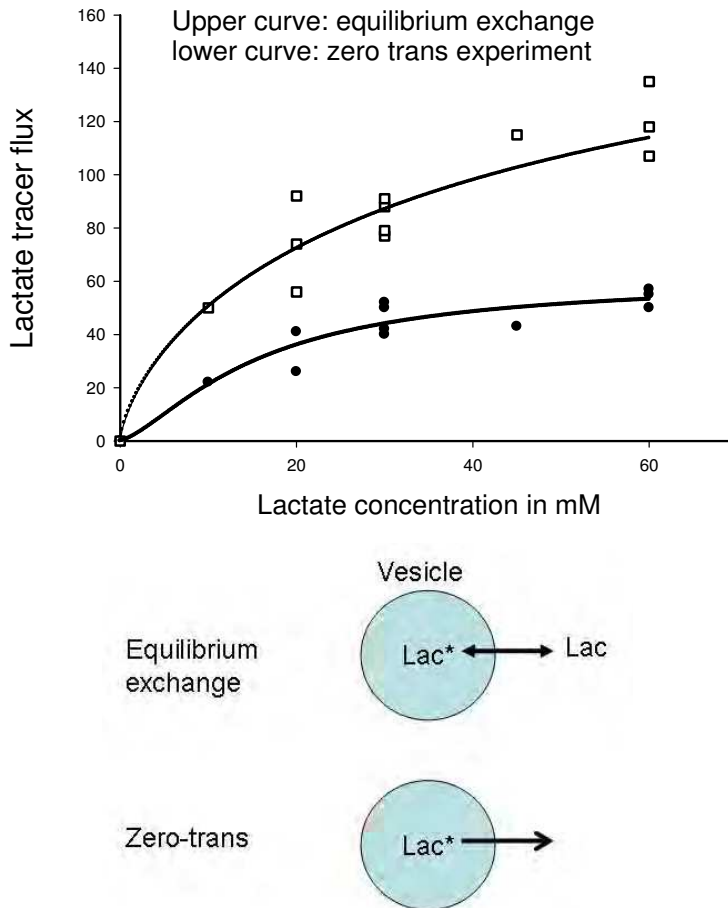


Fig. 2. Lactate transport measures in giant vesicles produced from human skeletal muscle biopsies. Data from zero-trans experiments (lactate initially present on one side of the membrane) and equilibrium exchange experiments (identical lactate concentration at both sides of the membrane, but initially with radio-labeled lactate only at one side). K_m was found to be 24 and 30 mM lactate, respectively. The lines represent Michaelis-Menten fits to the data. Adapted from Juel et al. 1994. Analysis of the pH changes in the same experiments revealed that lactate and H^+ were transported with a 1:1 ratio; lactate/ H^+ co-transport is therefore the correct name for this type of transport.

3.1.3 Lactate transport and training in humans

The proteins responsible for lactate/ H^+ co-transport were cloned in 1994, but antibodies for studies of the two isoforms MCT1 and MCT4 were first available in 1998 (Wilson et al. 1998). However, with the use of biopsy material and giant vesicles it was possible to investigate the effect of training before the proteins were identified. Biopsies were obtained from

subjects with different training status and the rate of lactate transport was quantified from tracer fluxes across vesicular membranes (Figure 3).

