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Born to run; the story of the PEPCK-C^{mus} mouse*

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Abstract

In order to study the role of the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK-C) in skeletal muscle, PEPCK-C^{mus} mice were created by introducing the cDNA for the enzyme, linked to the human α-skeletal actin gene promoter, into their germ line. Two founder lines generated by this procedure were bred together, creating a line of mice that have 9.0 units/g skeletal muscle, as compared to 0.080 units/g in muscle from control animals. The mice were more active than controls in their cages and could run for up to 5 km, at a speed of 20 m/min without stopping (control mice run for 0.2 km at the same speed). Male PEPCK-C^{mus} mice are extremely aggressive, as well as hyperactive. During strenuous exercise, they use fatty acids as a fuel more efficiently than do controls and produce far less lactate than do control animals, perhaps due to the greatly increased number of mitochondria in their skeletal muscle. PEPCK-C^{mus} mice also store up to five-times more triglyceride in their skeletal muscle, but have only marginal amounts of triglyceride in their adipose tissue depots, despite eating 60% more than controls. The concentration of leptin and insulin the blood of 8 to 12 month of PEPCK-C^{mus} mice is far lower than noted in the blood of control animals of the same age. These mice live longer than controls and the females remain reproductively active for as long as 35 months. The possible reasons for the profound alteration in activity and longevity caused the introduction of a simple metabolic enzyme into the skeletal muscle of the mice will be discussed.

> We gotta get out while we’re still young, Cause tramps like us baby, we were born to run.
>
> Bruce Springsteen *Born to run*

Rationale for the generation of the PEPCK-C^{mus} mice

PEPCK-C is a well studied enzyme, whose role in intermediary metabolism is seemingly established; it is present in the liver, kidney cortex and brown and white adipose tissue and is involved in gluconeogenesis and/or glyceroneogenesis [1]. However, a closer look at the biology of this enzyme indicates anomalies that are not explained by our current understanding of its metabolic role. PEPCK-C is present in a wide variety of mammalian tissues that do not make glucose or where glyceroneogenesis has yet to be demonstrated [2]. Over the years, there have been very few studies aimed at delineating the function of the enzyme in these tissues.

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We chose to study the role of PEPCK-C in skeletal muscle, a tissue of importance in total energy metabolism and one in which previous research had suggested a role for the enzyme, but fell short of providing a definitive outcome. These studies focused mainly on the potential role of PEPCK-C in the generation of alanine, which occurs during fasting in skeletal muscle [3]. The rationale for this research was that PEPCK-C removes citric acid cycle anions by decarboxylating oxalacetate to PEP, which could in turn be converted to pyruvate via muscle-type pyruvate kinase and then transaminated to alanine. The other possibility, for which there is scant physiological evidence, is that PEPCK-C is involved in gluconeogenesis or glycogen synthesis in the skeletal muscle. In addition, research in our laboratory on the extent of glyceroneogenesis in several tissues of the rat, using *in vivo* isotope techniques, has demonstrated that glyceroneogenesis, not glycolysis, was the major source of glyceride-glycerol in both the soleus and gastrocnemius (Nye, Hanson and Kalhan unpublished data). This supports an alternative role of PEPCK-C in skeletal muscle, since the synthesis of triglyceride is a major metabolic pathway in this tissue. With this general background, we decided to produce lines of transgenic mice that over-express the gene for PEPCK-C in their skeletal muscle and then to determine the metabolic outcome.

