[Chem. Pharm. Bull. 36(10)4182—4186(1988)]

Age- and Exercise-Related Changes in the Activities of Thiol Proteinases in Rat Skeletal Muscle

TADAHIRO SHIKIMI*,[a] and SUMIO MASUMURA[b]

Department of Pharmacology [a] and Department of Physiology,[b] Shimane Medical University, Enya, Izumo, Shimane 693, Japan

(Received February 12, 1988)

The activities of thiol proteinases and their endogenous inhibitors in musculus rectus femoris were studied in control and exercised rats of various ages. Cathepsins B and H, both lysosomal thiol proteinases, showed different age-related changes in activity. Two forms of calcium-activated neutral protease (CANP), μCANP and mCANP, differ from the lysosomal thiol proteinases with regard to age-related changes in activity. The activities of endogenous inhibitors of CANP, cathepsins B and H are apparently not affected by age. Among CANP, cathepsins B and H, only the activity of CANP (the sum of μCANP and mCANP activities) in the 16-week-old rats was reduced after training. Exercise had no effect on the activities of endogenous inhibitors in the 16-week- and 98-week-old animals. The relative percentage of the activity of μCANP to the activities of μCANP plus mCANP did not change regardless of differences of age or exercise. These findings suggest that factors other than endogenous inhibitors may be involved in the age- and exercise-related changes in the activities of these thiol proteinases, and also that there is a close relationship between μCANP and mCANP in skeletal muscle.

Keywords——thiol proteinase; endogenous thiol proteinase inhibitor; ageing; exercise; skeletal muscle; calcium-activated neutral protease; cathepsin B; cathepsin H

Thiol proteinases such as calcium-activated neutral protease (CANP) and cathepsins B and H can degrade myofibrillar proteins *in vitro*: CANP degrades tropomyosin, troponin, myosin heavy chain and α-actinin.[1] Cathepsin B destroys myosin heavy chain, troponin and tropomyosin,[2] while cathepsin H degrades troponin T.[3] Immunocytochemical evidence showed that CANP is localized in the Z-bands of myofibrils.[4] In dystrophic or atrophic skeletal muscles, there are increased activities of CANP[5] or of cathepsins B and H.[6] Hence, these thiol proteinases may participate in the myofibrillar protein turnover in muscle tissue. An attempt to treat muscular dystrophy with a compound which inhibits thiol proteinases, has also been reported.[7] Since ageing is one cause of muscular atrophy, it is of interest to investigate whether there are age-related changes in the activities of these thiol proteinases in skeletal muscle. In the present study, the activities of CANP, cathepsins B and H and their endogenous inhibitors in skeletal muscle were studied in control and exercised rats of various ages.

Experimental

Exercise Experiment——Female Wistar rats (Jcl: Wistar), 4 or 86 weeks of age, were housed in two groups and maintained on laboratory chow (Orient Yeast Co., NF., Japan) and tap water *ad libitum*. One of these groups served as the control (sedentary rats) and the other group was forced to run for 1 h/d, 6 d per week on a treadmill with a slope of 10°. In the initial exercise, the young animals ran from 10—25 m/min and the aged animals ran from 5—20 m/min. After 4 weeks, the same rats were made to run at 25 m/min (young ones) and at 20 m/min (old ones). The entire experiment lasted 12 weeks. These animals, then 16 or 98 weeks of age, were decapitated 24 h after the last run. The skeletal muscle in the lower limb (musculus rectus femoris; MRF) was promptly removed, washed with chilled

physiological saline and blotted to remove the adherent fluid. After excluding the fasciae, the isolated muscle was frozen in liquid nitrogen and stored at −70°C until assay.

CANP and CANP Inhibitor——The preparation and assay of CANP were performed according to Ishiura *et al.*[8] For the determination of μCANP activity, the calcium concentration was lowered to 0.2 mM. mCANP activity was calculated by subtracting the activity found with 0.2 mM $CaCl_2$ from that found with 5 mM $CaCl_2$. One unit of CANP was the amount of the enzyme that catalyzes an increase of 1.0 absorbance unit at 280 nm in 1 h, as defined by Ishiura *et al.*[8] CANP inhibitor was extracted by the method of Takahashi-Nakamura *et al.*[9] The muscle tissue was homogenized with 3 volumes of 20 mM sodium bicarbonate–5 mM ethylenediaminetetraacetic acid (EDTA) solution. After centrifugation of the homogenate at 10000 *g* for 20 min, the supernate was heated at 90 °C for 10 min and cooled. This fraction was re-centrifuged at 10000 × *g* for 20 min to remove the precipitate. The supernate thus obtained was used for the assay of CANP inhibitor. Assay for CANP inhibitor was performed by a similar procedure except that the inhibitor preparation was incubated with 0.3—0.5 unit of partially purified CANP; 20—45% ammonium sulfate fraction prepared by the method of Tsuji and Imahori[10] from rat MRF, for 5 min at 30°C prior to the addition of casein and calcium. One unit of CANP inhibitor was defined as the amount of inhibitor which completely inhibits one unit of CANP.

