BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 88–93 (1996)
ARTICLE NO. 1472

Localization of Nitric Oxide Synthase in Human Skeletal Muscle

Ulrik Frandsen,*[,1] Manuel Lopez-Figueroa,† and Ylva Hellsten*

*Copenhagen Muscle Research Centre, The August Krogh Institute, University of Copenhagen, Denmark; and †Institute of Medical Anatomy, University of Copenhagen, Denmark

Received August 29, 1996

The present study investigated the cellular localization of the neuronal type I and endothelial type III nitric oxide synthase in human skeletal muscle. Type I NO synthase immunoreactivity was found in the sarcolemma and the cytoplasm of all muscle fibres. Stronger immunoreactivity was expressed in the sarcolemma as well as the cytoplasm of type I muscle fibres. NADPH diaphorase activity confirmed a higher level of NO synthase activity in the sarcolemma as well as the cytoplasm of type I muscle fibers. Histochemical staining for cytochrome oxidase showed a staining pattern similar to that observed for type I NO synthase immunoreactivity and NADPH diaphorase activity. Type III NO synthase immunoreactivity was observed both in the endothelium of larger vessels and of microvessels. The results establish that human skeletal muscle expresses two different constitutive isoforms of NO synthase in different cellular compartments and suggest that NO may have specific actions in relation to its site of production. The localization of type I NO synthase in the vicinity of mitochondria supports a specific action of NO on mitochondrial respiration, whereas the localization of type III NO synthase in vascular endothelium is consistent with a role for NO in the control of blood flow in human skeletal muscle.

Nitric oxide (NO) is a short lived substance known to participate in neuronal signalling (1), smooth muscle relaxation (2) and cytotoxic and bactericidal actions of activated macrophages (3). NO derives from L-arginine as the product of a complex enzymatic reaction (4) catalysed by at least three isoforms of NO synthase purified from cDNA cloning of brain (type I NO synthase), macrophage (type II NO synthase) and endothelial forms (type III NO synthase) (5). The neuronal type I and endothelial type III NO synthases are activated by Ca^{2+} influx, which in turn, is controlled by Ca^{2+} channels in the target cell. These channels open in response to substances such as glutamate, bradykinin and acetylcholine as well as shear stress of flowing blood (6).

In skeletal muscle it has been shown that NO potentially generated from vascular endothelium contributes to the control of blood flow (7, 8). Furthermore, NO generation in skeletal muscle may inhibit oxygen consumption by mitochondria through reaction with cytochrome oxidase (8). In addition, studies on rat diaphragm indicate that excitation-contraction coupling is altered by manipulating NO levels where inhibitors of NO formation increase twitch and submaximal tetanic force production, whereas NO donors decrease force production (9). In a recent study type I NO synthase immunoreactivity was found in the sarcolemma of rat type II muscle fibers (9). This association of type I NO synthase with skeletal muscle membrane has been proposed to be mediated by binding of type I NO synthase to syntrophin, a dystrophin associated protein (10, 11). As syntrophin and type I NO synthase are highly expressed in neuromuscular junctions, it has furthermore been suggested that NO may be important in synaptic signalling (11). Endothelial type III NO synthase has been demonstrated to be located in vascular endothelium, cytoplasm of mitochondria-rich fibers (12) and within mitochondria

[1] To whom correspondence should be addressed at Copenhagen Muscle Research Centre, The August Krogh Institute, Universitetsparken 13, DK- 2100 Copenhagen, Denmark. Fax: 45-35 32 15 67.

0006-291X/96 $18.00

of rat muscle (13). The immunohistochemical localization of type I and type III NO synthase has primarily been performed on rat and mouse skeletal muscle preparations. The precise localization of various NO synthase isoforms has not been described for healthy human skeletal muscle and the purpose with the current study was therefore to investigate the cellular source of the neuronal type I and endothelial type III NO synthase in healthy human skeletal muscle. Because NO is a short lived free radical, the initial action of NO are limited to the vicinity of the production site. Thus, the precise cellular localization of NO synthase may give additional information of the potential role of NO in human skeletal muscle.

MATERIALS AND METHODS

Tissue preparations. Muscle biopsies were obtained from the vastus lateralis muscle of 5 young students using a Bergstrøm needle with suction. The study conformed with the code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the local Ethics Committee. The biopsy samples were mounted in an embedding medium and frozen in isopentane cooled in liquid nitrogen for subsequent immunohistochemical and histochemical analysis. The biopsy material was stored at − 80°C until time of analysis.

