

## Proteolytic enzyme activity in rat hindlimb muscles in fetus and during post-natal development

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**ABSTRACT** The enzymatic activity of two lysosomal enzymes, acid phosphatase and cathepsin D, was determined in fetus and during post-natal development of the rat gastrocnemius muscle in comparison to the histological differentiation of this muscle. The specific activity of cathepsin D and acid phosphatase was 7 and 2.5 fold higher in the muscle during development until 20 days after birth, than that of mature muscle, respectively. A trend of gradual decrease in the activity of these enzymes was observed concomitantly with the differentiation and maturation of the muscle from mononucleated cells in the fetus to myotubes formation at day 1 after birth, followed by the formation of "young" and then striated myofibers in 10- and 20-day old neonates, respectively. However, no correlation could be found between the lysosomal enzyme activity and the developmental stages of the muscle until 20 days after birth. It is suggested that the elevated activity of lysosomal acid hydrolases may be associated with late developmental processes from young to mature myofibers in normal skeletal muscle and not only in various pathological conditions.

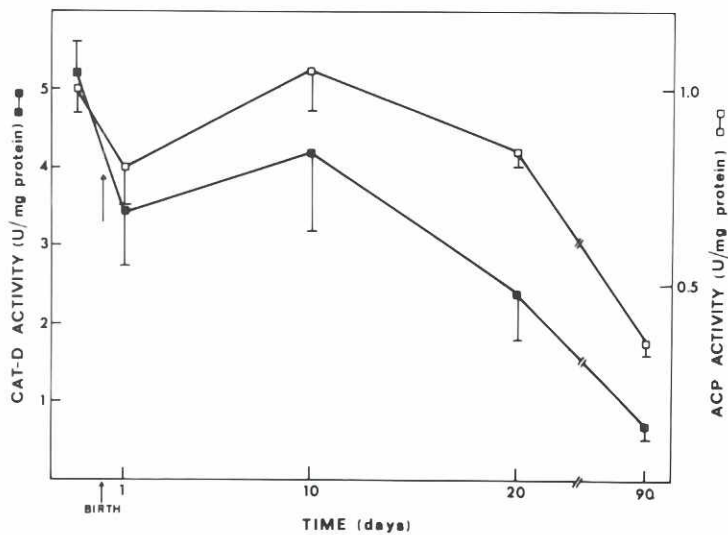
**KEY WORDS:** skeletal muscle, proteolytic enzymes, rat, development

Skeletal muscles undergo dramatic morphological, physiological and biochemical changes during embryonic development and post-natal growth (Obinata *et al.*, 1981; Rubinstein and Kelly, 1981). The lysosomal apparatus of muscle cells in the adult and its activation in pathological conditions have been well documented (Bird, 1975; Bird *et al.*, 1978; Libelius *et al.*, 1979; Lee *et al.*, 1984; Tagerud and Libelius, 1984). However, the possible role of proteolytic enzyme activity during development of skeletal muscles has been studied to a much lesser extent. Bird *et al.* (1981) have demonstrated that the specific activity of cathepsin D was higher in an order of magnitude in non-fusing cells than in post-fusing cells in chick cultured muscles and rat myogenic cell line L6 *in vitro*. They proposed that the lysosomal apparatus and its complement of enzymes may play a significant role in differentiation of muscle myotubes. Weinstock and Iodice (1969) compared the proteolytic enzyme activities in normal and dystrophic chicken breast muscle during development. It was found that while the activity of cathepsin D was elevated during 2 to 8 weeks post hatching, there was only a slight decrease in the activity of this enzyme during that period in the normal muscle.

The aim of the present study was to follow the enzymatic activity of some lysosomal enzymes in the rat hindlimb muscles in fetus and during post-natal development and to compare these findings with the morphological changes of these muscles during development.

