Mouse muscle regeneration: an in vivo 2D ^{1}H magnetic resonance spectroscopy (MRS) study

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Abstract Muscle degeneration and regeneration were studied by 2D ^{1}H magnetic resonance spectroscopy (MRS) and histological examination, in an experimental model of muscle injury using a myotoxic snake venom, notexin. The injured muscles produced a very specific MRS signal, corresponding to a tri-unsaturated fatty acid (linolenic acid-like) signal, from day 2 to day 9 after injury. The combination of MRS with histology showed that this signal was associated with a mechanism occurring during myoblast fusion to form myotubes. 2D ^{1}H MRS is thus a useful non-invasive tool for detecting muscle regeneration in vivo.

Key words: Muscle regeneration; Myoblast fusion; Notexin; Two-dimensional proton magnetic resonance spectroscopy; In vivo spectroscopy; Fatty acid

1. Introduction

Magnetic resonance spectroscopy (MRS) is a powerful non-invasive technique for the study of living material, such as skeletal muscle. Differences between normal and diseased muscles have been described in studies using ^{31}P MRS metabolism [1–3]. We used ^{1}H MRS to study skeletal muscle. The main advantages of ^{1}H over ^{31}P MRS are that ^{1}H MRS has higher proton sensitivity and detects a larger number of compounds. In a previous study of murine muscular dystrophy [4], we used 2D ^{1}H MRS to investigate the differences between dystrophic skeletal muscles from mdx mice and normal muscles from C57BL/10 mice. 2D ^{1}H MRS, which can be used to identify large molecules, such as fatty acid [5–9], allowed us to compare the lipid composition of mdx and C57BL/10 skeletal muscles in vivo.

A scalar correlation between saturated, mono-, and di-unsaturated fatty acids [4] was detected in all the COSY (COrrelation SpectroscopY) spectra of normal muscles in C57BL/10. The 2D spectra of dystrophic muscles from 2–6-week-old mdx mice had an additional cross-correlation peak, labelled 'KD', characteristic of the terminal part of the acyl chains of tri-unsaturated fatty acids (linolenic acid-like) [4]. This signal decreased as the mice became older and was not detected in mice older than 2 months of age. The correlation with linolenic acid-like fatty acids may be associated with muscle degeneration or regeneration because these processes are involved in most of the activity of the muscle in mdx during the first 2 months of life [10,11]. To determine the origin of the KD signal, we performed a series of in vitro experiments with cultured C2 myoblasts in replicating or fusing state. We found that the correlation with tri-unsaturated fatty acids appeared when myoblast fusion was taking place [12]. To confirm the relationship between this characteristic signal and myoblast fusion in vivo, we carried out a 2D ^{1}H MRS study of murine skeletal muscles injured by injection with a myotoxic snake venom, notexin. This experimental model is easier to assess than the dystrophic system, because notexin injection is followed by a single round of synchronous myofiber regeneration, whereas multiple rounds occur simultaneously in mdx muscle.

Notexin was injected into the hindlimb muscles of normal Swiss mice to cause muscle injury [13]. Muscle regeneration was assessed by a series of daily 2D ^{1}H MRS measurements, then the results were compared with the histological features.

2. Materials and methods

2.1. Animals and experimental design

Male Swiss mice weighing 14–18 g (CED, St Doulchard, France) were used in this study. All mice were anesthetized with 3.5% chloral hydrate (0.30 ml i.p. per animal) for surgery and MRS. For MRS experiments, the mice were randomly divided into four groups. Group 1, the control group, contained seven normal mice. Group 2 contained seven mice whose hindlimb muscles were injected with phosphate-buffered saline (PBS). Group 3 contained seven mice whose hindlimb muscles were injected with notexin (50 mg/ml) (Sigma V-0251). Injections were made into both the anterior and posterior compartments of the right leg using a 10 μl Hamilton microsyringe (Hamilton, Bonaduz, Switzerland). The needle was inserted through a skin incision (1 mm long), pushed down, and then slowly pulled up to deliver the solution (5 μl of either PBS or notexin) along the length of the leg. The mice in group 4 ($n = 3$) were injected with notexin and their hindlimb muscles were denervated by sectioning the sciatic nerve at the thigh, in order to assess the contribution of muscle reinnervation to the correlation with polyunsaturated fatty acids. The hindlimb muscles of other Swiss mice were injected with either PBS or notexin for histology studies, carried out in parallel with the MRS studies.

