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The specificity of the histochemical NADPH diaphorase reaction for nitric oxide synthase-1 in skeletal muscles is increased in the presence of urea*

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Summary

Nitric oxide synthase-1 (NOS-1) can be demonstrated in the sarcolemma region of myofibers in rodent skeletal muscles with the use of NADPH diaphorase histochemistry. Since other, especially intrafibrar enzymes also exhibit NADPH diaphorase activity, we tried to increase the specificity of the histochemical reaction for NOS-1. A qualitative and quantitative analysis was performed on cryostat sections of fast-twitch oxidative myofiber-rich tongue and fast-twitch glycolytic myofibers-rich tibialis anterior muscle derived from C57 mice and NOS-1 deficient knockout mice. All myofibers of both C57 mice and NOS-1 knockout mice contained significant intrafibrar NADPH diaphorase activity which was inhibited to almost background levels when 2 M urea was added to the incubation medium. On the other hand, myofibers of C57 mice but not of NOS-1-deficient knockout mice exhibited NADPH diaphorase activity in their sarcolemma region which was only weakly reduced in the presence of 2 M urea as was demonstrated by image analysis. Quantitative data on the activity of NADPH diaphorase(s) were obtained *in situ* by photometric analysis of formazan extracted from cryostat sections. The catalytic activity in tongue and tibialis anterior muscle was reduced in presence of 2 M urea to approximately 27% in C57 mice and to 7–17% in NOS-1 knockout mice, respectively. An *in vitro* NADPH diaphorase assay performed on homogenates of skeletal muscles also revealed an inhibitory effect of 2 M urea in both mouse strains and, additionally, indicated an upregulation of NADPH diaphorase activity in NOS-1 knockout mice. Finally, an immunodepletion analysis demonstrated that NOS-1 comprises 38% of the total NADPH diaphorase activity in tongue and approximately 59% in tibialis anterior muscle in C57 mice. In conclusion, we recommend the addition of 2 M urea to the incubation medium to increase the specificity of the NADPH diaphorase reaction to localise NOS-1 with the use of catalytic histochemistry.

Key words: nitric oxide synthase-1-NADPH diaphorase-skeletal muscle-image analysis-quantitative catalytic histochemistry

Introduction

Skeletal muscles of mice, rats, humans and many other mammalian species are important sources of nitric oxide synthase-1 (NOS-1) which produces the potent signalling molecule NO. The NOS-1/NO-system in skeletal muscle is involved in the establishment of the steady state in the energy metabolism at many levels (reviewed in Reid, 1998; Stamler and Meissner, 2001). NOS-1 is concentrated in the sarcolemma region of

* Dedicated to Professor Reinhart Gossrau on the occasion of his 61st birthday

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myofibers where it is particularly clustered in junctional parts such as costameres (Gossrau 1998; Baum et al., 2000), neuromuscular junctions (Kusner and Kaminski, 1996; Grozdanovic and Gossrau, 1998) and myotendinous junctions (Chang et al., 1996). The targeting of the enzyme to the sarcolemma is primarily mediated by its N-terminal PDZ domain which links NOS-1 to α1-syntrophin and, thus, integrates the enzyme into the dystrophin glycoprotein complex (Brenman et al., 1996). Not all myofibers in skeletal muscles contain equal amounts of NOS-1. In rats, the highest concentrations of NOS-1 are found in fast-twitch oxidative (FOG; type IIa) myofibers, whereas fast-twitch glycolytic (FG; type IIb) myofibers exhibit significantly lower levels of NOS-1 (Planitzer et al., 2001). Slow-twitch oxidative (SO; type I) myofibers contain the lowest amounts of NOS-1 (Grozdanovic et al., 1995; Kapur et al., 1997; Planitzer et al., 2001).

Qualitative and quantitative data on NOS-1 expression in skeletal muscles have been obtained with the application of various methodological approaches. Immunoblotting of tissue homogenates has been performed to determine the NOS-1 concentration in different skeletal muscles (Kapur et al., 1997; Hussain et al., 1997; Planitzer et al., 2001). Immunohistochemistry has demonstrated the presence of NOS-1 in the sarcolemma region (Kobzik et al., 1994; Grozdanovic et al., 1995) and possibly in the sarcosol of myofibers (Punkt et al., 2001).

Immunological methods such as immunoblotting and immunohistochemistry allow only the characterisation of the expression patterns of enzymes but do not yield information on their catalytic activity *in situ*. Various assays have been developed to monitor the biological activity of NOS (reviewed in Feelisch and Stamler, 1996). Approaches to directly quantify the NO-production in biological samples include methods based on the Gries reaction (Schmidt et al., 1990) or the oxyhemoglobin assay (Murphy and Noack, 1994). Alternatively, indirect methods such as ELISA-based or bioassay-based techniques to determine cGMP-levels produced by soluble guanylate cyclase(s) in response to NO activation have been successfully applied to measure NOS-activity (Ishii et al., 1991). The most specific and probably most popular method to determine NOS activity is the citrulline assay detecting the amount of radiolabelled citrulline co-generated with NO from L-arginine in homogenates of tissues or cultured cells with sensitivity in the picomolar range (Bredt et al., 1991). Recently, derivatives of 4,5-diaminofluorescein (DAF) have been introduced to detect NO generation in intact cultured cells or sections of neuronal tissues (Kojima et al., 1998). However, these methods to quantify the activity of NOS have specific disadvantages (reviewed in Feelisch and Stamler, 1996). They are either only semi-quantitative, indirect, require expensive equipment and/or are applicable only on cell extracts or homogenates so that the micro-environment of NOS-1 *in situ* is lost.

