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NMR SPECTROSCOPY AND OPTICAL MICROSCOPY STUDIES OF PYSIOLOGICAL STATES OF RAT SKELETAL MUSCLE OF DIFFERENT AGES

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Abstract

Skeletal muscle is a very good model for fundamental research of aging process due to its postmitotic character. In order to obtain new data concerning muscle contraction at the molecular level, the polar groups of contractile proteins have been investigated by means of ^1H NMR Spectroscopy. Glycerinated Sartorius muscle from 6 and 37 months old rats has been used for proton transverse relaxation time measurements in Ri, Co and Re at different [ATP]. The distribution of negative charges in contraction and relaxation has been measured by exposing glycerinated muscle from 6 to 37 months old rats to different [Mn^{2+}]. Our data have pointed out the existence of two proton relaxation tines: T2s and T21 accounted for two water compartments. The modifications in water state are related with modifications in contractile activity. The elongation odd proton transverse relaxation time is associated with a decrease in the degree of water molecules aggregation. T2s and T2l are correlated with a reduction in muscle hydration, contraction being a function of ions binding to the protein sites. These sites are implicated in determination of protein hydration state.

Key words: *^1H NMR, aging, glycerinated skeletal muscle, contraction, relaxation, rigor.*

INTRODUCTION

Skeletal muscle is a very good model for fundamental research of aging process due to its postmitotic character. It has been supposed that during involution process of muscle tissue, involves proteins and especially those contractile (Allnaqueeb, 1984; Marin et al., 2017).

Literature data (Dragomir, 1987) concerning muscular contraction phenomenon, have pointed out the appearance of long-range repulsive forces within contraction state, which tend to repel the myofilaments one from each other (Elliot, 1982). These forces are converted into active shortening tension through passive intervention of transverse myosin crossbridges with an oblique orientation between myofilaments (Eisenberg, 1980). The repulsive forces that take place during contraction are the consequence of the increase in the electric charge of myofilaments (Offer, 1984). Nuclear magnetic resonance (NMR) spectroscopy is an attractive technique due to its noninvasiveness with an increasing importance in determination of chemical structures, but only nuclei capable of transitioning between energy states, in the presence of an intense and constant magnetic field, could be studied and this includes abundant nuclei such as proton (^1H) and phosphorous (^{31}P), as well as stable isotopes such as deuterium (^2H) and carbon 13 (^{13}C) and for the *in vivo* study of metabolism (Alves, 2012; Marin et al., 2016).

^1H NMIR is a very useful method in biology because we can obtain very important data about mobility of some groups at the level of protein molecules, which provide information about conformation changes which result from the chemical modifications (Dragomir, 1992).

Ischemia induces changes in the distribution and polarization of tissue water and this may influence Nuclear Magnetic Resonance (NMR) relaxation times (Revnic, 2015).

The aim of our study was related with: In order to obtain new data concerning muscle contraction at the molecular level, the polar groups from the contractile proteins have been investigated by means of ^1H NMR Spectroscopy, to test the water state from the close proximity of myofilaments in different experimental conditions in contraction, relaxation from Sartorius muscle of Wistar rat.

1. The investigation of proton transverse relaxation times of water from glycerinated muscle in Ri, Re, Co at different ATP concentrations.
2. The distribution of charges in contraction and relaxation by exposing glycerinated muscle from 6 and 37 months old rats to different [Mn^{+2}], by means of ^{1}H NMR spectroscopy.

