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In vitro glucose uptake induced by electrical stimulation in *soleus* muscle. Does the stimulation frequency matter?

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Objective: Glucose uptake of skeletal muscle has been studied widely *in vitro* using electrical stimulation. High frequency stimulation resulted in higher glucose uptake. Since the physiological stimulation pattern for slow-twitch muscles is at low frequencies, this study aims to analyse the effects of low vs. high frequency stimulation on glucose uptake *in vitro* and on muscle structure in the slow-twitch rat *soleus* muscle. ***Design:*** Eighteen Wistar rats were assigned to two groups: stimulation at 10Hz and at 100Hz of isolated *soleus* muscles in an oxygenated Krebs buffer. Glucose uptake and the intrafiber localization of GLUT4 were determined. As an estimate for potential damage, the release of taurine into the buffer, cleaved caspase-3 occurrence and expression, and morphological signs of fiber damage were quantified. ***Results:*** Stimulation at 100Hz resulted in a more pronounced glucose uptake than at 10Hz. However, the muscles stimulated at 100Hz showed signs of cellular and membrane damage more frequently than the 10Hz-muscles. Therefore, cellular damage might account for an artificial glucose influx from the buffer by muscles stimulated at 100Hz. ***Conclusion:*** It does not seem meaningful to study glucose uptake in slow-twitch muscles with non-physiologically high stimulation frequencies.

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Key Words: muscle contraction, taurine, cleaved caspase-3, GLUT4

INTRODUCTION

Several studies have used *in vitro* muscle contraction protocols to elucidate the intracellular mechanism leading to exercise-induced glucose uptake (1, 38-40). Despite that the majority reported an enhanced glucose uptake after electrical stimulation, the great heterogeneity of the applied protocols is remarkable especially concerning the voltage used, pulse duration, and time of contraction (1-2, 19, 23, 39). However, regarding the stimulation frequency, most studies have applied high frequency of stimulation (100Hz) even with slow-twitch muscles such as the *soleus* muscle, which is activated at low frequencies of stimulation (10Hz) under *in vivo* physiological conditions (3, 37, 39).

The preference for using protocols with high stimulation frequencies may be linked with the highest rates of glucose uptake reported under these conditions (20). For instance, it was demonstrated in the *soleus* muscle of young Wistars rats that contraction-induced glucose uptake at 10Hz only reached 50-60 percent of the maximal uptake observed after insulin stimulation (26-27). In contrast, some studies demonstrated that high stimulation frequencies (50 to 100Hz) induced similar or higher rates of glucose transport compared to insulin stimulus alone in *soleus* muscle (18, 36). Additionally, Aslesen et al. (1) observed a higher rate of glucose uptake when the *soleus* muscle was stimulated with short tetanic contractions at 100Hz than when it was stimulated with single pulses of 10Hz.
Despite the apparent lack of concern in the literature

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about the unsystematic use of high stimulation frequencies in *in vitro* studies, it can be hypothesised that slow-twitch muscles such as the *soleus*, are physiologically not capable of sustaining high frequency stimuli and, consequently, the enhanced glucose uptake observed under these conditions might be the result of non-physiological mechanisms. In fact, compared to fast-twitch fibers, the phenotype of slow muscle fibers is characterized mainly by a fragile machinery to sustain fast and abrupt increases of sarcoplasmic calcium concentrations (reviewed in (3-4). The resulting loss of Ca^{2+} homeostasis could activate an assembly of catabolic enzymes such as calpains, caspases, and phospholipase A2, compromising the integrity and functionality of vital proteins and of the sarcolemma (6, 11, 16, 28, 35). Under these circumstances, it can be assumed that the release of intracellular proteins described in the literature (15) may parallel the entrance of extracellular substances into the fiber such as glucose. Therefore, it is possible that even with short periods of high frequency stimulation, the loss of the selective sarcolemmal permeability induced by abnormal calcium concentrations might facilitate glucose uptake in slow-twitch muscle fibers through a non-physiological mechanism not dependent on glucose transporter (GLUT4).

Based on these considerations, the aim of the present study was to verify, in rat *soleus* muscle, the effect of a high vs. low frequency *in vitro* electrical stimulation on glucose uptake and on several biochemical and histological markers of muscular damage closely associated with the loss of calcium homeostasis.

