RESEARCH COMMUNICATION

Localization of xanthine dehydrogenase mRNA in horse skeletal muscle by *in situ* hybridization with digoxigenin-labelled probe

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In situ hybridization was used to localize xanthine dehydrogenase (XDH) mRNA in horse skeletal muscle. Capillary endothelial cells were found to express XDH, but muscle cells did not give any signal. The digoxigenin-labelled probe was produced by

PCR with primers based on the cDNA sequence of mouse XDH and horse lung cDNAs. A 4.3 kb mRNA was detected in a Northern blot.

INTRODUCTION

In intensively contracting skeletal muscle, AMP is produced by the myokinase-catalysed reaction. Deamination of AMP leads to IMP, which is further metabolized via inosine, hypoxanthine, xanthine and uric acid to allantoin, the end-product of ATP catabolism in the horse. The activation of this cascade in skeletal muscle is shown by the veno–arterial concentration differences of hypoxanthine, xanthine and uric acid over exercising muscle in human athletes [1]. Similar increases in the plasma concentrations of hypoxanthine, uric acid and allantoin have also been reported in racehorses during and after exercise [2,3].

The reactions from hypoxanthine to xanthine and from xanthine to uric acid are catalysed by xanthine dehydrogenase (XDH). In many tissues, XDH [4,5] has been localized to capillary endothelial cells, but Ibrahim and Stoward [6] have found XDH activity in the sarcolemmae of aerobic skeletal muscle fibres in humans. Normally, XDH utilizes NAD⁺ as an electron acceptor, but under ischaemic conditions, e.g. during strenuous exercise, the enzyme can be converted to the xanthine oxidase form (XO), which transfers electrons directly to molecular oxygen and produces superoxide anions (O₂^{•−}). The conversion from XDH to XO is either reversible through thiol oxidation or irreversible in the case of proteolytic cleavage [7]. During the post-exercise recovery period XO could be a source of free radicals, which have been claimed to contribute to exercise-induced muscle damage [8]. The present study was aimed at identifying the cell type in which XDH is expressed in the horse skeletal muscle.

EXPERIMENTAL

Tissues

Muscle samples were biopsies from the middle gluteal muscle of clinically healthy horses. Additionally, samples from the masseter muscle and lung were obtained from a slaughterhouse. The tissue samples were immediately frozen in liquid nitrogen and kept at −70 °C until used.

Preparation of the probe

The nucleotide sequence of horse XDH was not available in the

data libraries and, therefore, the probe was synthesized using horse lung cDNAs as the template and mouse specific oligonucleotides as primers. The 20- and 26-mer oligonucleotides, synthesized according to the nucleotide sequence of a conserved region of the 5′-end of the mouse XDH mRNA [9], were purchased from the Institute of Biotechnology (University of Helsinki, Finland). The sequence of the 26-mer sense primer, representing residues 39–64 of the mouse XDH cDNA, was 5′-GTGAATGGCAAAAAGGT(GC)GTGGAGAA. The 20-mer antisense primer was complementary to the sequence from residue 271 to 252: 5′-GTTGTACAGCAACATG(AG)TG. These primers were used both in reverse transcription and in the PCR.

Horse lung mRNAs were isolated with a Quickprep micro mRNA purification kit (Pharmacia, Uppsala, Sweden). Reverse transcription (37 °C, 60 min) using Moloney Murine Leukemia Virus reverse transcriptase (Stratagene, La Jolla, CA, U.S.A.) was carried out using 1–3 µg of the polyadenylated lung mRNA as template and the 20-mer oligonucleotide as primer (80–160 nM).

