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ONTOGENETIC DEVELOPMENT OF CREATINE-PHOSPHOKINASE IN SKELETAL MUSCLES AND HEART FROM PIGS

By

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CEPICA, S. and P. FOGD JØRGENSEN: *Ontogenetic development of creatine phosphokinase in skeletal muscles and heart from pigs.* Acta vet. scand. 1977, *18*, 143—151. — Creatine phosphokinase (CPK) in striated muscles shows only small changes in activity before birth. After birth and during the first month of extrauterine life the activity increases rapidly. The largest increase is seen in muscles with a glycolytic energy metabolism (m. long. dorsi) and the smallest in muscles with an oxydative energy metabolism (m. flexor dig. ped. sup.). The differences between these groups of muscles are statistically significant. In heart tissue the increase in CPK activity is lower, the levels amounting to 40 to 47 % of those in striated muscles.

Early in fetal life only the BB isoenzyme is found in striated muscles. Synthesis of M subunits of CPK starts between day 76 and 65 before birth and increases rapidly after this time leading to disappearance of the BB isoenzyme 24 days prior to birth and of the MB isoenzyme at birth. In muscles with an oxydative as well as in muscles with a glycolytic metabolism all CPK activity after birth is caused by the MM isoenzyme.

All three isoenzymes are present in heart tissue at the earliest prenatal stage investigated, the pattern being dominated by the BB isoenzyme. During further differentiation the MM isoenzyme increases and the BB isoenzyme decreases. The development is completed during the first month after birth with a final isoenzyme composition of 81 % MM and 19 % MB isoenzyme.

pigs; ontogenesis; creatine phosphokinase; activity; isoenzymes.

Creatine phosphokinase (ATP: creatine phosphotransferase, E.C. 2.7.3.2., CPK) is mainly found in striated muscles, heart and nervous tissue, where it functions as a transferase for

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the important reaction: ADP + creatine phosphate ⇌ ATP + creatine.

Being a dimer, CPK from higher vertebrates exists as three isoenzymes (BB, MB, MM) each composed of two subunits B and M (*Burger et al.* 1963). The BB isoenzyme is characteristic for nervous tissue, while the MM isoenzyme is nearly the only one detected in adult skeletal muscles, but MB and sometimes BB isoenzymes have been found in skeletal muscles of different species (*Rosalki* 1965, *Allard & Cabrol* 1970). In adult heart tissue the MB isoenzyme is present in addition to the MM isoenzyme.

In striated muscles and heart the isoenzyme patterns of adult animals differ from those of the fetuses or immature animals (*Burger et al., Eppenberger et al.* 1964). The purpose of this investigation has consequently been to follow the ontogenetic development of activity and isoenzyme patterns of CPK in striated muscles and heart from pigs.

MATERIAL AND METHODS

The investigation comprised fetuses at seven different stages of prenatal development (at least two fetuses at each stage) and 21 pigs all of Danish Landrace.

Fetuses were obtained from pregnant sows at the slaughterhouse. Immediately after slaughter the fetuses were excised and age-determined according to *De Villiers et al.* (1958). The pigs were killed by exsanguination under thiomebumal anaesthesia. Samples from muscles and heart were removed, frozen and stored in liquid nitrogen until analyses were performed.

CPK activity was estimated after homogenization of tissues (0.2—0.4 g) in a Potter Elvehjem homogenizer with a buffer consisting of 0.05 mol/l triethanolamine, 0.05 mol/l magnesium sulphate, 2 mmol/l ethylene diamine tetraacetate and 1 mmol/l succinate, pH 7.4. Following centrifugation (18000 × g, 20 min., 4°C) the supernatant was diluted to 10 ml with the same buffer, 250 µl of this dilution was further diluted to 10 ml with 0.05 mol/l triethanolamine (pH 7.4), and CPK was determined spectrophotometrically as described by *Jørgensen* (1974). The analyses were performed at 25°C and the results expressed in international units per g of wet tissue (u/g).

Tissue extracts for CPK isoenzyme determinations were prepared by homogenizing fetal tissues in a five-fold volume and

tissues from older animals in a ten-fold volume of 0.1 mol/l tris buffer, pH 7.4, in a Potter Elvehjem homogenizer. The extracts were then centrifuged twice (18000 × g, 20 min., 4°C).