The catalytic histochemical assay based on the NADPH diaphorase reaction represents a simple method that was originally used to directly demonstrate the presence of NOS with high sensitivity and reliability in cryostat sections of the brain (Dawson et al., 1991; Hope et al., 1991). Image analysis allows the rapid quantification of the catalytic activity (Planitzer et al., 2001). All known NOS proteins/isoforms are able to reduce NADPH-dependently water-soluble tetrazolium salts such as nitroblue tetrazolium to water-insoluble formazan with their common C-terminal located reductase domain and are, therefore, identified as NADPH diaphorases (Bredt et al., 1991). However, the histochemical method is not specific for NOS-1 since several other enzymes catalyse the NADPH diaphorase reaction in many tissue including skeletal muscle (Stoward et al., 1991; Tracey et al., 1993; Blottner et al., 1995; Planitzer et al., 2000).

Several attempts had been made to increase the specificity of the histochemical NADPH diaphorase reaction for NOS-1. This can be semi-quantitatively achieved when the incubation is performed on fresh cryostat sections in the presence of formaldehyde (Nakos and Gossrau, 1994), thiol inhibitors, aldehydes, alcohols, oxidising agents and NADPH analogues (reviewed in Gossrau et al., 1996) or urea and other chaotropic agents (Gossrau, 1999). However, the effects of the inhibitory compounds on NOS-1 were not quantified in these studies, what is required if the histochemical NADPH diaphorase reaction is used for the analysis of catalytic activity.

We have performed a thorough quantitative study to optimise the specificity of the histochemical NADPH diaphorase reaction for NOS-1. With the application of image analysis to NOS-1-deficient knockout mice and corresponding control animals, we demonstrate a strong inhibitory effect of urea on all non-NOS-1 NADPH diaphorases as compared with only a weak influence on NOS-1-related NADPH diaphorase activity in NOS-1-rich FOG and FG myofibers. Consequently, we recommend the use of 2 M urea in the incubation medium to increase the specificity of the histochemical NADPH diaphorase reaction for NOS-1 in skeletal muscles.

Material and methods

Antibodies

All immunological methods that were applied to analyse NOS-1 expression in skeletal muscles (immunohistochemistry, immunoprecipitation and immunoblotting) were performed with a peptide-specific polyclonal anti-

serum raised against the C-terminal amino acids 1409–1429 of rat NOS-1 (Sigma, Munich, Germany) cross-reacting with mouse NOS-1.

Animals and tissue preparation

NOS-1 knockout mice, which were originally obtained from Jackson Laboratories (Bar Harbor ME, USA), and the corresponding C57/Bl6 control strain were bred under standardised conditions. Seven healthy male mice of both strains (6 months old) were used for this study.

For preparation of tissue samples, mice were sacrificed under deep ether anesthesia by excision of the heart. The tongue and tibialis anterior (TA) muscles were quickly removed. To prevent interference of NADPH diaphorase-dependent formazan production in epithelial cells with formazan production in myofibers, epithelial layers were pealed off from the tongues as much as possible. For histochemistry, muscle samples were mounted on cork plates with TissueTek, frozen in liquid nitrogen-cooled methylbutane and stored in closed plastic bags at –40 °C until use. For biochemical analysis, the samples were transferred to reaction tubes, frozen in liquid nitrogen and stored at –40 °C until use.

Catalytic histochemistry

NADPH diaphorase activity was visualised in cryostat sections as reported earlier (Planitzer et al., 2001) using an incubation medium containing 1 mg/ml β-NADPH (Biomol, Hamburg, Germany), 0.25 mg/ml nitroblue tetrazolium and 0.3% (v/v) Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.4. Variation of the concentrations of the compounds in the incubation medium (NADPH, nitroblue tetrazolium) and the addition of polyvinyl alcohol as diffusion protectant did not improve the discrimination between NOS-1 and non-NOS-1 NADPH diaphorase activity (G. Planitzer, thesis, Free University Berlin, in press). When indicated, this medium was supplemented with urea. Cryostat sections were incubated for 1 h in a moist chamber at 37 °C, rinsed several times in phosphate buffered saline, pH 7.4 (PBS) and then cover-slipped in glycerol jelly. Controls performed in the absence of β-NADPH did not produce formazan.

To obtain quantitative data on NADPH diaphorase activity in skeletal muscles *in situ*, formazan was extracted from cryostat sections as described before (Planitzer et al., 2001). Briefly, 40 cryostat sections of either tongue or TA muscles (thickness, 10 µm) were incubated to detect NADPH diaphorase activity in the absence or presence of 2 M urea as described above. Subsequently, the sections were scraped off the glass slides, transferred to reaction tubes and digested for 18 h with a solution of 1% (w/v) collagenase (Merck, Darmstadt, Germany). An almost homogeneous solution of dissolved formazan was obtained by ultrasonication for 30 min. The absorbance of this solution was measured in a standard laboratory photometer at 572 nm. Absorbance and mean protein content (determined in serial sections) were used to calculate the relative NADPH diaphorase activity expressed as ΔE/mg protein/30 min. Control experiments to obtain blank values for photometry were performed in the absence of β-NADPH.

Immunohistochemistry

The immunohistochemical detection of NOS-1 was performed as described before (Planitzer et al., 2000, 2001). Briefly, cryostat sections (thickness, 10 µm) were incubated after formaldehyde fixation in a solution of 5% (w/v) bovine serum albumin to block nonspecific binding and were then incubated with the primary anti-NOS-1 antibody in a dilution of 1:1,000 overnight. Immunopositive sites in skeletal muscle tissues were visualised with a secondary Cy3-conjugated antibody (Jackson ImmunoResearch, West Grove PA, USA). Control incubations were carried out by replacing the primary antibody with an IgG fraction of a pre-immune serum in the corresponding concentration (Sigma) and did not produce immunoreactivity.

