

Lamellar Metabolism

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An understanding of equine hoof laminar energetics is important for insight into normal foot metabolism and in turn better understanding of laminitis pathophysiology. Dietary glucose, like glucose synthesized within the body, is transported via rate-limiting, glucose transport proteins from the circulation into target cells. Cells extract glucose from blood using facilitative glucose transport proteins and once inside the cell rapid phosphorylation of free glucose into glucose-6-phosphate traps it inside the cell. There are thirteen known members of the facilitative sugar transport proteins and GLUT1 is the primary transporter in the blood-brain barrier, nerve sheath cells, skin, and red blood cells. Under aerobic conditions lactate can enter the citric acid cycle via pyruvate and thereby be used for ATP production. Lactate, instead of being a waste product, is an important metabolic intermediate that, under aerobic conditions, contributes to the energy production in several organs. In moderate exercise, lactate disposal through oxidation is believed to be around 80%. Sensors in pancreatic β cells trigger secretion of insulin, the hormone that stimulates glucose uptake into many types of cells. The pancreas can also be active in increasing the blood glucose levels. A sensor present in pancreatic α cells, responds to hypoglycemia and triggers secretion of glucagon to accelerate hepatic glucose production. Skeletal muscle is the principal tissue responsible for insulin-stimulated glucose disposal and thus the major site of peripheral insulin resistance. The insulin sensitivity of glucose transport in skeletal muscle can be increased by exercise. The hormone carries out its effect after binding to insulin receptors. The normal equine hoof consumes glucose and produces lactate. Hooves of horses at rest have a glucose consumption that exceeds that of the head. Since the hoof doesn't store glycogen, glucose consumption reflects glycolysis and oxidation; the high concentration of lactate in the digital veins indicates that most of the glucose is metabolized to lactate. Laminar tissues are rich in LDH thus suggesting that the laminar layer relies on glycolytically (anaerobic) generated ATP to a large extent. Laminar tissues are also rich in NADH-dehydrogenase a marker enzyme for mitochondria. This shows secondary epidermal laminae (SEL) have an oxidative capacity that resembles oxidative skeletal muscle cells. SEL basal cells, but not suprabasal cells, also react strongly for GLUT1 antibodies thus reflecting their dependence on glucose as an energy substrate.

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When transmitting the forces of weight bearing and locomotion, between body and ground, the hooves of a galloping horse are subjected to large workloads. To successfully survive these loads, without incurring damage, the hoof capsule and the soft tissues within continually stretch, bend and compress. To maintain this essential function the living cells of the hoof need energy. Even though understanding the energetics of the hoof is essential for performance, equine

exercise physiology has focused mainly on muscles. Recent research has been performed at the Australian Equine Laminitis Research Unit (AELRU) to give insight into the metabolism of hoof lamellae and in turn better understanding of laminitis pathophysiology.

Basic Metabolism: From Feedstuff to Cell

Horses are nonruminant herbivores and have the capacity for both enzymatic and microbial digestion.¹ Digestion of carbohydrates occurs primarily in the small intestine suggesting that sugars can be utilized directly from the feedstuff. This is important since carbohydrates, and especially glucose, are the main energy sources for most eukaryotic cells. Carbohy-

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urate uptake is affected by: luminal digestion of polysaccharides, digestion of tri- and disaccharides in the brush border and uptake of glucose by the intestinal epithelial cells. Because of its hydrophilicity, glucose cannot penetrate the lipid bilayer of the plasma membrane. Dietary glucose, like glucose synthesized within the body through gluconeogenesis and glycogenolysis, has to be transported via glucose transport proteins from the intestinal lumen and circulation into target cells. Glucose transport through these proteins is the rate-limiting step in glucose metabolism.² Glucose molecules enter enterocytes via Na⁺-dependent glucose transporter proteins (SGLT). SGLTs are located mainly in the small intestine and the kidney. These proteins can transport glucose into target cells, against its concentration gradient, by utilizing the Na⁺-electrochemical gradient provided by the Na⁺-K⁺ ATPase pump.³ In cells that get glucose from the blood, the transport mainly goes via facilitative glucose transport proteins which utilize the diffusion gradient across plasma membranes. These polytopic membrane proteins form an aqueous pore across the plasma membrane through which glucose can move. Glucose flux in one direction, ie, into the cell, is achieved by rapid removal of free glucose in the cell cytoplasm by its phosphorylation into glucose-6-phosphate. The negative charge of the phosphate prevents further passage of the sugar phosphate and traps it inside the cell.

There are thirteen known members of the facilitative sugar transport family of proteins capable of transporting glucose, fructose, galactose and mannose. They are GLUT1–GLUT12 and HMIT, and exhibit different hexos specificity and kinetic properties. The GLUT and HMIT family can be divided into three subclasses.⁴ Class I is comprised of the glucose transporters GLUT1 to GLUT4, which show a difference in distribution between and within tissues that corresponds to different patterns in glucose metabolism found in the different tissues.^{3,5} Class II is comprised of the fructose-specific transporter GLUT5 and three related proteins, GLUT7, GLUT9, and GLUT11. The glucose-transport activity of GLUT11 is markedly inhibited by fructose.⁶ Class III is comprised of GLUT6, 8, 10, 12 and HMIT, and is characterized by differences in protein structure. Even though tissues normally express high amounts of one of the glucose transport proteins several different transporters can be found in the one tissue. As an example, white adipose tissue expresses GLUT1, GLUT4, GLUT5, GLUT8, GLUT12 and HMIT, with GLUT 4 being the major one.