The reverse-transcribed cDNA (5 µl) was used as the template in the PCR. The concentrations in the 100 µl reaction mixture were as follows: primers, 2 µM each; dNTPs (Boehringer, Mannheim, Germany), 200 µM each; Fz-DNA polymerase (Finnzymes, Espoo, Finland), 1.4–3.5 units. The buffer was delivered by the polymerase manufacturer (Finnzymes). The reaction mixture was covered with 100 µl of mineral oil. The enzyme was added after heating the mixture to 70 °C in order to ensure that all of the cDNA was double-stranded and to minimize the risk of amplifying non-target sequences. A total of 30 reaction cycles were performed, each being composed of 60 s of denaturation at 94 °C, 60 s of annealing at 55 °C and 60 s of elongation at 72 °C. After the last cycle, the mixture was heated to 95 °C for 5 min and thereafter cooled to 4 °C.

The size of the PCR product was verified to be 234 bp by electrophoresis on a 2% NuSieve (3:1) agarose gel (FMC BioProducts, Rockland, ME, U.S.A.). pBR 322 plasmid digested with *Msp*I to 9–622 bp fragments (Biolabs, Beverly, MA, U.S.A.) was used as the molecular size standard.

Digoxigenin-labelled probe was generated by the second PCR with a DIG DNA labelling and detection kit (Boehringer) according to the instructions of the manufacturer and the PCR

Abbreviations used: XDH, xanthine dehydrogenase; XO, xanthine oxidase.

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procedure described above. The size of the PCR product was tested as before.

Dot-blot analysis

The effectiveness of digoxigenin labelling was determined by dot-blot analysis. Digoxigenin-labelled control DNA from the DIG DNA labelling and detection kit (Boehringer) was used as reference material.

Northern blot analysis of lung RNAs

Total RNA from horse lung was isolated according to Chomzynski and Sacchi [10]. A 5–30 μg sample of glyoxal/dimethyl sulphoxide-treated total RNA was fractionated by electrophoresis on a 1% agarose gel (Pharmacia). After removing glyoxal, the RNAs were blotted on to positively charged synthetic nylon membranes (Boehringer) according to Sambrook et al. [11]. For the detection of the 18 S and 28 S RNAs, part of the membrane was stained with Methylene Blue (0.04% in 0.5 M sodium acetate). The rest of the filter was hybridized with the digoxigenin-labelled probe (10 ng/ml) at 65 °C for 12 h and detected as instructed by the manufacturer of the DIG DNA labelling and detection kit (Boehringer).

In situ hybridizations

Skeletal muscles were cut into 7 μm sections in a cryostat, mounted on 3-aminopropyltriethoxysilane-pretreated slides and fixed with fresh 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS [12]. The fixed sections were dried in a graded alcohol series and kept at –70 °C until used. The dried sections were rehydrated in PBS containing 0.2% Tween 20 (PBST; Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubated at 37 °C for 30 min with proteinase K (3 $\mu\text{g}/\text{ml}$; Sigma). Proteinase K was inactivated with 0.2% glycine in PBST. The slides were refixed in 4% paraformaldehyde for 5 min and rinsed with PBST. The sections were prehybridized at 42 °C for 4–6 h under coverslips sealed with rubber cement (Sanford Co., Bellwood, IL, U.S.A.). The hybridization cocktail consisted of 5 $\times$ SSC (Sigma), 5 $\times$ Denhardt's reagent (Sigma), 50% formamide (Merck) and 1 mM EDTA (Sigma). Salmon sperm double-stranded DNA (Sigma) and calf pancreas tRNAs (Boehringer) were added to a concentration of 250 $\mu\text{g}/\text{ml}$ after denaturation. For the hybridization, the digoxigenin-labelled probe was denatured and added at a concentration of 0.1–5 ng/ml to a fresh reaction mixture. The sections were hybridized overnight at 42 °C. After hybridization, the sections were washed twice with 2 $\times$ SSC, once with 1 $\times$ SSC and twice with 0.5 $\times$ SSC.

The immunological detection of the digoxigenin-labelled sections was carried out according to the instructions of the manufacturer (Boehringer). The sections were mounted with Aquamount (BDH Limited, Poole, Dorset, U.K.).