MATERIAL AND METHODS

Animals

Male Wistars rats (Charles Rivers Laboratories, Barcelona, Spain) were housed, two animals per cage, in a room with constant temperature (21-23ºC) and exposed to a light cycle of 12h light/12h dark. Commercial chow and tap water were provided *ad libitum* during the quarantine period. After a fasting period of 12h, 18 rats (weighting 334.89±7.36 g, aged 11-14 weeks) were randomly divided into two different groups according to the frequency of electrical stimulation to be applied—the 10Hz group (n=9) and the 100Hz group (n=9). Rats were sacrificed by decapitation and both intact *soleus* muscles weighing 145.32±3.86 mg (n=36) were excised, with one muscle assigned to the electrical stimulation protocol (10Hz or 100Hz) while the contralateral muscle served as incubated rested control. Four extra animals with similar characteristics (age and gender) as those used in the *in vitro* protocol were used further to control potential *soleus* muscle alterations induced by the incubation process itself.

Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR 1996). The local ethics committee approved the study and the experiments complied with the current national laws.

Muscle preparation, stabilizing period, and experimental groups

The muscles were placed vertically suspended in an organ bath between two electrodes attached to an isometric transducer under a passive tension of 10 g. The muscles were incubated in a Krebs Buffer with the following composition (mM): 117 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 24.6 NaHCO3, 5.5 glucose, 2 pyruvic acid and 5 HEPES, gassed with a mixture of 95% CO2 and 5% CO2 and kept at 30ºC (1). After a stabilization period of 60 minutes, one muscle from each animal was submitted to electrical stimulation protocol ("stimulated") whereas the contralateral muscle was not stimulated and remained incubated during the time required for the stimulation protocol ("incubated controls"). Immediately before the application of electrical stimulation, the buffer of both muscles was refreshed with Krebs Buffer containing 0.25µCi/ml of 2-deoxy-D -[1-3H]-glucose (8.50 Ci/mmol; Amersham, UK) and 0.061 µCi/ml of [1-14C]-D mannitol (61 mCi/mmol; Amersham, UK). Soleus muscles obtained from the four animals not submitted to the in vitro protocol constituted the "non-incubated controls."

In vitro muscle electrical stimulation

The protocols of electrical stimulation consisted of trains of pulses (10V, 0.2 ms pulse width) delivered at 100Hz for periods of 10 seconds with an interval of 50 seconds during 10 minutes, or at 10Hz for periods of 10 seconds with an interval of 50 seconds during 30 minutes. After each protocol, muscles were assigned to biochemical (n=6 animals per group) or to morphological assays (n=3 animals per group).

Taurine release to Krebs Buffer

Samples from the incubation buffer were collected to assess the release of taurine from incubated controls and from muscles submitted to electrical stimulation. Taurine concentrations were determined by high-performance liquid chromatography (HPLC) as described by Queiroz et al. (31). The effect of electrical stimulation on taurine release was evaluated by calculating the release evoked after 10 minutes (t=0 being the onset of stimulation) in muscles stimulated at

100Hz and that evoked after 30 minutes in muscles stimulated at 10Hz; it is expressed as the percentage of respective non-stimulated controls after normalization by muscle weight.

Muscles biochemical assays

Stimulated and rested incubated muscles were blotted on filter paper and homogenized on ice in 1 ml of 10 mM Tris-HCl buffer containing 1 mM EDTA and protease inhibitors (500 µM of phenylmethylsulfonyl fluoride, 1 µg/ml of leupeptin, 1 µg/ml pepstatin). A part of the homogenate (500 µl) was frozen at -80ºC for further assay of cleaved caspase-3 while the remaining was digested in 2 ml of 1 M NaOH during 10 minutes at 80ºC for ^{3}H and ^{14}C quantification. Digested samples were neutralized with 2 ml of 1 M HCl mixed with 10 ml of scintillation cocktail (Optiphase HiSafe 3, PerkinElmer) and ^{3}H and ^{14}C in the samples was evaluated by scintillation spectrometry (Beckman LS 6500, Beckman Instruments, Fullerton, USA). Glucose uptake was determined from the intracellular accumulation of 2-[^{3}H]deoxy-D glucose and [1-^{14}C]-D mannitol was used to calculate the interstitial muscle volume. The intracellular muscle water content was calculated by subtracting the measured extracellular space water from the total muscle water, which was calculated for each animal based on the *gastrocnemius* muscle weight variations before and after liofilization (76.6±2.0% of the muscle weight, n=18). Glucose uptake was expressed as absolute values for each group of muscles and the effect of electrical stimulation on glucose uptake is represented as percentage of control incubated muscles.