Image analysis

As equipment, an Axioskop microscope (Zeiss, Oberkochen, Germany) in combination with a F10 CCD camera (Panasonic, Hamburg, Germany) was used which was connected via a PCI digitizer card (Miro, Braunschweig, Germany) to a G3 Desktop 233 computer (Apple, Cupertino CA, USA). The public domain software Object Image 1.62p2 (written by N. Fischer, University of Amsterdam, Netherlands, available from the web at http://simon.bio.uva.nl/object-image.html) was applied. Further details of image analysis to determine initial reaction rates (V_{init}) of NADPH diaphorase activity in cryostat sections have been described previously (Planitzer et al., 2001). Briefly, cryostat sections of tongues and TA muscle (thickness, 10 µm) were incubated to demonstrate NADPH diaphorase activity at room temp on the scanning stage of the microscope. A digital image was captured after every min during the first 10 min of the incubation, and then after every 5 min up to a total of 40 min. Tongues were used for the analysis of FOG myofibers, and TA muscles for the investigation of FG myofibers.

For the determination of fiber types, serial sections of the skeletal muscles were incubated for myosin heavy chain immunohistochemistry and succinate dehydrogenase (SDH) histochemistry. By semiquantitative evaluation of SDH-dependent formazan production, individ-

ual myofibers were identified to be either SO, FOG or FG fiber types as described earlier (Planitzer et al., 2001).

For analysis, 5 FOG myofibers were selected in each section and their area marked using the computer mouse. For the determination of NADPH diaphorase activity in the sarcolemma, a macro was written in the built-in macro language to select the area precisely. The time-dependent formazan generation in defined pixels was expressed in grey levels that was recorded and converted into optical density values (absorbance) with the use of a grey level standard (Zeiss). The data obtained were described as a quadratic function using a least square fitting method according to Jonker et al. (1995). The first derivative of this function at $t_0 = 0$ represented V_{init} and was taken as a measure of NADPH diaphorase activity.

In vitro assay of NADPH diaphorase activity

Tissue samples of tongue and TA muscle were homogenised with a micro-douncer (Braun, Melsungen, Germany) in a 10-fold volume of solubilisation buffer (0.1 M Tris-HCl, pH 7.4 containing 0.5% (v/v) Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and a standard protease inhibitor mixture). Homogenates were then centrifuged in a standard laboratory centrifuge (1 min at 1.000 g) to obtain a solubilisate fraction in the supernatant in which NOS-1 is found (Planitzer et al., 2001). An aliquot of the supernatant (500 µl) was mixed and incubated with 500 µl of a 2-fold concentrated NADPH diaphorase medium (20 mg/ml β-NADPH, 0.5 mg/ml nitroblue tetrazolium and 0.5% (v/v) Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.4) at 37 °C for 30 min. This medium was supplemented with 4 M urea when required (resulting in a 2 M final concentration). Absorbance of the formazan produced was measured at 575 nm immediately after the reaction had been stopped on ice. Blanks for photometry were obtained from incubations in the absence of NADPH. An additional aliquot of the solubilisate was used for determination of the protein concentration using the BCA method (Pierce, Rockford IL, USA). Combination of both values allowed the calculation of the relative NADPH diaphorase activity in the solubilisates expressed as ΔE/mg protein/30 min.

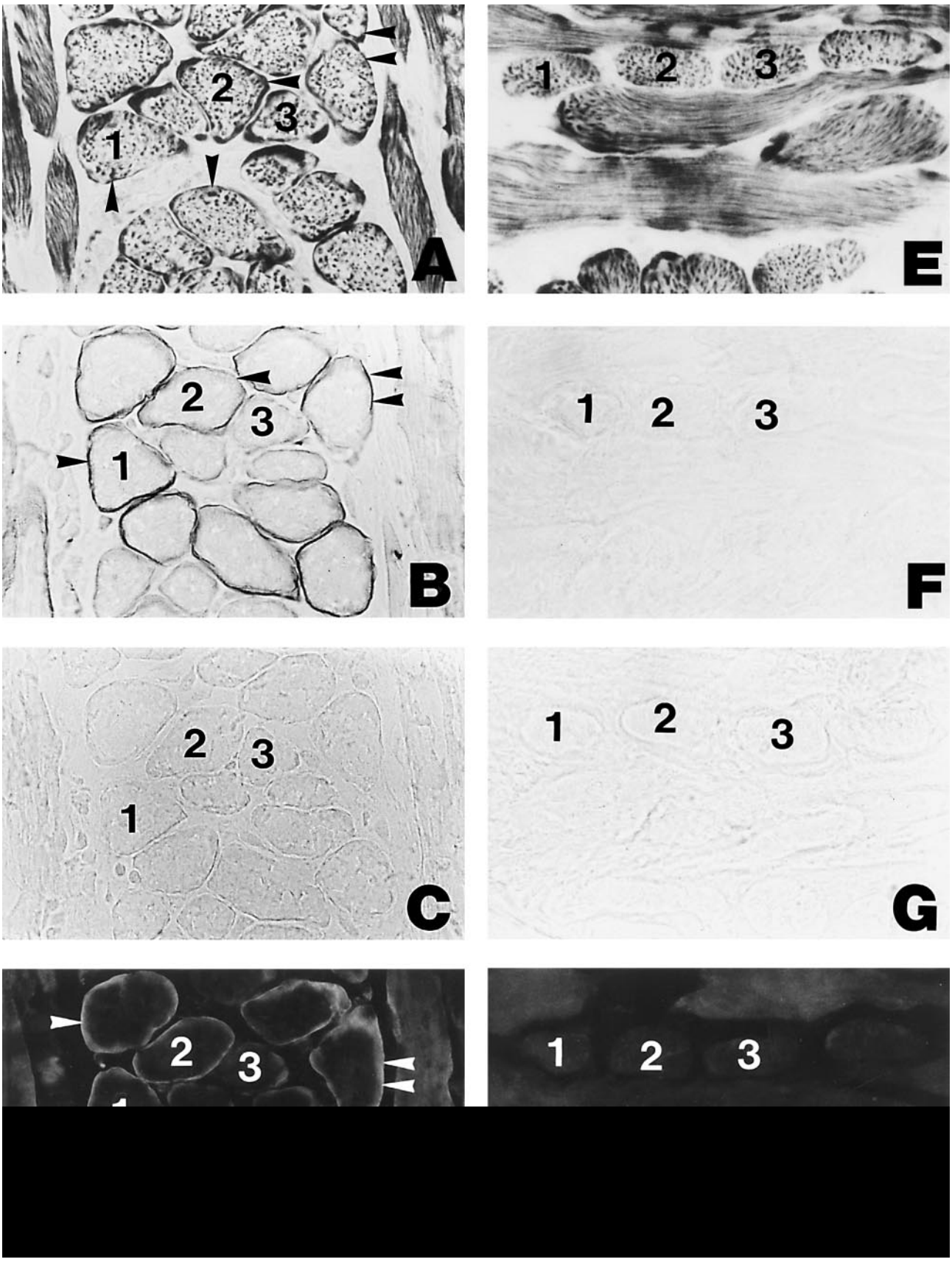
Immunoprecipitation and immunoblot analysis

