REVISTA ESPAÑOLA DE FISIOLOGIA, 35, 13-20. 1979

Amino Acids and Lactate Efflux From *in vitro* Incubated Soleus Muscles of Suckling Rats

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(Received on December 9, 1977)

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The net *in vitro* release of lactate, pyruvate, alanine, glutamate, glutamine and total amino acids from soleus muscles of suckling rats was studied. In all cases, the net output of metabolites was considerably higher in young animals than in 30 day old controls. Glutamine efflux of 1 day old pups was very low, probably due to lack of effectivity of the glutamine synthesizing system. The considerable output of amino acids in the younger animals was partly due to depletion of internal amino acid pools.

The importance of amino acid efflux from muscle has been increasingly acknowledged in the last years (13, 24, 34) as one of the main sources of gluconeogenic substrates in situations of glucose deprivation (22). Striated muscle has been considerably studied *in vitro*, and its ability to release amino acids has been widely assessed under very different conditions, physiological situations and hormonal treatments (14, 16, 21). The gluconeogenic pathway in liver has been found to develop rapidly after birth (27, 28, 34), achieving the definitive adult enzyme pattern between 20 and 30 days of age, when weaning takes effect (7, 11). The changes in the response to fasting in the activities of the glycolytic and citric acid cycle enzymes (4, 5) in the overall metabolic function of the animal.

In this work it has been intented to study the ability of the muscle from suckling rats to release and to retain different gluconeogenic substrates *in vitro*.

Materials and Methods

Albino rats from Eldridge stock were used throughout all experimental work. The animals used as controls (30 days of age) were weaned at 22 days and allowed to eat rat chow and drink tap water *ad libitum*. The younger groups (1, 5, 10 and 20 day old animals) were left with their mothers until sacrifice. The animals were killed by a blow in the back of the head, and both soleus muscles were subsequently exposed, dissected and extracted in less than 85 seconds. The weights of the animals and muscles used can be seen in table I.

Table I. *Weights of suckling rats of 1, 5, 10, 20 and 30 days and weight and protein content of their soleus muscles.*

(days) Age	(N)	Animal w. (g)	Muscle w. (mg)	Protein content (%)
1	71	7.92 ± 0.13	0.81 ± 0.01	7.12 ± 0.34
5	59	12.11 ± 0.27 *	1.61 ± 0.12 *	11.24 ± 0.71 *
10	68	17.72 ± 0.44 *	3.69 ± 0.22 *	11.61 ± 0.34 *
20	65	34.01 ± 0.48 *	10.51 ± 0.20 *	14.35 ± 0.66 *
30	70	87.28 ± 1.62 *	38.48 ± 0.54 *	19.80 ± 0.42 *

Significance between neighbouring groups: * = $p < 0.001$; * = $p > 0.05$.

Only intact muscles were used; damaged ones were discarded. Immediately after extraction, individual muscles were introduced in stoppered tubes and incubated at 37° C, in a gently shaking water bath. The tubes had individual aeration with water-saturated O_2/CO_2 (95:5 %) by means of poly-ethylene catheter tubing fitted with a hypodermic needle stuck through the tube plastic cap. The medium used was essentially the Krebs-Ringer bicarbonate buffer (KRB) (10), pH 7.4, containing additionally 0.05 mM dextran (Sigma, average molecular weight 250,000), 5.56 mM glucose (Fisher) and 0.025 % defatted bovine serum albumin (Sigma). When indicated, the medium was supplemented with a mixture of amino acids in the same proportion as in the rat plasma (26), at a final composite concentration of 10 mM.

Total amino acids were determined using a fluorescamine (Roche Diagnostics) method (30). Lactate, pyruvate, alanine, glutamate and glutamine were determined fluorometrically with enzymatic micromethods (17-19). All coenzymes, enzymes and standards were obtained from Sigma Chemical Co.

The incubations were ended at the exact time by fishing out the muscles with a wire loop, blotting them careflully on filter paper Whatman 1 and freezing them immediately with freon cooled with liquid nitrogen (12). The media were also frozen with dry ice kept at —80° C until the determination of metabolites. After being defrosted they were boiled 2 minutes in order to inactivate possible enzyme contaminants.

Frozen muscles were weighed in an electrobalance in the cold room. They were homogenized using a Potter-Elvejhem all-glass motor-driven homogenizer, in 1-1.5 ml of chilled 10 % Trichloroacetic acid (TCA). Supernatants were directly used for amino acid determination with fluorescamine, as TCA in low amounts do not interfere with this procedure (8). Protein precipitates were used for protein assay (20).

Muscles used for nucleotides determination were powdered frozen under liquid nitrogen, weighed and homogenized in chilled 5 % perchloric acid. Supernatants were neutralized with $KHCO_3$ and the new supernatants were used for enzymatic fluorometric ATP and ADP determinations (19).