MATERIALS AND METHODS

The study has been performed on 12 male Wistar rats divided in 2 groups of 6 rats each: young (6 months) and old (37 months).
The animals from boths groups have been anesthetized and then killed by cervical dislocation and then sample of fresh Sartorius skeletal muscle have been collected on ice bath and processed within one hour for optical measurements of sarcomere length and for (^{1}H) NMR measurements and the other Sartorius muscle samples were collected for glycerination procedure (kept for 3 weeks in glycerol 50% solution in order to remove biological membrane system and to expose the contractile apparatus which is a perfect biological working model for physiological studies of contraction and relaxation, according to the published technique (Revnic, 1992).
After 3 weeks, glycerinated muscle fragments of 2 cm long have been washed for 15 minutes in bidistiled water and then dried on filter paper. The next step was the placement of biological sample in solution at pH 7.2 in order to assure the ionic equilibrium for one hour according to the published method (Revnic, 1991).
Following preincubation, the muscle fragments have been incubated for 10 minutes in Ri, after that have been removed and have been dried on filter paper, and then introduced in special tested for (^{1}H) NMR measurements (Constantinescu, 1989).
The NMR measurements were performed in fresh skeletal muscle collected and kept on ice to prevent biochemical alteration within one hour from the end of experiment and on glycerinated samples after 3 weeks of glycerinating procedure, in contraction state (cross bridges are attached) and relaxing state (cross bridges are detached).
We tested glycerinated skeletal muscle fibrils in contraction and relaxing media, because these two situations imply different molecular surfaces toward the environment.
The measurements were performed with an AREMI Spectrometer, at 25 MHz frequency.
We used a Carr-Purcell-Meiboom-Gill pulse sequence with 32 spin echoes ranging from 8-256 milliseconds after 90 degree pulse.
After applying the natural logarithm to our experimental curves, the result has the aspect of a decay obtained from two exponential individual relaxations.
Therefore, we attributed the two relaxations times obtained from our data to the fractions of bound water that support the motion of the protein chain substrate and the free water in which are dissolved a large number of solutes.
Thus, the T2 values estimated from the relaxation curves obtained by NMR technique were calculated as two values: T2l (long) offering data about the state of free water and T2s (short) giving data about bound water. The values of T2l and T2s were calculated with a computerized program that could fit the relaxation curve with two exponentials.
The compartmentalization is not rigorously defined but, can help us in the interpretation of the damage generated by the attack of free radicals.
Statistical analysis. Data were expressed as means ± SE or as percentages from stabilization values. A one way analysis of variance was performed to test the differences among groups. If, the difference was significant, the groups were compared further. Significance was $p<0.05$.

RESULTS AND DISCUSSIONS

By optical measurements done with ML4 optical microscope on sarcomere lengths in contraction state, a decrease in the active shortening capacity of sarcomeres from 1.48 u in 6 months old rats to 1.69 u in 37 months old rats for 0.5 mM [ATP] has been recorded as we For 1 mM ATP, sarcomere length recorded in young rats was 1.64 u and for 37 months old 1.90 u. Concerning 2 mM ATP concentration, the mean value of sarcomere length was 1.62 u for young muscle and 1.89 for old muscle. The increase in sarcomere length with ageing is significant from statistical point of view for the three ATP concentrations.

There is a decrease in T2s proton transverse relaxation times in young rat versus old rat, as an expression of a decrease with aging in the active shortening capacity of sarcomeres.
Concerning relaxation for all three ATP concentrations, an age dependent reduction of sarcomere length without statistical significance has been recorded.

Table 1. Relationship between sarcomere length in Sartorius muscle of young and old rats in Contraction state and T2s and T2l values at three [ATP]

Speci-fication	Sartorius 6 months			Sartorius 37 months		
	T2s (ms)	T2l (ms)	Sarc. length (u)	T2s (ms)	T2l (ms)	Sarc. length (u)
Co (0.5 mM)	34	230	1.74	47	280	2.01
Co (1 mM)	33	180	1.72	45	270	1.74
Co (2 mM)	30	138	1.70	46	278	1.72

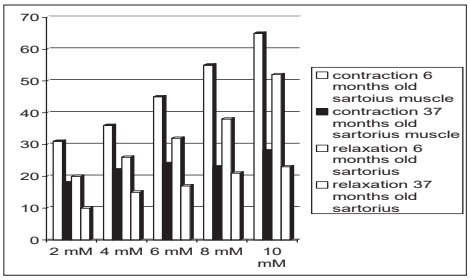

Figure 1. Relationship between sarcomere length in striated muscle of young and old rats in contraction and relaxation state at different ATP

