Plasminogen Activator in Mammalian Skeletal Muscle: Characteristics of Effect of Denervation on Urokinase-like and Tissue Activator

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Abstract. Analyses were made of the fibrinolytic, plasminogen-activating system in skeletal muscle to determine if a regulating influence of the nerve could be detected on these enzymes. Young male mice underwent right sciatic neurectomy. Extracts were prepared from denervated muscle at 2–17 d after axotomy and compared with controls. Using a cascade-style biochemical assay (Rönby, M., B. Nortman, and E. Wallén, 1982, Thromb. Res., 27:743–748) we found that low levels of plasminogen activator (PA) were present in adult, innervated mouse muscle, but that denervation resulted in a marked time-dependent increase in enzyme activity. Qualitative separation showed an eightfold increase in urokinase-like PA with moderate elevation of tissue PA activity after 10 d. Fibrin zymography (Granelli-Piperno, A., and E. Reich, 1978, J. Exp. Med., 148:223–234) revealed clear zones of lysis corresponding to molecular masses of 48 kD for urokinase-like PA and 75 kD for tissue PA, consistent with the molecular masses found for these enzymes in other tissues of the mouse (Dane, K., P. A. Andreasen, J. Grøndahl-Hansen, P. Kristensen, L. S. Nielsen, and L. Skriver, 1985, Adv. Cancer Res., 44:139–266).

In other studies we have shown that PA-activated plasmin readily attacks critical adhesive basement membrane molecules. The present results indicate that enzymes involved in plasminogen activation, particularly urokinase-like PA, rapidly increase after axotomy, suggesting they may have a role early in muscle denervation. Similar alterations in PA activity might underlie the elimination of polyneuronal innervation during mammalian muscle development. Certain neuromuscular diseases may also involve activation of these enzymes, resulting in degradation of basement membrane zone components and, therefore, warrant further study.

After injury, skeletal muscle undergoes a form of tissue remodeling that can result in relatively complete regeneration (1, 5, 6, 39, 40). Although the molecular mechanisms underlying this remodeling are not understood, the cellular basis appears to reside within the muscle tissue itself. For example, after mincing of mammalian muscle and replacing the minced tissue in the vacated site of the removed muscle (see reference 4) rather remarkable regeneration of form and function takes place. X-irradiation of the minced tissue before replacing completely blocks this muscle regeneration (20). This type of experiment eliminates the contribution of invading cells from the vascular system in the regeneration process (20). Experiments such as this and others (44, 51, 52) support the hypothesis that muscle regenerates from activation of quiescent "satellite" cells present within mammalian skeletal muscle (38). The mechanism(s) for activating these stem or satellite cells is unknown.

After experimental denervation of mammalian muscle a number of changes also occur. These include gross atrophy, nuclear alterations, and cellular proliferation, as well as metabolic and physiologic alterations (see references 27, 59). Early changes are located at the cell surface and include the loss of end plate–specific A12 (16 S) acetylcholinesterase (12–14, 16), reduction in fibronectin (16), and invasion of the end plate by Schwann cells (42). These changes occur within 1–3 d of severing the nerve, in advance of decrease in muscle bulk and reduction in muscle-specific sarcoplasmic proteins (27, 59). Reduction in the latter has been suggested to result from degradation by Ca÷+-activated (10), lysosomal (45, 48), or, possibly, membrane-bound neutral proteases (54). While conclusive data indicating increased synthesis and release of neutral proteases early in denervation are lacking, previous studies have suggested a role for such enzymes in reduction and release of A12 acetylcholinesterase (13, 14) and sarcocellular fibronectin (16) and in the internalization and partial degradation of acetylcholine receptors (29, 43,
Materials and Methods

Materials

Lys-plasminogen, a modified form of native glu-plasminogen with NH2-terminal lysine (Mr 85,000) was purchased from Choay Laboratories (Paris, France). The small quantity of plasmin present in the reagent was eliminated by prior chromatography on aprotinin agarose. Glu-plasminogen was kindly donated by Dr. O. Bertrand (Hôpital Beaujon, Clichy, France). tPA, isolated from human melanoma cells in tissue culture, was a generous gift of Drs. Lijnen and Collen (University of Leuven, Leuven, Belgium). The specific activity, expressed in uPA equivalent units, was 90,000 IU/mg. Chromogenic plasmin-specific S-2251 substrate was purchased from Kabi (AB) (Stockholm, Sweden). Ketamine (Ketalar) was from Parke, Davis & Co. (Chicago, IL) and acepromazine (Vetranquil) from Clin-Midy (Paris, France). Plasminogen-free human or bovine fibrinogen was purchased from Kabi (AB) and bovine thrombin from Parke, Davis & Co. Fibrin monomer (FM) was prepared by clotting human fibrinogen (1%) in distilled water with reptilase (Stago, Asnières, France). The clot was squeezed out, washed in 0.15 M NaCl, and dissolved in 5 M urea.