Results

Muscle viability. — In order to check the degree of anoxia in the samples, lactate/pyruvate concentration ratios (15, 18, 29, 33) in the media were determined in each sample. The muscles incubated in KRB with or without added amino acids showed lactate/pyruvate concentration ratios between 5.5 and 26.7, with a mean for all groups of 19.4. With the purpose of checking further the metabolic status

of the incubated muscles, ATP and ADP levels measured in the biggest muscles (30 day old animals) immediately after muscle dissection (frozen in liquid nitrogen-chilled freon [12]) and also after one hour incubation in KRB under standard conditions. The resulting ATP/ADP concentration ratios were 6.51 ± 0.78 at zero time and 3.62 ± 1.03 after one hour incubation.

Lactate and pyruvate release. — Lactate and pyruvate efflux from the muscles incubated in KRB, expressed per unit of wet blotted weight are shown in figure 1. The oldest animals showed a steady rise during all the time studied regarding the release of both lactate and pyruvate. The amount of lactate released was greater in the muscles from all the younger groups than in the 30 day old controls. The 1 and 5 day old animals (and to a lesser extent the 10 day old ones) showed release curves considerably flattened after the initial 15 minutes. Nevertheless, after 60 minutes of incubation, the net lactate (and pyruvate) amount released into the medium by 1 day old muscles was about 3 to 5-fold greater than in 30 day old controls.

Amino acids release. — The alanine and total amino acids efflux from the muscles incubated in KRB are shown in figure 2. The alanine release in the 30 day old controls is considerably lower in the other groups. The shape of the release curves is similar to the lactate ones, being flattened the release curves corresponding to 1, 5 and 10 day old animals. The 1 day old group released less alanine than the 5 day old ones.

The pattern of total amino acid release is the same as that for alanine and lactate, but here the 1 day old muscles show a lower release of total amino acids than the 5 and 10 day old groups.

In figure 3 the glutamine efflux curves obtained with muscles incubated in KRB can be seen. The glutamate release shows two different sets of groups, the 10 and 20 day old groups, together with the 30 day old controls behaving in a rather similar way, and the 1 and 5 day old muscles producing a significantly higher glutamate yield than the controls.

Glutamine efflux follows, in a general way, the same flattened curve pattern with regard to the 1 to 10 day old animals; but the 1 day old animals show additionally a net glutamine release (after

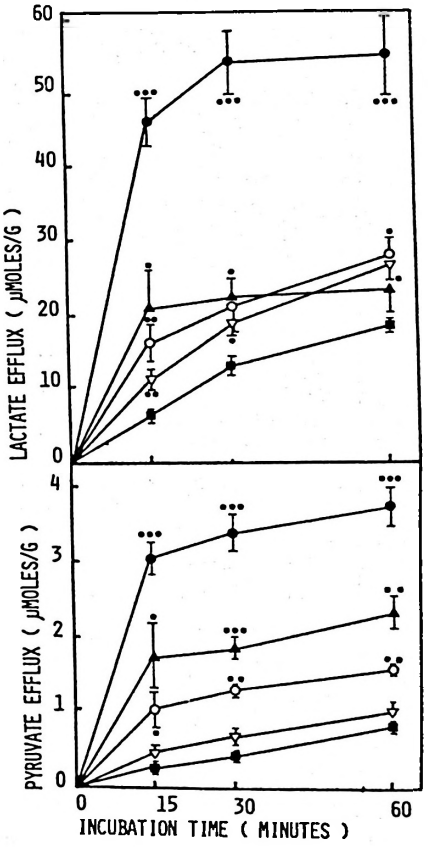

Fig. 1. *Lactate and pyruvate efflux (µmoles/g muscle weight) from soleus muscles of suckling rats incubated in vitro.*
All data are mean ± s.e.m. from 5 muscles: 1 (●), 5 (△), 10 (○), 20 (▽) and 30 (■) old day muscles. Significance versus 30 day old controls: O = $p < 0.05$; OO = $p < 0.01$; OOO = $p < 0.001$.

one hour) significantly lower than in controls.

Amino acid concentration in muscle. — Figure 4 shows the variations in the relative size of the «internal amino acid pool» (2, 3) of the muscle during the incubation, defined as the net content of amino acids, both in the intracellular and in the extracellular spaces; it also includes, probably, amino acids from the medium, stuck on the surface of the whole muscle due to the process used in their handling.