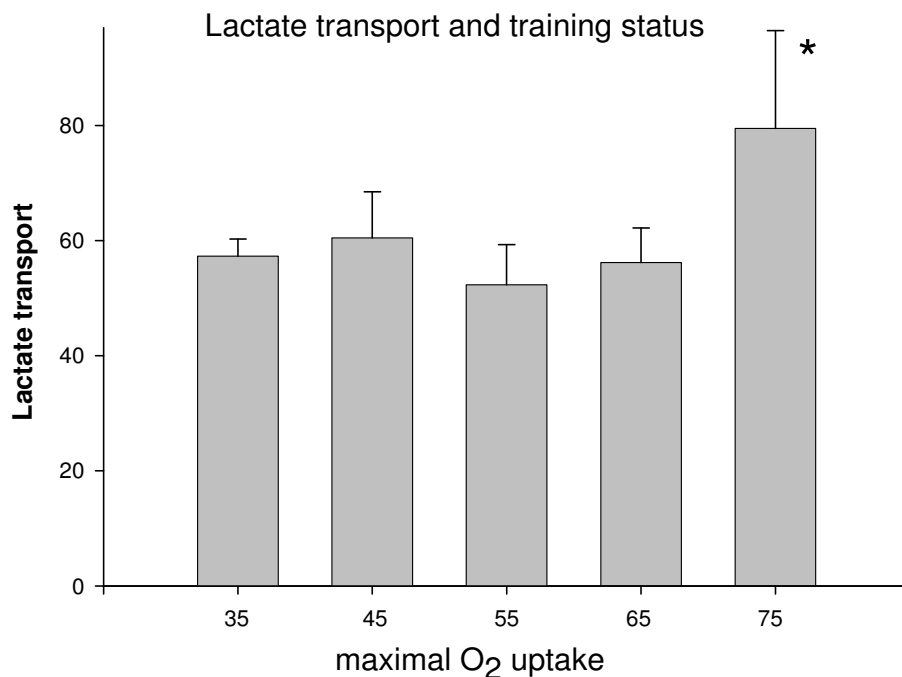


Fig. 3. Lactate transport in vesicles produced from human biopsy material. Lactate transport was quantified as tracer flux in vesicles incubated with radio-labeled lactate. Maximal O₂ uptake was used as an index of training status. It can be seen that some individuals with high training status had an improved lactate transport capacity (Data from Pilegaard et al. 1994).

It was concluded that some well trained sprinters had an increased lactate transport capacity. Later it was confirmed with antibodies and biopsy material that training can increase the protein density of the proteins involved (Pilegaard et al. 1999). Lactate/H⁺ co-transport is mediated by two isoforms called MCT1 and MCT4 (for monocarboxylate transporter 1 and 4).

3.2 Exercise induced changes in transport proteins studied with muscle biopsies

3.2.1 Exercise

Muscle biopsies have also been an exceptional tool to investigate the effects of exercise training. In combination with the western blotting technique it has been possible to quantify the changes at the protein level of many transport proteins, for instance the glucose transporter, the lactate H⁺ co-transporter, the Na⁺/H⁺ exchanger and the Na,K-pump. For a review on exercise-induced changes in muscle membrane transport systems see (Juel 2006) and Figure 4.

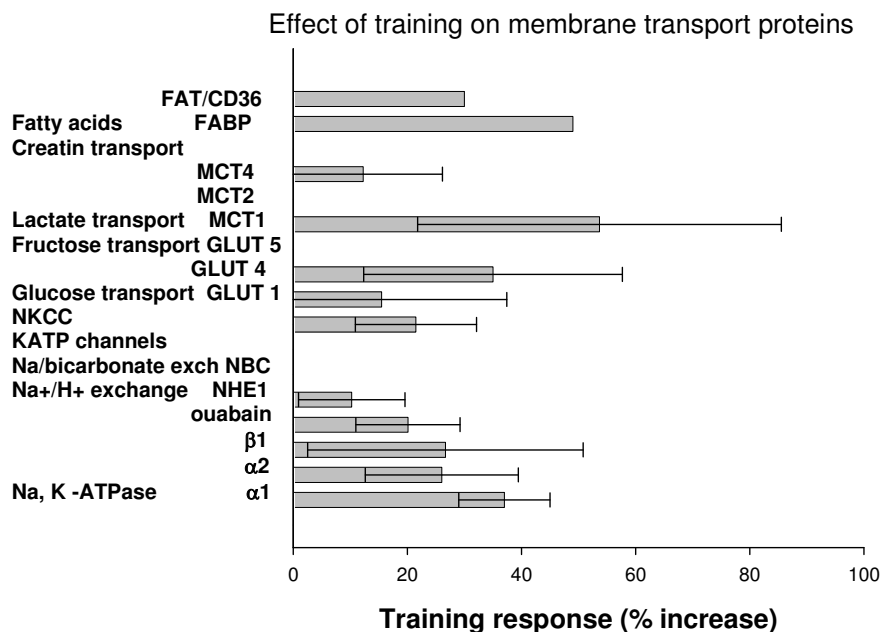


Fig. 4. Changes in membrane proteins involved in membrane transport; effect of training. All data were obtained with human muscle biopsies taken before and after a training period of various durations (weeks) and intensities. Mean \pm SD if more than one study. Please note that these data are collected from training studies with different training intensities and durations. Modified from Juel 2006.

It can be concluded from Figure 4 that all membrane transport proteins studied in humans can undergo changes with training. Although this is a well known phenomenon, the exact signaling pathways from physical activity to gene transcription are only partly known.