Cathepsins B and H and Their Inhibitors——For estimation of the cathepsin activity, the muscle tissue was homogenized with 4 volumes of distilled water containing 0.1 mM EDTA. The homogenate was centrifuged at 12000 × *g* for 20 min at 4°C, and the supernate was used as an enzyme sample for the cathepsin assay. The extract for the inhibitor assay was prepared with the supernatant fraction, according to Lenney *et al.*[11] The activities of cathepsin B and cathepsin H were determined with carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide and L-arginine 4-methylcoumaryl-7-amide, respectively, according to Barrett.[12] One milliunit (mU) of cathepsin activity was defined as the amount releasing 1 nmol of 7-amino-4-methylcoumarin per min. Cathepsin B and cathepsin H used in the inhibitor assay were purified from rat liver according to Lenney *et al.*[11] The inhibitor was assayed under the conditions used for the assay of cathepsins B and H, replacing some of the buffer with inhibitor solution. One unit of inhibitor was defined as the amount that decreased cathepsin activity by one unit.

Protein Determination——Protein concentration was measured by the method of Lowry *et al.*[13] using bovine serum albumin as a standard.

Statistical Analysis——Statistical analyses were performed by using Student's *t*-test.

Results and Discussion

Figure 1 shows the activities of CANP, cathepsins B and H and their endogenous inhibitors in the sedentary rats.

Two forms of CANP, one of which requires μM order Ca^{2+} for its activity (μCANP) and the other of which requires mM order of Ca^{2+} (mCANP), are present in most mammalian tissues.[14] In MRF, higher activities of μCANP and mCANP were observed in the 16-week- and 98-week-old rats than in the 4-week-old rats ($p<0.001$). There was no significant difference between the activities of CANP in the 16-week- and 98-week-old rats. The mean values ± S.E.M. of the relative percentage of the activity of μCANP to the activities of μCANP plus mCANP at the ages of 4, 16 and 98 weeks were 24.1 ± 1.9 ($n=5$), 25.9 ± 2.1 ($n=10$) and 24.0 ± 1.6 ($n=5$), respectively; that is, there is no age-related change. The cathepsin B activities in the 16-week- and 98-week-old rats decreased by 45.5% and 32.2%, respectively ($p<0.001$), in comparison with that estimated in the 4-week-old rats. A higher value was observed in the 98-week-old animals than in the 16-week-old animals ($p<0.05$). As for cathepsin H, no significant difference was observed between the activities in the 4-week- and 16-week-old rats. A lower activity was observed in the 98-week-old rats than in the 4- or 16-week-old rats ($p<0.001$). The activities of endogenous inhibitors of CANP, cathepsin B and cathepsin H were unchanged, irrespective of age.

The present results show that the effects of age on the activities of cathepsin B and cathepsin H are different. Differential localization in tissue[15] and a different mode of action on myofibrillar proteins[3] have also been noted for cathepsins B and H. These findings suggest that cathepsins B and H, both of which are lysosomal thiol proteinases, may have different roles in skeletal muscle. Regarding the age-related change in the activity, the two forms of CANP are in marked contrast to the lysosomal thiol proteinases. The latter proteinases