2.2. MRS experiments

All the animals in groups 2, 3 and 4 were examined daily by MRS, from day 1 to day 13 after injection. The control group was also examined over this period. Only 10 measurements were taken per animal over this period, to minimize the risk of death. The MRS experiments were carried out at 400 MHz using an AM Wide Bore Bruker Spectrospin spectrometer (9.4 T). Each mouse was anesthetized and introduced into a custom-built probe and a 'loop-gap' coil was placed around the right hindlimb [14]. Body temperature and respiration were monitored throughout the MRS procedure. The COSY spectra were obtained using water presaturation. Acquisition of eight 1 K FIDs in the time domain (t2) for each of the 256 increments in the time domain (t1) resulted in a total acquisition time of about 40 min. The data were multiplied by an unshifted sine-bell function in two dimensions and were then Fourier transformed. The acyl chain correlations in the muscle COSY spectra were identified directly by superimposition with the spectra of standard saturated and unsaturated fatty acids.

We performed two types of quantification. First, the presence or

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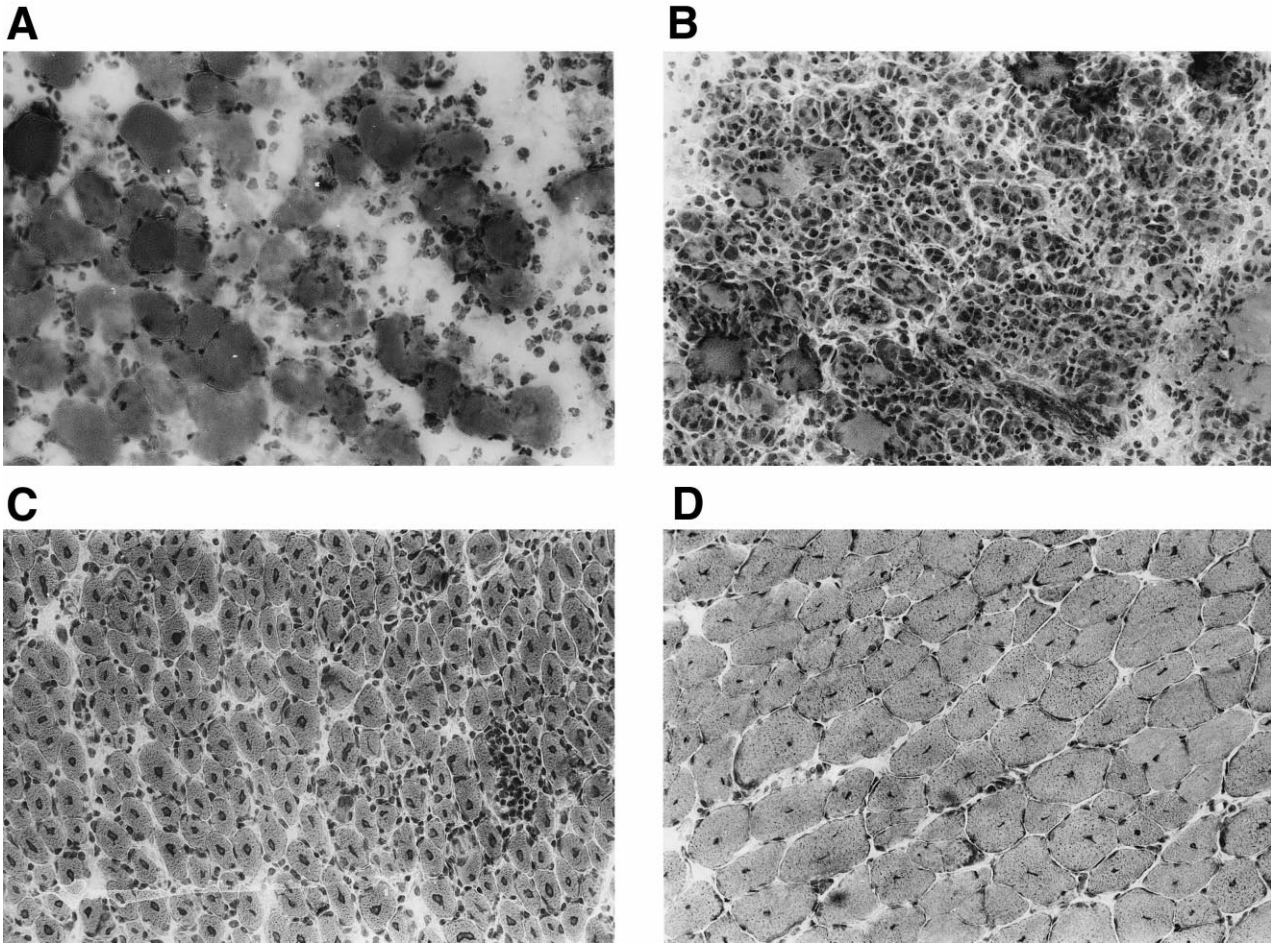