Immunohistochemical procedure. The distribution of NO synthase immunoreactive cells was determined immunohistochemically with the use of a monoclonal mouse antibody specific to endothelial type I NO synthase obtained from Transduction Laboratories (Lexington, KY) and a polyclonal rabbit antibody specific to neuronal type I NO synthase obtained from Euro Dianogstica (Malmø, Sweden). Frozen muscle biopsies cut at 8 μM were fixed in 2% formaldehyde for 5 min. The sections were then carefully rinsed in 0.01 M TBS and incubated for 30 min with 0.01M TBS containing 1% bovine serum albumin. After rinsing in 0.01 M TBS the sections were incubated with the primary antibody specific to type I NO synthase (5 μg/ml) and type I NO synthase (1:6000) overnight at 4 C. After rinsing of the excess antibody with 0.01 M TBS, biotinylated goat-anti mouse (DAKO Code No. E 433) or goat-anti rabbit immunoglobulin (DAKO Code No. E 433) diluted 1:400 and 1:600 in 0.01 M TBS was applied for 45 min. After rinsing of the excess secondary antibody with 0.01 M TBS, Avidin Biotinylated Alkaline Phophatase (ABComplex/AP DAKO Code No. K 376) was applied for 30 min or Streptavidin-sulforhodamine diluted 1:500 (Boehringer Mannheim GmbH, code No 1131575) was applied for 30 min or 60 min, respectively. Biotinylated alkaline phophatase was visualised by addition of New Fuchsin chromogen (DAKO Code No K 698) for 5 min. Levamisol was added to block the activity of endogenous alkaline phosphatase. The sections were mounted in glycerine. Specificity of the staining for the antibodies used was assessed by staining with streptavidin-sulforhodamine, without primary antibody and staining without secondary antibody. Immunoreactive cells were examined and photographed under bright field illumination in a photo microscope (Nikon Microphot-FXA, Nikon corporations, Tokyo). Nomarski optics were used to facilitate identification of the structures. Triton X-100 was avoided in the TBS/BSA buffer in order not to release NO synthases from membrane components.

NADPH diaphorase activity. Frozen muscle biopsies cut at 8 μm were fixed in 4% formaldehyde for 10 min and rinsed with PBS. The cryostat sections were then covered with a reaction mixture consisting of 0.1 mg/ml nitro blue tetrazolium, 1mg/ml β-NADPH and 0.3 % Triton X-100 in PBS pH 7.4 for 60 min at 37°C. After rinsing in PBS, sections were mounted and analysed as described above. Triton X-100 was added to the incubation media as Triton X-100-containing media leads to more extraction of formazan produced by NO synthase related NADPH diaphorases than of formazan generated by NADPH diaphorases not related to NO synthase (14). In fact, when substituting Triton X-100 (0.3%) with saponin (0.1 %) in the incubation media, lack of NO synthase related NADPH diaphorase activity was observed in sarcolemma of skeletal muscle fibers. Specificity of the staining was assessed by staining without NADPH in the incubation media.

Cytochrome oxidase activity. The method described by Burstone (15) was used to demonstrate cytochrome oxidase activity. Frozen muscle biopsies cut at 8 μm were covered with a reaction medium consisting of 0.2 mg/ml p-aminodiphenylamine, 0.2 mg/ml 8-hydroxy -1,4-naphthoquinone dissolved first in 50 μl absolute ethanol/mg reagent, and then in TRIS buffer, pH 7.4. Cytochrome C (0.2 mg/ml) was added to the solution after filtration. The sections were incubated for 30 min and then covered with a chelating and fixing solution consisting of 100 mg/ml Cobalt's acetate in 4% formaldehyde. After rinsing in PBS, sections were mounted and analysed as described above. Specificity of the staining was assessed by inhibiting cytochrome oxidase with 10^{-3} M sodium azide in the incubation media.

Muscle fiber ATPase activity. The method described by Brooke & Kaiser (16) was used to classify type I and type II muscle fibres.

RESULTS

In human vastus lateralis muscle, neuronal type I NO synthase immunoreactivity was found in the sarcolemma and cytoplasm of all muscle fibers (Fig. 1 A.). Stronger type I NO synthase immunoreactivity was evident in the sarcolemma as well as the cytoplasm of type I compared