3.2.2 Biopsies in exercise and training physiology with special focus on membrane transport in humans

The use of biopsies as snapshots of the ion and metabolite content in muscle is used in training and exercise physiology. In addition, biopsies can be used to monitor changes in proteins of importance for function. The combined used of other measurements (typically blood analysis) and biopsies in training and exercise physiology is demonstrated in the example below.

Human subjects trained with one leg for 8 weeks. The daily training consisted of fifteen 1-min high intensity bouts (150 % VO_2 max) separated by 3 min rest. After the training periods an exercise test was carried out both with the trained and the untrained leg. The test consisted of incremental exercise to exhaustion. Blood samples were obtained before, during the experiment, and in the recovery period. The releases of lactate and H^+ were calculated from the blood concentrations and blood flow. The effect of training on lactate release is illustrated in Figure 5.

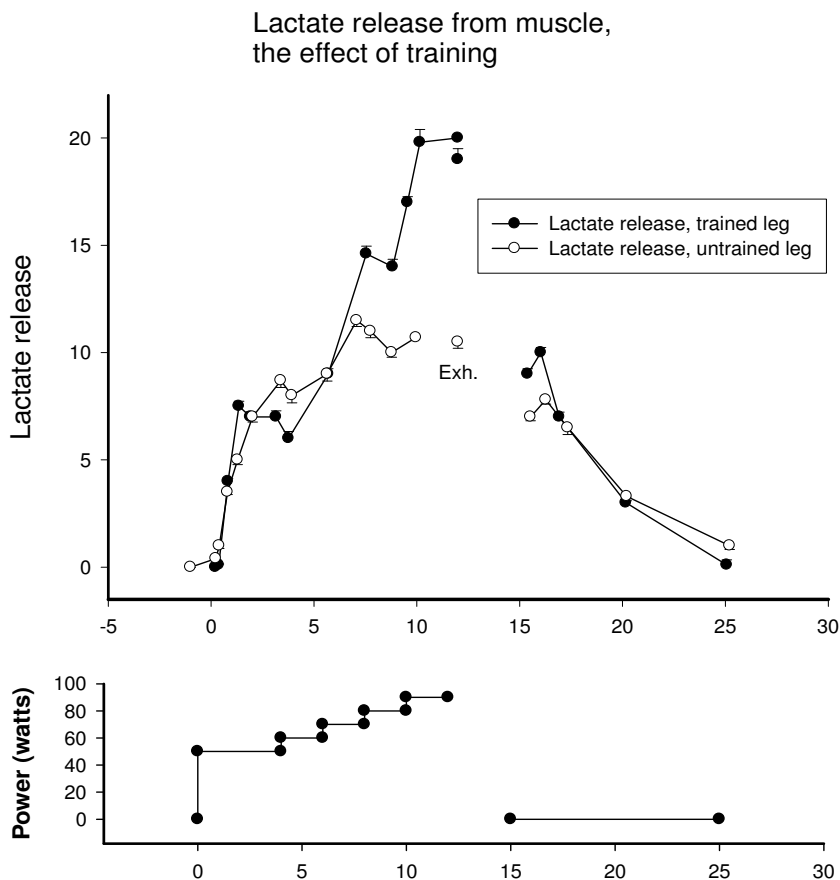


Fig. 5. Lactate release from exercising leg muscle, the effect of training. Values were calculated from arterial and venous blood lactate concentration, and blood flow. Exh: value at exhaustion. The exercise intensity (in Watts) is indicated in the diagram below. Adapted from Juel et al. 2004.

It can be concluded that lactate release was nearly doubled in the trained leg compared to the untrained leg (Juel et al. 2004). The question is now, what is the underlying mechanism? Changes in lactate release could be due to changes in the amount of lactate accumulated in the active muscle. Biopsy samples were therefore analyzed for total lactate content. It was found that the lactate concentration at exhaustion was higher in untrained muscle compared to trained muscle; therefore, the increased release in trained muscle can not be explained by a higher gradient, on the contrary the gradient from muscle to plasma was lower in trained muscle.

The improved release of lactate could also be due to an increased content of lactate/H⁺ co-transporter proteins called MCT1 and MCT4. To investigate this possibility, biopsy material from trained and untrained legs were analyzed for MCT content by western blotting (Figure 6).

