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Increase of Neuronal Nitric Oxide Synthase in Rat Skeletal Muscle during Ageing

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Nitric oxide synthases (NOS) are different widely expressed enzymes which produce the molecular messenger nitric oxide. The neuronal isoform of NOS (nNOS) is involved in several processes of the cell metabolism, most of which are, at present, not fully understood (neurotransmission, smooth muscle motility, myoblast and myocyte biology and others). In skeletal muscle nNOS is present mainly at the plasmalemma, where it is attached to the dystrophin-related proteins; in fact, in pathologies involving dystrophin, nNOS is altered as well. We report that in aged rats the nNOS amount in skeletal muscle increases both in the soluble and microsomal fractions and that an additional intracytoplasmic localisation appears.

Nitric oxide synthases are enzymes which produce nitric oxide (NO) from L-Arginine. Three major isoforms of this enzyme have been isolated and characterized: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). nNOS occurs in several cell types: neurons, skeletal muscle, epithelial and macula densa cells (1,2).

In rat skeletal muscle nNOS has been reported to be localised at the sarcolemma of both type I and II fibers and to be enriched at the neuromuscular endplates (2,3,4). nNOS has been found in association with the dystrophin-related proteins, in particular with α1 and β1 syntrophins (5,6). Moreover in muscular dystrophies involving dystrophin absence or alteration (Duchenne and Becker forms) and in animal models lacking dystrophin (mdx mice), sarcolemmal nNOS is decreased or absent (7, 8, 9).

In mature skeletal muscle nNOS has been hypothesized to be involved in the regulation of contractile force, probably through NO-mediated activation of cyclic GMP-dependent protein kinase type I (cGKI) (2). Recently it has been demonstrated that in skeletal muscle nNOS is present in an alternatively spliced form, referred to as nNOSμ. This isoform has the same catalitic activity of nNOS and a slightly different molecular size (164 kDa instead of 160 kDa); nNOSμ has been reported to be present only in skeletal muscle and myocardium (10).

In this study we demonstrate an increase in the amount of nNOS in rat skeletal muscle during ageing. In skeletal muscle cells we found by immunohistochemistry an additional intracytoplasmic nNOS localisation; by immunoblotting the increased nNOS level could be attributed both to the microsomal and soluble fractions. A modification in the electrophoretic mobility in old rats is also present. This may suggest a different nNOS activation and, probably, a variation in transcription regulation, with ageing.

MATERIALS AND METHODS

Animals. The experimental protocol was performed using 10 adult (6 months) and 10 old (24 months) male Wistar rats from the aging colony of I.N.R.C.A. (Ancona, Italy). The animals were previously inspected to exclude those affected by tumors or other diseases.

The rats were anesthetized with diethyl ether and sacrificed; the *musculus gracilis* was quickly removed and frozen in liquid nitrogen.

Antibody. Polyclonal rabbit anti-nNOS (NOS1 R20 by Santa Cruz Biotechnology) was used. This antibody is directed against the C-terminal domain of the protein (aa 1400–1419) and it recognises both the cerebellar nNOS and the muscular nNOSμ isoforms. nNOSμ differs from the nNOS because of the presence of 34 aminoacids inserted at position 839–840 of the cerebellar form. In differentiated skeletal muscle only the μ form is detected (10).

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Abbreviations used: nNOS: neuronal nitric oxide synthase; eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; cGKI: cyclic GMP-dependent protein kinase type I.

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Tissue extraction and Western blot analysis. Skeletal muscle samples were homogenized in 10 vol (w/v) of lysis buffer containing 25 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF. Heavy microsomes and soluble fraction were prepared by a standard protocol (Brenman, cell). The protein concentration was determined by Comassie blue staining and tissue extracts was boiled in SDS-containg sample buffer.