For immunoprecipitation of NOS-1, 10 µg of the polyclonal anti-NOS-1 antibody were coupled to 15 mg protein A-sepharose 4B (Sigma, Munich, Germany) in buffer A (0.5 M Tris-HCl, pH 7.4 containing 0.5% (v/v) Triton X-100, 1 mM EDTA, 1 mM PMSF) for 1 h at room temp. Subsequently, the isolated antibody-sepharose-conjugate was added to 0.1 ml of solubilised tissue (tongue or TA muscle) derived from C57 mice for 16 h at 4 °C. The antigen/antibody-sepharose complexes were then pelleted in a standard laboratory centrifuge (1 min at 1.000 g) to obtain NOS-1-depleted extracts in the supernatant. Aliquots of the original solubilised tissues, the NOS-1-depleted extracts and the antigen/antibody-sepharose pellet were incubated to determine the NADPH diaphorase activity in the *in vitro* assay. Other aliquots were analysed by immunoblotting of NOS-1 which was performed as described before (Planitzer et al., 2001) using an ECL detection kit (Amersham, Braunschweig, Germany).

Results

The histochemical analysis of NADPH diaphorase activity in the absence of urea in cryostat sections of the tongue (used as a muscle which consists exclusively of FOG myofibers) from C57 mice showed strong intracellular and sarcolemma-associated formazan production in all myofibers (Fig. 1A). In cross sections, intracellular formazan-positive sites appeared as dots that were significantly larger than formazan granules connected by fine lines. These structures were identified as mitochondria and endoplasmic reticulum on the basis of immunohistochemistry with antibodies against defined markers (data not shown; described in Planitzer et al.,

Fig. 1. Histochemical localisation of NADPH diaphorase activity in cryostat sections of tongue derived from C57 mice and NOS-1 knockout mice in the absence or presence of urea. Serial cryostat sections of tongue from either C57 mice (**A–D**) or NOS-1-deficient knockout mice (**E–H**) were incubated to demonstrate NADPH diaphorase activity in the absence (**A, E**) or presence of 2 M (**B, F**) or 4 M (**C, G**) urea, respectively. In C57 mice (**A**), the diaphorase-dependent formazan production in the absence of urea is present in the sarcolemma (arrowheads) and inside the myofibers as dots and fine lines representing mitochondria and the endoplasmic reticulum. In NOS-1 knockout mice (**E**), mitochondria and endoplasmic reticulum but not the sarcolemma showed formazan precipitation in the absence of urea. Addition of 2 M urea to the incubation medium inhibited almost completely the non-sarcolemma diaphorase activity in both mouse strains (**B, F**). In the presence of 4 M urea (**C, G**), NADPH diaphorase-produced formazan was not detectable. Immunohistochemistry with a polyclonal antibody demonstrated NOS-1 expression in the sarcolemma region (arrowheads) of myofibers of C57 mice (**D**) but not of NOS-1 knockout mice (**H**). The numbers 1–3 were added to allow the identification of defined myofibers in serial sections. **C** and **G** were photographed with double refraction. Bar in D, 20 µm.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2000). Outside myofibers, NADPH diaphorase activity was detected in neurons only. Other cell types in tongue (satellite cells, fibroblasts, pericytes, endothelial cells) did not express any significant NADPH diaphorase activity.

The addition of 2 M urea to the NADPH diaphorase incubation medium resulted in an almost exclusive sarcolemma-associated staining since only low levels of formazan were observed in the myofibers (Fig. 1B). Higher concentrations of urea such as 4 M completely abolished the generation of formazan in the tongue (Fig. 1C). Immunohistochemistry revealed that the sarcolemma-associated NADPH diaphorase-positive sites corresponded with those that were positive for NOS-1 (Fig. 1D).

When tongue from NOS-1 knockout mice was incubated to demonstrate NADPH diaphorase activity in the absence of urea, only mitochondria and endoplasmic reticulum were formazan-positive in myofibers whereas the sarcolemma was formazan-negative (Fig. 1E). The presence of 2 M or 4 M urea in the incubation medium abolished formazan production in all myofibers (Fig. 1F-G). As expected, NOS-1 immunohistochemistry showed that NOS-1 was not present (Fig. 1H).