The surprising results of over-expressing PEPCK-C in skeletal muscle

Four founder lines of transgenic mice, which contained a chimeric gene composed of the α-skeletal actin gene promoter, driving the expression of the cDNA for PEPCK-C, linked to the 3′ end of the bovine growth hormone gene (see reference [4] for details of this construct), did not have an overtly different phenotype than littermate controls. This was not surprising, since we expected that the major effect of over-expressing the gene would be subtle and would require detailed biochemical analysis of alterations in the rate of citric acid cycle flux, due to the additional PEPCK-C in the skeletal muscle, and/or of an increased rate of glyceroneogenesis in the tissue. The four founder lines expressed from 1.5 to 3 units of PEPCK-C/g muscle (in comparison, a liver from a fasted mouse has from 2 to 3 units of PEPCK-C/g). We next decided to breed two of these lines together to increase the overall activity of the enzyme in skeletal muscle, in addition to studying the original founder lines. The result of this breeding was a line of animals that we refer to as *PEPCK-C^{mus} mice*. From the earliest time of observation (about 2 weeks of age), we noted that these animals had a remarkable degree of hyperactivity. In fact, this phenotype was so obvious that it allowed us to select the PEPCK-C^{mus} mice without the need to genotype them extensively (i.e. hyperactivity always corresponded to a mouse that contained both transgenes). The PEPCK-C^{mus} mice were 7 to 10 times more active in their home cages and had the ability to run for long distances (5 km at 20 m/min) on a mouse treadmill without stopping. The animals were not as large as control littermates, but ate considerably more. An MRI analysis of the PEPCK-C^{mus} mice demonstrated that they had only marginal levels of adipose tissue and a biochemical analysis of the skeletal muscle indicated high levels of triglyceride in that tissue. In fact, the concentration of triglyceride in the skeletal muscle of the PEPCK-C^{mus} mice was directly proportional to the activity of PEPCK-C in the muscle, suggesting the importance of glyceroneogenesis in the tissue. Skeletal muscle triglyceride is clearly the major fuel driving the hyperactivity in the PEPCK-C^{mus} mice; they have many more mitochondria in their skeletal muscle than found in the muscle of controls, supporting the enhanced rates of fatty acid metabolism noted in the mice.

The PEPCK-C^{mus} mice have extended longevity and reproductive capacity

A second surprising result was the apparent extend longevity of the PEPCK-C^{mus} mice; they lived almost two years longer than the controls and had normal litters of pups at 30 to 35 months of age (most mice stop being reproductively active at 12 to 18 months). We use the word “apparent” because we have not as yet carried out a detailed aging study, involving multiple

mice, which are followed at regular intervals over their life time; this type of study is currently in underway in our laboratory so hopefully we will be able to state unequivocally that the PEPCK-C^{mus} mice do live longer than controls. However, the available evidence is strong enough to warrant some speculation as to why these mice have such an extended life span. This is especially important since the mice violate one of the pillars of aging research, namely that limiting food intake increases longevity. This general principle is supported by studies with species from flies to rats, and seems well grounded in the literature. If correct, the results with the PEPCK-C^{mus} mice imply that it is not the number of calories consumed, but what happens to these calories one they are consumed! The mice eat almost twice as much as control animals but because of their hyperactivity, utilize the excess calories to satisfy their energy demands. *Perhaps the new paradigm in aging research should be that sustained activity extends life span* At this point, both the extended life span and the prolonged reproductive capacity of the PEPCK-C^{mus} mice need detailed study before hard conclusions, rather than speculation, will be forthcoming.

Changes in hormone and cytokines in the blood of PEPCK-C^{mus} mice and the possible metabolic consequences

We have carried out a detail analysis of the levels of hormones and cytokines in the blood of the PEPCK-C^{mus} mice and noted very low levels of insulin, leptin and MCP-1 as compared to control animals (Hakimi, Berger, Tracy and Hanson, unpublished data). These factors all suppress appetite by directly influencing the hypothalamus, so that a markedly reduced circulating concentration of these proteins would explain the higher level of food intake in the PEPCK-C^{mus} mice, as compared to controls. The hyperactivity noted in the mice increases fuel consumption, especially in the skeletal muscle, resulting in dramatically less adipose tissue and thus less leptin. The high level of activity also results in increased insulin sensitivity and the recruitment of GLUT-4 to the cell surface [5] [6]. We thus assume that the very low concentration of insulin in the blood of the PEPCK-C^{mus} mice reflects increased insulin sensitivity of the skeletal muscle, which in turn results in decreased insulin secretion.