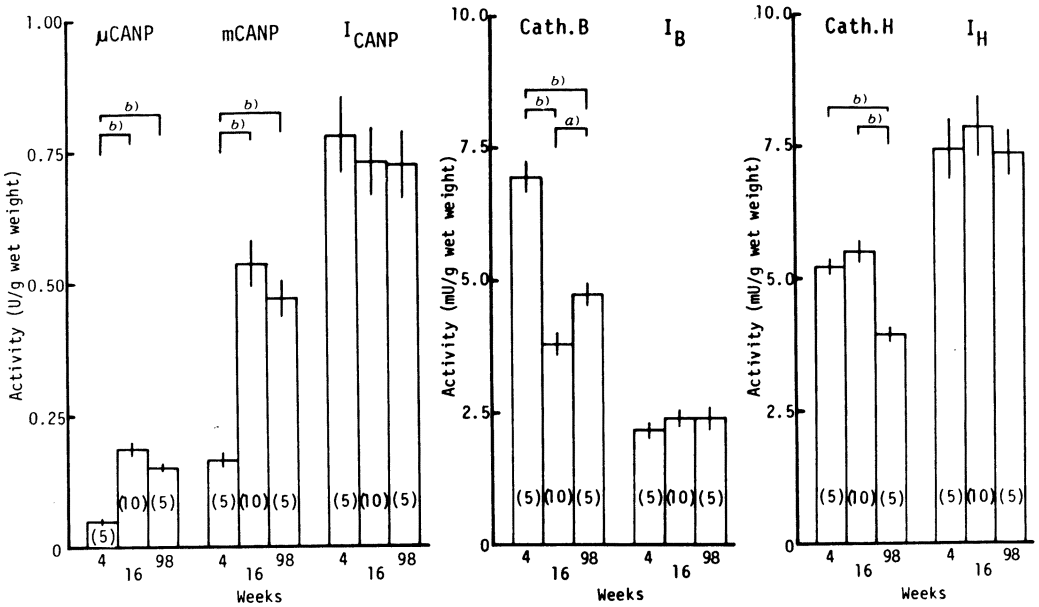

Fig. 1. Activities of CANP, Cathepsins B and H and Their Endogenous Inhibitors in Skeletal Muscle of Rats of Different Ages

CANP activity was measured with alkali-denatured casein as a substrate in the presence of 0.2 or 5 mM Ca^{2+}. mCANP activity was calculated by subtracting the activity found with 0.2 mM Ca^{2+} (μCANP activity) from that found with 5 mM Ca^{2+} (the activity of the sum of μCANP and mCANP). Cathepsins B (Cath. B) and H (Cath. H) activities were measured using methylcoumarylamide substrates. Inhibitory activity against CANP (I_{CANP}) in the sample was measured using a partially purified CANP obtained from rat musculus rectus femoris in the presence of 5 mM Ca^{2+}. Inhibitory activity against cathepsin B or H (I_B or I_H) in the sample was measured with rat liver cathepsin B or H. Numbers of determinations are shown in parentheses. Results show the mean ± S.E.M., and the data were analyzed by using Student's *t*-test. *a*) $p<0.05$, *b*) $p<0.001$.

decreased in activity, whereas the former increased in activity with ageing, when data for 4-week-old and 98-week-old rats were compared. Atrophic changes of skeletal muscle due to ageing are accompanied with a progressive decline in the rates of both protein synthesis and breakdown.[16] Although CANP and cathepsins B and H can degrade myofibrillar proteins *in vitro*,[1-3] the extent of participation of these thiol proteinases in the physiological turnover of myofibrillar proteins in relation to ageing requires further study.

Figure 2 shows the effect of exercise on the activities of CANP, cathepsins B and H and their endogenous inhibitors.

Prolonged, exhausting exercise causes not only activation of the lysosomal system but also necrotic and inflammatory change, in skeletal muscle.[17] Invading phagocytes contain lysosomal thiol proteinases and high levels of lysosomal thiol proteinase inhibitors.[18] In the present study, exercise-induced activation of lysosomal enzyme, cathepsin B or cathepsin H, was not observed. No difference in the activities of cathepsins B and H and their inhibitors per tissue wet weight, as shown in Fig. 2, or per protein content (data not shown), was observed between the control and trained rats. Thus, the exercise programme used in this study did not induce edema or any similar alterations in MRF, and macroscopic examinations confirmed this. The exercise reduced the body weight in the 98-week-old rats; mean values ± S.E.M. of the body weight of control group and trained group were 338.6 ± 11.5 g ($n=5$) and 290.8 ± 6.5 g ($n=5$), $p<0.01$, respectively, whereas in the 16-week-old rats, no significant difference in the body weight between the control and trained rats was observed; mean