To assess the muscular expression of cleaved caspase-3, samples were centrifuged at 14.000g at 4ºC for 10 minutes and the supernatant protein concentrations were determined according to the Lowry protocol (25). Supernatant volumes containing 30 µg of protein were separated on 12.5% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked in Tris Buffered Saline – Tween (TBS-T), pH 7.6 containing 5% dry milk and incubated with rabbit anti-cleaved caspase-3 antibody (1:1000, Cell Signaling Technology) followed by incubation with anti-rabbit IgG HRP conjugated (Amersham Biosciences). Bands were detected using a chemiluminescence ECL-kit (Amersham Biosciences), quantified by the densitometry, and expressed in percentage of the expression observed in incubated muscles.

Morphological assays

Muscles from three animals of each group (and from the four animals not submitted to electrical stimulation—the non-incubated controls) were fixed overnight in 4% formaldehyde/phosphate-buffered saline (PBS pH 7.4) buffer. Then, muscles were dehydrated through a graded series of ethanol to xylene and imbedded in paraffin following the conventional protocol. Transversal and longitudinal sections of 4 µm were prepared for immunohistochemistry of cleaved caspase-3 and GLUT4 or were stained with hematoxylin/eosin for light microscopy. Briefly, after being deparaffinised and rehydrated, slices were placed in 10 mM citrate buffer in a microwave oven (700W) during 20 min for antigen retrieval. Then, tissues were submitted to a block solution of 3% bovine serum albumin (BSA) in TBS-T at 37º C during 60 minutes. After several washes, sections were incubated overnight at 4ºC with cleaved caspase-3 primary antibody (rabbit polyclonal, 1:100 Cell Signaling, Technology) or with GLUT4 primary antibody (rabbit polyclonal 1:200 Chemicon, International) at 37ºC for 1h. After washing, all sections were incubated at 37ºC for 1 hour with alkaline phosphatase conjugated anti-rabbit antibody (1:100, Santa Cruz Biotechnology). Fast Red reagent (Sigma-Aldrich) was used to detect the alkaline-phosphatase from secondary antibodies and slides were counterstained with hematoxylin. Negative controls were prepared by replacing primary or secondary antibodies by TBS-T. For cleaved caspase-3, the results were expressed as the percentage of fibers stained red in at least 300 muscle fibers/skeletal muscle. The analysis of GLUT4 slides was merely qualitative, taking into consideration the distribution of red stain within muscle fibers. In slides stained with hematoxylin/eosin, the percentage of swelled and eosinophilic fibers was quantitatively assessed after observing at least 300 muscle fibers/skeletal muscle.

The non-incubated control muscles were used specifically for morphological analysis to control immunohistochemistry data and morphological alterations of the *soleus* muscle, which might have been induced by the incubation process itself.

Statistical analysis

Data are presented as means ± standard deviation (SD)**.** Statistical analyses were carried out using SPSS 15.0 for Windows software. Comparisons between groups were performed using the independent samples T-Test for variables with normal distribution (taurine release) and the Mann-Whithey U for variables with abnormal distribution (glucose uptake, cleaved caspase-3 expression, and morphological analysis). The correlation between rates of taurine release and glucose uptake was calculated with the Spearman's Rank correlation test. The significance level was set at $P<0.05$.

RESULTS

Glucose uptake

After the adjustment of muscular glucose content to the

respective incubation period of each protocol (10 or 30 minutes), the incubated controls for the muscles stimulated at 10Hz and 100Hz presented a basal glucose uptake rate of 42.5±8.6 (n=6) and 40.6±7.3 (n=6) nmol/ml/min, respectively. In muscles submitted to electrical stimulation these rates increased significantly to 61.3±15.5 (n=6) and 71.7±17.1 nmol/ml/min (n=6), which corresponds to an increment of 143±16 and 180±24% for muscles stimulated at 10Hz and 100Hz, respectively (Figure 1).

Taurine release

In control muscles incubated in parallel with those stimulated, basal taurine release was 20.00±7.41 (n=6) and 23.57±10.66 nmol/g/min (n=6) for 10Hz and 100Hz groups, respectively. The electrical stimulation of *soleus* muscle promoted a very small increased of taurine release in muscles stimulated at 10Hz (104.98±14%, n=6), whereas a significant increase of 149.32±34% (n=6) was observed in muscles stimulated at 100Hz (Figure 1). Considering the muscles analysed (incubated and stimulated), a significant correlation between the rate of glucose uptake and taurine release was found ($r=0.67$, $p<0.05$).