CPK isoenzymes were separated by agarose gel electrophoresis as described by *Hyldgaard-Jensen* (1971) with the following modifications. The gel buffer consisted of 0.035 mol/l veronal, 0.079 mol/l tris, 0.01 mol/l citric acid, pH 8.5, and duration of electrophoresis was reduced to 35 min.

After electrophoresis CPK isoenzymes were activated by reduced glutathione (5 mmol/l) soaked into filter paper and superimposed for 3 min. on the top of the gels (*Madsen* 1972). The gels were carefully dried by another piece of filter paper before staining.

CPK isoenzymes were stained by a method similar to that described by *Van der Veen & Willebrands* (1966). The staining mixture consisted of 6.2 μmol nicotinamide adenine dinucleotide phosphate, 143 μmol adenosine monophosphate, 18 μmol adenosine diphosphate, 100 μmol creatine phosphate, 120 μmol magnesium acetate, 66 μmol glucose, 0.8 μmol phenazine methosulphate, 6.6 μmol nitro blue tetrazolium, 15 μl hexokinase (2 mg/ml) and 15 μl glucose-6-phosphate dehydrogenase (1 mg/ml) dissolved in 6 ml 0.05 mol/l triethanolamine, pH 6.9. This mixture was added to 12 ml of 1 % agarose prepared in 0.05 mol/l triethanolamine, pH 6.9 and previously cooled to 45°C. The separation gel was overlayered by freshly prepared staining gel and incubated for 30 min. at 37°C. After fixation, washing with destilled water, and drying (*Hyldgaard-Jensen* 1971), percentages of M subunits were calculated from electropherograms on the basis of a dimeric structure of CPK.

RESULTS

Ontogenetic development of CPK activity was investigated in m. long. dorsi, m. gastrocnemius, m. soleus, m. flexor dig. ped. sup. and heart tissue. As no significant differences were found between m. long. dorsi, m. gastrocnemius, and m. soleus, only m. long. dorsi, m. flexor dig. ped. sup. and heart are represented in Table 1.

Before birth only small changes in activity occur. During the first month after birth the activity increases rapidly in m. long. dorsi while a smaller increase is found in m. flexor dig. ped. sup. leading to lower adult levels in this muscle.

Table 1. Developmental changes of creatine phosphokinase activity in pig heart and skeletal muscles. Means ± s.

Age (days)	Number of animals	Heart (u/g)	M. flexor dig. ped. sup. (u/g)	M. long. dorsi (u/g)
—83	4	29±9	—	—
—65	3	33±1	—	29±2
—24	4	34±5	—	80±4
—10	3	74±15	—	115±25
Birth	3	180±45	220±27	192±23
6	2	333±82	595±118	623±67
13	2	443±49	1047±33	1285±52*
31	2	578±26	1321±26	1807±110*
42	3	445±38	1434±158	1629±133
Adult[a]	9	660±110	1390±120	1660±290*

* Difference between m. flexor dig. ped. sup. and m. long. dorsi at the same age is signicifant ($P < 0.05$).

[a] *Jørgensen* (1975).

Contrary to striated muscles the increase in CPK activity in heart tissue after birth is slow, leading to adult values constituting 40 to 47 % of the levels in striated muscles.

CPK isoenzyme patterns were investigated in m. long. dorsi from day 76 before birth, in m. gastrocnemius and in m. flexor dig. ped. sup. from day 24 before birth and in m. soleus from birth.

Table 2. Developmental changes in percentage of M subunits of creatine phosphokinase in pig heart and skeletal muscles.

Age (days)	Heart (% M)	M. flexor dig. ped. sup. (% M)	M. long. dorsi (% M)
—83	43	—	—
—76	50	—	0
—65	58	—	54
—58	55	—	50
—51	61	—	62
—24	74	94	95
—10	82	95	97
Birth	78	100	100
6	82	100	100
13	86	100	100
31	91	100	100
42	89	100	100
Adult	90	100	100