The kinetics of cathepsin D and acid phosphatase during development is presented in Fig. 1. In both enzymes there was no significant difference in the specific activity in the gastrocnemius muscle during post-natal development from 1 day up to 20 days. However, the specific activity of cathepsin D in the muscles of the 19-day old fetus tends to be higher ( $P < 0.1$ ) if compared to the pooled results of activities until 20 days after birth. A significant decrease in the activity of cathepsin D ( $P < 0.05$ ) and acid phosphatase ( $P < 0.01$ ) was observed between the muscles of the 20-day old rat and the muscle of the adult (90-day old) rat. In adult gastrocnemius muscle the activity comprised only 15% of the activity found in this muscle at stages up to 10 days after birth. However, there is less of a reduction in acid phosphatase activity, and in the adult muscle the activity comprises approximately 40% of the activity during development and early post-natal period.

The sequential histological changes during the gastrocnemius muscle development is presented in Fig. 2. In the fetus only mononucleated cells (presumptive myoblasts and fibroblasts) were noticed (Fig. 2a) while in the 1-day old neonate fusing myoblasts and oriented myotubes were intermingled between the mononucleated cells (Fig. 2b). Ten and 20 days after birth a gradual maturation of the muscle was noticed. In the 10-day old neonate only "young" muscle fibers were visible, characterized by small fiber diameter and central and peripheral nuclei (Fig. 2c). In the 20-day old neonate



**Fig. 1. Specific activity of cathepsin D (CAT-D) and acid phosphatase (ACP) in the gastrocnemius muscle during embryonic and post-natal development.** Each point is mean  $\pm$  SEM of 3-5 samples. In each sample the muscles were collected and pooled together from 20-40 embryos or neonates. In the adult the activity is the mean of 5 individuals.

these fibers are more mature with well organized contractile proteins but still smaller in diameter than mature muscle fibers and containing relatively more central and peripheral nuclei (Fig. 2d).

The results of the present study clearly demonstrate a higher (2.5- to 7-fold) activity of lysosomal enzymes during muscle development and maturation than in mature muscle. These changes most probably reflect true alterations in the activity of those enzymes in the muscular tissue rather than in non-muscle cells (macrophages, granulocytes, etc.) that may be confined within the muscle. Bird *et al.* (1981) have shown positive histochemical staining for lysosomal enzymes and activity of several catheptic enzymes in homogeneous populations of cultured muscle cells. In addition, it was recently shown (Maltz and Oron, 1990) that during skeletal muscle regeneration the activity of cathepsin D did not correlate with the presence of high concentrations of granulocytes in the regenerated area one day post injury (Roth and Oron, 1985), indicating that granulocytes did not contribute to lysosomal activity within the muscle.

During early developmental stages of the gastrocnemius muscle no correlation could be made between the process of embryonic myogenesis as revealed from histological evaluation and the levels of lysosomal enzymes. For example, fusion of myoblast to myotubes took place during the first day after birth and yet the level of lysosomal enzyme activity in the gastrocnemius muscle was similar in fetus and one day after birth. Therefore, the possibility that these enzymes may play a significant role in the process of fusion of myoblasts to myotubes *in vivo* seems unlikely. During skeletal muscle regeneration after partial excision to the gastrocnemius muscle, similar results were obtained (Maltz and Oron, 1990). Fusion of myoblast to myotubes takes place during the first week post-injury (Roth and Oron, 1985) and yet no alteration in the level of activity of cathepsin D was detected. However, in cultured muscle cells the activity of cathepsin D was higher in non-fused myoblasts than their activity post-fusion, and both in an order of magnitude higher than in an adult (Bird *et al.*, 1981). Thus, it may be postulated that the specific role of these enzymes during muscle development *in vitro* may be different from the situation *in vivo*. The further maturation of the gastrocnemius muscle from young myofibers at 20 days after birth to adult fibers was associated with a significant decrease of