Below is a discussion of the Class I members GLUT1, GLUT 3 and GLUT 4, which all have a high affinity for glucose. GLUT1 and GLUT 3 are localized mainly in the plasma membrane and are insulin-independent and considered responsible for basal glucose transport. GLUT4 is normally found within organelles, including the plasma membrane, endosomes, *trans*-golgi network and GLUT4 storage vesicles.⁷

GLUT1 is the primary transporter in the blood-brain barrier, choroid plexus, ependyma, and glia,⁸ nerve sheath cells,⁹ skin,¹⁰ and red blood cells.¹¹ The glucose transport mediated by the GLUT1 isoform is supposed to be insensitive to normal variations in the plasma glucose concentration and shows only a minor acute response to hormones and local stimuli.¹² Nevertheless, GLUT1 expression in fibroblasts has

been reported to be rapidly, but transiently, increased by growth factors such as EGF, PDGF, and FGF.¹³ After it is produced GLUT1 needs to be activated. This can be done in response to inhibition of oxidative phosphorylation.¹⁴

GLUT3 is the neuronal glucose transporter⁸ and its expression is increased under conditions of energetic stress such as diabetes.¹⁵

GLUT4 is insulin-sensitive and can rapidly be translocated from the intracellular compartments to the plasma membrane and thereby allow these tissues to increase their rate of glucose transport, 10- to 40-fold, within minutes. In skeletal muscle, this translocation is triggered by two separate and distinct signaling pathways, namely (1) insulin and (2) metabolic stress such as muscle contractions, hypoxia, nitric oxide and chemical hypoxia, ie, pharmacological inhibitors and uncouplers of mitochondrial respiration.^{2,16,17} The actin cytoskeleton together with a protein called TC 10 is thought to play a role in the insulin-stimulated translocation process, and agents that depolymerize actin inhibit GLUT4 translocation.¹⁸ Insulin-like growth factor (IGF-1) also increases glucose uptake via GLUT4 translocation.¹⁸ Exercise increases the total GLUT4 content in muscle in both man and horse.^{16,19,20}

Aside from glucose, GLUT1, GLUT3 and GLUT4 also transport dehydroascorbate,^{21,22} which, when entering the cells, is converted into and stored as ascorbic acid. Ascorbic acid has a central role in the antioxidant defense system. It has been emphasized that vitamin C could be excluded from cells in certain tissues during hyperglycemia, due to its competition with glucose of the common transport mechanism²³ and thereby increase the risk of tissue damages if for example this coincides with reperfusion after ischemia.

Metabolism Within the Cell

Energy is extracted from food molecules by a process of gradual oxidation. Since the most energetically stable form of carbon in the presence of oxygen is as CO₂, and that of hydrogen as H₂O, mammalian cells are able to obtain energy from sugars by allowing their carbon and hydrogen atoms to combine with oxygen to produce these forms. Oxidation means not only the addition of oxygen atoms in chemical reactions it also means removal of electrons. Since there is no loss or gain of the total number of electrons in a chemical reaction, a reduction (addition of electrons) always occurs simultaneously with an oxidation. When a molecule picks up an electron it usually also picks up a proton (H⁺) since they are freely available in water.²⁴ Dehydrogenation reactions are reductions of oxidations and hydrogenation reactions. Enzymes are used to catalyze the oxidation steps. The energy released by the oxidation of food molecules is transferred to and stored in carrier molecules such as ATP, NADH and NADPH by coupled reactions. A coupled reaction means that an energetically favorable reaction is used to drive an energetically unfavorable one. The addition of a phosphate group to ADP to synthesize ATP is an energetically unfavorable (phosphorylation) reaction. When required, such as when the cell wants to join two molecules together and build something, ATP releases its stored energy through an energetically favorable hydrolysis to ADP. Macromolecules of the cell are polymers that are formed from subunits by a condensation

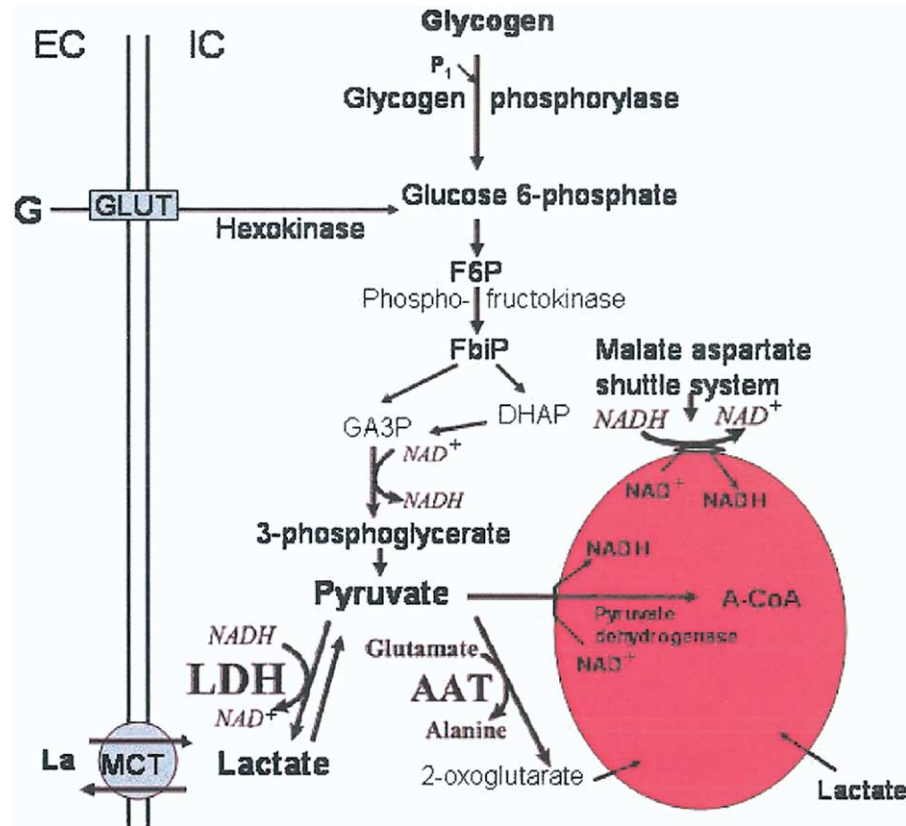


Figure 1 Simplified scheme of the cell metabolism with substrates, different enzymes and carrier molecules. EC, extracellular; IC, intracellular; G, glucose; La, lactate. The red ring illustrates mitochondria.

reaction (energetically unfavorable) and broken down by hydrolysis (energetically favorable).