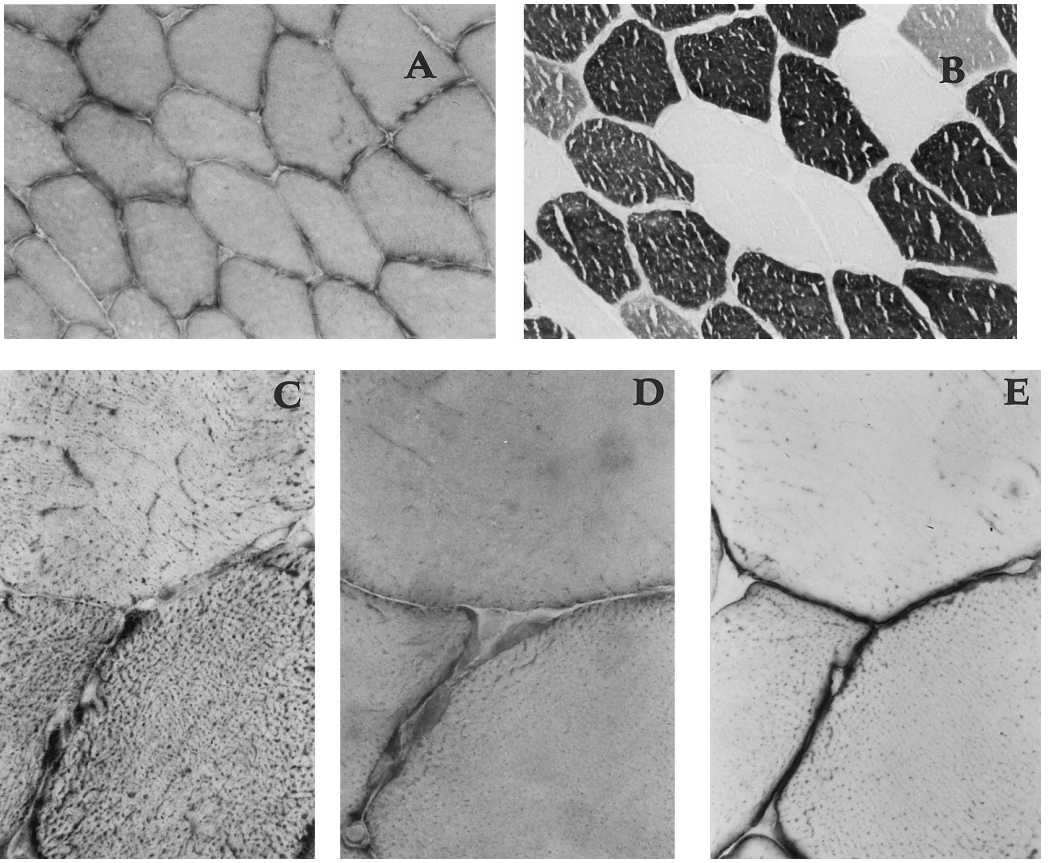

FIG. 1. Micrographs of immunohistochemically and histochemically stained serial sections from the human vastus lateralis muscle. (A) Neuronal type I NO synthase immunoreactivity shows a greater expression in the cytoplasma as well as the sarcolemma of muscle fiber type I (B; darkly stained fibers). Original magnification, 250×. (C) Cytochrome oxidase staining shows a close relationship to (D) neuronal type I NO synthase immunoreactivity and (E) histochemical NADPH diaphorase activity. Original magnification, 750×.

to type II muscle fibers (Fig. 1 A.), as assessed by histochemical ATP′ase staining of serial sections (Fig. 1 B.). A greater activity of NO synthase in the sarcolemma and the cytoplasm of type I compared to type II fibers was found when staining for NADPH diaphorase activity. Histochemical staining for cytochrome oxidase, an enzymatic marker of mitochondria, showed presence of mitochondria in both the type I and type II muscle fibers (Fig.1 C.). Cytochrome oxidase activity (Fig. 1 C.) appeared in a granular pattern similar to that observed with the type I NO synthase immunohistochemical stain (Fig. 1 D.) and the NADPH diaphorase stain (Fig. 1 E.). Endothelial type III NO synthase immunoreactivity was observed in the endothelium of larger vessels (Fig. 2 A.) as well as microvessels (Fig. 2 B.). None of the negative controls showed staining.

DISCUSSION

The present findings show that type I and type III NO synthase are expressed in the muscle cell and the vascular endothelium of human skeletal muscle, suggesting that NO is generated by both of these cell types. Our finding that human skeletal muscle cells express type I NO synthase in locations where also mitochondria are distributed is consistent with the proposed