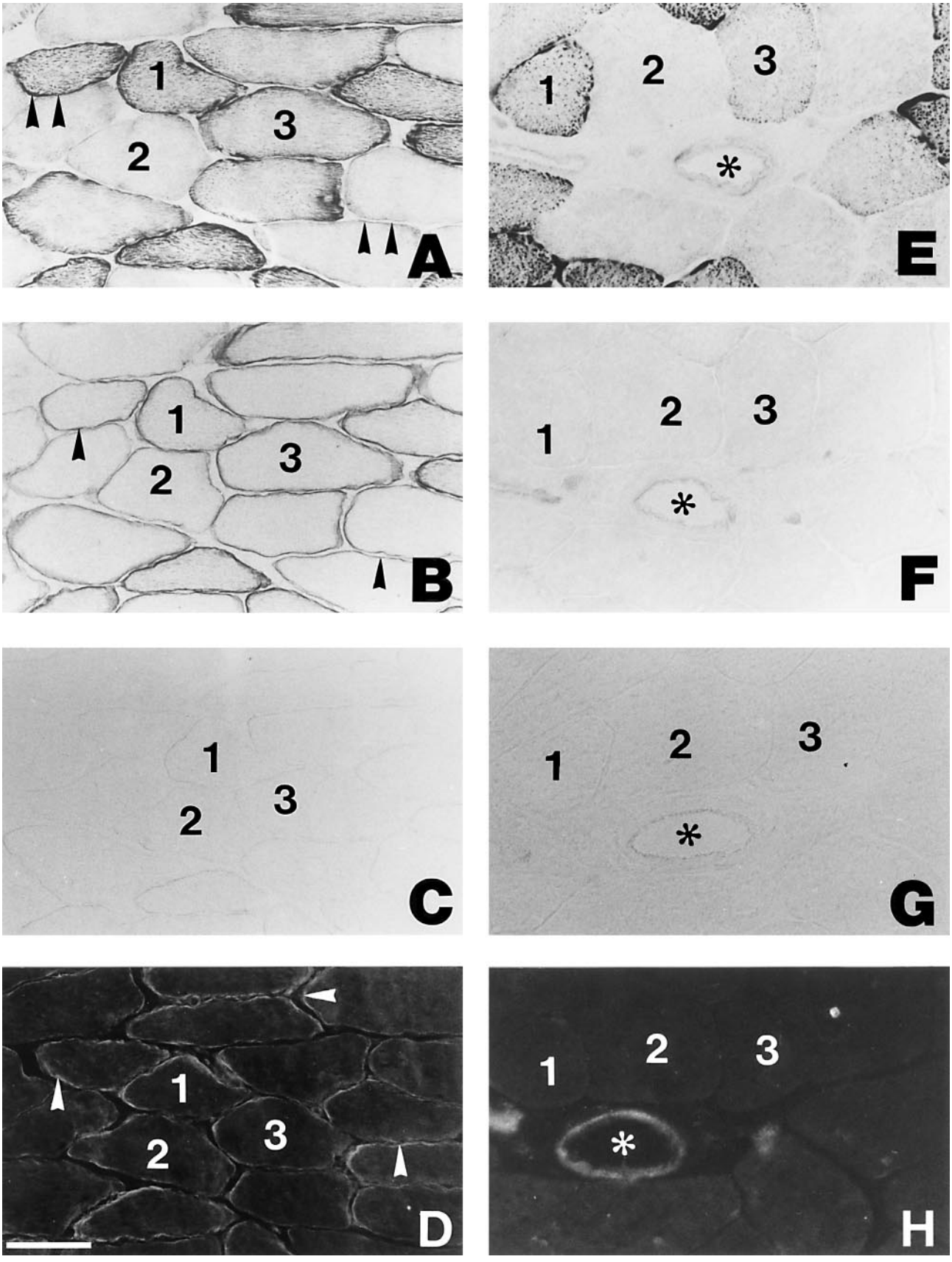
The histochemical analysis was performed on cryostat sections of TA muscle as well (Fig. 2). This type of skeletal muscle consists of approximately 50% FG myofibers, which can be identified in serial sections by the combination of myosin heavy chain immunohistochemistry and succinate dehydrogenase histochemistry (data not shown; described in Planitzer et al., 2001). In the absence of urea, strong NADPH diaphorase activity was present in the sarcolemma region of TA muscle from C57 mice. Additionally, mitochondria and endoplasmic reticulum in the myofibers showed formazan production. However, FG myofibers contain less mitochondria as compared with FOG and SO fibers and, consequently, contained only low intracellular NADPH diaphorase activity. Therefore, a variable pattern of intrafibrar formazan production was observed: FOG and SO myofibers showed strong NADPH diaphorase activity whereas FG myofibers were characterised by their low levels of formazan production in C57 mice (Fig. 2A) and NOS-1 knockout mice (Fig. 2E).

When 2 M urea was added to the incubation medium, the intrafibrar NADPH diaphorase activity was almost completely inhibited in TA muscles of both mouse strains. Accordingly, only very low levels of formazan were present in NOS-1 knockout mice (Fig. 2F), whereas a strong sarcolemma-associated staining was found in C57 mice (Fig. 2B). The presence of 4 M urea in the incubation medium abolished formazan production in all myofibers of both mouse strains (Fig. 2C,G). Specific NOS-1 immunoreactivity was restricted to the sarcolemma in C57 mice and was absent in NOS-1 knockout mice (Fig. 2D,H).

Although catalytic histochemistry demonstrated that the addition of at least 2 M urea to the incubation medium inhibited almost completely the non-sarcolemma NADPH diaphorase activity in both FOG and FG myofibers, it remained unclear whether the catalytic activity was also affected in the sarcolemma region. To address this question, we applied image analysis to monitor differences in the amount of NADPH diaphorase-dependent generation of formazan (expressed as absorbance) in the sarcolemma region of individual myofibers in either the absence or presence of 2 M urea. Fig. 3 shows the nonlinear reaction as exemplified for tongue of C57 mice. Therefore, V_{init} were taken as parameter for the velocity of the reaction (Table 1). In C57 mice, the V_{init} of NADPH diaphorase activity was higher in the absence than in the presence of 2 M urea in the sarcolemma region (in FOG myofibers, 31% inhibition and in FG myofibers, 26% inhibition was found). In the non-sarcolemma region, the inhibition rates were 81% and 85%, respectively. These results demonstrate a strong inhibitory effect of urea on all non-sarcolemma-associated NADPH diaphorases (to almost background levels) as compared with a remarkably lower effect on the sarcolemma-associated NADPH diaphorase activity.