When glucose has entered the cell it is phosphorylated by ATP to form glucose 6-phosphate (Fig. 1). This is the first of the ten reactions in the first stage of glycolysis. During the glycolysis, each molecule of glucose (6 carbon atoms) is converted into two molecules of pyruvate (3 carbon atoms) and the net products are two ATP molecules and two NADH molecules. Sugars other than glucose are similarly converted to pyruvate after being converted to one of the sugar intermediates in the glycolytic pathway. The pyruvate passes from the cytosol into mitochondria, where it is converted into CO_2 plus acetyl coenzyme A with the reduction of NAD to NADH. This reaction is catalyzed by the pyruvate dehydrogenase (PDH) complex which is the first irreversible reaction in glucose oxidation.²⁵ PDH is present in an active dephosphorylated form and an inactive phosphorylated form.²⁶ Activation of the PDH complex is a major event regulated by insulin in most cells.²⁷ The next series of reactions that take place within the mitochondria are the citric acid cycle and the oxidative phosphorylation, the only step in the oxidative metabolism of foodstuffs that directly requires O_2 from the atmosphere.²⁴ Each molecule of glucose, completely oxidized within the mitochondria, produces an additional 28 molecules of ATP. About 10^9 molecules of ATP are believed to be in solution in a typical cell at any instant. The turn over time for all this ATP is 1 to 2 minutes in most cells.²⁴

The most important cytoplasmic pathway of pyruvate metabolism is the second stage of glycolysis in which lactate dehydrogenase (LDH) converts pyruvate to lactate with the oxidation of NADH to NAD. LDH is present both within the

cytoplasm and within the mitochondria. Lactate will be produced if the amount of pyruvate and NADH production exceeds the PDH capacity or the malate–aspartate shuttle system that reconverts NADH to NAD. This is because these events determine how active the second stage of glycolysis needs to be in meeting the demand for energy. A major cellular fuel sensor is 5' AMP-activated protein kinase (AMPK). This enzyme is activated by phosphorylation during physiological conditions that deplete cellular ATP such as exercise and hypoxia. AMPK activation largely depends on the AMP/ATP ratio. Once activated in muscle, AMPK has been shown to inhibit anabolic reactions, promote catabolic reactions and increase glucose transport by increasing GLUT4 expression.^{28,29}

The electron transfer and oxidative phosphorylation steps in the mitochondria are most likely controlled by the level of their substrates, O_2 , NADH, free ADP and inorganic phosphate.³⁰ If the production of lactate exceeds the efflux, the pH of the cytosol will decrease. This leads to inhibition of phosphofructokinase and hence glycolysis.³¹

Lactate

Lactate, a product of anaerobic glycolysis, has usually been considered to be an end product of metabolism.³² However, under aerobic conditions lactate can enter the citric acid cycle via pyruvate and thereby be used for ATP production. So instead of being a waste product, lactate is now regarded an important metabolic intermediate that, under aerobic conditions, contributes to the energy production in several organs such as heart, muscles and brain.^{33,34} LDH is a tetramer com-

posed of either M (muscle) or H (heart) types that may be combined to form five LDH isozymes (LDH₁₋₅). In general, it has been suggested that the “heart type” LDH_{1,2} favors the reaction of lactate to pyruvate whereas the LDH_{4,5} isoforms favor lactate formation.³⁵ However, the LDH binding to different cellular structures, such as plasma membrane, peroxisomes and mitochondria, has recently been found to alter its kinetics. Its association with different cellular organelles has therefore been suggested to play a more predominant role than the isoform in whether the LDH reaction proceeds toward lactate or pyruvate formation.³⁶ In resting mammals, oxidation accounts for approximately half of lactate disposal and gluconeogenesis for approximately 20%. In moderate exercise, the disposal through oxidation is believed to be around 80%.^{33,37} It has also been shown that chronic hypoxia, ie, being at high altitude, increases the ability of muscle cells to transport and oxidize lactate by having a tissue-specific effect on MCTs and LDH.³⁸ During both rest and exercise skeletal muscle is a major site of lactate oxidation as well as production.^{31,33}

Although it is lactic acid that is both produced and utilized by metabolism, tissue pH ensures that it dissociates almost entirely to the lactate anion. This charged species cannot cross the plasma membrane by free diffusion, but requires transport via proton-linked monocarboxylate transporters (MCTs). These transporters catalyze the facilitated diffusion of lactate with a proton. There is no energy input other than that provided by the concentration gradients of lactate and protons. The rate-limiting step for this lactic acid flux is the return of the free carrier across the membrane that is required for the completion of the translocation cycle. MCT transmembrane proteins can be found both in the plasma and the mitochondria membrane. MCTs are also essential for the transport of other monocarboxylates such as pyruvate which gives them a central role in mammalian metabolism.³¹ However, pyruvate is capable of diffusing across phospholipid membranes at an appreciable rate, even in the absence of a transporter.^{25,39} Nine MCT-related sequences have so far been identified in mammals, each having a different tissue distribution. Direct demonstration of proton-linked lactate and pyruvate transport has been demonstrated for mammalian MCT1–MCT4.³¹

MCT1 is ubiquitously expressed and believed to have a role in lactic acid oxidation and is also believed to be the mitochondrial lactate/pyruvate transporter.⁴⁰ In heart and red muscle, where it is especially prominent, it is upregulated in response to increased work. MCT1 expression in individual muscle cells correlates with their mitochondrial content. MCT2, which has a higher affinity for substrates than MCT1 and MCT4, is expressed in cells where rapid uptake at low substrate concentrations may be required, such as sperm tails, neurons and proximal kidney tubules. MCT3 is uniquely expressed in the retinal pigment epithelium. MCT4 is considered to be expressed where lactic acid efflux predominates since it is most evident in cells with a high glycolytic capacity such as type II B muscle fibers.³¹ Studies of the intestinal distribution of MCTs have been restricted to the cecum of hamster and the colon of humans and pigs. In both cecum and colon MCT1 has been present and is therefore believed to play an important role in the uptake of lactate, pyruvate and short-chain fatty acids, such as butyrate and

propionate, from the intestinal lumen into the blood.³¹ LDH and MCTs together form the lactate shuttle that is proposed to be involved in the shuttling of lactate between cells.³⁸ There is also a proposed intracellular lactate shuttle that directs endogenous lactate toward oxidation by mitochondria, and it has been suggested that lactate is the predominant monocarboxylate oxidized by some tissue mitochondria *in vivo*.³³

After being produced, the activity of an enzyme is often highly regulated, and it is its activity that represents the function of an enzyme.⁴¹⁻⁴³ Therefore, large amounts of inactive, ie, non-functional enzymes, can be accumulated in a tissue. Nevertheless, the inactive enzyme can rapidly be converted to its active form on demand. Using biochemistry, localization and quantification of an active enzyme can be studied by either the production or the consumption of endogenous molecules, such as NADPH and NADH, or the formation of detectable products from substrates that have access to the enzyme.⁴⁴ LDH activity in the equine hoof has now been assessed (see below).