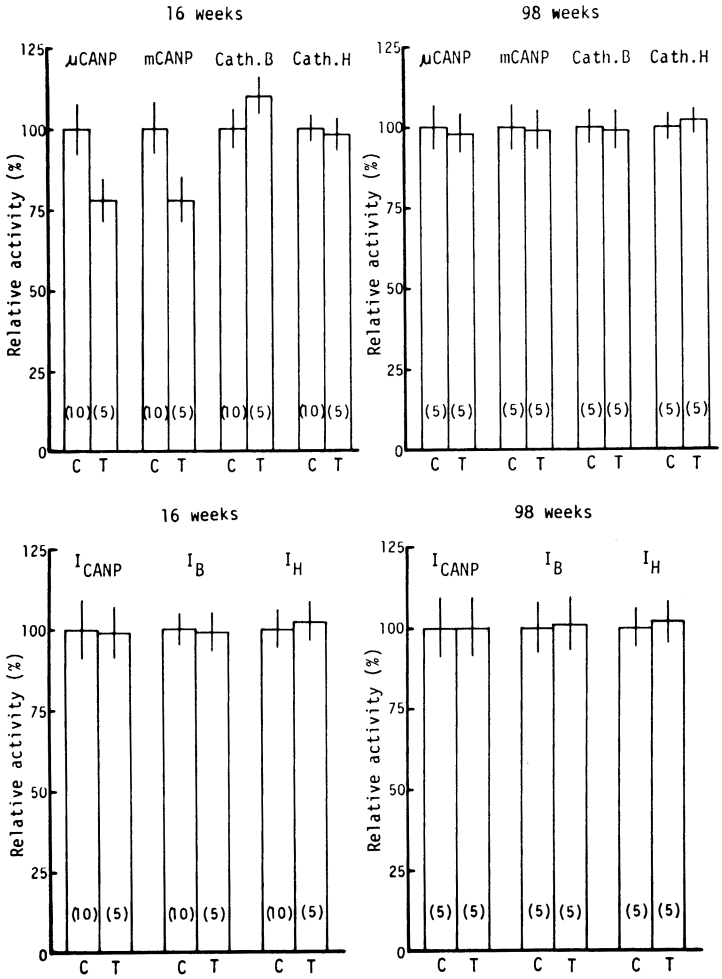

Fig. 2. Effect of Exercise on the Activities of CANP, Cathepsins B and H and Their Endogenous Inhibitors in Skeletal Muscle of Rats

Upper and lower panels show the effect of exercise on the enzymatic activities and on the inhibitor activities at different ages, respectively. The mean value of the enzymatic or inhibitory activity of the control (sedentary rats) group shown in Fig. 1 was taken as 100, and the relative percentage of the activity of the trained group was calculated from the corresponding enzymatic or inhibitory activity. Numbers of determinations are shown in parentheses. The mean ± S.E.M. are given, and the data were analyzed by using Student's *t*-test. C, control group; T, trained group.

valsues ± S.E.M. of the body weight of control group and trained group were 202.5 ± 3.0 g ($n=10$) and 198.6 ± 3.3 g ($n=5$), respectively. The wet weight and protein content of MRF were not affected by the exercise in the 16-week- and 98-week-old rats (data not shown). An exercise-induced response was observed in the activity of non-lysosomal enzyme, CANP, in adult (16 weeks) rats, but not in the senescent (98 weeks) rats. In the 16-week-old rats, mean values ± S.E.M. of CANP activity, the activity of the sum of μCANP and mCANP, of the control group and trained group were 0.722 ± 0.042 U/g wet weight ($n=10$) and 0.563 ± 0.042 U/g wet weight ($n=5$), respectively. The difference between them is statistically significant ($p<0.05$). The activity of μCANP or mCANP was reduced after training, but the difference in activity between the control group and trained group is not significant ($0.05< p<0.1$). In the case of exercise-induced lysosomal enzyme activation, young adults respond

better than do senescent animals,[19] and this difference may be caused by a partial loss of adaptive capacity of muscle cells during ageing.[20] The present result suggests that CANP is more susceptible than lysosomal thiol proteinases to physiological stimulus such as exercise in MRF. The mean values $\pm$S.E.M. of the relative percentage of the activity of μCANP to the activities of μCANP plus mCANP in the trained group at the ages of 16 and 98 weeks were 25.9 ± 2.3 ($n=5$) and 23.9 ± 1.5 ($n=5$), respectively. These values were similar to those of the control group. Since the physiological calcium ion concentration is of μM order, μCANP is an active form *in vivo*. With regard to the relationship between μCANP and mCANP, there are reports that mCANP is the precursor of μCANP, and the conversion occurs by limited autolysis of the former.[21] On the other hand, it has also been reported that these two forms of CANP are quite different in nature and are not in a simple relationship, *i.e.*, one of them is not derived from the other by autolysis or modification.[22] The present finding that the relative percentage of the activity of μCANP to the activities of μCANP plus mCANP did not change regardless of differences of age or exercise suggests a close relationship between μCANP and mCANP in skeletal muscle. The factors, other than endogenous inhibitors, which are implicated in the age- and exercise-related changes in the activities of the thiol proteinases are subjects of ongoing study.

Acknowledgements The authors thank Messrs. M. Hashimoto, H. Furui and I. Kihara for help with the program.