GLUT4 localization

As depicted in Figure 2, a notorious difference between the incubated control and stimulated muscles regarding the location of GLUT4 within the muscle fibers was observed. This transporter protein was spread all over the fiber in incubated control muscles while it was positioned mainly near the sarcolemma in stimulated muscles. In both protocols of stimulation (10Hz and 100Hz), there was no qualitative difference concerning the GLUT4 distribution at the periphery of the fibers, suggesting that both frequencies of stimulation have a similar effect on the translocation of this protein toward the muscle fiber membrane. Compared to non-incubated control muscles, the incubated control muscles also showed a discrete increase in concentration of GLUT4 near the periphery of fibers, suggesting that the incubation process itself might have constituted a weak stimulus for GLUT4 translocation (Figure 2).

Morphological alterations

The *in vitro* protocol induced several morphological alterations in *soleus* fibers. The incubated control muscles demonstrated the presence of a few swelled fibers (3.2±1.8%, n>300 fibers/muscle) located manly in the centre of the muscle (Figure 3A), whereas such alteration was not found in non-incubated control muscles. Both electrical stimulation protocols at 10Hz and 100Hz induced a significant increase of swelled fibers compared to the incubated muscle. Quantitative analysis revealed highest numbers of swelled fibers in muscles stimulated at 100Hz (44.6±11.5%, n>300 fibers/muscle), while only 18.2±6.6% (from n>300 fibers/muscle) of the fibers showed this feature in muscles stimulated at 10Hz (Figure 3B). Concerning the damage of fibers, as evaluated by eosinophilic staining, such was only observed in the stimulated muscles (Figure 3A) and the percentage of fibers affected was significantly higher in muscles stimulated at 100Hz (5.2±2.2%, n>300 fibers/muscle) compared to 10Hz (0.8±0.8%, n>300 fibers/muscle, Figure 4B).

Cleaved caspase-3

The absence of cleaved caspase-3 inside the fibers of non-incubated and incubated control muscles demonstrated that the incubated process per se did not induce any proteolysis signal. However, the discrete presence of this protein in its active form in the interstitial space of the incubated muscles suggests the occurrence of some proteolysis in endothelial cells (Figure 4A). In contrast, the fibers of muscles stimulated either at 10Hz or 100Hz showed the presence of cleaved caspase-3 (Figure 4A). This was more frequent in the muscles stimulated at 100Hz (38.3%±7.6%, n>300 fibers/muscle) than in the 10Hz-stimulated muscles (5.1±0.8%, n>300 fibers/muscle; Figure 4B). To confirm the immunohistochemistry results, the western blot technique was used to detect the cleaved caspase-3 content in soleus muscles. In agreement with immunohistochemical data, muscles submitted to electrical stimulation presented a higher expression of cleaved caspase-3 than incubated control muscles. Furthermore, the expression of cleaved caspase-3 was significantly higher in muscles stimulated at 100Hz than in muscles stimulated at 10Hz (Figure 5).

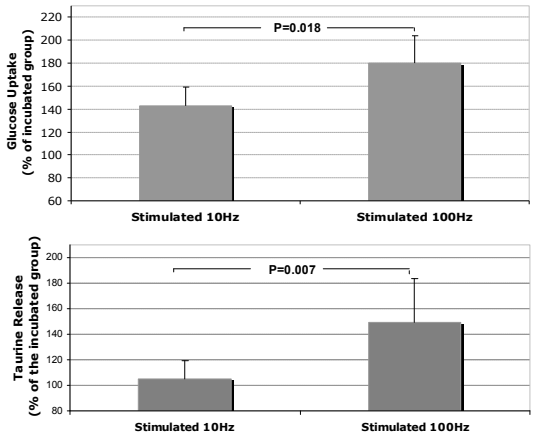

Figure 1. Muscle contraction induced muscle glucose uptake and taurine release to the incubation medium at two different frequencies of electrical stimulation: 10Hz and 100Hz. Values are means ± SD from n=6 and were calculated as a percentage of incubated controls, $p<0.05$ vs. stimulated 10Hz.