Denervation and Preparation of Extracts

4-6-wk-old male C57/BL mice (tifa-Credo, l'Arbresle, France) were anesthetized with intraperitoneal ketamine (10 mg/ml) and acepromazine (9:1 vol/vol), 10 μg/g. Then mid-point (sciatic notch) axotomy, with removal of a 5-mm segment of sciatic nerve, was performed. At varying times thereafter, after cervical dislocation, denervated and contralateral (unoperated) control hind-limb, sciatic-innervated muscles were cleaned of blood vessels and connective tissue and homogenized in 100 mM Tris-Cl, 2 mM EDTA, pH 7.6 (1:10, wt/vol) with a Polytron (Brinkmann Instruments Co., Switzerland) tissue homogenizer, all at 4°C. Homogenates were centrifuged at 12,000 g for 30 min (Sorvall Instruments Div., Wilmington, DE). The supernatant was further centrifuged at 100,000 g for 1 h (Beckman Instruments, Inc., Palo Alto, CA). After all procedures, fractions were aliquoted, flash-frozen in liquid N2, and stored at −80°C until used.

Amidolytic Assay of PA Activity in Muscle

Activation of plasminogen to plasmin was followed by determination of plasmin activity on the synthetic substrate S-2251 (49). To 0.1 ml (85 μg/ml) of lys-plasminogen in assay buffer (0.1 M sodium phosphate, pH 7.3; containing 10 mM EDTA, 0.01% NaN3, and 0.01% Triton X-100) were added either 0.1 ml of muscle extract at different dilutions or 0.1 ml of purified tPA or uPA diluted in assay buffer containing 0.01% Tween 80 (Sigma Chemical Co., St. Louis, MO) to avoid nonspecific adsorption. After 2 h at 37°C, 0.1 ml S-2251 (3 mM) in distilled water. Incubation was performed at 37°C in flat-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). The release of para nitroaniline from S-2251 was determined in each well by measuring the absorbance at 405 nm using a micro-ELISA autoreader (Dynatech Laboratories, Inc.). Color development was linear for 6 h after a 2-h lag, or if expressed to the fourth power (60). Controls, including buffer alone and extracts without plasminogen, were performed in parallel wells. Generally, 2 or 3 replicate wells were included in each assay. Our current results are expressed in absorbance units after 4 h incubation at 37°C.

Fibrin Zymography

SDS PAGE was performed according to Laemmli (34) in 10% gels and was followed by fibrin plate zymography according to the method of Granelli-Piperno and Reich (21). After SDS PAGE and elimination of SDS in 2.5% Triton X-100, the acrylamide gels were transferred onto protocol fibrin plates and incubated in a moist atmosphere at 37°C for 24 h. The resultant lytic areas were then observed. Photography of the fibrin plates was performed with indirect illumination on a black background. Molecular mass markers allowed for calculation of relative molecular masses.

Fibrinolytic Activity Localization by Fibrin Overlay

Localization of fibrinolytic activity in 8-μm cryostat sections of mouse sternomastoid sections was performed as previously described (30, 53). Briefly, both end plate and non-end plate regions of muscle were frozen in isopentane pre-cooled in liquid N2 and kept at −80°C until sectioned. Cryostat sections were briefly air dried and then a mixture of 60 μl plasminogen-free human fibrinogen (1%) and 10 μl of bovine thrombin (20 NIH U/ml) at 4°C was added and quickly spread thin. The thin clot was then allowed to become stabilized at 22°C for 20 min before incubation of sections in a moist chamber at 37°C for varying times. To determine if plasminogen-dependence of muscle section fibrinolysis was present, 50 μg/ml (20–50 μl) of purified human glu-plasminogen was added. After incubation, sections were fixed in 10% formaldehyde, washed, and then stained with Harris' hematoxylin.

Protein determination was estimated by the method of Bradford (3) using Coomassie Brilliant Blue-G (Sigma Chemical Co.) as the color indicator. All other chemicals were of reagent grade. Statistical analysis was performed using the two-tailed Student's t test.

Results

Atrophy and Protein Measurements

Table 1 shows the expected decrease in bulk of sciatic-innervated leg muscles after denervation (27, 59). The earliest atrophy was not detected until 4 d and the greatest decrease occurred between 4 and 7 d (10–27% reduction). The rate of muscle atrophy plateaued after 10 d, with little difference between 10 and 17 d. In addition to bulk atrophy, decrease in noncollagenous protein was also found, as has been demonstrated previously (27, 59).