Oxygen radicals may directly alter the proteins, causing cross linking and scission of aromatic amino acids and oxidation of SH groups.
If the 3rtiary and 4 ternary structure of the protein chains is effectively altered, H bonds became unshielded or exposed to the bulk phase water. As a consequence, we anticipate a decrease of T2s because of the increased polar sites directly exposed to the bulk phase water.
T21 is not as sensitive as T2s because only the fractions of random fluctuations that induces spin flip processes contribute to its values.
There is no great discrepancy between T2 values for fresh and glycerinated tissue, with the later about 18% shorter, may be because of the extra cellular fluids present in fresh tissue.
Our data emphasizes that muscle cell water is polarized and the contractile apparatus is responsible for this structure of polarized multi layers because of its proteins, which are partly or completely in the fully extended conformation and their NH and CO groups are free to interact with bulk phase water.
Our data considering T2 shortening in Co state show a possible increase of myofibril Ca^{2+} sensitivity that leads to more cross brides attached in CO groups.
We have calculated T2 by fitting the relaxation curve with two exponentials instead of one, which in our opinion is closer to its shape and to the water distribution in biological systems (free water having a longer T2, bound water a significantly smaller T2).
In such a way, T2s can give more information about the active centers distributed on proteins and responsible for water polarization
We investigated the behavior of contractile apparatus from sartorius muscle from 6 and 37 months old rats in the presence of different [ATP] concentrations.

Table 2. Mean values of sarcomere length (u) in Sartorius muscle from rats of different ages in relaxation state [ATP]

[ATP]	6 months	37 months
	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$
0.5 mM	2.38±0.02	2.20±0.01
1 mM	2.40±0.02	2.22±0.0l
2 mM	2.24±0.02	2.24±0.02

There is a reduction in relaxation capacity of sarcomeres in old rats versus young rats for 0.5 mM [ATP] and 1 mM [ATP], but for 2 mM [ATP] there is no difference between the sarcomere length between young and old rat Sartorius muscle.
Another objective of our study was to measure the proton transverse relaxation times within intracellular compartment in the presence of water exchange between intracellular and extracellular compartment doped with paramagnetic ions such as Mn^{2+} obtaining the apparent relaxation time T_{2a}'.
The representation as a function of transverse magnetization M(t) is:

$$M(t) = A^* \exp(-t/T_{2a}') + B^* \exp(-t/T_{2B}) \quad [1]$$

where the slow component of magnetization with the apparently T_{2a}', is significantly separated from the decreasing one, after introducing experimental data in a filtration program with two exponentials.

Table 3. Proton relaxation times T2s in contraction in the presence of Mn^{2+} from Sartorius muscle from 6 and 37 months old rats

[Mn+2]	6 months old rat		37 months old	
	T2s	T2l	T2s	T2l
2 mM	50	100	99	150
4 mM	65	150	-	-

Our studies concerning ionic charges distribution in Contraction and Relaxation using glycerinated skeletal muscle from young and old rats exposed to different concentrations of Mn^{2+} have pointed out an increase in T2s in contraction in old rats.

As it can be seen, the elongation of proton transverse relaxation times is proportional with the concentration of Mn^{2+} which are accommodated supplementary in Contraction at the level of contractile proteins due to their fixation at the level of negative charges on contractile protein filaments.

In ageing muscle there is an elongation of proton transverse relaxation times T2s and T2l both for Contraction state in the presence of an increased quantity of Mn^{2+}. T2s and T21 are correlated with a reduction of muscle hydration in case of old muscle, contraction being a function of ions binding to the protein sites; these sites being important in determination of hydration of proteins.

Dragomir (1980) has studied the level of fixed charges in rabbit muscle, and he concluded that the level of fixed charges increases with the external electrolyte. For example, in the presence of 100 mM KC1 the concentration of fixed charges is approximatively 75 mM for rabbit psoas muscle in Rigor.

^1H NMR studies in presence of Mn^{2+} related with negative charge density in glycerinated sartorius muscle from 6 and 37 months old rats have revealed an elongation of T2s and T2l as a function of Mn^{2+}, this being more reduced in Co than in Re which accounts for supplementary accumulation of Mn^{2+} in Co at the level of contractile proteins negatively charged.

CONCLUSIONS

Our data have pointed out the existence in glycerinated muscle of two proton relaxation times: T2s and T2l accounted for two water compartments. The modifications in water state are related with modifications in contractile activity. The elongation of proton transverse relaxation times is associated with a decrease in the degree of water molecules aggregation.

T2s and T21 are correlated with a reduction in muscular hydration, contraction being a function of ions binding to the protein sites. These sites are implicated in determination of protein hydration.

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