In the negative controls, either the probe was omitted or the digoxigenin-labelled control DNA from the kit (Boehringer) was used.

RESULTS AND DISCUSSION

Characterization of the probe

The product of PCR following reverse transcription of horse lung mRNA was a single fragment of approx. 240 bp. The size of the fragment was the same after the digoxigenin labelling procedure. This agree well with the length of the corresponding fragment, 234 bp, in the murine XDH cDNA [9]. In Northern

blot analysis the XDH probe hybridized to a 4.3 kb mRNA (Figure 1). This is similar to the size of the mouse and rat XDH cDNAs [9,13].

In situ hybridization

Hybridization of muscle sections from m. gluteus medius and m. masseter with the digoxigenin-labelled XDH probe resulted in staining of only capillary endothelial cells, whereas the muscle fibres did not give any signal (Figure 2). This is in accordance with earlier studies in which XDH/XO was localized to the capillary endothelial cells of several other tissues [4,5], but contrasts with the finding that the activity of XDH was detected

Figure 1 Northern blot analysis of XDH mRNA

Glyoxal/dimethyl sulphoxide-treated RNA from horse lung (10 μg) was separated on a 1% agarose gel, transferred to a positively charged synthetic nylon filter and hybridized with a XDH cDNA probe as detailed in the Experimental section. Each lane contains horse lung total RNA (25, 18.6, 17.7 and 32.6 μg samples).

Figure 2 Localization of XDH mRNA in skeletal muscle by *in situ* hybridization

Paraformaldehyde-fixed cryostat sections of horse masseter muscle were pretreated for *in situ* hybridization with a digoxigenin-labelled cDNA probe for horse XDH mRNA. After hybridization, the sections were washed and the probe was detected using an anti-digoxigenin antibody. The XDH mRNA is located in the capillary endothelial cells (arrowed). Magnification $\times 310$.

histochemically in the sarcolemmae [6]. *In situ* hybridization at the mRNA level increases both the specificity and the sensitivity of detection in comparison with the previously used enzyme histochemical and immunochemical methods. A review of the literature revealed that the results from studies using enzyme histochemistry or immunohistochemistry are conflicting [4,5,14–16]. False positive results may be obtained due to the use of polyclonal antibodies in immunohistochemical experiments or to the poor specificity of the substrates in enzyme histochemistry.

At the mRNA level, it is also possible to locate the actual cell type that expresses XDH. mRNA that is detected by *in situ* hybridization is made by the cell itself, whereas the enzyme protein detected by immunochemical and histochemical methods may originate from another tissue. XDH/XO activity is present in the serum of several species [7], and a recent report showed that XO may attached to the plasma membranes of endothelial cells from the circulating blood [17]. Thus the presence of XDH/XO protein does not necessarily specify the cell type in which it was synthesized. It is concluded that, when the site of XDH synthesis is examined, *in situ* hybridization gives more reliable results than the immunochemical or enzyme histochemical methods used in earlier studies.

Superoxide radical that are formed in the reactions catalysed by XO have been claimed to be the cause of vascular injury during the post-ischaemic reperfusion [18,19]. Damage to the capillary endothelial cells could serve as a stimulus for further tissue damage through the infiltration of inflammatory cells [20]. During strenuous exercise the working muscle of an athlete (human or horse) may become ischaemic, which leads to the conversion of XDH to XO. The highest activities of XDH + XO have been found in the soleus muscle [21]. This muscle consists mainly of slow-twitch oxidative type I fibres, which in the horse have a high number of contact capillaries relative to the fibre area [22]. These findings, together with the expression of XDH in the capillary endothelial cells only, are consistent with the view that the slow-twitch fibres are the prime site of the injury caused by exercise [23]. In another study, however, the exercise-induced damage was found to be concentrated in the fast-twitch fibres, probably due to their low oxidative capacity [24]. These findings emphasize that the mechanism of exercise-induced muscle damage is complex, and the capillary density together with the activity of XO cannot solely explain this phenomenon.

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