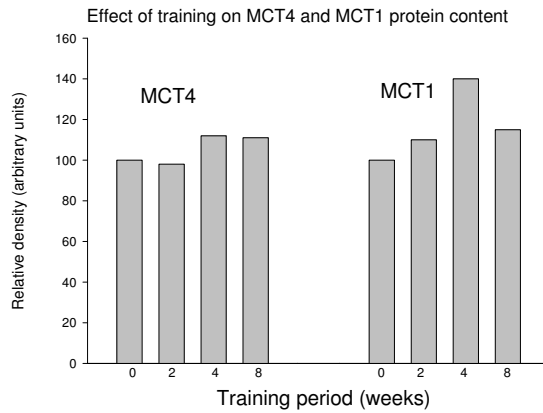


Fig. 6. Effects of 2, 4, and 8 weeks of high intensity training on muscle contents of the lactate/ H^+ co-transporter proteins MCT4 and MCT1. Adapted from Juel et al. 2004)

Indeed, analyses of MCT content in biopsies obtained before, during and after the training period, demonstrated an increase in MCT content. However, the increase was moderate, and can only partly explain the dramatic increase in lactate release in the test experiments comparing trained and untrained leg (Figure 5). Could other mechanisms be involved?

Again biopsy material was used. The improved lactate release could be due to a higher blood flow in the trained compared to untrained muscle, which could be the underlying mechanism for a better wash-away of lactate, which maintains the gradient and facilitates a higher release. Biopsies were analyzed for number of capillaries per fiber (Jensen et al. 2004). The analysis demonstrated a considerable increase in the number of capillaries in the trained leg compared to untrained leg (Figure 7).

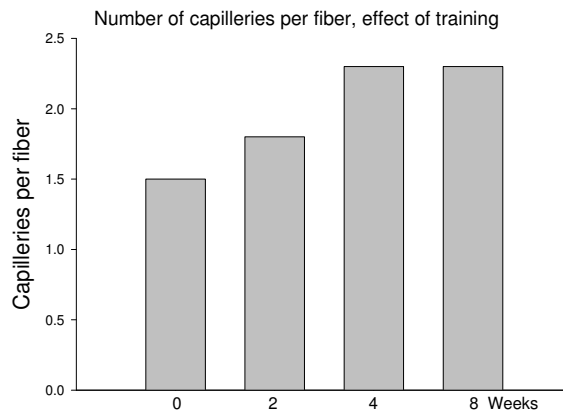


Fig. 7. Effect of training on number of capillaries per muscle fiber. Values determined in human biopsy material (data from Jensen et al. 2004).

Thus, the improvement in lactate release was partly due to a higher number of transporter molecules, and partly due to an improved blood flow. The improvement in blood flow was

mainly mediated by an increased number of capillaries. In conclusion, analysis of biopsy material contributed with information about the underlying mechanisms.

3.2.3 Biopsies from patients

It is obvious that muscle biopsies are used for diagnostic purposes. Analyses of biopsy material can give information about cellular changes including metabolic changes. Analysis of pathological changes is outside the scope of the present review.

But biopsies have also been used for comparing patients and healthy control groups. One example is given below.

Diabetic patients and normal healthy control subjects differ in their insulin sensitivity and glucose handling. The use of biopsies has revealed other differences.

In a training study comparing diabetic patients and healthy control subjects it was found that type 2 diabetic patients had a lower muscle content of Na,K-pump subunits. For both groups strength training resulted in an increased density of pumps, but the diabetic patients still had a lower level of pump proteins (Dela et al. 2004). Likewise, studies of biopsy material from these groups demonstrated that type 2 diabetes is associated with a lower capacity for lactate and H^+ transport, and that the transport capacity could be increased with training in both groups (Juel et al. 2004a). In conclusion, type 2 diabetes is not only associated with reduced insulin sensitivity/reduced glucose transport, other membrane transport systems are also affected.

3.3 pH regulation in muscle

3.3.1 pH regulation in general

The concentration of H^+ , which determines pH, is regulated by a number of mechanisms including several membrane transport systems. The sum of all these mechanisms is called pH regulation. The main problem in all living cells is that the negative membrane potential influences the distribution of H^+ across the outer membrane. The internal concentration of H^+ therefore tends to increase, and as a consequence there is a tendency towards a low intracellular pH (cellular acidification). The pH regulating system therefore has to remove free H^+ from the cell; this is either done by intracellular buffering or by transporting H^+ out (or OH^- in). For a review about pH regulation in human skeletal muscle see Juel (2008). The main components in pH regulation are outlined below.