A model to explain the metabolic alterations observed in the PEPCK-C^{mus} mice

From the available information on the PEPCK-C^{mus} mice, it is possible to speculate on a potential mechanism to explain this complex phenotype (see Figure 1). This requires several assumptions that need validation, but there is enough evidence in hand to make this proposed mechanism plausible. The first assumption is that the over-expression of PEPCK-C occurs only in skeletal muscle and that it functions as an enzyme and not as a transcription factor; this limits the effect directly to a metabolic perturbation and not to a global alteration in gene transcription such as occurs when PPARδ [7] or PGC-1α [8] were over-expressed in the skeletal muscle. Next, the greatly increased activity of PEPCK-C in the skeletal muscle results in an elevation of glyceroneogenesis and then in the concentration of triglyceride in the tissue. This is reasonable based both on the known role of PEPCK-C in glyceroneogenesis [9] and on our observation of a direct relationship between the activity of PEPCK and the amount of triglyceride in the muscle of several lines of transgenic mice [4]. This triglyceride would provide the major source of the fatty acid used to support muscle contraction during strenuous exercise in the mice.

Another major assumption, for which we have no direct evidence, is that the hyperactivity noted in the PEPCK-C^{mus} mice is directly related to the increased number of mitochondria in skeletal muscle. We do know that the PEPCK-C^{mus} mice are hyperactive as early as 10 days after birth, which suggests that whatever mechanism is responsible, is likely to have been

patterned by the over-expression of PEPCK-C in skeletal muscle during development *in utero*. In this regard, the gene for α-skeletal actin gene is first transcribed at about 14 days of fetal life in the mouse [10], when it replaces cardiac actin as the major form of actin in this tissue. There is thus ample opportunity for elevated PEPCK-C activity to alter mitochondrial biogenesis, either directly by increasing citric acid cycle flux or by stimulating glyceroneogenesis and triglyceride synthesis, thus altering fatty acid metabolism in newborn PEPCK-C^{mus} mice. The fatty acids required to support the observed hyperactivity would be derived from the very high level of fat present in rodent milk, which would be used in triglyceride synthesis via glyceroneogenesis and as a direct source of acetyl CoA for mitochondria oxidation. We do not know if the PEPCK-C^{mus} mice eat more while suckling, but the early onset of hyperactivity observed in nursing pups would support this possibility.

After weaning, the PEPCK-C^{mus} mice eat 60% more than control littermates but weigh almost half as much. They also have a marginal amount of white adipose tissue, suggesting that the hyperactivity channels the calories normally deposited as triglyceride in adipose tissue to support muscle contraction. The diminished adipose tissue would result in a very low concentration of leptin in the blood. This, together with the low level of insulin, explains the increased appetite noted in the mice. The low concentrations of insulin in the blood of the PEPCK-C^{mus} mice could be due to the hyperactivity of the mice, which would be expected to up-regulate GLUT-4 [6], thereby facilitating glucose transport in the absence of secreted insulin. We suspect that the major factor responsible for the longevity of the PEPCK-C^{mus} mice is the very low concentration of insulin in the blood of the mice that is maintained over their lifetime of hyperactivity. The factors responsible for the striking prolongation of reproductive capacity noted in female PEPCK-C^{mus} mice is most likely related to their physical activity, but the mechanism of this effect remains to be studied.