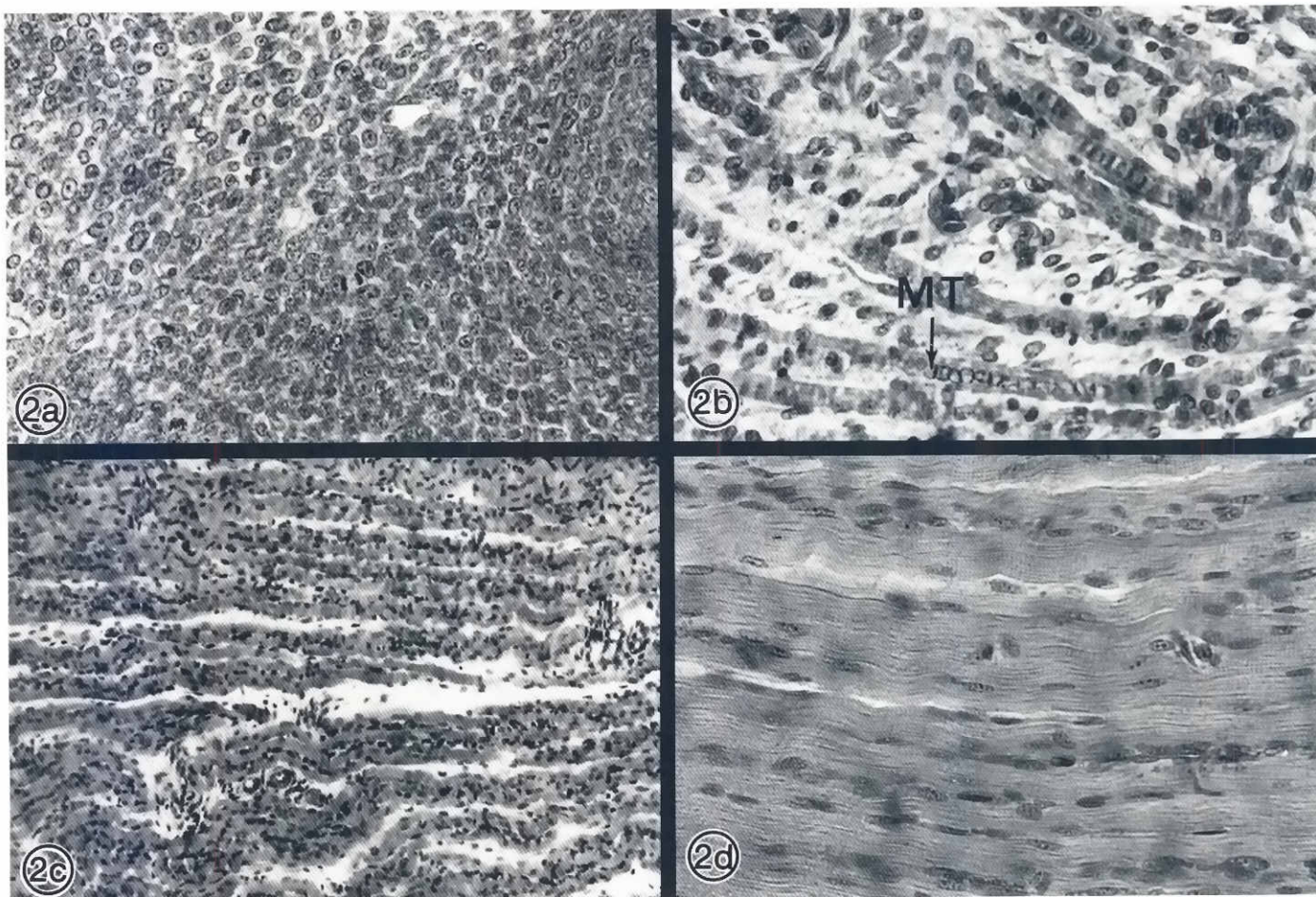
both cathepsin D and acid phosphatase. During skeletal muscle regeneration of the gastrocnemius muscle, higher levels of cathepsin D than control were detected during the first two weeks post injury when young myofibers were formed in the injured zone, and thereafter a decrease was noticed (Maltz and Oron, 1990). A gradual decrease in cathepsins A and D was found also in chicken with time after hatching (Weinstock and Iodice, 1969). The association of elevated activity of lysosomal enzymes with catabolic processes in skeletal muscle is not confined only to muscle development and maturation. In chicken muscle it was found that the proteolytic enzyme activity was enhanced by 40%-90% during stretching for 7 days which resulted also in a 50% increase in muscle weight (Lee *et al.*, 1984).