For the electrophoresis a 1.5 mm thick SDS polyacrilamide minigel (Mini-protean II; Bio-Rad, Richmond, CA, USA) was casted according to Leammli (11). The resolving gel contains 7.5% acrylamide and 5% stacking gel. Proteins were transferred to nitrocellulose membranes (Hybond-C; Amersham, UK); these were incubated in TBST (4% powdered milk, 0.05 Tween 20 in 10 mM Tris pH 8, 150 mM NaCl) for 1 h at room temperature to saturate non specific binding sites. Immunoreaction with 1μg/ml primary polyclonal antibody was performed for 1 h at room temperature. A second incubation was carried out with a goat anti-rabbit IgG-HRP (Amersham, UK) which was diluted 1:5000 in TBST. TBST was also used for washing cycles at each step. The blot was developed by the Amersham ECL detection system. Immunoreactive bands were analysed by densitometry (Phosphor Imager, BioRad).

Immunohistochemistry. Small pieces of skeletal muscle were dissected from *musculus gracilis* and immediately frozen in liquid nitrogen cooled isopentane. Transverse cryostat sections were cut (10 μm thick) and incubated with polyclonal rabbit anti-nNOS antibody diluted 1: 50 in Tris-HCl-phosphate buffer-saline (TBS) containing 1% bovine serum albumine (BSA) overnight at 4°C. After washing with PBS containing 0.1% Tween 20, the samples were incubated with fluorescein-conjugated anti rabbit secondary antibody (Dako) diluted 1:100 in TBS- 1% BSA for 1 hour, at room temperature, in the dark. Observation was done with a Zeiss Axiophot epifluorescence microscope.

Further cryostat sections were examined with a standard series of histochemical stainings.

RESULTS

In adult (6 months) rats the nNOS is detected as a single band by immunoblotting with the polyclonal anti-nNOS antibody. The comparison with cerebellar nNOS shows that the protein which is specifically recognized in skeletal muscle by the antibody is slightly heavier, as it is reported to be the μ form of nNOS (fig1).

In old (24 months) rat skeletal muscle the amount of nNOS dected by immunoblotting is definitely higher than in adults (fig2).

The muscle sections, examined by light microscopy, showed a normal variability of fiber size in adult rats; only rare hypotrophic fibres were observed. In old rats a marked increase of the fiber size variation was observed, associated with frequent atrophic fibres. Furthermore, in old rat muscles, adenosine triphosphatase reaction was uniform under all conditions (data not shown).

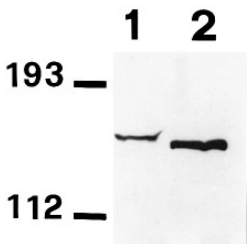


FIG. 1. Immunoblotting showing the specific detection of nNOS in total homogenate from rat skeletal muscle (lane 1), which correspond to an heavier isoform than in cerebellum (lane 2).

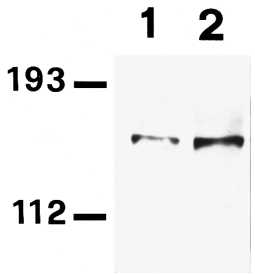


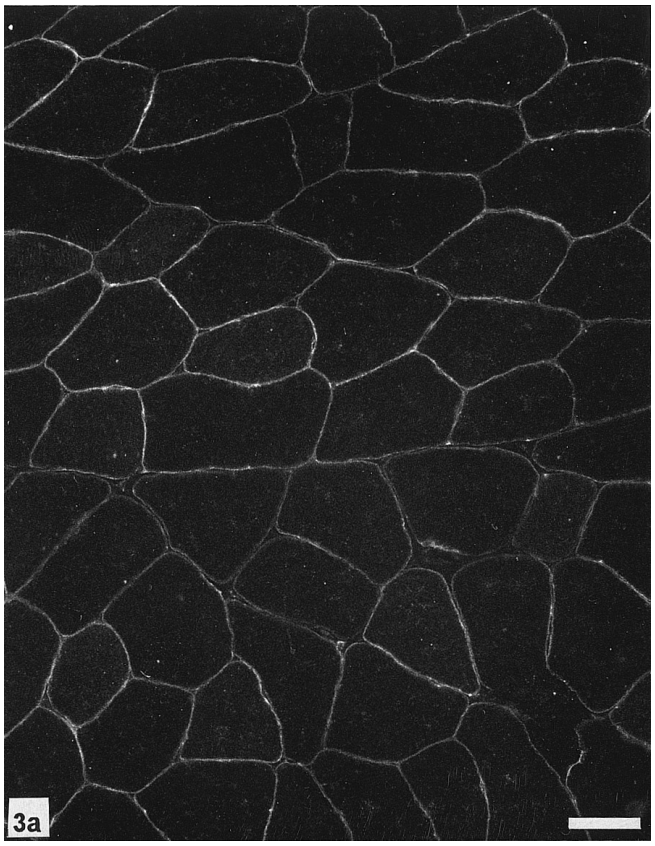
FIG. 2. Immunoblotting of nNOS in rat skeletal muscle total homogenate showing a comparison between adults (6 months) (lane 1) and old subjects (24 months) (lane 2).