PEPCK-C and energy metabolism

The general schema outlined above does not answer a fundamental question concerning the PEPCK-C^{mus} mice; how does a single enzyme, expressed at exceedingly high levels in skeletal muscle, initiate such a global alteration in energy metabolism? What is initiating event? Our current thinking on this issue focuses on the key role of PEPCK-C in citric acid cycle dynamics. Hans Krebs first described the citric acid cycle in 1937, yet the metabolic role of the cycle continues to intrigue biochemists. Of special interest is the concept of *anaplerosis* and *cataplerosis*, which essentially defines the metabolic role of PEPCK (Note: depending upon the species, both PEPCK-C and/or PEPCK-M may be involved in these processes). *Anaplerosis*, the refilling of the citric acid cycle, is required whenever a cycle intermediate leaves the mitochondria during biosynthetic events. Pyruvate carboxylase is the archetypical anaplerotic enzyme. *Cataplerosis* serves the opposite function by removing citric acid cycle anions that may accumulate. PEPCK-C is the major cataplerotic enzyme, since it removes these intermediates by converting oxalacetate (derived from malate) to PEP. This is an often overlooked, but critical cellular process, since the carbon skeletons of amino acids, virtually all of which enter the citric acid cycle in the liver and other tissues, are not oxidized completely to carbon dioxide in the mitochondria and will accumulate unless they are removed. The metabolism of glutamine by the kidney cortex is an excellent example of both anaplerosis and cataplerosis (Figure 2). Glutamine carbon enters the citric acid cycle as α-ketoglutarate (anaplerosis) and exits as malate, which is then oxidized to OAA and decarboxylated to PEP by PEPCK-C (cataplerosis). The PEP can either be converted to glucose or to pyruvate via pyruvate kinase and then oxidized to carbon dioxide in the citric acid cycle. In animals in which PEPCK-M is present at substantial activity (most mammalian species), OAA from the citric acid cycle can be decarboxylated directly to PEP and transported from the mitochondria [11].

It is well established that several citric acid cycle anions accumulate during strenuous exercise [12], so that an increased activity of PEPCK-C could be an important factor in insuring that the high rates of flux required to support exercise continues by removing these intermediates before they accumulate at too high a level and act as inhibitors of carbon flow into the cycle. The importance of PEPCK-C for citric acid cycle flux has been well demonstrated in studies using mice in which the gene for PEPCK-C has been deleted, either in the whole animal [13] [14] or in the liver [14] [15] [16] [17]. Of course, removing citric acid cycle intermediates as PEP (via PEPCK-C) implies that the PEP itself will be further metabolized. In this regard, both gluconeogenesis and glyceroneogenesis are cataplerotic pathways, since they convert citric acid cycle anions to PEP, which is then used to make either glucose or 3-glycerol phosphate (see reference [18] for a review of this concept). Based on the above discussion, we would predict that a 100-fold increase in PEPCK-C activity in skeletal muscle would dramatically alter citric acid cycle dynamics, allowing a greater flux of intermediates. This may be reflected in an elevated flow of carbon to glyceride-glycerol via glyceroneogenesis or an increased rate of recycling of the carbon skeletons of amino acids through the citric acid cycle in the muscle. The biochemistry of the PEPCK-C^{mus} mice awaits detailed analysis.

Final words

We are often asked if the remarkable physical activity of the PEPCK-C^{mus} mice, their longevity and reproductive vigor has direct application to human performance. Can we introduce the gene for PEPCK-C into human skeletal muscle and see a similar alteration in metabolism and behavior? Performance sports, such as bicycling, clearly would benefit from the type of activity noted in our PEPCK-C^{mus} mice. To be able to use fatty acid as a fuel for long periods of strenuous exercise while generating little lactate, is the metabolism that wins the *Tour de France* [19]! However, there is a down side to all of this. The PEPCK-C^{mus} mice are very aggressive; our world needs *less*, not more aggression. Most importantly, there is currently no way to over express PEPCK-C in all of the skeletal muscles without introducing the gene into the germ line of humans, as we do with the transgenic mice; this is neither ethical nor possible. Alternatively, current techniques for gene therapy do not insure the very high and prolonged expression of PEPCK-C in every skeletal muscle, which is required for this phenotype. One thing is for sure, if *Mother Nature* wanted 9 units/g of PEPCK-C in our skeletal muscle, instead of 0.08 units/g, she would have put it there!