The results of the present study (and others) clearly demonstrate that the association of high levels of lysosomal enzyme activity with metabolic equilibria between synthesis and degradation of muscle proteins during normal skeletal muscle embryonic and post-natal development is complex. Indeed, it was found that protein turnover rates during this period are different for various muscles (Lewis *et al.*, 1984). In addition, it was shown that there is no general trend in the levels of various cathepsins and acid hydrolases during development of the skeletal muscle (Bird *et al.*, 1981), regeneration (Maltz and Oron, 1990) and pathological conditions like denervation atrophy (Tagerud and Libelius, 1984).

In conclusion, the present study suggests the association of high levels of lysosomal enzymes with muscle maturation during development. The functional mechanisms involved in this phenomenon need to be further elucidated.

### Experimental Procedures

Gastrocnemius muscles were collected from fetus (19 days gestation) and 1-, 10- and 20-day old neonatal and adult (90 days old) Wistar rats under chloroform anesthesia. In early developmental stages (fetus and 1-day old neonates), it was difficult to isolate the gastrocnemius muscle and therefore muscles attached to the Achilles tendon were collected. About 500 mg of tissue was collected from 20-40 fetuses and neonates for each sample that served for enzymatic analysis. The tissue was briefly washed with saline, minced and rinsed in ice-cold 0.01 M phosphate buffer, pH 7.2 to give a 30% (W/V) homogenate. The homogenization was performed using a teflon-glass



**Fig. 2.** Light micrographs of gastrocnemius muscle of a fetus and 1-, 10- and 20-day old neonates. Note mononucleated cells in the fetus (a) and many myotubes (MT) between the mononucleated cells in the 1-day old neonate (b). In the 10-day old neonate (c) young myofibers with many central and peripheral nuclei are noticeable, while in the 20-day old neonate (d) striation is evident in the myofibers with a larger diameter. All figures are  $\times 350$ . H & E stain.

Potter Elvehjem homogenizer revolving at 430 rpm. The homogenate was then centrifuged at 6900 g for 10 min and the supernatant served for analysis.

Cathepsin D activity was determined according to Barrett and Heath (1977) with 2% hemoglobin as substrate. The incubation mixture contained 0.2–0.3 ml muscle homogenate (2–4 mg protein), 0.25 ml of 0.5 M sodium acetate buffer, pH 3.8 and the substrate. The incubation was carried out for 60 min at 37°C and stopped by the addition of 5 ml cold 3% trichloroacetic acid (TCA). Proper blanks were incubated without homogenate which was added momentarily before the introduction of the TCA. After centrifugation (6900 g for 10 min at 4°C), the absorbance of the supernatant was measured at 280 nm. The results were expressed as the difference between test and blank samples for each homogenate. One unit of cathepsin D was defined as the amount of enzyme producing a change of 1 O.D. per minute.

Acid phosphatase was determined using the p-nitrophenol phosphate method. Ten to 20  $\mu$ l of the homogenate was incubated at 37°C for 10 min in 2.5 mM sodium acetate buffer, pH 5.0 and 0.5 mM p-nitrophenol phosphate. The reaction was stopped by adding 0.2 ml NaOH 5N. One unit of the enzyme was defined as the amount of enzyme that liberates 1  $\mu$ mol of p-nitrophenol phosphate per hour.

Protein was measured according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

For light microscopy three rats per developmental stage were lightly anesthetized with chloroform, the muscles were dissected out and fixed in Bouin's fixative for three days. After dehydration in alcohol and embedding in paraffin, 8  $\mu$ m longitudinal sections were prepared. The sections were stained with H & E and photographed with a Zeiss microscope.

The data were statistically analyzed using the single classification analysis of variance and the Student-Newman-Keuls test (SNK test) for multiple comparisons among means (Sokal and Rohlf, 1969).

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