3.3.2 Kinetics of pH regulation

Information about the kinetics of skeletal muscle pH regulation has been obtained both from whole body experiments and from experiments involving membrane vesicles.

3.3.3 Whole body experiments

Interstitial pH in human skeletal muscle can be measured with the microdialysis technique combined with the use of pH sensitive dyes (Street et al 2001). With this technique it has been possible to measure the time course of the pH changes associated with leg exercise with different intensities, see Figure 9.

pH regulation in muscle. The concentration of H^+ (pH) is dependent on metabolic processes and membrane transport systems.

NHE: Na^+/H^+ exchange.

MCT: lactate/ H^+ cotransport

NBC: bicarbonate/ Na^+ cotransport

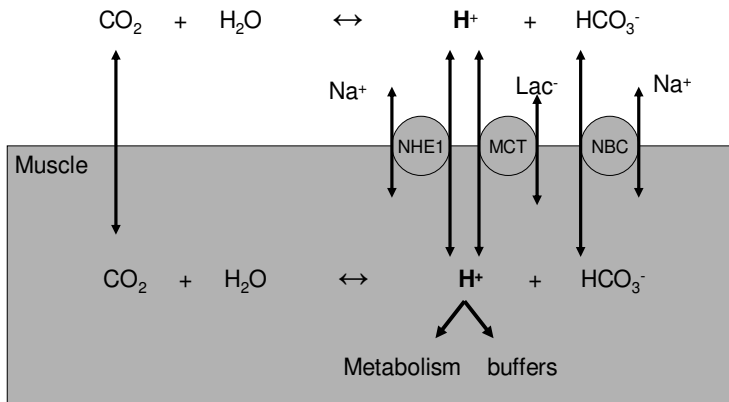


Fig. 8. Membrane transport proteins involved in pH regulation in skeletal muscle. Adapted from Juel et al. 2003.

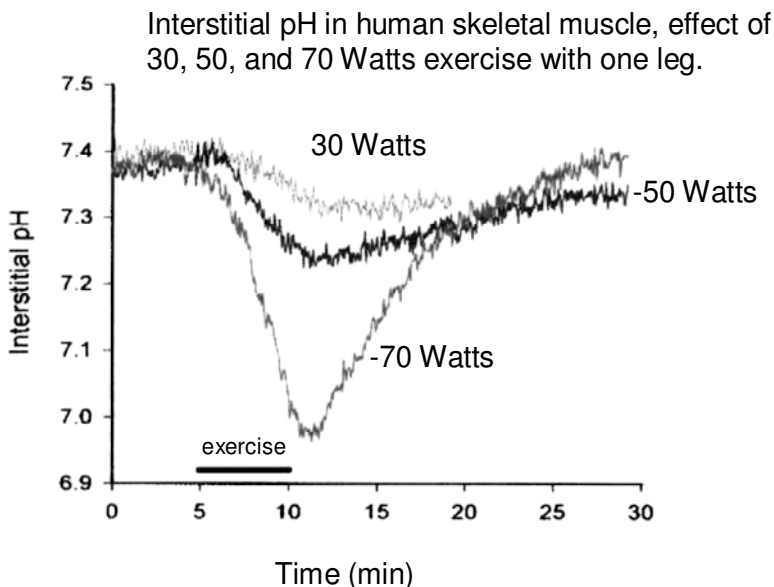


Fig. 9. Interstitial pH in human skeletal muscle during leg exercise with three different work intensities 30, 50 and 70 Watts. It can be seen that pH is reduced during exercise (acidification), and that recovery of pH takes place with a half-time of approximately 5 min. Data from Street et al. 2001.

3.3.4 Vesicle studies

Regulation of muscle pH has also been studied with sarcolemmal giant vesicles.