Control of Blood Glucose Levels

Glucose homeostasis requires that glucose sensors constantly monitor blood glucose concentrations and modulate food intake and glucose use or production. Pancreatic β cells possess glucose sensors, which are activated by rises in blood glucose that induces the secretion of insulin. Insulin is the primary hormone involved in the control of blood glucose. It stimulates glucose uptake into many types of cells but after a meal it enhances uptake of glucose mainly into fat and muscle and inhibits the production and release of glucose from the liver.¹⁸ The pancreas can also be active in increasing the blood glucose levels. A sensor present in pancreatic α cells, which responds to hypoglycemia, triggers secretion of glucagon to accelerate hepatic glucose production. The secretion activity in the pancreas is also under the control of the autonomic nervous system, which in turn is directed by glucose sensors located in the hypothalamus and the brain stem, which regulate feeding and glucose homeostasis. The hepatoportal vein also contains a metabolic sensor that is activated when a glucose gradient is established between the portal vein and the hepatic artery. The hepatoportal sensor contributes to the whole body glucose clearance by inhibiting food uptake,⁴⁵ stimulating uptake of glucose in liver⁴⁶ and muscle, through insulin receptor-independent uptake via GLUT4.⁴⁷

The equine blood glucose values vary frequently during the day but remain within physiological values thanks to these regulatory mechanisms (Fig. 1). Variations with a cycle of about 24 hours (circadian rhythms) have been demonstrated in equine plasma glucose and insulin.⁴⁸

Insulin

Skeletal muscle is the principal tissue responsible for insulin-stimulated glucose disposal and thus the major site of peripheral insulin resistance.⁴⁹ The insulin sensitivity of glucose transport in skeletal muscle can be increased by exercise.⁴⁹

The hormone carries out its effect after binding to insulin receptors. The insulin receptor, which in humans is expressed in two isoforms with different tissue distributions, is

synthesized from a single chain pro-receptor. The synthesis takes 1.5 to 3 hours and the receptor has a half-life time of about 7 to 12 hours.⁵⁰ The B isoform is predominantly expressed in insulin target tissues, ie, fat, muscle, and liver, while the A isoform predominates in tissues such as pancreatic β cells and neural tissue. Of these the A isoform has higher affinity for insulin-like growth factor (IGF) than the B isoform and an over expression of it results in insulin resistance.⁵¹ Insulin is very active within the body and has:

Metabolic effects: stimulation of glucose transport and metabolism, stimulation of glycogen synthesis, inhibition of production and release of glucose from the liver, stimulation of lipogenesis, inhibition of lipolysis, stimulation of ion flux.

Growth promoting effects: stimulation of DNA synthesis, stimulation of cell growth and differentiation.

Mixed metabolic and growth effect: stimulation of amino acid influx, stimulation of protein synthesis, inhibition of protein degradation, stimulation of RNA synthesis.⁵⁰

Metabolism in Different Components of the Hoof

Study of the Normal Metabolism of the Hoof

The uptake of glucose and the production of lactate in the equine hoof of normal horses has been studied by comparison of simultaneously taken blood samples from the superficial *arteria temporalis*, *vena jugularis* and a digital vein at the level of the pastern of one of the front legs.^{52,53} Differences between arterial and venous blood reflect the metabolic processes in the tissues drained by the two veins. The glucose levels in the digital veins showed that the hooves in horses at rest have a glucose consumption that exceeds that of the head. The hoof consists mainly of epidermis (hoof capsule), dermis (including the connective tissue consisting of fibroblasts, blood vessels and nerves), bone and cartilage. Since the hoof doesn't include tissue with the glycogen storage capacity of striated muscles, it is reasonable to believe that the glucose consumption reflects glycolysis and oxidation through the citric acid cycle. The levels of lactate were higher in blood from the digital veins indicating that, at physiological glucose concentrations at rest, a large part of the glucose that is taken up by the living cells in the hoof is metabolized to lactate.

The anaerobic glycolysis and the cell pyruvate \leftrightarrow lactate capacity was examined in hoof lamellae by assessing LDH activity on cryostat sections (Fig. 2A). Although it is common to regard LDH as an enzyme that produces lactate and NAD⁺ from pyruvate and NADH depending on the availability of substrates it nevertheless works in both directions. Thus, to study LDH activity the substrate lactate, the coenzyme (= electron acceptor) NAD⁺, exogenous electron carriers, and tetrazolium salt were added to a cryostat tissue section.⁵⁴ Controls were performed in the absence of substrate and coenzyme and with the addition of pyruvate. Aside from staining for LDH activity, the laminar layer was also examined for NADH-dehydrogenase with the method of Novikoff and coworkers.⁵⁵ NADH-dehydrogenase is frequently used

as a marker enzyme for mitochondria. The results of NADH-staining showed that the secondary epidermal laminae (SEL) have an oxidative capacity that resembles oxidative skeletal muscle cells rather than anaerobic type IIB muscle fibers. SELs were rich in LDH thus resembling hoof blood vessels suggesting that the laminar layer, like blood vessels (as discussed below), is able to rely on glycolytically generated ATP to a high extent under normal oxygenated conditions. The SEL basal cells showed a very strong reaction for GLUT1 antibodies while the suprabasal cells only expressed a very mild reaction (Fig. 2B). The SEL expressed small amounts of GLUT3 and GLUT4. The equine skin showed a similar expression of GLUT thus resembling the skin of man and rodents (see below). However, the expression of GLUT1 in the SEL basal cells seems to be much stronger compared with the expression in skin.