In the absence of amino acids added

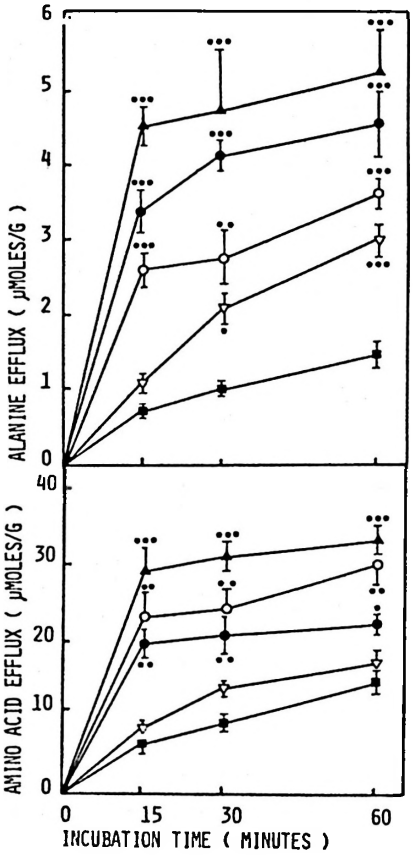

Fig. 2. *Alanine and total amino acid efflux (µmoles/g muscle weight) from soleus muscles of suckling rats incubated* in vitro. (The legend is the same as for figure 1.)

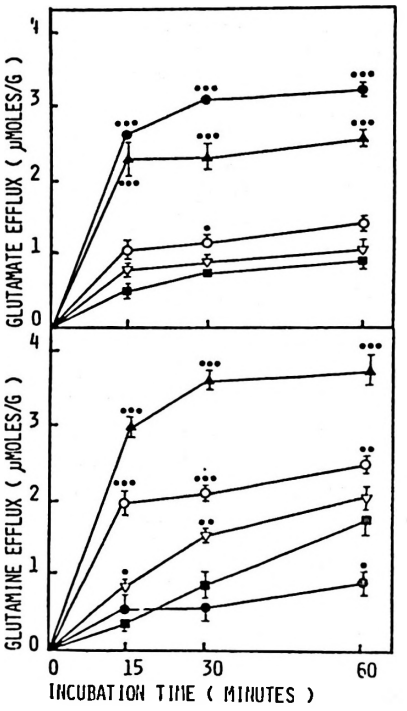

Fig. 3. *Glutamine and glutamate efflux (µmoles/g muscle weight) from soleus muscles of suckling rats incubated* in vitro. (The legend is the same as for figure 1.)

to the medium, the decrease of the amino acid content in muscles of 1 day old animals is less spectacular, and the 5 day old muscles do not change significantly their concentration with incubation. The same can be said of the 10 and 20 day old animals, in which there can be recognized a slight tendency to increase their amino acid content with incubation time. The 30 day old controls keep a steady rise in their amino acids concentration along the incubation time.

Discussion

The lactate/pyruvate concentration ratios for each group of muscles were rather constant with time, but in the 30 day old muscles there was a slight tendency towards the increase of this ratio with in-

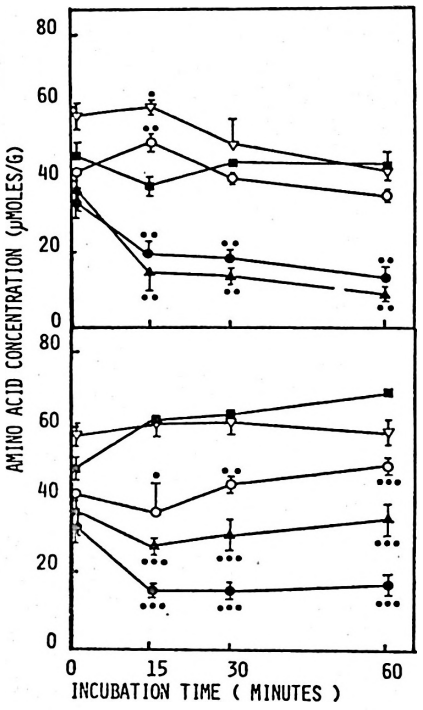

Fig. 4. *Amino acid concentration (μmoles/g muscle weight) during the* in vitro *incubation of soleus muscles from suckling rats.*
In the upper part of the graph, are shown the data obtained in the incubation of the muscles in plain KRB; in the bottom part of the figure, the data correspond to the incubation in KRB plus a 10 mM mixture of amino acids added. (The legend is the same as for figure 1.)

cubation time. Values indicated here for lactate/pyruvate concentration ratios are in general agreement with other data found in the literature (15, 18, 29) or lower (14). This fact, united to the known ability of both lactate and pyruvate to permeate across the cell wall easily (15), makes measurements of medium lactate/pyruvate ratios directly applicable to the muscle. The ATP/ADP ratios found in the oldest animals (the ones that possesed the heaviest muscle mass, apt therefore to develop anoxyc, symptoms) were not too different, after 1 hour incubation, from the initial values found at zero time. The ATP/ADP concentration ratios obtained were comparable to those found in the bibliography (14). All these results indicated that very probably there is no important alteration in the muscle metabolism in the 30 day old muscles as a consequence of the experimental conditions. It is supposed then, that the other groups, with greater muscle mass/muscle surface area ratios, and thence a better aeration, would not be affected by anoxia. This point is further stressed by the fact that their lactate/pyruvate concentration ratios are considerably low and no significantly different from those of the controls.