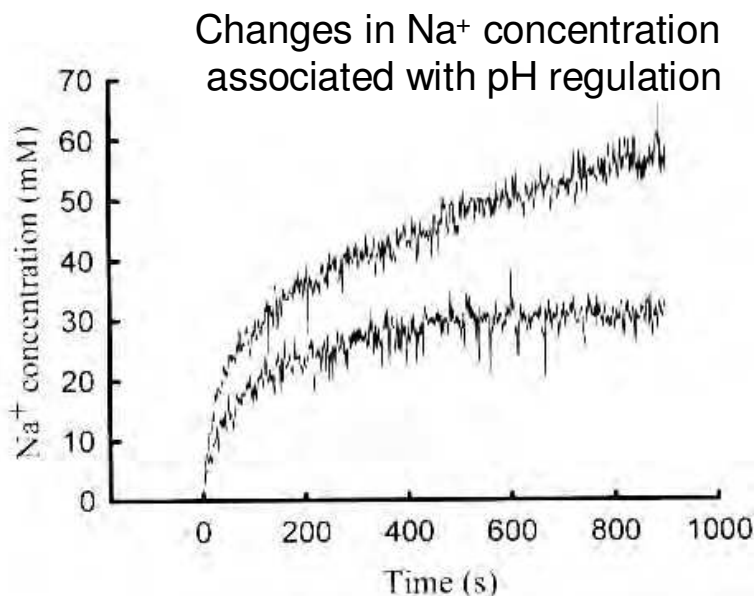


Fig. 10. pH regulation studied with vesicles obtained from rat skeletal muscle. At time zero external pH is changes 0.5 unit. Vesicular Na⁺ concentration is measured with an ion sensitive dye. The changes in vesicular Na⁺ concentration reveal that Na⁺ fluxes across the membrane takes place during pH regulation. By the use of a specific inhibitor, amiloride, (lower trace) it was demonstrated that part of pH recovery is mediated by Na⁺/H⁺ exchange (Juel 2000).

3.3.5 The use of biopsy material to study other components of pH regulation

The sections below focus on the components involved in pH regulation.

3.3.6 Sodium/bicarbonate transport

Only a few studies have investigated the functional significance of sodium/bicarbonate co-transport in skeletal muscle in general and especially in human muscle. However, two isoforms of the Na⁺/bicarbonate co-transporters NBCs have been identified in rat muscle and human muscle. For humans the experiments were based on biopsy material. Studies with vesicles made from rat have demonstrated that the NBCs contribution is approximately half of the total capacity for pH regulation in resting muscle (Kristensen et al. 2004).

3.3.7 Na⁺/H⁺ exchange

This exchanger is the classical pH regulating system found in most cell types. The kinetics of Na⁺/H⁺ exchange in skeletal muscle have been studied with rat muscle (Juel 1998, 1998,

2000). The effect of high-intensity training on human Na^+/H^+ exchange protein NHE1 has been studied with biopsy material obtained from trained and untrained muscle (Juel et al. 2004). Data from an animal study of Na^+/H^+ exchange in pH regulation is shown above, figure 10.

3.4 Ion homeostasis

Activation of skeletal muscle is associated with small displacements of ions; each action potential give rise to a small Na^+ influx and a small K^+ efflux due to opening if specific channel. During repeated activation Na^+ is accumulated in the muscle cell and K^+ is accumulated outside the cell. Analysis of blood samples obtained in association with muscle activity show an increased plasma K^+ concentration during muscle activity and in the first minute of the recovery period. The activity of the Na^+/K^+ -pump counteracts these concentration changes, but it is obvious that the Na^+/K^+ -pump can not keep pace with the K^+ efflux during muscle activity, which is the underlying mechanism for the extracellular accumulation of K^+ (Figure 11).

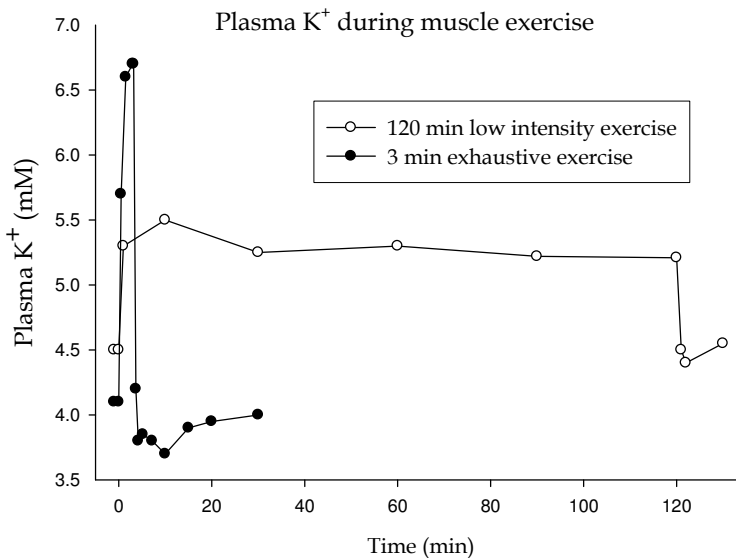


Fig. 11. Accumulation of K^+ in blood plasma during short intense muscle activity and during long lasting low intensity exercise. Potassium release was calculated from the arterial and venous blood K^+ concentration and blood flow. Data From Juel et al. 1990 and Sjøgaard 1986.