Fig. 1. Histological features of the tibialis anterior muscle in Swiss mice following notexin injection: 1 day (A); 3 days (B); 5 days (C) and 13 days (D) after injection. A: Phagocytosis of a degenerating myofiber. B: Young myotubes. C: Newly formed myofibers with central nuclei. D: Mature regenerated myofibers with persistence of central nuclei. Magnification: ×125.

lack of a KD signal in each spectrum was recorded, and the percentage of mice producing the KD signal was calculated for each day. Second, when a KD signal did appear, its intensity was measured using 'purpose-written' software to quantify signal density.

2.3. Histological studies

Normal and injected hindlimb muscles were examined in parallel with the MRS experiments (1–13 days after injection). The muscles of the mice subjected to MRS were also examined by histology after the MRS experiments. Hindlimb muscles were removed, mounted on a cork with tragacanth gum, and frozen in isopentane chilled in liquid nitrogen. Sections were cut on a cryostat and stained with hematoxylin-eosin for histological examination.

3. Results

3.1. Histological results

Injection of PBS resulted in no significant muscle damage: PBS-injected muscles were similar to non-injected control muscles.

In contrast, injection of notexin rapidly caused extensive infiltration of the entire muscle by inflammatory cells. On day 1, many myofibers had histological features typical of necrosis (Fig. 1A). Phagocytosis reached its maximum level on day 2. On days 2 and 3, the myoblasts proliferated and differentiated. The first myotubes that resulted from the myoblast fusion were detected on day 3 after notexin injection. Myoblast fusion took place from day 2 to day 9 (Fig. 1B,C), with a maximum on days 3 and 4, and was not detected after day 9. Reinnervation of the regenerating myofibers began 1 week after injury, and on day 13, muscle fiber maturation was well under way but not yet completed (Fig. 1D). Fig. 2

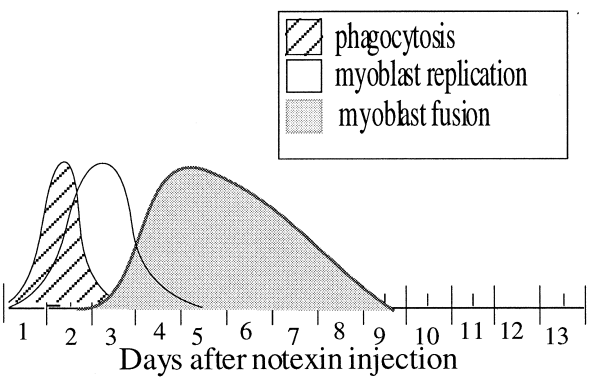


Fig. 2. Schematic time course of the cellular events occurring during muscle degeneration-regeneration following notexin injection.

outlines the events occurring during muscle degeneration and regeneration.

3.2. MRS results

Records of spectra for several fatty acids enabled us to identify the signals for the muscle spectra. The 2D spectra of linoleic and linolenic acid are shown in Fig. 3b, Fig. 4b respectively. BC- and CE-type correlations occurred in the spectra of saturated, mono-, di- and polyunsaturated fatty acids. The AB correlation was present in the spectra of all these acids except those of linolenic acid-like polyunsaturated acids in which there was a KD correlation. BD-, HD-, and HF-type correlations indicate the presence of further unsaturated bonds in the acyl-chains.

The COSY spectra of the muscles from groups 1 (not injected) and 2 (injected with PBS) contained all the fatty acid correlations except the KD signal (Fig. 3a) whereas COSY spectra of groups 3 (notexin injected) and 4 (notexin injected