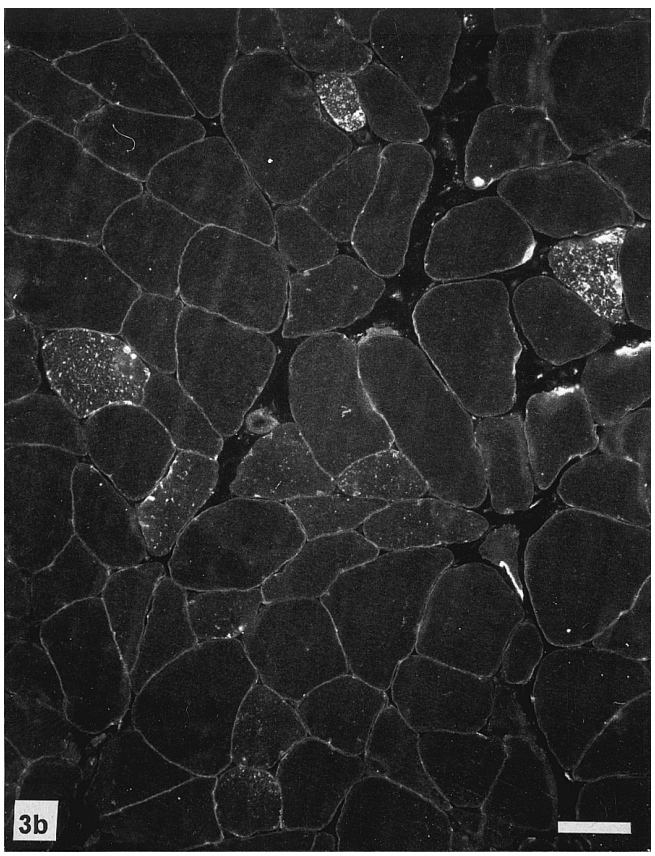
Immunohistochemical reactions revealed that in adult rats the fluorescence was localised exclusively at the sarcolemma. In old rats a positive staining was observed at the sarcolemma of muscle fibres, which show both normal and reduced size; in addition several muscle fibres showed a granular and diffuse sarcoplasmic fluorescence; this definite intracellular positivity was visible both in grouped and scattered fibers (fig3a,b).

Fractionation analysis of skeletal muscle showed that the increase of nNOS in old rats, in comparison with adults, was evident in both the heavy microsome and soluble fraction (fig 4). The densitometric analysis of the immunoreactive bands indicated an increase in old rats of about five fold in the total homogenate, of 71% and 36% in the heavy microsome and the soluble fraction respectively (Tab.1). All bands detected from skeletal muscle fractions were slightly heavier than the brain nNOS isoform. Nevertheless all the fractions from old rats appeared to migrate faster than the ones from adults. Moreover a double (specific) band was present in the heavy microsome fractions from old rats (Fig.4).