It can be concluded from Figure 11 that both short and long-lasting exercise is associated with a continuous potassium loss from muscle. It has been confirmed with muscle biopsies that the extracellular accumulation of K^+ is paralleled by a similar decrease in intracellular K^+ concentration.

The intracellular Na^+ increase and the extracellular K^+ concentration increase have been associated with muscle fatigue. The underlying mechanism is impaired muscle excitability,

which results in reduced force; muscle fatigue. The Na^+, K^+ -pump is the main membrane transport system responsible for ion homeostasis. The pump counteracts the ion displacements. Regulation of the pump is therefore important for development of fatigue. Stimulation of the pump by hormones and other mechanisms delays the development of fatigue.

3.4.1 Membrane purification, Western blotting

Analysis of sarcolemmal giant vesicle material with the use of specific membrane marker antibodies, has revealed that vesicular membranes exclusively consist of outer membranes with no contribution from T-tubuli and endoplasmic reticulum membranes. This fact has been used in studies of protein distribution. The use of vesicles as a method to purify the outer membrane seems to be more efficient than the traditional methods, which include several spinning steps. This fact has been used to determine the cellular localization of membrane transport proteins, an example is given below.

3.4.2 Translocation of pumps

It was suggested before 2000, that the cell-surface Na, K -ATPase protein density in rat could be increased by insulin and exercise. But it was not known whether this mechanism is also present in human muscle. This was probably due to the large amount of muscle tissue needed for membrane purification. However, the development of the sarcolemmal giant vesicle technique and the optimization for the small amount of vesicles obtained from human muscle biopsies, made it possible to study the distribution of the Na, K -ATPase (Na, K -pump) in human skeletal muscle. In the first study to use this technique 6 human subjects performed one legged exercise until fatigue, and needle biopsies were obtained before exercise and immediately after fatigue. The amounts of Na, K -ATPase isoforms in the sarcolemmal membrane were measured with antibodies. It was demonstrated that exercise significantly increased the amount of α_2 , total α , and β subunit proteins by 70, 35 and 26 %, respectively (Juel et al. 2000). These values clearly indicated that pump subunits can be translocated from one store to the outer membrane during exercise. However, the underlying nature of this translocation remained unknown. It was later demonstrated with sarcolemmal material from rat muscle that the translocation of pump subunits to the outer membrane is reversible with a half-time of approximately 20 minutes (Juel et al. 2001).

The translocation mechanism has later been studied with other techniques including biotin labeling of surface membrane proteins in combination with sarcolemmal giant vesicles used as a membrane purification method (Kristensen et al. 2008). These studies confirmed that Na, K -ATPase subunits can translocate to the outer membrane. In addition it was demonstrated that changes in caveolae pump content could be part of the mechanism. This is another example of the use of biopsy material; these experiments clearly brought new knowledge.

3.4.3 Glucose transport

The use of sarcolemmal giant vesicles produced from biopsy material was a success in studies of lactate transport. It was therefore logic to use the same method for the study of glucose transport and glucose transporters. The first studies used vesicles from rat muscle. It

was possible to demonstrate that glyucose transport across the vesicular membranes was affected by insulin and muscle contractions (Ploug et al. 1993). Furthermore, the effect of pH and glucose-6-phosphate was studied (Kristiansen et al. 1994).

Studies of glucose transport using vesicles from human biopsies have also been published. Since vesicles exclusively consist of material from the outer membrane it is possible to investigate if the glucose transporters GLUT4 are translocated to the outer membrane during exercise. Changes in glucose transport and translocation of GLUT4 with endurance training have been investigated with vesicles produced from biopsies from human muscle (Richter et al. 1998) However, in spite of the first promising experiments with vesicles; this technique has not been used in newer experiments. The reason is that regulation of glucose transport is dependent on internal signaling pathways, which are lost during the preparation of vesicles.