We are also asked “if direct application to improve human physiology is not feasible, of what value are the PEPCK-C^{mus} mice?” Several answers come to mind and others are likely. The hyperactivity, but robust health of the mice, provides an excellent model to study the effect of activity on the etiology of diseases, such as the development of cancer in susceptible lines of mice that have been crossed with the PEPCK-C^{mus} mice. The apparent increase in longevity also provides a model for determining the biochemical and metabolic factors that contribute to aging. If hyperactivity leads to longer life, what are the underlying mechanisms involved? Finally, the biological basis for the extended reproductive capacity of the mice provides a unique model to study this process. All of this reminds one of Shakespeare’s [20] admonition that “Certainties….. are past remedies.”

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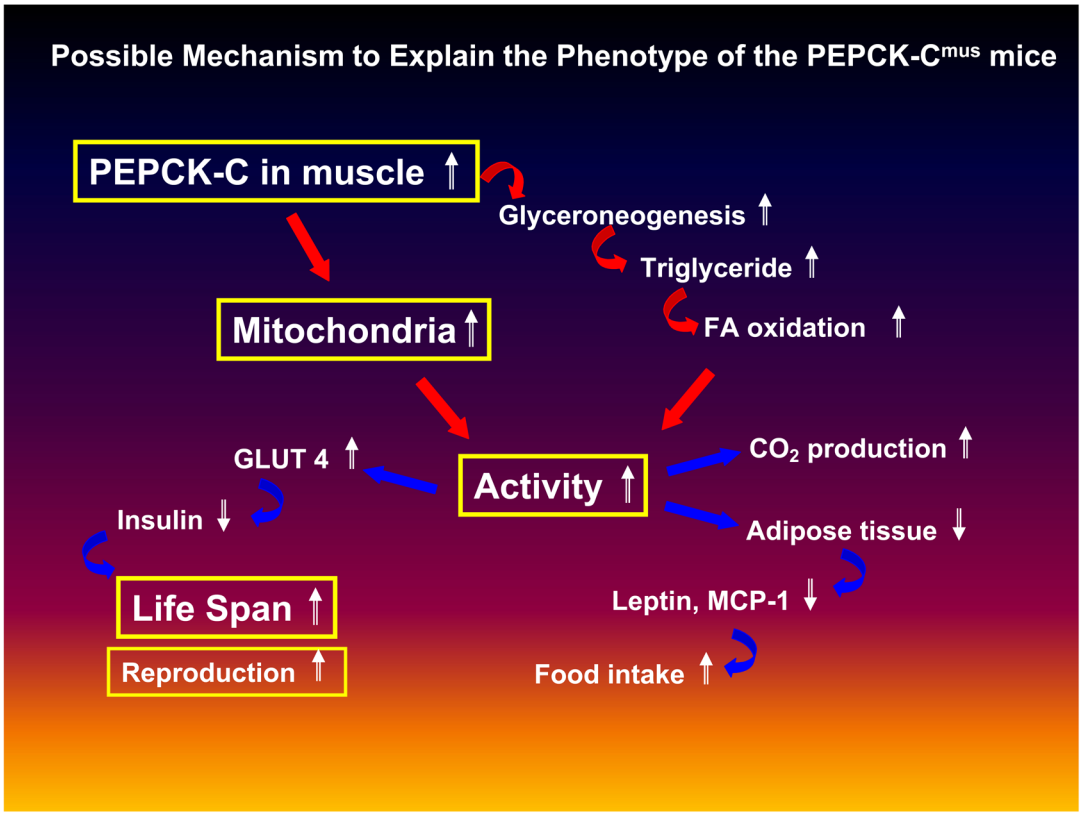

Figure 1. Possible mechanism to explain the phenotype of the PEPCK-C^{mus} mice

The over-expression of PEPCK-C in skeletal muscle leads to a greatly enhanced rate of glyceroneogenesis in the tissue and the accumulation of triglyceride. This results in increased fatty acid oxidation in the tissue and the subsequent stimulation of mitochondrial biogenesis. The increased mitochondria, and the availability of ample fatty acids as a fuel in the muscle, are responsible for the hyperactivity noted in the PEPCK-C^{mus} mice. The hyperactivity results in a marked reduction adipose tissue mass in the animals, which results in a lower level of leptin secretion, increasing food intake in the mice. The hyperactivity increases the insulin sensitivity of the muscle, recruiting GLUT-4 to the cell surface in the absence of insulin. The result is a lowered concentration of insulin over the life span of the mice. The reason for the prolongation of reproductive capacity in female PEPCK-C^{mus} mice is not clear. Finally, an understanding of the cause of the behavioral changes noted with the PEPCK-C^{mus} mice will require investigation