Quantitative data on the activity of NADPH diaphorase(s) in skeletal muscles of both C57 mice and NOS-1 knockout mice *in situ* were obtained by extrac-

Fig. 2. Histochemical localisation of NADPH diaphorase activity in cryostat sections of tibialis anterior (TA) muscle from C57 mice and NOS-1 knockout mice in the absence or presence of urea. Serial cryostat sections of TA muscle from either C57 (**A–D**) or NOS-1-deficient knockout mice (**E–H**) were incubated to demonstrate NADPH diaphorase activity in the absence (**A, E**) or presence of 2 M (**B, F**) or 4 M (**C, G**) urea, respectively. Immunohistochemistry with a polyclonal antibody showed NOS-1 expression in the sarcolemma region of myofibers (arrowheads) of C57 mice (**D**) but not NOS-1 knockout mice (**H**). The numbers 1–3 were added to allow identification of defined myofibers in serial sections: numbers 1 and 3 represent FOG myofibers and number 2 a FG myofiber in A–D, while number 1 is a FOG myofiber, number 2 a FG myofiber and number 3 a SO myofiber in E-H. Note the lower amount of sarcolemma-associated formazan in myofibers of C57 mice with the lowest non-sarcolemma NADPH diaphorase activity (e.g. number 2) which are FG myofibers. The asterisk in E–H marks a vessel that was non-specifically positive for NOS-1 (**H**) with the secondary antibody as demonstrated in control experiments (data not shown). C and G were photographed with double refraction. Bar in D, 20 µm.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tion of formazan from cryostat sections and subsequent photometric measurement (Fig. 4). Formazan production was specific for the catalytic activity in myofibers since other cell types in skeletal muscles contain only negligible NADPH diaphorase activity as was demonstrated by histochemistry. Amounts of formazan extracted from sections of tongue derived from C57 mice and NOS-1 knockout mice were reduced to 27% and 7%, respectively, in the presence of 2 M urea. Residual NADPH diaphorase activity in TA muscle was 27% for C57 mice and 17% for NOS-1 knockout mice in the presence of 2 M urea. When NADPH diaphorase activity in NOS-1 knockouts was compared with that of C57 mice, tongue expressed 63% and TA muscles 45%, respectively. In both mouse strains, TA muscles showed NADPH diaphorase activity that was half of the activity in tongue.

An *in vitro* assay was established to measure NADPH diaphorase activity in homogenates of skeletal muscles. The values obtained in this assay (Fig. 5) could be directly compared with those determined in the *in situ* assay since both were performed under identical experimental conditions and were, in general, several-fold higher. Again, the presence of urea significantly decreased NADPH diaphorase activity, in both muscles: tongue from C57 mice and NOS-1 knockout mice exhibited 65% and 57% residual catalytic activity, respectively, whereas in TA muscles of C57 mice and NOS-1 knockout mice 31% and 6% rest activity, respectively, was found. In the *in vitro* assay, NADPH

Table 1. Determination of initial velocities (V_{init}) of NADPH diaphorase activity in FOG and FG myofibers of C57 mice and NOS-1-deficient knockout mice**.** Cryostat sections of tongue and tibialis anterior muscle were analysed histochemically for NADPH diaphorase activity either in the absence or presence of 2 M urea. Formazan production in the sections was recorded using image analysis exclusively in FOG myofibers in the tongue and FG myofibers in tibialis anterior muscle as identified in serial sections on the basis of succinate dehydrogenase activity and myosin-heavy chain fiber-typing (data not shown). Absorbance values are plotted against incubation time. Standard deviations of the V_{init} values were less than 10% in all cases and omitted for reasons of clarity.

Mouse strain	Fiber type (muscle)	V_{init} without urea (ΔE/min)		V_{init} with 2 M urea (ΔE/min)	
		Sarcolemma region	Non-sarcolemma region	Sarcolemma region	Non-sarcolemma region
C57/Bl6	FOG (tongue)	$3.75 \cdot 10^{-2}$	$1.76 \cdot 10^{-2}$	$2.59 \cdot 10^{-2}$	$0.33 \cdot 10^{-2}$
	FG (tibialis anterior)	$2.72 \cdot 10^{-2}$	$0.67 \cdot 10^{-2}$	$2.01 \cdot 10^{-2}$	$0.31 \cdot 10^{-2}$
NOS-$1^{-/-}$-knockout	FOG (tongue)	$<0.1 \cdot 10^{-2}$	$1.91 \cdot 10^{-2}$	$<0.1 \cdot 10^{-2}$	$<0.1 \cdot 10^{-2}$
	FG (tibialis anterior)	$<0.1 \cdot 10^{-2}$	$0.71 \cdot 10^{-2}$	$<0.1 \cdot 10^{-2}$	$<0.1 \cdot 10^{-2}$

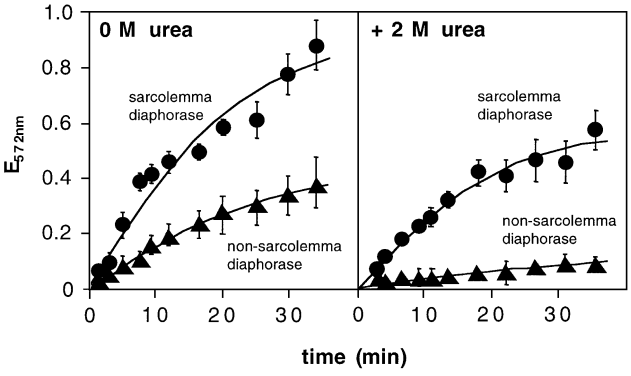

Fig. 3. The presence of 2 M urea in the incubation medium reduces the NADPH diaphorase activity in the sarcolemma region and the non-sarcolemma compartment of tongue myofibers of C57 mice. Serial cryostat sections of tongue from C57 mice were incubated to detect NADPH diaphorase activity histochemically in the absence or presence of 2 M urea. Generation of formazan was recorded with the use of image analysis in a number of selected myofibers and, subsequently, photometrically quantified in both sarcolemma and non-sarcolemma regions in time as indicated. The data are the mean values of 20–30 myofibers analysed.