PA Amidolytic Activity

Using a sensitive amidolytic assay with a synthetic pentidyl chromogenic substrate (S-2251) specific for plasmin (49), activity under our assay conditions was entirely dependent upon the addition of highly purified human plasminogen (Fig. 1). Plasminogen-independent activity was barely above the zero value even after 6 h incubation. This was true even for control muscle but was strikingly seen in the denervated muscle samples. Using the S2251-fibrin plate assay (56), previous studies with neutral protease enzymes secreted from clonal mouse muscle (18) and rat muscle in organ culture (Festoff, B. W., unpublished results) had indicated consider-
Table 1. Effects of Sciatic Neurectomy on Sciatic-innervated Muscle Weight and Protein Content*

| Days after denervation | No animals | Wet weight per animal | Decrease | Protein | Decrease |
|------------------------|------------|-----------------------|----------|---------|----------|
|                        |            | g                     | %        | mg/g    | %        |
| 2                      | 13         | Control 0.30           |          |         |          |
|                        |            | Denervated 0.29        | 0        | 13.0    | 0        |
| 4                      | 5          | Control 0.30           | 0        |         |          |
|                        |            | Denervated 0.27        | 3        | 12.8    | 2        |
| 7                      | 20         | Control 0.33           | 0        |         |          |
|                        |            | Denervated 0.24        | 10       | 11.8    | 6        |
| 10                     | 22         | Control 0.35           | 0        |         |          |
|                        |            | Denervated 0.22        | 37       | 10.3    | 38       |
| 17                     | 6          | Control 0.33           | 0        |         |          |
|                        |            | Denervated 0.19        | 42       | 7.5     | 45       |

* Values are averages of three experiments and were within 5–8%.
† Noncollagenous proteins, determined as described in Materials and Methods.

able PA activity but plasminogen-independent activity, especially in muscle homogenates, was also detected. Moreover, in the current series of experiments, the addition of FM (49) was used to distinguish fibrin-dependent (i.e., tPA) and fibrin-independent (i.e., uPA) PA activities in muscle extracts.

Confirmation that amidolysis of S-2251 was dependent on the presence of lys-plasminogen is further demonstrated in Fig. 2 where the plasminogen concentration is varied. Amidolysis increased with the plasminogen concentration in a linear fashion and plateaued above 40 μg/ml, consistent with other reports (49). Amidolysis was also critically dependent on the pH of the medium with maximum activity at pH 7.6, and was completely inhibited by aprotinin at 5 KIU/ml (not shown). This study indicated that amidolytic activity in muscle extracts represented only PA.

Effects of Denervation on Muscle Plasminogen-dependent Amidolytic Activity

The effect of mid-sciatic neurectomy on muscle PA activity at different times after nerve section is shown in Fig. 3. The PA activity measured in the presence of FM increased almost 10-fold by 7 d after neurectomy. The most rapid increase in PA activity occurred between 2 and 4 d. After this time, PA activity, determined in the presence of FM, appeared to plateau. PA activity in the absence of FM rose more gradually but also plateaued during the course of this temporal study of denervation. As shown, little or no change occurred in the contralateral, unoperated leg muscle activity, either in the absence or presence of FM.

Figure 1. Effects of plasminogen on amidolytic activity of control and denervated muscle extracts. Little or no amidolytic activity using S-2251 (see Materials and Methods) is found for control or 10-d denervated muscle in absence of plasminogen.

Figure 2. Concentration dependency of muscle amidolytic activity on lys-plasminogen. Purified human lys-plasminogen (see Materials and Methods) was added, in increasing concentration, to denervated muscle extracts, and activity after a 4-h incubation was compared. The linear portion of the curve, 10–40 μg/ml, correlates with that of PA enzymatic activity.
Figure 3. Time course of PA activity after denervation. Fibrin monomer-dependent PA activity was assayed for control (●) and denervated (○) muscle extracts as well as fibrin-independent PA activity in control (●) and denervated (○) extracts according to techniques described in Materials and Methods.

Fibrin Zymography of Muscle Extracts

We used fibrin zymography to more specifically identify the type of PA that increased in denervated muscle. Fig. 4 shows a typical fibrin zymogram of control and denervated muscle extracts, obtained at varying times after denervation. This technique enabled us to extend our biochemical results and offered the possibility to detect the presence of PA–inhibitor complexes (37, 55). The dark field photograph of a fibrin zymogram shows control and denervated (at three different times) muscle extract along with purified human tPA (lane 1) and uPA (lane 2), the latter showing the high and low molecular mass human uPAs. Clear lysis zones at 48 kD are seen with mouse muscle extracts from all times of denervation (lanes 4, 6, and 8). In contrast, lysis zones were not observed with control muscle extracts (lanes 3, 5, and 7), at equivalent protein loads. Lysis was also not seen when threefold-overloaded control samples were added (not shown).