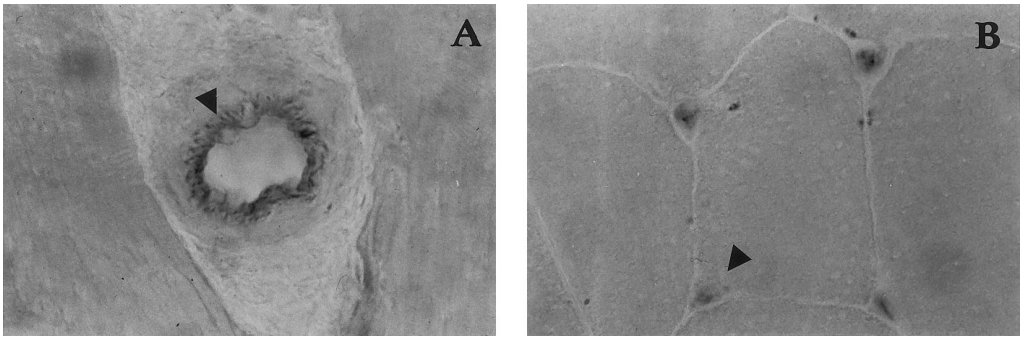

FIG. 2. Micrographs of immunohistochemically stained sections from the human vastus lateralis muscle. (A) Endothelial type III NO synthase immunoreactivity in the endothelium of a large vessel ► and (B) microvessels ►. Original magnification, 250×.

role for NO in mitochondria. It is known that NO exerts some of its physiological functions by suppressing mitochondrial respiration through competitive interaction with the oxygen-binding site of cytochrome oxidase (17, 18). This inhibition of mitochondrial phosphorylation in skeletal muscle would cause decreased ATP generation with a consequent attenuation in muscle oxygen consumption and muscle contractility. In accordance, it has been shown that oxygen uptake increased 30% in rabbit hindlimb (19) and 55% in dog hindlimb (8) following inhibition of NO synthesis by N^G-monomethyl-L-arginin and nitro-L-arginine, respectively. Additionally, it has been shown that blocking of NO synthesis in conscious dogs during treadmill running leads to an increase in oxygen extraction and consumption as compared to the control situation without blockage (20). Furthermore, inhibitors of NO formation may increase twitch and submaximal tetanic force production, whereas NO donors decrease force production (9). It has been proposed that NO formed in the microvascular endothelium is responsible for the inhibition of mitochondrial respiration and thus, the regulation of tissue oxygen consumption (21). Our present findings of a localization of type I NO synthase in the proximity of mitochondria in human skeletal muscle suggest that muscle respiration may be affected by NO generated in the close vicinity of mitochondria, which would allow for a shorter diffusion distance and possibly a more precise action of NO.

Human (7) and animal (20) studies have shown that NO may be involved in blood flow regulation in skeletal muscle through its vasodilatory action. A role for NO in blood flow-regulation can probably explain the present observation of localization of ((type III)) NO synthase in the microvascular endothelium as well as the endothelium of larger vessels in human muscle. The location of NO synthase in the vascular wall would appear optimal for rapid control of blood flow due to the direct contact between the endothelium and blood and through the proximity of the smooth muscle cells. The stimulus for NO release from vascular endothelial cells in skeletal muscle may be shear stress of flowing blood (22). In accordance, it has been suggested that an initial increase in blood flow mediated by an elevated metabolic demand of the muscle, causes an increase in shear stress which consequently stimulates NO production by vascular endothelium resulting in further vasodilation (23, 20). Thus, an important action of endothelium derived NO may be to act as an amplifier of vasodilation as previously suggested by Segal (22).

In contrast to findings on rat extensor digitorum longus (EDL) muscle (9), type I NO synthase was in the current study found to be not only expressed in the sarcolemma of type II muscle fibers but also in type I muscle fibers. Furthermore, type III NO synthase was not detected in cytoplasm of mitochondria-rich muscle fibers as previously described (12). The

discrepancy between previous findings on rat and the present could be due to species differences. However, preliminary results from our studies on rat EDL muscle have shown that the antisera used in the present study expresses type I NO synthase immunoreactivity in a granular pattern to the sarcolemma and the cytoplasm of type I, IIA and IIX muscle fibers but not type IIB muscle fibers, whereas it expressed type III NO synthase immunoreactivity on endothelium as well as the cytoplasm of mitochondria rich muscle fibers as previously observed (12); (U. Frandsen & Y. Hellsten, unpublished observation). In our studies on rat EDL muscle we, furthermore found that in accordance with our findings on human muscle, the distribution of type I immunoreactivity was similar to that of NO synthase activity as demonstrated by histochemical NADPH diaphorase staining, and similar to mitochondrial distribution as demonstrated by histochemical staining for cytochrome oxidase ((U. Frandsen & Y. Hellsten, unpublished observation)). Thus, our observations on rat EDL muscle support our findings on human skeletal muscle. It has been demonstrated (24) that the antisera used in the present study, raised against synthesized C- terminal fragments of the cloned type I NO synthase (25) has a higher specificity for type I NO synthase than the antisera raised against the whole enzyme (25), which was used in the immunohistochemical studies on rat skeletal muscle (9). Thus, differences in specificity between the antibody used by Kobzik et al. (9) and the antibody used in the present study could explain the different findings on cellular localization of type I NO synthase in rat compared to human skeletal muscle in the current study.