Blood Vessels

Endothelial and smooth muscle cells in the peripheral vascular beds can use amino acids and glucose as a source of energy and some endothelial cells also utilize fatty acids.⁵⁶ Amino acids with the highest rates of oxidation (glutamate, glutamine, alanine, asparagine) are degraded by no more than three intermediate steps before entering the citric acid cycle.⁵⁷ Glucose is used by the endothelial cells for both anaerobic and aerobic metabolism but at normal glucose concentration >98% of incorporated glucose is metabolized to lactate. When glucose concentrations are low oxidation of glucose via the citric acid cycle is higher. Consequently, glucose has an inhibitory effect on the mitochondrial respiration of endothelial cells. The high glycolytic activity and the capacity to use different fuels make endothelial cells adaptable to hypoxia and variations in blood glucose levels.⁵⁸ GLUT1 is the most abundant glucose transport protein in endothelial cells. However, endothelia may express GLUT 4 to some extent.^{59,60} Hypoxia is believed to increase GLUT1⁶¹ transport and hypoglycemia has been reported to up-regulate GLUT 1 expression. Hyperglycemia on the other hand is not considered to up-regulate the expression but histamine has been shown to stimulate glucose transport, at least in cultured endothelial cells.⁵⁸ Endothelial cells have insulin receptors but, it seems, not for promoting glucose uptake, but more for carrying out an insulin effect on local vascular tone.⁵⁸ Endothelial cells are actively involved in the local control of homeostasis⁶² and the amino acid transporter protein CAT-1 which can transport the nitric oxide (NO) precursor L-arginine is colocalized with nitric oxide synthase in plasmalemma caveolae. NO is a vasodilator and its production has been shown to be up-regulated by insulin and other factors such as hypoxia. Therefore, insulin resistance in endothelial cells might lead to decreased perfusion of the peripheral vascular bed.⁶³ Other factors that can modulate transport of L-arginine as well as glucose in endothelial and smooth muscle cells are endotoxin and certain cytokines and lipids. A most likely effect of insulin on the peripheral vascular bed in horses has been reported in the form of postprandial arterial vasodilation in the distal thoracic limb.⁶⁴ The change in homeostasis coincided with increases in plasma glucose and insulin in this study.⁶⁴

The smooth muscle cells of the blood vessels rely to a large

extent on glycolytically generated ATP, even under fully oxygenated conditions.^{65,66} Glycogen is considered to contribute with 10%, glucose 50% and fatty acids and/or amino acids with 40% of the total substrates entering the citric acid cycle in vascular smooth muscle cells (VSMC).⁶⁷ It is reasonable to believe that lactate is also included in the 40%. Insulin, IGF-1, endotoxin, LPS, TNF- α , interleukin 1 β , and interferon- γ are some substances considered to stimulate glucose uptake in VSMC.⁵⁸ Glucose transport via GLUT1 and both nonoxidative and oxidative glucose metabolism are up-regulated after injury. Moreover, there is also a link between upregulated glucose metabolism and an antiapoptotic cellular signaling pathway in VSMC.⁶⁸ Immunohistochemical staining with GLUT1 antibodies stain the vessels weakly, while the red blood cells stains in human but not in rat⁶⁹ or horse.⁵²

Epidermis

Glucose uptake by muscle accounted for 36% of the total glucose uptake in normal rats and for approximately 20% of the whole body glucose uptake in man under basal conditions. In rat 23-28% and in man 20% of the total peripheral glucose uptake is removed by the splanchnic tissues, liver and gut while the skin accounted for approximately 15% of the total glucose uptake under basal conditions. Consequently, the skin plays an important role in the whole body glucose utilization, as well as lactate production.⁷⁰ The metabolism of the skin is believed to depend mostly on glycolysis, especially in the basal layers and small soluble molecules such as glucose diffuse readily from the underlying connective tissue into the basal two-thirds of the epithelium.⁶⁹ GLUT1 is highly expressed in the basal cell layer of the skin and, to a lesser extent, in the first suprabasal layer with immunohistochemical staining being more intense at the top of the connective tissue papillae in human and rat⁶⁹ as well as in the horse.⁵²

GLUT1 expression in epidermis seems to be regulated by keratinocyte differentiation¹⁰ and glucose transport rate of proliferating and differentiating keratinocytes is down regulated at high glucose levels and increased at low glucose levels.⁷¹ Moreover, hyperglycemia has been shown to inhibit insulin action in skin. This inhibition is thought to be a result of serine phosphorylation through a PKC-mediated mechanism as well as by activation of protein tyrosine phosphatases, which deactivates the insulin receptor function.⁷¹ Barely detectable levels of GLUT4 mRNA have been found in skin epidermis, explaining why insulin resistance doesn't lead to decreased glucose uptake in this tissue.

Glucose affects cellular morphology of keratinocytes. Skin keratinocytes incubated in 2 mmol/l glucose levels become small and organized, whereas cells maintained at 20 mmol/l glucose concentration become large and flattened and lose some of their orientation toward each other.⁷¹ Based on the cell's expression of cytokeratins, high glucose concentrations are also believed to enhance Ca²⁺-induced differentiation of keratinocytes. Insulin and IGF-I increase cellular proliferation under normal glucose levels but in the presence of high glucose concentrations, the effects of both hormones, but mainly of IGF-I, are reduced.⁷¹

Peripheral Nerves

GLUT 1 is the major GLUT expressed in perineural sheath.^{9,52,69}

Connective Tissue

The cell surface of fibroblasts contains GLUT1 and its expression has been reported to increase in obese people.^{69,72,73} Antibodies toward GLUT1 did not react with fibroblasts in the study of Wattle and Pollitt.⁵²

Glucose Uptake Under Abnormal Conditions

Hypoxia

Both activity and expression of GLUT1 increase in response to hypoxia,^{14,74,75} and this characteristic has been used as a marker of inhibition of oxidative phosphorylation.⁷⁶ Hypoxia also triggers GLUT4 translocation up to the plasma membrane from the intracellular compartments.^{16,17,19}