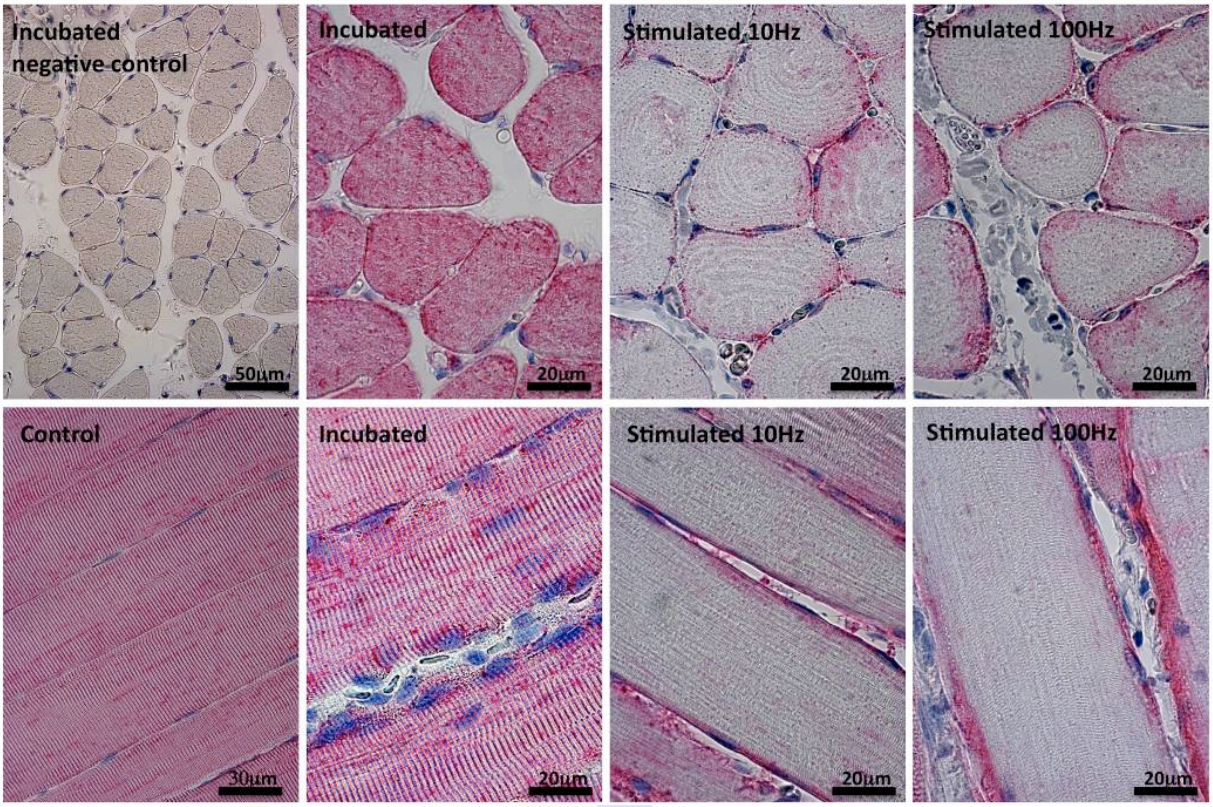

Figure 2. Representative light micrographs with GLUT 4 immunoreactivity of all groups studied stained with fast red and counterstained with hematoxylin. In non-incubated control muscles and incubated control muscles the red stain was spread within muscle fibers; however, in muscles stimulated with 10Hz and 100Hz, the red stain was located at the periphery of fibers without noticeable qualitative differences between these two groups.

DISCUSSION

The present study clearly demonstrates that muscle contraction triggered by electrical stimulation at frequency of 100Hz induces higher rates of glucose uptake in the isolated *soleus* muscle than when the muscle is stimulated at 10Hz. Our results could be merely explained through physiologic mechanisms associated both with the higher GLUT4 vesicle translocation to the sarcolemma or with the higher number of activated fibers triggered by stimuli at 100Hz. However, the percentage of damaged muscle fibers, the amount of taurine released to the incubation medium, the expression of caspase-3 and the similar distribution of GLUT4 in both stimulation groups suggest that other mechanisms related to increased sarcolemma permeability may explain the different glucose uptakes between muscles stimulated at 100Hz and 10Hz, respectively.

It has been demonstrated that muscle contractions triggered by different protocols promote the translocation of GLUT4 with a consequent enhancement of glucose transport (1, 24, 26, 31). None of these studies concomitantly analysed the effect of the *in vitro* contraction protocol on muscular integrity. However, our data demonstrated that the enhanced glucose uptake was coincident with several morphological and biochemical modifications in skeletal muscle, suggesting a reasonably compromised integrity and probably function of numerous muscle fibers.