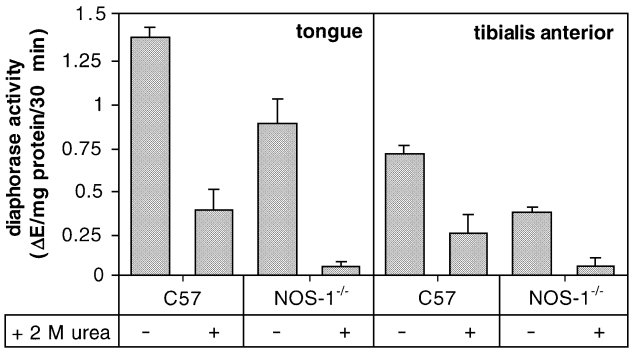

Fig. 4. Determination of NADPH diaphorase activity in cryostat sections of tongue and tibialis anterior (TA) muscle in situ. Fourty cryostat sections of tongue and TA muscle from either C57 mice or NOS-1-deficient knockout mice were incubated to detect NADPH diaphorase activity histochemically in the absence or presence of 2 M urea. Subsequently, the sections were transferred to reaction tubes and treated with collagenase to dissolve the formazan produced in the tissue sections. Photometric determination of both formazan and protein concentrations allowed the calculation of formazan produced per mg protein per unit time. Each bar is the mean value ± SD of 3 measurements.

diaphorase activities as measured in homogenates of tongue and TA muscle from NOS-1 knockout mice were higher than in those from C57 mice.

The proportion of NOS-1 in the pool of NADPH diaphorases in tongue and TA muscle was determined in an immunodepletion assay (Fig. 6). After immunoprecipitation with anti-NOS-1 antibodies, detergent extracts of tongue derived from C57 mice contained approximately 62% NADPH diaphorase activity as compared with complete homogenate. The immunoprecipitation fraction comprised 38% of the NADPH diaphorase activity. Depletion of NOS-1 from TA muscle homogenates reduced NADPH diaphorase activity to approximately 41% whereas NOS-1 comprised approximately 59%.

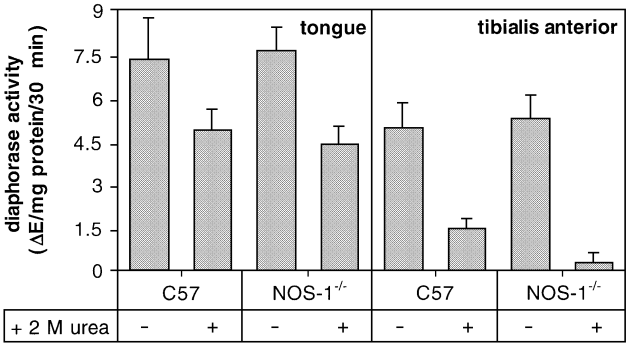

Fig. 5. Determination of NADPH diaphorase activity of tongue and tibialis anterior (TA) muscle *in vitro*. Detergent extracts of tongue and TA from either C57 mice or NOS-1 knockout mice were incubated to detect NADPH diaphorase activity *in vitro* either in the absence or presence of 2 M urea. Photometric determination of both formazan and protein concentrations allowed calculation of formazan produced per mg protein per unit time. Each bar is the mean value ± SD of 6 measurements.

Discussion

Although catalytic profiles of the different NADPH diaphorases have not been characterised, various reports suggest that the histochemical NADPH diaphorase reaction is not specific for a defined enzyme (Stoward et al., 1991; Tracey et al., 1993; Blottner et al., 1995). However, characterisation of their catalytic profiles is important since it finally may lead to the development of (a) method(s) that is (are) specific for single NADPH diaphorases and allow quantification studies. We have performed a qualitative and quantitative characterisation of the histochemical NADPH diaphorase reaction in skeletal muscle derived from C57 mice and NOS-1 knockout mice. We have determined V_{init}'s of the NADPH diaphorase(s) in the sarcolemma and the intrafibrar compartment applying image analysis and quantified specific NADPH diaphorase activities using an *in situ* assay. In summary, our results reveal a strong inhibitory effect of 2 M urea on intrafibrar NADPH diaphorase activity as compared with a significant weaker inhibition of the activity in the sarcolemma region. This compartment-selective inhibitory effect of urea was, in principle, observed in all myofibers investigated albeit FOG myofibers contained significantly more non-sarcolemma (intrafibrar) NADPH diaphorase activity than FG fibers in the absence of urea.