Sepsis

Maximal glucose uptake in response to insulin has been shown to decrease 30-40% in the *musculus gastrocnemius* during sepsis, while the uptake of glucose in the heart, diaphragm, and abdominal muscle was not affected.⁷⁷ This difference between muscles might be explained by a difference in expression of GLUT4. The basal glucose levels in skin increased 52% in septic rats. Also in the spleen, ileum and lung the basal glucose level is increased by sepsis.⁷⁸ Furthermore, in the study of Lang and Dobrescu,⁷⁸ sepsis induced increase in glucose uptake was still pronounced at low insulin levels and reducing the plasma glucose concentration did not alter the rate of glucose uptake. It seems that a Gram-negative infection, that induces whole body insulin resistance, increases the absolute rate of glucose uptake by insulin-independent GLUTs even under hypoglycemic insulinopenic conditions.^{78,79}

Sepsis and endotoxemia stimulate the release of numerous macrophage secretory products, which are capable of modulating glucose homeostasis. Among these, tumor necrosis factor, interleukin-1, and nonsuppressible insulin-like activity have been shown to increase insulin-independent glucose uptake under certain experimental conditions.

Hematological Changes Associated with Laminitis

In experimentally induced laminitis, hyperglycemia, and an increase in plasma cortisol have been documented,^{80,81} with peak levels for cortisol occurring shortly after the onset of lameness.⁸² A rise in blood D-lactate concentration has also been documented during the developmental phase.⁸³ Moreover, there is an increase in the enzymatic activity of creatine phosphokinase, LDH and AST.⁸⁴

Glucose Metabolism and Laminitis

Glucose has been shown to be important for the integrity of the hoof lamellar layer. In experiments with hoof explants,⁸⁵ it was necessary to add glucose to the media every 2 days to

maintain lamellar integrity. If glucose was excluded from the media or if 2-deoxyglucose (2-DG) was added to it in a concentration of ≥ 50 mmol/l the epidermal and dermal lamellae started to separate, ie, in vitro laminitis. 2-DG is believed to inhibit the glycolytic pathway by being converted to 2-DG-6-phosphate which competitively inhibits the metabolism of glucose-6-phosphate by phosphoglucose isomerase in the cell cytoplasm.⁸⁶ The results of Pass and coworkers⁸⁵ showed that decreased glucose metabolism causes separation of lamellae in vitro and that the hoof tissue is reliant on glucose for maintenance of the adhesion between the epidermal basal cells and the basement membrane for more than 2 days. As the time progressed in the study⁸⁵ the explant cells appeared to adapt to an alternative, unidentified, substrate for energy synthesis. However, it can be difficult to perform longer metabolic studies on explants since as time elapses the morphology of the basal cells of the cultured tissue changes. The nucleus becomes more condensed, indicating a change in the cells metabolic needs. Pass and coworkers⁸⁵ also inhibited the explants glucose consumption by adding aminophenylmercuric acetate (APMA) to the media. APMA (aminophenylmercuric acetate) is an organomercurial compound, which is recognized as an activator of MMP. Organomercurials are also known to inhibit enzymes of the glycolytic pathway, an effect inhibited by dithiothreitol (DTT).⁸⁶⁻⁸⁸ DTT also prevented the lamellar separation induced by APMA in the study of Pass and coworkers.⁸⁵

Insulin resistance decreases glucose uptake in cells that have insulin-sensitive glucose transport proteins, and has been discussed as a predisposing factor in equine laminitis.^{89,90} In terms of blood glucose values, ponies with laminitis⁹¹ and fat ponies⁹² are less sensitive to insulin and exhibited a far greater plasma insulin response, after glucose loading, than control ponies and horses. Moreover, ponies have been shown to have a positive correlation between the amount of backfat thickness and the degree of insulin resistance.⁹³ Insulin sensitivity can however be improved in ponies by exercise and loss of body weight.⁹⁴ It is also affected by the feeding regimes, that is, fasted animals exhibit insensitivity to insulin whereas fed ponies show normal response to glucose loading.⁹⁵ With insulin resistance it follows that triglycerides are more readily mobilized and the animal is therefore susceptible to hyperlipemia in a situation of negative energy balance⁸⁹ such as starvation, pregnancy, parasitism, lactation or transportation.⁹⁶

Insulin resistance can be defined as decreased uptake of glucose in skeletal muscle, liver and fat cells, either by reduced production of insulin, reduced effect of insulin at the receptor level or on the reactions that take place downstream of the receptor.⁶³ Fat, overweight, and unfit ponies are reported to have an increased risk for developing laminitis.⁹⁷ Adipocytes produce several products such as free fatty acids FFA, tumor necrosis factor- α (TNF- α)⁹⁸ and the adipocyte specific cytokines leptin,⁹⁹ and resistin¹⁰⁰ that have been associated with insulin resistance. Obesity leads to elevated circulating levels of FFA and increased plasma levels of FFA in turn inhibit insulin-stimulated glucose transport, insulin-stimulated glycogen synthesis, and insulin-stimulated glucose oxidation.¹⁰¹ Leptin regulates feeding behavior and energy metabolism and even though it has been suggested that leptin resistance may contribute to development of obesity,

administration of leptin decreases insulin resistance, hyperglycemia, and hyperinsulinemia in man.¹⁰² Serum leptin levels correlate with body condition in horses, ie, fat ponies/horses tend to have greater serum levels of leptin than thinner ones. Therefore, leptin has been put forward as a possible endocrine signal of nutritional status and body fat-mass in horses.¹⁰³

Hypothyroidism has also been suggested as a causative for laminitis in obese horses. However, no signs of laminitis have been documented in horses that have had their thyroid gland removed.^{104,105}

Glucocorticoid excess causes insulin resistance in skeletal muscle by directly inhibiting the translocation of the GLUT4 glucose transporters to the plasma membrane in response to insulin.¹⁰⁶

It has been proposed that a reduction of insulin effectiveness can predispose the animal to peripheral vasoconstriction and thereby laminitis.⁸⁹ However, when given insulin⁹¹ the decline in diastolic, systolic and mean blood pressure values was significantly greater in laminitic ponies than in controls. This result indicates that laminitic animals still respond with vasodilatation when given insulin. Nevertheless, it remains to be investigated if this also holds for the blood vessels in the laminar layer of the hoof.