For instance, the presence of eosinophilic fibers, a good marker of fiber damage (17), was observed only in muscles submitted to electrical stimulation, with a higher percentage of affected fibers in muscles stimulated at 100Hz. Additionally, the presence of swelled fibers, suggesting an increased sarcolemma permeability (33), was another one of the changes observed, in particular in muscles stimulated at 100Hz. These results clearly demonstrate that the *in vitro* stimulation is harmful to muscles and the contraction evoked by stimulation at 100Hz is more deleterious to cell structure and membrane permeability than the contraction evoked by 10Hz stimulation. It appears noteworthy to mention that even the *in vitro* incubation itself has slightly harmful effects on muscle fibers.

It is widely accepted that the loss of cellular membrane integrity promotes an efflux of intracellular substances, which includes taurine, the most abundant amino acid within the excitable muscle cells (22, 29). This small molecule is concentrated inside muscle fibers by a transport system coupled to Na^+ and Cl^- ions (34). In previous studies it has been suggested that taurine is released from the contracted muscle as observed in our

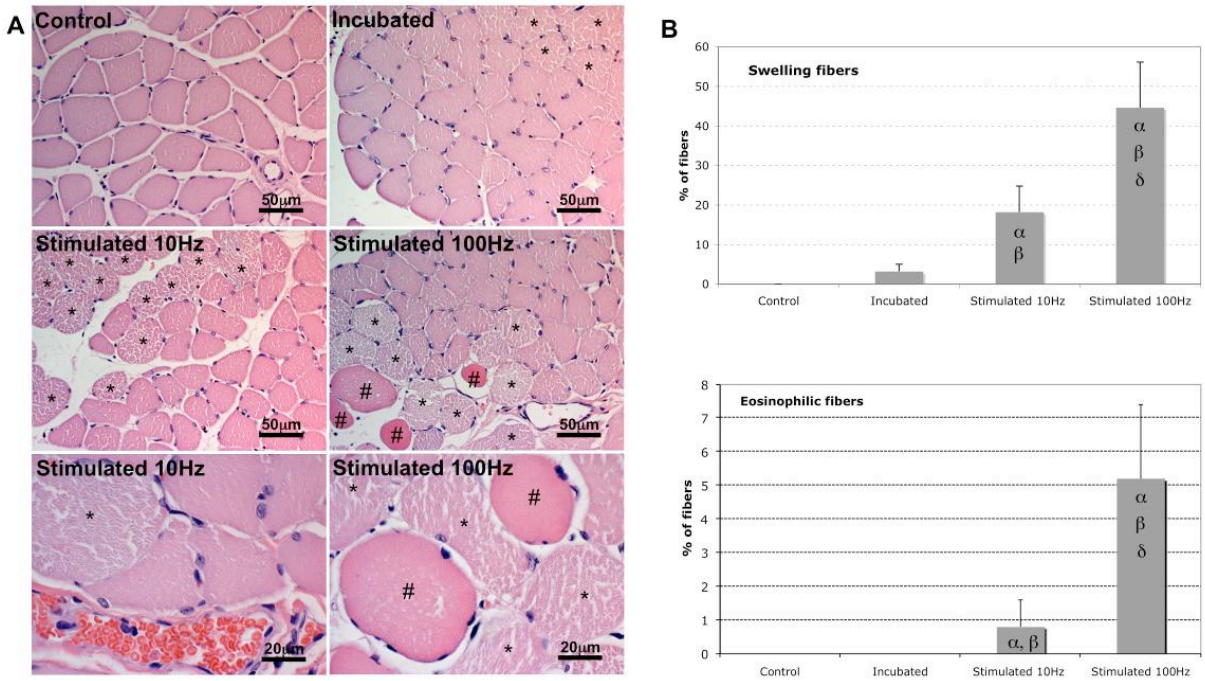

Figure 3. (A) Representative light micrographs stained with Hematoxylin/Eosin and (B) percentage of muscle fibers showing swelling and an eosinophilic character in the groups studied. The incubated control muscles evidenced a slight swelling particularly at the centre of the muscle, depicted in the photograph at the upper position fibers; swelled fibers (*) are more evident in muscles stimulated with 10Hz and 100Hz and several round huge eosinophilic fibers (#) are depicted in 100Hz stimulated muscles. In (B) the values are mean ± SD from n>300 fibers/analyzed muscle. α- $p<0.05$ vs. non-incubated control. β - $p<0.05$ vs. incubated control, and , δ - $p<0.05$ vs. stimulated 10Hz.