DISCUSSION

The results we show demonstrate that the enzyme nNOS is more abundant in skeletal muscle of 24 months-old rats in comparison to 6 months-old rats. Moreover we show that nNOS in older rats is often localised also in the cytoplasm of muscle fibers, in addition to the normal plasmalemmal localisation. The granular intracytoplasmic fluorescence is clearly evident in grouped fibres of different size at low magnification, but at higher magnification a definite intracellular granular signal is visible also in several scattered





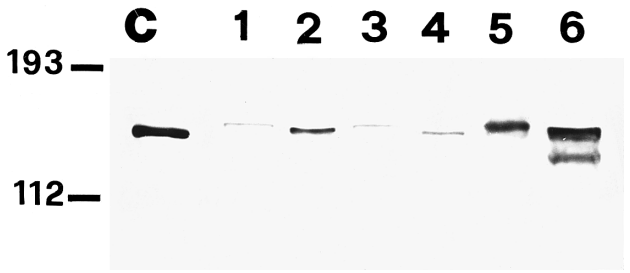


FIG. 4. Immunoblotting of skeletal muscle lysates from different fractions. Total homogenates from adults (lane 1) and old rats (lane 2); soluble fractions from adults (lane 3) and old rats (lane 4); heavy microsome fractions from adults (lane 5) and old rats (lane 6). C: cerebellum total homogenate. Increase of nNOS level is detectable in all fractions from old (24 months) rats in comparison with adults (6 months). A faster electrophoretic motility characterises all fractions from old subjects, whose heavy microsomal fraction shows a double specific band.

fibers. The granular pattern of nNOS probably reflects a localisation near the mitochondria, according to recent results (12, 6).

The histological pattern found in old rat muscle sections corresponds to a normal situation in aged individuals. In fact, a wide fibre size variation is reported as normal during ageing (13). Moreover a progressive loss of contractile fibrils leads to fibre atrophy, while axonal degeneration with subsequent regeneration produces type grouping in mixed fibres-type muscles in old individuals (14).

The increase of nNOS in skeletal muscle, however, is conceivably not caused by axonal degeneration. In fact neurogenic diseases, as well as experimental axotomy, are demonstrated either to reduce the protein level or to leave its expression unaffected (2,15,16).

Increase of nNOS has been described during rat ageing in the nucleus gracilis (17) and in the vas deferens (18); moreover, in the ventral horns of the lumbar cord, a number of NOS positive motoneurons appear (and increase) during ageing (19). These findings were interpreted as: I) a possible involvement of increased nNOS expression in the physiopathological processes of ageing in the gracile nucleus, II) a possible androgen dependence of nNOS regulation and III) a possible role of nNOS in the production of NO-dependent degeneration in the motoneurons between 24 and 32 months of age.

Control of nNOS transcription is specifically modulated in different tissues and times of development. This regulation process requires multiple steps which have been hypothesized to be dependent on alternative

FIG. 3. Immunohistochemitry. a) nNOS labelling in adult (6 months) rat skeletal muscle tranverse section. Positivity is specific for the sarcolemmal region. b) nNOS labelling in old (24 months) rat skeletal muscle tranverse section. Positivity is not restricted to the sarcolemmal region, diffuse intracytoplasmic granular fluorescence is present in grouped fibres. Bar: 20μm.

TABLE 1

Densitometric Analysis of the Immunoreactive Bands Detected after Fractionation

	Adults (o.d.)	Olds (o.d.)	Relative increase (%)
Total homogenate	0.44	2.35	430
Soluble fraction	0.61	0.83	36
Heavy microsome fraction	5.78	9.89	71

Note. The values (o.d.: optical density) represent the mean of five experiments. In the total homogenate a five fold increase is found. Heavy microsome and soluble fractions show an increase of about 71% and 36%, respectively.

splicing and multiple transcription factors (20,21); in fact several alternatively spliced isoforms have been described (22,10). It has also been reported that nNOS can be phosphorylated by several protein kinases at different sites, resulting in different electrophoretic mobility (23) and that alternative nNOS isoforms may exist (24). These reported data could support our finding of increased nNOS during ageing, as well as the finding of a constant small difference in electrophoretic mobility of nNOS between old and adult subjects; they also may explain the presence of a second smaller size immunoreactive band in the heavy microsome fraction from aged rats.