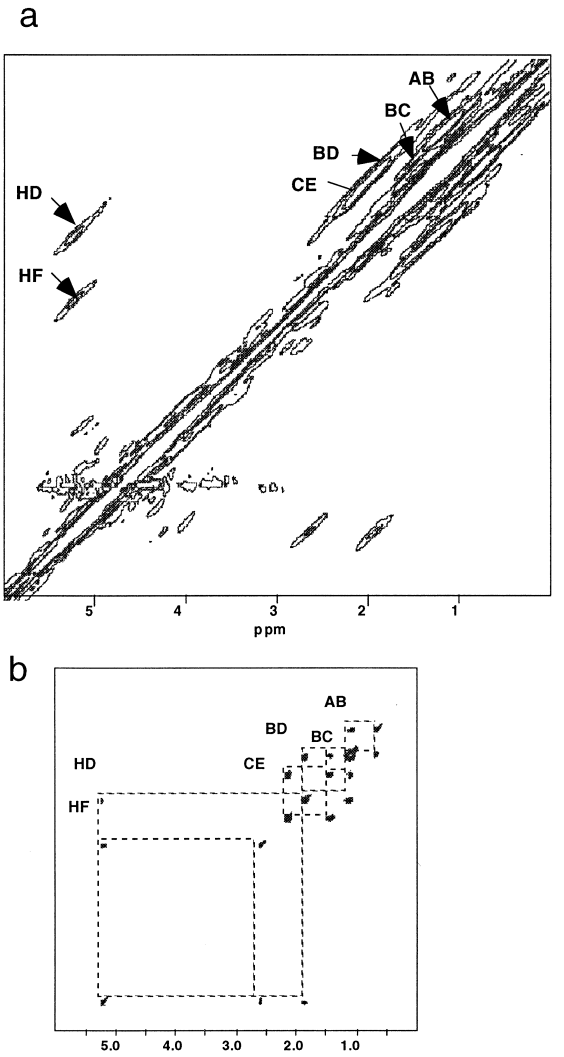
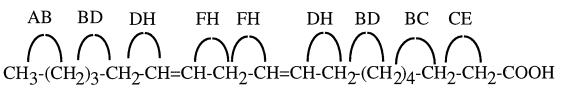


Fig. 3. a: In vivo ^{1}H COSY spectrum of PBS-injected hindlimb muscles (group 2). b: ^{1}H COSY spectrum of linoleic acid



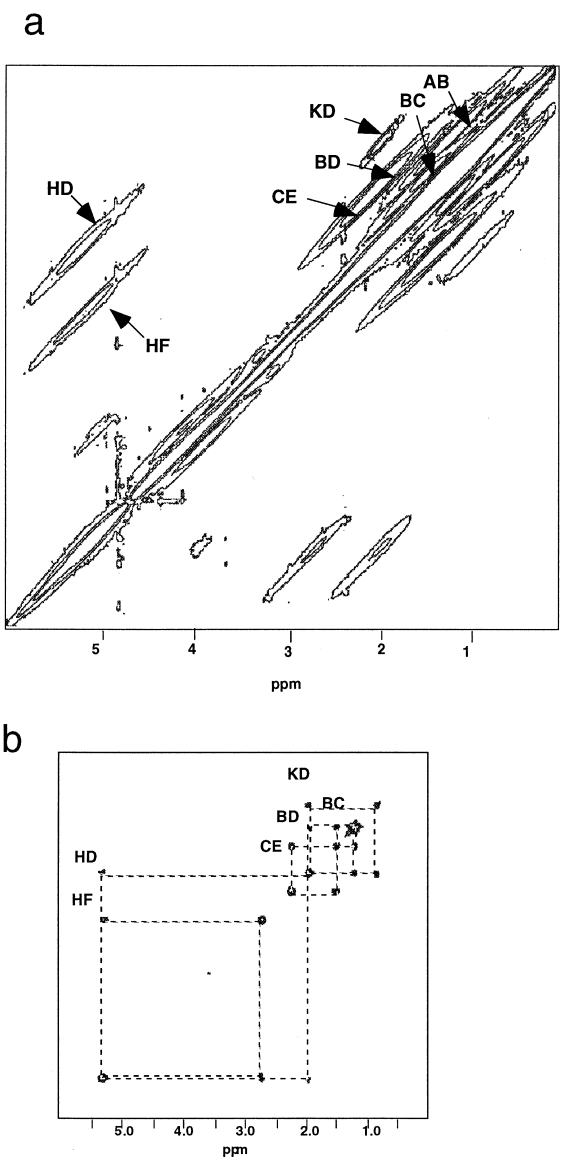
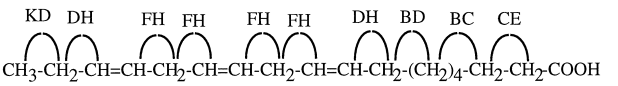


Fig. 4. a: In vivo ^{1}H COSY spectrum of notexin-injected hindlimb muscles (group 3). b: ^{1}H COSY spectrum of linolenic acid



and denervated) showed the KD signal several days after notexin injection (Fig. 4a).