experiments, with the purpose of osmoregulation (8, 33). However, taurine leakage might also be explained by reversal of Na^+-dependent transport (32) and under certain circumstances may be correlated to membrane damage (29) since the hidrophylic properties of taurine prevent its diffusion by a passive process through the phospholipids of an intact membrane. In support of this last concept, a high relationship between the plasmatic taurine concentration and other standard markers of tissue injury was observed, such as creatine kinase and lactate dehydrogenase (29). Moreover, our results also revealed a higher rate of taurine release at 100Hz stimulation, which also caused higher damage of muscle fibers (Figure 3). Therefore the substantial release of taurine detected in the incubation medium of muscles contracted by stimulation at 100Hz may indicate a loss of membrane integrity. Accordingly, if taurine leaks out of the fiber by disruption of membrane permeability, it might be expected that substances with similar molecular weight located in the incubation medium, i.e., glucose, might also passively cross the membrane according to is gradient—opposite to that of taurine—and enter into the fibers without the interference of the GLUT4 carrier. The similarity of GLUT4 distribution at the periphery of fibers in both 100Hz and 10Hz stimulated muscles also reinforces the assumption that mechanisms other than GLUT4 might have contributed to the enhanced rate of glucose uptake observed in muscles stimulated at 100Hz. Therefore, the enhanced permeability of the sarcolemma, allowing a passive diffusion of glucose from the incubation medium into the muscle, is supported by the increase in the percentage of damage fibers and taurine release observed only at 100Hz. This mechanism is non-physiological and may account for the enhanced rate of glucose uptake observed in muscles stimulated at 100Hz.

The arguments supporting this non-physiological mechanism are strengthened by the results of cleaved caspase-3 assays. Electrical stimulation acutely induces an increased expression of cleaved (activated) caspase-3, as it had been previously demonstrated (5). Our study also showed that when a frequency of 100Hz is applied, the presence of cleaved caspase-3 is observed in nearly half of the muscle fibers, suggesting a reasonable catabolic state in muscles stimulated at 100Hz. Although the activation of caspase-3 might be associated closely with the phenomenon of apoptosis (7, 14) it also may be the consequence of an abnormal concentration of Ca^{2+} within muscle fibers. Compared to fast twitch fibers (which contract at high frequencies), slow twitch muscle fibers, like those present in *soleus*, are characterized by a lower quantity of sarcoplasmic reticulum (SR)-Ca^{2+} pumps and lower

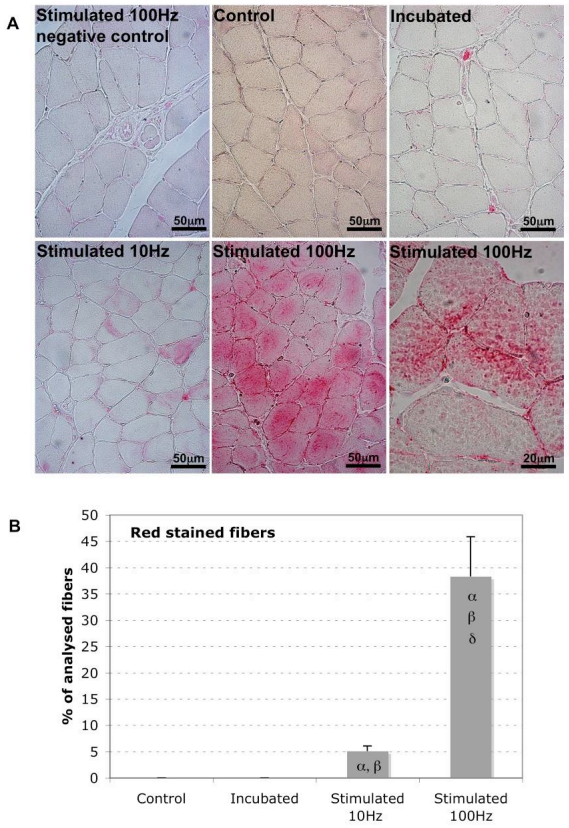

Figure 4. (A) Representative light micrographs with cleaved caspase-3 immunoreactivity of all studied groups stained with fast red (counterstained with hematoxylin) and (B) percentage of red stained muscle fibers. The non-incubated control muscles did not show any red staining; in the incubated control muscles several red stained interstitial cells (apparently endothelial cells) were observed. The red staining affecting muscle fibers from 10Hz and 100Hz stimulated muscles showed a granular pattern of caspase-3 distributed over the fiber. In (B) the values are mean ± SD from n>300 fibers/analyzed muscle; α - $p<0.05$ vs. non-incubated and incubated control muscles; β - $p<0.05$ vs. stimulated 10Hz.