References

1) S. Ishiura, H. Sugita, K. Suzuki and K. Imahori, *J. Biochem.* (Tokyo), **86**, 579 (1979).
2) T. Noda, K. Isogai, H. Hayashi and N. Katunuma, *J. Biochem.* (Tokyo), **90**, 371 (1981).
3) N. Katunuma and T. Noda, "Muscular Dystrophy," ed. by S. Ebashi, University of Tokyo Press, Tokyo, 1982, pp. 225—237.
4) S. Ishiura, H. Sugita, I. Nonaka and K. Imahori, *J. Biochem.* (Tokyo), **87**, 343 (1980); W. R. Dayton and J. V. Schollmeyer, *J. Cell. Biol.*, **87**, 267*a* (1980).
5) N. C. Kar and C. M. Pearson, *Clin. Chim. Acta*, **73**, 293 (1976); W. R. Dayton, J. V. Schollmeyer, A. C. Chan and C. E. Allen, *Biochim. Biophys. Acta*, **584**, 216 (1979); T. Toyo-oka, *FEBS Lett.*, **117**, 122 (1980).
6) M. G. Pluskal and R. J. Pennington, *Biochem. Soc. Trans.*, **1**, 1307 (1973); T. Noda, K. Isogai, N. Katunuma, Y. Tarumoto and M. Ohzeki, *J. Biochem.* (Tokyo), **90**, 893 (1981).
7) K. Imahori, H. Sugita, T. Miyatake, T. Ishihara, N. Miyatani and T. Kuwabara, *Rinsho Yakuri*, **16**, 749 (1985).
8) S. Ishiura, H. Murofushi, K. Suzuki and K. Imahori, *J. Biochem.* (Tokyo), **84**, 225 (1978).
9) M. Takahashi-Nakamura, S. Tsuji, K. Suzuki and K. Imahori, *J. Biochem.* (Tokyo), **90**, 1583 (1981).
10) S. Tsuji and K. Imahori, *J. Biochem.* (Tokyo), **90**, 233 (1981).
11) J. F. Lenney, J. R. Tolan, W. J. Sugai and A. G. Lee, *Eur. J. Biochem.*, **101**, 153 (1979).
12) A. J. Barrett, *Biochem. J.*, **187**, 909 (1980).
13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) R. L. Mellgren, *FEBS Lett.*, **109**, 129 (1980); W. R. Dayton, J. V. Schollmeyer, R. A. Lepley and L. R. Cortes, *Biochim. Biophys. Acta*, **659**, 48 (1981); A. Kishimoto, N. Kajikawa, H. Tabuchi, M. Shiota and Y. Nishizuka, *J. Biochem.* (Tokyo), **90**, 889 (1981); T. Murachi, M. Hatanaka, Y. Yasumoto, N. Nakayama and K. Tanaka, *Biochem. Intern.*, **2**, 651 (1981).
15) K. Li, K. Hizawa, E. Kominami, Y. Bando and N. Katunuma, *J. Histochem. Cytochem.*, **33**, 1173 (1985).
16) S. E. M. Lewis, F. J. Kelly and D. F. Goldspink, *Biochem. J.*, **217**, 517 (1984).
17) V. Vihko, J. Rantamaki and A. Salminen, *Histochemistry*, **57**, 237 (1978).
18) E. Kominami, T. Tsukahara, K. Li, K. Hizawa and N. Katunuma, *Biochem. Biophys. Res. Commun.*, **123**, 816 (1984); E. Kominami, Y. Bando, K. Li, K. Hizawa and N. Katunuma, *J. Biochem.* (Tokyo), **96**, 1841 (1984).
19) A. Salminen, H. Kainulainen and V. Vihko, *Experientia*, **38**, 781 (1982).
20) A. Salminen and V. Vihko, *Acta Physiol. Scand.*, **112**, 89 (1981).
21) K. Suzuki, S. Tsuji, S. Kubota, Y. Kimura and K. Imahori, *J. Biochem.* (Tokyo), **90**, 275 (1981); K. Suzuki, S. Tsuji, S. Ishiura, Y. Kimura, S. Kubota and K. Imahori, *ibid.*, **90**, 1787 (1981); D. R. Hathaway, D. K. Werth and J. R. Haeberle, *J. Biol. Chem.*, **257**, 9072 (1982).
22) M. Inomata, M. Hayashi, M. Nakamura, K. Imahori and S. Kawashima, *J. Biochem.* (Tokyo), **93**, 291 (1983); M. Inomata, M. Nomoto, M. Hayashi, M. Nakamura, K. Imahori and S. Kawashima, *ibid.*, **95**, 1661 (1984).