We measured changes in KD signal intensity during muscle regeneration for each mouse of groups 3 and 4. The individual responses of two mice (one from group 3 and one from group 4) are shown in Fig. 5. The maximum KD signal occurred between days 3 and 6 for each mouse. The intensity of the signal before and after these particular days depends on the animal tested but no KD signal was detected on day 1 or after day 9 after notexin injection.

In group 3 as a whole (Fig. 6a), the KD signal was detected from day 2 to day 9 after notexin injection, with a maximum positive response on days 3 and 4. This signal was never detected after day 11. We obtained similar results with group 4 (Fig. 6b), showing that denervation did not affect this response.

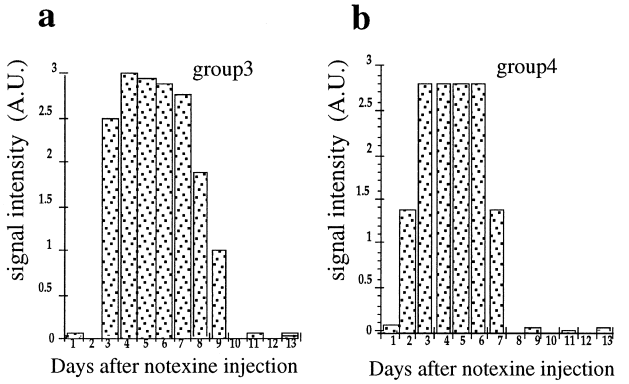


Fig. 5. Changes in the KD signal intensity during muscle regeneration (a) for one mouse from group 3 (notexin injected) and (b) for one mouse from group 4 (notexin injected and denervated).

4. Discussion

The KD signal was not detected during the intense inflammatory response or phagocytosis of the necrotic myofibers (day 1 after injection) (Figs. 2 and 6). The tri-unsaturated (linolenic acid-like) fatty acid KD signal only occurred between days 2 and 9 after notexin injection. This corresponds to the myoblast fusion period which results in myotube formation or lengthening. The similarity of the results obtained for groups 3 and 4 shows that this signal does not depend on the reinnervation of the newly formed myofibers. Thus, the KD correlation is linked to muscle regeneration, and particularly to the process of myoblast alignment and fusion. This is consistent with our previous work on myoblast fusion in vitro [12]. Results also show that the correlation observed in vivo in the COSY spectra of 2–6-week-old mdx mouse muscles was not specific for muscular dystrophy, but probably due to the process of muscle regeneration [4] and came from muscular cells.

In our study of myoblasts in replication and fusion states, we found a large increase in lipid signal during fusion. The presence or lack of high resolution signals from lipids has been reported for other mammalian cells including cancerous, stimulated and stressed cells [14]. The KD correlation seems to be present in the spectra of some cancerous [5,15,16] and stimulated cells [17,18] but was only clearly identified in the spectrum of stimulated human lymphocytes [19]. The origin of these high-resolution ^{1}H MRS signals is still a matter of debate. The most recent studies suggest that these signals arise from neutral triglycerides in the plasma membrane bilayer or from the formation of cytoplasmic lipid droplets depending on the cell line [20,21].

In conclusion, we have shown that the specific signal produced by 'linolenic acid-like' fatty acids on 2D ^{1}H MRS occurs at the same time as myoblast fusion. As a consequence, the presence of this correlation in 2D spectra of muscle is probably a marker for muscle regeneration. This study shows that 2D ^{1}H MRS is of value for non-invasive assessment of muscle regeneration.

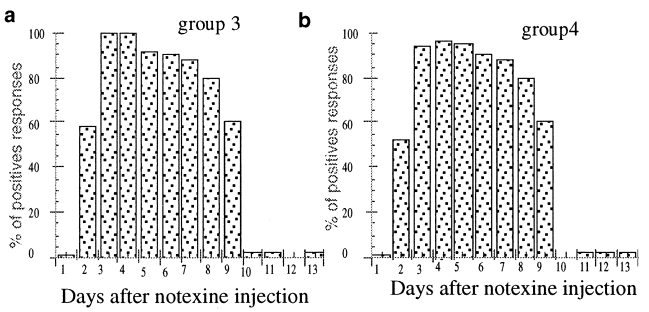


Fig. 6. Percentage of mice producing a KD signal during muscle regeneration after injection of notexin alone (group 3), or after notexin injection and sciatic nerve section (group 4).

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