3.4.4 Fri fatty acid transport

Long-chain fatty acids, LCFA, are important as an energy source in many tissues including skeletal muscle. It was originally believed that long chain-fatty acids can freely diffuse across the plasma membrane. It was early recognized that LCFA can bind to the outer membrane; the binding proteins were simply called fatty acid binding proteins. The details could only be studied in a model system; again vesicles were used. The early studies of fatty acid membrane transport were carried out with sarcolemmal giant vesicles produced from rat muscle (Bonen et al, 1999; Luiken et al., 2001; Turcotte et al., 2000; Bonen et al., 1998; Bonen et al., 2000). It was demonstrated that the transport showed saturation and could be inhibited with specific antibodies, which clearly indicates that a transport system is involved (Turcotte et al. 2000).

These studies in combination with the use of antibodies and western blotting revealed three groups of transport systems: fatty acid binding proteins (FABPm), fatty acid translocase (FAT), and fatty acid transporter proteins (FATP)(Bonen et al. 1998a). These early transport studies were not based on human membrane material. But human biopsy material was later used to identify the transporter proteins in human skeletal muscle (Roepstorff et al. 2004). In addition, biopsies taken before and after training were used to characterize the effect of endurance training on the FABPm protein density, which was increased by 49 % after three weeks of training (Kiens et al. 1997).

4. Conclusion

Whole body experiments in humans have given much information about membrane transport. These data have been combined with information obtained by the use of biopsies.

Biopsies from human skeletal muscle have been used to give a snapshot of metabolite content and ion composition in connection with muscle activity and training. It is also possible to study dynamic aspects of gene regulation in human biopsy material.

The present review has focused on studies of membrane transport based on biopsy material. It has been shown that muscle treated with collagenase and high K^+ concentrations induce formation of membrane vesicles. Single vesicles were originally used for microelectrode recordings of ion currents (Burton et al. 1990). A method to purification vesicles made from

animal muscle was later developed (Juel 1990), and the method was further developed allowing vesicles to be produced from human skeletal muscle biopsies (Juel et al. 1994). These vesicles were demonstrated to be a new and unique method to investigate membrane transport processes in human skeletal muscle. In combination with the use of radio-labeled tracers these vesicles allowed quantification of the transport kinetic parameters K_m and V_{max} (per square cm of membrane).

The first membrane transport system to be investigated in humans with the biopsy based vesicle technique was the lactate/proton co-transport system. This transport system was characterized first in rat skeletal muscle membranes later in membranes from human skeletal muscle (Juel 1991, Pilegaard et al. 1993, Juel et al. 1994). With this technique it was possible to measure lactate/proton co-transport in muscle fiber types and to show that this transport system could be up-regulated with training and decreased with inactivity. It must be noted that the first discovered adaptive changes was quantified before the transport protein was cloned and before antibodies were available. Later studies have used vesicles as a method to purify muscle membranes. These studies were combined with information obtained by whole body experiments.

Sarcolemmal giant vesicles have later been used to study:

- glucose transport (Kristiansen et al. 1994)
- pH regulation (Juel 1995)
- translocation of Na,K-pump subunits (Juel et al. 2001)
- Na^+/H^+ exchange
- K^+ displacement during exercise
- membrane transport of free fatty acids (Bonen et al. 1998)

Data from biopsies were also used in exercise and training experiments. These studies gained from the fact that a biopsy represents a snapshot of the metabolic and ionic content of an active muscle.

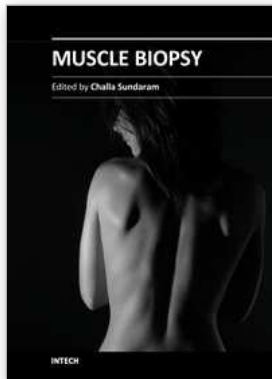
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Investigation of muscle diseases has changed dramatically with the understanding of genetic basis of a wide range of muscle diseases. Muscle biopsy has become a powerful tool not only to provide diagnosis but to make tissue available for genetic studies and to basic scientists for biomedical research. Accurate interpretation of muscle biopsy to detect cell dysfunction/ damage/death or absence / abnormality of a protein or genetic defect by the sophisticated technologies is important to guide treatment of various muscle diseases. In this book on muscle biopsy various chapters deal with the procedure and interpretation of muscle biopsy, its use in the culture of myotubes and membrane transport studies. Muscle biopsy is an important technique to investigate mitochondrial dysfunction and the mitochondrial DNA integrity in oxidation. Phosphorylation in various metabolic diseases like obesity, type 2 diabetes mellitus and peripheral vascular disease is explored in the other chapters with detailed descriptions on methodology. This book provides the advances in the basic techniques of muscle biopsy for a neuroscientist.

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