The *in vitro* assay was applied as an alternative methodological approach to quantify NADPH diaphorase activity in tissues. The values determined in this assay were, in general, higher than those measured with the *in situ* assay. This discrepancy could be the result of the improved availability of compounds of the incubation medium to the solubilised enzymes or, alternatively, may be caused by the destruction of

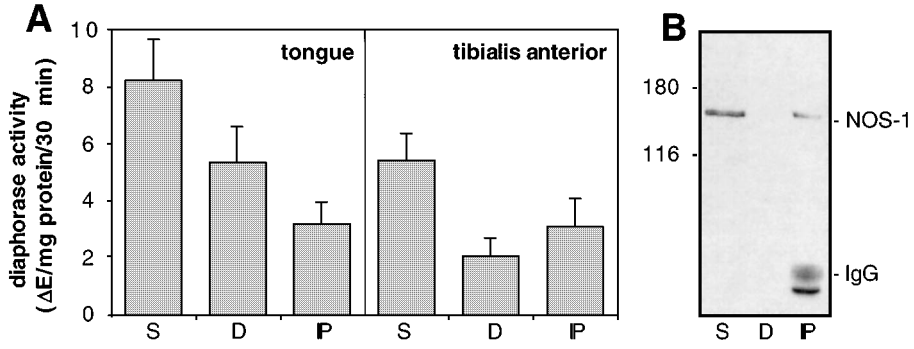

Fig. 6. Immunodepletion analysis to determine the proportion of NOS-1 in NADPH diaphorase activity in homogenates of tongue and tibialis anterior (TA) muscle. Detergent extracts of tongue and TA muscle of C57 mice were subjected to immunoprecipitation with an anti-NOS-1 antibody. Subsequently, aliquots of the original solubilisates (S), the NOS-1-depleted extracts (D) and the NOS-1-containing immunoprecipitates (IP) were incubated *in vitro* to determine NADPH diaphorase activity (**A**) or analysed by immunoblotting to control the effectiveness of immunoprecipitation as shown for one tongue preparation (**B**). Bars in A represent mean values ± SD of 3 immunoblotting measurements.

the microenvironment of the enzymes in the intact tissue. In both assays, FOG myofiber-rich tongue contained approximately twice as much NADPH diaphorase activity than FG myofiber-rich TA muscle and, again, the addition of 2 M urea inhibited significantly the generation of formazan in all samples investigated. However, the inhibition rates of the catalytic activity *in vitro* were significantly lower in tongue than in TA muscle (of both mouse strains) as compared with the respective values determined in the *in situ* assay. We suggest that the mitochondria (which are rich in NADPH diaphorase activity; Lind et al., 1990; Olausson et al., 1995) of FOG myofibers are less sensitive to the treatment with urea under the *in vitro* assay conditions (where they are solubilised) than under *in situ* assay conditions. The most striking difference between both assays applied was the higher NADPH diaphorase activity *in vitro* in both tongue and TA muscle homogenates of NOS-1 knockout mice which contained even more activity than the homogenates of C57 mice. This observation can only be explained by an upregulation of the activity of the non-NOS-1 NADPH diaphorase(s) in NOS-1 knockout mice. The molecular basis for this upregulation is not known yet.

To our knowledge, there is only one report that investigated the effect of urea on the reductase activity of NADPH diaphorase(s) so far (Narayanasami et al., 1997). Urea (in concentrations up to 4 M) and guanidine hydrochloride were found to mimic the binding of calmodulin to NOS-1 in an *in vitro* assay. As a consequence, the NADPH-dependent cytochrome c reductase activity of NOS-1 (corresponding to NADPH diaphorase activity) was temporarily increased. These results are apparently in contradiction with our results showing an inhibition of V_{init}'s and activity of all NADPH diaphorases by urea in catalytic histochemistry. Interestingly, when potassium thiocyanate, which is another chaotropic agent, is applied in concentrations higher than 200 mM, it causes an irreversible loss of the FAD cofactor and denaturation of DT diaphorase (Byron et al., 1997). Therefore, it seems reasonable that the presence of high concentrations of chaotropic agents such as urea may either activate or inhibit individual NADPH diaphorases leading to selective staining patterns in catalytic histochemistry. With respect to the histochemical NADPH diaphorase reaction in cryostat sections of skeletal muscle, it could mean that the cell surface of myofibers provides a microenvironment protecting NADPH diaphorases of the sarcolemma more efficiently from denaturation than that/those present inside the myofibers.

In NOS-1 knockout mice, NADPH diaphorase-dependent generation of formazan in the sarcolemma region was completely abolished suggesting that in C57 mice all NADPH diaphorase activity present in this site is related to NOS-1. Since, in addition, the intrafibrar V_{init} values were almost identical in skeletal muscles of C57 and NOS-1 knockout mice, one may speculate that comparable amounts of formazan are produced in the non-sarcolemma compartment in both mouse strains. Consequently, the higher catalytic activities as determined histochemically with the *in situ* assay in skeletal muscles of C57 mice should reflect NOS-1-specific NADPH diaphorase activity. Calculations revealed that the proportion of NOS-1 in total NADPH diaphorase activity is approximately 37% in tongue and 52% in TA muscle. These values correspond very well with those obtained in the immunodepletion assay of muscle of C57 mice showing that in tongue 38% and in TA muscle 59% of the total NADPH diaphorase activity is produced by NOS-1.

The fact, that NOS-1-specific NADPH diaphorase activity was always restricted to the sarcolemma allows the conclusion that previously reported intrafibrar NADPH diaphorase activity (Kobzik et al., 1995; Frandsen et al., 1996) and NOS-1 immunoreactivity (Frandsen et al., 1996; Punkt et al., 2001) were either not related to NOS-1, were caused by methodological variations or reflect species-specific differences in NOS-1 expression.

Preliminary investigations (data not shown) indicate that the addition of urea to the NADPH diaphorase incubation medium also inhibits the intrafibrar staining in skeletal muscles of rats, guinea pigs, gerbils and hamsters. The urea concentration needed for complete suppression of intrafibrar activity of NADPH diaphorase(s) seemed to be dependent on the species investigated. Therefore, we conclude that urea as supplement to the incubation medium increases the specificity of the NADPH diaphorase reaction associated with NOS-1 in skeletal muscle not only of mice (as shown here) but also of rodents in general. In contrast, human quadriceps muscles did not contain intrafibrar NADPH diaphorase activity as demonstrated with catalytic histochemistry (even in absence of urea). Thus, further research should be performed to clarify the molecular basis of the different species-specific profiles of the NADPH diaphorase activity in presence of urea (or other chaotropic reagents) as applied in catalytic histochemistry.

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