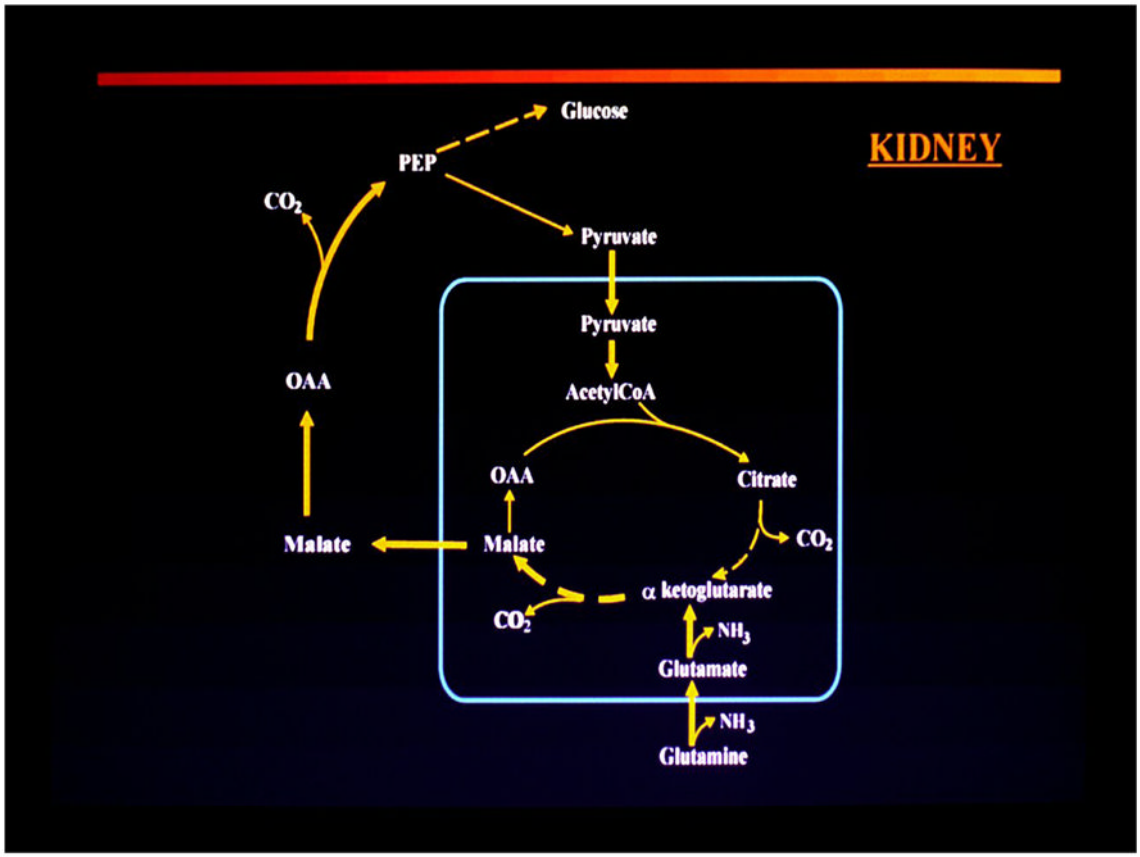

Figure 2. The metabolism on glutamine in the kidney: the role of anaplerosis and cataplerosis in citric acid cycle flux

The conversion of glutamine to glucose in the kidney cortex illustrates the metabolic role of PEPCK-C as a cataplerotic enzyme (see text for details)