The major band of fibrinolytic activity at 48 kD migrates just between the positions of high (55 kD), and low (33 kD) molecular mass human uPA. A smaller band, evident in the later denervation extracts (lanes 6 and 8), at 75 kD, co-migrates with pure human tPA. In neither the control nor denervated muscle extracts could a high molecular weight lytic zone, comparable to activity arising from a complex of tPA and tPA inhibitor at 100 kD (37) or uPA and uPA inhibitor at 68 kD (9) be detected.

Cellular Localization of Muscle Tissue PA

To attempt to localize the PA activity in control and denervated muscle, we used the fibrin overlay technique (30, 53). This technique has previously been used to detect PA in sciatic nerve and spinal cord from several species (53) as well as in a number of vascular tissues (30). In mouse muscle, we found a relative dependence of fibrinolysis on plasminogen on cryostat sections. When overlays of plasminogen-free human fibrinogen were used, incubation for at least 3 h at 37°C was required to demonstrate lytic zones for both

Figure 4. Fibrin zymogram of control and denervated muscle extracts. Electrophoresis of extracts from control (lanes 3, 5, and 7) and denervated (lanes 4, 6, and 8) muscle at 7 (lanes 3 and 4), 10 (lanes 5 and 6) and 17 (lanes 7 and 8) d after denervation applied to fibrin layer as described in Materials and Methods. (Lane 1) Human tPA obtained from recombinant DNA techniques; (lane 2) human uPA (purified from urine).
denervated and control muscle, but this was seen only overlying blood vessels (not shown). In contrast, when purified human plasminogen was added, after only 30 min, large lytic zones were present over denervated muscle fibers, in areas that also included muscle fascicles and interfascicular blood vessels (Fig. 5, a and b). Reproducibly, lysis was found over denervated fibers where no vessels were present (5 b, lower right) as well as when vessels were underlying the lytic zones. However, lysis occurred only over blood vessels after 3 h in control muscle cryostat sections, even in the presence of plasminogen (Fig. 5, c and d). In a number of sections of denervated muscle, lysis was also detected over the connective tissue and at the transition zone between denervated fibers and the interfascicular matrix (not shown).

Discussion

Our present results indicate that adult, innervated mammalian skeletal muscle contains detectable amounts of plasminogen-dependent amidolytic activity (Figs. 1 and 2). After sciatic neurectomy, with removal of a 5-mm segment of nerve, PA activity rises dramatically in the muscle, in advance of the expected decrease in muscle bulk (Table I).

Lysosomal and cathepsin-like enzymes were not measured in these studies, since little or no amidolytic activity could be detected in the absence of plasminogen in the incubation mixture. However, other reports have indicated that significant increases of these plasminogen-independent enzymes occur somewhat later than the presently reported rise in PA (41, 45, 46). The time course of amidolytic activity shows more than twice the PA activity in control muscle as early as 2 d after neuroectomy (the earliest time point in these studies) in denervated muscle which progressively increases until day 7 and plateaus afterwards. The dependence of PA activity on fibrin was also studied. In the absence of FM, there was a consistent increase in PA activity after denervation indicating an increase in fibrin-independent PA activity. In the presence of FM, the steady increase in PA activity was higher than that observed in the absence of FM, indicating an additional increase in fibrin-dependent PA activity. Because fibrin is required for tPA-induced plasminogen activation (i.e., tPA), these results were compared with those obtained by the fibrin zymography by evaluating the intensity of the fibrin lysis area as well as the Mr of the activator. Using this technique, the presence and increase in the 48-kD lytic band occurred first after nerve section (Fig. 4, lanes 4,
transplanted muscle where these same components of the basement membrane zone first disappear and are then synthesized during muscle regeneration. However, they differ considerably for the findings in allografts where the muscle graft is rejected and basement membrane zone components are not degraded, but persist (23).

The role of the nerve in controlling muscle PA is likely not to be passive. It may function to regulate PA synthesis, at either transcriptional or translational levels, and/or may also function posttranslationally, to inactivate the PA after it is released. A similar model was previously proposed to be the mechanism for the normal turnover of basement membrane components, augmentation of which might occur experimentally, or in the fatal human neuromuscular disorder, amyotrophic lateral sclerosis (15). In this regard, it has recently been shown that nerves also secrete PAs (31, 47, 57) and that concentration of PA is found at axon growth cones (32, 33). Study of the PA-plasmin system, its inhibitors (2, 11, 36), and other factors involved in both thrombosis and fibrinolysis in the peripheral and central nervous systems may help to elucidate some of the questions raised by the current study.

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