In conclusion, the present data demonstrate that two different isoforms of NO synthase are located in healthy human skeletal muscle. Type I NO synthase is located in the sarcolemma and the cytoplasm of all muscle fibers but shows a greater expression in type I muscle fibers. Type III NO synthase is found in microvascular endothelium and the endothelium of larger vessels. Thus in human muscle, NO may be generated in sites that allow for direct release of NO into blood, interstitium and the muscle cell, particularly nearby mitochondria and the sarcolemma. The generation of NO in the vicinity of mitochondria would allow for a close diffusion distance to mitochondria, which would be beneficial for the action of NO on mitochondrial respiration as previously observed in animal muscle. The specific localization of type III NO synthase in vascular endothelium, a location that allow for a release of NO into the blood and the surrounding smooth muscle cells is consistent with the proposed role for NO in the control of blood flow in human skeletal muscle.

ACKNOWLEDGMENT

This study was supported by Grant 504-14 from the Danish National Research Foundation.

REFERENCES

1. Schumann, E. M., and Madison, D. V. (1994) *Annu. Rev. Neurosci.* **17,** 153–183.
2. Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987) *Nature* **327,** 524–526.
3. Hibbs, J. B., Jr., Taintor, R. R., and Vavrin, Z. (1987) *Science* **235,** 473–476.
4. Nathan, C., and Xie, Q. (1994) *Cell* **78,** 915–918.
5. Förstermann, U., Schmidt, H. H. H. W., Pollock, J. S., Sheng, H., Mitchell, J. A., Warner, T. D., Nakane, M., and Murad, F. (1991) *Biochem. Pharmacol.* **42,** 1849–1857.
6. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
7. Vallance, P., Collier, J., and Moncada, S. (1989) *The Lancet* 997–1000.
8. Shen, W., Hintze, T. H., and Wolin, M. S. (1995) *Circulation* **92,** 3505–3512.
9. Kobzik, L., Reid, M. B., Bredt, D. S., and Stamler, J. S. (1994) *Nature Lond.* **372,** 546–548.
10. Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., and Bredt, D. S. (1995) *Cell* **82,** 743–752.
11. Brenman, J. E., Chao, D. S., Gee, D. S., Magee, 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.
12. Kobzik, L., Stringer, B., Balligand, J.-C., Reid, M. B., and Stamler, J. S. (1995) *Biochem. Biophys. Res. Commun.* **211,** 375–381.
13. Bates, T. E., Loech, A., Burnstock, G., and Clark, J. B. (1996) *Biochem. Biophys Res. Commun.* **218,** 40–44.
14. Blottner, D., Grozdanovic, Z., and Grossrau, R. (1995) *Histochemical Journal* **27,** 785–811.

15. Burstone, M. S. (1961) *Journal of Histochemistry and Cytochemistry* **9,** 59–65.
16. Brooke, M. H., and Kaiser, K. K. (1970) *J. Histochem. Cytochem.* **18,** 670–672.
17. Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., and Schapira, A. H. (1994) *FEBS Lett.* **345,** 50–54.
18. Brown, G. C., and Cooper, C. E. (1994) *FEBS Lett.* **356,** 295–298.
19. King, C. E., Melinyshyn, M. J., Mewburn, J. D., Curtis, S. E., Winn, M. J., and Cain, S.
20. Shen, W., Lundborg, M., Wang, J., Stewart, J. M., Xu, X., Ochoa, M., and Hintze, T. H. (1994) *J. Appl. Physiol.* **77**(1), 165–172.
21. Shen, W., Zhang, X., Zhoa, G., Wolin, M. S., Sessa, W., and Hintze, T. H. (1995) *Med. Sci. Sports. Exerc.* **27,** 1125–1134.
22. Pohl, U., Herlan, K., Huang, A., and Bassenge, E. (1991) *Am. J. Physiol.* **261,** H2061–2023.
23. Segal, S. (1992) *News Physiol. Sci.* **7,** 152–156.
24. Alm, P., Larsson, B., Ekblad, E., Sundler, F., and Andersson, K.-E. (1993) *Acta Physiol. Scand.* **148,** 421–429.
25. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. *Nature* **351,** 714–718.