Moreover, elevation of plasma lactate suppresses glycolysis and thereby affects insulin-stimulated glucose uptake.¹⁰⁷ Research on rodents has shown that high-sucrose and high-fructose diets can decrease insulin sensitivity. However, experiments in humans have produced very conflicting results on insulin sensitivity at higher intakes of fructose or sucrose.¹⁰⁸

When considering the expression of the insulin-independent GLUT1 as the major glucose transport protein in the laminar basal cells^{52,53} it seems reasonable to believe that insulin resistance will not cause a shortage of glucose in these cells. It is still not known if it is a decrease in glucose metabolism that causes laminitis in vivo and if so, what triggers this.

Lactate Metabolism and Laminitis

L-lactate produced by the bacterial flora in the cecum and large colon after a carbohydrate overload and taken up through a damaged intestinal mucosa has been proposed to play a role in the development of alimentary laminitis.^{109,110} However, the rise in blood lactate is relatively mild during the developmental phase of laminitis.¹¹¹ It is much higher when skeletal muscles produce L-lactate during exercise. Lactic acid has also experimentally been injected into digital arteries without causing clinical signs of laminitis¹¹² The results of Wattle and Pollitt⁵³ have also shown that the laminar basal cells are rich in LDH indicating that lactate, in the presence of oxygen, is an important metabolite in this tissue.

Corticosteroids and Laminitis

Corticosteroids and insulin are mutually antagonistic in key areas of energy metabolism and hyperglycemia can be observed following administration of glucocorticoids and prednisone.^{106,113} The inhibition of glucose uptake, protein synthesis, and cell division are possible mechanisms explaining the acute effect of dexamethasone.¹¹⁴

Both prednisone and corticosterone (CT), the rodent

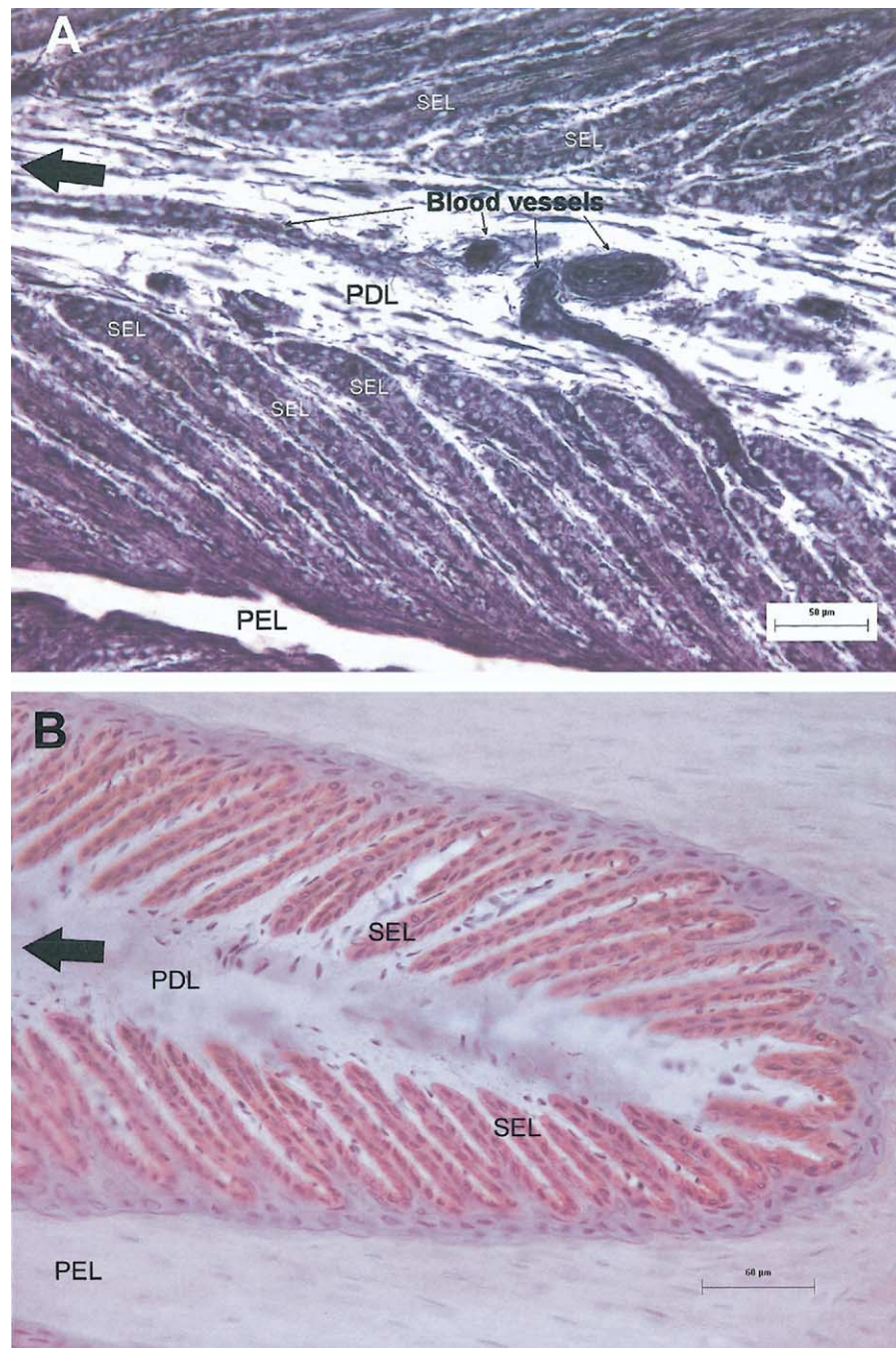


Figure 2 (A and B) Cross section of the lamellar layer at the mid level of the hoof wall. PEL, primary epidermal laminae; SEL, secondary epidermal laminae (small arrows); PDL, primary dermal laminae. (A) Cryostat section stained for LDH with dark blue staining indicating presence of the enzyme. (B) Cryostat section stained with GLUT1 monoclonal antibody and ABC-elite immunohistochemical method, using Mayers hematoxylin counter stain. Brown stain indicates presence of GLUT 1. Big black arrow points toward the inner parts of the hoof.