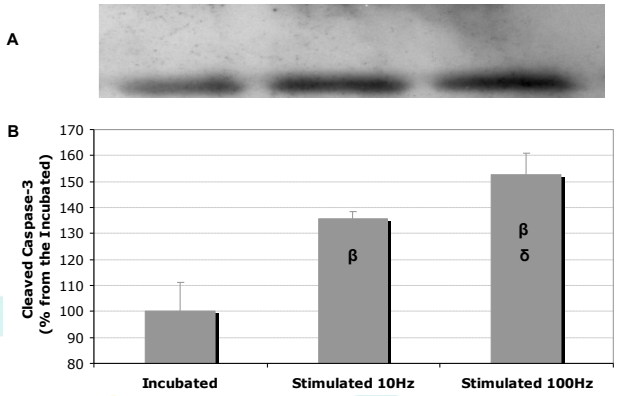

Figure 5. The representative effect of electrical stimulation triggered by 10Hz and 100Hz protocols on cleaved caspase-3 expression in *soleus* muscle. A represents immunoblot against cleaved caspase-3 on incubated control 10Hz and 100Hz muscles. B represents cleaved caspase-3 quantified by scanning densitometry. Data are means ± SD (n=4) in incubated control, 10Hz and 100Hz muscles; β $p<0.05$ vs. incubated control group and δ $p<0.05$ vs. stimulated 10Hz group.

levels of calcium buffering proteins such as parvalbumin, which reduce the ability of these muscle fibers to re-establish Ca^{2+} homeostasis during high-frequency induced contractions (3-4). Therefore, it appears feasible that Ca^{2+}-overload in the fiber also explains the loss of muscle integrity and the release of intracellular proteins in accordance with previous reports (12-13). Calcium overload also activates phospholipase A_2 (PLA_2) which metabolizes membrane phospholipids to arachidonic acid and lysophospholipid, thereby promoting the loss of membrane integrity and function (9).

Since this phenomenon was previously associated with the taurine efflux (30), the loss of calcium homeostasis with further activation of PLA_2 may trigger the increased rates of taurine release and glucose uptake observed in muscles stimulated at 100Hz.

As to our experimental protocol, stimulation at 10Hz lasted 30 minutes while at 100Hz it only lasted 10 minutes. This protocol was chosen in accordance with other research groups working on this topic (10, 19-20, 38). The difference in the duration of the stimulation period reinforces our considerations about the harmful effects of high frequency stimulation of slow-twitch muscles. Tripling the duration of physiological stimulation (stimulation at 10Hz, see above) did not bring about a reasonable "structural failure" like that observed in muscles stimulated at 100Hz. Moreover, in our study the option to use the complete and intact *soleus* instead of small splits of this muscle, as used in other glucose transport studies (20, 38), was chosen because cutting the muscle for this procedure would certainly damage many muscle fibers with a consequent taurine release to the buffer. Additionally, the split muscle procedure is recommended for easy comparisons of muscles with different sizes and fiber-types (21), which were not used in our experimental design. In fact, in all groups of our study the same muscle was always used without any significant size difference between groups. We cannot exclude the possibility of limited substrate diffusion into different zones of the muscle, especially for the centre of the muscles as suggested by the morphological analysis of incubated muscles. However, if such occurred in our muscles, the existence of a control group in the experiment allowed neglecting this condition.

In summary our findings demonstrate that the *in vitro* contraction at 10Hz promotes only a slight injury to *soleus* fibers, but stimulation at higher frequencies

(100Hz) is definitely harmful to these muscle fibers. The morphological results and biochemical assays of taurine release and cleaved caspase-3 allow us to conclude that the increased intramuscular glucose concentration in slow twitch muscles triggered by electrical stimulation at 100Hz might not only be explained by glucose uptake promoted through GLUT4 but also by artificial modifications of the muscle membrane permeability.

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