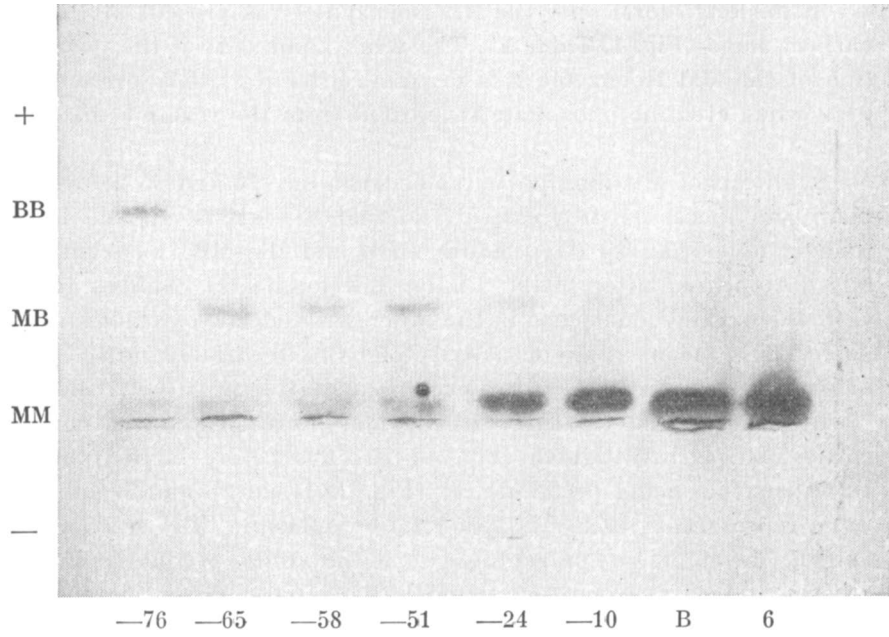

Figure 1. CPK isoenzymes in m. long. dorsi during ontogeny. Age of fetuses and animals in days, B = birth.

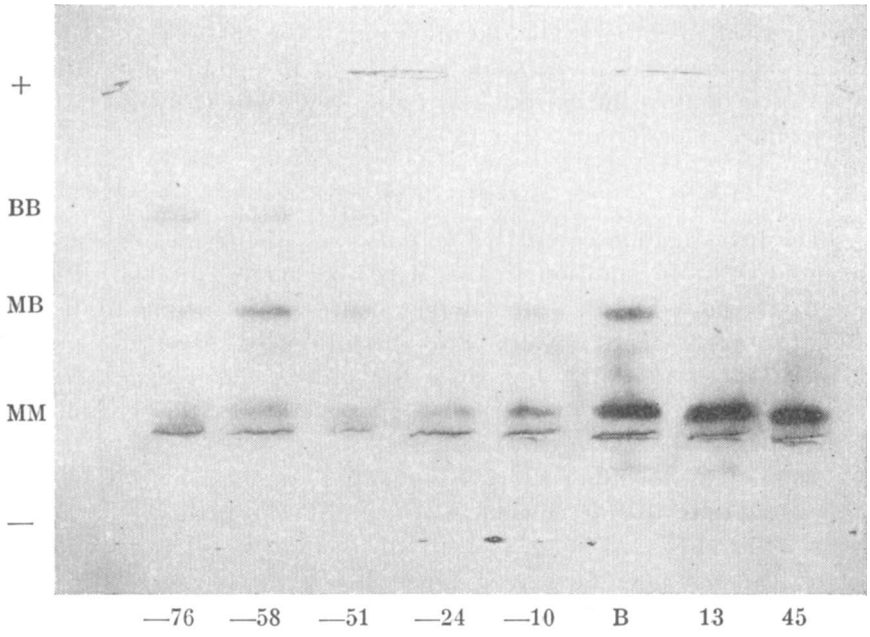

Figure 2. CPK isoenzymes in heart during ontogeny. Age of fetuses and animals in days, B = birth.

In m. long. dorsi only the BB isoenzyme was present at the earliest stage (Fig. 1, Table 2). The weak band seen at the position of the MM isoenzyme is a myokinase band as it is present even when creatine phosphate is omitted from the reaction mixture.

Synthesis of M subunits begins between day 76 and 65 before birth and increases progressively so that the BB isoenzyme is undetectable from 24 days before birth and the MB isoenzyme has disappeared after birth. Similar developmental changes in CPK isoenzymes took place in the other striated muscles investigated. This means that the activity of CPK in striated muscles of pigs after birth is merely represented by the MM isoenzyme.

In heart tissue all three isoenzymes were present at the earliest stages investigated (83 and 76 days before birth), the BB isoenzyme being predominant (Fig. 2, Table 2) and B subunits representing 50 to 57 % of total cytoplasmic CPK activity. During development percentage of M subunits gradually increases and adult isoenzyme composition is reached at the end of the first month after birth. As a result of these changes the staining intensity of the BB isoenzyme gradually decreases and after birth it is hardly detectable. The BB isoenzyme was detectable in adult heart as a trace only if a sufficient amount of CPK was applied to the gel. On the other hand the intensity of MM isoenzyme increases and accounts for 81 % of cytoplasmic CPK activity in mature heart, the rest being represented by the MB isoenzyme.