equivalent of human cortisol, have been shown to depress the release of glucose-induced insulin secretion and affect the glycogen synthesis and glucose oxidation in response to insulin.^{113,114} The mechanism for the decreased glucose uptake in skeletal muscle seems to be a direct inhibition of the translocation of the GLUT4 glucose transporters to the plasma membrane in response to insulin.¹⁰⁶ Treatment of rats with dexamethasone reduced insulin-stimulated increases in GLUT4 in the soleus muscle by about 60%. The rate of lactate formation did not decrease indicating that glucose metabolism was shifted toward glycolysis to maintain a normal rate

of lactate formation. Dexamethasone also seems to decrease the sensitivity of glycogen synthesis and glucose oxidation to insulin.

Aside from the antiinflammatory effect of corticosteroids and its effects on glucose uptake, metabolism and cell growth and differentiation, it also increases the vasoconstrictive effects of epinephrine and serotonin.¹¹⁵ Glucocorticoids are used in treating a variety of diseases but in the horse appear to occasionally precipitate laminitis.¹¹⁶ However, there are no reports of laminitis being induced experimentally with glucocorticoids. Nevertheless, in one study,¹¹⁷ the clinical signs

were enhanced in horses that were subjected to an extended treatment with glucocorticoids before induction of laminitis. Moreover, laminitis together with hirsutism are the most common clinical signs in horses with equine Cushing's Disease.¹¹⁸ Lowering blood ACTH concentration in horses with equine Cushing's Disease has been reported to improve clinical signs of laminitis.¹¹⁸

It is currently recommended not to treat laminitic horses with corticosteroids,¹¹⁹ even though glucocorticoids were regularly used for horses with laminitis, even locally by injection in the digital artery¹²⁰ in the 1960s and 1970s. Theoretically the use of corticosteroids can increase the risk of developing laminitis since: it has effects on glucose metabolism and causes insulin resistance,¹²¹ it influences the differentiation of keratinocytes,¹²² it increases the vasoconstrictive effects of epinephrine and serotonin.¹¹⁵ Reduced digital perfusion and ischemia have been described as the main hypothesis for development of laminitis.¹²³ However, the role of corticosteroids in the development and treatment of laminitis, including its effect on the vascular bed, remains to be investigated.

Ischemia in Combination with Hyperglycemia and Increased Levels of Corticosteroids

The epidermal cells of the laminar layer seem to mainly depend on insulin-independent glucose transport proteins for their supply of energy. The uptake of glucose will increase in the case of hyperglycemia or sepsis. Both insulin resistance^{89,90} and ischemia have been put forward as hypotheses for the development of laminitis.¹²³ Ischemia in combination with hyperglycemia and increased levels of corticosteroids has for decades been an area for extensive research with regards to brain disorders.¹²⁴⁻¹²⁶ This research has mainly been performed on rodents and there are major differences between brain tissue and the epidermal and dermal tissues in the hoof. However, there are points of similarity. Like the brain, the hoof epidermal cells are of ectodermal origin and are rich in the insulin-independent glucose transport proteins GLUT1 and to a certain extent GLUT3. Both the hoof and the brain are well vascularized, they have insulin receptors and express minor amounts of GLUT 4 and seem to have the capacity to use lactate as a substrate. Based on the understanding that preischemic hyperglycemia aggravates the neural damages seen postischemia,¹²⁶⁻¹²⁸ it has been postulated that lactic acid that accumulates in the brain during an ischemic event will contribute to neuronal damage in cerebral ischemia.¹²⁵ However, this concept has been challenged recently¹²⁹ after studies showed that the extent of postischemic neuronal damage correlates with plasma corticosterone (CT), the rodent equivalent of human cortisol level, not glucose level, at the onset of ischemia¹³⁰ and that glucose, when given between 90 and 240 minutes before a hypoxic or ischemic insult, can be neuro-protective both in vitro¹³¹ and in vivo.¹³² Moreover, lactate accumulated during a hypoxic event has been suggested to be the main energy substrate for the brain immediately after reperfusion. In experiments where lactate transport has been blocked experimentally, delayed ischemic neuronal damage is exacerbated.^{34,133} However, the conclu-

sion that the influence of glucose on an ischemic transient is dependent on the duration of hyperglycemia is still claimed to be unsubstantiated.¹³⁴ Regardless of the hypothesis, it has earlier been concluded that there is no role for steroids in the treatment of global brain ischemia.¹³⁵ Use of corticosteroids in cases of brain ischemia has resulted in either no improvement or a worsening of neuronal damages if given before or during ischemia, but not if administered after reperfusion. Likewise, postischemic hyperglycemia has been shown not to worsen the postischemic outcome.¹³⁶⁻¹³⁸

Whether it is the steroid^{130,139} or the lactic acidosis^{140,141} (resulting from the enhanced anaerobic glucose metabolism following the corticosteroid induced hyperglycemia), that is responsible for the aggravation of the ischemic damages, it is yet to be decided. However, it is noteworthy that glucose administration itself can induce a short-lived, significant elevation in plasma levels of CT that peaks at 15 to 30 minutes and returns to baseline levels by 60 to 120 minutes.^{34,137} Nevertheless, it is hard to draw any final conclusion regarding what factors cause aggravation of delayed neuronal damage in preischemic hyperglycemia since such an aggravation cannot be reproduced in vitro. Moreover, elevated glucose levels in vitro can protect against hypoxic damage, but this is an outcome that has seldom been reproduced in vivo.³⁴

Conclusion

Despite extensive research on mammalian cellular and tissue metabolism little has been done to elucidate how the equine hoof wall utilizes its energy substrates to maintain its integrity. Clearly, an effort should be made to clarify the metabolic changes that take place in the living cells of the laminar layer during the developmental phase of laminitis and how these changes are affected by alterations in energy supply.

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