Lactate can be used as a gluconeogenic substrate by the young pup (32), and gluconeogenesis is known to be considerably enhanced in suckling rats compared with adults, using both lactate and alanine as substrates (31). The release of lactate by the muscle is more marked in the younger animals than in the 30 day old ones, and the pyruvate efflux follows closely this same pattern. This could be the consequence of a transit from the immediate fetal life, in which the lactate production by the animal is considerable (9) to the more independent extrauterine life. The gluconeogenesis from pyruvate is also enhanced in the sukling rat, dropping to the adult levels at 30 days of age (7). The pyruvate release curve follows the same pattern as lactate, being maximal in the younger animals.

Alanine is the main gluconeogenic amino acid substrate released by muscles, as described elsewhere (13, 23). The composite alanine, glutamine and glutamate figures accounted for a mean 40.9 ± 0.7 % of the total amino acid efflux found in all studied groups. This value is in agreement with the data found in the bibliography for adults (25). Thus indicating that the individual composition of the amino acid output of muscle cells does not change significantly with age.

The glutamine release at 1 day of age is minimal as can be seen in figure 3; as glutamine is synthesized by glutamine synthetase from glutamate, when the composite value of glutamate plus glutamine is plotted, the relative increase in glutamate efflux found in the 1 day old muscles is greatly suppressed, giving a composite figure very much similar to that of the other groups studied.

From the data of figure 1, 2 and 3, it is apparent that the efflux of muscle amino acids, lactate, pyruvate and total amino acids show a considerable change during the first month of postnatal life in the suckling rat, but the real possibility of comparison between so many differently sized animals remains to be demonstrated.

The data shown here obout pool changes indicate that there is a considerable depletion of the internal amino acids pool in the younger animals with incubation time. This situation is best seen in the 1 and 5 day old animals, and seems to indicate that they have not yet developed adequate systems for preventing their internal pool amino acids from total depletion. In the older animals, from 10 day old onwards, changes are not too much important after one hour incubation, indicating a better retaining mechanism. This fact, together with the timing of amino acid release, that is maximal in the initial minutes of incubation, suggests that probably most of the amino acids released in the 1 and 5 day old animals come from the depletion of the internal pool of amino acids, instead of from metabolic action. It has also to be taken into account that the possibility of artifactual error in the weighing of the muscles and of carrying attached media amino acids (in spite of the blotting) is higher in the smaller muscles than in the big ones.

In the other animals, the contribution of this internal pool source seems to be minimized, as there are no significant changes in pool size with incubation time. Thus there is a considerable part of the amino acids released from the muscles of the younger animals that must come from their internal pools; in the older animals there must be also a *de novo* production of these amino acids, probably through proteolysis, as the internal pool remains rather constant and there is a net amino acids efflux. The presence of added amino acids in the medium practically do not induce changes in the 5, 10 and 20 day old muscles, but in the oldest ones, it is appreciable a rise in the amino acid content of the muscle. This increase is related with active uptake by the muscle of the amino acids present in the medium, and was ascertained in several cases by adding radioactive phenylalanine to the medium, retrieving 30 day old muscles at different times and counting the radioactivity incorporated into them after NCS digestion.

In the 1 day old muscles, the amino acid net loss is maintained even in the presence of added amino acids to the medium, but showing a lower relative loss.

This lack of ability to retain gluconeogenic substrates in the youngest rat muscles must be correlated with important changes in membrane permeability; nevertheless, the high glutamate and alanine content of the total amino acids released by these muscles is exactly the same as in the older muscles, this it cannot be totally ruled out the possibility that, at least partly, some of these amino acids came from physiological interconversion in the muscle rather than coming totally from the washing out of the internal amino acid pools.

Resumen

Se ha estudiado la liberación de lactato, piruvato, alanina, glutamina, glutamato y aminoácidos totales en músculos soleus aislados de ratas lactantes. En todos los casos la liberación neta de sustratos gluconeogenéticos era considerablemente mayor en los animales más

jóvenes que en los mayores de 30 días. La liberación de glutamina por los músculos de las crías de un día era muy baja, debido probablemente a la relativa falta de funcionalidad del sistema de síntesis de la glutamina. La considerable liberación de aminoácidos en los animales más jóvenes se debe en parte al vaciado de sus acervos internos de aminoácidos.

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