DISCUSSION

The investigation comprised activity and isoenzymes of cytoplasmic CPK. In addition to the M-B isoenzymes another CPK isoenzyme bound to the mitochondria is present in cardiac tissue. This form represents about 6 % of the total CPK activity in pig heart (*Vial et al.* 1972), but since the mitochondria have been removed during the preparation of tissue extracts this enzyme is not detected in the present material.

In pigs like in other species sequential changes in CPK isoenzymes occur during muscle maturation (*Eppenberger et al.* 1964, *Ziter* 1974). At an early fetal stage only the BB isoenzyme is present, but later on a very rapid shift towards the MM isoenzyme occurs. The time of such pronounced changes, however, differs in different species. In guinea-pigs the shift takes place

from the 27th to the 40th day of intrauterine life (*Prochazka & Wachsmuth* 1972) i.e. at approximately the same prenatal stage of development as in pigs. Contrary to this the changes appear relatively earlier in human muscles (*Goto et al.* 1969), while in rats the isoenzyme shift occurs from the 10th day before until the 10th day after birth (*Ziter*).

According to in vitro experiments performed on rat muscle cultures the BB isoenzyme is present in myoblasts, while the specific muscle form, the MM isoenzyme, appears in myotubes. The isoenzyme shift from BB to MM parallels the fusion of cells into multinucleated, cross-striated, contractile muscle fibers (*Delain et al.* 1973).

More or less the same time course and the same direction of developmental changes were observed in muscles with a glycolytic and muscles with an oxydative energy metabolism. After birth exclusively the MM isoenzyme was found in both groups of muscles, in contrast to human skeletal muscles where MB and incidentally the BB isoenzymes are detectable in adults (*Rosalki* 1965, *Smith* 1972, *Goto* 1974).

The only difference observed between the above-mentioned groups of muscles was that m. flexor dig. ped. sup. in adult animals reached significantly lower levels of CPK than the other muscles investigated. This difference appeared two weeks after birth. Similar differences between oxydative ("red") and glycolytic ("white") muscles are also found in mature rats (*Ziter*).

The ontogenetic development of CPK isoenzyme patterns in pig heart differs considerably from that in skeletal muscles. A more complete isoenzyme pattern is present early in fetal life. The isoenzyme shift is much slower and is finished later after birth when the MB isoenzyme accounts for about 19 % of the total cytoplasmic CPK activity. These findings are in agreement with a 15—20 % relative amount of the MB isoenzyme in adult rat heart (*Ziter*), whereas the MB isoenzyme from human adult heart represents 34 % of total CPK activity (*Goto*) and only 5 % or less are found in the heart of rabbits and mice (*Hall & De Luca* 1975).

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SAMMENDRAG

Ontogenetisk udvikling af kreatinfosfokinase i tværstribede muskler og hjerte hos svin.

CPK aktiviteten i tværstribede muskler hos svin er kun underkastet små ændringer før fødslen. Ved overgangen til det ekstrauterine liv indtræder en stigning, der fortsætter i den første måned efter fødslen. Stigningen er størst i muskler med et glykolytisk stofskifte og mindst i muskler med et oxydativt stofskifte. Dette medfører et signifikant lavere indhold af CPK i oxydative muskler. I hjertevæv er stigningen i CPK aktiviteten mindre end i tværstribede muskler resulterende i et indhold af CPK på 40 til 47 % af indholdet i tværstribede muskler.

I de tidligste undersøgte fosterstadier findes kun BB isoenzymet i tværstribede muskler. Syntesen af M subunits begynder mellem 76 og 65 dage før fødslen og stiger markant herefter, således at BB isoenzymet ikke er til stede 24 dage før fødslen, og MB isoenzymet forsvinder ved fødslen. Dette medfører, at CPK aktiviteten i såvel oxydative som glykolytiske muskler efter fødslen udelukkende udgøres af MM isoenzymet.

Alle tre isoenzymer er til stede i hjertevæv i det tidligste undersøgte stadium med BB isoenzymet som det dominerende. Under den videre udvikling stiger indholdet af MM isoenzymet, samtidig med at BB isoenzymet falder. I løbet af den første måned efter fødslen afsluttes isoenzymændringerne, således at den endelige isoenzymsammensætning af hjertevæv udgøres af 81 % MM og 19 % MB.

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