Studies are in progress in order to verify the hypothesis that, in old rats, muscular nNOS could be less phosphorylated (faster migration) than in adults (slower migration), and that different phosphorylation levels could influence the enzymatic activity variations during ageing.

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REFERENCES

1. Kobzik, L., Reid, M. B., Bredt, D. S., and Stamler, J. (1994) *Nature* **372,** 546–548.
2. Chao, D. S., Silvagno, F., Xia, H., Cornwell, T. L., Lincoln, T. M., and Bredt, D. S. (1997) *Neuroscience* **76(3),** 665–672.
3. Grozdanovic, Z., Nakos, G., Dahrmann, G., Mayer, B., and Gossrau, R. (1995) *Cell Tissue Res.* **281,** 493–499.
4. Grozdanovic, Z., Gosztony, G., and Gossrau, R. (1996) *Acta histochem.* **98,** 61–69.
5. Peters, M. F., Marvin, E., and Froehner, S. C. (1997) *J. Cell Biol.* **138(1),** 81–93.
6. Wakayama, Y., Inoue, M., Murahashi, M., Shibuya, S., Jimi, T., Kojima, H., and Oniki, H. (1997). *Acta Neuropathol.* **94,** 455–464.
7. Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., and Bredt, D. S. (1995) *Cell* **82,** 743–752.
8. Chao, D. S., Gorospe, J. R. M., Brenman, J. E., Rafael, J. A., Peters, M. F., Froehner, S. C., Hoffman, E. P., Chamberlain, J. S., and Bredt, D. S. (1996) *J. Exp. Med.* **184,** 609–618.
9. Chang, W. J., Iannaccone, S. T., Lau, K. S., Masters, B. S. S., McCabe, T. J., McMillan, K., Padre, R. C., Spencer, M. J., Tidball, J. G., and Stull, J. T. (1996). *Proc. Natl. Acad. Sci. USA* **93,** 9142–9147.
10. Silvagno, F., Xia, H., and Bredt, D. S. (1996) *J. Biol. Chem.* **271(19),** 11204–11208.
11. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
12. Frandsen, U., Lopez-Figueroa, M., and Hellsten, Y. (1996) *Biochem. Biophys. Res. Comm.* **227,** 88–93.
13. Alnaqueeb, M. A., and Goldspink, G. (1987) *J. of Anat* **153,** 31–45.
14. Brown M. (1987) *Age and Ageing* **16,** 244–248.
15. Grozdanovic, Z., Christova, T., Gosztony, G., Mellerowicz, H., Blottner, D., and Gossrau, R. (1997) *Histochem. J.* **29,** 97–104.
16. Tews, D. S., Goebel, H. H., Schneider, I., Gunkel, A., Stennert, E., and Neiss, W. F. (1997) *J. Neuropathol. Exp. Neurol.* **56(12),** 1283–1289.
17. Ma, S. X., Holley, A. T., Sandra, A., Cassel, M. D., and Abboud, F. M. (1997) *Neuroscience* **76(3),** 659–663.
18. Ventura, S., and Burnstock, G. (1996) *Cell Tiss. Res.* **285,** 427–434.
19. Kanda, K. (1996) *Neurosci. Lett.* **219,** 41–44.
20. Lee, M. A., Cai, L., Huebner, N., Lee, Y. A., and Linpainter K. (1997) *J.Clin. Invest.* **100(6),** 1507–1512.
21. Xie, J., Roddy, P., Rife, T. K., Murad, F., and Young, A. P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11242–1246.
22. Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996) *Cell* **84,** 757–767.
23. Bredt, D. S., Ferris, C. D., and Snyder, S. H. (1992) *J. Biol. Chem.* **267(16),** 10976–10981.
24. Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C. L., and Marsden, P. A. (1994) *J. Biol. Chem.* **269(52),** 33082–33090.